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# Connecting the dots on vertical transmission of SARS-CoV-2 using protein-protein interaction network analysis – Potential roles of placental ACE2 and ENDOU

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A B S T R A C T

We conducted a protein-protein interaction (PPI) network study searching for proteins relevant to pregnancy-associated COVID-19 in pregnancy complicated with severe preeclampsia (sPE) and intra-amniotic infection and/or inflammation (Triple-I). PPI networks from sPE and Triple-I were intersected with the PPI network from coronavirus infection. Common proteins included the SARS-CoV-2 entry receptor ACE2 and ENDOU, a placental endoribonuclease homologous to Nsp15, a protein produced by the virus to escape host immunity. Remarkably, placental ENDOU mRNA expression far exceeded that of ACE2. Immunohistochemistry confirmed ENDOU localization at the hemochorial maternal-fetal interface. Investigation of ENDOU's relevance to vertical transmission of SARS-CoV-2 is further warranted.

## 1. Introduction

SARS-CoV-2 is the etiologic agent of COVID-19 which phenotypically manifests with fever, malaise, severe pneumonia and lethal systemic inflammatory syndrome. Experiences from the 1918–1919 influenza epidemic and most recently from the 2015 Zika virus outbreak support the hypothesis that pregnant women may be more vulnerable during viral pandemics [1–3].

Placenta is generally considered a barrier to vertical transmission. Only a few viruses are proven to cause fetal infection [4]. SARS-CoV-2 is an emergent viral pathogen whose ability to infect the human fetus remains unknown. A case report of COVID-19 associated with preeclampsia symptoms showed that SARS-CoV-2 is able to infect the placental syncytiotrophoblast [5]. COVID-19 infection carries overlapping phenotypic features with preeclampsia as surveilled by clinical cohorts worldwide [6,7]. These preliminary observations led us to explore possible molecular intersections between coronavirus infection and pregnancy-specific complications such as preeclampsia and Triple-I. This report uses protein-protein interaction (PPI) network analysis of our proteomics data and published placental RNAseq datasets to gain new insight into proteins that may modulate vertical transmission of SARS-CoV-2.

## 2. Methods

Protein-protein interaction (PPI) network analysis was conducted using Cytoscape software (v.3.7.2) on bottom-up proteomics mass spectrometry data generated from 62 biological samples collected from pregnant women with well-defined diagnoses of pregnancy complications: Triple-I (n = 25), preeclampsia (n = 4) and idiopathic preterm birth (iPTB, n = 33) [8–10]. The samples included maternal blood, urine, amniotic fluid, cord blood, placenta and fetal membrane tissue lysates. Serum, amniotic fluid and lysates were treated with Pierce Abundant Protein Depletion Spin Columns (Thermo Fisher Scientific, Rockford, Illinois) and normalized for protein concentration before tryptic digestion. Tandem MS/MS spectra were acquired using a timsTOF Pro mass spectrometer (Bruker, Bremen, Germany), and a Velos Pro mass spectrometer (Thermo) coupled to a PepMap Easy-Spray C18 column on a 1D nano Acquity UPLC (Waters, Milford, Massachusetts), and matched against Uniprot database (Homo sapiens) for protein identification. Protein IDs from all samples of women sharing the same clinical diagnosis were aggregated to generate the most inclusive *in silico* proteome for each condition. These PPI networks were intersected using Cytoscape's DyNet Analyzer with the PPI network of coronavirus infection from STRING disease database [11,12]. The placental RNAseq datasets were generated earlier by our group and are publically available (GEO accession number GSE73714). Normalization and

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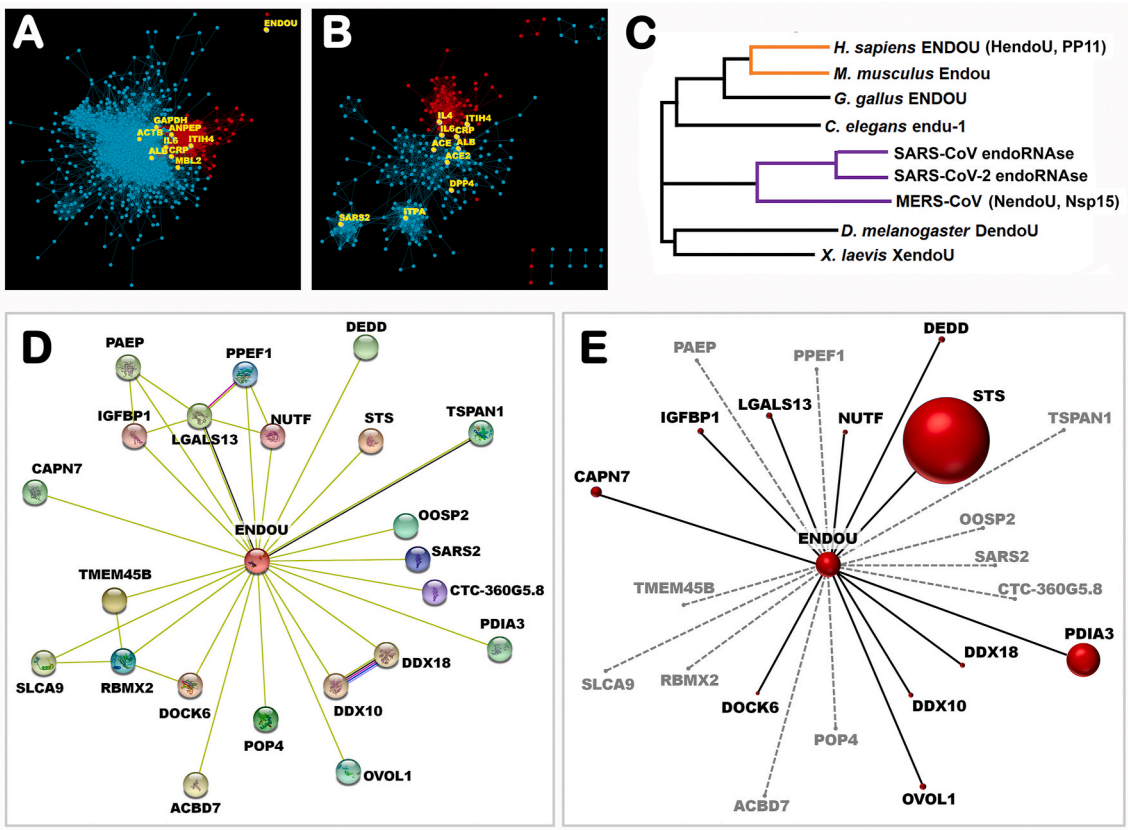


**Fig. 1. Intersection of coronavirus infection, Triple-I and preeclampsia protein-protein interaction (PPI) networks. A.** The Triple-I PPI network comprised 816 protein IDs (cyan) was intersected with coronavirus infection network (red) and common IDs are highlighted in yellow. **B.** Preeclampsia PPI network comprised 207 protein IDs (cyan) intersected with coronavirus infection network (red) and common IDs are highlighted in yellow. **C.** Dendrogram showing the phylogenetic relationships among select XendoU family members. Amino acid reference sequences were downloaded from the NCBI database and subjected to multiple sequence alignment, followed by pairwise distance (Fitch) matrix generation (based on degree of sequence similarity) and neighbor-joining tree estimation. **D.** In this network, minimum required interaction score was set at medium confidence of 0.4, with max number of interactors for 'first shell' and 'second shell' setting to 22 and 0, respectively. The STRING network analysis for protein-protein interaction (PPI) obtained an average node degree of 2.78; average local clustering coefficient of 0.919; and PPI enrichment p-value: 0.0487, indicating that these proteins can be at least partially associated as a group regarding their biological function. **E.** Placental RNAseq transcript abundance mapped to putative PPI of ENDOU in **D**.

downstream analysis were performed using the DESeq2 statistical package in R. Gene expression was quantitated with RT-PCR, and immunohistochemistry (IHC) was used for further validation of protein expression and immune-localization in the placenta.

## 3. Results

The PPI network intersection between the Triple-I proteome and coronavirus infection highlighted 9 shared proteins: ACTB, ALB, ANPEP, CRP, ENDOU, GAPDH, IL6, ITIH4 and MDL2 (Fig. 1A) while the pre-eclampsia proteome and coronavirus infection shared 10 proteins: ACE, ACE2, ALB, CRP, DPP4, IL4, IL6, ITIH4, ITPA and SARS2 (Fig. 1B). Of these, Placental Endonuclease, Poly(U) Specific (ENDOU, placental protein 11 or PP11), which has high homology with the coronavirus endoribonuclease Nsp15 [13] (Fig. 1C), and human Angiotensin-Converting Enzyme 2 (ACE2), the cellular entry receptor for SARS-CoV-2 [14,15] were specifically selected for validation by RT-PCR and IHC. Additionally, placental RNAseq transcript abundance mapped to ENDOU's PPI network suggests putative interactions of ENDOU (Fig. 1D) with the mapped higher abundance transcripts (Fig. 1E).

RT-PCR confirmed mRNA expression of ACE2 and ENDOU in placenta with a significantly higher ENDOU abundance both in iPTB and term placenta (Fig. 2A). ACE2 mRNA expression was downregulated in Triple-I and term placenta (Fig. 2B) and IHC confirmed the decrease in signal intensity in syncytiotrophoblast and inflammatory cells in Triple-I (Fig. 2 D and E). Conversely, ENDOU mRNA expression was upregulated in preeclampsia (Fig. 2C) and ENDOU staining was highly increased in syncytiotrophoblast of preeclamptic placenta (Fig. 2 F and G).

## 4. Discussion

To our knowledge, a potential involvement for ENDOU in modulating COVID-19 vertical transmission has not yet been proposed. Our results highlighting the abundant placental expression of ENDOU and its high homology to coronavirus Nsp15 suggest ENDOU may potentially play a role in vertical transmission of SARS-CoV-2. Nsp15 is a cleavage product of coronavirus replicase polyprotein (pp1ab) and is essential for effective viral replication and increased infectivity [16,17]. ENDOU, or PP11, is a scarcely studied protein. PP11 was first purified in the 1980s and thought to function as a serine protease [18]. Subsequent work demonstrated that ENDOU is an RNA endonuclease capable of generating small RNA fragments in a $Mn^{2+}$-dependent fashion [17]. Aside from placenta, ENDOU is expressed ectopically in several malignant tumors [19]. The observed increased ENDOU expression in the pre-eclamptic placentas is intriguing in the context of COVID-19 often being a clinical imitator of preeclampsia [20]. Furthermore, ENDOU's endoribonuclease roles may be linked to cell-free RNA signatures released by placenta in maternal plasma [21]. As for COVID-19, it is important to determine if placental ENDOU cooperates in any way with

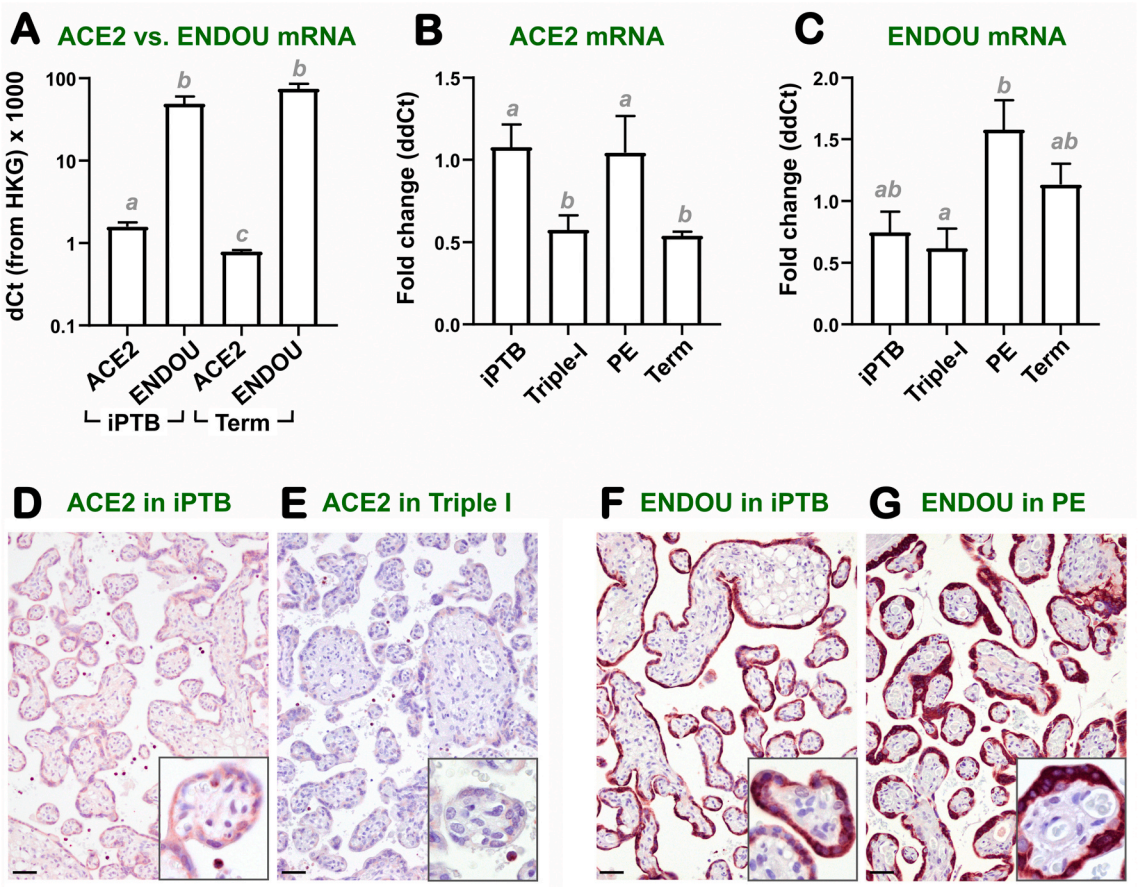


**Fig. 2. mRNA expression and protein localization of ACE2 and ENDOU in placental villous tissue.** Real time quantitative RT-PCR using TaqMan gene expression assays (Hs00962470_m1 and Hs01085333_m1, Life Technologies) were performed with ABI StepOnePlus RT-PCR system, and immunohistochemistry on matched formalin-fixed paraffin-embedded placental tissue was done using anti-ACE2 (ab15348) and anti-ENDOU (ab121367) antibodies (Abcam). Tissues were retrieved from 22 women with idiopathic preterm birth (iPTB), intra-amniotic infection and inflammation (Triple-I) with histological chorioamnionitis, severe preeclampsia (PE), or term normal pregnancy (n = 5–6/group). **(A)**. ENDOU mRNA was more abundant than the ACE2 mRNA in both preterm and term placenta. Relative quantitation (RQ) dCT values are reported relative to expression of housekeeping genes RPL30 and B2M. ddCT RQ values for ACE2 (**B**) and ENDOU (**C**) expression are reported relative to a reference RNA pool. Data presented as mean + SEM and analyzed by 1-way ANOVA followed by post-hoc Shapiro-Wilk tests. Means marked with different superscripts are statistically significant ($p < 0.05$). Representative photomicrographs showed placental villi stained immunohistochemically for ACE2 (**D-E**) or ENDOU (**F-G**) using rabbit polyclonal primary antibodies. In iPTB placenta, ACE2 localized to syncytiotrophoblast and inflammatory cells in maternal vascular spaces **(D)**. Staining intensity appeared decreased in placenta of women with Triple-I **(E)**. In iPTB placenta, the syncytiotrophoblast stained strongly for ENDOU **(F)** and this pattern was further increased in PE placenta **(G)**. Vector NovaRed was used as chromogen and slides were counterstained with hematoxylin. Negative control slides exposed to non-immune rabbit serum were devoid of staining. Magnification: 200× for panels (scale bar: 50 μm) and 600x for insets.

coronavirus Nsp15 to enhance or diminish the effectiveness of the placental barrier. We observed that, in the human placenta, ENDOU is significantly more abundant than ACE2. This is interesting because it suggests that while the cellular gate receptor for SARS-CoV-2 (ACE2) is less represented, the endoribonuclease activity favoring viral infectivity is present [22]. Collectively, our data suggest a potentially active mechanism that may favor SARS-Cov-2 trans-placental transmission that may be more active in preeclampsia patients or in inducing preeclampsia-like symptoms in mothers with COVID-19.

## Declaration of competing interest

No conflicts of Interest.

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