COVID-19 and vertical transmission: assessing the expression of ACE2 / TMPRSS2 in the human fetus and placenta to assess the risk of SARS-CoV-2 infection.

Max A. Beesley*[1], Joseph R. Davidson*[1,2], Francesco Panariello[3], Soichi Shibuya[1], Dominic Scaglioni[1], Brendan C. Jones[1], Katarzyna Maksym[2], Olumide Ogunbiyi[5], Neil J. Sebire[1,4], Davide Cacchiarelli[3,4], Anna L. David[2,7], Paolo De Coppi[†,1,5,6], Mattia F.M. Gerli[†,1,8]

1. Great Ormond Street Institute of Child Health, University College London, UK
2. EGA Institute for Women's Health, University College London, UK
3. Telethon Institute of Genetics and Medicine (TIGEM), Armenise / Harvard Laboratory of Integrative Genomics, Pozzuoli, Italy
4. Department of Translational Medicine, University of Naples "Federico II", Naples, Italy
5. NIHR Great Ormond Street Biomedical Research Centre, London, UK
6. Great Ormond Street Hospital for Children, London, UK
7. Fetal Medicine Unit, University College London NHS Foundation Trust, London, UK
8. UCL Division of Surgery and Interventional Science, Royal Free Hospital, London, UK

*Contributed equally to this work

†Correspondence to be addressed to Mattia Francesco Maria Gerli (m.gerli@ucl.ac.uk) and Paolo De Coppi (p.decoppi@ucl.ac.uk)

Abstract

Objective: While pregnant women have been identified as a potentially at-risk group concerning COVID-19 infection, little is known regarding the susceptibility of the fetus to infection. Co-expression of ACE2 and TMPRSS2 has been identified as a pre-requisite for infection, and expression across different tissues is known to vary between children and adults. However, the expression of these proteins in the fetus is unknown.

Methods: We performed a retrospective analysis of single cell data repositories. This data was then validated at both gene and protein level by performing qRT-PCR and two-colour immunohistochemistry on a library of second-trimester human fetal tissues.

Results: TMPRSS2 is present at both gene and protein level in the predominantly epithelial fetal tissues analysed. ACE2 is present at significant levels, only in the fetal intestine and kidney and is not expressed in the fetal lung. The placenta is also negative for the two proteins both during development and at term.

Conclusions: This dataset indicates that the lungs are unlikely to be a viable route of SARS-CoV2 fetal infection. The fetal kidney, despite presenting both the proteins required for the infection, is anatomically protected from the exposure to the virus. However, the GI tract is likely to be susceptible to infection due to its high co-expression of both proteins, as well as its exposure to potentially infected amniotic fluid.

Funding: This work was made possible by an MRC / UKRI COVID-19 Rapid response initiative grant (MR/V028480/1).

Keywords: COVID-19, SARS-CoV2, ACE2, TMPRSS2, Vertical Transmission, Fetal Infection

Introduction

The COVID-19 pandemic has brought about unprecedented research efforts in attempts to prevent and treat infection across all ages. SARS-CoV-2 viral entry mechanisms into the human body have been well established, highlighting the importance of tissue-level co-expression of ACE2 and TMPRSS2 (along with potential roles for further surface markers, such as NRP1[1,2]). The risk profile and infection mechanisms of SARS-CoV-2 appear significantly different in young children compared to older individuals. Clinical reports of confirmed perinatal infection in the neonate have led to concerns of a potential mechanism for vertical transmission. Recent data suggest that the fetus may have some susceptibility to SARS-CoV-2 infection[3–5], alongside a plausible increased risk of preterm birth. However, the observational data included in these studies is unable to infer the specific risk of COVID-19 infection over general risk of spontaneous preterm labour with severe maternal illness. Documented cases of confirmed vertical transmission indicate that this occurs around the time of birth, with low neonatal morbidity[6]. While neonatal SARS-CoV-2 infection is rarely of significant severity, there remains concern surrounding disruption of the pregnancy during the 2nd trimester, as was observed during outbreaks of similar coronaviruses SARS and MERS[7,8]. Additionally, there are concerns regarding the impact of fetal infection during critical developmental phases, which is observed in other viral infections such as cytomegalovirus. Therefore, understanding the potential for, and impact of early gestation fetal infection remains an ongoing public health question.

Various mechanisms of vertical transmission have been hypothesised: I) direct infection of syncytiotrophoblast with subsequent transmission through the cytotrophoblast; II) infection via trafficked maternal cells; III) ascending infection through the vagina and cervical canal; and IV) infection acquired during delivery via direct infant contact with maternal virus, as

might be assumed most likely in cases of peripartum infection with resultant neonatal positivity[9]. To explore the susceptibility of the mid-gestation human fetus to SARS-CoV-2 infection, we studied multiple fetal tissues at different gestational stages. We initially probed publicly available single-cell RNA sequencing data from fetal tissues across gestation. We then validated our findings by exploring gene expression levels of ACE2 and TMPRSS2 via quantitative PCR analysis of the fetal tissues that could be exposed to virus *in utero* and examined expression at protein level using multicolour immunohistochemistry. This enabled us to explore both expression of proteins implicated in SARS-CoV-2 infection and their tissue colocalization. Finally, we studied amniotic membrane and placental samples of matching developmental stages and at term, to identify potential viral entry points at the maternal-fetal interface.

Methods

Samples and Ethics

Human fetal tissues were obtained with consent through the Human Developmental Biology Resource (HDBR; REC 18/LO/0822 – IRAS 244325; Project ID 2000478). Fetal samples were obtained from fetuses ranging from 14 to 22 Post Conceptual Weeks (PCW, 12 – 20 Post Menstrual Weeks of Gestation). Fetal staging was calculated based on foot length (mm) and referred to as PCW. The tissue library included fetal lung, intestine, kidney, bladder, skin, amniotic membrane and placenta (CV - Chorionic villi). Placental samples were obtained at delivery of an uncomplicated, full-term pregnancy (median 39 weeks PCW [Range 38+1 - 39+4] for 6 patients recruited through the EVERREST Prospective Study as normal controls (National Research Ethics reference 13/LO/1254), NCT02097667 registered 31st October 2013[10]. Tissue staining and RNA expression level was referenced to that of human adult lung controls, commercially obtained from Insight Biotechnology and Takara respectively.

Single-Cell RNA Sequencing

Suitable Single-Cell RNA sequencing datasets were identified in public repositories from broad high-impact Single-Cell studies (available at www.proteinatlas.org and https://descartes.brotmanbaty.org/, [11,12]). These covered human fetal specimens from multiple gestational ages, as well as human adult reference samples. For the adult data, a normalised count matrix (i.e. TPM normalised) was downloaded, containing samples from different origins, annotated by cell-type. Fetal data was downloaded as a raw count matrix with a random sampling of 5,000 cells per cell type per organ, to allow easier exploration, which was then CPM normalised, and log transformed using Seurat R package. A custom R

script was then used to extract and plot (using the ggplot2 package by tidyverse, ggplot2.tidyverse.org) the expression data for ACE2 and TMPRSS2 across a range of tissues (colon, eye, heart muscle, kidney, liver, lung, pancreas, PBMC, placenta, rectum, skin, small intestine, testis) and time-points (between 10 and 18 PCW).

Gene expression Analysis via qRT-PCR

Whole tissue gene expression was analysed by Real-Time quantitative Polymerase Chain Reaction (qRT-PCR). Firstly, RNA was extracted from formalin fixed paraffin embedded tissue blocks (RNAEasy FFPE Kit; QIAGEN 73504). cDNA was then generated upon retro-transcription following the manufacturers recommendation (Super Script VILO IV Master Mix; Life Technologies 11756050). qRT-PCR was performed using an Applied Biosystem Step One v23 machine, utilising SYBR Green (Agilent, 600882) and predesigned KiCqStart primers for ACE2, TMPRSS2 and GAPDH (Merck). Relative expression of the analysed genes was calculated based on ΔC_T values, compared to the adult lung expression level as positive control, using the $\Delta\Delta C_T$ Livak method[13]. Statistical significance was assessed by non-parametric one-sample Wilcoxon signed rank test against the positive adult lung control.

Two-colour immunohistochemistry to assess protein localisation.

Tissue slides were analysed by two-colour immunohistochemistry. Staining was performed utilising primary antibodies for ACE2 (R&D MAB933, RRID:AB_2223153; Mouse anti-Human 1:200 with red chromogen) and TMPRSS2 (Abcam ab109131, RRID:AB_10863728; Rabbit anti-Human 1:500 with Brown chromogen). The Leica refine polymer kit (DS9800, RRID:AB_2891238) and refine red kit (DS9390) were utilised to stain the antibody-antigen complex. Sections were counterstained with Haematoxylin to highlight the localization of the

nuclei. Staining specificity and quality were evaluated by the Department of Histopathology of Great Ormond Street Hospital. Qualitative analysis of the staining localisation within each tissue was performed to assess localisation and co-localisation of the staining. Quantitative analysis was performed using QuPath [14]: specifically, tissue sections were segmented using a semi-automated pixel classifier. Positively stained areas for each chromogen were measured using machine-learning based pixel classifiers and the percentage of staining area relative to total tissue area was computed. Double positive expression was defined by the percentage of staining areas that co-expressed ACE2 and TMPRSS2. This was then compared between samples across gestational ages and compared to adult lung, prostate and kidney sections as positive control reference.

Statistical analyses

Gestational expression changes were explored using linear regression modelling comparing expression level and developmental stages. We obtained and analysed 7 tissues from 6 fetal samples, in addition to 7 term placenta samples. Statistical significance between each tissue and the adult lung expression threshold was assessed using the non-parametric one-sample Wilcoxon signed rank test, the value of the adult lung was set to 1 and used as the positive control for the qPCR. The changes in ACE2 and TMPRSS2 levels are presented in a combined graph depicting the average expression in each tissue. The temporal variation for each tissue is also presented as individual graphs, simple linear regression was performed to identify temporal trends with 95% confidence intervals (CI). Statistical analyses were performed using Microsoft Excel and GraphPad Prism v9.0. Data are displayed as Mean ±SEM unless otherwise stated, and a p-value of <0.05 was accepted as denoting statistical significance.

Results

Analysis of scRNA-sequencing data

Our analysis of public repository data identified the human fetal intestine as highly expressing both ACE2 and TMPRSS2. Upon interrogation of expression in the different specific cell types, we identified the intestinal epithelial cells as being the main contributors to overall intestine positivity for these genes (Figure 1). This analysis also indicated that while other tissues, notably kidney and lung, showed expression of ACE2, the lack of co-expression with TMPRSS2 suggests these tissues are unlikely to be susceptible to SARS-CoV-2 infection during gestation. Co-expression of ACE2 and TMPRSS2 in the intestine increased during gestation in an exponential manner across the timepoints studied (10 to 18 PCW). None of the other tissues analysed showed such significant increase in gene expression over the gestational age range for which data were available. In line with this observation, the adult intestine also showed the greatest co-expression. This was further supported by analysis of cell-specific expression, with greatest expression in adult intestinal enterocytes. Overall, this analysis of open access RNA sequencing atlas data suggests that transcription of ACE2 and TMPRSS2, differ markedly in human fetal subjects compared to what reported for children and adults.

Analysis of gene expression at tissue level across gestation

RT-qPCR gene expression analysis was carried out on a library of fetal tissues (ranging from 14 to 22 PCW) that are potentially susceptible to SARS-CoV-2 infection (Figure 2 and Table 1). Using the adult lung as reference: I) the fetal intestine demonstrated the highest relative expression of ACE2 and TMPRSS2, at levels compatible with SARS-CoV-2 infection (Fold change: ACE2 15.49 ±4.54, p=0.0243 and TMPRSS2 10.80 ±2.61, p=0.0132); II) the fetal lung expressed significant TMPRSS2 expression but showed significantly lower ACE2, far below

that observed in the adult lung; III) the fetal kidney showed increased expression of ACE2 and TMPRSS2, the latter not reaching statistical significance; IV) the skin and bladder both showed ACE2 expression significantly below that of the adult lung, and TMPRSS2 levels similar to that of the adult lung; V) Placental tissues showed significantly lower expression than adult lung for both ACE2 and TMPRSS2.

Analysis of protein expression at tissue level across gestation

Two-colour immunohistochemistry was used to investigate expression and localisation of ACE2 and TMPRSS2 proteins in multiple fetal tissues across gestation. Tissues positively expressing the antigens showed localisation of TMPRSS2 (Brown) across the plasma membrane of the epithelial cells, whereas ACE2 (Red) localised specifically to the apical membrane of the epithelia (Figure 3). This data validates the molecular data confirming little to no ACE2 expression in fetal skin, bladder and lung, with lung and bladder expressing detectable levels of TMPRSS2. The fetal intestine and kidney specimens showed a strong protein co-expression, with the intestine having the most signficant co-localisation on the mucosal epithelium, highlighted in the high magnification images (Figure 3A, Right). Placental samples expressed low to absent ACE2, while TMPRSS2 seemed to be present at variable levels in the chorionic villi; a finding that is consistent across the gestational window of study but which did not match the gene expression data by tissue-level PCR. The lack of co-localisation with ACE2 was also true for the term placenta samples (Figure 3B). Semi-automated quantification of the ACE2 and TMPRSS2 staining corroborate the observation obtained with visual assessment (Table 2).

Discussion

Main findings: In this study, we assess the expression of ACE2 and TMPRSS2 at the gene and protein level, across a library of human fetal tissues obtained from the second trimester (14 to 22PCW) to term. Our findings demonstrate that most of the fetal tissues analysed lack expression (or co-localisation) of the proteins required for SARS-CoV-2 infection. However, two tissues (fetal kidney and intestine) manifested co-expression of both target proteins. As the fetal kidney is not anatomically directly exposed to the amniotic fluid, we believe it is unlikely to be a relevant route for viral infection, especially since haematogenous spread has not been proposed as a mechanism of infection and transmission across the placenta appears unlikely. The fetal intestine, directly exposed to the amniotic fluid via fetal swallowing, shows an increase in the expression of ACE2 throughout gestation with a high level of TMPRSS2 expression in the mucosa. Results from publicly available fetal single cell atlas support our findings[12], allowing extension of these observations to 8PCW, before the gestational age that fetal swallowing is known to begin. The intestinal findings are also in line with recent demonstration that the human fetal stomach highly co-expresses proteins required for SARS-CoV-2 infection and that fetal stomach-derived organoids are susceptible to viral infection[15]. ACE2 expression is also reported in fetal ileum and rectum samples from 15 weeks of gestation[16]. Taken together, these results suggest that both the upper and lower gastrointestinal tract may be a potential entry route for SARS-CoV-2 into the second-trimester human fetus. This is also consistent with the additional retrospective analysis performed on all the other fetal tissues present the reference single cell atlas [12], indicating that the co-expression of the proteins is also present in the stomach (Figure S1).

Strengths and limitations: Previous studies have assimilated data available from expression profiles on scRNA atlas in the public domain to demonstrate ACE2/TMPRSS2 expression in human fetal tissues and at the maternal-fetal interface[17]. However, these have not explored expression within the fetal intestine, and lacked validation at a protein level[18]. To the best of our knowledge, this is the first work to explore ACE2/TMPRSS2 both at gene expression and protein level in the human fetus, and specifically in the second trimester of pregnancy. While this study contains novel data that corroborates real world observations, it is important to recognise a few limitations to the data we present. The Human Developmental Biology Resource includes human fetal tissues for research purposes only up to 22 weeks of gestation, therefore we are unable to investigate the expression of ACE2 and TMPRSS2 in fetal tissues past this time point. Furthermore, we have not directly validated the susceptibility to the infection in the tissues of interest with infection assays; work with live SARS-CoV-2 virus is beyond the scope of this study.

Interpretation: Our findings support clinical observations from meta-analyses that the incidence of COVID-19-related fetal complications during pregnancy is low given the number of adult COVID-19 cases worldwide (182 million confirmed cases, 3.95 million deaths at the time of writing - https://github.com/CSSEGISandData/COVID-19), including large numbers of pregnant women. Comparatively, in previous coronavirus epidemics, far fewer cases were reported but with a higher frequency of fetal adverse events[19]. The UK Obstetric Surveillance System reports that pregnant women in later gestation are not protected from severe infection[20]; with an increased frequency of iatrogenic preterm birth, possibly due to severe maternal illness. There have been speculative data published suggesting that women

may be at risk of severe SARS-CoV2-related illness in the peripartum period, which has led to regional policy that the non-compromised fetus should remain undelivered if possible[21,22]. Previous collaborative studies from our group have demonstrated infection in tissue and organoids derived from human fetal stomach demonstrating that a high expression at both RNA and protein level corresponds to a significant propensity for viral infection[15]. Hence, the proposed route of infection via the gastrointestinal tract is reasonable, in keeping with others' published work on the adult intestine [23]. Furthermore, this corresponds to proposed mechanisms for spread among younger children, since gastrointestinal symptoms and fecal shedding are more characteristic of infection in younger patients[24–26], and possibly associated with a milder disease phenotype[27].

The hypothesis of intrauterine infection through the gastrointestinal tract requires viral entry to the gastrointestinal lumen through fetal by swallowing of infected amniotic fluid and presumes that the amniotic fluid can contain infectious viral particles. Our data indicate that term placenta expresses low to absent ACE2, a key protein for SARS-CoV-2 infection, making it difficult for the virus to pass from the maternal blood to the fetus or amniotic fluid. In one study of 31 mothers with COVID-19, there were only two cases of vertical transmission in which the viral genome was detected in term placentas, and these were associated with strong maternal pro-inflammatory response [28]. In contrast, single cell transcriptomic studies of early placenta (6-14 post menstrual age gestational weeks) reported co-expression of ACE2 and TMPRSS2 in stromal and perivascular cells in decidua, and villous cytotrophoblast and syncytiotrophoblast [22,29]. It is therefore plausible that women infected early in pregnancy could potentially pass SARS-CoV-2 to the fetus but with low fetal infection rates due to the minimal ACE2 and TMPRSS2 co-expression in the fetal tissues studied, including the gastrointestinal tract, early in pregnancy. Regarding perinatal transmission, there have

been several studies reporting an absence of detectable virus in genital tract swabs in women with severe symptoms[30], or in pregnant women with mild symptoms[31,32]. However, given the immunological changes occurring around the time of labour[33], we would suggest that there is currently inadequate evidence to rule this out as the potential route for infection of neonates who are known to have been infected[3–5].

Conclusion

In conclusion, we propose that maternal viraemia-associated presence of virus within the amniotic fluid and birth canal, as reported [17,22], may produce an environment where the fetus is susceptible to infection through the gastrointestinal tract. Our data indicate that this susceptibility may be present, increasingly, across the whole second trimester.

Author contributions

MFMG and PDC conceived the study and designed the experiments with the help of ALD. MAB, JRD contributed equally to this work, conducted the experiments and analysed the data with the help and support of MFMG. FP and DC helped MAB in performing the retrospective sequencing analyses. SS, DS, BCJ, OO and NJS helped with staining optimisation, interpretation and imaging. KM procured the term placental RNA. MAB, JRD, MFMG and PDC wrote this manuscript. All authors contributed to manuscript revision and approved the final version.

Declaration of interests

The authors declare no conflicts of interest related to this work or its developments. DC is founder, shareholder, and consultant of Next Generation Diagnostic SRL.

Acknowledgements

We are thankful to Yau Mun Lim from UCL IQPath and UCL Institute of Neurology for his help with the semi-automated image analysis. We acknowledge the use of fetal tissues obtained through the Human Developmental Biology Resource. This work was supported by a UKRI COVID-19 Rapid response initiative (MR/V028480/1). MB is supported by a UCL Child Health Research PhD Studentship. JRD is supported by a Clinical Research Training Fellowship from the MRC. DS is supported by a PhD studentship from the British Heart Foundation. BCJ is supported by the General Sir John Monash Foundation. FP and DC work was supported by Fondazione Telethon Core Grant, Armenise-Harvard Foundation Career Development Award, European Research Council (759154, CellKarma), and the Rita-Levi Montalcini program from MIUR. MFMG and PDC receive support from the NIHR GOSH BRC. MFMG holds a European Commission H2020 Marie Skłowdoska-Curie Action Individual Fellowship (843265 AmnioticID). ALD is supported by the NIHR UCLH BRC. PDC, JRD and ALD have been supported through an Innovative Engineering for Health award by the Wellcome Trust (WT101957) and the Engineering and Physical Sciences Research Council (EPSRC)(NS/A000027/1).

References

[1] Cantuti-Castelvetri L, Ojha R, Pedro LD, Djannatian M, Franz J, Kuivanen S, et al. Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity. Science (80-) 2020;370:856–60. doi:10.1126/science.abd2985.

[2] Daly JL, Simonetti B, Klein K, Chen K-E, Williamson MK, Antón-Plágaro C, et al. Neuropilin-1 is a host factor for SARS-CoV-2 infection. Science (80-) 2020;370:861–5. doi:10.1126/science.abd3072.

[3] Goh XL, Low YF, Ng CH, Amin Z, Ng YPM. Incidence of SARS-CoV-2 vertical transmission: a meta-analysis. Arch Dis Child - Fetal Neonatal Ed 2021;106:112–3. doi:10.1136/archdischild-2020-319791.

[4] Khalil A, Kalafat E, Benlioglu C, O'Brien P, Morris E, Draycott T, et al. SARS-CoV-2 infection in pregnancy: A systematic review and meta-analysis of clinical features and pregnancy outcomes. EClinicalMedicine 2020;25:100446. doi:10.1016/j.eclinm.2020.100446.

[5] Kotlyar AM, Grechukhina O, Chen A, Popkhadze S, Grimshaw A, Tal O, et al. Vertical transmission of coronavirus disease 2019: a systematic review and meta-analysis. Am J Obstet Gynecol 2021;224:35-53.e3. doi:10.1016/j.ajog.2020.07.049.

[6] Gale C, Quigley MA, Placzek A, Knight M, Ladhani S, Draper ES, et al. Characteristics and outcomes of neonatal SARS-CoV-2 infection in the UK: a prospective national cohort study using active surveillance. Lancet Child Adolesc Heal 2021;5:113–21. doi:10.1016/S2352-4642(20)30342-4.

[7] Alfaraj SH, Al-Tawfiq JA, Memish ZA. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection during pregnancy: Report of two cases & review of the

literature. J Microbiol Immunol Infect 2019;52:501–3. doi:10.1016/j.jmii.2018.04.005.

[8] Diriba K, Awulachew E, Getu E. The effect of coronavirus infection (SARS-CoV-2, MERS-CoV, and SARS-CoV) during pregnancy and the possibility of vertical maternal-fetal transmission: a systematic review and meta-analysis. Eur J Med Res 2020;25:1–14. doi:10.1186/s40001-020-00439-w.

[9] Komine-Aizawa S, Takada K, Hayakawa S. Placental barrier against COVID-19. Placenta 2020;99:45–9. doi:10.1016/j.placenta.2020.07.022.

[10] Spencer R, Ambler G, Brodszki J, Diemert A, Figueras F, Gratacós E, et al. EVERREST prospective study: a 6-year prospective study to define the clinical and biological characteristics of pregnancies affected by severe early onset fetal growth restriction. BMC Pregnancy Childbirth 2017;17:43. doi:10.1186/s12884-017-1226-7.

[11] Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science 2015;347:1260419. doi:10.1126/science.1260419.

[12] Cao J, O'Day DR, Pliner HA, Kingsley PD, Deng M, Daza RM, et al. A human cell atlas of fetal gene expression. Science (80-) 2020;370:eaba7721. doi:10.1126/science.aba7721.

[13] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8. doi:10.1006/meth.2001.1262.

[14] Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep 2017;7:16878. doi:10.1038/s41598-017-17204-5.

[15] Giobbe G, Bonfante F, Zambaiti E, Gagliano O, Jones B, Luni C, et al. SARS-CoV-2

infection and replication in human fetal and pediatric gastric organoids. BioRxiv n.d.

[16] Faure-Bardon V, Isnard P, Roux N, Leruez-Ville M, Molina T, Bessieres B, et al. Protein expression of angiotensin-converting enzyme 2, a <scp>SARS-CoV</scp> -2-specific receptor, in fetal and placental tissues throughout gestation: new insight for perinatal counseling. Ultrasound Obstet Gynecol 2021;57:242–7. doi:10.1002/uog.22178.

[17] Li M, Chen L, Zhang J, Xiong C, Li X. The SARS-CoV-2 receptor ACE2 expression of maternal-fetal interface and fetal organs by single-cell transcriptome study. PLoS One 2020;15:1–12. doi:10.1371/journal.pone.0230295.

[18] Weatherbee BAT, Glover DM, Zernicka-Goetz M. Expression of SARS-CoV-2 receptor ACE2 and the protease TMPRSS2 suggests susceptibility of the human embryo in the first trimester. Open Biol 2020;10:4–7. doi:10.1098/rsob.200162.

[19] Di Mascio D, Khalil A, Saccone G, Rizzo G, Buca D, Liberati M, et al. Outcome of coronavirus spectrum infections (SARS, MERS, COVID-19) during pregnancy: a systematic review and meta-analysis. Am J Obstet Gynecol MFM 2020;2:100107. doi:10.1016/j.ajogmf.2020.100107.

[20] Knight M, Bunch K, Vousden N, Morris E, Simpson N, Gale C, et al. Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: National population based cohort study. BMJ 2020;369. doi:10.1136/bmj.m2107.

[21] Kadiwar S, Smith JJ, Ledot S, Johnson M, Bianchi P, Singh N, et al. Were pregnant women more affected by COVID-19 in the second wave of the pandemic? Lancet 2021;397:1539–40. doi:10.1016/S0140-6736(21)00716-9.

[22] Maeda M de FY, Brizot M de L, Gibelli MABC, Ibidi SM, Carvalho WB de, Hoshida MS, et al. Vertical transmission of SARS-CoV2 during pregnancy: A high-risk cohort. Prenat

Diagn 2021:pd.5980. doi:10.1002/pd.5980.

[23] Lamers MM, Beumer J, van der Vaart J, Knoops K, Puschhof J, Breugem TI, et al. SARS-CoV-2 productively infects human gut enterocytes. Science (80-) 2020;369:50–4. doi:10.1126/science.abc1669.

[24] Puoti MG, Rybak A, Kiparissi F, Gaynor E, Borrelli O. SARS-CoV-2 and the Gastrointestinal Tract in Children. Front Pediatr 2021;9. doi:10.3389/fped.2021.617980.

[25] Akobeng AK, Grafton-Clarke C, Abdelgadir I, Twum-Barimah E, Gordon M. Gastrointestinal manifestations of COVID-19 in children: a systematic review and meta-analysis. Frontline Gastroenterol 2020:flgastro-2020-101529. doi:10.1136/flgastro-2020-101529.

[26] Parasa S, Desai M, Thoguluva Chandrasekar V, Patel HK, Kennedy KF, Roesch T, et al. Prevalence of Gastrointestinal Symptoms and Fecal Viral Shedding in Patients with Coronavirus Disease 2019: A Systematic Review and Meta-analysis. JAMA Netw Open 2020;3:1–14. doi:10.1001/jamanetworkopen.2020.11335.

[27] Wang X, Zheng J, Guo L, Yao H, Wang L, Xia X, et al. Fecal viral shedding in COVID-19 patients: Clinical significance, viral load dynamics and survival analysis. Virus Res 2020;289:198147. doi:10.1016/j.virusres.2020.198147.

[28] Fenizia C, Biasin M, Cetin I, Vergani P, Mileto D, Spinillo A, et al. Analysis of SARS-CoV-2 vertical transmission during pregnancy. Nat Commun 2020;11. doi:10.1038/s41467-020-18933-4.

[29] Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, et al. Single-cell reconstruction of the early maternal-fetal interface in humans. Nature 2018;563:347–53. doi:10.1038/s41586-018-0698-6.

[30] Qiu L, Liu X, Xiao M, Xie J, Cao W, Liu Z, et al. SARS-CoV-2 Is Not Detectable in the Vaginal Fluid of Women With Severe COVID-19 Infection. Clin Infect Dis 2020;71:813–7. doi:10.1093/cid/ciaa375.

[31] Aslan MM, Uslu Yuvacı H, Köse O, Toptan H, Akdemir N, Köroğlu M, et al. SARS-CoV-2 is not present in the vaginal fluid of pregnant women with COVID-19. J Matern Neonatal Med 2020:1–3. doi:10.1080/14767058.2020.1793318.

[32] Chen H, Guo J, Wang C, Luo F, Yu X, Zhang W, et al. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. Lancet 2020;395:809–15. doi:10.1016/S0140-6736(20)30360-3.

[33] Stelzer IA, Ghaemi MS, Han X, Ando K, Hédou JJ, Feyaerts D, et al. Integrated trajectories of the maternal metabolome, proteome, and immunome predict labor onset. Sci Transl Med 2021;13:eabd9898. doi:10.1126/scitranslmed.abd9898.

Figure Legends

Figure 1 The image represents a retrospective analysis performed on public repository single cell RNA sequencing data, investigating the expression of ACE2 (Cyan) and TMPRSS2 (Purple). The UMAPs on the Left depict the clusters of cells expressing ACE2 and TMPRSS2 in the fetal intestine, kidney, lung and placenta. The plots on the right, show a regression analysis on the variation in the expression levels of the two genes of interest, across the gestational stages analysed.

Figure 2 The graphs depict the results of a quantitative real-time PCR analysis performed on human fetal and placental tissues (CV - Chorionic Villi; AM - Amniotic membrane) at different developmental stages. (A) The gene expression of ACE2 and TMPRSS2 is presented as the log fold change relative to the adult lung, averaged across the gestational ages studied. Data are presented asd Mean ±SEM. (B) The panel shows a linear regression analysis performed on the expression data shown in A, to determine variations in expression across gestation. The data is presented as relative expression to the adult lung.

Figure 3 The panel shows a two colour immunohistochemical staining for ACE2 (Red) and TMPRSS2 (Brown). Sections were counterstained with Haematoxylin (Purple) to highlight the localisation of the cell nuclei. (A) The panel shows antigen localisation in the fetal lung, kidney, intestine and skin. The last column shows a higher magnification to highlight the staining localisation (Scale bars: 100µm). (B) The panel shows antigen expression in the placental tissues across the second trimester (14 - 22 PCW), as well as at term. Placental sampling was performed to investigate expression in the amniotic membrane and chorionic villi structures (Scale bar: 100µm).

Table 1 Summary of the RT-qPCR data depicted in Figure 2, showing; Mean, Standard Deviation (SD), Standard Error of Means (SEM), Sample Number (N), Statistical Test (one sample t- and Wilcoxon test).

Table 2 Summary of the image quantification analyses performed using QuPath highlighting the gestational age (GA), percentage of tissue expressing TMPRSS2 and ACE2 as well as their co-localisation (%) across the total area analysed (mm^2).

Figure S1 The image represents a retrospective analysis performed on public repository single cell RNA sequencing data, investigating the expression of ACE2 (Cyan) and TMPRSS2 (Purple). The UMAPs depict the clusters of cells expressing ACE2 (left) and TMPRSS2 (right) for all the fetal tissues present in the dataset. The bar plots show a regression analysis on the mean expression level of the two genes of interest, for each of the gestational stages analysed.