Title page

Title: Antibody levels to SARS-CoV-2 spike protein in mothers and children from delivery to six months later

Running heading: Maternal and neonatal antibody levels to SARS-CoV-2

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Abstract

Introduction: Pregnant women are vulnerable to Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2) infection. Neutralizing antibodies against the SARS-CoV-2 spike (S) protein protect from severe disease. This study analyzes the antibody titers to SARS-CoV-2 S protein in pregnant women and their newborns at delivery, and six months later.

Methods: We conducted a prospective study on pregnant women with confirmed SARS-CoV-2 infection and newborns. Antibody (IgG, IgM, and IgA) titers were determined using immunoassays in serum and milk samples. An angiotensin-converting enzyme 2 (ACE2) receptor binding inhibition assay to the S protein was performed on the same serum and milk samples.

Results: At birth, antibodies to SARS-CoV-2 spike protein were detected in 81.9% of mothers' sera, 78.9% of cord blood samples, and 63.2% of milk samples. Symptomatic women had higher antibody titers (IgG, IgM, and IgA) than the asymptomatic ones ($p<0.05$). At six months postpartum, IgG levels decreased drastically in children's serum ($p<0.001$) but remained high in mothers' serum. Antibody titers correlated positively with its capacity to inhibit the ACE2-spike protein interaction at baseline in maternal sera ($R2=0.203$; $p<0.001$), cord sera ($R2=0.378$; $p<0.001$), and milk ($R2=0.564$; $p<0.001$); and at six-month in maternal sera ($R2=0.600$; $p<0.001$).

Conclusions: High antibody levels against SARS-CoV-2 spike protein were found in most pregnant women. Due to the efficient transfer of IgG to cord blood and high IgA titers in breast milk, neonates may be passively immunized to SARS-CoV-2 infection. Our findings could guide newborn management and maternal vaccination policies.

Keywords

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is expanding all over the world, causing devastating diseases in humans (coronavirus disease 2019 or COVID-19) with a tremendous impact on global health and the economy (1). The most common symptoms of mild infections include fever, fatigue, and dry cough. However, in severe cases, pneumonia and acute respiratory distress syndrome, both with high mortality rates, are developed one to two weeks after the onset of the illness (2). In the severe COVID-19, a deregulated pro-inflammatory response, known as a "cytokine storm", leads to systemic inflammation and multiple organ failure (3).

Pregnant women are at increased risk of severe COVID-19 (4). Furthermore, the inflammatory response induced by SARS-CoV-2 may damage the placenta, raising the probability of viral vertical transmission (5), and the inflammatory condition may be extended to the fetus, compromising its normal development (5). Nonetheless, placental and fetus infections have been reported only in a few studies, and data on vertical transmission are not conclusive because false-positive results or postnatal infection can not be excluded (6). The mother experiences substantial physiologic and immunologic changes during normal pregnancy to ensure proper fetal growth (7). These changes include the overexpression in the placenta and fetal organs of the angiotensin-converting enzyme 2 (ACE2) (8). ACE2 promotes an anti-inflammatory, vasodilatory and antithrombotic response that favors the implantation and development of the fetus (9). However, ACE2 is the SARS-CoV-2 receptor, and its upregulation in the placenta and fetal organs may increase the risk of SARS-CoV-2 infection in these organs (3). Moreover, when the virus binds to the ACE2, this receptor is down-regulated, and its levels remain low during infection (10), promoting vasoconstriction, inflammation, and pro-coagulopathic effects resembling preeclampsia (11).

Specific neutralizing antibodies against the "spike" or S glycoprotein of SARS-CoV-2 protect from severe COVID-19. Those antibodies are raised mainly against the receptor-binding domain, which mediates the attachment to ACE2 on the host cells (12, 13). A few studies have shown that SARS-CoV-2 specific immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies are present in the blood of pregnant women, while immunoglobulin A (IgA) predominates in breast milk (14-18). SARS-CoV-2 IgG is transferred to the fetus through the placenta (15, 17). However, IgM and IgA do not cross the placenta, and their presence in the fetus's umbilical cord blood could indicate a possible infection (5, 19).

In the present study, we aimed to analyze the antibody levels to SARS-CoV-2 S protein at delivery in the serum and milk of SARS-CoV-2 infected pregnant women and their newborns' umbilical cord serum. Six months after delivery, antibody levels were also quantified in the serum of the women and their infants.

Methods

Study design

We carried out a prospective study on pregnant women with laboratory-confirmed SARS-CoV-2 infection before childbirth or at childbirth and their newborns. At delivery, we studied the antibody levels to SARS-CoV-2 S protein in the maternal serum (n=104), milk (n=46), and umbilical cord serum (n=71). Six months later, we also studied the paired serum samples available from those patients [mothers (n=33) and children (n=23)] who attended their routine medical examination.

All participants were recruited at the Hospital General Universitario Gregorio Marañón (HGUGM) from Madrid, Spain, between March 2020 and November 2020, inside the GESNEO-COVID Cohort (20). The follow-up study (six months after delivery) was carried out in the Department of Paediatrics of the HGUGM, where the routine medical examination of children exposed to or infected by SARS-CoV-2 was performed. However, some children were lost because they were examined in other hospitals or primary care centers. Only children (and their mothers) that attended the pediatric care of the HGUGM six months after delivery were included in the follow-up study. This follow-up study

was stopped on May 15, 2021. The study was approved by the HGUGM Ethics Committee (Ref.: IRB 00006051) and was conducted following the Declaration of Helsinki. All participants gave their informed consent before enrollment.

Clinical data and samples

Epidemiological, clinical, and microbiological data (PCR test and serological analysis for COVID-19 diagnosis) of mothers and newborns were collected from the hospital's electronic medical history through Research Electronic Data Capture (REDCap) platform, hosted on a secure server at the Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain.

Mothers were tested for SARS-CoV-2 infection by polymerase chain reaction (PCR, TaqPath 1-Step Multiplex Master Mix, Applied Biosystems) on a respiratory specimen (nasopharyngeal swab) or by detecting IgG or/and IgM antibodies by immunoassay (IgG/IgM against SARS-CoV-2 by SARS-CoV-2 IgG Reagent Kit, Abbott) in maternal serum before childbirth or on the day of childbirth. Newborns were tested for SARS-CoV-2 infection (nasopharyngeal PCR) at birth and 15 days later. Both mother and child were followed up for six months.

For anti-SARS-CoV-2 S antibody detection, blood samples were collected in ethylene diamine tetra-acetic acid tubes from the mothers during childbirth and six months later. At delivery, cord blood samples were collected for the same purpose. For some children, blood samples were also collected six months after birth. Breast milk was collected in sterile sample bottles during the first 48 hours after delivery and, when not possible, after 15 days. All samples were received, processed, and stored at the Spanish HIV BioBank at -20°C until use (21).

Immunoassay for antibody quantification

The plasmid pαH coding for the S protein ectodomain (residues 1-1208) of SARS-CoV-2 2019-nCOV (GenBank: MN908947) stabilized in the prefusion conformation was kindly provided by Dr. Jason McLellan (the University of Texas at Austin-USA). Mutagenesis was conducted to obtain a HexaPro construct that allowed a high-yield production of a stabilized prefusion spike protein. The following substitutions were included at the ectodomain: glycine at residue 614 (D614G), a "GSAS" substitution at the furin cleavage site (residues 682–685), and proline at residues 817, 892, 899, 942, 986, and 987. A plasmid coding for residues 1-165 of ACE2, the cell receptor for the SARS-CoV-2 S protein, fused to a StrepTag was also built. The expression vector coding for the ectodomain of the SARS-CoV-2 S protein was used to transfect FreeStyle 293F cells transiently. The S protein domain was purified from filtered cell supernatants using Ni-NTA resins followed by size-exclusion chromatography. The ACE2 receptor was expressed in the same way and purified by the Insights into the Strep-tag system followed by size-exclusion chromatography (full description in **Supplementary file 1**).

Antibody titers against the S protein were determined in an ELISA assay by incubating serial dilutions of serum samples with the purified S protein ectodomain in 96-well plates, followed by successive incubations with a secondary peroxidase-conjugated anti-human IgG, IgM, or IgA (Jackson Immunoresearch, West Grove, PA, USA) and the OPD substrate (Sigma Aldrich, San Luis, MO, USA). One phase exponential decay least-squares fit curves, and the area under the curve (AUC) were calculated using GraphPad Prism 9.0. The capacity of the antibodies to inhibit the binding of the soluble ACE2 receptor to the SARS-CoV-2 S protein was determined by ELISA (full description in **Supplementary file 1**). A pool of sera from individuals negative for anti-S antibodies, collected in 2016, was used as a control. After subtraction of the background, the percentage of inhibition was calculated as [1 - (OD_{493} test serum / OD_{493} control serum)] x 100 %. The percentage of inhibition relative to ACE2 in which the StrepTag was removed was further calculated as (% inhibition of serum samples / % inhibition of ACE2) x 100 % (full description in **Supplementary file 1**).

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Statistical analysis

Statistical analysis was carried out with IBM SPSS Statistics 25.0 (SPSS INC, Armonk, NY, USA), and figures were generated by GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA). The level of significance was defined as p<0.05 (two-tailed).

Data were shown as absolute counts (percentages) for categorical variables and median (interquartile range, IQR=P25th; P75th) for continuous variables. The Mann-Whitney U test calculated differences between independent groups and the Wilcoxon signed-rank test for dependent groups. Correlation analysis was performed using the Pearson test.

Results

Patient characteristics

The characteristics of the study population are described in **Table 1**. Most mothers (83.7%) were PCR positive for SARS-CoV-2 during pregnancy or childbirth. However, 16.3% of pregnant women had a negative SARS-CoV-2 PCR but positive anti-SARS-CoV-2 antibodies. COVID-19 diagnosis was performed at childbirth (30.8%), two weeks before childbirth (35.6%), or more than two weeks before childbirth (33.7%). Only 43.3% had symptoms (9.6% were severe), and 4.8% needed oxygen therapy. Newborns had a mean gestational age of 36.7 weeks, 82.7% were born vaginally, and 93.8% were breastfed at hospital discharge. In general, anthropometric measurements and health status were within normal ranges. Finally, 9.7% were admitted to the Neonatal Intensive Care Unit by prematurity, and three newborns had a positive PCR for SARS-CoV-2.

Table 1. Characteristics of mothers and babies.

Parameters	Values
A) Mothers	
No.	104
Age (years)	34.3 (29.4; 36.9)
Country of birth	
Spain	52 (50%)
Latin American Countries	40 (38.5%)
Other	12 (11.5%)
Comorbidities	
Obesity	10 (9.6%)
Hypertension	4 (3.8%)
Diabetes	6 (5.8%)
COPD	4 (3.8%)
Hypothyroidism	4 (3.8%)
COVID-19	
Diagnosis (+)	
> Two weeks before childbirth	35 (33.7%)
Two weeks before childbirth	37 (35.6%)
In childbirth	33 (30.8%)
SARS-CoV-2 PCR (+)	
Pregnancy	87 (83.6%)
Childbirth	66 (63.4%)
Gestational age (weeks) at diagnosis	38 (33.3; 39.4)
Symptoms	46 (44.2%)
Severe symptoms	10 (9.6%)

Hospitalization	15 (14.9%)
Treatment	
Oxygen therapy	5 (4.8%)
Lopinavir/r	9 (8.7%)
Hydroxychloroquine	8 (7.7%)
Corticosteroids	3 (2.9%)
Heparin	14 (13.5%)
Antibiotics	13 (12.5%)
B) Newborn	
No.	71
Gestational age (weeks)	36.7 (38.4; 40.4)
Mode of delivery	
Vaginal	86 (82.7%)
Caesarean	18 (17.3%)
Gender (male)	55 (52.9%)
Breastfeeding	91 (93.8%)
Admitted to NICU by prematurity	10 (9.7%)
Condition at birth	
APGAR1	9 (9; 9)
APGAR5	10 (10; 10)
Weight (Kg)	3.3 (2.9; 3.5)
Height (cm)	50 (48; 51)
Head circumference (cm)	34 (33; 35)
SARS-CoV-2 PCR (+)	
Pre-perinatal	1 (1%)
Postnatal	2 (1.9%)

Statistics: Values are expressed as the median (Q1; Q3) and absolute count (percentage).
Abbreviations: COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; PCR, polymerase chain reaction; APGAR, Appearance, Pulse, Grimace, Activity, and Respiration; APGAR1, 1-minute APGAR; APGAR5, 5-minute APGAR.; NICU, Neonatal Intensive Care Unit.


Antibody titers in mothers and neonates

At birth, antibodies to SARS-CoV-2 S protein were detected in 81.9% of mothers' sera, 78.9% of cord sera samples, and 63.2% of milk samples. A significant relationship between the positive antibodies to SARS-CoV-2 S protein and the time of infection was only found in maternal sera. Mothers diagnosed more than two weeks before delivery had higher percentages of positive sera for IgG (94.3% vs. 68.6%; p = 0.003), IgM (65.7% vs. 36.8%; p = 0.009) and IgA (65.7% vs. 45.7%; p = 0.056) than mothers diagnosed later (at two weeks before delivery or at the childbirth). The presence/absence of antibodies to SARS-CoV-2 S protein correlated between mother sera, cord sera, and milk. Thus, 15 newborns had undetectable IgG, and 13 were born to mothers diagnosed with COVID-19 near delivery (<30 days). Except for one, IgG antibodies were detected in all preterm infants (less than 37 weeks gestation). Among the newborns with positive IgG cord sera, 94.6% were delivered by mothers who also had positive IgG sera. Concerning negative IgG cord sera, 86.7% of the newborns with negative results were delivered by mothers with negative sera (p <0.001). Among the mothers with IgA positive serum samples, 61.5% also had IgAs in their milk, while 73.7% of mothers with no detectable IgAs in their serum also were IgA negative in milk (p = 0.021).

Levels of anti-SARS-CoV-2 S protein IgG in maternal serum (264.4 [IQR = 11.5; 697.2]) were higher than levels of IgM (1 [IQR = 1; 29.6]; p<0.001) and IgA (2.4 [IQR = 1; 22.6]; p<0.001) (**Figure 1A**). In breast milk (**Figure 1B**), levels of anti-SARS-CoV-2 S IgA (4 [IQR = 1; 141.1]) were higher than IgM levels (1 [IQR = 1; 7.32]; p<0.001) and IgG levels (1 [IQR = 1; 1]; p<0.001). Regarding umbilical cord serum (**Figure 1C**), 78.9 % of samples had IgG antibodies (280.8 [IQR = 7.4; 1,031]), but only a few samples had detectable IgMs or IgAs. The average ratio of IgG antibody levels between the umbilical cord and maternal serum was 1.4.

There were three children with a positive PCR for SARS-CoV-2. A neonate was diagnosed at the time of delivery, whose mother was ill and had no antibodies to SARS-CoV-2 S protein in serum and milk. The other two neonates were diagnosed 15 days after delivery. However, only one of them had anti-SARS-CoV-2 IgG antibodies in the umbilical cord and six months later in the serum, and whose mother also had antibodies against SARS-CoV-2 (IgG, IgM, and IgA) at delivery and six months later.

There were also two umbilical cord samples with low levels of anti-SARS-CoV-2 S IgM and three with low anti-SARS-CoV-2 S IgA levels, which corresponded to neonates with a negative PCR for SARS-CoV-2. These children had, however, high levels of anti-SARS-CoV-2 S IgG antibodies.
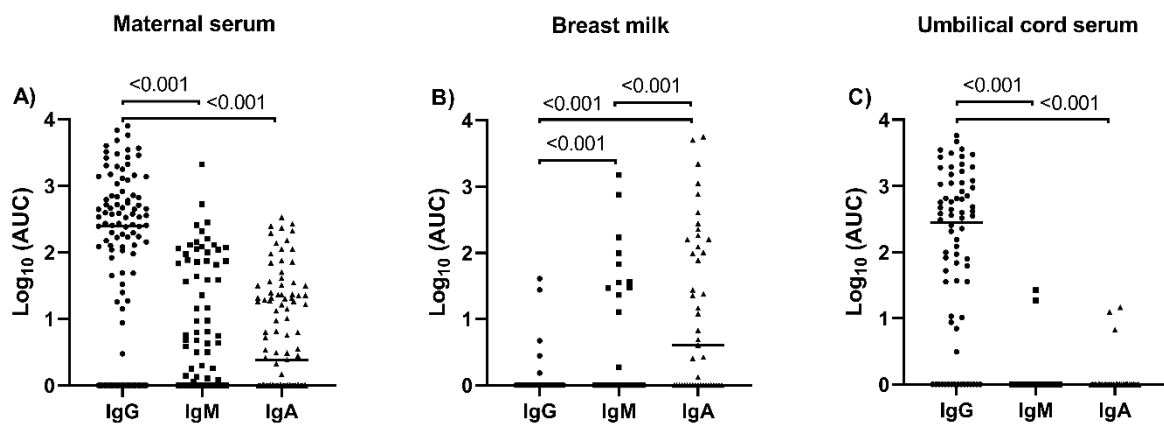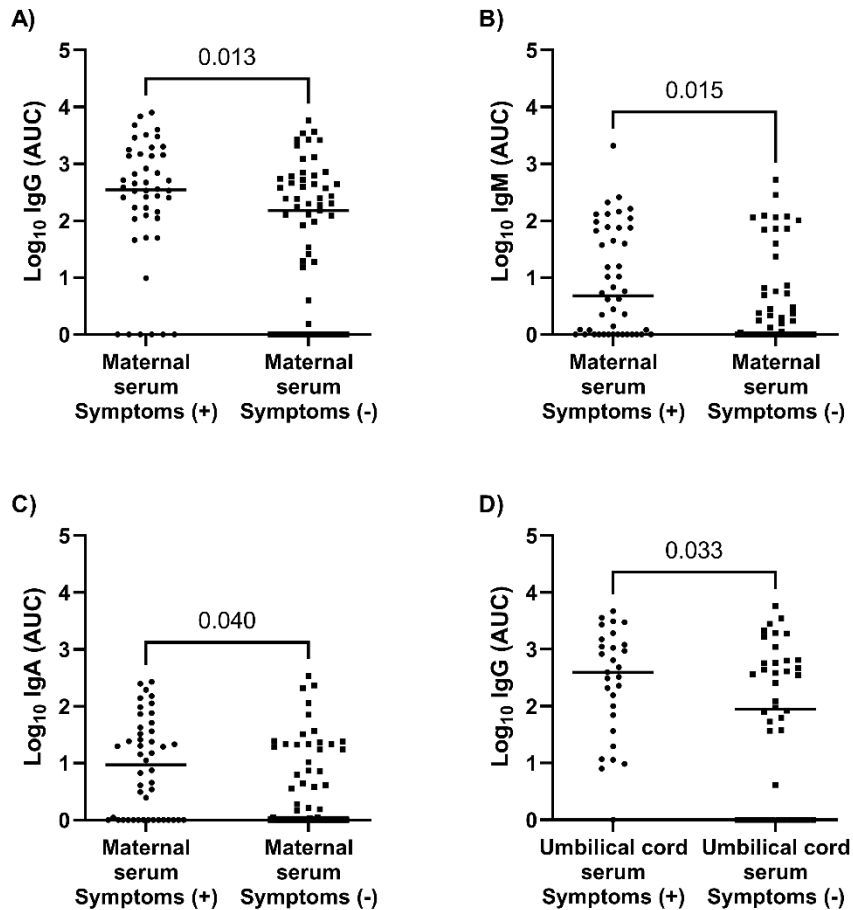

Figure 1. Antibody levels against SARS-CoV-2 S protein in maternal serum (A), breast milk (B), and umbilical cord serum (C). **Statistics**: Differences were calculated by the Wilcoxon signed-rank test, and only p-values <0.05 are shown. Medians are represented by a horizontal bar. In some cases, the horizontal bar is not visible because it overlaps with the X-axis. **Abbreviations**: AUC, the area under the curve; IgG, anti-SARS-CoV-2 S IgG; IgM, anti-SARS-CoV-2 S IgM; IgA, anti-SARS-CoV-2 S IgA.

Antibody titers according to COVID-19 symptoms

Women with COVID-19 symptoms had higher serum values of anti-SARS-CoV-2 S protein IgG (350.3 [IQR = 110.1; 1,449] vs. 150.6 [IQR = 1; 523.6]; p=0.013; **Figure 2A**), IgM (4.8 [IQR = 1; 75.1] vs. 1 [IQR = 1; 5.8]; p=0.015; **Figure 2B**) and IgA (9.4 [IQR = 1; 37.7] vs. 1 [IQR = 1; 19.3]; p=0.040; **Figure 2C**) than women without COVID-19 symptoms. Additionally, newborns of women with COVID-19 symptoms had higher serum IgG values than newborns of women without COVID-19 symptoms (395.9 [IQR = 53.2; 1,353] vs. 88.8 [IQR = 1; 592.3]; p=0.033; **Figure 2D**).
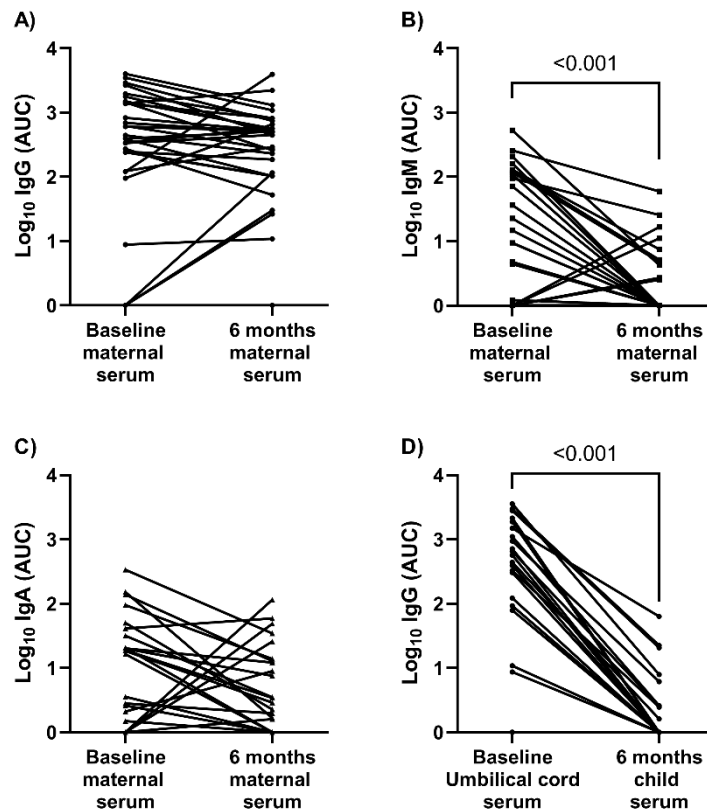
Figure 2. Antibody levels against SARS-CoV-2 S protein in maternal serum (A, B, and C) and umbilical cord serum (D) according to COVID-19 symptoms. **Statistics**: Differences were calculated by the Mann-Whitney U test and p-values are shown. Medians are represented by a horizontal bar. **Abbreviations**: AUC, the area under the curve; IgG, anti-SARS-CoV-2 S IgG; IgM, anti-SARS-CoV-2 S IgM; IgA, anti-SARS-CoV-2 S IgA.

## Antibody titers at six months postpartum

For some participants with paired samples at baseline and six-month of follow-up, serum antibody levels against SARS-CoV-2 S protein were determined (**Figure 3**). IgG levels remained relatively high in most mothers (**Figure 3A**). In contrast, in 33 mothers, we observed a pronounced decrease in serum IgM levels during the follow-up (4.4 [IQR= 1; 102.2] vs. 1 [IQR= 1; 3.47]; p<0.001; **Figure 3B**). In 23 children, serum IgG levels decreased drastically at six months postpartum (440 [IQR= 79.1; 1,501] vs. 1 [IQR= 1; 2.6]; p<0.001; **Figure 3D**).

Figure 3. Evolution of antibody levels against SARS-CoV-2 S protein from delively to six months postpartum in maternal serum (A, B, and C) and newborn serum (D). **Statistics**: Differences were calculated by the Wilcoxon signed-rank test, and only p-values <0.05 are shown. **Abbreviations**: AUC, the area under the curve; IgG, anti-SARS-CoV-2 S IgG; IgM, anti-SARS-CoV -2 S IgM; IgA, anti-SARS-CoV-2 S IgA.

Antibody titers and their capacity to inhibit ACE2-S protein interaction

We found a positive and significant correlation between serum antibody titers against SARS-CoV-2 S protein and the percentage of inhibition of ACE2 binding to the S protein at baseline in maternal serum (R^2=0.203; p<0.001; **Figure 4A**), umbilical cord serum (R^2=0.378; p<0.001; **Figure 4B**), and breast milk (R^2=0.564; p<0.001; **Figure 4C**). Moreover, a positive and significant correlation, much higher than at baseline, was found in maternal serum samples six months after delivery (R^2=0.600; p<0.001; **Figure 4D**).
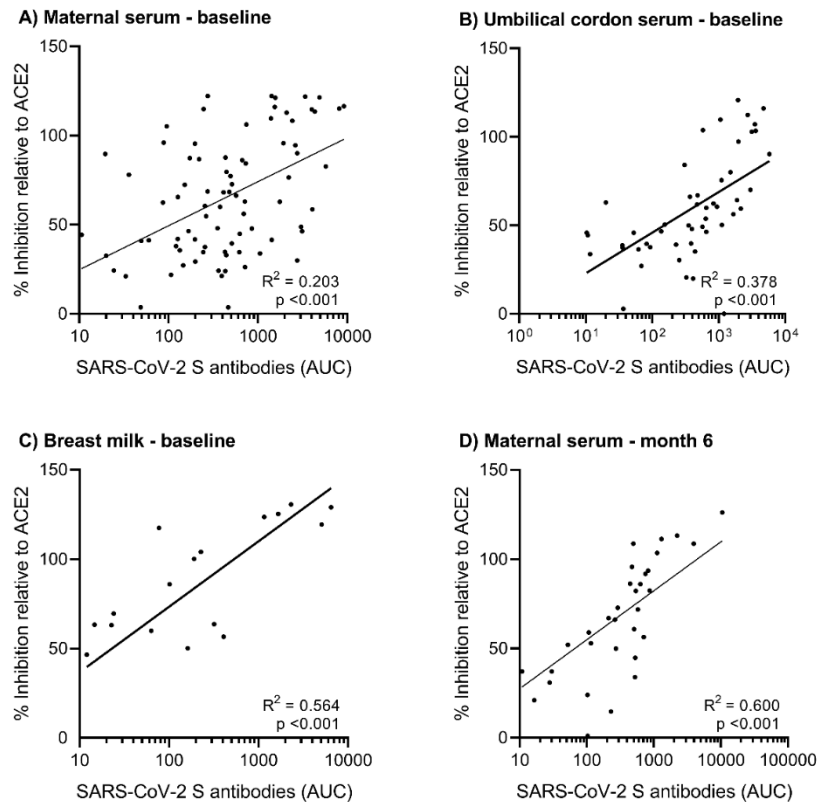
Figure 4. Correlation between antibody levels against SARS-CoV-2 S protein (sum of the AUC of IgG, IgM, and IgA) and percentages of inhibition of ACE2 receptor binding to the S protein. Antibodies at baseline against S protein in maternal serum (A), umbilical cord serum (B), breast milk (C), and maternal serum six months later (D) are represented. **Statistics**: Correlation analysis was performed using the Spearman test. **Abbreviations**: AUC, the area under the curve; ACE2, angiotensin-converting enzyme 2.

Discussion

This study analyzed the antibody levels against the SARS-CoV-2 S protein in pregnant women and neonates. The main findings were: 1) most pregnant women displayed high antibody levels in serum and milk; 2) IgG was transferred efficiently from mother to fetus, while the presence of IgM antibodies in the cord blood was exceptional; 3) symptomatic women had higher titers of antibodies than the asymptomatic ones; 4) IgG titers remained relatively high in mother serum after six months of delivery while they decreased drastically in children; 5) antibody titers correlated positively with its capacity to inhibit the binding of the ACE2 receptor to the S protein.

We observed high antibody levels against SARS-CoV-2 S protein in the serum and milk of pregnant women. However, according to a recent report, these levels may be lower than in nonpregnant women (22), although other studies have not confirmed this result (23). In line with previous findings, we also noted that antibody levels were higher in symptomatic women than in asymptomatic ones, indicating a correlation between COVID-19 severity and antibody titers (18, 24). Besides, antibody titers against SARS-CoV-2 S protein, primarily to the receptor-binding site, correlate with neutralization titers (13, 25, 26). According to this, we have seen a positive correlation between antibody titers by ELISA and inhibition of ACE2 binding to the S protein in pregnant women. This positive correlation was much better after six months of follow-up than at baseline, indicating antibody affinity maturation during this period.

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Neonatal protection from infections depends on transplacentally acquired IgG antibodies from the mother. We observed that the mean IgG ratio of umbilical cord serum to maternal serum was 1.4, indicating an efficient transplacental transfer of anti-S antibodies. This is in line with previous reports on SARS-CoV-2 and other pathogens (23, 27-30). The transfer ratio increases with increasing time between maternal infection and delivery, regardless of the severity of the disease (29). However, inefficient transplacental antibody transfer specific for SARS-CoV-2 has been observed in other studies (6, 17, 23). This may be due to perturbed Fc glycosylation of antibodies caused by SARS-CoV-2 infection. However, this effect was observed when SARS-CoV-2 infection occurred in the third trimester but not in the second trimester, suggesting that glycosylation alterations in anti-SARS-COV-2 specific antibodies normalize over infection time (23). Although most mothers in our study were diagnosed with SARS-CoV-2 in the third trimester, we cannot precisely define when they were infected. Despite this, we observed that most mothers with very low antibody levels were diagnosed with COVID-19 close to delivery (<30 days). Consequently, 13 out of 15 infants born to these mothers had undetectable IgG in the cord serum. Therefore, rather than prematurity or dysfunctional transplacental transport, the low levels of IgG antibodies in the mothers' serum seem to cause the non-detection of antibodies in their infants. Moreover, other factors, such as ethnicity and accompanying comorbidities, may affect the transplacental transfer of antibodies (17). Those conflicting results raise some concerns about the efficacy of vaccination during pregnancy. In this sense, efficient transplacental transfer of IgG after SARS-CoV-2 vaccination has been observed recently (31, 32), but it remains to be elucidated whether the antibody levels transferred to the fetus are high enough to protect against infection. However, even in the case of low transplacental antibody transfer, vaccination should be helpful to protect pregnant women, which are at risk for severe COVID-19 and related pregnancy complications.

IgM immunoglobulins cannot cross the placenta, and their presence in cord blood suggests fetus infections (5, 6). However, detection of IgM in the umbilical cord does not necessarily imply intrauterine infection since it may be due to assay errors, sample contamination, or increased permeability of the syncytiotrophoblast barrier by infection-induced inflammation (33). Thus, low IgM levels have been detected in the umbilical cord without fetal infection in some cases (6, 29, 34, 35). Likewise, there was no concordance between SARS-CoV-2 positive PCR of newborns and the presence of IgM antibodies in their umbilical cord in our study. Thus, three infants were PCR positive for SARS-CoV-2, but only two of them had specific anti-SARS-CoV-2 IgM antibodies in the cord serum. A similar interpretation can be made for the presence of IgA in the umbilical cord.

Anti-SARS-CoV-2 S protein IgM peak around 14 days after symptom onset and then decline. IgG titers peak around 21-28 days and remain high for several weeks (36-39). Similarly, we have found that anti-SARS-CoV-2 S protein IgM in mother serum decreased almost to undetectable levels in most cases at six months after delivery, while IgG and IgA levels remained high. On the contrary, IgG antibodies dropped drastically in children at that point, which agrees with previous reports (40, 41), indicating that potential IgG-mediated protection of neonates is limited in time.

Milk antibodies also may protect infants from infections via breastfeeding. Secretory IgA (sIgA) derives mainly from the gut-associated lymphoid tissue and is actively secreted into the milk via the polymeric immunoglobulin receptor (42). We have observed high antibody titers in most breast milk, principally IgA, that correlated with inhibition of ACE2 binding to the S protein, indicating neutralizing activity. A strong sIgA neutralizing response against SARS-CoV-2 S protein has also been described in milk from infected women (14-16). These results encourage breastfeeding of SARS-CoV-2 infected mothers to protect infants from COVID-19.

Finally, it should be noted that this study has a small sample size and, despite the study's prospective design, biases that are difficult to control may have been introduced. Besides, some samples have been lost at six months, although these are complicated samples to obtain. Additionally, a control group of nonpregnant women was not included.

In conclusion, high antibody titers against SARS-CoV-2 S protein were found in most pregnant women, and fetus infection, if any, was rare. Due to the efficient transfer of IgG to cord blood and high IgA titers in breast milk, neonates may be passively immunized to SARS-CoV-2 infection. Our findings could guide newborn management and maternal vaccination policies (43).

Declarations

Ethics approval and consent to participate
The study was approved by the HGUGM Ethics Committee (Ref.: IRB 00006051) and was conducted following the Declaration of Helsinki. All participants gave their informed consent before enrollment.

Consent for publication
Not applicable

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Author contributions
Data curation: MMV, IC, MJMG, AHL, SVV, LTD, MMC, RA, and IM.

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Critical revision of the article: MMV, DSC, and MLN.

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Supplementary file 1

Cloning of the S protein and receptor domains

The plasmid pαH coding for the S protein ectodomain (residues 1-1208) of SARS-CoV-2 2019-nCOV (GenBank: MN908947) stabilized in the prefusion conformation was kindly provided by Dr. Jason McLellan (the University of Texas at Austin-USA) [1]. Mutagenesis was carried out to obtain a HexaPro construct that allowed a high-yield production of a stabilized prefusion spike protein [2]. The following substitutions were included at the ectodomain: glycine at residue 614 (D614G), a "GSAS" substitution at the furin cleavage site (residues 682–685), and proline at residues 817, 892, 899, 942, 986, and 987. For trimerization and purification, the C-terminal end of the S protein ectodomain was fused to the T4 fibritin trimerization motif (foldon), an HRV3C protease cleavage site, and an 8XHisTag.

As a control, a locked closed conformation of S, unable to bind the ACE2 receptor, was also made by introducing in the HexaPro backbone a double cysteine mutant, S383C D985C [3].

Finally, a plasmid coding for the residues 1-165 of ACE2, the cell receptor for the SARS-CoV-2 S protein, fused to a StrepTag was also built.

Expression and purification of soluble domains of the S protein and its receptor

The expression vector coding for the ectodomain of the SARS-CoV-2 S protein was used to transiently transfect FreeStyle 293F cells (Life Technologies, Carlsbad, CA, USA) using polyethyleneimine. The S protein domain was purified from filtered cell supernatants using Ni-NTA resins (Cityva, Uppsala, Sweden). Following a wash with 20 mM Na_2HPO_4 7.4, 200 mM NaCl 20mM Imidazole, protein elution was performed using a gradient from 0 (wash buffer) to 100% 20 mM Na_2HPO_4 7.4, 200 mM NaCl 300mM Imidazole. Fractions collected were concentrated with Amicon (Millipore) and exchanged to buffer (20 mM Na_2HPO_4 7.4, 200 mM NaCl) without imidazole before being loaded onto a Superose 6 10/300 gel filtration column (Cytyva, Uppasala, Sweden), equilibrated and eluted with the same buffer. Finally, protein purity and integrity were checked by SDS-PAGE and Coomassie-blue staining under reducing conditions (Supplementary figure 1).
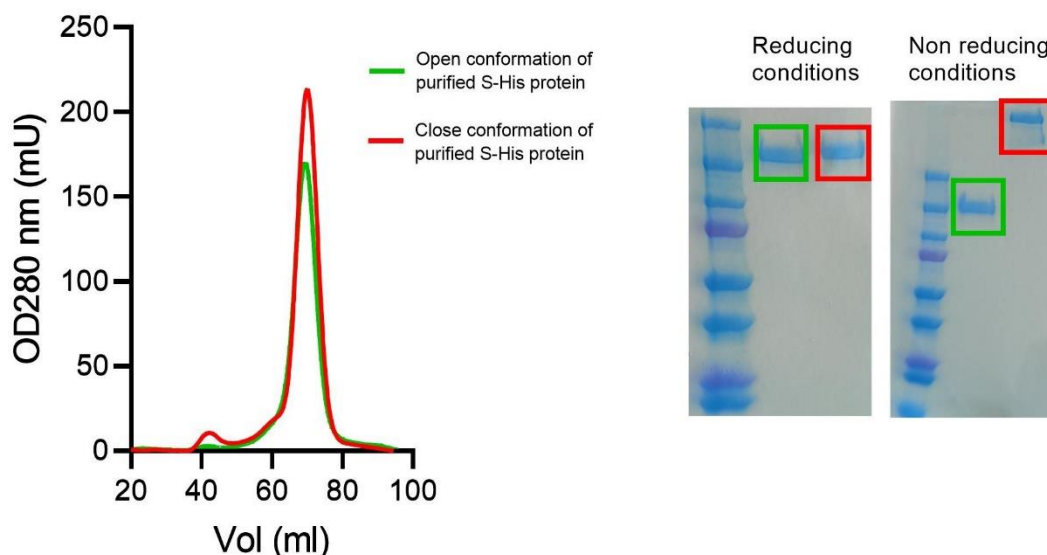
Supplementary figure 1: Gel filtration trace of S protein construct. Inset shows a Coomassie blue-stained SDS-PAGE for the major peak (trimer) for each protein of the chromatogram run under reducing and non reducing conditions.

The ACE2 receptor was expressed similarly and purified by the Insights into the Strep-tag system (IBA Lifesciences, Gottingen, Germany), followed by Superdex 200 (Cytiva, Uppsala, Sweden).

Antibody titration

Antibody titers quantification against the S protein were determined by incubating serial dilutions of serum samples (starting at a 1:50 dilution) with the purified S protein ectodomain. Ninety-six well plates were coated with 200 ng per well of the S protein ectodomain. The following day, serum samples were added, and the binding to the S protein was determined by successive incubations with a secondary peroxidase-conjugated anti-human IgG, IgM, or IgA (Jackson Immunoresearch, West Grove, PA, USA) and the OPD substrate (Sigma Aldrich, San Luis, MO, USA). After each step, extensive washing was done, and optical density (OD) was read at 493 nm. After subtracting the background for each sample (OD from non-coated wells and incubated with the lower serum dilution), values were fitted to a one-phase exponential decay least-squares curve, and the area under the curve (AUC) was calculated using GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA) (peaks less than 10% of the distance from minimum to maximum Y were ignored; all peaks must go above the baseline). The AUC is expressed as X units times the Y units. A positive control from the sera of patients with previous SARS-CoV-2 infection was used to normalize the results.

ACE2 binding inhibition assay

An ELISA binding inhibition assay of soluble ACE2 receptor to the SARS-CoV-2 S protein was performed as follows: Fifty nanograms per well of the S protein ectodomain were captured by a chimeric version of a monoclonal anti-Foldon antibody [4] previously coated in 96 well plates. Next, a 1:10 dilution of the different serum samples was added, and plates were incubated for 45 min at room temperature (RT). Then, one µg of the cell receptor ACE2 complexed with StrepTactin-peroxidase (Bio-Rad; Halle, Germany) was added to each well, incubated for 15 min at RT, revealed with the OPD substrate (Sigma Aldrich, San Luis, MO, USA), and the OD_{493} was measured in a spectrophotometer. The assay background was determined in parallel plates coated with a locked closed conformation of the S protein unable to bind the ACE2 receptor. A pool of sera from individuals negative for anti-S antibodies, collected in 2016, was used as a control. After subtraction of the background, the percentage of inhibition was calculated as [1- (OD_{493} test serum / OD_{493} control serum)] x 100 %. The percentage of inhibition relative to ACE2 in which the StrepTag was removed was further calculated as (% inhibition of serum samples / % inhibition of ACE2) x 100 %.

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