Cell Systems Transparent Peer Review Record

Summary

Received: Aug 29, 2025
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Scientific editor: Yuan Hu, Ph.D.

Revision invited: Nov 06, 2025

Revision received: Apr 24, 2026

Accepted: Nov 03, 2026

This Transparent Peer Review Record is not systematically proofread, type-set, or edited. Special characters, formatting, and equations may fail to render properly. Standard procedural text within the editor's letters has been deleted for the sake of brevity, but all official correspondence specific to the manuscript has been preserved.

Editorial decision letter with reviewers' comments, first round of review

Dear Dr. Herse,

I hope this email finds you well. The reviews of your manuscript are back and I've appended them below. On balance, the reviewers appreciate the goals of the work presented here; they've provided constructive comments that are aligned with our hopes for the paper. Accordingly, we're happy to invite a revision.

To help guide this revision, here are a few points that I see as being potentially make-or-break as we move forward:
We would encourage including experimental validation for the prediction of SPP1-integrin pair as a key ligand-receptor mediator of the pro-inflammatory response in HBCs, but this is not a requirement.

I hope you find this feedback helpful. If you have any questions or concerns, I'm always happy to talk, either over email or by phone. More technical information and advice about resubmission can be found below my signature. Please read it carefully, as it can save substantial time and effort later.

If any changes to the authorship are needed (addition, removal, or a change in order of authors), we require that an authorship change form be provided with the revised manuscript that indicates the

reason for the change and provides written consent for the change from all authors, including any that were removed.

I look forward to seeing your revised manuscript.

All the best,

Yuan Hu, Ph.D.
Scientific Editor, Cell Systems

Reviewers' comments:

Reviewer #1: The researchers used single nucleus RNAseq, combined with deep visual proteomics and differentiated trophoblast stem cells, to detail the responses of first trimester human placental cels to smoking. While the comprehensive analysis included placental villous cell types, most differences between smokers and non-smokers were found in placental Hofbauer and in syncytiotrophoblast substyles (defined by snDEG). Not surprisingly, they found enhanced oxidative stress, with no evidence for enhanced hypoxia, apoptosis or ferroptosis. Interestingly, many of the changes seemed compensatory, with activation of AHR-dependent xenobiotic response, increased STB mitochondrial accumulation, enhanced STB differentiation and fusion, upregulation of STB pro-angiogenic effectors, upregulation of HMOX1, and separately increased deposition of fibrin on STBs.

Overall, the experiments are well designed and executed, and the informatics-based analyses are compelling and well-presented. The data illuminate key pathways to placental cells response to a common injury and has clear translational relevance. Addressing the comments below may improve the analysis and its impact.

Comments:
1. The authors mention their analyses of cytotrophoblast cell columns, but they may want to elaborate more on the effect of smoking on the diverse extravillous trophoblasts in their samples.
2. Fig 4: the laser capture acquisition of single STBs requires better explanation. How were scSTB defined?
3. In the discussion the authors correctly focus on changes that may underlie the decreased incidence of preeclampsia amount smokers. Although the expression changes in PGF and ADM are statistically significant, they are relatively small. Yet, when combined with the other changes, as described in the discussion (please add the downregulation of ENG, Fig 5D), these may be important.
4. The entire text should be edited for syntax.

Minor comments:
* Line 85: Placental development occurs primarily in the first half of pregnancy, not only in the first trimester.
* Lines 116-117: "At term, the morphological effects of maternal smoking… include a higher placental weight…". This is controversial, and many authors report lower placental weight or no change (see review in PMID: 17900829)
* Might be good to add to Fig. S1 the average number of cigarettes smoked, as reported by the participants. Cotinine levels are most useful, but the number of cigarettes may be of interest to some of the readers.
* Supplementary Fig. S8 does not provide the scale represented by the bars.

Cell Systems Transparent Peer Review Record

Reviewer #2: Through snRNA-seq and deep visual proteomics of matched placental tissues, this manuscript identifies the syncytiotrophoblast (STB) and Hofbauer cells as the placental cell types most affected by maternal smoking in early pregnancy. Integrative analyses revealed dysregulation in oxidative stress, xenobiotic detoxification, mitochondrial function, and angiogenic signaling pathways. Complementary in vitro trophoblast stem cell experiments and independent cohort validations supported the in vivo findings. The work provides novel insight into how smoking-induced molecular perturbations contribute to placental dysfunction and fetal growth restriction (FGR) early in gestation. However, the analytical design and statistical rigor remain inadequate and require urgent improvement.

Major comments:

1)In the snRNA-seq analysis, the authors mention that "batch correction" was performed. To ensure the reproducibility of the analysis, they should specify the exact algorithm or software function used—for example, Harmony, CCA, or another integration method.

2)The results indicate an increased proportion of STB nuclei in smokers (mean difference = 9.1%, p=0.06), a trend approaching statistical significance. We recommend performing a subgroup analysis by gestational age (e.g., 5-7 weeks vs. 8-11 weeks) to verify whether this trend is more pronounced in a specific period, thereby avoiding uncertain conclusions due to limited sample size.

3)In the independent validation cohort, significant differences exist between smokers and non-smokers for both maternal BMI (21.9±2.8 vs. 25.3±3.7, p=0.03) and maternal age (26.3±6.0 vs. 34.7±6.9, p=0.002). Since both BMI and age are potential confounders of placental function, it is necessary to perform a multivariable regression analysis.

4)While CellChat predicts the "SPP1-integrin" pair as a key ligand-receptor mediator of the pro-inflammatory response in HBCs, this finding requires experimental validation. It is recommended to use a co-culture model with SPP1 blockade or integrin inhibition to definitively confirm its role in driving the inflammatory phenotype.

5)The study classified subjects only as "smokers" or "non-smokers" without stratifying by smoking dose. It is recommended to perform a dose-stratified analysis to clarify the dose-dependent relationship of maternal smoking.

Minor comments:

6)In the abstract, the term "deep visual proteomics" is used for the first time without defining its abbreviation ("DVP"). The abbreviation should be introduced upon its first mention.

7)The authors infer fetal sex based on the expression of sex chromosome-associated genes. To validate this methodological choice, citations are needed for studies that have previously established the utility of these particular genes for sex determination in similar research contexts (e.g., human placental or single-cell studies).

8)In the Discussion, the term "failed-maternal fetal tolerance" is incorrect. It should be replaced with the standard terminology "failed maternal-fetal tolerance".

9)The function name "FindintegrationAnchors" is misspelled. Please use the correct form, "FindIntegrationAnchors" (capital "I"), as defined in the Seurat package.

10)The format of the abbreviation is inconsistent (e.g., "snRNAseq" and "snRNA-seq"). Please use one consistent form throughout the manuscript.

11)The word "dysregulation" is repeatedly misspelled as "dysregulaiton" throughout the manuscript. Please correct it.

Cell Systems Transparent Peer Review Record

Authors' response to the reviewers' first round comments

Attached.

Editorial decision letter with reviewers' comments, second round of review

Dear Dr. Herse,

I hope this email finds you well. The reviews are back on your manuscript and I've appended them below. You'll see that the reviewers find the manuscript compelling and their comments are intended to strengthen an already strong piece of work. We're happy to invite a revision. Below are specific points I would like to highlight;
1. While we do not strongly require direct evidence that the identified DEGs/proteins affect fetal growth, please revise the manuscript to ensure that all claims are fully supported by the data presented. Any statements that go beyond what the evidence shows should be qualified or removed.
2. Please make sure to address major point 6.
If you have any questions or concerns about the revision, I'd be happy to talk about them over email. More technical information and advice about resubmission can be found below my signature. Please read it carefully, as it can save substantial time and effort later.

If any changes to the authorship are needed (addition, removal, or a change in order of authors), we require that an authorship change form be provided with the revised manuscript that indicates the reason for the change and provides written consent for the change from all authors, including any that were removed.

I look forward to seeing your revised manuscript.

All the best,

Yuan Hu, Ph.D.
Scientific Editor, *Cell Systems*

Reviewer comments:

Reviewer #3: This work brings an important development in perinatal biology and pregnancy. The study has generated a lot of data.

Major issues that repeatedly appear throughout the paper:
1) The write up requires appropriate toning. Where opinions are reflected based on prior literature, a clear citation is needed, but is frequently missing. At times, as in the Abstract, a reader cannot tell whether a sentence is referring to results or evidence from literature. At the end of each sub-section of the Results section, there are summary paragraphs---some require citation of prior literature, some need toning down not to overstate beyond the presented data, and some are unnecesary
2) While there is claim regarding value of the findings to fetal growth, no data has been provided in support. I urge the investigators to include evidence.
3) A lot of edits are required throughout to make it accessible to readers.

Some specific comments:
1) A major limitation that is in disagreement with the paper's tone is absence of evidence linking the signals (DEGs, proteins etc) with fetal growth outcomes. Otherwise, all statements from Abstract to Discussion need thorough revision. I encourage the authors to at least make some connection with existing research on birth outcomes (DEG, GWAS, EWAS etc)
2) Discussion (line 739): What is the evidence from the study regarding "STB fusion efficiency..." Claims such as these should be supported by data; I do not see direct data in support of this.
3) Different cut-off for cotinine levels was used for the discovery and validation cohorts. Why? Discuss implications.
4) Line 72-74: "We provide insight into placental dysfunction contributing to FGR early in pregnancy, before clinical symptoms appear." No such evidence was provided with regards to links to FGR or clinical complications
5) Line 221-223: These changes influence the development and function of this organ and underpin the pathophysiology of causally-related pregnancy complications to smoking. >>>no data to show influence on organ function not on complications. This is an over-step
6) Line 366-368: We microdissected and collected approximately 200 cells (or their equivalent area) across the whole tissue section per phenotype technical replicate from six placental tissues (n = 3 smokers, n = 3 non-smokers, all female fetuses). How does the cell type % and DEG expression levels compare between these female-specific subsample and the bigger cohort? What is the implication on generalizability? This is important because there is sex-specificity in placental function and gene expression profiles.
7) The specific elements of the analysis involving CellChat is missing. Add this in the Methods section, citing the original paper.

Other comments:
1) Abstract: "These molecular changes affect placental development with important consequences for fetal growth." This is stated without supporting data from the study. A reader would think that the study has also looked into fetal growth outcomes, which is not the case when reading the full paper.
2) Line 87: "The most abundant phenotypes in the placenta..... ". Replace 'Phenotypes' by 'cell types'. Phenotype is non-specific
3) Line 102: associated with the pathophysiology like fetal growth restriction (FGR), pre-term labor.... (replace 'like' by 'of')
4) Line 132: Reports largely focusing on global tissue dysregulation in the third trimester (...replace by "…have been largely focused on...")
5) Line 134-135: "...understanding of the transcriptional and proteomic changes of maternal smoking in first trimester placental tissue." (replace 'changes of" by "consequences of")
6) Line 176: placental (Hofbauer cells, HBC). I think you mean placental macrophages (Hofbauer cells, HBC)
7) Line 261: Human Proteomic Atlas....It is Human Protein Atlas (not proteomics)
8) Line 399-403: Trophoblast differentiation modeling revealed a subtle yet preferential shift towards the STB lineage in smokers. Smoking-associated dysregulation was linked to regulatory and effector genes governing cell signaling and cytoskeletal remodeling. These data support the notion that smoking disrupts core molecular programs of placental function and may influence trophoblast fate decisions. Both sentences require appropriate citation
9) Citations are missing in the Discussion section for the first paragraph sentences in lines 656-660

Cell Systems Transparent Peer Review Record

Authors' response to the reviewers' second round comments

Attached.

CellPress

CELL-SYSTEMS-D-25-00520 Revision 1 point-by-point response letter

Reviewers' comments:

Reviewer #1: The researchers used single nucleus RNAseq, combined with deep visual proteomics and differentiated trophoblast stem cells, to detail the responses of first trimester human placental cels to smoking. While the comprehensive analysis included placental villous cell types, most differences between smokers and non-smokers were found in placental Hofbauer and in syncytiotrophoblast substyles (defined by snDEG). Not surprisingly, they found enhanced oxidative stress, with no evidence for enhanced hypoxia, apoptosis or ferroptosis. Interestingly, many of the changes seemed compensatory, with activation of AHR-dependent xenobiotic response, increased STB mitochondrial accumulation, enhanced STB differentiation and fusion, upregulation of STB pro-angiogenic effectors, upregulation of HMOX1, and separately increased deposition of fibrin on STBs.

Overall, the experiments are well designed and executed, and the informatics-based analyses are compelling and well-presented. The data illuminate key pathways to placental cells response to a common injury and has clear translational relevance. Addressing the comments below may improve the analysis and its impact.

Comments:

1. The authors mention their analyses of cytotrophoblast cell columns, but they may want to elaborate more on the effect of smoking on the diverse extravillous trophoblasts in their samples.

We thank the reviewer for this insightful comment regarding extravillous trophoblasts (EVTs). Our study focused specifically on first-trimester villous samples, which are enriched for cytotrophoblast (CTB) cells, the syncytiotrophobalst (STB) and cell columns (CCTs), yet do not contain decidual tissue harboring mature EVTs.

While we observed a very moderate CCT transcriptomic dysregulation in smokers, there is evidence suggesting nicotine exposure impairs CTB proliferation and differentiation into column intermediates (https://pubmed.ncbi.nlm.nih.gov/7579909/). These column-forming CTBs represent the progenitors of invasive EVTs. Our trajectory analysis did not show a dysfunctional transition towards the CCT state (Fig 3H). Smoking-induced CCT dysregulation may impair subsequent EVT invasion capacity, future studies incorporating paired villous-decidual sampling will be needed to dissect mechanistic EVT lineage responses to maternal smoking.

2. Fig 4: the laser capture acquisition of single STBs requires better explanation. How were scSTB defined?

We thank the reviewer for this comment. Because the STB forms a continuous multinucleated syncytium without discrete cell boundaries, the isolation of single-cell STBs is not possible. Rather, we isolated STB syncytium areas for proteomic profiling. We have amended the results section have expanded the description in lines 454-465 to clarify how STB regions were identified for laser capture microdissection:

“The main cell types of interest based on snRNAseq findings included HBCs that constitutively express cluster of differentiation CD163 (filled arrow), CTBs characterized by epithelial cadherin (E-cadherin, coded by the *CDH1*; empty arrow) and the STB layer that is E-cadherin negative, and on the apical side of CDH1 positive villous structures, where maternal blood circulates (dotted arrow) (Fig 4B, C). To exclude potential contamination from the cell column trophoblast, we used serial section histocompatibility antigen, class I, G (HLA-G) immunostaining and selected only HLA-G negative regions. We annotated and collected STB regions (apical surface, CDH1neg/HLAGneg) as well as single HBC (CD163pos) and CTB (CDH1pos) cells for proteomics measurement. Notably, this technique allowed for the deep proteomic profiling of the multinucleated STB layer, which has been historically impossible due to the inability to derive this syncytium into a single cell suspension.”

This inclusion in the main reflects our described methods for the approach, line 1127: “Serial tissue sections were stained with HLA-G to exclude the selection of cell column trophoblasts as regions of interest.”

3. In the discussion the authors correctly focus on changes that may underlie the decreased incidence of preeclampsia amount smokers. Although the expression changes in PGF and ADM are statistically significant, they are relatively small. Yet, when combined with the other changes, as described in the discussion (please add the downregulation of ENG, Fig 5D), these may be important.

We thank the reviewer for this comment. It is correct, that the differences of PGF and ADM expression are relatively low. We therefore carefully validated these factors on different levels as described in the manuscript. In addition to the snRNA-Seq data, we validated PGF and ADM in the validation cohort by qRT-PCR and the PLGF level on protein level from maternal circulation. On all levels, we were able to detect the regulation

by smoking. We must point out here that we analyzed tissue and blood from first trimester (5-11 week of gestation). Our hypothesis is that even small differences at this early time points in pregnancy have a certain effect on the development of the placenta and the further progression of pregnancies. We are in line with the published circulating PLGF levels in the progression of preeclampsia (DOI: 10.1056/NEJMoa031884). It is important to note that at this stage of pregnancy, the gap between the control and the preeclamptic course is only just beginning.

We agree with the reviewer about the importance of the endoglin and included it in the discussion part that links preeclampsia and smoking:

Line 772-775: “Endoglin, a transmembrane co-receptor of transforming growth factor β was downregulated on protein level by smoking in STB and is described as an elevated anti-angiogenic factor involved in the development of preeclampsia. (https://doi.org/10.1038/nm1429)”

4. The entire text should be edited for syntax.

We have made several modifications throughout the text to improve the syntax of the manuscript.

Minor comments:

* Line 85: Placental development occurs primarily in the first half of pregnancy, not only in the first trimester.

We have changed the wording from “Placental development occurs primarily in the first trimester of pregnancy” to “Placental development occurs primarily in the first half of pregnancy” (line 87).

* Lines 116-117: "At term, the morphological effects of maternal smoking… include a higher placental weight…". This is controversial, and many authors report lower placental weight or no change (see review in PMID: 17900829)

We have changed the wording from “a higher” to “an altered” placental weight in the introduction and included the cited review as resource.

* Might be good to add to Fig. S1 the average number of cigarettes smoked, as reported by the participants. Cotinine levels are most useful, but the number of cigarettes may be of interest to some of the readers.

We agree it may be of interest to have information regarding the self-reported numbers of daily cigarette consumption per participant. We have added this to "Table 1. Characteristics of included study subjects" in an additional row as follows:

	Omics cohort			Independent validation cohort mRNA PlGF ELISA		
Parameter	Non-smokers	Smokers	p	Non-smokers	Smokers	p
Sample size (n)	11	5	-	15 24	24 36	- -
Cotinine (ng/mL)	n.d.	147.2 ± 42.1	0.002*	n.d. n.d.	147.4 ± 153.5 105.2 ± 97.0	<0.0001* <0.0001§
Self-reported cigarettes/day (n participants)	0	15-20 (n=3) 20-30 (n=2)	-	0	1-5 (n=9) 5-10 (n=16) 10-20 (n=8) Not specified (n=3)	-

* Supplementary Fig. S8 does not provide the scale represented by the bars. We have added the scalebar dimension (1mm) to the panel and legend of Supplementary Figure 8.

Reviewer #2: Through snRNA-seq and deep visual proteomics of matched placental tissues, this manuscript identifies the syncytiotrophoblast (STB) and Hofbauer cells as the placental cell types most affected by maternal smoking in early pregnancy. Integrative analyses revealed dysregulation in oxidative stress, xenobiotic detoxification, mitochondrial function, and angiogenic signaling pathways. Complementary in vitro trophoblast stem cell experiments and independent cohort validations supported the in vivo findings. The work provides novel insight into how smoking-induced molecular perturbations contribute to placental dysfunction and fetal growth restriction (FGR) early in gestation. However, the analytical design and statistical rigor remain inadequate and require urgent improvement.

Major comments:

1) In the snRNA-seq analysis, the authors mention that "batch correction" was performed. To ensure the reproducibility of the analysis, they should specify the exact algorithm or software function used—for example, Harmony, CCA, or another integration method.

We thank the reviewer for emphasizing reproducibility.
We have included our batch correction and integration approach in our methods section under 'Data integration and cell type and state annotation' in line 1252. It reads:
“Dataset integration was performed to model and correct for gene expression heterogeneity in samples attributable to differences in biological (sample ID, fetal sex) and technical (batch, sequencing depth) variation using the ‘sctransform’ package (v0.3.3). A zero-inflated negative binomial distribution was used to model gene expression. Anchor features across datasets were computed with the ‘FindIntegrationAnchors’ function using the 4,000 most variable genes computed with the ‘SelectIntegrationFeatures’ function. With integration anchors as foundation, datasets were integrated after normalization and variance stabilization with the ‘SCTransform’ function without regressing mitochondrial counts or sequencing depths. Cell cycle state was annotated using cell cycle scores with the ‘CellCycleScoring’ function that predicts S, G2M and G1 phases based on expression correlation of canonical marker sets. Gene expression counts from the integrated dataset was used for cell type and state annotation. Gene expression counts from non-integrated data were used for downstream analyses.“

Our approach follows the standard Seurat sctransform workflow as described in doi:10.1186/s13059-019-1874-1. This procedure omits the need for heuristic steps including pseudocount addition or log-transformation and improves common downstream analyses.

2) The results indicate an increased proportion of STB nuclei in smokers (mean difference = 9.1%, p=0.06), a trend approaching statistical significance. We recommend performing a subgroup analysis by gestational age (e.g., 5-7 weeks vs. 8-11 weeks) to verify whether this trend is more pronounced in a specific period, thereby avoiding uncertain conclusions due to limited sample size.

We thank the reviewer for this suggestion. We performed the requested subgroup analysis stratifying by gestational age (5-7 weeks vs 8-11 weeks) and found that the increased STB nuclei proportion in smokers (mean difference = 9.1%, p=0.06 overall) is indeed driven primarily by samples from the later first trimester (8-11 weeks; mean difference = 12.8%, p=0.025, Figure 1 for reviewer's inspection). We included this new finding in our manuscript figure 1F, and have edited results to reflect this.

Line 211, "We found increased STB nuclei proportions in placentas from smokers (mean difference = 12.8%, p=0.025, Fig 1F) and no differences for any other cell type. This was restricted specifically to the late first trimester (weeks 8 – 11) and was not observed in the earlier period (weeks 5 – 7, mean difference = -0.5%, p = 0.9)."

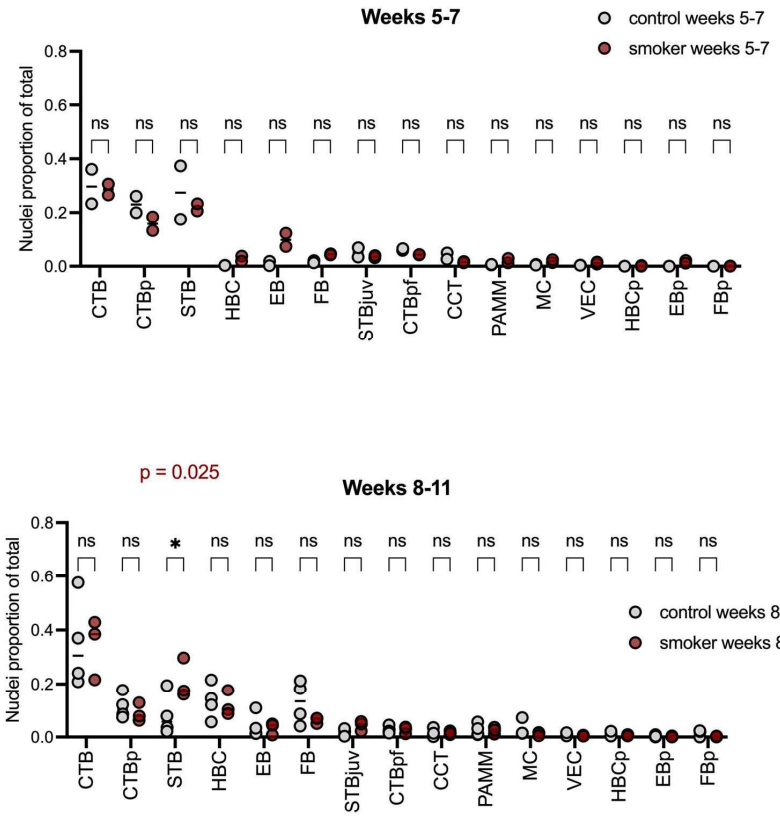


Figure 1 for reviewer inspection. Comparison of relative abundance per cell type between smokers and non-smokers, separated by gestational age. Differences per gestational age were calculated using a two-way ANOVA with a single pooled variance and using a Sidak's multiple comparisons correction.

3) In the independent validation cohort, significant differences exist between smokers and non-smokers for both maternal BMI (21.9±2.8 vs. 25.3±3.7, p=0.03) and maternal age (26.3±6.0 vs. 34.7±6.9, p=0.002). Since both BMI and age are potential confounders of placental function, it is necessary to perform a multivariable regression analysis.

We thank the reviewer for highlighting these baseline characteristics. Maternal age and BMI are well established confounders of placental function. Because significant differences exist in the validation cohort, we agree that modelling these variables as potential confounders helps enhance our findings.

Thus, we performed multiple linear regression analyses with marker expression as dependent variable, and smoking status, maternal age, maternal BMI, and gestational age (GA) as covariates. The model used was: Gene/Protein ~ Intercept + Smoke status + maternal age + maternal BMI + gestational age. This is now described in the methods in line number 1077-1080.

Smoking status remained significantly associated with altered gene expression across our previously significantly dysregulated genes after adjustment for these covariates. Consistent with our snRNAseq and previous uncorrected findings in the validation cohort, all markers are still significantly upregulated by smoking (except CYP1A1 that was not significant before). Maternal age and BMI were non-significant in all models.

To reflect these enhancements, we have amended the existing uncorrected 'P Previous smoke' p-values in Figure 3D,E with the new 'P Model smoke' values, as shown in Table 1 for reviewer inspection.

***Gene* / Protein**	**β Smoke (95% CI)**	**Model P**	**R2 Smoke**	**P Model smoke**	**P Previous smoke**
CYP1B1	1.09 (0.45, 1.74)	0.0012	0.38	**0.0017**	**<0.001**
ADM	0.53 (0.17, 0.89)	0.037	0.39	**0.0047**	**0.002**
PGF	0.46 (0.09, 0.83)	0.039	0.33	**0.017**	**0.0009**
PlGF (pg/mL)	5.96 (0.41, 11.5)	<0.001	0.15	**0.036**	**0.03**
CYP1A1	0.38 (-1.09, 0.87)	0.35	0.61	0.12	0.17

Table 1 for reviewer inspection. Multivariable regression evaluating differences between smokers and non-smokers, using maternal age, BMI and gestational age as covariates.

4) While CellChat predicts the "SPP1-integrin" pair as a key ligand-receptor mediator of the pro-inflammatory response in HBCs, this finding requires experimental validation. It is recommended to use a co-culture model with SPP1 blockade or integrin inhibition to definitively confirm its role in driving the inflammatory phenotype.

The reviewer raises an important point by emphasizing the need for experimental validation of the predicted SPP1–integrin interaction. While our CellChat analysis provides a hypothesis-generating framework, we agree that functional evidence is essential to support a causal role in driving the pro-inflammatory phenotype in HBCs.
In this context, we performed several complementary experiments that support the relevance of SPP1 in this system. Secreted phosphoprotein 1, also referred to as osteopontin, coded by the SPP1 is elevated in the placental macrophages (Hofbauer cells, HBC) of smokers (Fig 2a for reviewer inspection). This multifaceted glycoprotein is an important component of the extracellular matrix and plays a role as cytokine, associated with a pro-inflammatory state in other organs (10.1146/annurev.pharmtox.41.1.723, https://doi.org/10.1161/ATVBAHA.107.144824, https://doi.org/10.1007/s12079-009-0068-0). In line with this, we find SPP1 to be elevated in M1-polarized macrophages in-vitro, compared to M2-polarized macrophages (Fig 2b for reviewer inspection).
In the manuscript, we identified the SPP1-integrin interaction as a key ligand-receptor mediator of immune response. We now did a validation approach. In our current validation, we observed a dramatic shift in the proteome composition of M2-polarized bone marrow derived mouse macrophages following in-vitro stimulation with SPP1 (Fig 2 c,d for reviewer's inspection). The most highly upregulated proteins are strongly linked to immune response, including enrichment of interferon gamma response, interferon alpha response, and allograft rejection, as well as pathways related to MTORC1 signaling and cholesterol homeostasis (Fig 2e for reviewer inspection). These findings suggest that SPP1 not only marks inflammatory macrophages but can also actively shape their functional state. Interestingly, we also found CD74 as one of the most upregulated proteins (top 20). CD74 was identified by our group as a mediator of preeclampsia. We described CD74 was downregulated in preeclamptic placental macrophages, leading to impaired macrophage–trophoblast interactions, a shift toward a pro-inflammatory phenotype, and consequent placental dysfunction (Przybyl et al., Circ Res, 2016, DOI: 10.1161/CIRCRESAHA.116.308304). We already discussed in the manuscript "Maternal smoking constitutes the sole environmental exposure known to reduce the risk of gestational hypertension and preeclampsia (PE) by up to 50%".
Furthermore, we validated the cytotrophoblast cells (CTB) response to SPP1 as reviewer advised. In response to smoking components (stimulation by cigarette smoke extract; CSE), primary-derived cytotrophoblast cells (CTB) increase SPP1 expression and, as predicted by our CellChat analysis, integrin receptor alpha 5 (TGA5) as receptor partner for the interaction (Fig 2f for reviewer inspection).

In addition, we observed an upregulation of interleukin 6 (IL-6) and Intercellular Adhesion Molecule 1 (ICAM1) in response to SPP1 stimulation in in-vitro CTBs (Fig 2g for reviewer inspection). Both molecules are critical markers for immune recruitment following infection or injury (10.1002/JLB.2MR0220-549R, https://doi.org/10.1093/intimm/dxaa078). Our findings show that the SSP1-integrin pair may act as a key modulator for immune recruitment during smoking.

Thus, we have included our new data in the supplement of the manuscript in supplemental figure 8. We also changed the wording of our findings on page 23, lines 588-592 to "Within the tropho-immune comparison (CTB-HBC), we identified osteopontin (SPP1)-integrin signaling a driver of the HBC immune response (**Fig 5C)**. This secreted glycoprotein, an important component of the ECM, promotes an in-vitro immune response in murine macrophages and human trophoblast cells **(Suppl Fig 8)**. Further functional studies are essential to establish a causal role for predicted interactions."

Finally, we have changed the category label on Fig 5C from 'inflammation' to 'immune response' pertinent to the SPP1-integrin findings.

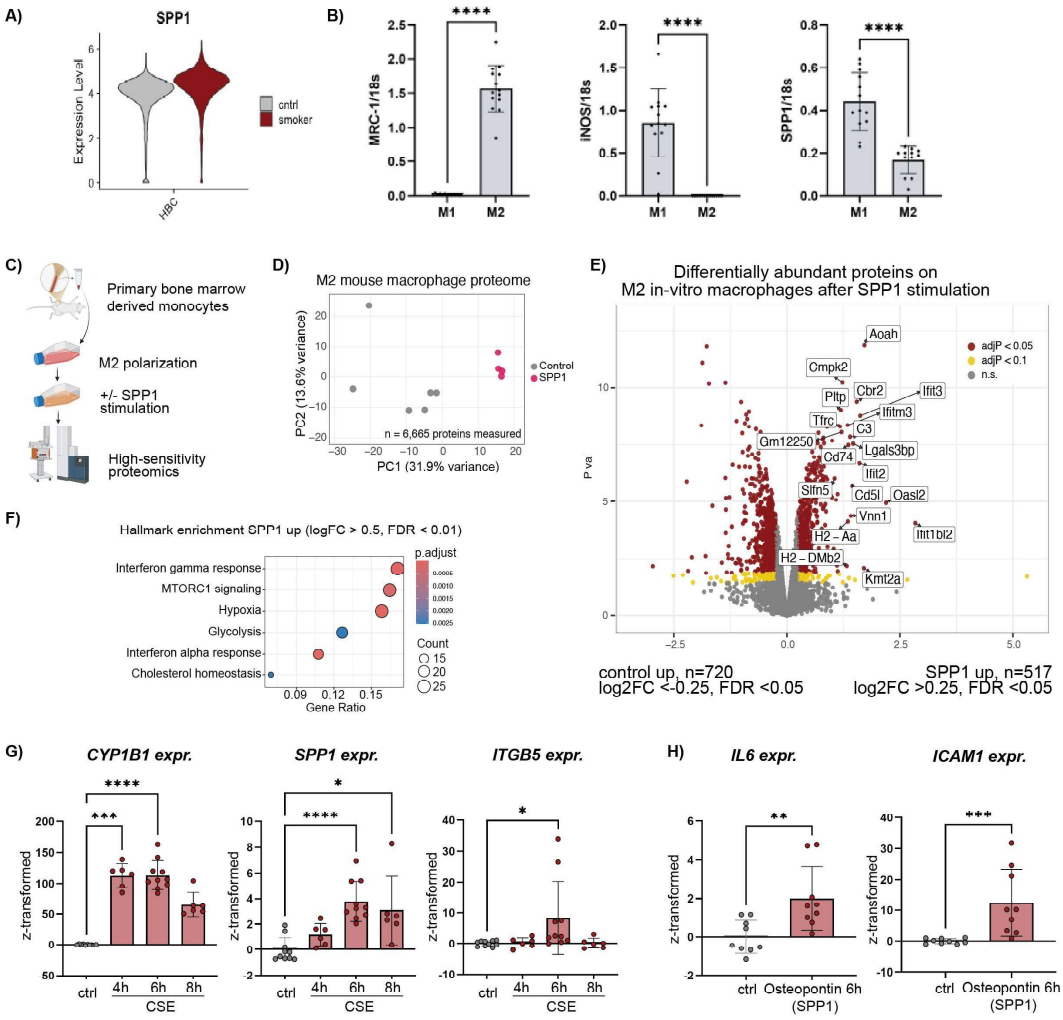


Figure 2 for reviewer's inspection: Osteopontin upregulation in smoking induces immune response and tissue recruitment. Panels C-H are included in the manuscript as Suppl Fig8 A-F.

A) snRNAseq expression of SPP1 (Osteopontin) in the Hofbauer cell nuclei from controls and smokers.

B) Mouse bone marrow-derived monocytes were polarized towards M1-phenotype by LPS (100ng/ml) or M2-phenotype by IL-4 (10 ng/mL) and IL-13 (10 ng/ml) for 24h respectively. Cells were analyzed by qRT-PCR. Classical marker (MRC-1 and iNOS) demonstrated their phenotypes. SPP-1 expression is higher in LPS-polarized M1 phenotype macrophages, indicating the role of SPP-1 in the pro-inflammatory state. (n=3 mice per condition).

C) Principal component analysis of the proteome of M2-phenotype mouse bone marrow derived macrophages (n=665 proteins measured).

D) Volcano plot of differentially abundant proteins in M2-phenotype mouse bone marrow derived macrophages following in-vitro SPP1 stimulation. The top 20 most dysregulated proteins are shown.

Differentially abundant proteins were calculated by linear regression and Benjamini-Hochberg correction in the limma package.
E) Summary of pathway enrichment analysis of in-vitro upregulated proteins by SPP1 stimulation of mouse macrophages (absolute log2FC > 0.5, FDR < 0.05). Enrichment was done based on ranked log2fold changes spanning the gene set enrichment analysis (GSEA) and Hallmark databases.
F,G) Gene expression of selected markers as measured via quantitative PCR of primary derived cytotrophoblast (CTB) cells following exposure to cigarette smoking extract (CSE [1:200 of 100%], F) and osteopontin (SPP1 [2.5ug/ul], G) in-vitro for variable timepoints as indicated. Differences calculated by two-tailed Welch's t-test.

5) The study classified subjects only as "smokers" or "non-smokers" without stratifying by smoking dose. It is recommended to perform a dose-stratified analysis to clarify the dose-dependent relationship of maternal smoking.

We thank the reviewer for this important suggestion. We fully agree that smoking exhibits dose-dependent effects across multiple organs (https://doi.org/10.1038/s41591-022-01978-x).
In this study, our cotinine sampling approach precluded a reliable dose-dependent analysis. Thus, we used cotinine values for smoking status classification between smokers and non-smokers.
Two main factors contributed to this. Firstly, maternal blood was collected on the day of elective pregnancy termination following an instructed 24-hour abstinence protocol, which systematically depresses habitual levels and introduces a procedure-related bias to measured cotinine values. Secondly, the degree of this systematic bias is uncertain, as it depends on individual adherence to smoking cessation instructions. For these reasons, conclusions of this manuscript pertain specifically to effects of moderate to heavy smoking on the early human placenta.
We have expanded on this limitation in the Discussion on page 31, lines 783-788 to clarify: "Smoking is known to exhibit dose-dependent effects across multiple organs. In this study, dose-response analyses were precluded by procedure-mandated smoking abstinence and self-reported compliance variability, both of which may bias cotinine measurements. Additional prospective studies with pre-procedure biomarker collection are needed to establish quantitative exposure-response relationships between maternal smoking and findings described herein."

Minor comments:

6) In the abstract, the term "deep visual proteomics" is used for the first time without defining its abbreviation ("DVP"). The abbreviation should be introduced upon its first mention.

We have added the “DVP” abbreviation to the abstract, in line 62 of the manuscript.

7) The authors infer fetal sex based on the expression of sex chromosome-associated genes. To validate this methodological choice, citations are needed for studies that have previously established the utility of these particular genes for sex determination in similar research contexts (e.g., human placental or single-cell studies).

We included two publications that worked and described these genes in placental tissue in the relevant results section, line 189.

doi: 10.1186/s13293-022-00470-y

doi: 10.1186/s13293-018-0165-y.

8) In the Discussion, the term "failed-maternal fetal tolerance" is incorrect. It should be replaced with the standard terminology "failed maternal-fetal tolerance".

The term has been amended to reflect the standard terminology of “failed maternal-fetal tolerance” in the discussion, line 680.

9) The function name "FindintegrationAnchors" is misspelled. Please use the correct form, "FindIntegrationAnchors" (capital "I"), as defined in the Seurat package.

The correct spelling for the function “FindIntegrationAnchors” is now included in the Methods section.

10) The format of the abbreviation is inconsistent (e.g., "snRNAseq" and "snRNA-seq"). Please use one consistent form throughout the manuscript.

The format of abbreviation “snRNAseq” has been harmonized throughout the manuscript. This includes Figure 4 legend for panel H (line 547), and the Methods ‘Data analysis’ section.

11) The word "dysregulation" is repeatedly misspelled as "dysregulaiton" throughout the manuscript. Please correct it.

All instances of the incorrect misspelling of “dysregulaiton” have been corrected, including Fig 2 legend for panels B,E (line 323), Fig 4 legend for panel H (line 543), Supplementary Fig 7 legend for panels C (line 1851) and F (line 1867).

CELL-SYSTEMS-D-25-00520R1 point-by-point response letter

Reviewers' comments:

Reviewer #3: This work brings an important development in perinatal biology and pregnancy. The study has generated a lot of data.

Major issues that repeatedly appear throughout the paper:
1) The write up requires appropriate toning. Where opinions are reflected based on prior literature, a clear citation is needed, but is frequently missing. At times, as in the Abstract, a reader cannot tell whether a sentence is referring to results or evidence from literature. At the end of each sub-section of the Results section, there are summary paragraphs---some require citation of prior literature, some need toning down not to overstate beyond the presented data, and some are unnecessary.

Response:

We thank the reviewer for this helpful comment and agree with the points raised. We have carefully revised the manuscript to ensure more appropriate and balanced toning throughout. Specifically, we have worked through each of the mentioned points by adding appropriate citations wherever statements are based on prior literature, and by clarifying the distinction between findings from the present study and evidence or interpretations derived from previous studies, particularly in the Abstract. We have also carefully reviewed the summary paragraphs at the end of each Results subsection. Where appropriate, we have added citations, toned down statements that extended beyond the data presented, and removed summaries that were not necessary.

2) While there is claim regarding value of the findings to fetal growth, no data has been provided in support. I urge the investigators to include evidence.

Response:

According the reviewer comment, we toned down our statements and direct links to FGR all over the manuscript. We now rather focus on insufficient placenta development that is leading to pregnancy complications. Please also follow-on the next comment.

3) A lot of edits are required throughout to make it accessible to readers.
Some specific comments:
1) A major limitation that is in disagreement with the paper's tone is absence of evidence linking the signals (DEGs, proteins etc) with fetal growth outcomes. Otherwise, all statements from Abstract to Discussion need thorough revision. I encourage the authors to at least make some connection with existing research on birth outcomes (DEG, GWAS, EWAS etc)

Response:

We thank the reviewer for this constructive comment. We agree that our study does not directly test associations between molecular signals and fetal birth weight or growth trajectories, and we have revised from Abstract to Discussion accordingly to avoid implying direct outcome linkages where it was not measured. Our intention is to frame our findings as potential mechanistic early pregnancy perturbations upstream of smoking-related growth

restriction. We therefore included further evidence (including additional references) to support claims made throughout the discussion:

Lines 680685:

“Our data supports that high transcript and protein turnover in the HBC coupled with its pro-inflammatory profile in maternal smoking conforms to this auto-amplifying vicious cycle that may ultimately contribute to placental insufficiency at term. Furthermore, smoking induced regulated genes we identified in the HBC are CCL4, CCL3L1 and PBX1, all of which have been previously implicated in the pro-inflammatory induction of FGR 62–64.”

Lines 716-719

“Activation of maternal platelets in placenta associated pregnancy pathologies directly underpins or propagates the underlying pathophysiology53, while increased fibrin deposition in the perivillous space is strongly associated with fetal growth restriction71,72.”

2) Discussion (line 739): What is the evidence from the study regarding "STB fusion efficiency..." Claims such as these should be supported by data; I do not see direct data in support of this.

Response:

We agree that without a functional readout the term efficiency is not supported. Our molecular findings speak only of known fusion markers. We have removed the word ‘efficiency’ from this sentence and rewritten it to tone down this statement. It now reads (line 739-741): “Our molecular findings suggest that STB fusion is indirectly affected by biochemical activator dysregulation, not directly via aberrances in morphological fusion drivers.”

3) Different cut-off for cotinine levels was used for the discovery and validation cohorts. Why? Discuss implications.

Response:

We used different cotinine thresholds by design. In the discovery cohort (snRNA-seq and DVP), smokers were defined as >50 ng/mL to capture heavy smoking and maximize signal detection in small, cell-type-resolved datasets. In the independent validation cohort, smokers were defined as >9 ng/mL to include moderate-to-heavy smokers and test whether core findings generalize. Non-smokers were defined consistently in both cohorts (<1 ng/mL), with status confirmed by concordance between self-report and cotinine.

This approach prioritizes sensitive discovery and broader validation, with findings supporting that smoking-associated placental dysregulation is not limited to the heaviest smokers. Effect sizes are smaller in the validation cohort because of exposure heterogeneity, which we interpret as expected rather than as evidence against reproducibility.

In accordance with the reviewer comment, we included a sentence to the result section. It now reads: (line 162-170): “Maternal smoking status was stratified by concordance between self-reporting and circulating cotinine levels, a stable metabolite from nicotine and clinical gold standard for assessing smoking status. In the cohort used for snRNAseq and DVP techniques, cotinine cut-offs of > 50 ng/mL (heavy smokers, range 88–183 ng/mL) and < 1 ng/mL (non-smokers) were used to stratify samples, to capture effects of heavy smoking. For

the independent validation cohort, cotinine cut-offs of > 9 ng/mL (smokers, range 9-536 ng/mL) and <1 ng/mL (non-smokers) were used to include moderate-to-heavy smokers and test whether core findings generalize to the broader smoking population."

4) Line 72-74: "We provide insight into placental dysfunction contributing to FGR early in pregnancy, before clinical symptoms appear." No such evidence was provided with regards to links to FGR or clinical complications

Response:

We have toned down this statement, as we agree that the established causal relationship between maternal smoking and FGR alone does not merit such a claim. The part now reads (line 76-79): "We provide insight into placental dysfunction potentially contributing to pregnancy complications early in pregnancy, before clinical symptoms appear. We anticipate this data to advance further research for diagnostics and therapies that improve fetal and maternal health."

5) Line 221-223: These changes influence the development and function of this organ and underpin the pathophysiology of causally-related pregnancy complications to smoking. >>>no data to show influence on organ function not on complications. This is an over-step

Response:

We deleted this sentence.

6) Line 366-368: We microdissected and collected approximately 200 cells (or their equivalent area) across the whole tissue section per phenotype technical replicate from six placental tissues (n = 3 smokers, n = 3 non-smokers, all female fetuses). How does the cell type % and DEG expression levels compare between these female-specific subsample and the bigger cohort? What is the implication on generalizability? This is important because there is sex-specificity in placental function and gene expression profiles.

Response:

We thank the reviewer for raising this important point. Spatial microdissection coupled with proteomics was feasible only in a limited set of six placental tissues (n = 3 smokers, n = 3 non-smokers), reflecting technical and resource constraints of this workflow. Within this design, we decided to restrict analysis to female placentas to minimize fetal sex confounding. Fetal sex effects were treated as covariates in the transcriptomics dysregulation analysis, as described in the beginning of the results (lines 190-193): "We evaluated the transcriptomic dysregulation of populations (> 500 nuclei) using a pseudo-bulk approach (edgeR) via a likelihood ratio test generalized linear model framework that accounted for fetal sex and sequencing batch as covariates and corrected for multiple comparisons (Fig 1D left)."

In the larger snRNA-seq dataset, sample-level analyses showed no significant differences in cell type proportions of STB and HBC Figure 1 for reviewers inspection) or in proteomics hub-gene expression between fetal sexes (Figure 2 (STB) and Figure 3 (HBC) for reviewers inspection). We therefore propose that the smoking effects highlighted here are likely generalizable across fetal sexes. This generalizability is evidenced by validations performed in an independent and larger cohort (n=39, 15 non-smokers, 24 smokers). We cannot exclude the possibility of sex-specific effects induced by maternal smoking, which would require further work for their elucidation.

We have added a statement in the results section to address this point (lines 468-470): “While this design increases power to detect smoking-associated changes, it precludes elucidation of sex-specific effects of maternal smoking, for which further studies are needed.”

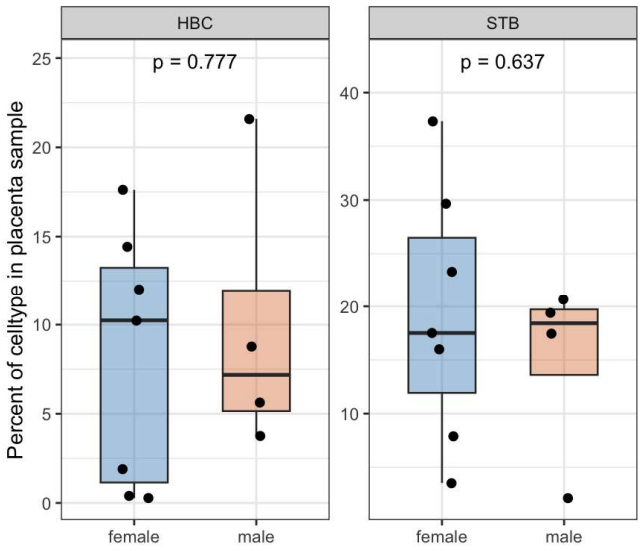


Figure 1 for reviewers inspection. STB and HBC proportions by fetal sex. Wilcoxon rank-sum on sample-levelproportions.

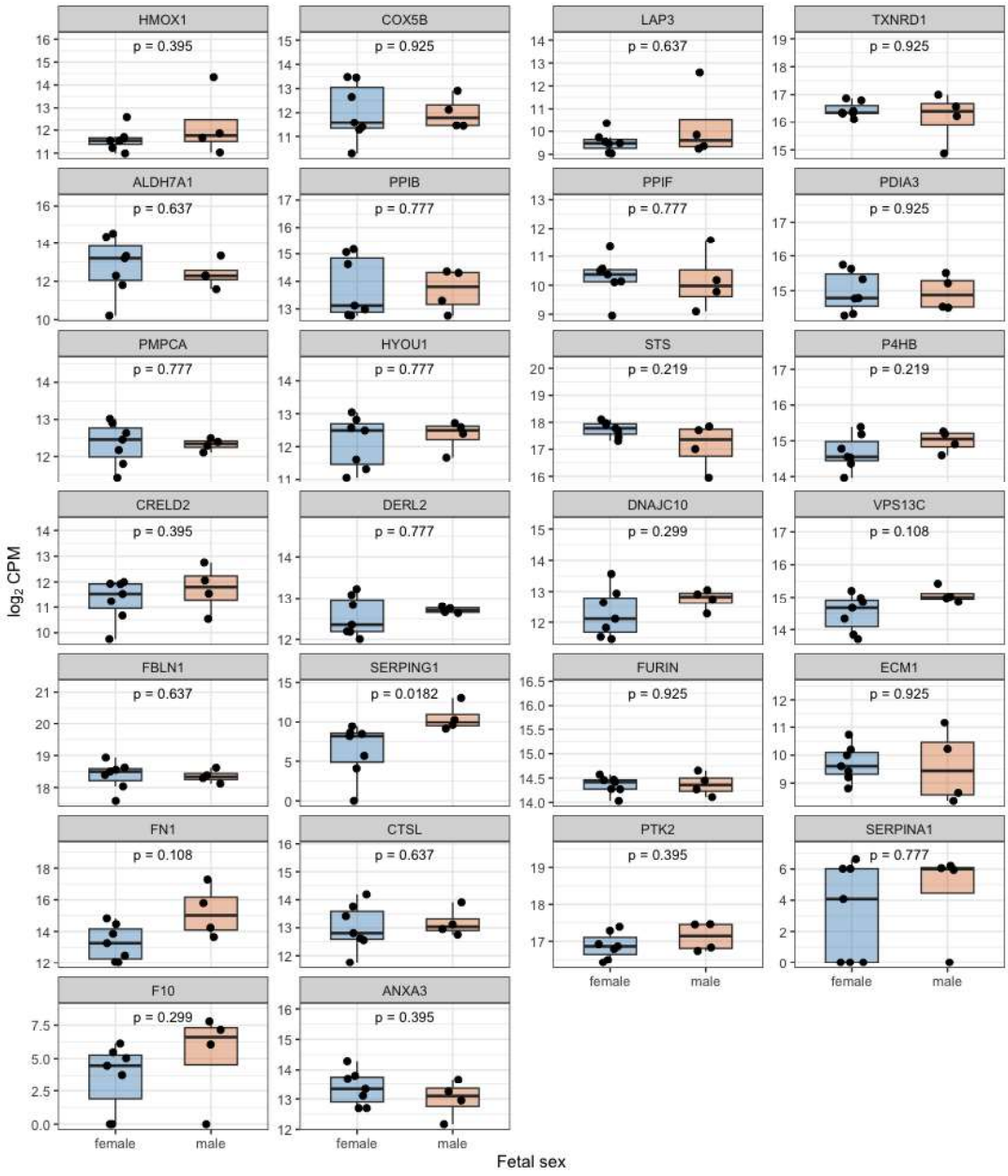


Figure 2 for reviewers inspection. DVP hub genes in STB from sample-level pseudobulk snRNAseq expression. STB non-quantifiable DVP hub genes (i.e. absent from the snRNAseq matrix): FGA, FGB, FGG, SERPINA3

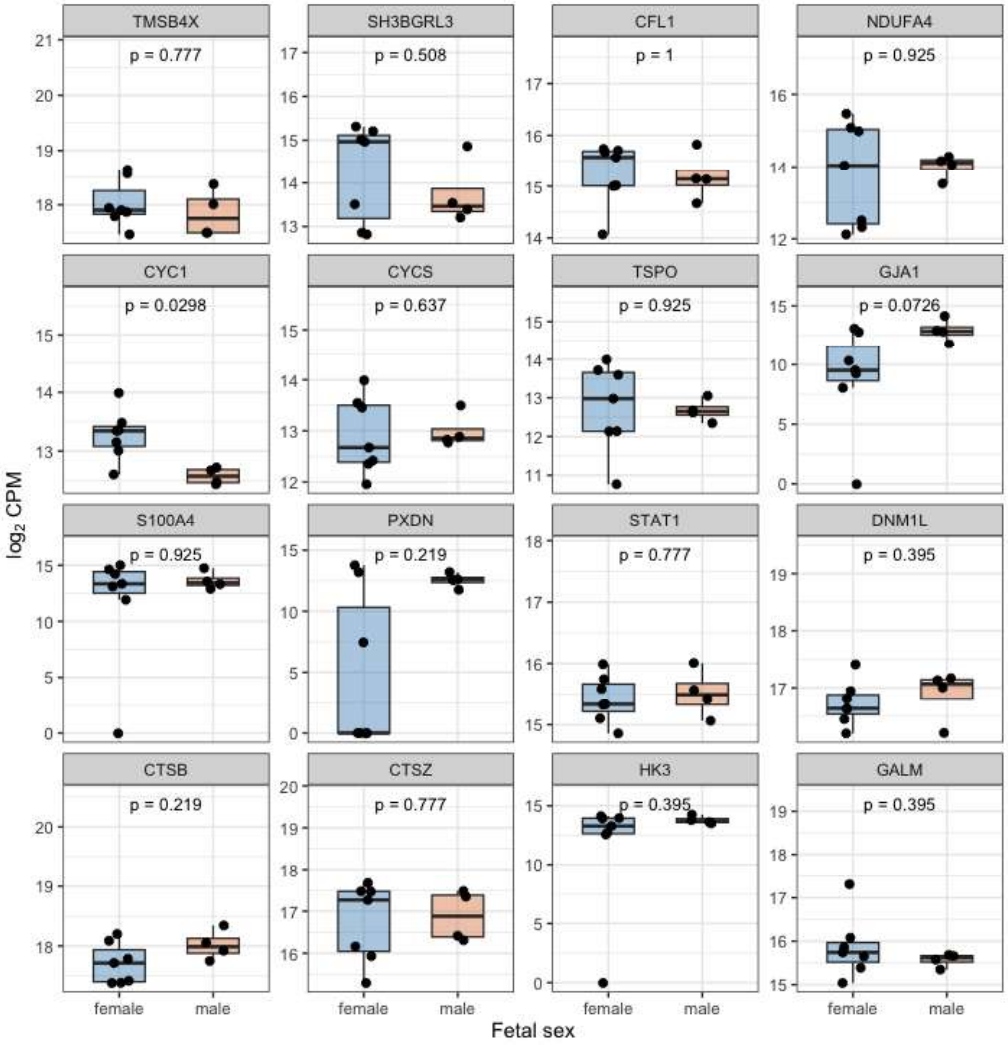


Figure 3 for reviewers inspection. DVP hub genes in HBC from sample-level pseudobulk snRNAseq expression. HBC non-quantifiable DVP hub genes: CALML5

7) The specific elements of the analysis involving CellChat is missing. Add this in the Methods section, citing the original paper.

Response:

We thank the reviewer for flagging this and included it in the methods under lines 1709-1732.

Other comments:
1) Abstract: "These molecular changes affect placental development with important

consequences for fetal growth." This is stated without supporting data from the study. A reader would think that the study has also looked into fetal growth outcomes, which is not the case when reading the full paper.

Response:

We included reference and toned down this statement.

2) Line 87: "The most abundant phenotypes in the placenta..... ". Replace 'Phenotypes' by 'cell types'. Phenotype is non-specific

Response:

We did.

3) Line 102: associated with the pathophysiology like fetal growth restriction (FGR), pre-term labor.... (replace 'like' by 'of')

Response:

We did.

4) Line 132: Reports largely focusing on global tissue dysregulation in the third trimester (...replace by "…have been largely focused on...")

Response:

We did.

5) Line 134-135: "...understanding of the transcriptional and proteomic changes of maternal smoking in first trimester placental tissue." (replace 'changes of" by "consequences of")

Response:

We did.

6) Line 176: placental (Hofbauer cells, HBC). I think you mean placental macrophages (Hofbauer cells, HBC)

Response:

Yes. Thank you!

7) Line 261: Human Proteomic Atlas....It is Human Protein Atlas (not proteomics)

Response:

We have corrected the name for the HPA abbreviation.

8) Line 399-403: Trophoblast differentiation modeling revealed a subtle yet preferential shift towards the STB lineage in smokers. Smoking-associated dysregulation was linked to regulatory and effector genes governing cell signaling and cytoskeletal remodeling. These

data support the notion that smoking disrupts core molecular programs of placental function and may influence trophoblast fate decisions. Both sentences require appropriate citation

Response:

We have included appropriate citations for both sentences.

9) Citations are missing in the Discussion section for the first paragraph sentences in lines 656-660

Response:

We have added citations for the first paragraph sentences of the Discussion.