 doi:10.20944/preprints202209.0063.v1

SARS-CoV-2 Infection in Unvaccinated High Risk Pregnant Women in the Bronx, NY is Associated with Placental Abnormalities, Shorter Gestation and Lower Apgar Scores

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Key Words: SARS-CoV-2, Placental Pathology, Apgar Scores, Gestational Age, Under-resourced Patient Population

 doi:10.20944/preprints202209.0063.v1

Abstract. Babies born to severe acute respiratory syndrome corona virus-2 (SARS-CoV-2) infected mothers are at greater risk for perinatal morbidity and more likely to receive a neurodevelopmental diagnosis in the first year of life. However, the effect of maternal infection on placental function and neonatal outcomes varies depending upon the patient population. We set out to test our hypothesis that maternal SARS-CoV-2 infection in our underserved, socioeconomically disadvantaged, predominantly African American and Latina population in the Bronx, NY would have effects evident at birth. Fifty-five SARS-CoV-2 positive and 61 negative third trimester patients were randomly selected from Montefiore Medical Center (MMC), Bronx, NY. In addition, two positive cases from Yale New Haven Hospital, CT were included as controls. All 55 placentas delivered by SARS-CoV-2 positive mothers were uninfected by the virus, based on immunohistochemistry, in-situ hybridization, and qPCR analysis. However, placental villous infarcts, mild preeclampsia, shortened gestational periods and lower Apgar scores were observed in the infected cases. These findings suggest that even without entering the placenta, SARS-CoV-2 can affect various systemic pathways culminating in altered placental development and function, which may adversely affect the fetus, especially in a high-risk patient population such as ours. These results underline the importance of vaccination among pregnant women, particularly in low resource areas.

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Introduction. While the link between prenatal exposure to stress and adverse effects on health has been established both historically and biologically [1,2], the effects that coronavirus disease 2019 (COVID-19) has on pregnancy, the placenta, the *in-utero* environment, and neonatal outcomes are only now coming to light. A recent study of SARS-CoV-2 infected placentas associated with intrauterine fetal demise has established the histopathologic features of SARS-CoV-2 placentitis [3]. Most cases of maternal infection, however, do not lead to infection of the placenta or vertical transmission to the fetus [4-7]. Nevertheless, maternal SARS-CoV-2 infection even in the absence of infection of the placenta or fetus has been associated with various adverse obstetrical outcomes, including shorter gestational period [8-10], lower Apgar scores [11], increased rates of intrauterine fetal demise [11], increased rates of neurocognitive deficits in the first year of life [12] and various placental histopathologic findings [13-15], depending on the patient population studied.

At the height of the COVID-19 pandemic, New York City was considered the epicenter of the pandemic. Within New York City, the Bronx has been the most affected borough, with over 455,753 cases and more than 7916 deaths to date [16]. Within the Bronx, the Department of Obstetrics and Gynecology and Women's Health at the Montefiore Medical Center (MMC) serves an under-resourced predominantly Latinx and African American patient population who have increased risk factors for pregnancy complications, including hypertensive disorders of pregnancy and diabetes. We hypothesized that SARS-CoV-2 infection in unvaccinated pregnant women in the third trimester in this patient population would have an unfavorable effect on placental morphology and function, resulting in compromise of the *in-utero* environment and adverse neonatal outcomes when fetal demands for oxygen and nutrients are highest. Given the prevalence of infection in pregnant women at the height of the pandemic, a better understanding

 doi:10.20944/preprints202209.0063.v1

of how COVID-19 affects the milieu of the developing fetus, which may lead to the identification of histopathological biomarkers for predicting long-term outcomes of exposed offspring, is critical to meet the health needs of the next generation. Here we evaluate the placenta, which performs the vital functions of nutrient, gas and waste exchange, as well as perinatal clinical outcomes, both of which set the stage for lifelong health and susceptibility to disease.

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Methods.

Study Approval. IRB approval was obtained from MMC (Approval Number 2020-11651) and from Yale University Medical Center, New Haven, CT (Yale) (HIC no. 2000027690).

Patient Population. Fifty-five third trimester placentas from SARS-CoV-2 positive obstetrical patients and 61 third trimester placentas from SARS-CoV-2 negative patients were randomly selected from the MMC pathology archives with approval from the Albert Einstein College of Medicine Institutional Review Board (IRB). Patients in the two groups were well matched for maternal age, race and pre-existing clinical conditions, as shown in Table 1. In addition, one previously described [17] SARS-CoV-2 positive mid-trimester placenta and one newly identified SARS-CoV-2 positive third trimester from Yale New Haven Hospital, New Haven, CT were included, with approval from the Yale IRB. All patients were unvaccinated for SARS CoV-2.

Histopathologic Evaluation. The 55 placentas from SARS-CoV-2 positive mothers were compared to the group of 61 randomly selected third trimester placentas delivered by uninfected mothers histopathologically, as detailed in Table 2. Five Hematoxylin and Eosin (H&E) stained sections of formalin fixed paraffin embedded (FFPE) tissue from each placenta from the MMC pathology archives (n=55 from SARS-CoV-2 positive patients and n=61 from SARS CoV-2 negative patients) were examined by a practicing pathologist at MMC, using routine light microscopy. Sections included at least one extra-embryonic membrane roll, two sections of umbilical cord, at least one section of villous parenchyma including the chorionic plate and at least three sections of villous parenchyma including the decidua basalis. All placentas were staged and graded for chorioamnionitis and funisitis if present. Placentas were also evaluated for

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decidual vasculopathy in the decidua capsularis and decidua basalis, increased syncytial knotting and villous crowding, villous congestion, villous hypervascularity/chorangiosis, intervillous thrombi, distal villous hypoplasia, reactive amnionic epithelium, chronic villitis and infarcts. No cases showed evidence of fetal vascular malperfusion, chronic villitis or chronic histiocytic intervillositis.

Quantitative Polymerase Chain Reaction (qPCR).

FFPE tissue acquisition

Unstained 10 µm sections from one FFPE tissue block from each of the 55 MMC SARS-CoV-2 positive study subjects and SARS-CoV-2 infected lung tissue as a positive control were obtained. The corresponding H&E-stained sections were reviewed by one of the study pathologists (SER) for uniform representation of the placental cells on the sections. Unstained tissue sections in their entirety were subjected to RNA extractions, prior to qPCR analyses, using the Taqman® 2019-nCov Assay Kit.

FFPE RNA extraction and quantification

Total RNA extractions from FFPE clinical specimens were performed using a simultaneous RNA/DNA extraction protocol described previously, where RNA is extracted prior to DNA [18, 19]. In brief, a total of 3 consecutive unstained 10 µm tissue sections were scraped off the slides and transferred to individual siliconized Eppendorf tubes. The tissues were de-paraffinized using Citri-Solv (Thermo Fisher Scientific, Inc.) at room temperature on a Thermomixer (Eppendorf), followed by ethanol washes on ice, one 1xPBS (RNase-free) wash, and a re-hydration in the presence of RNase-inhibitors in 1xPBS (RNase-free). The tissues were subjected to proteinase K (3 mg/ml) digest at 59°C for 1 h. The digested tissues underwent butanol-1 extraction to obtain a

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final volume of 100 µl, which was homogenized in 1 ml of TRIzol (Invitrogen) following the manufacturer's instructions. The RNA was recovered from the upper phase of the TRIzol solution, transferred to a new siliconized Eppendorf and precipitated with 0.1 µl /µl linear acrylamide (1 µl), 3M sodium acetate (18 µl), and 660 µl of isopropanol. The tubes were stored at −20°C overnight, and centrifuged the next day at 14,000 rpm for 30 min at 4°C. The RNA pellets were washed with 200 µl of 70% RNase-free ethanol, dried and re-suspended in 12 µl of RNase-free 1x Tris/EDTA solution and incubated for 30 min at 70°C. The RNA was quantified on a total RNA chip on a Bioanalyzer (Agilent). Genomic DNA was extracted from the lower phase of TRIzol and stored for future experiments [20].

SARS-CoV-2 reverse transcription and qPCR analyses**.** Quantitative PCR experiments were performed using Taqman® 2019-nCov Assay Kit v1, Taqman® 2019 nCov Control Kit v1 and Taqman® Fast Virus 1-Step Master Mix (Applied Biosystems, USA). For each clinical specimen, 100 ng of total RNA was used for each qPCR analysis. Reagents for each RNA specimen were combined in a MicroAmp Fast Optical 96 well-plate following the manufacturer's instructions. In brief, 6.25 µl of Taqman® Fast Virus 1-Step Master Mix (4x), 1.25 µl nCov assay (20x) (either ORF1ab, S Protein, or N Protein), 1.25 µl of RNaseP assay (20x) and 11.25 µl of nuclease-free water were combined in each well. For each RNA sample, 5 µl of total RNA (20 ng/ml) was added to respective sample wells of a 96 barcoded well-plate (Applied Biosystems). For these analyses, following the manufacturer's recommendations, control wells containing 1 µl of either ORF1ab, S Protein, or N Protein with 4 µl of nuclease-free water were also prepared for each 96 well-plate. The real-time PCR quantifications for each sample were monitored on a StepOnePlus instrument (ABI) and the comparative thresholds were determined after sample plates underwent the recommended cycles as follows: 5 minutes at 50°C, 20 seconds at 95°C , 3 seconds at 95°C, and

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30 seconds at 60°C for 40 cycles. The fold change differences between the clinical specimens and the control specimens were calculated using the $2^{(\Delta\Delta CT)}$ formula.

Immunohistochemistry (IHC). FFPE 4 μm sections from the 55 MMC SARS-CoV-2 positive study subjects and the two positive control Yale cases were stained with anti-SARS-CoV-2 nucleocapsid protein antibody (Thermofisher, mouse monoclonal antibody clone B46F, dilution 1:200), as previously described [8]. The nucleocapsid antibody has been previously validated with positive and negative controls. The antibody immunostains SARS-CoV-2 infected skin, lung, liver and kidney autopsy tissue [21]. In addition, the antibody used in this study immunostains control infected placental tissue [8], which contains SARS-CoV-2 RNA detectable by two different spike protein antibodies (Sino Biological, 40150-T62-COV; and GeneTex, SARS-CoV/SARS-CoV-2 spike antibody clone 1A9), by PCR and by ISH [8]. The nucleocapsid antibody has also been shown not to stain placental tissue that is negative for SARS-CoV-2 by PCR and by ISH and does not cross react with human coronaviruses 229E and OC43 [21].

In-situ hybridization (ISH). ISH was performed on sections from the 55 MMC SARS-CoV-2 positive study subjects and the two positive control cases from Yale with a probe directed against the spike protein (V-nCoV2019-S; Advanced Cell Diagnostics), using appropriate positive and negative controls [21]. Positive control placental tissue was proven to be infected with SARS-CoV-2 by PCR and by immunohistochemistry, using two different antibodies directed against the spike protein (Sino Biological, 40150-T62-COV; and GeneTex, SARS-CoV/SARS-CoV-2 spike antibody clone 1A9) and one antibody directed against nucleocapsid protein (Thermofisher, mouse monoclonal antibody clone B46F) [21]. Negative controls were proven to be uninfected, based on

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PCR and IHC, using the antibodies listed [21]. Probes directed against the bacterial gene dapB and probes for the housekeeping gene peptidylprolyl isomerase B were included as negative and positive technical controls, respectively. Sections were counterstained with periodic acid-Schiff.

Statistical Analysis.

Statistical analyses were performed using JMP software version 7.0 (SAS Institute, Cary, NC) or Graph Pad Prism software version 5.04 for Windows (Graph Pad Software, San Diego, CA). For continuous variables, data are presented as the means ± SEM. Analysis of variance was used to test for significant differences between the means of two (*t*-test) or more groups, and Bonferroni's posthoc analysis was performed. Pearson's χ^2 test was the statistical test applied to sets of categorical data to evaluate how likely it is that any observed difference between the sets arose by chance. When appropriate, the two-tailed *U*-test was used. Acceptable study power was agreed *a priori* to be ≥ 80% (type I error of ≤0.20). $P < 0.05$ was considered statistically significant.

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Results.

Maternal SARS-CoV-2 infection affects length of gestation and Apgar scores. Fifty-five SARS-CoV-2 positive and 61 SARS-CoV-2 negative MMC third trimester obstetrical patients were compared for an extensive list of sociodemographic, obstetrical, and medical parameters, as detailed in Table 1. No cases of neonatal death or neonatal asphyxia occurred. None of the neonates developed clinical, radiologic, hematologic, or biochemical evidence of COVID-19. While both groups have a high prevalence of obesity, hypertensive disorders and diabetes, no significant differences between the two groups were found in age, body mass index (BMI), ethnic origin, homelessness, cigarette smoking, gravidity, parity, presenting obstetric symptoms, mode of delivery, diabetes, clinical chorioamnionitis, placentation, prescription drug use, intrauterine fetal demise, or other maternal disease (Table 1). Mild preeclampsia was more prevalent in the infected group. Consistent with other reports [8-10], the length of gestation was significantly shortened in infected patients ($P = 0.02$). Importantly, Apgar scores were significantly lower in the infected group than in the uninfected group at one minute (7.6 ± 2.6 vs. 8.6 ± 0.6, $P = 0.03$) and five minutes ($8.1 + 2.5$ vs. $8.9 + 0.2$, $P = 0.04$).

Maternal SARS-CoV-2 infection increases placental villous infarcts. Next, we tested our hypothesis that maternal SARS-CoV-2 infection would affect placental histopathology. The prevalence of placental histopathology was very high in the cases examined, not surprisingly, given the high frequencies of obesity, hypertensive disorders, diabetes, and placental findings in our high-risk patient population. All the placentas from SARS-CoV-2 negative patients were submitted for pathological examination due to significant obstetrical, medical, or neonatal

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concerns. In contrast, all placentas from SARS-CoV-2 positive mothers regardless of maternal risk factors or clinical outcome had placental evaluations. Nevertheless, a statistically significant difference between the two groups was seen in the number of villous infarcts with placentas from the infected mothers being more frequently infarcted ($P = 0.03$, Table 2).

The placenta is an effective barrier for SARS-CoV-2. Three methods were used to test for the presence of SARS-CoV-2 in the placentas of the positive MMC patients: qPCR; ISH, using probes directed against SARS-CoV-2; and IHC, using an antibody directed against the nucleocapsid protein. All 55 cases were subjected to IHC and qPCR and a subset of 19 cases were subjected to ISH. All 55 cases were consistently negative for SARS-CoV-2. This finding is consistent with the low rate of placental infection reported by other investigators [4-7], as well as data suggesting that ACE2 and transmembrane protease serine 2 (TMPRSS2) rarely co-localize in the placenta [22].

However, two control infected placentas from Yale clearly showed positive staining in the syncytiotrophoblast, using IHC (Figures 1A and 1D, respectively) and ISH (Figures 1B and 1E, respectively). The mid-trimester placenta used as a positive control (Figures 1A and 1B) was delivered by dilation and evacuation at 22 weeks' gestation by a 35-year-old, who had had one previous full-term pregnancy and one aborted pregnancy. She presented in this pregnancy with severe preeclampsia complicated by placental abruption and disseminated intravascular coagulation. Microscopic findings in this placenta were significant for a marginal retroplacental hematoma and overlying villous infarct (consistent with placental abruption) and for diffuse intervillous fibrin and CD68 and CD3 positive mononuclear cell infiltrates, consistent with macrophages and lymphocytes, respectively, and with SARS CoV-2 placentitis. Electron

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microscopy of this placenta revealed cytoplasmic viral particles in the villous trophoblast cells [8]. The third trimester placenta used as a second positive control (Figures 1C and 1D) was delivered at 34 weeks and 6 days' gestational age by a 40-year-old who had had 3 previous full-term pregnancies and one previous preterm birth with no significant obstetrical history other than preterm labor in a previous pregnancy. She was treated prophylactically with 17-alpha-hydroxyprogesterone (Makena). Microscopically, this placenta was significant for increased intervillous fibrin and multiple acute villous infarcts. In addition, like the first positive control case, there was CD68 positive histiocytic intervillositis, consistent with SARS CoV-2 placentitis. SARS-CoV-2 was confirmed in both positive control patients by PCR of nasopharyngeal swabs.

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Discussion.

While the effect of maternal SARS CoV-2 infection on pregnancy has been investigated in various patient cohorts [9,11,12], we evaluate here the effect of maternal infection in an under-resourced, predominantly African American and Latinx, high-risk patient population. One of the significant findings in our study is that maternal SARS-CoV-2 infection is associated with Apgar scores that are approximately 1 point lower at 1 and 5 minutes. Decreases in Apgar scores of this magnitude predict greater rates of neonatal morbidity and mortality [23]. Interestingly, in a recent study that examined a more affluent, predominantly Caucasian patient population in Boston, MA, investigators found no significant change in Apgar scores associated with maternal SARS CoV-2 infection [12], suggesting that social determinants of health may exacerbate health risks for offspring of infected pregnant women.

One weakness of our study is the prevalence of placental pathology in our control cases. Nevertheless, even in the context of a high background level of placental histopathology, placental villous infarcts were significantly increased in placentas delivered by infected mothers. Villous infarcts represent the terminal lesion affecting the placental parenchyma in the setting of maternal vascular malperfusion. Infarcted villous parenchyma is no longer able carry out gas exchange, nutrient delivery and waste removal between the mother and fetus. While there is "placental reserve," the presence of infarcts also serves as a biomarker for poor placental perfusion and function. Poor placental function increases the risk for adverse perinatal outcomes, including intrauterine growth restriction and fetal demise. In addition, suboptimal intrauterine conditions resulting from poor placental function may have long term effects for the fetus. It is now well established that a suboptimal *in-utero* environment such as one in which the placenta is

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insufficient results in altered fetal epigenetic programming and lifelong increased risk for cardiometabolic disease [24].

Although the neonates in the SARS-CoV-2 positive group were not infected, maternal SARS-CoV-2 infection altered their *in-utero* environment. In addition to potential effects on the fetal milieu produced by the maternal immune response to the virus, changes in levels of ACE2, the SARS CoV-2 receptor, are directly linked to maternal vascular malperfusion of the placenta and obstetrical complications [25-29]. The long-term consequences of these complications will become apparent in the decades ahead but based on our current understanding of the effect of the *in-utero* environment on lifelong fetal programming, it is likely that offspring born to SARS-CoV-2 positive mothers will be at increased risk for cardiometabolic disease and cancer [30], particularly among high-risk patient populations such as ours. Importantly, the group of SARS-CoV-2 positive patients in this study were infected at the time of delivery and most likely not infected in the first weeks of gestation, when placentation and critical uteroplacental vascular remodeling is taking place. The effect of COVID-19 and loss of ACE2 in early pregnancy on placental morphology and function and fetal wellbeing remains to be investigated.

While placental SARS-CoV-2 infection is rare, COVID-19 pneumonia or infection of other non-gynecologic organs was common in pregnant women in our center with 30% of patients presenting to our Labor & Delivery service testing positive for SARS-CoV-2 by nasopharyngeal swab PCR at the height of the first wave of the pandemic. Our infection rate was higher than those in nearby institutions in greater New York serving different patient populations, which averaged only 14% infection rates [31-33]. Both the rate of maternal infection and the impact of maternal COVID-19 on offspring is likely to be more pronounced in patient populations like ours, where underlying risk factors for obesity, hypertension and

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diabetes are common. The results of this study underline the importance of vaccination for SARS-CoV-2 among pregnant women, particularly in low resource areas.

Author Contributions: SER, MJC, PMV, SAF and FH conceived of the study and contributed to its design. MH contributed the IHC and ISH data. OL performed the qPCR. RK collected the clinical data. RB collected the pathology cases. SER drafted the manuscript and made edits following input from all co-authors.

Funding: Dr. Reznik received an internal seed grant from the MMC Department of Pathology to support histological and molecular studies. Dr. Reznik is supported by a grant from the NIH (1R16GM145586). Dr. Charron is supported by a grant from the NIH (1R01HD092533).

Institutional Review Board Statement: Institutional Review Board approval was obtained from both the Albert Einstein College of Medicine and the Yale University Medical Center IRB.

Data Availability Statement: Not applicable.

Acknowledgments. We are grateful to James M. Pullman, M.D. for developing the MMC COVID-19 placenta registry, Ijeoma Green for mining the pathology database, Wa Shen for preparing unstained slides, Mary Helie for performing IHC and ISH, Fangping Chen for performing IHC and Christina Liu for performing qPCR.

Conflicts of Interest: The authors declare no conflicts of interest.

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	SARS-CoV-2 Negative (n=25)	SARS-CoV-2 Positive (n=19)	*P* Value
Mean age ± SEM (Years)	30.92 ± 1.20	29.42 ± 1.08	NS
Mean BMI ± SEM (kg m^{-2})	32.76 ± 1.76	32.00 ± 1.46	NS
--BMI > 50 (%)	8.0	16	
--BMI > 40 and ≤ 50 (%)	16	16	
Race (%)			NS
--Black	52	47	
--Latina	40	42	
--Non-Latina white	4.0	5.3	
--Asian	4.0	5.3	
Homeless (%)	8.0	16	NS
Cigarette smoking (%)	12	0	NS
Mean gestational age	37.5 ± 0.7	37.2 ± 0.7	
Gravidity ≥ 5 (%)	20	21	NS
Parity ≥ 5 (%)	4.0	11	NS
Presenting obstetric symptoms (%)			
--Labor	8.0	16	NS
--Ruptured membranes	12	21	NS
--Vaginal bleeding	4.0	0	NS
--Decreased fetal movement	20	16	NS
Mode of Delivery (%)			
--Cesarean Section	4.0	0	NS
--Induction	40	47	NS
Hypertensive disease of pregnancy (%)			
--Severe preeclampsia/eclampsia	20	26	NS
--Mild/unspecified preeclampsia	4.0	11	NS
--Gestational hypertension	16	21	NS
--Chronic hypertension	20	0	NS
Diabetes mellitus (%)			
--Gestational diabetes	4.0	16	NS
--Pre-gestational diabetes mellitus	4.0	0	NS
Clinical chorioamnionitis (%)	8.0	11	NS
Other maternal disease (%)			
--Pre-existing cardiac disease	4.0	0	NS
--Systemic lupus erythematosus	4.0	0	NS
--HIV	4.0	0	NS
--Asthma	24	21	NS
--Anemia	72	53	NS
Maternal medications			
--Inhaled steroids	8.0	21	NS
--Aspirin	8.0	26	NS
--Antihypertensives	12	11	NS
Abnormal Placentation (%)	4.0	16	NS
Fetal Death (%)	12	0	NS

Table 1. Clinical features of SARS-CoV-2 negative and positive patients.

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	COVID Negative (n=25)	COVID Positive (n=19)	*P* Value
Maternal vascular malperfusion (%)	84	74	NS
--Small for gestational age placenta	32	16	NS
--Increased syncytial knotting/Tenney-Parker change	32	42	NS
--Distal villous hypoplasia	16	16	NS
--Villous congestion	0	5.3	NS
--Villous hypervascularity/chorangiosis	16	5.3	NS
--Villous infarct	4.0	26	**0.03**
--Intervillous thrombus	16	16	NS
--Villous crowding	4.0	5.3	NS
--**Decidual vasculopathy**	24	32	NS
----Decidual capsularis vasculopathy	20	11	NS
------Hypertrophy	20	11	NS
------Fibrinoid necrosis	0	5.3	NS
------Vasculitis	0	5.3	NS
----Decidua basalis vasculopathy	12	26	NS
------Fibrinoid necrosis	12	21	NS
------Thrombosis	8.0	16	NS
------Atherosis	0	5.3	NS
------Vasculitis	0	5.3	NS
------Incomplete remodeling	0	5.3	NS
Large for gestational age placenta (%)	0	5.3	NS
Inflammatory responses (%)	40	47	NS
--**Maternal inflammatory response**	40	47	NS
----Acute chorioamnionitis	36	32	NS
----Necrotizing chorioamnionits	0	5.3	NS
----Chronic deciduitis	4.0	0	NS
----Chronic villitis	0	11	NS
--**Fetal inflammatory response**	20	11	NS
----Acute phlebitis	16	5.3	NS
----Acute funisitis	4.0	5.3	NS

Table 2. Histopathologic features of placentas from SARS-CoV-2 negative and positive patients.

 doi:10.20944/preprints202209.0063.v1

Figure Legends.

Figure 1. Positive control cases of SARS-CoV-2 placentitis. (A) SARS-CoV-2 IHC staining in a 22-week infected placenta. Sections were stained using an antibody directed against the nucleocapsid protein. **(B)** SARS-CoV-2 ISH staining of the same placenta as in Panel A. Hybridization was directed against the Spike protein. **(C)** SARS-CoV-2 IHC staining in a 34-week-and-6-day infected placenta. **(D)** SARS-CoV-2 ISH in the same placenta as in Panel C. Microscopic slides were originally photographed at a magnification of 200X. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

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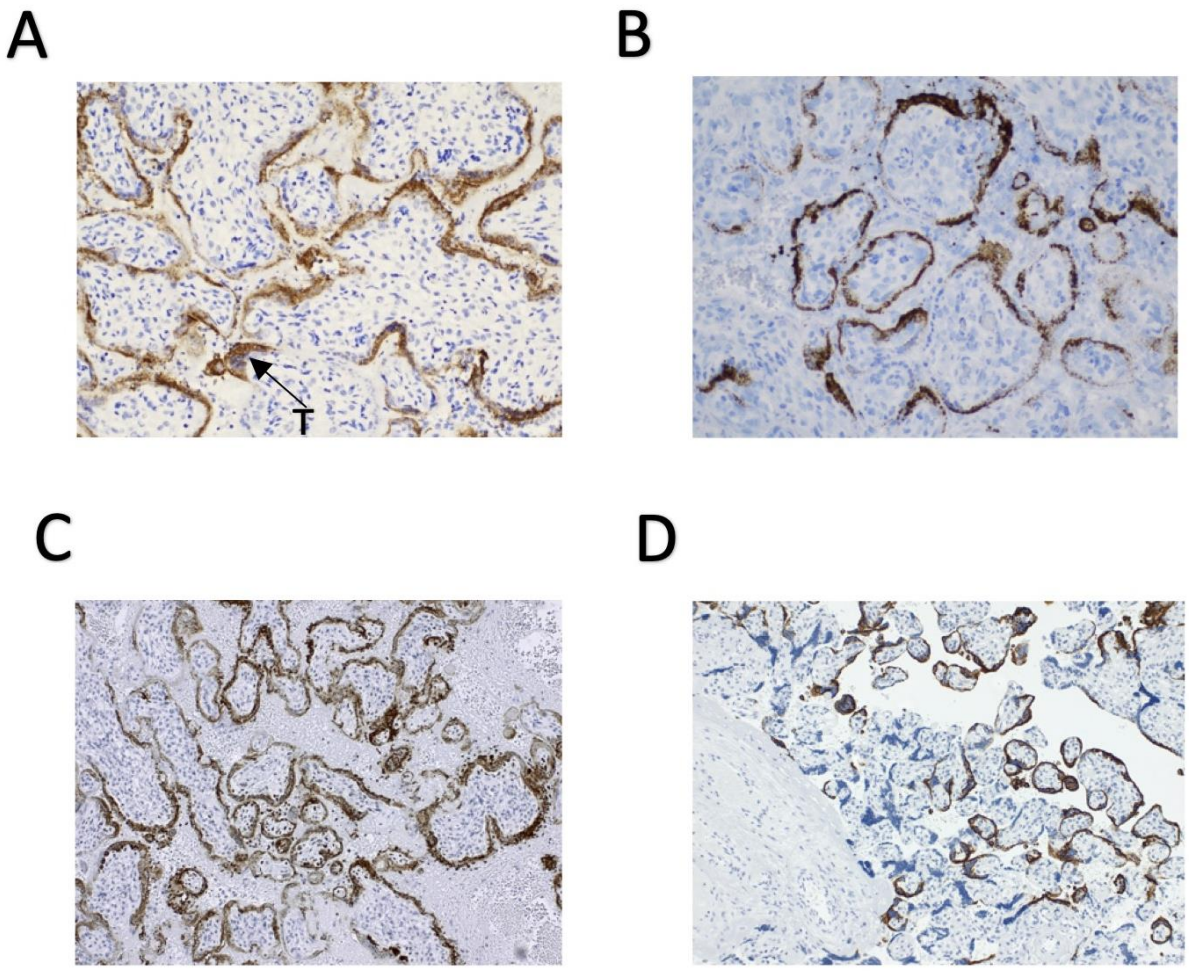

Figure 1