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RESEARCH ARTICLE

Characteristics of RSV-Specific Maternal Antibodies in Plasma of Hospitalized, Acute RSV Patients under Three Months of Age

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Abstract

Respiratory syncytial virus (RSV) is the leading cause for respiratory illness that requires hospitalization in infancy. High levels of maternal antibodies can protect against RSV infection. However, RSV-infected infants can suffer from severe disease symptoms even in the presence of high levels of RSV-specific antibodies. This study analyzes several serological characteristics to explore potential deficiencies or surpluses of antibodies that could relate to severe disease symptoms. We compare serum antibodies from hospitalized patients who suffered severe symptoms as well as uninfected infants. Disease severity markers were oxygen therapy, tachypnea, oxygen saturation, admission to the intensive care unit and duration of hospitalization. Antibodies against RSV G protein and a prefusion F epitope correlated with *in vitro* neutralization. Avidity of RSV-specific IgG antibodies was lower in RSV-infected infants compared to uninfected controls. Severe disease symptoms were unrelated to RSV-specific IgG antibody titers, avidity of RSV-IgG, virus neutralization capacity or titers against pre- and postfusion F or G protein ectodomains and the prefusion F antigenic site Ø. In conclusion, the detailed serological characterization did not indicate dysfunctional or epitope-skewed composition of serum antibodies in hospitalized RSV-infected infants suffering from severe disease symptoms. It remains unclear, whether specific antibody fractions could diminish disease symptoms.

Introduction

Human respiratory syncytial virus (RSV) infections are a major burden for infants [1]. The symptoms of RSV infection range from a common cold to severe bronchiolitis and

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pneumonia. Children below 3 months of age are at risk for developing severe symptoms that require admission to the hospital, whereas the vast majority shows only mild disease [2].

Early in life, the infant's immune system relies mostly on innate immunity and the presence of maternal, transplacentally transferred immunoglobulin G (matAbs). Consistently, high levels of RSV-specific matAbs in the cord blood are reported to delay the time point of primary RSV infection [3, 4]. Intriguingly, primary infections occur even though high levels of matAbs are present. This raises the question to what extent serum antibodies play a role in infants suffering from severe symptoms during RSV infection and whether antibodies are any different from those of infants with a mild course of disease or uninfected controls. To answer these questions, we investigated whether properties of matAbs such as the level of prefusion F protein-specificity or levels of non-neutralizing antibodies, could relate to severity of disease.

Here, we studied the properties of anti-RSV IgG in plasma of infants that were hospitalized with an acute RSV-infection before 3 months of age. Antibodies observed in a peer group of uninfected individuals represent a base line for: (1) RSV-IgG antibody titers, RSV-IgG avidity, and neutralization capacity, (2) antibody titers against G protein, prefusion F protein, postfusion F protein and (3) antibody titers against prefusion antigenic site Ø and postfusion antigenic site I. The composition of antibodies and/or their combination with properties might represent a signature that is typical for severe RSV disease. We analyzed the antibodies for a correlation with multiple severity parameters, including oxygen therapy, age, respiratory rate, transcutaneous oxygen saturation, admission to an intensive care unit and duration of hospitalization. A better understanding of how antibody properties relate to disease progression is essential to develop safe and effective immunization strategies.

Materials and Methods

Study design

Hospitalized children below 3 months of age with PCR-confirmed RSV infections were included during 2011–2013. Within 24 h after admission, a blood sample was taken. Patients with congenital heart or lung disease, immunodeficiency or glucocorticoid use and infants born at a gestational age below 35 weeks were excluded. For primary analyses, patients were classified into two severity groups based on the necessity for oxygen therapy. For secondary analyses, additional disease severity parameters were used to categorize patients: the presence of tachypnea, transcutaneous oxygen saturation ($\geq 93\%$ or $< 93\%$), admission to an intensive care unit and duration of hospitalization. The correlation with age was investigated for all analyses to determine whether age would be a potential confounding factor. Infants below 3 months of age requiring surgery for an inguinal hernia repair were included as healthy, uninfected controls. Nasopharyngeal aspirates from all uninfected individuals were RSV negative. The study protocols were approved by the Regional Committee on Research involving Human Subjects Arnhem-Nijmegen (serving as the IRB) and were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from the parents of all children.

Plasma collection

Coagulation of blood samples was prevented by sodium heparin (BD Vacutainer). After centrifugation at 800 x g, the plasma fraction was diluted with an equal volume of phosphate-buffered saline (PBS) and stored at -80°C .

Culture of virus and neutralization assay

Recombinant RSV-A2 expressing green fluorescent protein (rgRSV) was kindly provided by Dr. Peebles and cultured on HeLa cells as described previously [5]. RSV was purified by ultracentrifugation over a 30% sucrose layer for 1.5h at 20,000 x g. RSV was quantified by end point titration assays on HeLa cells.

Recombinant RSV-X expressing green fluorescent protein (RSV-X) has been described earlier as E1-rRSV_X [6]. RSV-X is a more recent clinical isolate of genotype GA2 with 98.5% amino acid sequence identity with RSV-A2 F protein and 86.2% amino acid sequence identity with RSV-A2 G protein. A derivative of recombinant RSV-X that is lacking the G protein was generated by replacing the G protein reading frame by a gene encoding green fluorescent protein (RSV-X-dG) [7]. RSV-X and RSV-X-dG (Dutch genetically modified organism license IG-99-210) were propagated in Vero cells (CCL-81, American Type Culture Collection) using Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 5% FCS and Pen Strep Glutamine (Life Technologies). The cell culture supernatants were concentrated by polyethylene glycol-6000 precipitation and stored with 10% sucrose. Virus titration by end point dilution and plaque reduction neutralization assays were performed as described earlier [6]. The 50% plaque reduction neutralization titers (PRNT) were normalized using a correction factor derived from the intra-assay variation of the WHO513 standard (BEI Resources) and half maximal inhibitory concentrations calculated by fitting a non-linear curve (GraphPad Prism).

RSV-specific IgG titer and avidity ELISA

RSV-specific IgG concentrations in plasma samples were determined by ELISA. ELISA plates were coated with rgRSV at a concentration of 2×10^5 U/ml in PBS or PBS as negative control. Plates were washed with PBS containing 0.05% Tween-20 and blocked with PBS containing 1% BSA (Sigma-Aldrich). Plasma samples were incubated for 2 h at room temperature (RT). After washing, plates were incubated with alkaline phosphatase (AP)-conjugated anti-human IgG in PBS containing 1% BSA for 2 h (1:10,000; Southern Biotech). Detection of AP activity was performed with 10 mM diethanolamine with 0.5 mM $MgCl_2$ using an ELISA plate reader. Optical density at 450 nm wavelength (OD) from PBS-coated wells was subtracted from parallel samples coated with RSV. OD values from serial dilutions of healthy adult pooled plasma ranging 1:10 to 1:10,240 were used as standard to calculate arbitrary units (AU). To determine the IgG avidity of RSV-specific antibodies, wells were treated with sodium thiocyanate (NaSCN, 2 mM) for 10 min to weaken antibody binding and compared to untreated wells [8]. The IgG avidity index was calculated as follows: (RSV-IgG titer without NaSCN (AU) / RSV-IgG titer with NaSCN (AU) * 100).

Recombinant RSV F and G ELISA

Expression constructs, production, and purification of the postfusion F ectodomain (Fwt, called postfusion F protein hereafter), the trimeric, furin cleavage site-mutated, heptad repeat B domain-deleted, prefusion F ectodomain (Flys.ΔHRB-GCN, called prefusion F protein hereafter), and a tetrameric subtype A RSV G ectodomain (residues 64 to 298) have been described previously [9–11]. The antibody response to RSV F or G_A was determined using ELISA. NUNC multisorp plates were coated with 25 ng of recombinant protein at 4°C overnight. After washing with PBS containing 0.05% Tween-20, wells were blocked with 3% BSA in PBS containing 0.1% Tween-20 at RT. Two-fold serial dilutions of human patient plasma in blocking buffer were incubated for 1h at RT. After washing, antibodies were labeled with HRP-conjugated goat anti-human IgG (ITK Southern Biotech) for 1h at RT. HRP activity

was detected by tetramethylbenzidine substrate (BioFX). The IgG titer for RSV F or G were determined by calculating the corresponding dilution at OD = 1. Antibody response against antigenic site Ø and I was assessed by competition ELISA as previously described [11]. Two-fold serial dilutions of plasma samples or non-labeled monoclonal antibodies (D25 directed at the specific prefusion F antigenic site Ø, and 131-2a directed at a specific postfusion F antigenic site I) in blocking buffer were added and incubated for 1h at RT. After washing, wells were incubated with 0.6 µg/ml biotin-D25 or 0.2 µg/ml biotin-131-2a to enable binding to prefusion F after preincubation with the diluted plasma samples. After 1h at RT, the biotinylated antibodies were labeled with 1 µg/ml HRP-conjugated Streptavidin (Thermo Scientific) and detected by adding tetramethylbenzidine substrate (BioFX) using an ELISA plate reader (EL-808, Biotek). Wells incubated with biotinylated antibodies alone represent uncompleted, positive controls and wells blocking buffer alone served as negative control. Percentage inhibition was calculated for every dilution using the following equation: $(OD \text{ biotinylated MAb} - OD \text{ plasma samples}) / OD \text{ biotinylated MAb} \times 100$. Using a nonlinear 4-parameter fit curve analysis (GraphPad) the inhibition titer for each plasma samples were determined as the plasma dilution that resulted in 25% inhibition of D25 or 15% inhibition of 131-2a binding.

Statistical analysis

Normality tests were performed on all parameters using the Shapiro-Wilk normality test and showed non-parametric distribution. Comparison between two groups was analyzed by two-tailed Mann-Whitney *U* test and comparison between more than two groups was analyzed by the Kruskal-Wallis test and if significant followed by two-tailed Mann-Whitney *U* test. Spearman correlation test was used for correlation tests. Tests were considered significant if $P < 0.05$. All statistical analyses were done with GraphPad Prism.

Results

Study population

Plasma IgG from thirty-three hospitalized RSV patients was compared to eleven uninfected infants. In our primary analysis, the distinction between severe or moderate RSV disease is determined by whether or not patients received oxygen therapy. Medical doctors make the decision to hospitalize patients based on most severe symptoms that indicate oxygen therapy or on moderate symptoms in combination with other indicators, for example frailty or age, but not requiring oxygen. Consistent with more severe disease, the group that received oxygen was hospitalized for a longer period, as compared to RSV-infected infants without oxygen (Table 1). Gender, gestational age, presence of breastfeeding and presence of parental smoking were comparable between all groups (Table 1). Only children below three month of age were considered for this study. The group of uninfected infants was slightly older and had a lower birth weight compared to the RSV-infected groups (Table 1). There was no significant difference in age and duration of symptoms before hospitalization between RSV-infected infants with or without oxygen therapy.

Level and avidity of RSV-specific IgG in infant sera

Age is correlated with a decay of matAbs and young age is a risk factor for severe RSV disease [12]. In this study, we observed no correlation between age at RSV infection and the levels of RSV-specific IgG (Fig 1A). Moreover, there was no significant difference in RSV-IgG levels between RSV patients and healthy children (Fig 1B). Next, we asked how antibody levels relate

Table 1. Study population.

	Healthy (n = 11)	No oxygen (n = 11)	Oxygen (n = 22)	P value
Age [days ± IQR]	68 [61–80]	58 [36–66]	39.5 [23–70]	<0.05*
Male gender (%)	9 (82)	6 (55)	11 (50)	NS
Gestational age [wk ± IQR]	37.3 [34.5–39.0]	39.4 [37.3–40.4]	38.6 [36.9–39.9]	NS
Birth weight [kg ± IQR]	2.6 [2.13–3.01]	3.16 [2.79–3.62]	3.27 [2.96–3.97]	<0.05 [#]
Breastfeeding (%)	6 (55)	8 (73)	13 (59)	NS
Parental smoking (%)	0 (0)	0 (0)	2 (9)	NS
Symptomatic [days ± IQR]	NA	3.0 [3.0–4.0]	3.5 [2.0–5.0]	NS
Duration hospitalization [days ± IQR]	NA	3.0 [2.0–6.0]	10.0 [8.0–11.3]	<0.001 ⁺

Plasma from infants with RSV infections and healthy controls were included and divided by disease severity based on oxygen therapy. Values are depicted as medians ± interquartile range (IQR) or number with percentage for categorical data. Testing was performed with Kruskal-Wallis and if significant followed by two-tailed Mann Whitney *U* test.

* Healthy versus no oxygen *p*<0.05, healthy versus oxygen *p*<0.01, oxygen versus no oxygen NS.

[#] Healthy versus no oxygen *p*<0.05, healthy versus oxygen *p*<0.05, oxygen versus no oxygen NS.

⁺ oxygen versus no oxygen <0.001.

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to severity of disease. We observed similar levels of RSV-specific IgG in RSV-infected children with or without oxygen therapy (Fig 1C). In addition, similar serum RSV-IgG levels were found when comparing other measures of severity (Table 2). The presence of low-avidity RSV-specific IgG enhances disease symptoms in animal models [13]. RSV-specific IgG in plasma from RSV-infected infants had a lower RSV-IgG avidity compared to healthy controls, respectively 45 [41–51] versus 55 [48–68] (Fig 1D). However, no difference in RSV-IgG avidity was observed when categorizing RSV patients based on oxygen therapy or other disease severity measures (Fig 1E and Table 2).

RSV neutralization capacity of infant sera

Next, we investigated whether the RSV neutralization capacity *in vitro* relates to RSV infection or severity of disease. We determined the capacity of patient plasma to neutralize infection by RSV-X and the G protein-deficient RSV-X-dG. Plasma from healthy controls and RSV-infected infants neutralized RSV-X and RSV-X-dG to a similar extent (Fig 2A and 2C). Also, the median PRNT against both viruses was similar between RSV-infected infants with or without oxygen therapy (Fig 2B and 2D). In addition, no difference in neutralization of RSV-X and RSV-X-dG was observed when categorizing RSV-infected infants based on other disease severity parameters (Table 2). Furthermore, we analyzed whether the contribution of anti-G antibodies to neutralization was different between infant groups. To that end, ratios were calculated from the PRNT using RSV-X and RSV-X-dG. Average PRNT ratios of 0.9 indicated that 10% of the neutralizing activity could be contributed by anti-G antibodies (Fig 2E). These average PRNT ratios were significantly different from 1, which would occur if RSV-X and RSV-X-dG were neutralized equally well. A similar PRNT ratio of 0.9 was observed when comparing RSV-infected infants based on oxygen therapy and healthy infants (Fig 2E). The functionality of this assay was verified by measuring the ratio of RSV-X and RSV-X-dG neutralization by palivizumab, a monoclonal antibody that neutralizes RSV by binding only to the F protein. Thus, palivizumab is independent of the absence or presence of the G protein. Correspondingly, the ratio of PRNT for RSV-X and RSV-X-dG for palivizumab was 1.01.

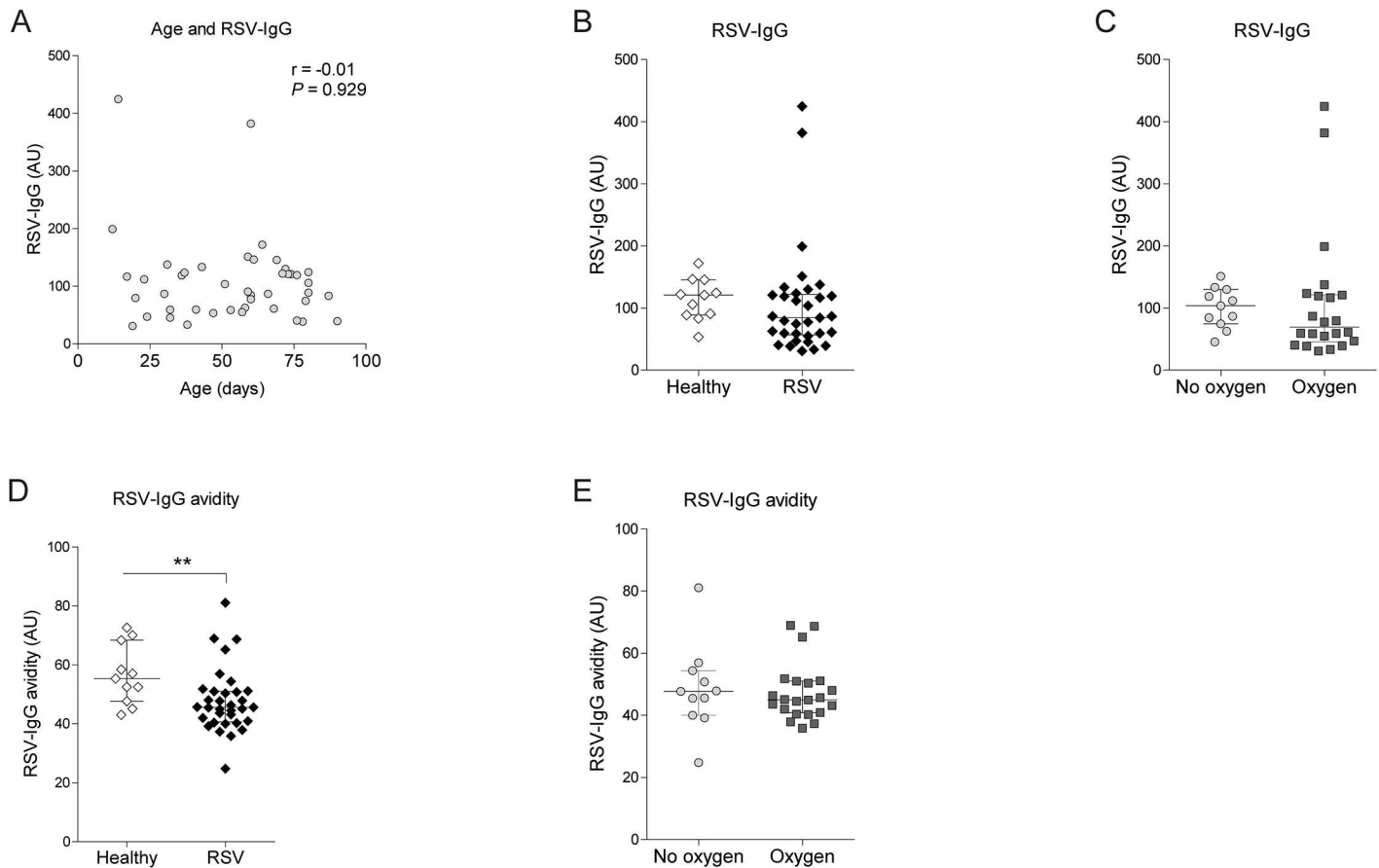


Fig 1. RSV-specific IgG titer and RSV-IgG avidity do not correlate with disease severity. RSV-specific IgG levels in plasma of infants was determined by ELISA using virus particles. RSV-IgG avidity was assessed by supplementing NaSCN during ELISA. (A) RSV-specific IgG levels were displayed versus age. (B-C) Median RSV-specific IgG levels (\pm IQR) were compared between healthy and RSV-infected infants as well as between RSV patients with and without oxygen therapy. (D-E) Median (\pm IQR) avidity of RSV-IgG in healthy infants was compared to RSV-infected infants or between RSV patients with or without oxygen therapy. Associations were assessed by Spearman correlation test. Statistical analyses employed Mann Whitney U test. (** $P < 0.01$).

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RSV-specific IgG against viral surface antigens

An excess of anti-G antibodies have been linked with the failure of the formalin-inactivated RSV vaccine in the 1960s and enhanced disease in mice [14, 15]. Also, antibodies specific for the postfusion F protein seem not to contribute to RSV neutralization [16]. We tested whether antibodies against individual antigens would correlate with disease severity. The levels of antigen-specific IgG present in plasma were determined using soluble, recombinant G protein, pre- and postfusion F protein ectodomains in ELISA. Median antibody levels against G protein were comparable in plasma derived from healthy infants and RSV infections (Fig 3A). Similarly, we did not find a difference of antibody levels against pre- or postfusion F proteins between healthy infants and RSV-infected infants (Fig 3C and 3E). The same was true for median antibody levels when categorizing RSV infections based on oxygen therapy or other disease severity parameters (Fig 3B, 3D, 3F and Table 2). To investigate whether the balance between different antibodies is associated with disease severity, we analyzed the difference between pre- and postfusion F and G-specific antibodies as well as pre- and postfusion F-specific antibodies by subtracting antibody levels against G protein or postfusion F protein from those

Table 2. Antibody properties do not correlate with disease severity parameters.

	RSV-IgG (AU) [IQR]	P value	RSV-IgG avidity (AU) [IQR]	P value	Neutralization RSV-X (PRNT log2) [IQR]	P value	Neutralization RSV-X (PRNT log2) [IQR]	P value
Tachypnea (absent vs present)	112 [68–138] vs 79 [52–121]	0.299	47 [42–51] vs 45 [40–51]	0.828	9.4 [7.9–10.4] vs 9.1 [8.3–9.7]	0.828	9.4 [7.9–10.4] vs 9.1 [8.3–9.7]	0.560
Saturation (>=93% vs <93%)	87 [55–130] vs 77 [60–122]	1.000	46 [40–52] vs 46 [43–51]	0.782	9.1 [8.2–9.4] vs 9.5 [7.9–9.9]	0.782	9.1 [8.2–9.4] vs 9.5 [7.9–9.9]	0.434
ICU admission (absent vs present)	87 [60–127] vs 70 [40–119]	0.303	48 [41–51] vs 45 [40–50]	0.359	9.1 [8.0–9.5] vs 9.6 [8.9–10.0]	0.359	9.1 [8.0–9.5] vs 9.6 [8.9–10.0]	0.184
Duration hospitalization (correlation)	No correlation	0.101	No correlation	0.108	No correlation	0.108	No correlation	0.918
Age (correlation)	No correlation	0.929	No correlation	0.070	No correlation	0.070	No correlation	0.089
	Neutralization RSV-X-dIG (PRNT log2) [IQR]	P value	G protein IgG (AU) [IQR]	P value	Prefusion F protein IgG (AU) [IQR]	P value	Prefusion F protein IgG (AU) [IQR]	P value
Tachypnea (absent vs present)	7.9 [6.8–10.0] vs 7.8 [6.7–9.0]	0.469	216 [151–383] vs 209 [127–478]	0.754	179 [119–263] vs 215 [110–365]	0.754	179 [119–263] vs 215 [110–365]	0.717
Saturation (>=93% vs <93%)	7.6 [6.7–9.0] vs 8.0 [6.8–9.1]	0.490	161 [122–272] vs 261 [186–572]	0.072	202 [111–303] vs 225 [138–418]	0.072	202 [111–303] vs 225 [138–418]	0.357
ICU admission (absent vs present)	7.6 [6.7–8.7] vs 8.5 [7.1–9.1]	0.303	200 [134–414] vs 207 [80–416]	0.667	202 [138–356] vs 262 [103–430]	0.667	202 [138–356] vs 262 [103–430]	0.955
Duration hospitalization (correlation)	No correlation	0.884	No correlation	0.116	No correlation	0.116	No correlation	0.698
Age (correlation)	No correlation	0.129	No correlation	0.409	No correlation	0.409	No correlation	0.109
	Postfusion F protein IgG (AU) [IQR]	P value	D25 inhibition (25%inhibition) [IQR]	P value	131-2A inhibition (15%inhibition) [IQR]	P value	131-2A inhibition (15%inhibition) [IQR]	P value
Tachypnea (absent vs present)	647 [236–888] vs 529 [245–894]	0.828	5.1 [4.2–6.0] vs 5.4 [4.7–6.4]	0.334	5.0 [4.6–5.7] vs 5.4 [5.2–5.5]	0.334	5.0 [4.6–5.7] vs 5.4 [5.2–5.5]	0.328
Saturation (>=93% vs <93%)	590 [296–1415] vs 546 [242–770]	0.645	5.1 [4.7–5.9] vs 5.4 [4.7–6.8]	0.685	5.4 [5.0–6.0] vs 5.2 [4.6–5.5]	0.685	5.4 [5.0–6.0] vs 5.2 [4.6–5.5]	0.397
ICU admission (absent vs present)	590 [238–1078] vs 388 [262–743]	0.640	5.5 [4.7–6.2] vs 5.3 [4.7–7.0]	0.829	5.4 [5.0–5.8] vs 5.4 [4.7–5.8]	0.829	5.4 [5.0–5.8] vs 5.4 [4.7–5.8]	0.762
Duration hospitalization (correlation)	No correlation	0.101	No correlation	0.504	No correlation	0.504	No correlation	0.980
Age (correlation)	No correlation	0.450	No correlation	0.268	No correlation	0.268	No correlation	0.243

Antibody properties were assessed for correlation with multiple disease severity markers. Values are depicted as medians ± IQR. Statistical analyses employed Mann Whitney U test. Testing for correlation was performed with Spearman correlation test. P values are depicted.

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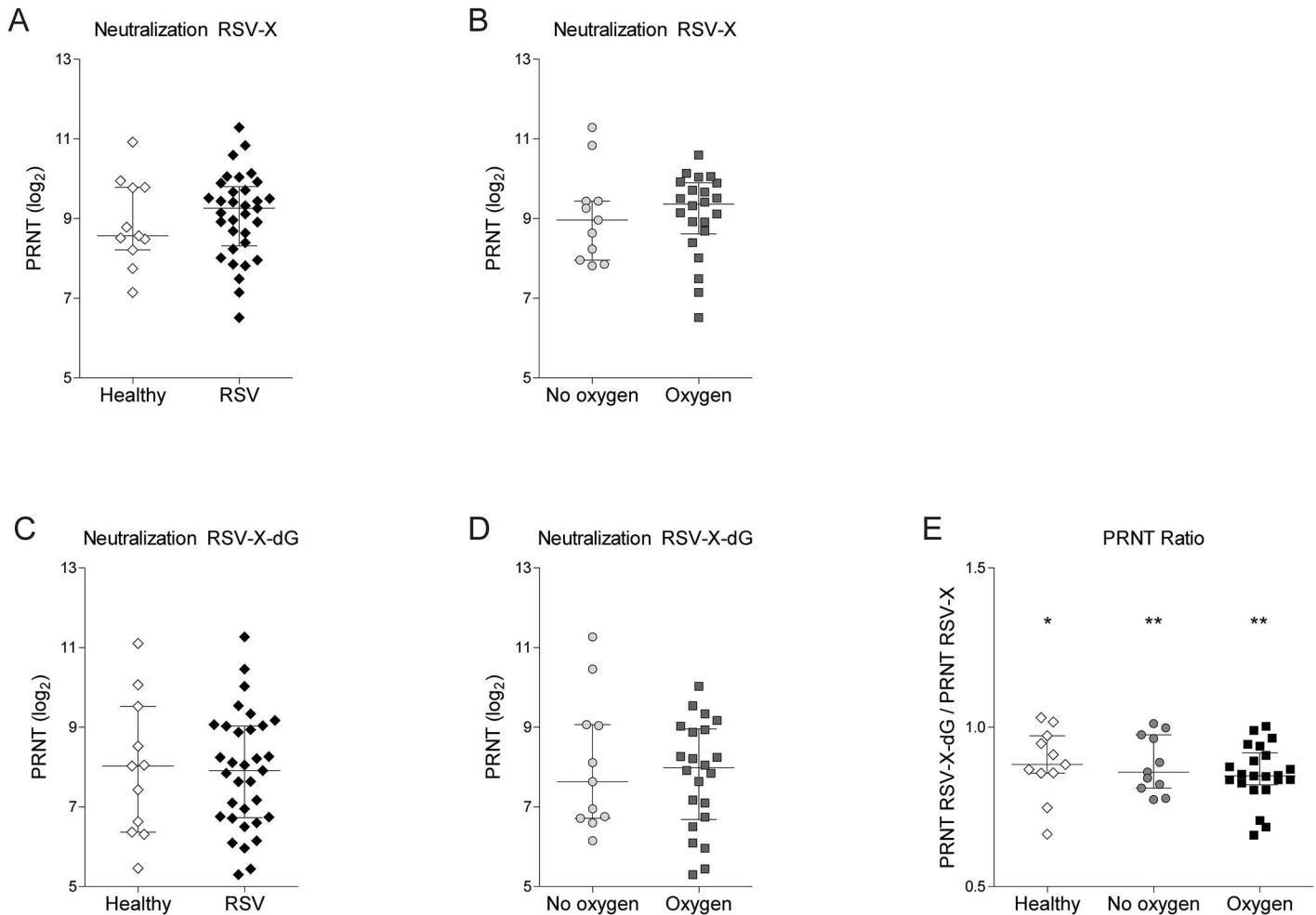


Fig 2. RSV neutralization does not correlate with severity of disease. 50% plaque reduction neutralization titers (PRNT) were determined against RSV-X and RSV-X-dG, the latter is lacking the G protein. (A-B) Median (\pm IQR) PRNT against RSV-X were compared between healthy and RSV-infected infants as well as between RSV patients with and without oxygen therapy. (C-D) Median (\pm IQR) PRNT against RSV-X-dG were compared between healthy and RSV-infected infants as well as between RSV patients with or without oxygen therapy. (E) The PRNT against RSV-X was divided the PRNT against RSV-X-dG, so that values below 1.0 indicate a contribution of anti-G antibodies to the neutralization. The median (\pm IQR) ratio was compared between plasma from healthy infants and RSV patients with or without oxygen therapy and all were significantly different from 1.0. Statistical analyses employed Mann Whitney *U* test for comparison between two groups and Kruskal-Wallis for comparison between more than two groups. (* $P < 0.05$, ** $P < 0.01$).

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against prefusion F protein. The relative abundance of RSV-antigens was comparable in all groups (Fig 3G and 3H), thus no symptom-related antibody composition was identified.

RSV-specific IgG against a prefusion F epitope

Antibodies against prefusion F protein have received much attention recently, because they correlate best with RSV neutralization *in vitro*. We refined our analysis to detect antibodies against epitopes that discriminate between pre- and postfusion F [16, 17]. Sera from healthy and RSV-infected infants were titrated to compete with the monoclonal antibody D25 for pre-fusion-specific antigenic site \emptyset or with the monoclonal antibody 131-2a for the postfusion-specific epitope I [9, 11, 16, 18]. The median competition titers were similar between healthy infants and RSV-infected infants for D25 (Fig 4A) and for 131-2a (Fig 4C). We observed no difference in D25 competition or 131-2A competition when categorizing RSV infections based

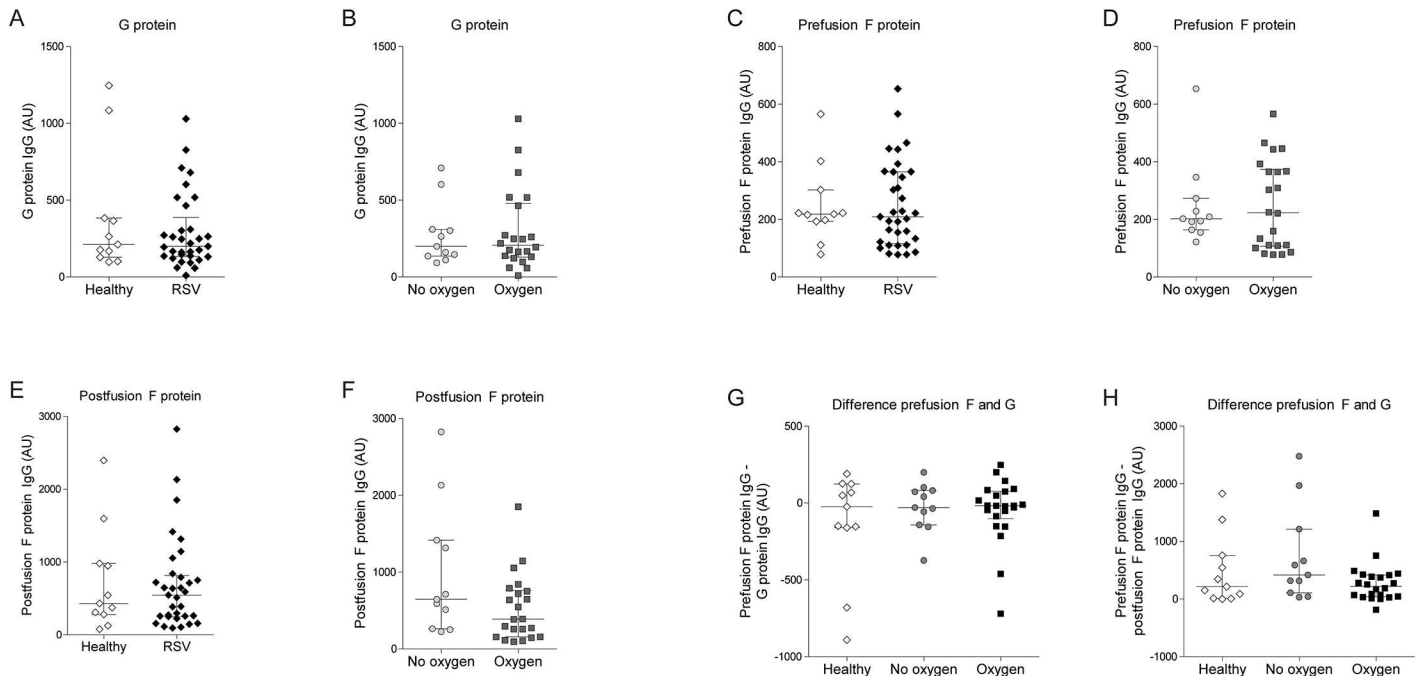


Fig 3. RSV-specific IgG against individual RSV glycoproteins do not correlate with disease severity. RSV glycoprotein-specific antibody levels were determined by ELISA against recombinant, soluble ectodomains. Median IgG levels (\pm IQR) were compared between healthy and RSV-infected infants as well as between RSV patients with and without oxygen therapy. Glycoproteins used for ELISA coating were (A-B) RSV G protein, (C-D) prefusion F protein, and (E-F) postfusion F protein. (G-H) The relative abundance of antibodies against prefusion F compared to G protein as well as pre-compared to postfusion F protein were categorized assessed by subtracting their levels. The median (\pm IQR) difference was compared between plasma from healthy infants and RSV patients with or without oxygen therapy. No significant differences were observed by Mann Whitney *U* test for comparison between two groups and Kruskal-Wallis for comparison between more than two groups.

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on oxygen therapy (Fig 4B, 4D and Table 2). A previous study showed that prefusion F protein-specific antibodies determine the neutralization activity of seroconverted individuals older than 6 years of age [16]. To assess whether this is represented in our measurements of RSV-specific matAbs, we considered the relationship between antigen- and epitope-specific antibody titers and neutralizing capacity (PRNT). Antibodies against postfusion F and 131-2a competition titers represent a negative control and were not associated with neutralization (Fig 5A and 5B). Prefusion F-specific antibodies and D25 competition titers correlated with PRNT (Fig 5C and 5D). Furthermore, antibody titers against G protein also correlated positively with PRNT (Fig 5E).

Discussion

Infants encounter primary RSV infections even though matAbs are present in their blood. We investigated whether individual serologic properties of antibodies or any signature combinations thereof would be related to the observed disease symptoms. Our results indicate that (i) disease severity is not associated with differences in RSV-specific IgG titers, RSV-IgG avidity, or virus neutralization and (ii) plasma titers of RSV-specific IgG against the G protein and the prefusion F protein correlate with neutralizing capacity. In addition, (iii) IgG titers directed against the G protein, prefusion F protein, postfusion F protein, F-antigenic site \emptyset and site I, or any combination of thereof could not be associated with severity of symptoms.

Plasma from infants below 3 months of age was selected because of the high incidence of severe RSV infection even though high levels of matAbs are present in this group. Indeed, we

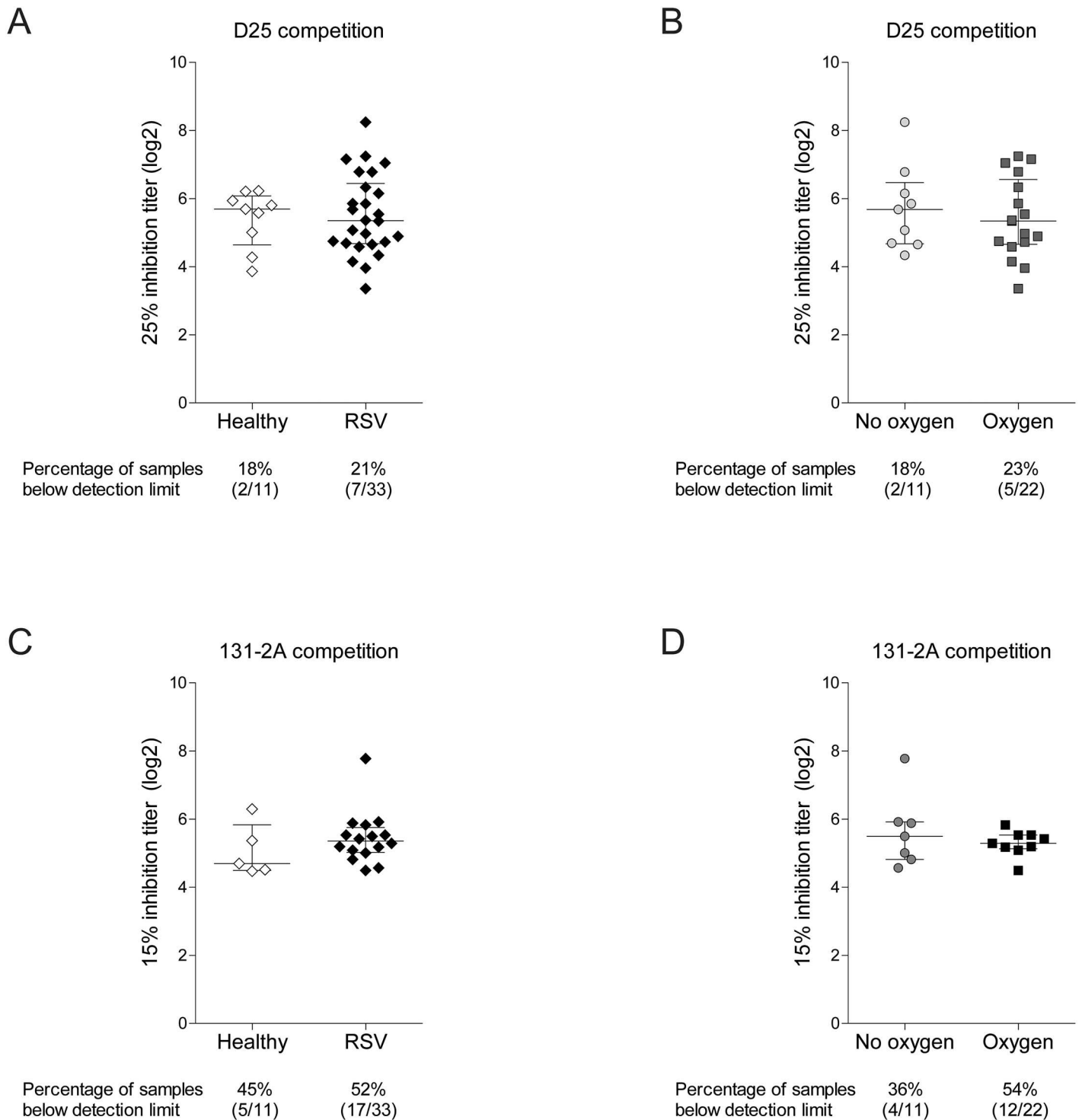


Fig 4. RSV-specific IgG against F protein antigenic sites Ø and I not correlate with disease severity. The abundance of antibodies in human infant plasma that bind to the prefusion F protein antigenic site Ø or the postfusion F protein antigenic site I was determined by competition with site-specific monoclonal antibodies in ELISA. (A-B) Median (\pm IQR) IgG titer that blocks 25% binding of D25 (site Ø) were compared between healthy and RSV-infected infants as well as between RSV patients with and without oxygen therapy. (C-D) Median (\pm IQR) IgG titer that blocks 15% binding of 131-2A (site I) were compared between healthy and RSV-infected infants as well as between RSV patients with and without oxygen therapy. No significant differences were observed by Mann Whitney *U* test.

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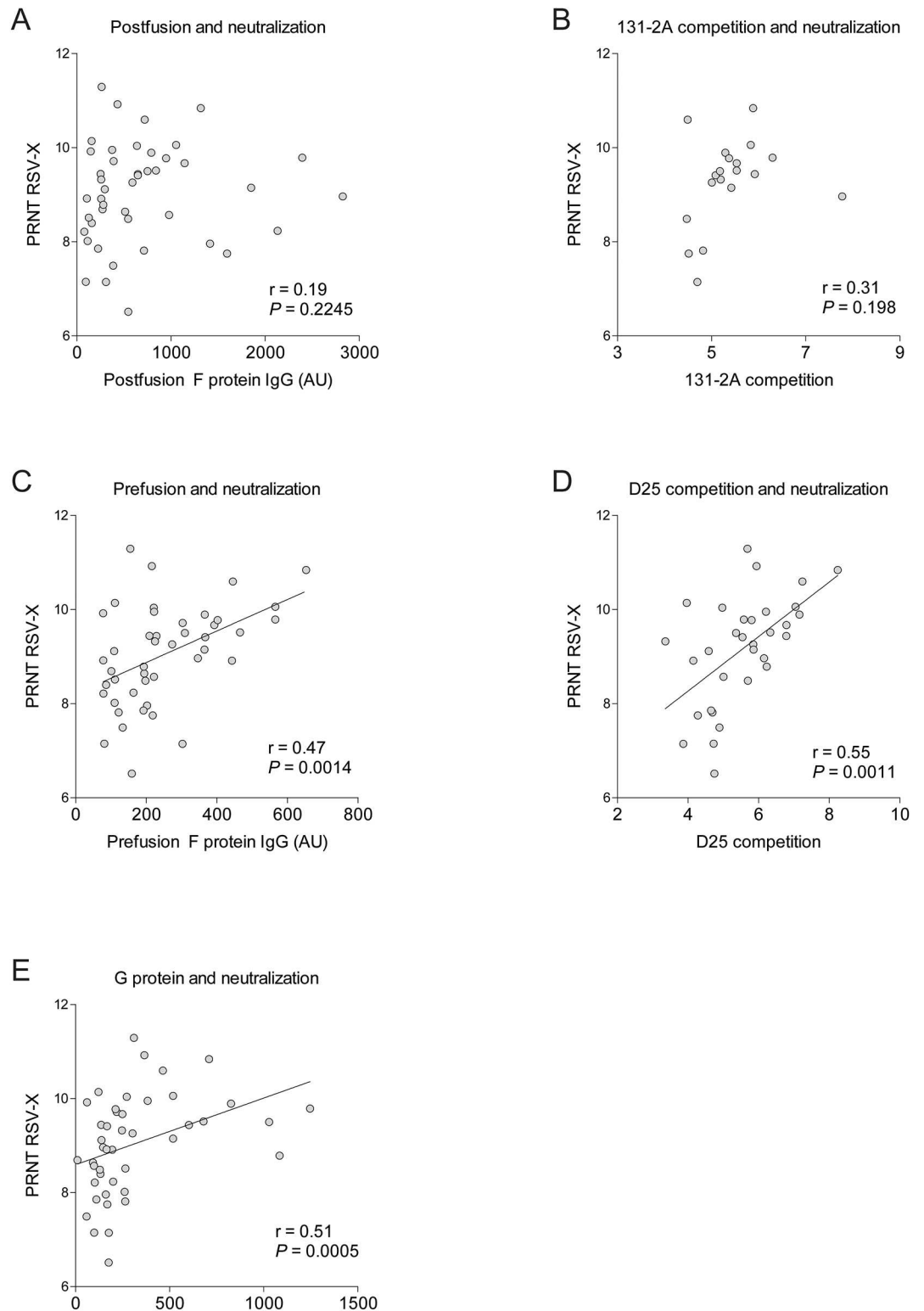


Fig 5. RSV-specific IgG against prefusion F epitope and G protein correlate with neutralization.

Neutralization titers against RSV-X (PRNT) were compared to IgG levels against (A) postfusion F protein, (B) site I (131-2A competition titer), (C) prefusion F protein, (D) site Ø (D25 competition titer) and (E) G protein. Testing for correlation was performed with Spearman correlation test.

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found a median RSV neutralization titers of about 1:512 for all infants (Fig 2), which is similar to what is reported for cord blood in some studies [19, 20]. We omitted age as a confounder for different severity of symptoms, as the age of RSV-infected infants without oxygen therapy was comparable to that of oxygen-treated children. A potential limitation of our study is the difficulty to separate the presence of matAbs from endogenous RSV-induced antibodies of the infant during infection. With a median onset of disease of three days and the short time between symptoms and hospitalization, production of endogenous RSV-IgG is improbable. Moreover, as Dutch newborns in general suffer their first respiratory infection after 3–7 months of age and only 11% of respiratory infections within the first year of life are caused by RSV, infants in our study most likely experienced a primary RSV infection [21]. Therefore, it is plausible that we detect matAbs [22]. Our plasma selection facilitates the comparison of antibodies from hospitalized patients with symptoms ranging from moderate to severe. As this is not a prospective study, the ideal control group of RSV-infected children with mild symptoms was not available, because blood of these children is not routinely sampled. A common practice in RSV research is to use age-matched infants as control. One assumes that this group is not protected and if healthy infants become RSV infected, a vast majority of 98–99% would develop only mild symptoms [23]. We too, substituted the group with mild disease by the analysis of antibodies from uninfected infants. This has no significant impact on our findings and conclusions. The sample size of our control group is relatively small and definitive conclusions in the context of correlation with infection warrant future studies. Nevertheless, the median levels of antibody properties are in close proximity with the median levels of RSV-infected infants. Therefore, it seems unlikely that an addition of more control samples would significantly affect our interpretation. Future studies may include mild infections or prospectively follow healthy infants until RSV infections occurs to investigate the protective effect of RSV-specific antibodies.

High titers of IgG matAbs against RSV have been inversely associated with prevention of hospitalization for RSV disease [24], whereas mucosal IgA reduces the propensity to become infected with RSV [25]. We did not study IgA-mediated prevention of infection, because RSV-IgA is not transplacentally transferred during gestation. We specifically investigated IgG antibodies during an acute infection and observed no correlation between RSV-IgG titer or RSV neutralization and severity of symptoms. Differences in the design of studies investigating prevention of RSV infection versus prevention of severe disease symptoms in hospitalized infants may explain the different conclusion. However, our data is in line with some studies that describe that hospitalization during RSV disease is not correlated to matAb levels [22, 26]. Furthermore, we found RSV-IgG avidity from uninfected infants was significantly higher than from RSV patients, but was not associated with disease severity. Previous studies in mice suggest that non-neutralizing and low affinity antibodies could contribute to the enhanced pathology [13, 14, 27]. The biological relevance of the small but significant differences in RSV-IgG avidity between uninfected infants and infants with RSV infection should be carefully interpreted, because the threshold for antibody avidity to be efficacious during RSV infections is currently unknown. These results should be validated with a prospective study to determine the association of avidity and the protection against severe RSV infections in infants.

As global antibody properties such as titers and neutralization capacity did not correlate with severity of disease, we analyzed whether this was also the case for antibodies against specific RSV antigens. Anti-G protein antibodies have been suggested to facilitate immunopathology in children [14, 27]. In contrast, RSV neutralizing antibodies are usually directed against prefusion F and can be administered as prophylaxis [16, 28]. By comparing the PRNT of patient plasma against full length RSV-X and RSV-X-dG, which lacks the G gene, we observed that anti-G protein antibodies contribute to neutralization *in vitro*. This is

supported by a positive relationship between anti-G protein antibody titers and PRNT. However, the median anti-G antibody titers were similar across all infant sera, regardless of disease and severity. Our serology using RSV A-derived materials does not account for potential impact of the RSV genotype on disease. We believe this is not affecting the interpretation of our results, because only a single serotype for RSV exists. Plasma antibodies from human infants prior to RSV infection, i.e. maternal antibodies, generally show very similar activity against major RSV subtypes regarding the conserved F protein but also the more variable G protein [29]. Moreover, different RSV genotypes have not been convincingly or continuously linked to the epidemiology of RSV outbreaks. We next asked whether the ratios between antibodies against G, pre- and postfusion F would represent a specific signature in severely sick infants compared to mildly sick children and controls. We found that the balance between pre-fusion F and other antibodies was similar in all infants and independent of their disease severity.

Recent data suggests that especially antibodies targeting the pre-fusion F protein epitope Ø efficiently neutralize RSV [30, 31]. To determine whether infants suffering from severe disease symptoms have different epitope Ø binding antibodies compared to controls, we analyzed patient plasma from all groups in a competition assay with D25 and 131-2A. As we did not find any difference, we argue that sera from mildly sick RSV patients have average epitope Ø-specific antibody titers just like children with severe symptoms. We did observe an association between recognition of the D25 epitope and the PRNT of matAbs, like previously shown in seroconverted individuals above 6 years of age [16]. We tested for this pre-fusion F specific antibody because it is the focus of current therapeutic developments. We note that there are more pre-fusion epitopes that could theoretically correlate with disease.

Apart from interacting with viral antigens on virions, antibodies also have Fc region-mediated immunological functions during natural infection. For example, antibodies form immune complexes that can reshape the cytokine and chemokine response of human immune cells [32, 33] or facilitate complement deposition. We did not study antibody effector functions that could relate to severity of disease.

In conclusion, this study investigated the properties of naturally occurring RSV-specific matAbs hoping to provide clues about a specific signature of antibodies that is needed to prevent severe RSV infections. Our detailed characterization did not find any difference in matAbs between infants with severe RSV disease symptoms, controls with mild RSV disease and uninfected infants. This means that the matAb properties we studied do not indicate whether a child would be protected from severe symptoms. It will be very important to find such properties or signatures in order to design effective vaccination strategies in the future.

Supporting Information

S1 Table. Supporting information depicting all individual data.
(XLS)

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