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Pregnancy alters IL-1β expression and anti-viral antibody responses during SARS-CoV-2 infection

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Condensation: Pregnant women exhibit an early inflammatory response and a reduced antiviral antibody response against SARS-CoV-2 as compared with non-pregnant women.

Short Title: COVID-19 and Pregnancy

AJOG at a Glance:

A. Why was the study conducted?

- Inflammatory and humoral responses during SARS-CoV-2 infection of pregnant women have not been extensively evaluated.

B. What are the key findings?

- Pregnant women who delivered <14 days after positive SARS-CoV-2 test expressed more IL1β mRNA in their blood compared to pregnant women who were uninfected or delivered >14 days after a confirmed test.
- Pregnant women with confirmed infection had lower anti-spike-receptor binding domain IgG titers and were less likely to have detectable neutralizing antibodies compared to non-pregnant women.
- Protein concentrations of placental FcRn, a receptor essential for maternal transfer of antibodies to the fetus were not affected by SARS-CoV-2 infection during pregnancy.

C. What does this study add to what is already known?

- Our results demonstrate potential differences in the pathogenesis of SARS-CoV-2 between pregnant and non-pregnant women, including inflammatory and antibody responses to the virus.

Key Words: SARS-CoV-2, COVID-19, pregnancy, maternal infection, antibody, cytokine

Abstract

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the disease-causing pathogen of the COVID-19 pandemic, has resulted in morbidity and mortality worldwide. Pregnant women are more susceptible to severe COVID-19 disease and are at higher risk for preterm birth compared to uninfected pregnant women. Despite this evidence, the immunological effects of SARS-CoV-2 infection during pregnancy remain understudied.

Objective: To assess the impact of SARS-CoV-2 infection during pregnancy on inflammatory and humoral responses in maternal and fetal samples and compare antibody responses to SARS-CoV-2 among pregnant and non-pregnant women.

Study Design: Immune responses to SARS-CoV-2 were analyzed using samples from pregnant (n=33) and non-pregnant (n=17) women who had either tested positive (pregnant n=22; non-pregnant n=17) or negative for SARS-CoV-2 (pregnant n=11) at Johns Hopkins Hospital. We measured proinflammatory and placental cytokine mRNAs, neonatal Fc receptor (FcRn) expression, and tetanus antibody transfer in maternal and cord blood samples. Additionally, we evaluated anti-spike (S) IgG, anti-S-receptor binding domain (RBD) IgG, and neutralizing antibody (nAb) responses to SARS-CoV-2 in serum or plasma collected from non-pregnant women, pregnant women, and cord blood.

Results: SARS-COV-2 positive pregnant women expressed more *IL1β*, but not *IL6*, in blood samples collected within 14 days versus > 14 days after a confirmed SARS-CoV-2 test. Pregnant women with confirmed SARS-CoV-2 infection also had reduced anti-S-RBD IgG titers and were less likely to have detectable nAb as compared with non-pregnant women. Although SARS-CoV-2 infection did not disrupt FcRn expression in the placenta, maternal transfer of SARS-CoV-2 nAb was inhibited by infection during pregnancy.

Conclusions: SARS-CoV-2 infection during pregnancy was characterized by placental inflammation and reduced antiviral antibody responses, which may impact the efficacy of

COVID-19 therapeutics in pregnancy. The long-term implications of placental inflammation for neonatal health also requires greater consideration.

Introduction

The ongoing coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in over 75 million infections and over 1.5 million deaths worldwide, as of December 2020[1]. Despite global efforts to characterize the pathogenesis of SARS-CoV-2 infection, the effects of infection on immunity during pregnancy remain undefined. Due to pregnancy-associated immune and endocrine fluctuations, pregnant women and their fetuses are at greater risk for severe complications caused by infectious diseases[2]. Most pregnant women with COVID-19 are asymptomatic or experience mild disease. The U.S. Center for Disease Control (CDC), however, reports that one in four women, aged 15–49 years, hospitalized for COVID-19 during March 1–August 22, 2020 were pregnant, and these women were more likely to require mechanical ventilation compared to nonpregnant women[3]. Pregnant women also are at increased risk of mortality following SARS-CoV-2 infection[4], prompting the CDC to revise their guidelines and include pregnant women as an at risk population for severe COVID-19 disease. SARS-CoV-2 surveillance of pregnant women in Washington state further reveals greater morbidity and mortality in pregnant women with SARS-CoV-2 infection, and suggests possible underreporting in nationwide surveillance data[5]. In addition to maternal morbidity and mortality, the CDC reports that women infected with SARS-CoV-2 during pregnancy are at higher risk for preterm birth[6]. Because maternal immune activation can be associated with adverse fetal outcomes, including preterm birth [7,8], it is possible that SARS-CoV-2 during pregnancy may have detrimental effects on the developing fetus.

During pregnancy, a typical inflammatory response to pathogens includes the secretion of proinflammatory cytokines, such as IL-1β and IL-6, not only at the site of infection but in the placenta as well; these cytokines can readily enter the amniotic cavity and interfere with normal fetal development[7–9]. Thus, even in the absence of severe maternal symptoms or fetal viral infection, the maternal immune response to SARS-CoV-2 could lead to short and long-term

consequences in the fetus and neonate[2,10–12]. At the same time, the maternal immune response can also have a protective effect on neonatal health, including the placental Fc receptor (FcRn)-mediated transfer of SARS-CoV-2-specific antibodies transplacentally[13,14].

In the present study, we investigated the inflammatory and humoral responses to SARS-CoV-2 using maternal blood, cord blood, and placenta samples collected from pregnant women who had either tested positive or negative for SARS-CoV-2 prior to admission and delivery at the Johns Hopkins Hospital (JHH). We measured maternal and cord blood serum or plasma anti-spike (S) and anti-S-receptor binding domain (RBD) IgG and neutralizing antibody (nAb) responses to SARS-CoV-2, whole blood proinflammatory cytokine mRNA expression, as well as placental cytokine and FcRn expression. Furthermore, we compared antibody responses to an outpatient non-pregnant cohort of women with confirmed COVID-19.

Materials and Methods

Study Participants, sample collection, and storage

Pregnancy Cohort. Pregnant women were recruited by convenience sampling through Johns Hopkins Hospital outpatient obstetric clinics and the JHH Labor & Delivery unit prior, or after delivery of the patient. We utilized discarded maternal blood, discarded neonatal cord blood, and a small placental sample collected during admission for delivery. Patients were contacted, informed of the study, and consented by phone to decrease face to face exposure due to concern of SARS-CoV-2 spread/infection. Basic demographic information and clinical data, including info on the history of SARS-CoV-2 testing (usually via the POCT nasopharyngeal swab) was collected from the patient's medical record. Blood samples were collected into gold top SST tubes and purple top EDTA tubes. The top SST tubes were inverted several times, before being centrifuged for 10 minutes at 3000 rpm at 22°C. Then, both maternal and cord whole blood and serum samples were aliquoted and stored at -80°C. Placental samples were collected after delivery and were not treated with any preservatives or reagents. Samples were

processed using two different methods for both the maternal and fetal sides; placental tissue was either frozen at -80°C immediately or was placed in RNAlater for 48 hours prior to -80°C storage. To obtain tissue that was representative of the placental sample, half thickness samples using a disc tissue punch were taken from two different locations on each side of the placenta. Thus, ultimately, each method of processing placental tissue had two tissue punches from different locations on a given side of the placenta.

Non-Pregnant Cohort. A convenience sample of non-hospitalized participants were recruited and provided informed consent by phone between April 21 and August 13, 2020 after receiving a positive SARS-CoV-2 RT-PCR test from an outpatient or emergency department facility within the Johns Hopkins Health Sytem[15]. One participant requested participation in the study via the Johns Hopkins HOPE (Hopkins Opportunities for Participant Engagement) COVID-19 registry. Samples from adult women of reproductive age, 18-49 years[16], with positive RT-PCR results for SARS-CoV-2 were included in this study. Basic demographic information and clinical data, including that regarding the history of SARS-CoV-2 testing, was collected from the patient and the patient's medical record. Participants in this study attended a research clinic visit on average 42.2 days after COVID-19 symptom onset (range 29-92 days), at which blood was drawn. Approximately 25 ml of whole blood was collected in Acid Citrate Dextrose glass tubes. Peripheral blood mononuclear cells were separated, and the remaining plasma was stored in 1 ml aliquots at -80^{o}C. Plasma was defrosted and then heat inactivated at 56^{o}C for 30 minutes prior to serologic assays. The study was approved by the Johns Hopkins School of Medicine Institutional Review Board.

Gene Expression Analysis

Total RNA was extracted from placental tissue samples using the RNeasy Plus Mini Kit (Qiagen) or from whole blood using NucleoSpin RNA Blood Kit (Macherey-Nagel).

Complementary (c) DNA synthesis in a 40-µL reaction was performed using Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad). TaqMan® (Thermo Fisher Scientific) mRNA assays were run for analysis. The primers used were *IL-1β* (Table 1; Integrated DNA Technologies) and *IL-6* (Table 1; Integrated DNA Technologies). mRNA expression was calculated relative to housekeeping genes: 18S (Hs99999901_s1; Applied Biosystems) and Actin (Table 1; Integrated DNA Technologies).

Indirect enzyme-linked immunosorbent assays (ELISAs)

The protocol was adapted from a published protocol from Dr. Florian Krammer's laboratory[17], as described in Klein et al., 2020[18]. Briefly, ninety-six-well plates (Immulon 4HBX, Thermo Fisher Scientific) were coated with either full-length S protein or S-RBD at 4oC overnight. Coating buffer was removed, and plates were washed and then blocked for 1 hour at room temperature. All plasma samples were heat inactivated at 56oC on a heating block for 1 hour before use. Negative control samples were prepared at 1:10 dilutions and plated at a final concentration of 1:100. A mAb against the SARS– CoV-2 S protein was used as a positive control (1:5000; catalog 40150-D001, Sino Biological). For serial dilutions of plasma on either S- or S-RBD–coated plates, plasma samples were prepared in 3-fold serial dilutions starting at 1:20. Blocking solution was removed, and 10 µL diluted plasma was added in duplicate to the plates and incubated at room temperature for 2 hours. Plates were washed 3 times with PBST wash buffer, and 50 µL secondary antibody was added to the plates and incubated at room temperature for 1 hour (Fc-specific total IgG HRP 1:5000 dilution, catalog A18823, Invitrogen, Thermo Fisher Scientific). Plates were washed and all residual liquid removed before addition of 100 µL SIGMAFAST OPD (o phenylenediamine dihydrochloride) solution (MilliporeSigma) to each well, followed by incubation in darkness at room temperature for 10 minutes. To stop the reaction, 50 µL 3M HCl (Thermo Fisher Scientific) was added to each well. The OD of each plate was read at 490 nm (OD490) on a SpectraMax i3 ELISA Plate Reader (BioTek Instruments). The positive

cutoff value for each plate was calculated by summing the average of the negative values and 3 times the SD of the negatives. All values at or above the cutoff value were considered positive.

Microneutralization assay

The plasma neutralizing antibody (nAb) protocol was adapted from Dr. Andrew Pekosz's laboratory[19], as described in Klein et al., 2020[18]. Briefly, infectious virus (SARS-CoV-2/USA-WA1/2020) was added to two-fold diluted plasma at a final concentration of 1 × 104 TCID50/mL (100 TCID50 per 100 µL). Samples were added to VeroE6-TMPRSS2 cells in sextuplet for 6 hours at 37˚C. The inocula were removed, fresh IM was added, and the plates were incubated at 37˚C for 2 days. Cells were fixed by the addition of 150 µL of 4% formaldehyde per well, incubated for at least 4 hours at room temperature, and then stained with Napthol Blue Black (MilliporeSigma). The nAb titer was calculated as the highest serum dilution that eliminated the cytopathic effect in 50% of the wells.

Western blot

Western blotting was used to measure the protein expression of FcRn in placenta. To prepare tissue lysate, tissue was homogenized on ice in RIPA lysis buffer (Sigma‐Aldrich) with proteinase inhibitor (Sigma‐Aldrich) and phosphatase inhibitor cocktail 2 (Sigma‐Aldrich). The homogenized specimens were then placed on ice for 15 min and centrifuged at 14,000 rpm for 20 min at 4°C. The resulting supernatants were collected for further experiments. Total protein was separated by sodium dodecyl sulfate‐polyacrylamide gel electrophoresis (SDS‐PAGE, Bio‐Rad) using 4%–15% gels (Bio‐Rad), and then, transferred onto nitrocellulose membranes (Bio‐Rad) using semidry transfer device (Trans‐Blot® Turbo™, Bio‐Rad). Membranes were blocked with 5% of bovine serum albumin (BSA, Sigma‐Aldrich) in Tris‐buffered saline (Corning) plus 0.1% of Tween‐20 (Sigma‐Aldrich) (TBST) for 15 min at room temperature and incubated with

primary antibodies in 5% of BSA at 4°C overnight, then, washed using TBST. FcRn antibody (1:1000, Santa Cruz) and GAPDH (control marker, 1:1000, Abcam) were used for primary antibodies. ECL (GE Healthcare) was used for detection using the ImageQuant LAS 500 (GE Healthcare), and densitometric analysis was performed using ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Statistical Analysis

Descriptive statistics stratified by pregnancy state (SARS-CoV-2 positive pregnant, SARS-CoV-2 positive non-pregnant) are presented as medians and IQRs. Comparisons of demographic characteristics were tested via exact Wilcoxon two-sample test, Pearson's chi-squared test, or Fisher's exact test, where appropriate dependent on variable structure as continuous, binary, or categorical and sample size within individual cells. Prior to conducting any inferential statistics, AUC values for anti-S IgG and anti-S-RBD IgG titers were computed by plotting normalized OD values against sample dilution for ELISAs. The AUC for microneutralization assays used the exact number of wells protected from infection at each plasma dilution. For each assay, samples with titers below the limit of detection were assigned an AUC value of half of the lowest measured AUC value. Due to the non-normal distribution of cytokine and antibody data, comparisons between SARS-CoV-2 positive pregnant and SARS-CoV-2 positive non-pregnant women were examined via exact Wilcoxon two-sample tests. Correlations between antibody isotypes and assays with days since the initial SARS-CoV-2 positive test or days since symptom onset were assessed using the Spearman correlation coefficient. The data were then log transformed for visualization. Finally, a generalized linear model was used to determine if the association between days since the initial SARS-CoV-2 positive test or days since symptom onset and antibody responses differed by pregnancy status (pregnant, non-pregnant). All analyses were two-tailed tests with a significance threshold of $p < 0.05$.

Results

Cohorts. Two cohorts were included in this study: the pregnant cohort, consisting of 33 pregnant women who either tested positive (n=22) or negative (n=11) for SARS-CoV-2 prior to delivery (in inpatient or Labor and Delivery settings) at the JHH, and the non-pregnant cohort, consisting of women within reproductive age (18-48 years of age), as defined by WHO [16] (n=17) who tested positive for SARS-CoV-2 at an outpatient clinical testing site within the JHH Health System. Comparing demographic characteristics between SARS-CoV-2 positive and negative pregnant women revealed differences in maternal age at delivery, race, and ethnicity. SARS-CoV-2 positive pregnant women gave birth at a younger age (median=27; IQR 23-34) compared to SARS-CoV-2 negative pregnant women (median=32; IQR 29-35) (p<0.05, Table 2), were more likely to identify as Other (63.64%) or Black/African American (22.73%) than SARS-CoV-2 negative pregnant women (p<0.001, Table 2), and were more likely to identify as being of Hispanic/Latina ethnicity (50%) (p<0.05; Table 2). No significant differences were found between SARS-CoV-2 positive and negative pregnant participants in pre-pregnancy BMI, BMI at delivery, gestational age at birth, neonate late-onset sepsis, chorioamnionitis, gestational nicotine use, time between membrane rupture and delivery, preeclampsia, gestational diabetes, gestational hypertension, delivery type (cesarean vs vaginal), size of neonate, sex of neonate, NICU stay, or neonatal readmission (Table 3). In comparing SARS-CoV-2 positive pregnant women and non-pregnant women, pregnant women were younger (pregnant median age =27 IQR 23-34; non-pregnant median age=34 IQR 28-41) (p<0.05; Table 2), less likely to identify as White/Caucasian (14% vs. 47%) (p<0.05; Table 2), and more likely to identify as Hispanic or Latina (50% vs. 6%) (p<0.05; Table 2) than non-pregnant women.

Cytokine expression after SARS-CoV-2 infection during pregnancy. Increased inflammation caused by infection during pregnancy can be detrimental for long-term fetal and neonatal outcomes[2,12,20]. We assayed cytokine mRNA expression during SARS-CoV-2 infection as a biomarker for inflammation. Because IL-1β activation during pregnancy can cause adverse fetal

outcomes[2,21,22], we measured *IL1β* mRNA expression in maternal blood (total, n=27; positive, n=18; negative, n=9), cord blood (total, n=29; positive, n=20; negative, n=9), and the maternal (total, n=11; positive, n=8; negative, n=1) and fetal (total, n=26; positive, n=19; negative, n=7) sides of placentas, which did not differ between SARS-CoV-2 positive and negative pregnant women (Figure 1A-D). To assess whether the expression of *IL1β* differed depending on the number of days between a pregnant woman's PCR test and blood sample collection, maternal blood *IL1β* mRNA expression was compared based on the time window between diagnosis and blood collection (total, n=27; positive, n=18; negative, n=9). Day 14 was chosen for analysis based on the incubation period of SARS-CoV-2, which extends to 14 days after symptom onset[23]. *IL1β* expression in maternal blood was higher in samples collected within 14 days of a positive SARS-CoV-2 test compared with samples collected > 14 days after test, representative of an acute, as opposed to chronic, inflammatory response ($p<0.05$; Figure 1E).

We measured *IL6* mRNA expression in maternal and fetal blood and tissue from our SARS-CoV-2 positive and negative pregnant cohort. It is important to note that all SARS-CoV-2 positive women experienced mild to moderate disease from SARS-CoV-2 infection. In contrast to the elevation observed among severe COVID-19 cases in non-pregnant individuals[24–26], there was no change in the expression of *IL6* in blood or placentas based on SARS-CoV-2 infection status (Figure 2A-D) or duration of time between a positive SARS-CoV-2 test and sample collection (Figure 2E). These data provide evidence that *IL1β* mRNA, in particular, is upregulated early after infection and on the fetal side of the placenta in non-severely ill pregnant women with SARS-CoV-2 infection.

Antibody responses to SARS-CoV-2 in pregnant and non-pregnant women. To evaluate the impact of pregnancy on humoral responses to SARS-CoV-2, antibody responses measured in serum or plasma samples were collected at a median of 34 (IQR: 31.5 – 40) days since confirmed infection, from pregnant (18.91$\pm$29.57 days post confirmed infection) (n=17) and non-

pregnant (37.29±12.66 days post confirmed infection) (n=17) women who tested positive for SARS-CoV-2. Pregnant and non-pregnant women showed similar titration of IgG (i.e., area under the curve [AUC]) recognizing the full-length SARS-CoV-2 spike (S) protein (**Figure 3A**). In contrast, pregnant women had significantly lower anti-S-RBD IgG titers than non-pregnant women ($p<0.05$, **Figure 3B**). Titers of nAb, however, which correlate with anti-S-RBD antibodies[27], were measured and were not significantly different between pregnant and non-pregnant women (**Figure 3C**). We observed, however, that significantly fewer pregnant women (8/17) had detectable nAb titers (i.e., ≥ 1:20 titer) compared with non-pregnant women (16/17) ($p<0.05$; **Figure 3C**), indicating reduced production of neutralizing antibodies in a subset of pregnant women.

To further explore how pregnancy altered the relationship between anti-S-RBD IgG and nAb, titers were directly compared and revealed that anti-S-RBD IgG titers were higher than nAb titers in both pregnant and non-pregnant women ($p<0.001$ **Figure 4A,B**). Among pregnant women only, a dichotomy in nAb titers was evident. Consistent with this observation, pregnant women with low nAb titers <1:20 (i.e., no detectable nAb) also had lower anti-S-RBD IgG titers ($r= 0.9023$, $p<0.001$). Further, pregnant women with <1:20 nAb titers had significantly lower anti-S-RBD IgG responses than pregnant women with nAb titers >1:20 ($p<0.05$; **Figure 4A**)**.** To determine whether time since a SARS-CoV-2 positive test or time since symptom onset could predict antibody responses, we analyzed responses over time. Variation in anti-S-RBD IgG or nAb responses among pregnant women with non-detectable as compared with detectable nAb titers could not be explained by the length of time since a positive SARS-CoV-2 positive test (**Figure 4C,D**). Furthermore, time since symptom onset did not explain variation in anti-S-RBD IgG or nAb responses among pregnant women with non-detectable as compared with detectable nAb titers (**Figure 4E,F**). Differences in the number of days between a PCR+ test or symptom onset and sample collection also did not statistically explain variation in either anti-S-

RBD IgG or nAb responses between pregnant and non-pregnant women (**Figure 4C-F**). These data suggest that, independent of time, pregnancy may reduce the quality of antiviral antibodies against SARS-CoV-2; (pregnant, n=17; non-pregnant, n=17).

Antibody transfer in SARS-CoV-2 infection. To assess whether antibody transfer from mother to fetus was broadly affected by SARS-CoV-2 infection, SARS-CoV-2-specific antibody levels in maternal (n=17) and cord blood (n=17) serum, FcRn expression, and anti-tetanus IgG titers were assessed in SARS-CoV-2 positive (n=22) and negative women (n=11). Anti-S and anti-S-RBD IgG titers did not differ between maternal and cord blood serum samples (**Figure 5A,B**); titers of nAb in maternal serum, however, were significantly greater than in cord blood serum ($p < 0.05$; **Figure 5C**). Semi-quantitative protein concentrations of placental FcRn, used as a biomarker of IgG transfer, were not affected by SARS-CoV-2 infection during pregnancy **(Figure 5D)**. To further evaluate whether SARS-CoV-2 infection altered the transfer of other antibodies from mother to fetus, maternal and cord blood serum anti-tetanus IgG titers were measured and were not inhibited by SARS-CoV-2 infection during pregnancy **(Figure 5E,F).** These data suggest that while maternal transfer of SARS-CoV-2-specific nAb may be reduced, SARS-CoV-2 infection does not impact semi-quantitative protein concentrations of placental FcRn or maternal transfer of anti-tetanus IgG.

Structured Discussion

1. Principal Findings

Our study provides preliminary evidence that pregnant women exhibit an inflammatory response in maternal blood within 14 days of a PCR+ test, exhibit lower anti-S-RBD IgG titers, and are less likely to have detectable nAb compared to non-pregnant women. Protein concentrations of placental FcRn, a receptor essential for maternal transfer of antibodies to the fetus were not affected by SARS-CoV-2 infection during pregnancy; reduced nAb responses

against SARS-CoV-2, however, were detected in cord blood. These results suggest that during pregnancy there is an acute increase in *IL-1β* mRNA expression and reduced antiviral antibody responses during SARS-CoV-2 infection.

2. Results

The inflammatory response of pregnant women who experienced mild to moderate COVID-19 was characterized by greater *IL-1β*, but not *IL-6,* mRNA expression as has been reported in severe male and non-pregnant female COVID-19 patients[25,26]. Current studies highlight differences in clinical manifestations between SARS-CoV-2 positive pregnant and non-pregnant women, with some studies reporting differences in presenting symptoms, such as lower incidence of fever and cough in pregnant women[28,29]. There is growing evidence that SARS-CoV-2 infected pregnant women face greater risk of hospitalization, intensive care unit admission, invasive ventilation, and death compared to non-pregnant women[4,5,30]. Studies in SARS-CoV-2 positive pregnant and non-pregnant women report higher frequencies of neutrophils and D-dimer concentrations and lower percentages of lymphocytes, CD4+/CD8+ ratios, and IgG levels in pregnant than non-pregnant women infected with SARS-CoV-2[31–34]. Thus, our study adds to the growing literature demonstrating enhanced inflammatory responses and reduced humoral responses during SARS-CoV-2 infection of pregnant compared to non-pregnant women.

The antiviral response to SARS-CoV-2 includes development of antibodies that recognize the S-RBD as well as neutralize virus[35]. Detection of anti-SARS-CoV-2 IgG antibodies in maternal and neonatal blood following infection has been reported[36–40]; how pregnancy status, however, affect detection (qualitative) and titers (quantitative) of both anti-SARS-CoV-2 IgG and nAb responses has not been previously investigated. Here, we demonstrate that pregnant women infected with SARS-CoV-2 had lower titers of anti-S-RBD IgG compared to non-pregnant women. Although nAb titers were similar between pregnant and non-pregnant women, pregnant women were significantly less likely to have detectable nAb responses.

Furthermore, SARS-CoV-2 infected pregnant women who had non-detectable nAb responses had significantly lower anti-S-RBD IgG titers. Reduced antiviral antibody responses in pregnant women infected with SARS-CoV-2 were independent of time since infection. Other longitudinal studies evaluating antibody responses across gestational timepoints illustrate that neutralizing antibody is detectable in only 52.9% of SARS-CoV-2 positive pregnant women, with no changes over gestation; thus, reduced nAb titers in a subset of pregnant women is independent of time since infection[40]. Furthermore, pregnant women with low antibody titers do not present with worse symptoms or experience worse disease outcomes, similar to studies in non-pregnant adults[41,42]. We hypothesize that reduced antiviral antibody titers could increase the potential for reinfection following pregnancy, especially to variant viruses. While we observed reduced titers of anti-S-RBD IgG in pregnant compared with non-pregnant women, other studies report no difference in anti-S-RBD IgG titers between pregnant and non-pregnant women[43]. Without complete details about how assays are standardized, it is difficult to compare results. The serological assays used in this study have been well-characterized and validated[17,18,44]. It is well-established that nAb titers are correlated with anti-S-RBD titers in nonpregnant individuals[18]. Our observation that nAb titers and anti-S-RBD titers are correlated not only in nonpregnant, but in pregnant women is clinically novel and adds to the growing literature in this field.

Despite reduced SARS-CoV-2 nAb titers in cord blood, semi-quantitative protein concentrations of placental FcRn, responsible for placental IgG transfer, were not affected by SARS-CoV-2 infection during pregnancy. Similar results have been found in other cohorts, in which reduced SARS-CoV-2-specific placental antibody transfer is observed in infected pregnant women, without differences in overall placental FcRn expression between SARS-CoV-2 positive and negative pregnant women being reported[38,43]. The Fc-glycosylation in the third trimester of SARS-CoV-2 positive women was also perturbed, which could impact the transfer of SARS-CoV-2-specific antiboides[43]. In addition to tetanus-specific antibodies, influenza and

pertussis-specific antibody transfer was not affected by SARS-CoV-2 infection[43]. Overall, these results reiterate that non-SARS-CoV-2-specific antibody transfer is intact in SARS-CoV-2 positive women, but that SARS-CoV-2-specific antibody transfer mechanisms may be compromised by infection.

3. Clinical Implications

These preliminary observations suggest that pregnant women who are infected with SARS-CoV-2 may have an altered cytokine and humoral response compared to non-pregnant women, which must be verified in a larger clinical cohort. Specifically, we report reduced anti-S-RBD IgG responses and a reduction of nAb production in a subset of pregnant women, suggesting that humoral immunity to SARS-CoV-2 infection during pregnancy is reduced as compared to non-pregnant individuals. Increased cytokine activation at the maternal-fetal interface can have adverse implications for the developing fetus[45]; therefore, children born to mothers infected with SARS-CoV-2 during pregnancy should be longitudinally observed to assess long-term outcomes. With mRNA-based vaccines against SARS-CoV-2 now available, the unique biological state of pregnancy needs be considered[46]. None of the SARS-CoV-2 vaccine candidates included pregnant women in their Phase III trials. The CDC acknowledges the lack of data for vaccine efficacy in pregnant women and urges women to consult with their healthcare provider prior to vaccination[47]. This is a real burden to place on pregnant women. We urge greater use of animal models to assess the immunogenicity and reactogenicity of the approved SARS-CoV-2 vaccine platforms to provide some indication of how pregnancy may or may not alter responses, adverse reactions, and protection from infection and disease[46].

4. Limitations

Limitations of this study include the small sample size as well as significant differences in age, race, and ethnicity between SARS-CoV-2-infected pregnant and non-pregnant women. These differences are attributable to our reliance on convenience sampling and are a result of differences in participant recruitment, in which sample collection from pregnant women was

based on time of delivery, and sample collection from non-pregnant women was based on symptom presentation. While there was a significant difference in age between the cohorts, all women in this study were within reproductive ages[16]. Due to our inability to know precisely when each participant was infected with SARS-CoV-2, we used the number of days between a SARS-CoV-2 PCR test and blood collection as the metric to assess cytokine responses, and additionally used the number of days since symptom onset to evaluate humoral responses over time. These metrics may not accurately represent the time since initial infection, as symptom onset is self-reported and studies have reported PCR positivity for extended periods of time past the initial infection[48,49]. Further, differences in blood volume between individuals, and throughout gestation in pregnant women could lead to variability in antibody titers.

5. Conclusions

Our results demonstrate potential differences in the pathogenesis of SARS-CoV-2, including inflammatory and antibody responses to the virus, between pregnant and non-pregnant women. It is well-established that immune responses change dramatically during pregnancy in order to accommodate the developing fetus[50]. Therefore, understanding the impact of SARS-CoV-2 infection during pregnancy on the maternal immune system, and how these changes alter maternal and fetal susceptibility to disease is crucial for the development of vaccines and other therapeutics for COVID-19. In addition to further investigations of short- and long-term consequences of SARS-CoV-2 infection in pregnancy, the safety, immunogenicity, and efficacy of SARS-CoV-2 vaccines in pregnant women must be considered.

Contributions. IB, SK, AP, JSS, AJS, WCG, and KJ-B conceived of the study and experimental questions, TB, RR, AARA, and YCM collected and provided samples, MJ, TB, DMB, SNW, and RR obtained and organized clinical data, MLS, JL, PC, KL, AP, H-SP, RLU, and AG processed blood samples, MLS, PC, JL, KV, and SO analyzed and graphed data, MLS, MJ, IB, and SK wrote the manuscript, all authors reviewed, edited, and approved the final submission.

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614

615 **Table 2.**

633 **Table 3.**

Pregnant Female Cohort

634

635 **Figure Legends**

636 **Figure 1: *IL1β* expression in maternal and fetal samples.** Maternal and fetal blood and
637 placentas were used to detect IL1β gene expression relative to the housekeeping genes (HKG),
638 18S and ACTB. (**A-D**) Maternal blood, cord blood, and maternal and fetal side placental IL1β
639 expression between SARS-CoV-2 positive (P(+)) and negative (P(-)) samples in the pregnant
640 cohort. (**E**) Maternal blood IL1β expression analyzed as a function of symptom expression and
641 days between SARS-CoV-2 PCR positive test and blood sample collection; dashed line located
642 at Day 14; significance denotes comparison of samples collected within 14 days of a positive
643 SARS-CoV-2 test with samples collected > 14 days after test. Maternal blood n= 27; cord
644 blood=29; maternal side placenta n=11; fetal side placenta n=26. *p<0.05 by Kruskal-Wallis,
645 Dunn's multiple comparisons or Mann-Whitney test.

646

647 **Figure 2. *IL6* expression in maternal and fetal samples.** Maternal and fetal blood and
648 placentas were used to detect IL6 gene expression relative to the housekeeping genes (HKG),
649 18S and ACTB. (**A-D**) Maternal blood, cord blood, and maternal and fetal side placental IL6
650 expression between SARS-CoV-2 positive (P(+)) and negative (P(-)) samples in the pregnant
651 cohort. (**E-H**) Maternal blood, cord blood, and maternal and fetal side placental IL6 expression
652 in pregnant women who were asymptomatic (P-A), symptomatic (P-S), or SARS-CoV-2
653 negative (P-N). (**I**) Maternal blood IL6 expression analyzed as a function of symptom expression
654 and days between SARS-CoV-2 PCR positive test and blood sample collection; dashed line
655 located at Day 14. Maternal blood n= 27; cord blood=29; maternal side placenta n=11; fetal side
656 placenta n=26.

657

658 **Figure 3. Anti-SARS-CoV-2 antibody titration in samples collected from pregnant and**
659 **non-pregnant women.** Peripheral serum or plasma was used to titer IgG antibodies against

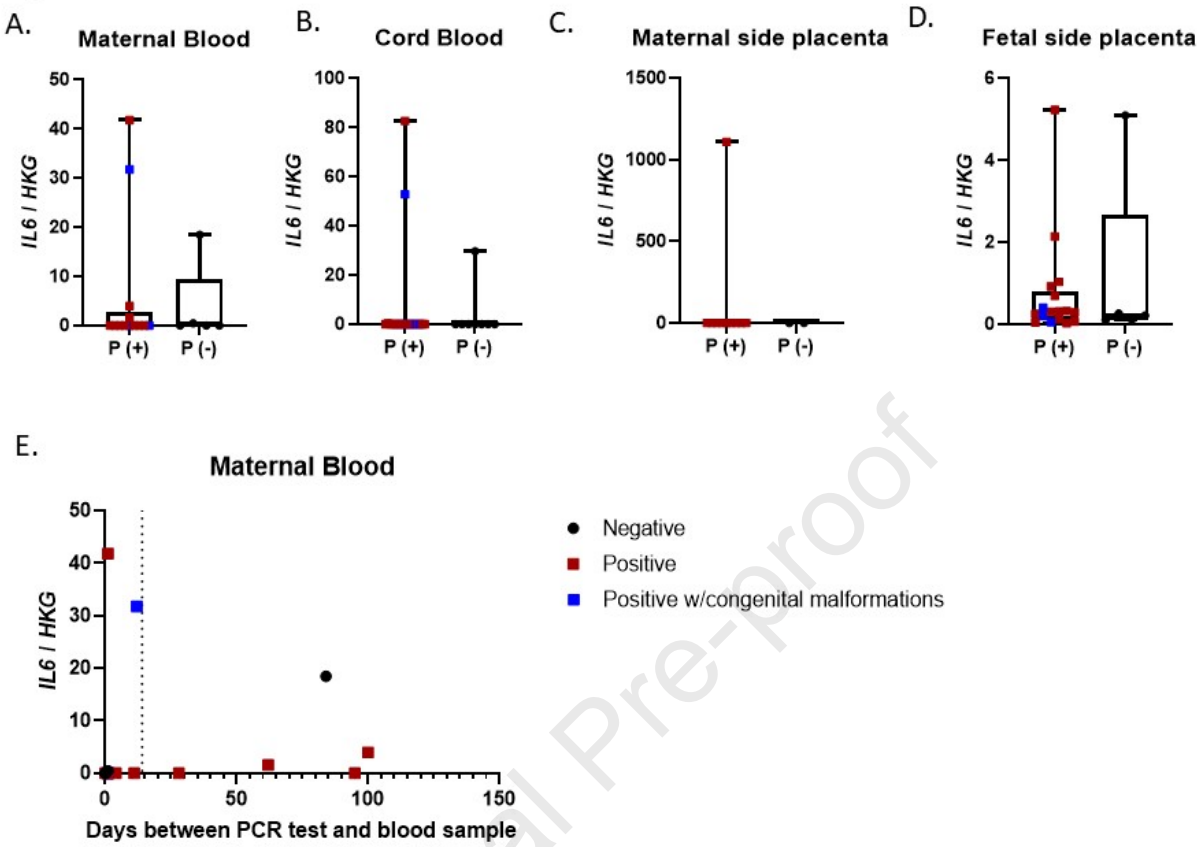
SARS-CoV-2 full-length spike (S), S-receptor binding domain (RBD), as well as whole virus neutralizing antibodies (nAb). (**A**) Anti-S IgG, (**B**) anti-S-RBD IgG, and (**C**) nAb area under the curve (AUC) titrations in serum or plasma from pregnant (P) (n=17) and non-pregnant (NP) (n=17) women. The dashed line denotes the median AUC for SARS-CoV-2 negative samples. Above each box-plot is the proportion of samples with detectable antibody; *p<0.05 by Kruskal-Wallis, Dunn's multiple comparisons, Wilcoxon exact, or Chi-square tests.

Figure 4. Association between anti-Spike-receptor binding domain (S-RBD) IgG and neutralizing antibody (nAb) titers in pregnant and non-pregnant women. (**A**) Comparison between anti-S-RBD IgG and nAb AUC in pregnant women, with additional comparison of anti-S-RBD IgG and nAb responses between pregnant with (nAb titer ≥1:20) and without (nAb titers <1:20) detectable nAb. (**B**) Comparison between anti-S-RBD IgG and nAb AUC in non-pregnant women. (**C,D**) Anti-S-RBD IgG AUC and nAb analyzed as a function of detectability of nAb and days between SARS-CoV-2 PCR positive test and blood sample. (**E,F**) Anti-S-RBD IgG AUC and nAb analyzed as a function of detectability of nAb and days since symptom onset and blood collection; missing data points due to unknown symptom onset date n=4. *p<0.05 by Wilcoxon exact.

Figure 5. Effects of SARS-CoV-2 infection on antibody transfer from mother to fetus. (**A**) Anti-S IgG, (**B**) anti-S-RBD IgG, and (**C**) nAb area under the curve (AUC) titrations in maternal serum and cord blood serum in SARS-CoV-2 positive pregnant women. (**D**) Western blot analysis for the neonatal Fc receptor (FcRn) protein in placentas from SARS-CoV-2 (+) and SARS-CoV-2 (-) women, n=35. (**E**) Quantification of FcRn western blot analysis relative to GAPDH was analyzed in placentas from SARS-CoV-2 positive (P(+)) and negative (P(-)) women, n=35. (**F)** Maternal and (**G**) cord blood serum anti-tetanus IgG titers in SARS-CoV-2

positive and negative samples in the pregnant cohort; maternal serum n=35, cord blood serum n=21.

Figure 2.



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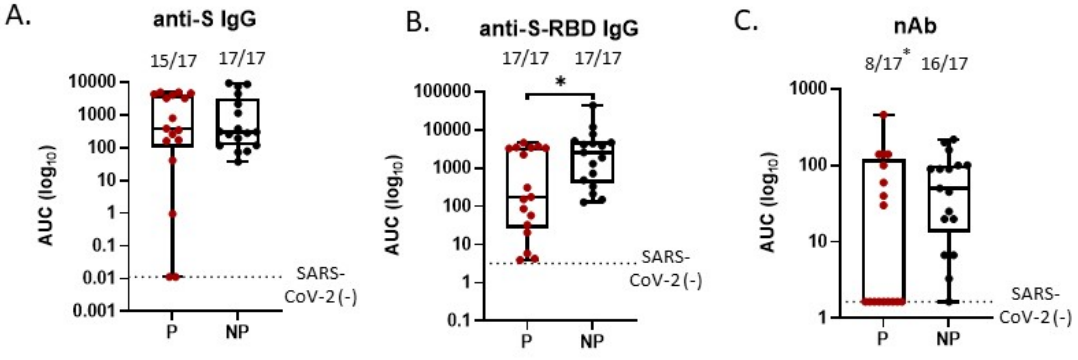
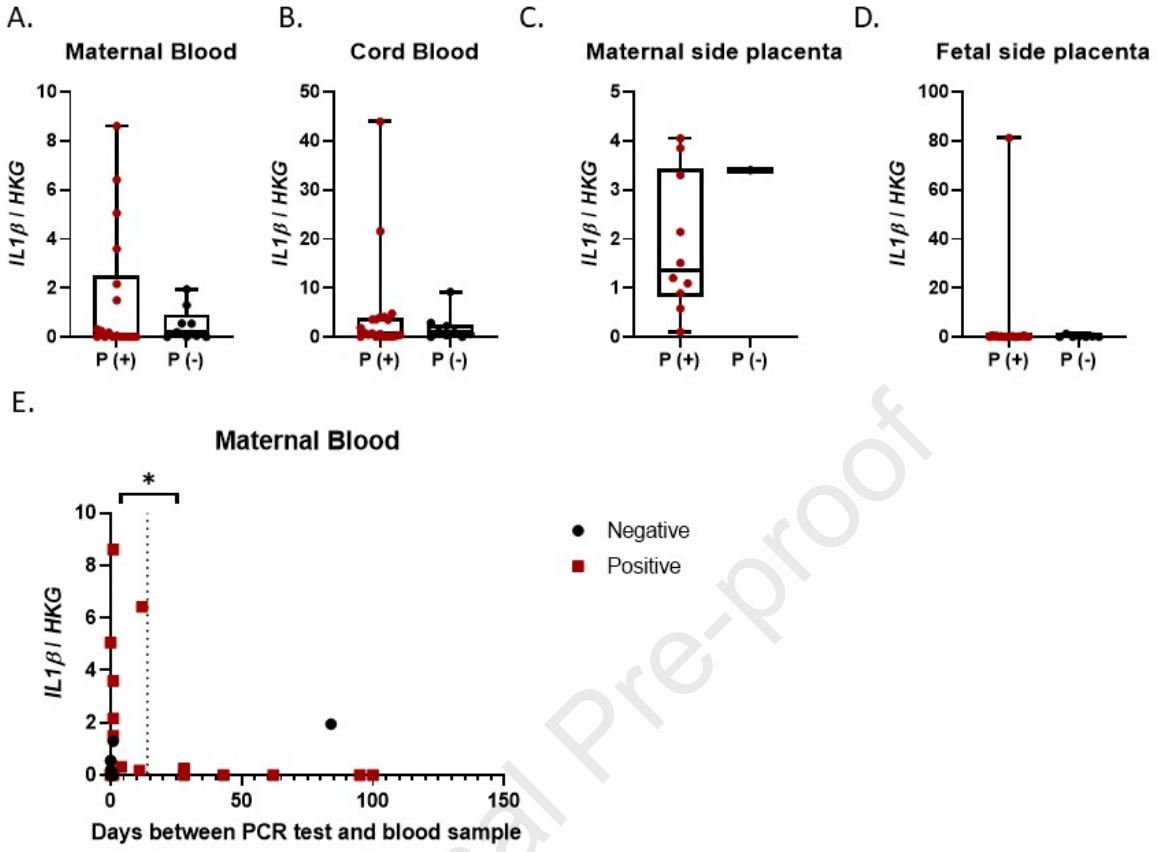


Figure 1.



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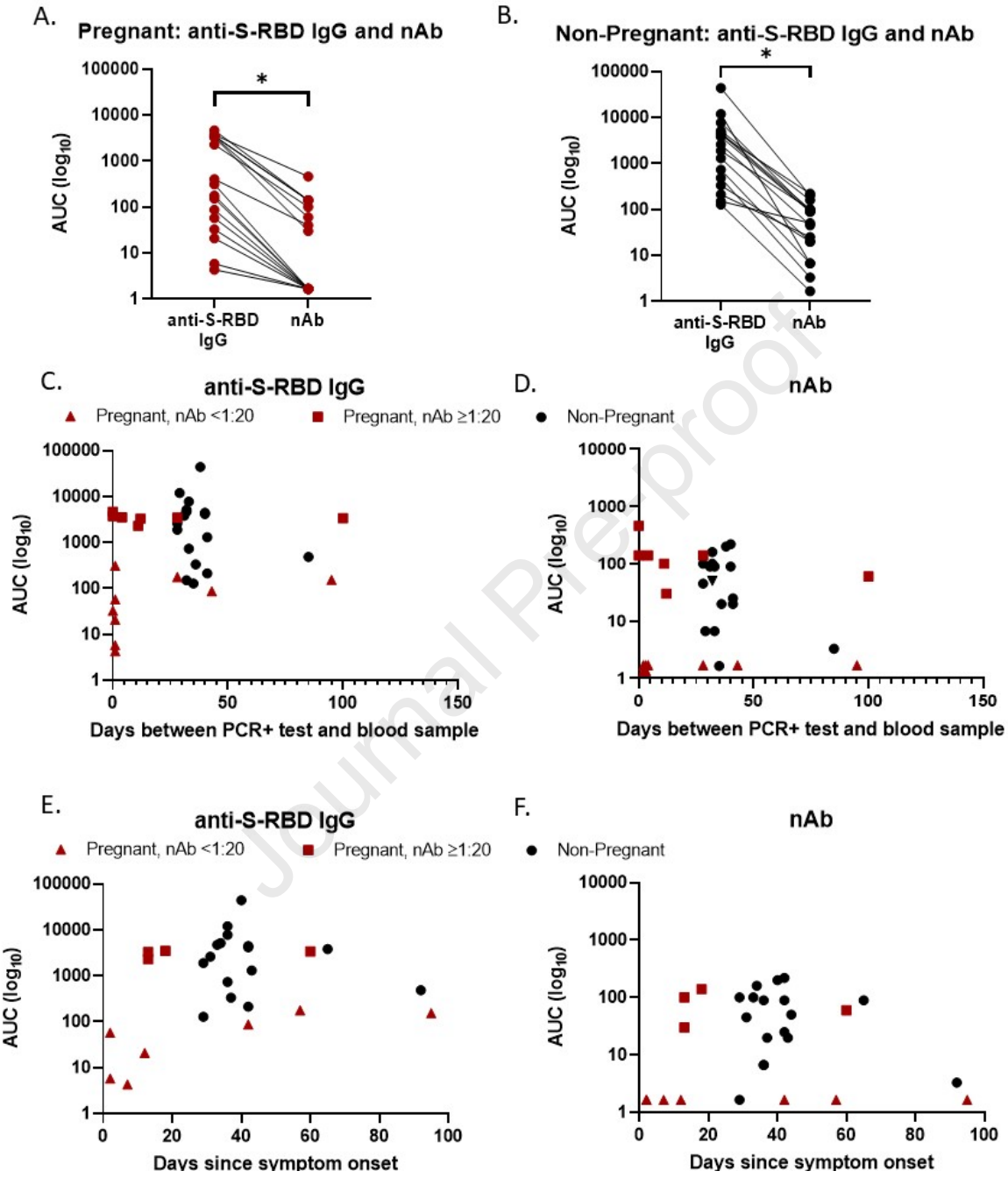

Figure 5.