Anatomical and timely assessment of protein expression of angiotensin-converting enzyme 2, SARS-CoV-2 specific receptor, in fetal and placental tissues: new insight for perinatal counseling

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Contribution

What are the novel findings of this work?

The protein expression of ACE2, SARS-CoV2 specific receptor, in the fetal-placental unit across gestation is unknown. There is marked expression of ACE2 in placenta, fetal bowel and kidneys but not in fetal brain and heart.

What are the clinical implications of this work?

Those findings provide a rationale for vertical transmission at cellular level. Organs with absence of ACE2 expression should not be the target of direct virus-related insult. The absence of ACE2 expression in fetal brain and heart is reassuring on the risk of congenital malformation.
Abstract

Infection with SARS-CoV2 does not spare pregnant women and the possibility of vertical transmission which might lead to fetal damages is pending.

Objective: We hypothesized that the observed low incidence of perinatal infection could be related to a low expression of the membrane receptor for SARS-CoV2, ACE2, in the fetal-placental unit. We evaluated protein expression of ACE2 both in placentas and fetal organs from non-infected pregnancies across gestation.

Methods: Discovery study. Immunocytochemistry analysis for ACE2 in organs and placentas were performed in May 2020, in samples from a registered biobank. Five cases of medical termination of pregnancy performed at between 15 and 38 weeks’ in healthy women. Paraffin-embedded tissues (kidneys, brain, lungs, intestinal tract, heart). Matching tissues from 8-year-old children (N=4) were tested as controls. Seven placentas including those of the 5 cases, 1 of a 7-week miscarriage and 1 of a symptomatic SARS-COV2 pregnancy at 34 weeks. Tissues’ sections were incubated with rabbit monoclonal anti-ACE2. Protein expression of ACE2 was detected by immunochemistry.

Results: ACE2 expression was detected in fetal kidneys, rectum and ileum across gestation and similarly in the pediatric control. It was barely detectable in lungs at 15 weeks’ and not found thereafter. In the pediatric control, ACE2 was only detectable in type 2 pneumocytes. No ACE2 expression was found in the cerebral ependymal, parenchyma nor in cardiac tissues ACE2 was expressed in syncitiotrophoblast and cytotrophoblast from 7th weeks’ onwards and across gestation but not in the amnion.
Similar intensity and distribution of ACE2 staining were identified in the mother’s SARS-CoV2 placenta.

**Conclusions:** Marked placental expression of ACE2 provides a rationale for vertical transmission at cellular level. Absence of ACE2 expression in the fetal brain and heart is reassuring on the risk of congenital malformation. Clinical follow-up of infected pregnant women and their children are needed to validate these observations.
Introduction

Infection with coronavirus SARS-CoV2 does not spare pregnant women, and the possibility of vertical transmission of the virus to the fetus is the subject of a vivid debate\(^1\text{-}^6\). The entry of SARS-CoV2 into target cells is conditioned by the presence of the angiotensin-converting enzyme 2 (ACE2) on the membrane of the host cell. The viral spike protein is primed by the transmembranous cellular serine protease, TMPRSS2, enabling membrane fusion and virus entry\(^7\text{(p2)}\). TMPRSS2 has a broader distribution than ACE2, suggesting that ACE2, rather than TMPRSS2, may be a limiting factor for viral entry\(^8\). Beyond theoretical assessment of risk and pathways for vertical transmission, the low incidence of perinatal infections might relate to a lower expression of ACE2 in the placenta and targeted organs.

We aimed to evaluate protein expression of ACE2 both in placentas and in all fetal organs from pregnancies not infected with SARS-CoV2 at various gestational ages. As a second objective, we also evaluated ACE2 expression in the placenta of a pregnant woman infected with SARS-CoV2 in order to assess whether ACE2 expression was modified in the context of SARS-CoV2 infection.
Methods

Immunochemistry analysis for ACE2 was performed on fetal and placental tissue samples from a biobank authorized by the National Biomedical Agency. All women gave informed consent for the use of fetal and placental specimens for research purposes.

Fetal and placental tissues

Samples were retrieved from five cases of medical termination of pregnancy from healthy, non-hypertensive women. Data about these five cases are in Table 1. Our three inclusion criteria for these cases were 1) The different stages of development of the fetoplacental unit had to be represented. We thus chose fetuses/placentas of different gestational ages: 3 cases from the 2nd trimester (beginning = case 1/middle = case 2 / end = case 3) and 2 cases from the 3rd trimester (beginning = case 4 and end = case 5) were selected; 2) The fetuses should not have any extracerebral organ involvement on fetopathological examination (in particular lung, kidney, digestive tract, heart). The placenta should also be eutrophic. It was impossible to include fetuses with no sign of brain damage since the reason for medical termination of pregnancy at these late stages was most often related to a late discovery of brain abnormalities that could affect the neurodevelopmental prognosis; 3) The pregnancy should not be terminated because the fetus had a congenital infection.
Gestational ages of these cases were 15+5, 20+1, 27+5, 29+4 (olfactory mucosa only) and 38+1 weeks’. Seven placentas were analyzed including 5 from the cases described above, 1 from a 7 weeks’-miscarriage, and 1 from a symptomatic infected pregnant woman with positive SARS-CoV2 RT-PCR delivered by cesarean section at 34 weeks’. This last placenta tested negative for SARS-CoV2 by RT-PCR. Regarding the clinical history of the placenta from the miscarriage, it was a spontaneous miscarriage in a 33-year-old woman with no sign of septic abortion. Ultrasound examination showed an aborted pregnancy and the patient requested surgical management by endo-uterine suction. The results of placental histological analysis were normal and no signs of intervillitis were found.

Paraffin-embedded tissues, including kidneys, brain, lungs, intestinal tract, heart, gonads, cornea, conjunctiva, and olfactory mucosa were used. Matching tissues (kidney, intestinal tract, lung and testis) from 8-year old children, without histological lesion in the analyzed sections were selected from the registered human tissue collection of the pathology department. The aim of choosing pediatric cases as controls was to obtain positive control to visualize the expected cell type and subcellular ACE2 localization regarding post-natal immunohistochemical published data on different organs known to express ACE2.

Immunohistochemistry for ACE2

An automated IHC stainer BOND-III (Leica Biosystems) was used for ACE2 detection by immunohistochemistry. Briefly, 4µm sections (7µm for brain) of paraffin-
embedded tissue were deparaffinized, rehydrated and submitted to heat-induced antigen retrieval (H1, PH6, Leica Biosystems). Tissues' sections were incubated with rabbit monoclonal anti-ACE2 (Abcam, [EPR4435(2)]) 1/200 for 30min. The primary antibody was visualized using diaminobenzidine, and counterstained with hematoxylin using the Leica Biosystems BOND detection kit. Sections processed with replacement of the primary antibody by tris-buffered saline were used as a negative control.
Results

Immunostaining of ACE2 in fetal organs (Figure 1 & 2)

Protein expression of ACE2 was detected in fetal testis, kidneys, rectum and ileum, from 15 + 5 weeks’ onwards. Similar results were found in pediatric controls. The fetal olfactory mucosa, the cornea and bulbar conjunctiva showed ACE2 protein expression in the one sample at 29+4 and at 38+1 weeks’ respectively.

The exact location of ACE2 immunostaining in these organs was:

- Testis: membrane and cytosolic staining within seminiferous tubules
- Kidneys: Membrane apical staining of the proximal convoluted tubule in the kidney cortex with mild cytosolic expression due to protein synthesis pathway. Membrane podocyte and parietal epithelial cells expression within glomeruli.
- Intestinal tract: Membrane apical staining of enterocyte and no signal within goblet cells.
- Olfactory mucosa: Bowman gland staining
- Eye: membrane apical staining within cornea and conjunctiva epithelium

Immunohistochemical staining was barely detectable in the lungs at 15+5 weeks’ and was not found at later gestations. This weak staining was in the membrane apical within type 2 pneumocyte and was not present at all in type 1 pneumocyte.

In the pediatric control, ACE2 protein expression was also only detectable in type 2 pneumocytes.
No expression of ACE2 was found in the cortex or the ependymal of the brain; and ACE2 expression was limited to choroid plexuses. Cytosolic and membrane staining for ACE 2 was positive in the inner stromal core of choroid plexus villi, possibly corresponding to stroma cells. These non epithelial cells, had less spindle shape nuclei than endothelial cells, and were negative for actin and CD 34 staining. Thus these cells are potentially not vascular endothelial cells, CD34 being quite specific of this lineage, nor are they myocyte or fibroblast cells (actin negative). Neither cardiac tissue nor coronary endothelial cells showed ACE2 staining.

**Immunostaining of ACE2 in the placentas (Figure 3).**

Staining with ACE2 was detectable in all non-COVID placentas as early as 7 weeks’ within both syncytiotrophoblast and cytotrophoblast but not in the vascular endothelium. In the basal plate, staining was in the intermediate trophoblast membrane, and in the placenta villi apical membrane staining was in both cytotrophoblast and syncytiotrophoblast. ACE2 expression was not detected in the amnion. ACE2 staining was of the same intensity and distribution in the placenta of the infected case.
Discussion

Main findings

Protein expression of ACE2 was marked in kidneys and gastrointestinal tract of all fetuses but not in brain ependyma nor parenchyma. Protein expression of ACE2 was also marked in placentas throughout pregnancy but not in the amnion. These results broaden the insight on likelihood, pathways and morbidities of vertical transmission of SARS-CoV-2, providing a unique anatomical and timely validation of the protein distributions in fetal organs and placentas.

Interpretation

Neonatal proven infection within 48 hours of birth raised the possibility of perinatal infection. Our findings support that SARS-CoV2 is able to cross into the placenta at any gestational age, either as a blood-borne transmission across the maternal-fetal interface or through ascending vertical transmission of the virus. Viremia is only detectable in around 1% of symptomatic adults and could not be demonstrated in any of the infected neonates, which makes blood-borne transmission unlikely. In symptomatic infected pregnant women, viral RNA could not be amplified from amniotic fluid nor vaginal secretions. This makes ascending infection through an intact amnion also unlikely, since the amnion does not bear ACE2. RT-PCR in placentas have yielded mainly negative results but for few cases following caesarean section and the diagnosis of chorioamnionitis related to SARS-CoV-2 has also
been suggested. Ascending colonization and infection of the placenta following prolonged rupture of the amniotic membranes therefore appears the most likely pathway for vertical transmission.

In the hypothesis of fetal infection with SARS-CoV2, our data are somehow reassuring regarding the risk of fetal complications. No expression of ACE2 was found in the brain or in the ependyma. The virus appears therefore unlikely to cause direct neurological sequelae as can be observed with cytomegalovirus for example. ACE2 was markedly expressed in the fetal kidneys and this could affect amniotic fluid production and kidney development. ACE2 expression in the fetal intestinal epithelium makes SARS-CoV-2 a candidate for enterocolitis, a condition broadly associated with perinatal infection and showing hyperechoic bowel on antenatal ultrasound examination. ACE2 expression in lungs is known to mainly involve type-2 pneumocytes. This is compatible with a transient expression at 15+5 weeks’, the end of the pseudoglandular phase of lung development. The absence of ACE2 expression in the third trimester is compatible with the scarcity of related respiratory symptoms found in infected neonates. The absence of ACE2 immunostaining in all cardiac tissues suggests that the heart would not be a direct target to the virus but rather be exposed to indirect pathogenicity of COVID including deregulated inflammation through the myocardial effects of cytokines storm. Finally, ACE2 expression was found in the Bowman glands of the olfactory mucosa involved in olfaction. This could contribute the understanding of olfactory defects in COVID patients.
A recent work of A. J. Vivanti et al. described a proven case of congenital infection associated with neurological manifestations. The authors reported that the neonate was complicated by white matter injury and provided insights to fetal neurological manifestations in the setting of SARS-CoV2 infection. However, we think that the white matter injury identified by MRI lacks specificity and could be triggered by several causes such as hypoxia, immune injury or endothelial cell activation (complement dysregulation, pro-inflammatory cytokines). Indeed, there were limited evidence for a direct effect of the virus on the brain: 1) the newborn has had several punctures of cerebrospinal fluid (CSF) and it has always been negative for SARS-CoV2; 2) no parenchymatous lesions were described on MRI (as it can be observed for congenital cytomegalovirus, toxoplasmosis, Zika infections); 3) all other brain tests remained normal (electroencephalogram, brain ultrasound). Finally, even with this one case describing neurological disorders in the neonatal period, there is no enough argument that SARS-CoV2 would provide long-term neurological sequelae: 1) the neurological examination returned to normal in the absence of any medical intervention 2) the newborn was discharged from hospital after a standard length of stay for a premature infant 3) the neurological examination was normal at 2 months of life. 4) This is the first and only case in the literature reporting neurological effects in children born to infected mothers.

Finally, these post-natal data of this case of proven congenital infection mostly support our histological data: direct placental infection by SARS-CoV2, detection of the virus.
in the nasopharyngeal and feces, a normal cardiac function in the newborn, a normal lung ultrasound with no respiratory signs, absence of the virus in the CSF.

**Strengths**

This provides a unique in vivo human anatomical and timely mapping of the distribution of SARS-CoV-2 receptor ACE2.

**Limitations**

We did not study the expression of TMPRSS2, the cofactor of ACE2, but its broader distribution suggest that it may not be a limiting factor for viral entry\(^8\). Furthermore, the multibasic cleavage site in the spike protein of SARS-CoV2 is activated by ubiquitously expressed proprotein convertases, including furin, suggesting that other membranous proteases than TMPRSS2 may trigger virus entry\(^{16}\).

**Conclusion**

The marked placental presence of the specific SARS-CoV2 receptor, ACE2, and its absence from the amnion suggests that ascending vertical transmission could mainly occur following rupture of the amniotic membranes. Absence of ACE2 expression in the fetal brain, lungs and heart is reassuring on the risk of congenital malformation and on the morbidity of perinatal infection. However, placenta-mediated fetal morbidity including chorioamnionitis-related prematurity and growth restriction should be subjected to follow-up studies of infected pregnancies.
References

1. Zeng L, Xia S, Yuan W, Yan K, Xiao F, Shao J, Zhou W. Neonatal Early-Onset Infection With SARS-CoV-2 in 33 Neonates Born to Mothers With COVID-19 in Wuhan, China. *JAMA Pediatr.* Published online March 26, 2020. doi:10.1001/jamapediatrics.2020.0878

2. Lamouroux A, Attie-Bitach T, Martinovic J, Leruez-Ville M, Ville Y. Evidence for and against vertical transmission for SARS-CoV-2 (COVID-19). *American Journal of Obstetrics and Gynecology.* Published online May 4, 2020. doi:10.1016/j.ajog.2020.04.039

3. Dong L, Tian J, He S, Zhu C, Wang J, Liu C, Yang J. Possible Vertical Transmission of SARS-CoV-2 From an Infected Mother to Her Newborn. *JAMA.* 2020;323(18):1846-1848. doi:10.1001/jama.2020.4621

4. Kimberlin DW, Stagno S. Can SARS-CoV-2 Infection Be Acquired In Utero?: More Definitive Evidence Is Needed. *JAMA.* 2020;323(18):1788-1789. doi:10.1001/jama.2020.4868

5. Algarroba GN, Rekawek P, Vahanian SA, Khullar P, Palai a T, Peltier MR, Chavez MR, Vintzileos AM. Visualization of SARS-CoV-2 virus invading the human placenta using electron microscopy. *American Journal of Obstetrics and Gynecology.* Published online May 2020. doi:10.1016/j.ajog.2020.05.023

6. Poon LC, Yang H, Kapur A, Melamed N, Dao B, Divakar H, McIntyre HD, Kihara AB, Ayres-de-Campos D, Ferrazzi EM, Di Renzo GC, Hod M. Global interim guidance on coronavirus disease 2019 (COVID-19) during pregnancy and puerperium from FIGO and allied partners: Information for healthcare professionals. *International Journal of Gynecology & Obstetrics.* 2020;149(3):273-286. doi:10.1002/ijgo.13156

7. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu N-H, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell.* 2020;181(2):271-280.e8. doi:10.1016/j.cell.2020.02.052

8. Sungnak W, Huang N, Bécavin C, Berg M, Queen R, Litvinukova M, Talavera-López C, Maatz H, Reichart D, Sampaziotis F, Worlock KB, Yoshida M, Barnes JL. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nature Medicine.* 2020;26(5):681-687. doi:10.1038/s41591-020-0868-6
9. Vivanti AJ, Vauloup-Fellous C, Prevot S, Zupan V, Suffee C, Do Cao J, Benachi A, De Luca D. Transplacental transmission of SARS-CoV-2 infection. Nat Commun. 2020;11(1):3572-3572. doi:10.1038/s41467-020-17436-6

10. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA. 2020;323(18):1843-1844. doi:10.1001/jama.2020.3786

11. Qiu L, Liu X, Xiao M, Xie J, Cao W, Liu Z, Morse A, Xie Y, Li T, Zhu L. SARS-CoV-2 Is Not Detectable in the Vaginal Fluid of Women With Severe COVID-19 Infection. Clin Infect Dis. doi:10.1093/cid/ciaa375

12. Penfield CA, Brubaker SG, Limaye MA, Lighter J, Ratner AJ, Thomas KM, Meyer J, Roman AS. Detection of SARS-COV-2 in Placental and Fetal Membrane Samples. American Journal of Obstetrics & Gynecology MFM. Published online May 2020:100133. doi:10.1016/j.ajogmf.2020.100133

13. Favre G, Pomar L, Musso D, Baud D. 2019-nCoV epidemic: what about pregnancies? The Lancet. 2020;395(10224):e40. doi:10.1016/S0140-6736(20)30311-1

14. Viner RM, Whittaker E. Kawasaki-like disease: emerging complication during the COVID-19 pandemic. The Lancet. Published online May 13, 2020. doi:10.1016/S0140-6736(20)31129-6

15. Butowt R, Bilinska K. SARS-CoV-2: Olfaction, Brain Infection, and the Urgent Need for Clinical Samples Allowing Earlier Virus Detection. ACS Chem Neurosci. 2020;11(9):1200-1203. doi:10.1021/acschemneuro.0c00172

16. Hoffmann M, Kleine-Weber H, Pöhlmann S. A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. Molecular Cell. 2020;78(4):779-784.e5. doi:10.1016/j.molcel.2020.04.022
Figure legends

Figure 1. ACE2 antenatal expression compare to 8-year-old control patients.

Immunochemistry using anti-ACE2 antibody in different fetal organs compare to 8-year-old control patients. A. Light microscopy (x200) showing strong ACE2 expression in fetal kidney within proximal tubule (black arrow) and glomeruli (red arrow) with parietal cells and podocytes expression. B. Light microscopy (x40, x40, x400, x200, x40) showing ACE2 expression in fetal intestinal tract (rectum and ileum). Red arrow revealing negative goblet cell surrounded by positive enterocytes. C. Light microscopy (x400 and x200) showing ACE2 expression in lung. Control patient showing positive ACE2 staining in type 2 pneumocyte. 15+5 weeks’ fetus showing weak apical expression in alveolar epithelium. No clear ACE2 expression was found after 15+5 weeks’. D. Light microscopy (x400) showing weak positive staining within fetal testis at 38+1 weeks’. WA: weeks of amenorrhea

Figure 2. ACE2 expression in fetal brain, heart, eye, and olfactory mucosa.

Immunochemistry using anti-ACE2 antibody A. Fetal brain expression of ACE2 at 20+1 weeks’. Upper layer, left image, light microscopy (x100) revealing no ACE2 expression in the frontal cortex, right image, light microscopy (x400) revealing no ACE2 expression in brain ependyma. Lower layer, left image, light microscopy (x40) and right image (x400) showing ACE2 expression in fetal choroid plexus. B. Cardiac expression of ACE2 at 38+1 weeks’. Light microscopy (x400) showing neither cardiac nor coronary artery expression of ACE2. C. Eye expression of ACE2 at 38+1 weeks. Light microscopy (x400) showing apical expression in cornea and bulbar conjunctiva. D.
Olfactory mucosa expression of ACE2 at 29+4 weeks’. Light microscopy (x200 right image and x400 left image) showing Bowman gland staining.

**Figure 3.** ACE2 expression in placental tissue of COVID and non-COVID patient.

Immunohistochemistry using anti-ACE2 antibody A. ACE2 expression in the placenta of non-COVID patients, upper layer corresponding to the basal plate, light microscopy (x100) revealing ACE2 expression in intermediate trophoblast. Lower layer corresponding to the placental villi, light microscopy (x400) showing ACE2 expression in cytotrophoblast (black arrow) and syncytiotrophoblast (red arrow). B. ACE2 expression in the placenta of a COVID patient showing similar staining compare to the placenta of non-COVID patients.

WA: weeks of amenorrhea

**Supplementary table legend:**

**Table 1.** Characteristics of the five cases of termination of pregnancy.
### Figure 1

| ACE2 postnatal expression | ACE2 antenatal expression |
|---------------------------|---------------------------|
| **Kidney**                |                           |
| 8 year-old                | 15+5 WA                   |
|                           | 20+1 WA                   |
|                           | 27+5 WA                   |
|                           | 38+1 WA                   |
|                           |                           |
| **Intestinal tract**      |                           |
| 8 year-old                | 15+5 WA                   |
|                           | 20+1 WA                   |
|                           | 27+5 WA                   |
|                           | 38+1 WA                   |
|                           |                           |
| **Testis**                |                           |
| 8 year-old                | 15+5 WA                   |
|                           | 20+1 WA                   |
|                           | 27+5 WA                   |
|                           | 38+1 WA                   |
|                           |                           |

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Frontal Cortex

Brain ependyma

Choroid plexus

Choroid plexus

Heart

Coronary artery

Cornea

Bulbar conjunctiva

Olfactory mucosa

Olfactory mucosa

Figure 2

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Figure 3