

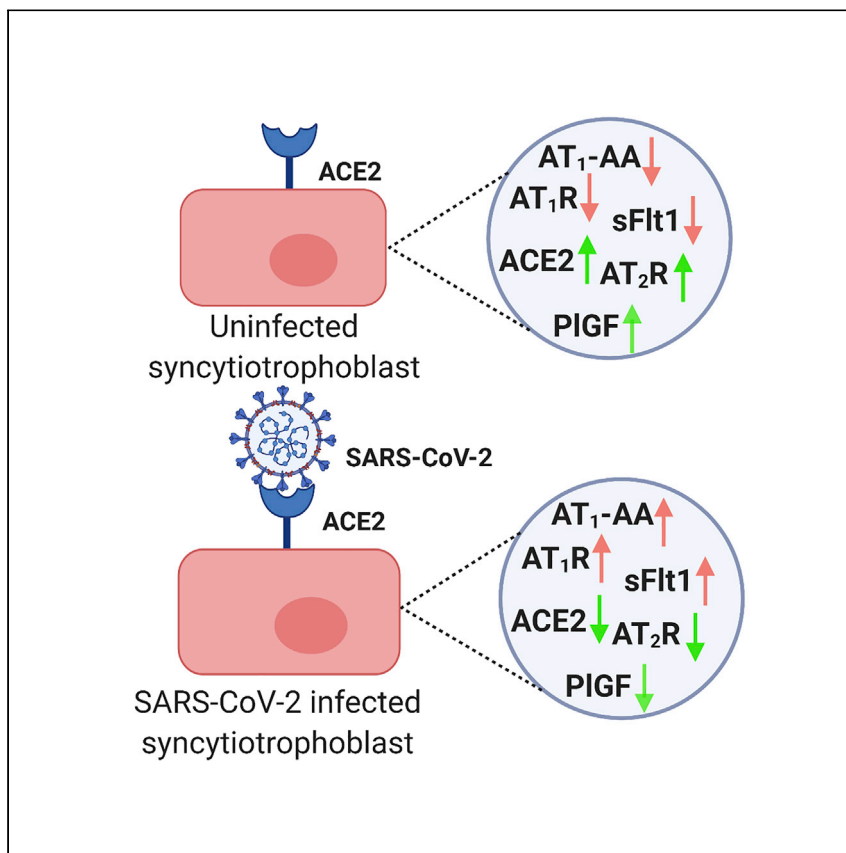


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Clinical and Translational Article

SARS-CoV-2 colonization of maternal and fetal cells of the human placenta promotes alteration of local renin-angiotensin system



Verma et. al. show that the novel coronavirus, SARS-CoV-2, infects maternal and fetal cells in the human placenta, and infection is associated with decreased angiotensin-converting enzyme 2 (ACE2) expression and alterations in the blood-pressure-regulating renin angiotensin system (RAS). Alterations in this system are implicated in hypertensive disorders of pregnancy, such as pre-eclampsia. These findings shed light on how SARS-CoV-2 infection may increase risk of adverse pregnancy outcomes.

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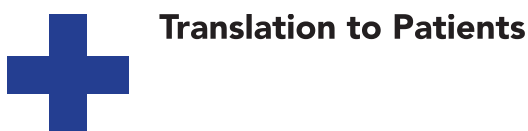
Highlights

SARS-CoV-2 colonizes multiple compartments at the maternal-fetal interface

Placental ACE2 expression decreases with SARS-CoV-2 infection

SARS-CoV-2 infection leads to dysregulation of placental renin-angiotensin system

SARS-CoV-2 infection is associated with markers of hypertensive disorders of pregnancy



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Clinical and Translational Article

SARS-CoV-2 colonization of maternal and fetal cells of the human placenta promotes alteration of local renin-angiotensin system

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SUMMARY

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection appears to increase the risk of adverse pregnancy outcomes, such as pre-eclampsia in pregnant women. The mechanism(s) by which this occurs remains unclear.

Methods: We investigated the pathophysiology of SARS-CoV-2 at maternal-fetal interface in pregnant women who tested positive for the virus using RNA *in situ* hybridization (viral RNA), immunohistochemistry, and hematoxylin and eosin staining. To investigate whether viral infection alters the renin angiotensin system (RAS) in placenta, which controls blood pressure, we treated human trophoblasts with recombinant spike protein or a live modified virus with a vesicular stomatitis viral backbone expressing spike protein (VSV-S).

Findings: Viral colonization was highest in maternal decidua, fetal trophoblasts, Hofbauer cells, and in placentas delivered prematurely. We localized SARS-CoV-2 to cells expressing angiotensin-converting enzyme 2 (ACE2) and demonstrate that infected placentas had significantly reduced ACE2. In response to both spike protein and VSV-S, cellular ACE2 decreased although angiotensin II receptor type 1 (AT₁R) increased with concomitant increase in soluble fms-like tyrosine kinase-1 (sFlt1). Viral infection decreased pro-angiogenic factors, AT₂R, and placental growth factor, which competitively binds to sFlt1. Sera from infected pregnant women had elevated levels of sFlt1 and angiotensin II type 1-receptor autoantibodies prior to delivery, both signatory markers of pre-eclampsia.

Conclusions: SARS-CoV-2 colonizes ACE2-expressing maternal and fetal cells in the placenta. Infection in pregnant women correlates with alteration of placental RAS. As RAS regulates blood pressure, SARS-CoV-2 infection may thus increase adverse hemodynamic outcomes, such as pre-eclampsia in pregnant women.

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INTRODUCTION

The outbreak caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019 has led to a global health crisis, causing more than 115 million infections and 2.5 million deaths worldwide. In March 2020, the World Health Organization (WHO) declared a pandemic of coronavirus disease 2019 (COVID-19). The burden of disease has not been borne equally among the population, with more severe disease noted in the elderly, men, and those with cardiovascular

Context and significance

SARS-CoV-2 infection appears to increase the risk of pre-eclampsia, a dangerous condition in pregnancy characterized by high blood pressure. However, the mechanisms by which it may do so are currently unknown.

Researchers at the Washington University School of Medicine in St. Louis, Missouri show that SARS-CoV-2 colonizes multiple cell types in the human placenta. In addition, infection is associated with alterations in the placental renin-angiotensin hormonal system, which regulates blood pressure leading to increased markers of pre-eclampsia. This study provides insights into the pathology of SARS-CoV-2 infection in the placenta and supports a link between SARS-CoV-2 infection and development of pre-eclampsia in pregnant women through changes in the renin-angiotensin system.



comorbidities^{1–4}. Initially the majority of studies to date suggest that COVID-19 did not cause increased mortality in pregnant women compared to non-pregnant women⁵. There are studies which do not support evidence of SARS-CoV-2 infection in the placenta and also disprove the possibility of vertical transmission and pregnancy-related complications.^{6–19} However, a case report published early on in the pandemic showed the death of 7 pregnant women (out of 9), as well as one who was critically ill and one who recovered after prolonged hospitalization due to severe COVID-19 disease. Comparison of severity of infection to family members of all 9 women showed disease severity in COVID-19-infected pregnant women.²⁰ Recent studies have reported that pregnant women are more likely to be positive for SARS-CoV-2 and have both asymptomatic and severe disease outcomes as compared to non-pregnant women.^{21–25} SARS-CoV-2 infection has been reported to put pregnant women and their fetuses at risk by increasing the possibility of pregnancy-related complications, including pre-eclampsia and intrauterine growth restriction (IUGR).^{23,26–31} A number of recent studies now indicate increased risk for perinatal complications in pregnant patients with severe COVID-19, including higher incidence of pre-eclampsia and preterm birth in SARS-CoV-2-infected women.^{18,32} That said, the risk for vertical transmission,^{33,34} although not nil, appears to be minimal.^{6,7,35–48} Thus, it remains imperative to understand and elucidate mechanisms of SARS-CoV-2 at the maternal-fetal interface dictating its pathogenesis during pregnancy.

Multiple studies have demonstrated the presence of SARS-CoV-2 in placental villi, mainly in syncytiotrophoblasts (STBs), placental submembranes (chorionic and amniotic membranes), maternal macrophages, and fetal macrophages (Hofbauer cells [HCs]) at different gestational stages of pregnancy.^{29,44,48–55} A second-trimester miscarriage was reported in a case study of a woman with SARS-CoV-2 infection, and virus was found in placental submembranes with immune cell infiltration in placental cotyledons.⁵⁶

SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as a cellular entry receptor via viral spike (S) protein, which binds the N-terminal peptidase domain of ACE2.^{57–59} ACE2 is a key component of the renin-angiotensin system (RAS), a cascade of vasoactive peptides controlling angiogenesis, blood pressure, and fetal development by regulating inflammation.⁶⁰ Viral endocytosis into host cells is also mediated by transmembrane protease, serine 2 (TMPRSS2), so maximum viral tropism for host cells depends on co-expression of ACE2 and TMPRSS2.^{57–59} Notably, placental trophoblasts are highly responsive to the changes in angiotensin receptor concentrations that occur during pregnancy.^{60,61} Expression of ACE2 and TMPRSS2 has been found during early pregnancy at the maternal-fetal interface in villous cytotrophoblasts (CTBs), STBs, and extravillous trophoblast cells (EVTs), but ACE2 expression decreases as the pregnancy progresses.^{44,62,63} Although some doubts have been raised regarding whether ACE2 is present in the placenta,⁶⁴ a number of recent studies have confirmed the expression of ACE2 in placental cells.^{49,50,65–68} The mechanism by which expression of ACE2 changes in the context of COVID-19 remains to be fully elucidated. The objective of our study was to determine whether SARS-CoV-2 affects expression of ACE2 in the placenta and, if so, whether alterations in ACE2 impacts the RAS system, which can contribute to pregnancy outcomes.

Here, we report SARS-CoV-2 colonization in term and preterm placentas from SARS-CoV-2-positive women. SARS-CoV-2 was found in all placental sections, colonizing the basal plate (BP) in EVT, villous tissue (VT) in STBs and HCs, subchorionic (SC)

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Table 1. Demographic and clinical features of COVID-19-negative and -positive women

No.	Maternal age, years	Symptoms	Gestational age at delivery (weeks and days)	Pre-eclampsia features	Mode of delivery	Maternal race
COVID-19-negative women						
P1	30	NA	37 and 3	NA	vaginal	white
P2	33	NA	38 and 1	NA	vaginal	white
P3	33	NA	39 and 2	NA	C-section	white
P4	19	NA	24 and 4 (preterm)	NA	vaginal	African American
P5	24	NA	37 and 1	NA	C-section	African American
COVID-19-positive women						
S1	17	anosmia (no cough or other symptoms)	37 and 1	NA	vaginal	white
S2	32	asymptomatic	37 and 5	chronic hypertension, pre-eclampsia with severe features	vaginal	African American
S3	23	asymptomatic	39 and 3	gestational hypertension	vaginal	African American
S4	28	asymptomatic	23 and 5 (preterm)	NA	C-section	African American
S5	32	cough, dyspnea, chest pain, n/v, chills	38 and 1	gestational hypertension	C-section	African American

region, amniotic epithelium of fetal membrane (FM), and tunica adventitia layer of umbilical cord (UC). Intracellular virus was found in placental trophoblasts and HCs expressing ACE2. Histopathological analyses showed that SARS-CoV-2-infected preterm placentas harbored inflammatory infiltrates, whereas inflammation was negligibly present in non-infected preterm or any term placentas. Transfection of human trophoblasts with SARS-CoV-2 S protein or infection with a live modified virus with a vesicular stomatitis viral (VSV) backbone expressing SARS-CoV-2 S protein triggers downregulation of ACE2 receptor expression while increasing expression of angiotensin II type 1 receptor 1 (AT₁R) and soluble fms-like tyrosine kinase-1 (sFlt1), which are associated with pre-eclampsia pathogenesis. These findings were confirmed in SARS-CoV-2-infected pregnant patients. Thus, SARS-CoV-2, likely via viral S protein, disrupts the placental RAS pathway. In sum, we demonstrate that SARS-CoV-2 colonizes fetal trophoblasts, stromal cells, and macrophages in the placenta, which express the ACE2 receptor. S binding to ACE2 leads to reduction of the receptor expression and results in alterations of the RAS pathway—changes that are similar to those typically noted in pre-eclampsia.

RESULTS

Clinical characteristics

In our study, we used five placentas each from SARS-CoV-2 uninfected and SARS-CoV-2-infected pregnant women. Of the five SARS-CoV-2-infected women, three were asymptomatic, one had only anosmia, and one had cough, dyspnea, chest pain, nausea/vomiting (n/v), and chills. SARS-CoV-2 infection was confirmed by qRT-PCR using nasopharyngeal swabs or saliva. There were no significant demographic differences between cases with positive maternal SARS-CoV-2 testing and our controls, and maternal age ranged from 19 to 33 years (uninfected) and 17 to 32 years (infected; Table 1). One patient (20%) in both the infected and uninfected groups delivered preterm and three (60%) in both groups delivered vaginally. With the exception of one patient, who tested positive and was symptomatic prior to delivery, the patients were positive at delivery. In addition, 3/5 (60%) pregnant patients were diagnosed with chronic hypertension, pre-eclampsia with severe features, or gestational hypertension.

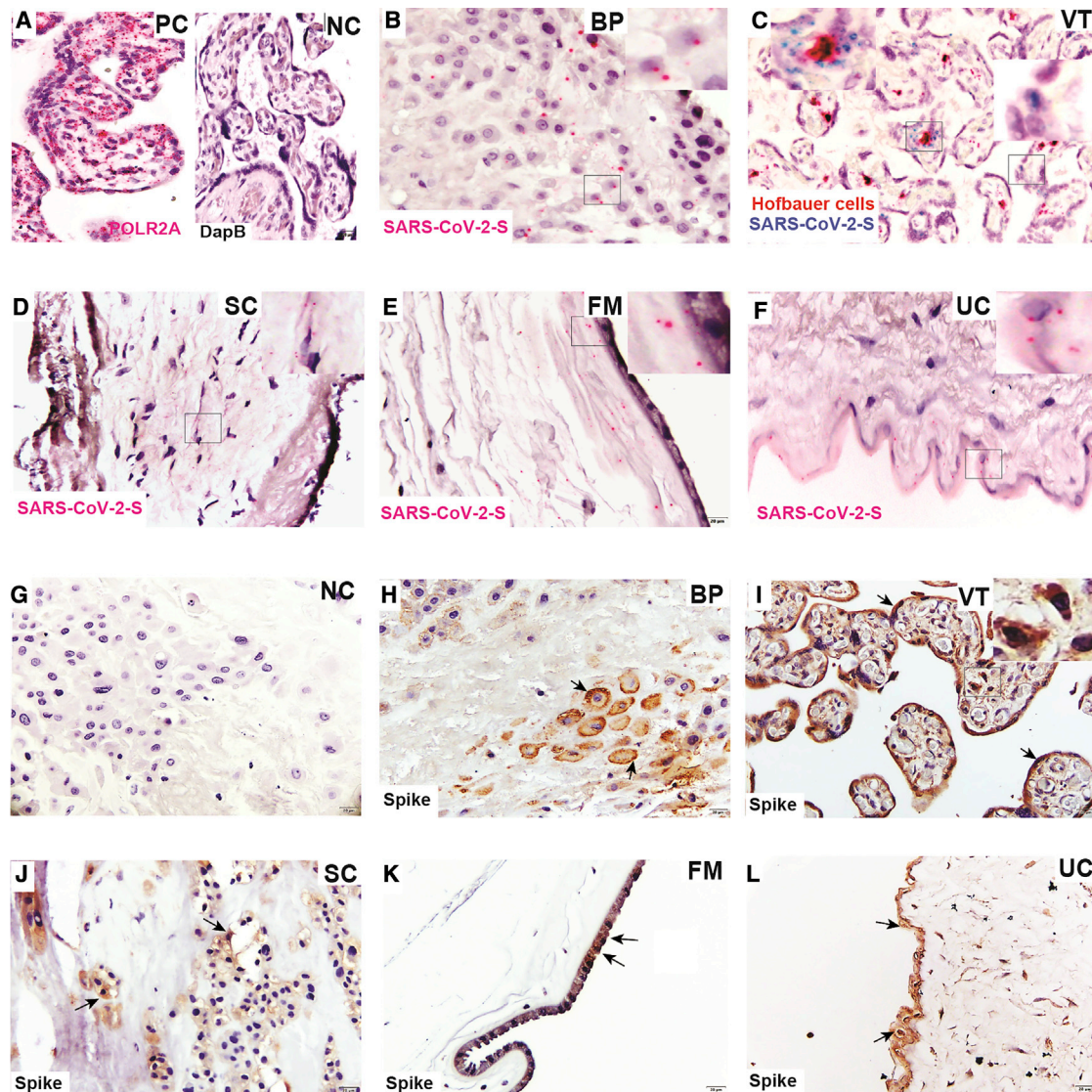


Figure 1. Term placenta from SARS-CoV-2-infected women shows SARS-CoV-2 localization

(A) RNA scope ISH positive control (PC) (RNA polymerase II subunit A [POLR2A]) and negative control (NC) (bacterial gene; DapB). (B–F) RNA-ISH images of SARS-CoV-2-S RNA (B) in the basal plate (BP) in extravillous trophoblast cells (EVTs); (C) villous tissue (VT); syncytiotrophoblasts (STBs) shown in right side inset and Hofbauer cells (HCs) in left side inset; (D) subchorion (SC); (E) fetal membrane (FM); and (F) umbilical cord (UC).

(G) Immunohistochemical (IHC) NC.

(H–L) IHC staining for the presence of viral spike protein in (H) BP; (I) VT, including STBs (black arrow) and HCs (right side inset); (J) SC; (K) FM; and (L) UC. Black arrows show the location of spike protein in respective tissues.

Scale bar represents 20 μ m.

SARS-CoV-2 colonizes multiple compartments in the maternal-fetal interface

Biopsies were taken from multiple sections: (1) BP (or decidua, which represents the maternal face of the placenta); placental VT (VT) (which represents the fetal compartment comprising STBs and CTBs); FMs; SC; and UC. The placental VTs also included fetal macrophages termed Hofbauer cells (HCs). The presence of SARS-CoV-2 was determined in term placental sections from SARS-CoV-2-positive women at RNA (Figures 1A–1F) as well as protein level (Figures 1G–1L) using RNA probes for SARS-CoV-2-S RNA and an antibody against the S protein using RNA scope *in situ*

hybridization (ISH) and immunohistochemistry (IHC), respectively. The corresponding controls for RNAscope ISH and IHC are presented in [Figures 1A and 1G](#), respectively.

SARS-CoV-2-S RNA and S protein localized to EVT_s in the BP and STBs in the villous placenta ([Figures 1B, 1C, 1H, and 1I](#)), supporting recent published studies showing SARS-CoV-2 in the placenta.^{49,50,69} However, we determined that the virus was also found in fetal macrophages present in villous capillaries ([Figure 1C](#), left inset, and [1I](#), right inset), stromal cells of the chorionic plate ([Figures 1D and 1J](#)), and amniotic epithelial cells of the FM ([Figures 1E and 1K](#)). Finally, we note evidence of the virus in the tunica adventitial layer of the UC ([Figures 1F and 1L](#)). Together, our findings indicate that the virus can colonize multiple compartments at the maternal-fetal interface, including trophoblasts, stromal cells, immune cells, and epithelial cells.

Preterm placenta harbors higher abundance of SARS-CoV-2 and histopathological abnormalities

The preterm placenta delivered at 23 weeks and 5 days harbored an abundance of S protein in EVT_s ([Figures 2A and 2G](#)) and in STBs and HCs ([Figures 2B, 2C, and 2H](#)). SARS-CoV-2 S RNA and protein were present in higher abundance in stromal cells of the SC ([Figures 2D and 2I](#)), amniotic epithelial cells ([Figures 2E and 2J](#)), and the UC ([Figures 2F](#), inset, and [2K](#)). These findings indicate that SARS-CoV-2 can colonize all compartments at the maternal-fetal interface even earlier in gestation. Indeed, severity of SARS-CoV-2 infection appears to be greater in preterm than term tissue as observed by RNA-ISH staining of SARS-CoV-2-S ([Figures 1 and 2](#)). Consistent with this, pathological changes were evident in the preterm placenta relative to term placentas. The term placentas did not show any significant histopathological changes ([Figures 3A–3E](#), left panel). However, the preterm placenta had trophoblast giant cells in the BP ([Figure 3A](#), right panel) but no changes in VT ([Figure 3B](#), right panel). The subchorion harbored infiltration of neutrophils indicative of increased inflammation ([Figure 3C](#), right panel). The FM showed amniotic edema and acute chorioamnionitis (inset, [Figure 3D](#), right panel) and the presence of necrotic amniocytes in addition to pigmented macrophages ([Figure 3D](#), right panel). The umbilical cord revealed acute funisitis or vasculitis ([Figure 3E](#), right panel). There was no histological correlation between asymptomatic versus symptomatic placentas of SARS-CoV-2-positive women.

SARS-CoV-2 entry receptor ACE2 is expressed in the placenta and is downregulated upon SARS-CoV-2 infection

SARS-CoV-2 uses ACE2 as entry receptor, which is abundantly expressed in different organs of the body.^{70–73} A few studies predating the pandemic have suggested that the placenta is no exception.^{74,75} Here, using IHC with antibodies to ACE2, we analyzed SARS-CoV-2-positive and negative and preterm and term placentas for localization of ACE2 in multiple compartments. We found ACE2 expression in EVT_s ([Figures 4A and 4D](#)), STBs ([Figures 4B and 4E](#)), HCs ([Figures 4B and 4E](#)), as well as in the FM ([Figures 4C and 4F](#)).

Recent studies have shown that, upon SARS-CoV-2 infection, interaction between viral S protein and an intact and cell-surface-localized ACE2 leads to the internalization of ACE2, which facilitates SARS-CoV-2 entry into host cells, resulting in infection.⁷⁶ Semi-quantitative analysis of IHC images of infected ([Figures 4D–4F](#)) versus uninfected placental sections ([Figures 4A–4C](#)) showed a significant reduction in the level of ACE2 expression after SARS-CoV-2 infection ([Figure 4G](#)). A similar observation was found after western blotting quantification of ACE2 levels in SARS-CoV-2-positive

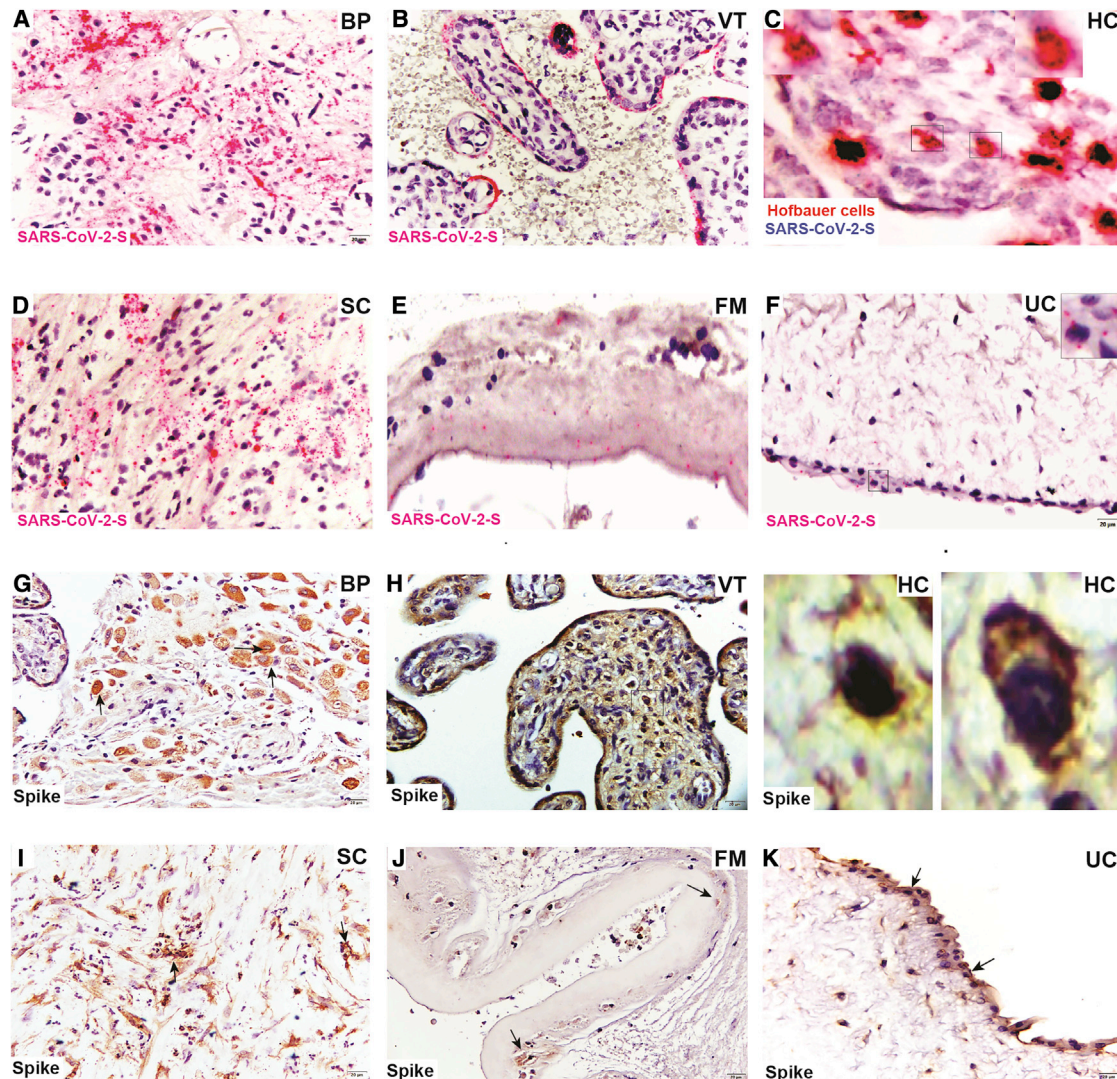


Figure 2. Preterm placenta from SARS-CoV-2-positive woman shows higher SARS-CoV-2 viral presence

(A–F) RNAscope ISH images depict presence of (A) SARS-CoV-2 spike RNA in BP (EVTs), (B) VT in STBs and (C) fetal HCs (insets), (D) SC, (E) FM, and (F) UC (inset).

(G–K) Immunohistochemical staining for SARS-CoV-2 spike protein demonstrating virus localization in (G) BP, (H) VT (including STBs and fetal HCs in insets), (I) SC, (J) FM, and (K) UC.

Scale bar represents 20 μm.

and uninfected term placentas (Figure 4H, left panel). Furthermore, ACE2 levels were relatively lower in preterm placenta, and severe infection was associated with a greater decrease (Figure 4H, right panel). Thus, not only does the placenta express the viral entry receptor ACE2, it is actively responsive to infection, which results in its downregulation, which is itself indicative of infection.

SARS-CoV-2 infection is associated with dysregulation of the RAS pathway

The RAS is a complex cascade of vasoactive peptides controlling angiogenesis, blood pressure, and fetal development and is made up of two major axes: inflammatory angiotensin II (Ang II)/(AT₁R; vasoconstrictive) and the anti-inflammatory protective axis ACE2/Ang-(1-7) (angiotensin II type 1 receptor 2 [AT₂R]; vasodilatory).⁷⁷ SARS-CoV-2 entry via ACE2 causes downregulation of membrane-bound ACE2⁷⁸ in

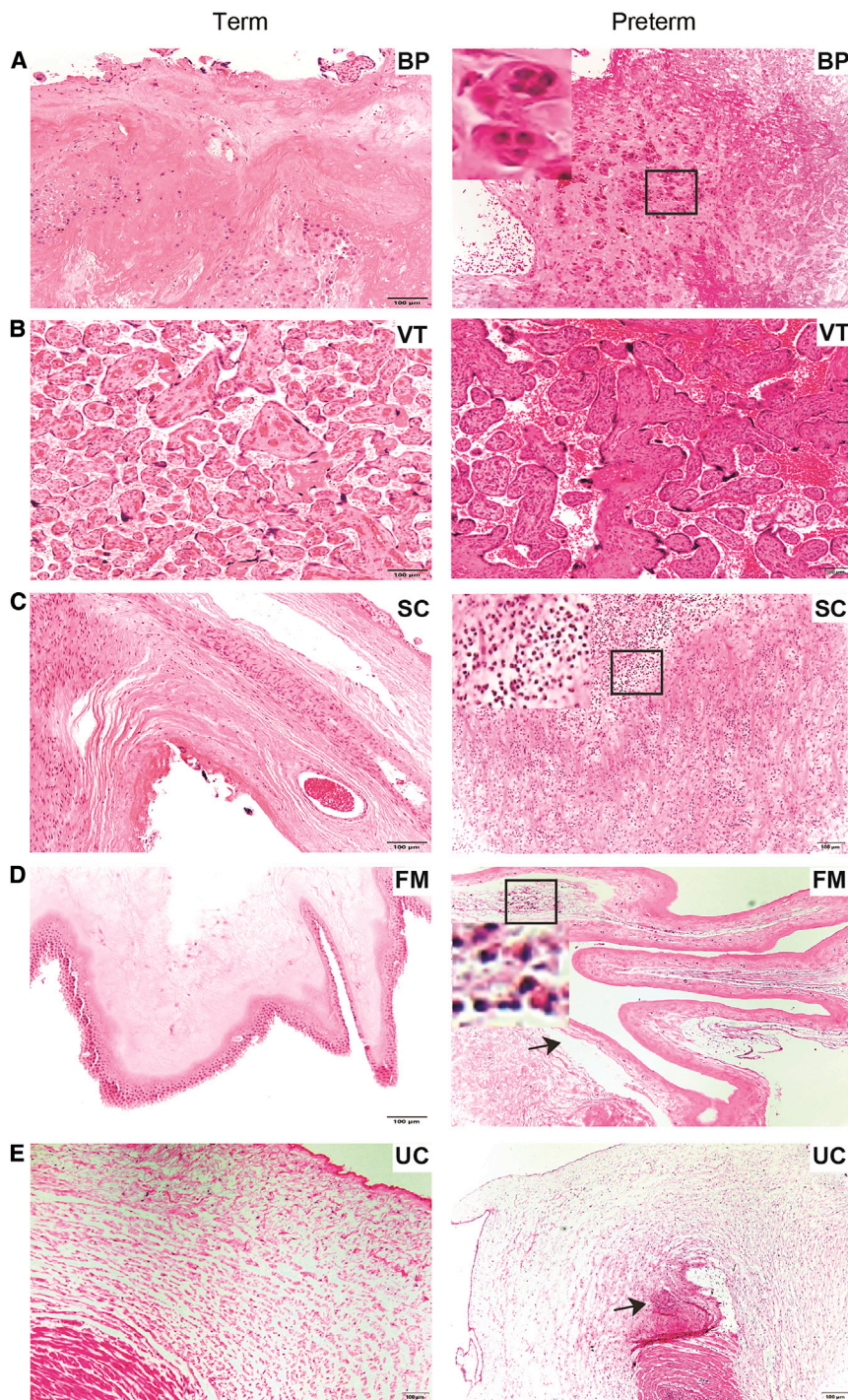


Figure 3. Histopathological findings in SARS-CoV-2-positive preterm placenta

(A–E) (Left) H&E staining of term placental compartments does not indicate obvious histopathological changes. (Right) H&E staining of preterm placental compartments is shown. (A) Trophoblast giant cells (inset) BP. (B) No histopathological defects found in VT. (C) Acute chorioamnionitis, infiltration of neutrophils (inset) in SC. (D) Necrotic amniocyte (inset), edema of amnion (arrow), and pigmented macrophages in FM. (E) Acute funisitis or vasculitis in UC. Black arrows indicate defects in respective tissue. Scale bar represents 100 μ m.

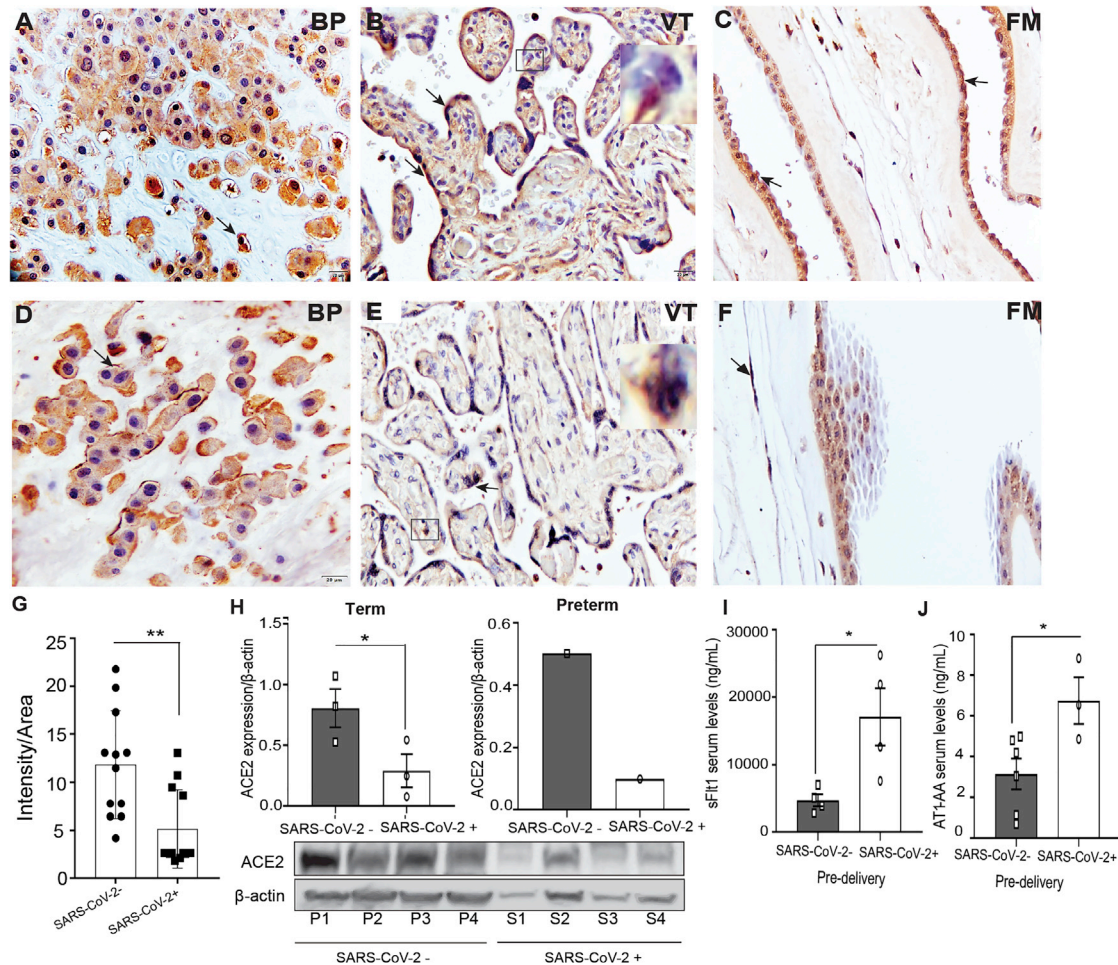


Figure 4. SARS-CoV-2 infection in pregnant women is associated with changes in RAS components

(A–F) Immunohistochemical (IHC) staining for (A) ACE2 in BP, (B) VT (STBs and HC; inset), and (C) FM in uninfected and (D) BP, (E) VT (STBs and HC; inset), and (F) FM in SARS-CoV-2-infected placenta. Arrows indicate expression of ACE2. Scale bar represents 20 μm.

(G) Semiquantitative analysis of ACE2 expression in uninfected and SARS-CoV-2-infected placenta.

(H) Densitometric analysis of ACE2 protein expression in term (left panel) and preterm (right panel) placenta of SARS-CoV-2-negative and positive pregnant women. Absolute quantification was performed after normalization with β-actin. The respective blots of ACE2 expression and loading control β-actin are shown.

(I and J) Pre-delivery soluble fms-like tyrosine kinase 1 (sFlt1) and angiotensin II type 1-receptor autoantibody (AT1-AA) levels in sera of uninfected and SARS-CoV-2-infected pregnant women.

P1–P3, SARS-CoV-2-negative placenta; P4, SARS-CoV-2-negative preterm placenta; S1–S3, SARS-CoV-2-positive placenta; S4, SARS-CoV-2-positive preterm placenta. Values are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

other tissues, an occurrence we now show is recapitulated in the placenta. We reasoned that reduced ACE2 due to SARS-CoV-2 infection could impact the RAS system. Impaired RAS function and increase in the inflammatory AT₁R could lead to increased production of pre-eclampsia markers, including soluble fms-like tyrosine kinase-1 (sFlt1).^{79–82} Consistent with this, we observed increased levels of sFlt1 in the pre-delivery sera of SARS-CoV-2-infected versus uninfected pregnant women (Figure 4I). Pre-eclampsia is associated with increased serum secretion of an autoantibody (AT₁-AA) that stimulates the AT₁R receptor on trophoblasts, where it synergistically acts with Ang II to impair RAS function, leading to increased production of sFlt1.^{79–82} Indeed, we note that the pre-delivery sera of SARS-CoV-2-infected women exhibited significantly increased levels of AT₁ autoantibodies AT₁-AA

(Figure 4J). Furthermore, the patients with increased AT₁-AA and sFlt1 were diagnosed with chronic hypertension or gestational hypertension.

It has been shown that the viral S protein can induce internalization of ACE2, which initiates the fusion of viral particles and host cells.^{76,77,83–85} To model the complex interaction between host receptor and viral S protein and investigate the mechanistic link with RAS pathway activity, we treated placental trophoblast cells (JEG-3 cells) with the recombinant SARS-CoV-2 S protein in culture. We noted a significant reduction in the expression of ACE2 at 3 and 6 h post-S protein treatment compared to untreated control cells (Figure 5A). Immunofluorescence staining and western blotting for ACE2 showed that S treatment leads to reduction of ACE2 levels in placental cells, corroborating our placental data (Figures 5C and 5D).

In addition, to model whole-virus interaction with ACE2 in placental cells, we also employed a replication-competent virus to study entry and neutralization of SARS-CoV-2.⁸⁶ This pseudovirus (VSV-EGFP-SARS-CoV-2-S) permits folding and presentation of SARS-CoV-2 S protein in its native form. Infection of placental cells with VSV-EGFP-SARS-CoV-2-S at 1 multiplicity of infection (MOI) shows a significant reduction in the expression of ACE2 at all the time points (1, 3, and 6 h) studied compared to 0 h control cells (Figure 5B). Immunofluorescence analysis confirmed that the expression of ACE2 is reduced after VSV-EGFP-SARS-CoV-2-S infection (Figure 5E). Together, these cellular findings confirmed that viral S protein interaction with ACE2 on placental cells leads to ACE2 downregulation. Next, we determined the consequences of ACE2 decrease on RAS activity in placental trophoblasts. We found a significant increase in expression of the inflammatory AT₁R (1 h) after the addition of recombinant SARS-CoV-2 S protein; expression of AT₁R was significantly high at all time points compared to 0 h JEG-3 cells (Figure S1A). We confirmed the same findings in the presence of VSV-EGFP-SARS-CoV-2-S infection at 1 MOI as compared to 0 h control (Figure S1B). In contrast, expression of the protective angiotensin II type 1 receptor 2 (AT₂R) and placental growth factor (PIGF) was significantly downregulated after S treatment (Figures S1C and S1E) and VSV-EGFP-SARS-CoV-2-S infection (Figures S1D and S1F). S protein treatment was sufficient to induce a significant increase in sFlt1 (Figure S1G). Furthermore, levels of sFlt1 were upregulated after VSV-EGFP-SARS-CoV-2-S at 1 and 3 h of infection compared to uninfected controls (Figure S1H). The data here strongly suggest that, even in the absence of viral nucleic acid, the presence of SARS-CoV-2 S antigen is enough to induce pre-eclamptic signatory markers in trophoblasts.

Together, our *in vitro* and tissue findings demonstrate that SARS-CoV-2 infection leads to reduced expression of ACE2, resulting in downregulation of the protective AT₂R and PIGF and upregulation of the proinflammatory AT₁R, AT₁-AA, and sFlt1 arms of the RAS cascade.

DISCUSSION

In this study, we detected both viral S RNA and protein in all five placentas (four term and one preterm) from SARS-CoV-2-infected women. We described the presence of SARS-CoV-2 S RNA and protein at the maternal-fetal interface, showing virus colonization in EVT_s, STB_s, HC_s, stromal cells, amniotic epithelium, and tunica adventitia layer of the UC. Earlier studies have shown evidence of SARS-CoV-2 S or nucleocapsid protein in STB_s, EVT_s, FM, and HC_s, which support our findings.^{44,49–51} The abundance of viral S RNA and protein was high in preterm placenta compared to term infected placenta of SARS-CoV-2-infected women. The colonization of virus was greater in the maternal decidua (BP) and fetal VT compared to other compartments of the placenta. Although

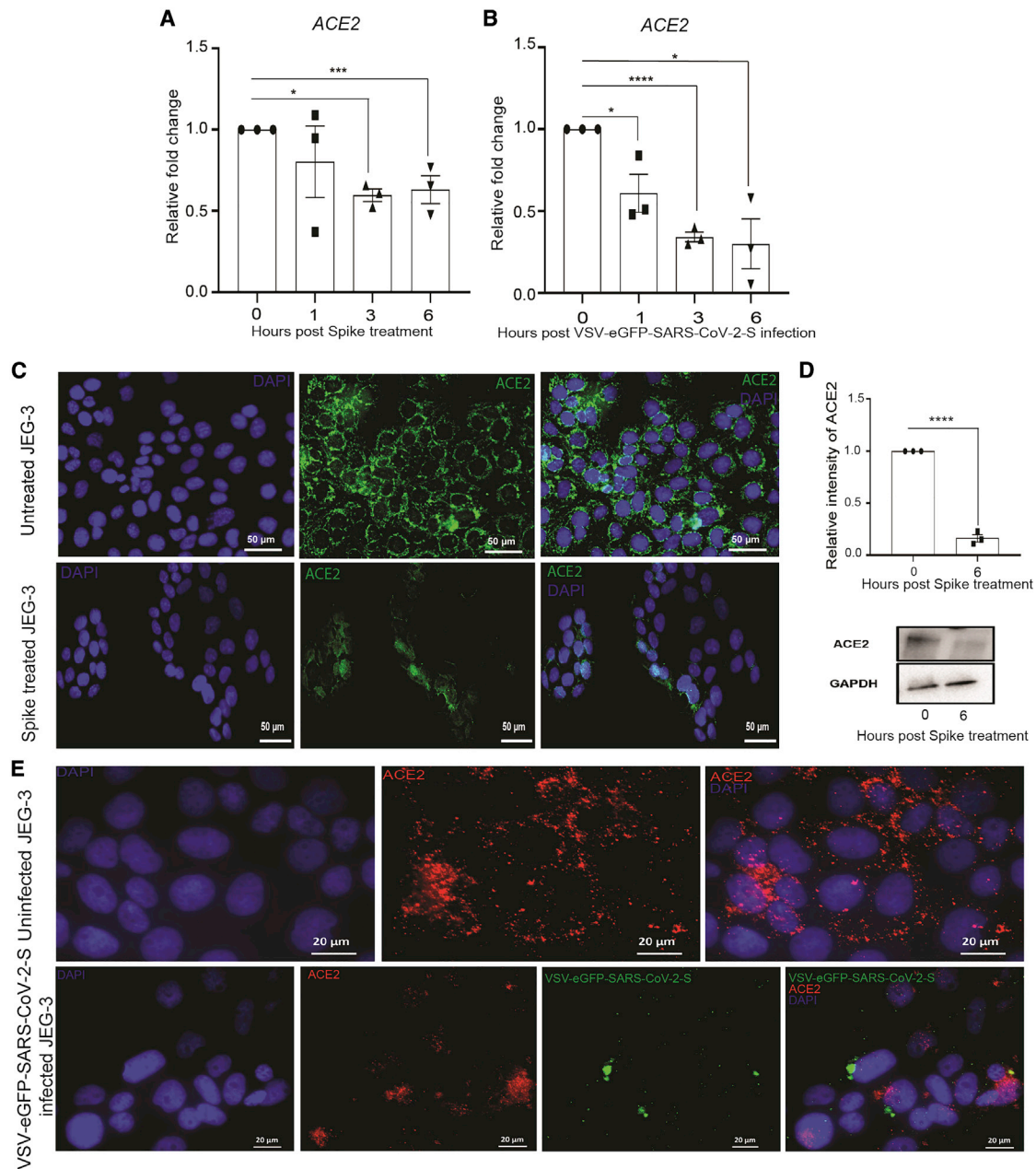


Figure 5. ACE2 expression decreases when treated or infected with spike or VSV-EGFP-SARS-CoV-2-S

(A and B) Relative expression of ACE2 normalized with GAPDH in JEG-3 cells treated or infected with spike or VSV-EGFP-SARS-CoV-2-S at 0, 1, 3, and 6 h treatment or post-infection.

(C and D) Expression of ACE2 in untreated and spike-protein-treated JEG-3 cells after 6 h of treatment by (C) immunofluorescence and (D) western blot, respectively (magnification scale 20 \times ; scale bar represents 50 μ m).

(E) Immunofluorescence images of uninfected and VSV-EGFP-SARS-CoV-2-S-infected JEG-3 cells after 6 h post-infection, showing expression of ACE2 (magnification scale 63 \times ; scale bar represents 20 μ m).

Values are expressed as mean \pm SEM of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

viral infection at term did not appear to be associated with significant histopathological changes consistent with a number of recent studies,^{7,8,44,45,49} the preterm placenta exhibited significant inflammation, including neutrophil infiltration. A second-trimester case study has also reported abnormalities in the placenta, including perivillous fibrin

deposition and inflammatory infiltrates.^{43,56} Together, these studies suggest the possibility that SARS-CoV-2 infection is more severe in preterm placenta. Whether the virus would also show similarly increased abundance in the term placentas examined earlier in gestation or whether there is a more causal relationship between the virus and the time of delivery remains unclear. Detection of virus in HCs supports the possibility of fetal infection as HCs, which serve as vectors that transmit many viruses (Zika virus [ZIKV], human immunodeficiency virus [HIV], and human cytomegalovirus [HCMV]), although HCs can also induce immunomodulatory cytokines that limit viral replication.^{87–91}

The high affinity of SARS-CoV-2 toward ACE2 aids in viral entry that subsequently triggers downregulation of ACE2 expression, disrupting the equilibrium of the RAS pathway.^{76,77,92} The importance of ACE2 and RAS in pregnancy is supported by several pieces of evidence. First, expression of renin and angiotensin increases as pregnancy progresses.⁹³ Second, mice lacking *Ace2* exhibit placental dysfunction, as evidenced by abnormal uterine remodeling, placental hypoxia, and uterine artery reactivity to vasoconstrictors.⁹⁴ Third, the circulating levels of renin and angiotensin 1 are lower in women with pre-eclampsia than in normotensive women.⁹⁵ Fourth, ACE2 is expressed in multiple cell types throughout the body, and its expression can be altered due to pregnancy. A recent study showed the downregulation of ACE2 in the nasal epithelium of pregnant rats compared with non-pregnant rats.⁹⁶ Finally, reduction in ACE2 can pivot the RAS to become more inflammatory with increased activity of AT₁⁹⁷ and a concomitant reduction in AT₂ receptor. Indeed, a 2005 study on SARS-CoV infection showed that reduced ACE2 levels and increased expression of AT₁R promotes disease pathogenesis in the lung.⁹⁸

In our studies, treatment or infection of trophoblast cells with SARS-CoV-2 S recombinant protein and modified virus (VSV-EGFP-SARS-CoV-2-S) led to reduced ACE2 expression levels. Furthermore, SARS-CoV-2-infected placentas harbored reduced ACE2 levels. Reduced ACE2 correlated with an imbalance in the RAS pathway reflected in increased expression of the proinflammatory AT₁R arm of the RAS pathway and concomitant downregulation of the AT₂R protective arm. Thus, the increased expression of sFlt1 and downregulation of PlGF found in SARS-CoV-2-infected patients further suggests a potential mechanism for the endothelial dysfunction associated with multi-organ failure.^{76,99,100} Given the critical role of balanced angiogenic (e.g., PlGF) and antiangiogenic (e.g., sFlt1) factors required for normal placental angiogenesis, the dysregulation of PlGF and sFlt1 in SARS-CoV-2-infected placentas specifically suggests a mechanism for pre-eclampsia in COVID-19 patients supported by increased secretion of AT1 autoantibodies. Therefore, targeting RAS and the various specific components dysregulated by viral infection could improve pregnancy outcomes in pregnant patients with COVID-19. However, a number of recent clinical studies are uncovering a strong link between SARS-CoV-2 infection and the probability of pre-eclampsia in SARS-CoV-2-infected severe and non-severe pregnant groups.^{31,47,80,101–105} Our study provides mechanistic support for this link. In addition, large and diverse cohort studies, including from the Eunice Kennedy Shriver National Institute of Child Health and Human Development Maternal-Fetal Medicine Units (MFMU) Network, suggest that patients with severe COVID-19 are at increased risk of perinatal complications.^{22,31} In sum, our study provides the foundation to understand the mechanism by which SARS-CoV-2 affects pregnancy by downregulating ACE2 expression in placental trophoblast cells and subsequently dysregulating the RAS pathway.

Limitations of study

Our study is limited by the small number of placentas analyzed, which are not powered to determine an association of infection and changes in ACE2 expression with

expression of pre-eclampsia markers. Further studies are warranted to validate the impact of SARS-CoV-2 on pregnancy and on adverse hemodynamic outcomes, such as pre-eclampsia in pregnant women.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.medj.2021.04.009>.

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AUTHOR CONTRIBUTIONS

S.V., E.B.C., and I.U.M. conceived the study; S.V. performed the principal experiments supported by C.S.J. and R.B.S.; M.H. provided expertise on placental pathology analysis; E.B.C. provided patient samples and clinical data; and S.V. and I.U.M. wrote the full draft of the manuscript. All authors edited and agreed with the final version.

DECLARATION OF INTERESTS

I.U.M. serves on the Scientific Advisory Board of Luca Biologics. The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-ACE2	Proteintech	Cat#21115-1-AP; RRID: AB_10732845
anti- SARS-CoV/SARS-CoV-2 Spike Protein S2	Thermo Scientific	Cat#MA5-35946; RRID: AB_2866558
anti-Rabbit IgG HRP	Cell Signaling	Cat# 7076S, RRID: AB_330924
anti-Mouse IgG HRP	Cell Signaling	Cat# 7074S, RRID: AB_2099233
anti-rabbit Alexa 594	Thermo Scientific	Cat# A11012, RRID: AB_2534079
anti-rabbit Alexa 488	Thermo Scientific	Cat# A11008, RRID: AB_143165
Bacterial and virus strains		
VSV-eGFP-SARS-CoV-2-S	Case et al. ⁸⁶	N/A
Chemicals, peptides, and recombinant proteins		
4% buffered formalin	Fisher Scientific	Cat# 316-156
Tissue Tek O.T.C. Compound	Fisher Bioreagents	Cat# BP1426
Hydrogen peroxide	Sigma Aldrich	Cat# 216763
Hematoxylin	Thermo Scientific	Cat# 7221
Eosin	Thermo Scientific	Cat# 7111
CytosealTM-60	Thermo Scientific	Cat# 8310-4
1X Phosphate Buffer Saline	GIBCO	Cat# 14190-136
Bovine Serum Albumin	Sigma Aldrich	Cat# 126593
3,3'-diaminobenzidine (DAB)	Dako	Cat# K346711-2
DMEM/F12 media	Sigma Aldrich	Cat# D6421
DAPI (4', 6'-diamidino-2-phenylindole)	Sigma Aldrich	Cat#32670
ProLong Diamond Antifade	Thermo Scientific	Cat# P36930
Paraformaldehyde	Sigma Aldrich	Cat#P6148
Xylene	Sigma Aldrich	Cat# 247642
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad	Cat# 1725275
SARS-CoV-2 Spike protein	Sino Biological	Cat #40589-V08B1
Experimental models: cell lines		
JEG3	ATCC	ATCC@HTB-36™
Oligonucleotides		
ACE2 Forward: 5'CAAGAG CAAACGGTTGAACAC 3'	Integrated DNA Technologies	N/A
ACE2 Reverse: 5' CCAGAGCCTCTATTGTAGTCT 3'	Integrated DNA Technologies	N/A
AT ₁ R Forward: 5' CAGAAAGT CGGCACCAGATGAAG 3'	Integrated DNA Technologies	N/A
AT ₁ R Reverse: 5' CTGAGCTG TGGAGCCTTTGGA 3'	Integrated DNA Technologies	N/A
AT ₂ R Forward: 5' CTGGCAACA ATGAGTCTACCTT 3'	Integrated DNA Technologies	N/A
AT ₂ R Reverse: 5' GCCACAGC GAGTTGAAGAT 3'	Integrated DNA Technologies	N/A
sFlt1 Forward: 5' GAAAACGCA TAATCTGGGACAGT 3'	Integrated DNA Technologies	N/A
sFlt1 Reverse: 5' GCGTGGTGTG CTTATTTGGA 3'	Integrated DNA Technologies	N/A
PIGF Forward: 5' CAGAGGTGGA AGTGGTACCCTTCC 3'	Integrated DNA Technologies	N/A
PIGF Reverse: 5'CGGATCTTTAGG AGCTGCATGGTGAC 3'	Integrated DNA Technologies	N/A
SPIKE Forward: 5' GCTGGTGCTGC AGCTTATTA 3'	Integrated DNA Technologies	N/A
SPIKE Reverse: 5' AGGGTCAAGT GCACAGTCTA 3'	Integrated DNA Technologies	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
VSV Forward: 5' GATAGTACCGGA GGATTGACGACTA 3'	Integrated DNA Technologies	N/A
VSV Reverse: 5' TCAAACCA TCCGAGCCATTC 3'	Integrated DNA Technologies	N/A
Critical commercial assays		
RNAscope® 2.5 HD Detection Reagent	ACD	Cat# 322360
RNAscope® 2.5 HD Duplex Detection Reagents	ACD	Cat# 322500
Human VEGFR1/Flt-1 DuoSet ELISA kit	R&D systems	Cat# DY321B
AT ₁ -AA ELISA kit	Mybiosource.com	Cat# MBS722452
QIAamp® Viral RNA kit	QIAGEN	Cat# 52904
RNeasy® plus Mini Kit	QIAGEN	Cat# 74134
Superscript II Reverse Transcriptase	Invitrogen	Cat# 18064-014
DNase Amplification Grade	Thermo Fisher Scientific	Cat# 18068-015
Bicinchoninic acid assay (BCA)	Thermo Fisher Scientific	Cat# 23225
Software and algorithms		
Prism 8.2.1	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Image Lab software (6.0.1)	Life science research Bio-Rad	https://www.bio-rad.com/en-us/product/image-lab-software
Fiji-ImageJ	ImageJ	https://imagej.net/Fiji
Others		
RNA-ISH Probe: RNA Polymerase II Subunit A, polr2a	ACD	Cat# 310451
Bacterial gene, dapB	ACD	Cat# 310043
SARS-CoV2-Spike	ACD	Cat# 848561
CD163	ACD	Cat# 417061

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Indira U. Mysorekar (imysorekar@wustl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study approval

This study was approved by Washington University School of Medicine Institutional Review Board (IRB #202003130). Informed consent has been signed by all the subjects involved in the study. Selected patient demographic data and institutional pathology reports were collected for all cases and controls. Universal testing for SARS-CoV-2 by PCR of nasopharyngeal swabs and later saliva was performed for all women admitted to our labor and delivery service at the Barnes Jewish Hospital. Placental samples and blood were collected from women who tested positive at the time of delivery, and contemporary controls who tested negative.

Placental tissue processing

Placentas were collected upon vaginal or C-section delivery. Multiple biopsies were isolated from basal plate (maternal decidua); villous placenta, fetal membranes, sub

chorion, umbilical cord and processed as follows: 1) placed in 4% buffered formalin and embedded in paraffin for histological analysis and 2) placed in Tissue-Tek O.C.T. compound (Sakura, Finetek, USA) and frozen at -80°C . The placenta was delivered to Pathology for formal grossing, staging and histological evaluation by a board-certified placental pathologist (Dr. Mai He).

Virus propagation and titration

VSV-eGFP-SARS-CoV-2-S was kindly provided by Dr. Sean P.J. Whelan, Washington University in Saint Louis. The VSV-eGFP-SARS-CoV-2-S was synthesized by incorporating SARS-CoV-2 S gene (Wuhan-Hu-1 isolate) in an infectious molecular clone of VSV⁸⁶. VSV-eGFP-SARS-CoV-2-S was propagated in Vero cells and titration of virus was performed following viral RNA isolation (QIAamp® Viral RNA kit, #52904), cDNA synthesis and qPCR using VSV and Spike specific primers¹⁰⁶. Experiments were performed under biosafety level 2 (BSL2) conditions.

Cell Culture and infection

JEG-3 cells were purchased from ATCC (HB-36) and grown in 10% DMEM/F12 media at 37°C , 5% CO_2 and 70% relative humidity as described in¹⁰⁷. JEG3 cells (0.15×10^6) were infected with VSV-eGFP-SARS-CoV-2-S at a multiplicity of infection (MOI) 1 for 1, 3 and 6 h. After each incubation supernatant was collected and cells were washed with PBS and harvested for RNA, protein, and immunofluorescence experiments.

METHOD DETAILS

RNA- *In situ* hybridization (RNA-ISH)

RNA-ISH was performed using RNAscope® 2.5 HD Detection Reagent – RED (#322360) and RNAscope® 2.5 HD Duplex Detection Reagents (#322500) as per manufacturer's instructions. Briefly, Formalin-Fixed Paraffin-Embedded (FFPE) tissues were deparaffinized followed by dehydration and rehydration using ethanol-water gradient followed by H_2O_2 incubation for 10 min for quenching peroxidase activity at room temperature (RT). Slides were boiled in target retrieval solution for 20 min followed by protease treatment for 30 min at 40°C . After protease treatment slides were incubated in SARS-CoV2-Spike probe (#848561), CD163 probe (#417061) Positive probe (RNA Polymerase II Subunit A, polr2a, #310451) and negative probe (bacterial gene, dapB, #310043) synthesized by ACD. Amplification of signal was performed using Amp1-6 probes for single staining and Amp1-10 for dual staining. Slides were counterstained using Hematoxylin and mounted using Eco mount solution. Slides were scanned using standard bright field Nikon Eclipse E800 microscope.

H & E staining and Immunohistochemical (IHC) analysis

Tissue section slides of various parts of the placenta were sequentially rehydrated using ethanol-water gradients. For H & E staining, slides were stained using hematoxylin and Eosin, mounted using Cytoaseal™-60 and scanned using a Nikon Eclipse E800 microscope.

For IHC, indigenous peroxidase activity was quenched using 1% H_2O_2 for 30 min. Sections were washed twice with 1X Phosphate Buffer Saline (PBS). Tissue sections were blocked with 1% Bovine Serum Albumin (BSA) at RT for 1 h. Next, sections were incubated with primary antibodies overnight at 4°C . The next day, sections were washed three times with 1X PBS and the respective secondary antibodies were incubated for 1 h at RT. Sections were developed using liquid 3,3'-diaminobenzidine (DAB) and counterstained with Hematoxylin. They were sequentially

dehydrated and cleared in Xylene and mounted using Cytoseal™-60. Finally, images were captured using a Nikon Eclipse E800 microscope.

For image analysis histology images were imported into ImageJ (1.53c), color deconvolution was used to process images, H-DAB module was used to isolate DAB and hematoxylin layer. DAB Intensity was analyzed and normalized to the area of the selected region of interest (ROI). One representative image of BP, VT and FM from four COVID-19 negative and COVID-19 positive placentas was selected. Parametric unpaired t test was applied to calculate statistical significance. $p < 0.05$ was considered significant.

sFlt1 and AT₁-AA Elisa

Pre-delivery serum samples from uninfected and SARS-CoV-2 infected pregnant women were used for Elisa analysis using Human VEGFR1/Flt-1 DuoSet (R&D systems, Minneapolis, MN, USA) and AT₁-AA (Mybiosource.com, Southern California, San Diego, USA) Elisa kits as per manufacturer's instruction. Optical density was measured using microplate reader at 450 nm and 540 nm. The concentration of sFlt1 and AT₁-AA was calculated using 4-parametric logistic curve-fit. The experiment was performed in duplicate.

Immunofluorescence (IF)

Indirect immunofluorescence was performed to check infection of VSV-eGFP-SARS-CoV-2-S and the expression of ACE2. Briefly, JEG-3 cells were seeded in 2-chamber slides and kept overnight at 37°C, 5% CO₂ and 70% relative humidity. The next day, cells were infected/treated with VSV-eGFP-SARS-CoV-2-S (1 MOI)/Spike protein (2 μg) and incubated for 6 h in the CO₂ incubator. After 6 h, cells were fixed with 4% PFA (15 min) at 4°C followed by blocking with 1% BSA in PBS for 1h at RT. After 1h, cells were incubated with ACE2 antibody (1:200) overnight at 4°C. The following day, cells were washed with PBS and incubated with anti-rabbit Alexa 594 or 488 (1:400) secondary antibody for 1h at RT. Further, cells were washed and counterstained with DAPI (4', 6'-diamidino-2-phenylindole) and fixed in ProLong Diamond Antifade mounting medium. The images were captured using an epifluorescence microscope using 20X or 63X objectives.

qPCR analysis

Total mRNA was isolated after VSV-eGFP-SARS-CoV-2-S infection/Spike protein treatment at 0, 1, 3, and 6 h using RNeasy® plus Mini Kit (QIAGEN kit #74134) as per manufacturer's instructions. The cDNA was made using 1000 ng of RNA and Superscript II Reverse Transcriptase (Invitrogen, #18064014). The qPCR reaction was performed in triplicates using primers shown in Table S1 using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, #1725275) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The fold change expression ($-\Delta\Delta C_t$) was calculated after normalization with GAPDH expression.

Western Blotting

Placental samples (BP, VT, FM, SC) from uninfected and SARS-CoV-2 infected women were collected and homogenized in RIPA buffer with protease and phosphatase inhibitor cocktails. Proteins isolated from individual section of placenta (BP, VT, FM, SC) were combined equally to study ACE2 expression.

JEG-3 cells were harvested after each time point in RIPA buffer with protease and phosphatase inhibitor cocktails. The total protein estimation was performed using Bicinchoninic acid assay (BCA) (Thermo Fisher Scientific, #23225) and separated

on 4%–20% Mini-PROTEAN precast TGX gels (Bio-Rad Laboratories, #4561093). The proteins were transferred on Polyvinylidene fluoride (PVDF) membrane for 2 h at 90 Volts. After transfer, the membrane was blocked in 5% skimmed milk followed by incubation in primary antibody for ACE2 (1:1000) overnight at 4°C. The next day the membrane was washed with 0.1% TBST 3 times followed by incubation in HRP conjugated anti-rabbit secondary antibody (1:3000) for 1 h at RT. After incubation, the membrane was washed and developed using immobilon® Western Chemiluminescent HRP substrate (Millipore corporations, #WBKLS0100). The blots were captured using Chemidoc (Bio-Rad Laboratories) and bands were quantified using ImageJ software and plotted after normalization with loading control (β -actin/GAPDH).

Chemicals

Rabbit polyclonal anti- ACE2 was purchased from Proteintech (#21115-1-AP, 1:100 dilution). anti- SARS-CoV/SARS-CoV-2 Spike Protein S2 (1:100 dilution) were purchased from Thermo Scientific (#MA5-35946) respectively. HRP labeled anti-mouse (#7076S) and anti-rabbit (#7074S) antibodies were procured from Cell Signaling. Hydrogen peroxide was purchased from Sigma Aldrich (#216763, 1:200 dilution). Liquid DAB was procured from Dako (#K346711-2, 1:200 dilution). Mounting medium Cytoseal™-60 was obtained from Thermo Scientific (#8310-4).

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments were performed at least three times, and results were presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed using unpaired t-Test for qPCR experiments. One way Anova was performed for western blotting and IHC experiments. Graphpad Prism 8.0 was used for all analyses. $p < 0.05$ was considered significant.