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Evaluating gestational stage of maternal SARS-CoV-2 infection on immune and inflammatory response & fetal outcomes

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EVALUATING GESTATIONAL STAGE OF MATERNAL SARS-COV-2 INFECTION ON IMMUNE AND INFLAMMATORY RESPONSE & FETAL OUTCOMES

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ABSTRACT

Despite the surge of research since the onset of the coronavirus disease 2019 (COVID-19) pandemic, the pathophysiology of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in the placental microenvironment during pregnancy is still unclear. The indirect effect of the virus via maternal immune and inflammatory activation is implicated in adverse outcomes. This study aims to characterize the viral response in light of the fluctuating inflammatory states natural to pregnancy.

Objective: To evaluate the impact of timing of maternal SARS-CoV-2 infection on systemic and placental immune and inflammatory response, and assess the risks and effects of infection at each gestational stage throughout pregnancy.

Design, Setting, and Participants: Longitudinal prospective cohort study across 12 Boston area hospitals taking place over 5 years involving a study group divided into pregnant people infected with SARS-CoV-2 during different trimesters, matched with control group of non-infected pregnant people.

Exposures: Maternal SARS-CoV-2 infection, as confirmed by polymerase chain reaction tests and antigen tests, during pregnancy.

Main Outcome Measures: immune and inflammatory systemic biomarkers, placental pathology at delivery and at dilation and curettage, pregnancy complications and mortality, maternal health (gestational diabetes, preeclampsia, HELLP - Hemolysis, Elevated Liver Enzymes, Low Platelets – syndrome), fetal mortality and health (size according to gestational age, birth weight, Apgar score, congenital abnormalities, neurodevelopmental disorders.)

Implications: pregnancy risk assessment, obstetrics clinical management, therapeutic targets, maternal health, fetal growth and development, public and mental health resources.

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LIST OF ABBREVIATIONS

ACE2 ..... angiotensin-converting enzyme 2

BMI.....body mass index

CDC .....Centers for Disease Control and Prevention

CMV..... cytomegalovirus

COVID-19 ..... coronavirus disease 2019

CRP..... C-reactive protein

CRS..... congenital rubella syndrome

CTBs..... cytotrophoblasts

DNA..... deoxyribonucleic acid

ELISA..... enzyme-linked immunoassay

ESR..... erythrocyte sedimentation rate

EVTs..... extravillous trophoblasts

FcRn..... neonatal crystallizable fragment receptor

GM-CSF..... granulocyte macrophage colony-stimulating factor

HELLP..... Hemolysis, Elevated Liver Enzymes, Low Platelets

HIV..... human immunodeficiency virus

HSP..... heat shock protein

ICD-10..... International Classification of Diseases-10

IFITM ..... interferon-induced transmembrane

IFN..... interferon

IL..... interleukin

IRB..... Institutional Review Board

ISGs..... interferon-stimulated genes

LDH..... lactate dehydrogenase

LGA..... large for gestational age

MIA..... maternal immune activation

nAb..... neutralizing antibody

NK ..... natural killer

NLR..... neutrophil to lymphocyte ratio

qRT-PCR..... quantitative Real Time-Polymerase Chain Reaction

RNA..... ribonucleic acid

RV..... Rubella virus

SARS-CoV-2 ..... severe acute respiratory syndrome coronavirus 2

SGA..... Small for gestational age

ST / STB..... syncytiotrophoblast

TMPRSS2..... transmembrane serine protease 2

TNF..... tumor necrosis factor

TORCH..... Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes Simplex

uDCs..... uterine dendritic cells

WHO .....World Health Organization

ZIKV..... Zika virus

INTRODUCTION

BACKGROUND

A novel coronavirus was identified at the end of 2019 and rapidly spread. By March 11, 2020, the World Health Organization (WHO) declared coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) a global pandemic.[1] In the two years since, over 538 million cases have been confirmed and over 6.3 million people have died worldwide. Just in the U.S., there are over 86 million cases with deaths surpassing one million.[2]

Nearly 150 births occur annually worldwide – the effects of the virus on pregnancy have yet to be fully determined.[3] Based on current population-level studies, pregnant people infected with SARS-CoV-2 are at increased risk of severe illness. Furthermore, there are reports of higher risk of pregnancy complications such as miscarriage, premature rupture of membranes, preterm birth , preeclampsia, and fetal growth restriction.[4] Lasting consequences of in utero exposure to the virus are just being uncovered with early data showing neurodevelopmental repercussions.[5] The mechanisms underlying these effects are unclear, but dysregulated immune and inflammatory response has been implicated.[6] The extent to which and how exactly SARS-CoV-2 disrupts the placental microenvironment remain unknown.

STATEMENT OF THE PROBLEM

Evidence from other viral infections such as rubella indicate that maternal infection and in utero exposure earlier in pregnancy have higher risk of congenital anomalies.[7] Early studies on virus receptor expression hint that placentas of women in

early stages of gestation are more vulnerable to SARS-CoV-2 infection.[8] With most existing studies focused on data from third trimester exposure and its consequences, there is a gap in understanding both the mechanisms and effects of infection during the first two trimesters. This is partly due to the difficulties in exacting time of infection, as well as detecting it during early weeks of pregnancy when a woman may not even know she is pregnant. It is also a challenge to obtain placental samples during early gestation due to the risks involved.

However, as the immune and inflammatory state fluctuates throughout pregnancy, it is important that studies acknowledge these changes and distinctions at the maternal-fetal interface during an infection. SARS-CoV-2 infection has both direct and indirect effects, yet it is not known whether these effects are cumulative throughout the course of a pregnancy and the extent to which the placenta is affected over time. This thesis aims to answer the questions of what the risks of viral infection as pregnancy progresses are, whether the placenta's defenses will hold up, and whether there is a threshold at which SARS-CoV-2 becomes teratogenic.

HYPOTHESIS

SARS-CoV-2 infection modulates the proinflammatory stages of pregnancy, disrupting processes necessary for successful implantation during the first trimester and diverting resources needed for fetal growth and parturition during the third trimester. Additionally, it transforms the anti-inflammatory stage of pregnancy into a more inflammatory state during the second trimester, affecting fetal development.

SARS-CoV-2 is likely to have cumulative effects dependent on timing of

maternal infection — from threatening the pregnancy at an early stage to congenital effects in exposed neonates.

OBJECTIVES and SPECIFIC AIMS

The objective of this study is to assess the impact of timing of maternal SARS-CoV-2 infection on systemic and placental immune and inflammatory response. Specific aims include:

- To assess the risks associated with viral infection during each of the 3 gestational trimesters;
- To evaluate the changes in immune and inflammatory markers at different gestational stages due to SARS-CoV-2 infection;
- To determine if the effects of SARS-CoV-2 infection during pregnancy are cumulative.

REVIEW OF LITERATURE

OVERVIEW

Covid-19

SARS-CoV-2 is transmitted between people in close contact via droplet and aerosol spread. The virus primarily affects the upper and lower respiratory tracts, but can also affect other organ systems. SARS-CoV-2 virus uses its surface spike (S) protein and gains entry into host cells via the angiotensin-converting enzyme 2 (ACE2) receptor.[9] ACE2 receptors are present in many cell types and tissues including the lungs, heart, gastrointestinal tract, kidneys, and liver; but are most abundantly found on the surface of type II alveolar cells of the lungs and epithelia of the small intestine. It is also present in vascular endothelium.[9] Host co-factors like transmembrane serine protease 2 (TMPRSS2), furin, and Interferon-induced transmembrane (IFITM) proteins are also found to be necessary for successful infection as they aid the fusion of virus membranes and host cells, and functional activation of the virus.[10]

Infection can cause direct damage to the alveoli or indirect damage through a local inflammatory response.[9] Damage to the epithelium and endothelium further promotes and amplifies inflammation and coagulation. Uncontrolled inflammation characterizes the more severe immunopathology of COVID-19.[9]

Infected people may be asymptomatic or symptomatic, though asymptomatic infections may also have objective clinical abnormalities. Symptoms include fever, cough, headache, fatigue, chills, myalgia, difficulty breathing, loss of smell and loss of taste. Onset of symptoms is acute (from 2-14 days) but may become chronic, though

studies on long-term effects are still underway.[11] There is a spectrum of symptom severity from mild to fatal – with more severe symptoms including pneumonia, hypoxia, respiratory failure, shock, or multiorgan dysfunction.[12]

Differences in severity of symptoms have been associated with different SARS-CoV-2 variants, with the later Omicron variants (B.1.1.529) appearing to cause milder symptoms than the earlier Delta variant (B.617.2.)[11] Hospitalization and mortality are also associated with vaccination status, resource shortages, and various comorbidities.

Pregnancy is included in the CDC's list of underlying medical conditions that elevates a person's risk of severe illness from COVID-19.[12] In a living systematic review and meta-analysis of 435 studies spanning the entire year 2020, it was found that pregnant and recently pregnant people with COVID-19 are more likely to be admitted to intensive care units, require mechanical ventilation or die compared with non-pregnant people of reproductive age with COVID-19.[13] Risk of severe illness is further increased by increasing maternal age, non-white ethnicity, high BMI, other pre-existing co-morbidities, and pregnancy-specific disorders such as pre-eclampsia and gestational diabetes.[13] Pregnant people infected with COVID-19 have increased likelihood of preterm birth, stillbirth, or admission of their neonates. There is also higher absolute risk for admission to intensive care units, preterm birth, and caesarian section, compared with pregnant people without COVID-19.[13] Hitherto, vertical transmission has been rare, and COVID-19 pregnancies do not seem to affect rates of miscarriage and congenital anomalies, nor does infection seem to negatively impact outcome of neonates.[14] However, there are still many unknowns as well as conflicting studies, with numerous

variables to be accounted for with vaccinations and the emergence of different virus variants.

Immunology of Pregnancy

A broader look at the role of the immune system and inflammation during pregnancy could be clarifying as to the pathophysiology of SARS-CoV-2 in pregnant people. Historically, the idea that pregnancy is associated with immune suppression and that pregnancy is a state of immunological weakness was pervasive.[15] The fetus was once perceived as a semi-allograft expressing paternal proteins that would be rejected under normal circumstances by the maternal immune system; therefore, successful pregnancy requires the dampening of the maternal immune response.[16] More recent research has challenged this idea, instead proposing that the maternal immune system is merely modulated, but not suppressed, by the placenta and growing fetus.[15] During normal pregnancy, the decidua contains many immune cells – macrophages, natural killer (NK) cells, dendritic cells, and regulatory T cells.[17-19] Mor et al. suggests that differentiation and function of immune cells infiltrating the implantation site depends on the placental microenvironment – that trophoblast cells can stimulate the differentiation of immune cells into a self-supporting phenotype.[15] In *in vitro* studies, they found that trophoblast cell-conditioned media is able to induce secretion of cytokines beneficial for its own development and function, as well as attract and recruit monocytes/macrophages around trophoblast-derived structures to aid its migration and invasion into the maternal endometrium.[19]

Pregnancy involves three distinct immunological phases. A proinflammatory

phase is necessary for implantation, placenta formation, and the first and early second trimesters of pregnancy. Successful implantation during the early phases of pregnancy requires the blastocyst to break through the epithelial lining and endometrial tissue, as well as take over the maternal vasculature in order to establish a blood supply between the placenta and the fetus.[20] Early implantation is characterized by a high level of proinflammatory T helper (Th)-1 and cytokines secreted by endometrial cells and immune cells recruited to the site of implantation.[21-22] It was shown that depletion of uterine dendritic cells (uDCs) resulted in serious impairment of implantation and resorption of the embryo.[23] This inflammatory environment is also needed for healing of the uterine epithelium and elimination of cellular debris.[24] Clinically, this phase is sometimes reflected as "morning sickness" as the mother's body struggles to adapt to the presence of the fetus and changes are further regulated by ovarian hormones.[15]

In the second immunological phase, an anti-inflammatory state predominates during the time of fetal growth and development, and mother-placenta-fetus are in symbiosis.[15] In the final phase, influx of immune cells into the myometrium and renewed inflammation is required to stimulate contraction of the uterus, delivery of the baby, and expulsion of the placenta during parturition.[25] Thus, pregnancy is both proinflammatory and anti-inflammatory, varying by gestational stage.

The Placenta and Viral Infection During Pregnancy

The human placenta is the fetus' defense against microbials. It begins as fetus-derived trophoblasts forming the trophectoderm – the earliest barrier an embryo has

against infection – and dramatically transforms in architecture through the trimesters of pregnancy.[26] Near the end of the first trimester, the placenta undergoes significant morphological change resulting in the growth of chorionic villi which connects the fetus-derived placenta with the maternal blood supply.[26] Trophoblast stem cells generate cytotrophoblasts (CTBs) and the syncytiotrophoblast (STB), which mature into proliferative mononuclear cells and multinucleated cells to establish the placenta and surface of the placental chorionic villi. Remodeling of the maternal microvasculature by extravillous trophoblasts (EVTs) aids the development of the villous structure, and allows for the maternal blood to come into direct contact with the fetal placenta.[26] This direct contact of maternal blood and the placenta is distinct to the first semester, versus the later stages when there is a division of blood supply with the establishment of the haemochorial placenta.[26] The maternal decidua further protects the placenta as a layer populated by various immune cells.[27] The composition of the decidua also changes with gestational age. In addition to these physical and immune barriers, villous trophoblasts create a chemical barrier against microbial vertical transmission by secreting immunomodulators such as antiviral interferons, antiviral microRNAs, and other cytokines. Interferon-stimulated genes (ISGs) have a strong cytotoxic and pro-inflammatory influence.[26] This chemical barrier too is altered throughout pregnancy with fluctuating levels of the different immunomodulators and cytokines. The variations in the placenta's defensive ability throughout gestational stage have been tested and observed through studies of other viruses.

Lessons from other Viruses

Review of other viral infections during pregnancy can provide insight into risk assessment, as well as hints on guidance on management and treatment of pregnant people infected with SARS-CoV-2. Like SARS-CoV-2, influenza, Zika (ZIKV), and rubella (RV) are RNA viruses.[28] Due to a lack of proofreading ability, RNA viruses have high mutation rates compared to DNA viruses.

Pregnant people, along with pediatric and geriatric populations, are at high risk of developing serious or possibly fatal influenza infections during both pandemics and seasonal epidemics. During the 1918 H1N1 and 1957 H2N2 pandemics, as well as the 2009 H1N1 pandemic, pregnancy increased risk of complications. In the more recent 2009 pandemic, approximately 5% of total deaths were pregnant people though they constituted only 1% of the total population.[29] Interestingly, the greatest risk of severe influenza was found for those at a later gestational stage. People in their last trimester of pregnancy are at higher risk of death due to infection than nonpregnant people during the 1918 H1N1 pandemic, 1974 and 1993 influenza seasons.[30] Furthermore, even pregnant people without pregnancy-associated cardiopulmonary comorbidities were shown to be at a greater risk of influenza-related hospitalization and death.[30] This suggests that regardless of the various anatomical changes related to pregnancy, a distorted immunological profile itself is sufficient to cause severe disease.[31] Death of the fetus, low weight, and preterm delivery were some of the negative fetal outcomes observed. Vertical transmission, however, was not observed suggesting that fetal outcomes are due to indirect effects of influenza infection.[31]

The TORCH pathogens are notably vertically transmissible and known to increase risks for miscarriages, stillbirth, preterm birth, fetal brain damage, intrauterine growth restrictions, and other fetal abnormalities.[26] The acronym TORCH comprises of toxoplasma, others, rubella (RV), cytomegalovirus (CMV), and herpes. "Others" includes parvovirus B19, human immunodeficiency virus (HIV), hepatitis viruses, Zika virus (ZIKV), and more.[26] TORCH infections can cause adverse effects that are pathogen or placenta mediated, and/or through inflammation-induced premature delivery, as well as effects that are not manifested till after delivery.[26]

Zika is an arbovirus that is transmitted by *Aedes* mosquitoes as well as vector-independent routes such as sexual, blood transfusion, and vertically from mother to fetus. ZIKV caused an outbreak of > 2,000 cases of congenital disease in 2015 – 2016, with its focal point in Brazil.[26] Maternal ZIKV infections are sometimes asymptomatic, but 4-7% of pregnancies result in miscarriage, and 5-14% are associated with microcephaly, intrauterine growth restriction (IUGR), hepatosplenomegaly, intrahepatic calcifications, ventriculomegaly, intracerebral calcifications, stillbirth/miscarriage, and developmental delays.[26] The wide range of neurological sequelae from infection is grouped as ZIKV syndrome. How the Zika virus (ZIKV) breaches the placenta is not fully known, however, evidence indicates that human placental trophoblast cells, endothelial cells, and Hoffbauer cells are susceptible to infection and replication by Zika virus. In animal models, maternal systemic inflammatory response is heightened with elevated levels of interleukins associated with inflammation such as IL-1, IL-2, IL-6, IL-7, IL-15, and IL-16. Infection with ZIKV has also been shown to suppress interferon-mediated antiviral

activity and obstruction of immune pathways.[26] A CDC report found that among pregnancies with confirmed ZIKV infection, Zika-associated birth defects were more prevalent among infants born to mothers exposed during the first trimester.[32] In U.S. states and territories, about 2 in 25 (8%) of pregnant people infected during the first semester had babies with Zika-associated birth defects, compared to 6% in the second trimester and 3.8% in the third.[32]

Also causing congenital disease and more well-known is rubella virus (RV), which caused a major epidemic in the US from 1964-1965.[33] The CDC estimates that 12.5 million people got rubella, with 11,000 pregnant people losing their babies and 2,100 newborn deaths.[33] 20,000 babies were born with congenital rubella syndrome (CRS) – a range of birth defects like growth delays, cataracts, glaucoma, deafness, congenital heart problems, defects in other organs, and intellectual disabilities.[33] RV enters the fetus through maternal blood and infects the epithelium of chorionic villi and the endothelium of the placental vasculature. It then spreads through the vascular system of the developing fetus, causing cytopathic effects to its organs. The effects of RV on the developing fetus are cumulative, beginning with disruption of gene expression leading to impaired development of sensory organs. In RV-infected endothelial cells, there were increased levels of inflammatory chemokines. Infection also resulted in significant changes in interferon stimulation in the placenta. In consistence, RV vaccination altered the cellular immune profile to be more proinflammatory with elevated levels of IL-6, TNF, and granulocyte macrophage colony-stimulating factor (CM-CSF), along with decreased level of IL-10.[28] Like in Zika, vertical transmission rate of RV from mother to

fetus is highest in the first trimester – as high as 81% - compared to 25% in the second trimester. Interestingly, the risk of fetal infection increases again in the third trimester from 35% at 27–30 weeks, to nearly 100% for fetuses exposed beyond 36 weeks.[33] However, congenital defects seem to be limited to pregnancies in which the mothers were infected during the first 16 weeks of pregnancy. For those infected after 20 weeks of gestation, there is rarely a risk of CRS.[33]

If Zika and Rubella serve as experience, it is likely that infection with SARS-CoV-2 also poses the greatest risks to the developing fetus during the first trimester. There remains, however, a lack of evidence.

EXISTING RESEARCH

COVID-19 Infection During Pregnancy

Vertical Transmission & Virus Receptor Protein Expression at the Maternal-Fetal Interface

Vertical transmission of SARS-CoV-2 is rare and currently estimated to about 2-3%.[34] There have been sporadic reports and documented cases of feto-placental infection with and without fetal sequelae that have a wide range of accuracy and reproducibility, and varying methods of virus detection.[35-38] A 2021 systematic review of current literature based on early RNA detection of SARS-CoV-2 post-delivery concluded that though vertical transmission is possible, it occurs only in a minority of births to mothers infected the third trimester.[34] However, there is a lack of first trimester data and no assessment could be made of vertical transmission rates in early pregnancy and the risks of subsequent fetal morbidity and mortality.[34]

For vertical transmission to occur, the pathogen must bypass the placenta, the maternal-fetal interface. In addition to the forementioned lungs, heart, gastrointestinal tract, kidneys, liver, and vascular endothelium, tissue tropism of SARS-CoV-2 extends to the placenta.[9] ACE2 is expressed in the placenta, specifically in the STB, CTB, endothelium, and vascular smooth muscle from primary and secondary villi.[39] It has been postulated that ACE2 alters vascular flow by activating the renin-angiotensin system (RAS) in chorionic villi and extravillous trophoblast, and aids the invasion of the trophoblast.[39, 40] Whether ACE2 co-factor TMPRSS2 functions similarly in the placenta as in the lungs is not yet confirmed.[41]

Edlow et al. at Massachusetts General Hospital sought to characterize mechanisms of SARS-CoV-2 infection at the placenta. In a prospective cohort study including 127 pregnancies from three tertiary care centers in Boston, 64 women with SARS-CoV-2 confirmed through nasopharyngeal swab RT-PCR were recruited from April to June 2020, and followed up through July 2020 along with 63 women negative for SARS-CoV-2.[42] Outcomes measured were SARS-CoV-2 viral load in maternal plasma or respiratory fluids, umbilical cord plasma, as well as SARS-CoV-2 RNA in the placenta. SARS-CoV-2 antibodies from maternal and cord plasma were also quantified.[42]
In their cohort, they found no maternal viremia, placental infection, or vertical transmission of SARS-CoV-2.[42] They proposed that these findings were due to patterns of placental SARS-CoV-2 receptor distribution. Pathologic examinations of 88 placentas (44 from women with SARS-CoV-2 and 44 from women without) and immunohistochemistry (IHC) on placental tissue sections showed reduced co-expression

and colocalization of placental ACE2 and TMPRSS2.[42] ACE2 expression was identified on membranous syncytiotrophoblast (ST) with bias to the stromal side of the cell.[42] Expression of TMPRSS2, however, was weak in the villous endothelium and not present on the ST membrane.[42] It is probable that virus adhesion to the ST and entry into the villous trophoblast are limited due to the polarity of ACE2 expression away from maternal blood, along with the widespread absence of TMPRSS in the ST. Additionally, they found compromised and ineffectual transplacental transfer of anti- SARS-CoV-2 antibodies which indicate that neonates could be left at risk of infection.[42]

Though this study had a large cohort with contemporaneous controls, it was limited by its recruitment of controls through convenience sampling. This resulted in demographic disparities between cases and controls, as the Latinx community was disproportionately affected by COVID-19 disease in the greater Boston area. Furthermore, all of the SARS-CoV-2 positive patients were diagnosed in late gestation, with the majority in the third trimester.[42] Of the SARS-CoV-2 positive participants, the majority had asymptomatic (36%) or mild (34%) disease. There were no participants with detectable viremia in the cohort, and it cannot be determined from this study whether absence of viremia truly correlates with pregnancy, or with lack of placental infection and vertical transmission.[42] Nonetheless, the speed at which this study was deployed – timed with the peak of the pandemic onset – allowed for a special opportunity to investigate transfer of COVID virus antibodies due to native infection during the last trimester.[42] Furthermore, it provided an explanation for the low rates of vertical transmission with pathological evidence at the placenta.

A follow-up to this study examined SARS-CoV-2 receptor protein expression patterns throughout gestation, from first through third trimester.[43] As the placenta changes throughout gestation, Roberts et al. aimed to determine whether the polarized expression pattern of ACE2 found in the previous study is present in first and second trimester placentas. Lack thereof may indicate increased susceptibility of the placenta to SARS-CoV-2 during early gestation, and greater risk of vertical transmission, possibly with damaging fetal effects.[43]

The authors studied 12 cases ranging in gestational age from 5.3 weeks to near term at 36.0 weeks. Only pre-pandemic women or women testing negative for SARS-CoV-2 infection who underwent a therapeutic pregnancy termination or preterm delivery between January 1, 2019 and December 1, 2020 with pathology samples at Massachusetts General Hospital were included. Placental and villous tissue along with pathology reports were reviewed, and immunohistochemistry studies were completed staining for ACE2 monoclonal antibody and TMPRSS2 antibody.[43]

Similar to the earlier study, they found that TMPRRS2 expression was usually not detectable in the villous endothelium or the extravillous trophoblast, and very rarely detectable in the ST (2 of 12 cases) or the placental endothelium (2 of 12 cases.)[43] The TMPRSS2 expression pattern was described as cytoplasmic to faintly membranous. ACE2 expression, however, was found to be mixed during gestation with circumferential ST expression more common in early gestation (2 of 12 cases) than in later gestation (1 of 12 cases). Overall, there was still a distinct ACE2 expression polarity in the ST with basal expression favored over apical expression (10 of 12 cases).[43] Based on this

evidence, it is likely that pregnancies in the earlier gestational stages may be more vulnerable to SARS-CO-V-2 infection and the effects of infection compared to those in the later stages of gestation.[43]

This study was very limited by its small sample size also did not include any SARS-CoV-2-infected placentas. Due to the rarity of SARS-CoV-2 infected placentas and none found from the first or second trimester, the authors instead chose to investigate the native expression pattern of the SARS-CoV-2 receptors.[43] Their finding however, is supported by experience with other TORCH and TORCH-like infections where infection during early gestation tends to cause more fetal morbidity than those in later gestation.[44, 45]

A study by Lu-Culligan et al. at Yale further corroborated the presence of ACE2 in ST cells of healthy placenta during early pregnancy, and rarity in normal placentas at full term.[46] In contrast, in the placenta of infected individuals with COVID-19 at term, they found widely expressed ACE2.[46] Their *in vitro* experiments confirmed that trophoblasts from healthy term placentas isolated from placental cells from elective cesarian sections are innately susceptible to SARS-CoV-2.[46] Both their *in situ* and *in vitro* analyses of ACE2 protein expression over the course of pregnancy suggest that low ACE2 expression could be protective of term placenta from SARS-CoV-2- infection.[46] Though ACE2 and TMPRSS2 were found to have low mRNA expression based on the study's transcriptomic data, high expression of *CTSL* — the gene encoding Cathepsin L protease — was found.[46] Furthermore, their elaborate study — described further in the sections below — examined immune activation and the inflammatory response at the

placenta following infection.

Inflammatory & Immune Response in SARS-CoV-2 Infected Pregnant People

Even in the absence of vertical transmission and infection of the placenta, maternal SARS-CoV-2 infection can trigger immune response and inflammation both at the maternal-fetal interface as well as systemically. The magnitude and appropriateness of the immune response is key — as it could be beneficial in fighting infection, but also detrimental as overresponse could lead to pregnancy complications and has implications for maternal and fetal health. Lu-Culligan et al. sought to elucidate the potential immune mechanisms shielding placental cells from infection in vivo during full-term pregnancy through bulk and single-cell transcriptomics analyses.[46]

In this prospective study by Lu-Culligan et al., 15 COVID positive women donated specimens during the month before or at delivery. In the majority of cases, the placenta did not have SARS-CoV-2 viral RNA found. No difference between the virus' S1 spike protein IgG and IgM antibodies was found in the plasma of the majority of virus infected women either. The ELISA absorbance results revealed no obvious differences between asymptomatic and symptomatic infected, or between pregnant and non-pregnant infected subjects.[46]

However, large scale RNA sequencing of placenta villi and assessment of differential placental gene expression in SARS-CoV-2 infected pregnant people and uninfected controls showed significant immune response revealed significant immune response to viral infection even when no placenta viral RNA was detected. Analyses by Gene Ontology describe a general upregulation of pathogen-response immune pathways,

and a downregulation in physiological pathways involved in maintaining a normal pregnancy. [46] IFN-regulated genes were particularly affected, highlighting the sensitivity and impact of even distal infection on the placenta.[46]

Examination of transcriptomic changes of cell interactions at the placenta also revealed unusual interactions between NK cells and T cells in placentas from SARS-CoV-2 infected women, but not in uninfected controls.[46] Based on this data, the authors believed that inappropriate stimulation of NK cells and their dysregulation in late gestation may increase risk of complications in SARS-CoV-2 infected pregnant people.[46] This is supported by the study's bulk sequencing data which implicated the HLA-C gene involved in regulating NK and T cell tolerance, as well as HSPA1A (Hsp70) which was upregulated at the maternal-fetal interface.[46] These results are shown in Figure 1 below. Hsp70 has been hypothesized as an alarmin shown *in vitro* to stimulate inflammation associated with parturition and pre-term birth. It is additionally correlated with endothelial activation in placental vascular disease, and its serum levels are elevated in cases of pre-eclampsia.[49] Extracellular Hsp70 stimulates proinflammatory cytokines and are increased in individuals displaying HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome.[50] This is supported by several reports such syndromes in pregnant people with COVID-19.[51]

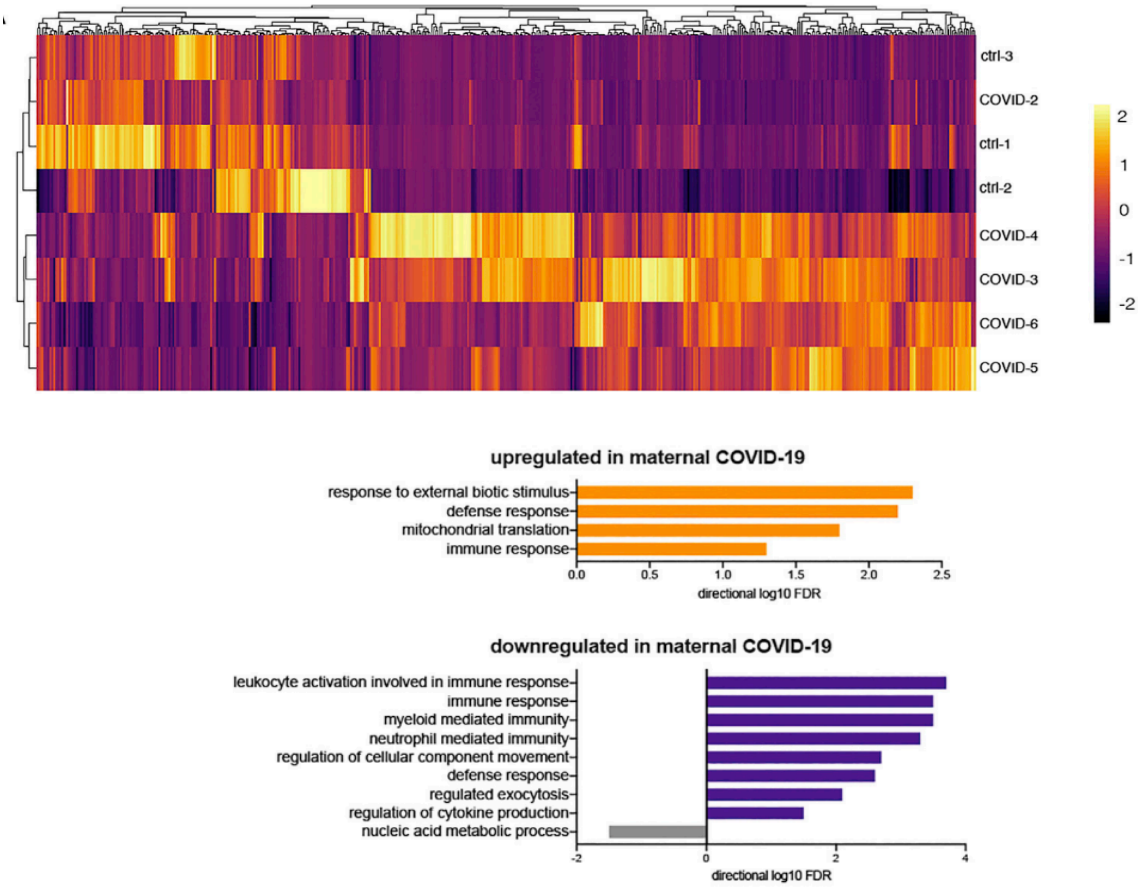

FIGURE 1: Bulk Sequencing Data of Upregulated and Downregulated genes in COVID-19 infected pregnancies vs uninfected controls. Figure amended from Figure 4 in Lu-Culligan et al.[46]

Like many of the previous studies, this study was also limited by assessing placentas only from women infected with SARS-CoV-2 near or at term.[46] It therefore does not explain pathological or inflammatory placental changes resulting from infection during the early trimesters. Additionally, SARS-CoV-2 nasopharyngeal screening did not take place throughout the entire duration of pregnancy, so the timing of infection is unclear.[46] Within this time gap, acute inflammatory and immune response could have affected the strength of the results. Nonetheless, Lu-Culligan et al. provided ample

evidence of immune activation and inflammatory response at the placenta even in the absence of measurable local SARS-CoV-2 viral invasion, which suggests elevated risk of complications in COVID-19 affected pregnancies.

Beyond the maternal-fetal interface, systemic immune and inflammatory response following SARS-CoV-2 infection during pregnancy was evaluated by Sherer et al. at Johns Hopkins Hospital in a pre-printed study.[52] They assessed the impact of COVID-19 infection on inflammatory and humoral responses in both fetal and maternal samples, as well as compared antibody responses to SARS-CoV-2 in pregnant and non-pregnant people. The study involved two cohorts – a pregnant cohort comprising of 33 pregnant people who either tested positive (n = 22) or negative (n = 11) for SARS-CoV-2 prior to delivery, and a non-pregnant cohort comprising of 17 women within reproductive age (18-48 years old) who all tested positive for SARS-CoV-2.[52] Immune responses to SARS-CoV-2 were analyzed, and proinflammatory and placenta cytokine mRNAs, neonatal Fc receptor (FcRn) receptor expression, and tetanus antibody transfer in maternal and cord blood samples were quantified. Anti-spike (S) IgG, anti-S-receptor binding domain (RBD) IgG, and neutralizing antibody (nAb) responses to SARS-CoV-2 in serum or plasma from the cohorts and cord blood were quantified.[52]

Sherer et al. found that SARS-COV-2 positive pregnant people expressed more IL-1β but not IL-6 in blood samples collected within 14 days (based on the incubation period of the virus) after a confirmed positive test.[52] Comparable patterns were observed in the fetal side of placentas, chiefly among asymptomatic pregnant people. Their data suggests selective IL-1β mRNA upregulation, especially early after

infection and on the fetal surface of the placenta in even non-severely ill pregnant people infected with SARS-CoV-2.[52] This is concerning because IL-1β activation can cause unfavorable fetal outcomes, especially neurodevelopmental disorders.[53, 54] Pregnant people with confirmed SARS-CoV-2 infection also had lower anti-S-RBD IgG titers and were less likely to have measurable nAb as compared to non-pregnant people.[52] This study found that maternal transfer of nAb during pregnancy was disrupted by SARS-CoV-2 infection even though placental FcRn expression remained unaffected.[52] Substantiating the previous study, Sherer et al. noted that COVID-19 infected pregnancies were characterized by inflammation at the placenta. Further, they added that decreased antiviral antibody responses may indicate reduced efficacy of viral therapeutics in pregnancy.[52]

This study was limited by its small sample size and reliance on convenience sampling, which resulted in substantial differences in age, race, and ethnicity between SARS-CoV-2-infected pregnant and non-pregnant people.[52] Sample collection was influenced by time of delivery as well as symptom presentation. There were also no results presented that stratified and accounted for differences in gestational stage of the pregnant people, and these findings are not generalizable to women in early stages of pregnancy. Due to the difficulty in establishing when each participant was infected with SARS-CoV-2, Sherer et al. also faced the challenge of a time gap which could have impacted their study results on the robustness of cytokine and humoral response. However, their study adds to the growing body of evidence of pregnancy-specific responses to viral infection, and concern for potential adverse outcomes for the fetus.

Tanacan et al. studied the systemic immune and inflammatory response following SARS-CoV-2 infection compared COVID-19 positive pregnant people (n = 90) against a gestational age-matched control group lacking risk factors (n = 90).[55] Each control and COVID-19 positive group enrolled 30 subjects per trimester and the demographic factors evaluated are shown in Table 1. [55] Various levels of interferons, interleukins, clinical labs, CRP levels and other parameters were assessed between groups and are shown in Table 2.[55]

TABLE 1: Demographic Features of Pregnant Women With and Without COVID Enrolled in Study Table taken from Tanacan et al. 2021. [55]

Variables	Pregnant women with COVID-19 infection (n = 90)	Pregnant women without any defined risk factor (n = 90)	P value
Maternal age (years) (median, IQR)[a]	28 (6)	27 (5)	0.64
Gravidity (median, IQR)[a]	2 (1)	2 (2)	0.07
Parity (median, IQR)[a]	1 (2)	1 (1)	0.08
BMI (median, IQR)[a]	25.6 (4.5)	25.4 (5.2)	0.53
Gestational age at hospital admission (weeks)(median, IQR)[a]	24 (21)	24 (22)	0.69
Pregnancy complication rate (n, %)[b]	15 (16.6%)	3 (3.3%)	0.01
Pregnancy complication type (n, %)[b]			0.03
Miscarriage (n, %)	2 (2.2%)	0 (0%)	
GDM (n, %)	3 (3.3%)	1 (1.1%)	
GHT (n, %)	3 (3.3%)	1 (1.1%)	
IHCP (n, %)	2 (2.2%)	0 (0%)	
Preeclampsia (n, %)	2 (2.2%)	0 (0%)	
Preterm delivery (n, %)	3 (3.3%)	1 (1.1%)	
Hb (g/dl)(median, IQR)[a]	11.5 (1.7)	12 (1.5)	0.003
Leukocyte (10^9/L) (median, IQR)[a]	6.1 (3.4)	8.8 (3.1)	<0.001
Platelet (10^9/L)(median, IQR)[a]	220 (84.5)	251 (81.7)	<0.001
Lymphocyte (10^9) (median, IQR)[a]	1.2 (6.6)	1.8 (5.8)	<0.001
ESR (mm/hr)(median, IQR)[a]	32 (12.5)	24.5 (17.5)	<0.001
CRP (mg/L)(median, IQR)[a]	11.5 (10.5)	4 (3.5)	<0.001
Procalcitonin (ng/ml) (median, IQR)[a]	0.03 (0.02)	0.01 (0.01)	<0.001
Ferritin (ng/ml)(median, IQR)[a]	21 (28)	12 (10)	<0.001
D-dimer (mcg/ml) (median, IQR)[a]	1.2 (1.1)	0.6 (0.5)	<0.001
LDH (IU/L)(median, IQR)[a]	200 (50)	180 (60)	0.01
NLR (median, IQR)[a]	3.7 (3)	3.4 (1.8)	0.56
IFN γ (ng/dl)(median, IQR)[a]	20 (18)	17.5 (5)	<0.001
IL-2 (pg/ml)(median, IQR)[a]	90 (20)	115 (33.75)	<0.001
IL-6 (pg/ml)(median, IQR)[a]	6.5 (7.8)	3.6 (1.25)	<0.001
IL-10 (pg/ml)(median, IQR)[a]	8.6 (4.6)	9.6 (2.9)	0.002
IL-17 (pg/ml)(median, IQR)[a]	76 (28)	86 (31.5)	0.03

BMI: Body-mass index, COVID-19: Coronavirus disease 2019, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, GDM: Gestational diabetes mellitus, GHT: Gestational hypertension, Hb: Hemoglobin, ICHP: intrahepatic cholestasis of pregnancy, IFN γ: Interferon gamma, IL: Interleukin, IQR: Interquartile-range, LDH: Lactate dehydrogenase, NLR: Neutrophil to lymphocyte ratio.

[a] Statistical analysis was performed by Mann-Whitney *U* test.

[b] Statistical analysis was performed by chi-square test.

TABLE 2: Levels of Interleukins in Control and Test Groups During Pregnancy. Shown are the different levels of various interleukins in each trimester. N=30 for control and pregnant women with COVID-19 infection. (Table taken from Tanaka et al., 2021). [55]

Variables	COVID-19 group first trimester (n = 30)	Control group first trimester (n = 30)	COVID-19 group second trimester (n = 30)	Control group second trimester (n = 30)	COVID-19 group third trimester (n = 30)	Control group third trimester (n = 30)	P value[a]
IFN γ (ng/dl) (median, IQR)	17.5 (5)	15 (3)	20 (10)	15 (2.5)	37.5 (35)	20 (17.5)	**<0.001**[c]
IL-2 (pg/ml) (median, IQR)	80 (20)	105 (25)	90(23.75)	145 (40)	95 (15)	110 (20)	**<0.001**[d]
IL-6 (pg/ml) (median, IQR)	6.1 (4.3)	3.3 (1.2)	6.3 (6.2)	3.5 (2.3)	8.1 (15)	3.8 (1.5)	0.30
IL-10 (pg/ml) (median, IQR)	7 (4.5)	10.8 (5)	8.8 (3.5)	9.6 (5)	9.6 (5.6)	9.3 (2.6)	**<0.001**[e]
IL-17 (pg/ml) (median, IQR)	68 (26)	104 (19)	80 (26)	76 (18)	84 (35)	80 (24)	**0.01**[f]

IFN γ: Interferon gamma, IL: Interleukin.

[b]Pairwise comparisions was performed by Mann-Whitney *U* test.

[a] Statistical analysis analysis was performed by Kruskal-Wallis test between the groups.

[c] Statistically significant difference was found between the study and control groups in the third trimester ($p = 0.001$).

[d] Statistically significant differences were found between the study and control groups in the first and second trimesters ($p < 0.001$ for both).

[e] Statistically significant differences was found between the study and control groups in the first trimester ($p = 0.01$).

[f] Statistically significant differences was found between the study and control groups in the first trimester ($p = 0.04$).

In their SARS-CoV-2 infected study group, Tanacan et al. found a higher pregnancy complication rate, more signs of inflammation with elevated ESR and CRP, as well as other markers of infection and injury such as procalcitonin, D-dimer, LDH, ferritin, IFN g, and IL-6. [55] In their uninfected control group, participants were found to have markedly elevated hemoglobin, leukocytes, platelets, lymphocytes, and IL-2, IL-10, and IL-17. [55] The decrease in some of these interleukins, as seen in the SARS-CoV-2 infected pregnancies, have been linked to reduced immune tolerance and associated with pregnancy complications and losses. [55] Furthermore, statistically significant differences in multiple interferon and interleukin levels were measured between the trimesters, and also correlated with disease severity.[55] Statistical significance is defined by $p < 0.05$. Of note, the elevated IL-6 finding in the infected group differed from the unchanged IL-6 level in Sherer et al.'s study.[55] It is possible that differences in disease severity of their infected groups may account for the incongruity. This study was also inconsistent with previous literature in finding significantly reduced levels of IL-2 in COVID-19-infected pregnant people, especially in the early trimesters.[55] Others have observed increased levels of IL-2 in COVID-19 infection in parallel with disease severity.[55] This may indicate a pregnancy-specific distorted immune response. Overall, these results supports the findings that SARS CoV2-2 infection leads to significantly increased inflammation in pregnant women, further complications and immunological changes.

This study was limited by its small population and relatively small number of cytokine types examined, as well as disproportionate number of mild cases. Furthermore, it lacked any supporting histopathological evidence and relied solely on body fluid

samples. However, this study was distinct in its evaluation of cytokine levels between pregnancy trimesters, and its correlation with disease severity.

Placental Histopathology

The effects of SARS-CoV-2 infection and subsequent immune response are often notably visible at the maternal-fetal interface, at the placenta, regardless of disease severity and successful vertical transmission. However, studies are inconsistent in their characterization of these effects. The most recent studies attribute these inconsistencies to the rapidly evolving and multiple emerging variants of SARS-CoV-2, each with distinct virulence and infectivity.

In a meta-analysis of literature up to April 2021, Hessami et al. found no correlation between SARS-CoV-2 and maternal or fetal placental malperfusion, nor maternal or fetal inflammatory response.[56] Aggregating 15 studies published on 19,025 COVID-19-infected placentas and 18,326 negative controls, the only pathological finding in the placentas of SARS-CoV-2-infected pregnant people was an increased prevalence of perivillous fibrin deposition.[56] In very similar meta-analysis of publications from December 2019 to August 2021, Suhren et al. likewise found that the frequency of both vascular and inflammatory lesions in COVID-19-infected placentas was comparable to those non-infected.[57] They concluded that there is no evidence of a characteristic COVID-19 associated pattern of placenta pathology.[57]

In stark contrast, Watkins et al. broadcasts a triad of pathologic features that define SARS-CoV-2 placentitis.[58] In a small retrospective study of 7 placentas from women with active SARS-CoV-2-infection, the authors sought to characterize SARS-

CoV-2-infected placentas (as demonstrated by RNA in situ hybridization) irrespective of vertical transmission and to evaluate the frequency of C4d activation in these cases.[58] Of the 7 placentas (1 of which was a non-fused diamniotic-dichorionic twin placenta), 5 of the 8 neonates (including a stillborn) were negative for SARS-CoV-2 and tested negative for neonatal infection.[58] All 7 placentas were positive for SARS-CoV-2 infection by RNA in situ hybridization and exhibited varying degrees of histiocytic intervillositis, perivillous fibrin deposition, and trophoblast necrosis.[58] Even without transplacental transmission, damage to the COVID-19-infected placentas was present. This damage was deemed likely to be mediated by complement activation as evidenced by C4d deposition lining the trophoblastic surface of villi.[58] It is not clear whether viral load or timing of infection played in a role preventing neonatal infection, or whether there is an intrinsic intra-placental antiviral response to maternal infection as posited by Lu-Culligan et al.[46, 58]

In the previously described study by Lu-Culligan et al. at Yale, histological analysis was also performed for those with confirmed COVID.[46] 22 of the 39 (56%) SARS-CoV-2-infected people identified were symptomatic, all but 1 resulted in live births, and 27 had placenta available (with one extra placenta from dizygotic twins.)[46] Pathology assessment of the placenta specimens was performed without knowledge of infection status, with attention to signs of villitis, intervillositis, chorioamnionitis, changes in decidual lymphocyte population, and vascular perfusion defects in the fetus and mother. [46] Compared to pathology from matched pre-pandemic controls, there were no significant differences found – with the only exception of intervillous fibrin, which

was seen in approximately one third of the infected cases and not at all in the controls. [46] However, there was no correlation of this finding with any clinical features.[46]

Intervillous fibrin builds with diminished maternal perfusion and increased coagulability, and impairs thrombolysis by of trophoblasts.[46] Lu-Culligan et al. posited that maternal COVID infection may stimulate the maternal endothelium, with endothelitis leading to ineffective lysis of fibrin which further causes excess fibrin build-up, similar to that in preeclampsia.[46] Concurrently, stimulation of immune cells at the placenta and circulating pro-inflammatory cytokines may activate pro-coagulation signals in the maternal-fetal microenvironment, including tissue factor synthesis from syncytiotrophoblasts causing an accumulation of fibrin.[46] This is supported by earlier described results from the study's single-cell transcriptomic analysis described earlier, which additionally suggests the occurrence of injury to placentas of infected individuals by oxidative damage.[46] These changes, too, are often seen in other pregnancy-related disorders like pre-eclampsia and pre-term labor.[46]

In two more recent studies focusing on data from the Delta wave, placentitis as defined by Watkins et al. was observed. Huynh et al. additionally noted intraparenchymal thrombohematomas along with SARS-CoV-2-placentitis among COVID-19-infected pregnancies.[59] Shook et al. further associated SARS-CoV-2-placentitis with fetal death (2) and distress (1) following confirmed maternal infection with the SARS-CoV-2 delta variant.[14]

Possible Congenital Effects of SARS-CoV-2 Infection

About 30 months since the beginning of the COVID-19 pandemic, data on follow-ups with offspring from SARS-CoV-2-infected pregnancies is just emerging. To assess whether in utero exposure to SARS-CoV-2 is linked with risk for neurodevelopmental disorders in the first year after birth, Edlow et al. performed a retrospective cohort study examining live offspring of all mothers who gave birth between March and September 2020 at 6 Massachusetts hospitals across two health systems.[5] The cohort incorporated 7772 live births (7466 pregnancies, 96% singleton) with 222 of those births to SARS-CoV-2 positive mothers.[5] The International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10) was used to code for neurodevelopmental disorders over the first year of life, along with sociodemographic and clinical features of mothers and offspring pulled from electronic health records.[5] They found that preterm delivery was more likely among infected mothers 14.4% (32) versus uninfected 8.7% (654) (P = .003).[5] In both unadjusted models (odds ratio [OR], 2.17 [95%CI, 1.24-3.79]; P = .006) and models adjusted for race, ethnicity, insurance status, offspring sex, maternal age, and preterm status (adjusted OR, 1.86 [95%CI, 1.03-3.36]; P = .04), maternal SARS-CoV-2 positivity during pregnancy was linked with higher rate of neurodevelopmental diagnoses.[5] Third trimester infection was also linked with consequences of greater magnitude (adjusted OR, 2.34 [95%CI, 1.23-4.44]; P = .01.)[5]

Results are still considered preliminary due to the limited duration of follow-up, and it is possible that additional neurodevelopmental effects will appear later. The

analyses in this study may be premature in rendering some diagnoses, as the offspring in the study are younger than the age at which some neurodevelopmental disorders are usually diagnosed.[5] The study authors also acknowledge possible ascertainment bias, as parents of offspring whose mothers were ill during pregnancy have a greater tendency to seek out evaluation. The study was also limited in its retrospective study design and dependence on ICD-10 codes, as a prospective cohort study would be more sensitive and allow for incorporation of neurocognitive phenotyping to help define any associations with maternal SARS-CoV-2 infection.[5] Misclassification of diagnoses also could not be excluded as the study was completed in an open health system. Furthermore, the study had a comparatively small sample size which precluded evaluation of maternal infection severity, and as their total rate of SARS-CoV-2 positivity in pregnancy was low.[5] Despite the limitations, this study was a necessary initial step given the plethora of data regarding congenital syndromes and neurodevelopmental outcomes as a result of infection of known viruses like Zika, cytomegalovirus, and rubella.

Shook et al. published a review on COVID-19-infected pregnancies and implications on fetal brain development.[60] Three possible pathways through which maternal COVID infection could influence fetal brain development are illustrated in Figure 2.

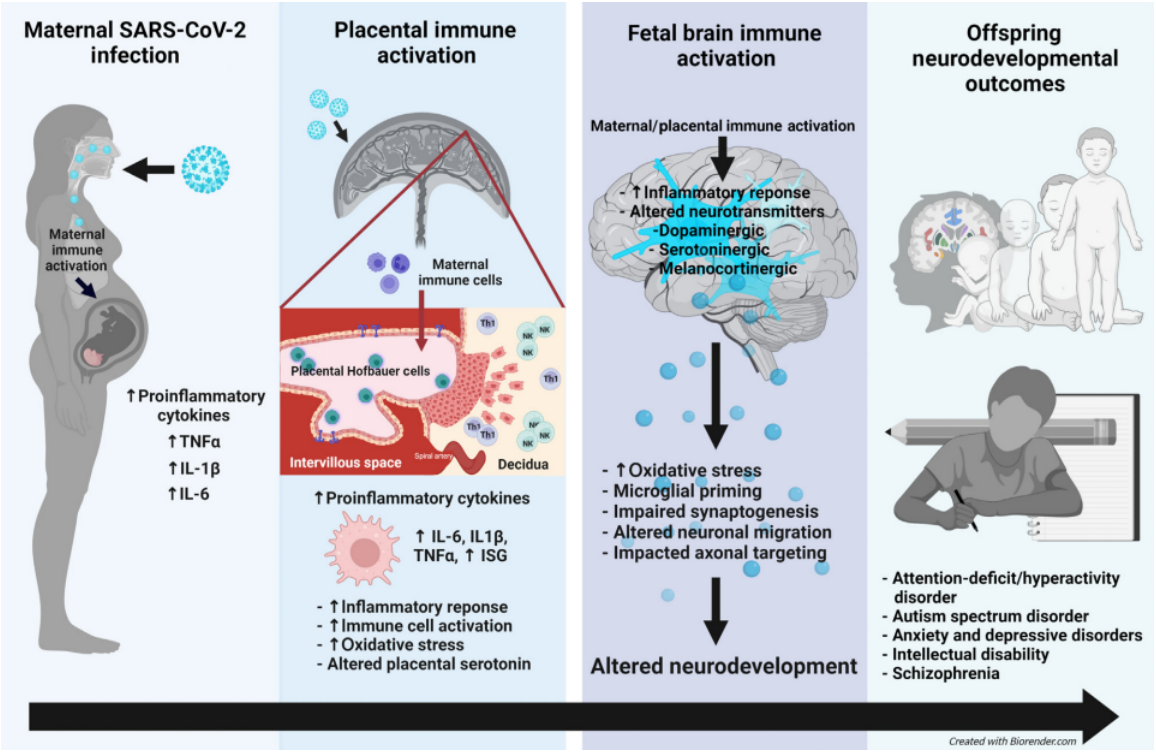

FIGURE 2: Possible pathways of SARS-CoV-2 impact on fetal neurodevelopment. Taken from Shook et al.[60]

Especially in light of the rarity of transplacental transmission of SARS-CoV-2 and direct fetal infection in observations so far, they contend that maternal and placental immune activation (MIA) in response to viral infection is the most common pathway by which maternal COVID infection confers morbidity to offspring.[60] This is supported by both clinical and pathological human evidence, as well as data from animal models. They illustrate that MIA increases proinflammatory cytokines, TNFα, IL-1β, as well as IL-6. This activates the immune cells and response within the placenta – further increasing levels of proinflammatory cytokines, IL-6, IL-1β, TNFα, and upregulating interferon-stimulated genes (ISG).[46] In the placenta, inflammatory response is triggered along with increased immune cell activation and oxidative stress.[60] Placental serotonin, which is the

principal source of serotonin for the developing fetal brain, is altered and dysregulated.[61] This leads to increased inflammatory response at the fetal brain, along with altered dopaminergic, serotoninergic, and melanocortinergic neurotransmitter signaling, and aberrant microglial priming.[62-64] Synaptogenesis could be impaired with altered neuronal migration and affected axonal targeting.[60] Along with increased oxidative stress, these changes lead to altered neurodevelopment which could manifest as attention-deficit/hyperactivity disorder, autism spectrum disorder, anxiety and depressive disorders, intellectual disability, and schizophrenia.[61] Shook et al. also note that timing of infection, variant strain, fetal sex, other prenatal exposures, and perinatal circumstances can all intersect to influence maternal-fetal defense and fetal outcomes.[60]

PROJECT METHODS

Study Design

The study design will be a longitudinal prospective cohort study to evaluate in utero exposure to SARS-CoV-2 during different gestational stages on maternal and fetal immune response, and fetal outcomes.

Study Population and Sampling

Participants will be recruited over a period of five years from 12 Boston area hospitals with obstetrics care. These sites will include but not be limited to hospitals affiliated with Boston University.

Inclusion criteria would be women aged 18–49 with known pregnancy with viable fetus and COVID-19 infection during pregnancy as verified by RT-PCR.

Exclusion criteria would be in vitro fertilization-conceived pregnancies, people unable to provide informed consent, and people with other major infection during pregnancy (e.g. influenzas A or B, cytomegalovirus, toxoplasma, rubella, syphilis, HIV.) An estimated sample size of 3000 patients with 1500 patients per group (COVID-19 positive and COVID-19 negative) will be used to achieve an alpha level of 0.05 and power of 0.80 based on an effect size of 0.2. Sampling method: non-probability sample.

Exposure

Over the study period of 5 years, it is expected that 8% of the study population will be exposed to COVID-19 based on the most recent test positivity rates in Massachusetts provided by the CDC.

Outcome

Patients that have a positive test for SARS-CoV-2 infection during pregnancy, including at the time of delivery, will be assigned to the study group. Study group will be divided based on initial positive test for SARS-CoV-2 during the first trimester (through 12 weeks), second trimester (13-27 weeks), or third trimester (28 weeks till birth). The control group will include patients who test negative for SARS-CoV-2 during pregnancy. Early detection can be aided by a SARS-CoV-2 screening antigen test.

Individuals in the study will have serum inflammatory biomarkers and pregnancy hormones (E2, E3, and progesterone) collected every 6 weeks up through the time of delivery to assess for changes throughout pregnancy.

Post-delivery follow-up for offspring outcomes of participants will occur every 3 months until the end of the study.

Study Outcomes and Measures

Primary outcome measures:

- Immune cell population at the placenta (maternal decidual natural killer and T cells) at the time of delivery
- Inflammatory biomarkers (e.g. TNF, CRP, IL-6, IL-17α, IL-1Rα, IL-1β, IL4, IL13, IFN γ, IL-2, and IL-10) in maternal blood every 6 weeks up through time of delivery, and in placental plasma and cord blood at the time of delivery
- Pregnancy-associated hormones estradiol (E2), estriol (E3), and progesterone in

maternal blood every 6 weeks

- Placental pathology taken time of delivery, or at the time of dilation and curettage with consent from patient.
- Pregnancy loss at less than 20 weeks' gestation
- Intrauterine fetal demise (IUFD), pregnancy loss at 20 weeks or more
- Maternal death
- Neonatal death
- Low birth weight (< 2500g)
- Preterm delivery (at gestational age < 37 weeks)
- Preeclampsia, eclampsia, or HELLP (Hemolysis, Elevated Liver Enzymes, Low Platelets) syndrome
- Small for gestational age (SGA)
- Large for gestational age (LGA)
- Diagnosis of a birth defect, malformation, developmental disorder per ICD-10 from birth through end of follow-up.

Secondary outcome measures (exposures): vaginal delivery, cesarian delivery, unscheduled cesarean delivery, Apgar scores at 1 and 5 minutes, and admission to NICU. Co-variate: offspring gender.[66]

Recruitment

All pregnant patients aged 18-49 who present for obstetrics care at participating sites will be flagged and considered for enrollment. Those who fit the study criteria will be

recruited and informed consent will be obtained. If the patient has not already had a COVID-19 RT-PCR test, they will immediately be tested once they agree to participate. Recruitment will continue for 5 years or before, if adequate sample size has been reached.

Data Collection

Participant demographic information including maternal age, race, ethnicity, and insurance status will be obtained from the electronic medical record. In cases where record or information is missing or not available, patients will be asked to provide it upon recruitment. With permission from the patient, other information that will be extracted from the medical record will include medical history such COVID-19 vaccination status, maternal gestational diabetes, maternal preeclampsia, maternal hemorrhage, history of autoimmune disease and/or serious infection. Where a diagnosis is unclear, the patient's provider will be contacted for clarification. All collected data will be entered and tracked via a secure electronic database.

Data Analysis

Analyses will be performed using R project for Statistical Computing. Statistical significance will be defined as uncorrected 2-tailed $P < 0.05$. One-way ANOVA will be used to compare clinical and demographic features between the groups.

Placental specimens will be examined by two independent pathologists blinded to the individual's SARS-CoV-2 infection status. For statistical comparisons of histologic features of cases versus controls, chi-square tests will be performed.

Fisher's exact test will be used to determine if there are non-random associations between the study's categorical variables. Mann-Whitney test will be used to compare the difference in the dependent variable between the two groups. and Kruskal-Wallis will also be used to validate the associations between variables and whether they are statistically significant.

Timeline and Resources

This study will span 5 years (or less, if sufficient sample size is reached) and be submitted to the Boston University Medical Center IRB for review and approval in June 2022. Following approval from the IRB, the recruitment process will begin in August 2022 and end August 2025 to allow for at least 1 year of follow-up of offspring born in 2026. Participants in both the study groups and control group will need to have blood collected every 6 weeks. Remaining specimen and sample collections will be done at the time of delivery. After birth, offspring of the participants will be evaluated by a designated study pediatrician at one of the participating institutions at 2 weeks, and then every 6 weeks following till the end of study in August 2027.

The study will require a primary investigator, a co-investigator, a study coordinator at each participating institution for project oversight and organization. Another study coordinator for the entire study is also needed to help manage operations and data collection from all the institutions. 5 research assistants at each site will be needed for data collection and tracking. A designated study pediatrician, a study obstetrician or maternal-fetal health specialist, and 2 pathologists are also necessary at each participating

institution to ensure patient safety and to aid with interpretation of clinical data. A statistician will perform the statistical analysis of the collected data.

Institutional Review Board

This study entails recruitment and participation of human subjects that will require full review and approval from the Boston University Medical Center Institutional Review Board, as well as IRBs from the participating hospital institutions. The study necessitates full board review as it includes collection of blood and tissue samples. Approval from the IRB will be obtained before the recruitment of participants.

Limitations

Due to the longitudinal nature and 5-year duration of the study, some participant attrition is expected for a variety of reasons, including the development of other health conditions that can impact participation or confound data. The high level of participant involvement requiring blood samples every 6 weeks is acknowledged.

Serum blood and hormone samples only provide a general systemic view and does not fully capture effects of interactions at the tissue level due to receptor dependence and the ability of SARS-CoV-2 to alter gene expression. Determining time of infection is challenging and a positive SARS-CoV-2 RT-PCR test may not be an accurate reflection due to lack of symptoms and/or sufficient viral load, and incubation period. Furthermore, as it is difficult to predict the course of the pandemic, the emergence of multiple variants within the duration of the study is likely to add confounders as well.

The COVID-19 pandemic continues to evolve and new variants continue to emerge. Mutations in SARS-CoV-2 are known to affect its ACE-2 receptor affinity, infectivity, viral replication, transmissibility, resistance to neutralizing antibodies and immune escape, disease severity, reinfection risk, and clinical outcomes. Replicating the same study in a mouse pregnancy model with the previously and currently circulating SARS-CoV-2 variants of concern would be elucidating for a more controlled comparison and to adjust for known differences in outcomes.

To better evaluate changes in pathology by gestational age following infection, an animal model would also be helpful due to the difficulty and risks of obtaining placentas or placental samples from earlier human gestational stages. Future studies could follow the method proposed by Nakayama et al. which was used to study the impact of timing of Zika virus infection at different embryonic stages on fetal and postnatal outcomes in mice.[65] Strains of Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) variants of SARS-CoV-2 could be used in place of the ZIKV strain.[12]

CONCLUSION

Discussion

This study aims to address the gaps in knowledge regarding the timing of viral infection during gestation as well as its impact on immune response and maternal-fetal outcomes. The majority of existing studies involve in utero exposure during the third trimester; however, it is not known whether teratogenic effects are more likely with earlier maternal infection, and whether the effects accumulate as pregnancy progresses. The evidence on whether and how infection during the first and second trimesters leads to pregnancy loss or fetal demise is still unclear. The pathogenesis of SARS-CoV-2 on implantation, placental perfusion and development, and the early embryo is to be determined.

As the inflammatory state changes with each trimester in pregnancy, it is important to study how SARS-CoV-2 viral infection may alter the immune and cytokine profiles at each gestational stage and what the maternal and fetal effects are. This study additionally tracks and aims to account for the immunomodulatory effects of pregnancy hormones, and possible viral effects on hormone levels.

This study's longitudinal design follows individuals through their pregnancy and post-delivery, enabling cohesion of care for patients and serving as another source of control. It also enables observation of more acute changes in maternal pregnancy hormones and inflammatory biomarkers, in addition to longer-term consequences and developmental outcomes of the fetus. The dynamic changes throughout pregnancy calls for increased granularity of timepoints and more frequent data collection which this study aims to provide.

Supplementing this human study with an animal model in the future would also allow for more complete experimental control and helps limit confounding factors while avoiding the risks due to placental sampling during pregnancy. This is especially important as all the reviewed studies may not be generalizable for different virus variants, and may be confounded due to the emergence of different variants during the course of their study.

Given the rarity of placental infection and vertical transmission, population-level studies will be needed. As the virus continues to evolve simultaneously with human immunity and with mixed vaccination efforts in every locality, it may also be difficult to generalize larger studies. Local-level studies may be beneficial as well.

The placental microenvironment is dynamic and complex in a healthy pregnancy, and how it is perturbed by SARS-CoV-2 infection is likely to have a cascade of direct and indirect effects.

Summary

The surge of research since the start of the COVID-19 pandemic has been impressive, but much remains unknown. There is a paucity of data on altered immune responses to SARS-CoV-2 infection during pregnancy, and few studies that recognize and address the changing immune and inflammatory states throughout gestation. The study proposed here aims to address this gap and to approach these questions in a comprehensive methodical way through its longitudinal prospective design in parallel with replication in an animal model.

Implications - Clinical and Public Health Significance

Results from this study would help with risk assessment based on timing of maternal infection with SARS-CoV-2 and in utero exposure. A better understanding of the impact of infection on the changing inflammatory state could improve medical management, the selection of pro versus anti-inflammatory therapeutics, at each stage of an infected pregnancy. Furthermore, it could shed light on new pregnancy-specific and safe drug targets based on immune and endocrine profiles.

This study would also have implications on public health, as knowledge on long term consequences of SARS-CoV-2 affected pregnancies would aid with allocation and development of neonatal, neurodevelopmental, and mental health resources. For each individual, knowing the risks and consequences could help them decide whether to pursue pregnancy if they become infected. This study would aid with patient counseling and family planning. The current pandemic presents an additional challenge in women's health, and it is with hope that this research helps improve understanding of the intrinsic dynamics of pregnancy and its response in the face of SARS-CoV-2 and future viruses.

LIST OF JOURNAL ABBREVIATIONS

AJP Rep	AJP Reports
Am J Epidemiol	American Journal of Epidemiology
Am J Obstet Gynecol	American Journal of Obstetrics and Gynecology
Am J Perinatol	American Journal of Perinatology
Am J Reprod Immunol	American Journal of Reproductive Immunology
Ann N Y Acad Sci	Annals of the New York Academy of Sciences
Arch Pathol Lab Med	Archives of Pathology & Laboratory Medicine
BMJ	BMJ: British Medical Journal
Br J Exp Pathol	British Journal of Experimental Pathology
Cell Stress Chaperones	Cell Stress & Chaperones
Clin Obstet Gynecol	Clinical Obstetrics and Gynecology
Int J Neuropsychopharmacol	International Journal of Neuropsychopharmacology
JAMA Netw Open	JAMA Network Open
J Clin Invest	Journal of Clinical Investigation
J Clin Virol	Journal of Clinical Virology
J Exp Med	Journal of Experimental Medicine
J Immunol	Journal of Immunology
J Infect Dis	Journal of Infectious Diseases
J Matern Fetal Neonatal Med	Journal of Maternal-Fetal and Neonatal Medicine
J Med Virol	Journal of Medical Virology
J Reprod Immunol	Journal of Reproductive Immunology
J Virol	Journal of Virology

Med (N Y)	New York Medical Journal
Nat Commun	Nature Communications
Nat Med	Nature Medicine
Nat Rev Microbiol	Nature Reviews. Microbiology
PLoS Med	PLoS Medicine
Prog Neurobiol	Progress in Neurobiology
Reprod Sci	Reproductive Sciences
Sci Rep	Scientific Reports
Sci Transl Med	Science Translational Medicine
Semin Fetal Neonatal Med	Seminars in Fetal and Neonatal Medicine
Trends Mol Med	Trends in Molecular Medicine: Cell Press

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