Placental metabolic and endocrine changes with onset of the maternal circulation

## Abstract

***INTRODUCTION***: The intraplacental environment undergoes major changes at the end of the 1st trimester associated with onset of the maternal arterial circulation. There is a three-fold rise in oxygenation and a switch from histotrophic to haemotrophic nutrition. We hypothesised major changes in placental metabolism and function across this transition.

***MATERIALS***: Placental villi were obtained by chorionic villous sampling prior to termination of pregnancy at 7-8 (n=8) and 13-14 (n=6) weeks gestational age with patient consent and local ethics approval.

***RESULTS***: Most differentially expressed genes were associated with protein synthesis, transport and secretion, multi-level immune response, signaling pathways (MAPK, ERK1/2, GPCR, Rap1, cGMP-PKG, cAMP-mediated, NIK/NF-kappaB, Oxytocin), vasculature development, lipid metabolism and reactive oxygen species metabolism. Kegg and GO analysis showed an increase in inflammatory/immune response in second trimester (ST) as well as increase in graft rejection, corresponding to placental role in evoking the immune system response. Protein processing in ER was a primary term enriched in first-trimester samples.

***CONCLUSION***: Paradoxically, transcriptomic analysis revealed high synthetic capacity for peptide hormones under the low oxygen conditions prevailing in the first trimester. A novel hormone spexin that may modulate maternal lipid metabolism was identified in the syncytiotrophoblast.

## Significance Statement

Authors must submit a statement of no more than 120 words about the significance of their research paper written at a level understandable to an undergraduate-educated scientist outside their field of specialty. The primary goal of the Significance Statement is to explain the relevance of the work in broad context to a broad readership. Significance statements are not required for Brief Reports.

## Introduction

The placenta is essential to a successful pregnancy and the life-long health of the offspring. Impaired placental function has both immediate obstetric consequences (1), including fetal growth restriction, pre-eclampsia and stillbirth, and long-term impact on the risk of chronic disease for the offspring through developmental programming (2). Recent advances in imaging and biomarkers indicate that the pathophysiology of many non-communicable complications of pregnancy starts during early pregnancy (3). The intrauterine environment in which the placenta develops undergoes a major transition towards the end of the first trimester with the switch from primarily histotrophic to haemotrophic nutrition (4). This transition, which involves the same placental structure being perfused from different sources, is unique to the human and great apes, and may explain why conditions such as pre-eclampsia are virtually restricted to our species.

During the first trimester, maternal arterial blood flow into the placental intervillous space is restricted by aggregates of endovascular extravillous trophoblast that migrate down the lumen of the endometrial spiral arteries. The trophoblast cells are loosely linked by desmosomes (5), creating a network of intercellular channels through which plasma may seep. Consequently, the placenta develops in a relatively low oxygen environment (6), supported principally by carbohydrate- and lipid-rich secretions from the endometrial glands (7). These secretions are also a potential source of mitogenic growth factors (8), including epidermal growth factor (EGF) that stimulates proliferation of cytotrophoblast cells when applied to explant cultures (9). Metabolism of the placental tissues is heavily glycolytic, and the phylogenetically ancient polyol pathways are highly active (10).

With onset of the maternal circulation towards the end of the first trimester there is a three-fold rise in the intraplacental oxygen concentration (6). Oxygen has been implicated in the induction of many changes that take place at the transition between the first and second trimesters, including trophoblast proliferation and invasion, hormone production and transporter expression (11-13). The transition from histotrophic to haemotrophic nutrition involves other potential influences, such as the dilution of growth factor support and increased biomechanical forces, e.g. shear stress. Previous studies have compared gene expression in the first trimester placenta as assessed by microarray analysis with that at term (14, 15) when many other changes, such as placental senescence and preparation for delivery, will be taking place. In order to focus on the critical changes taking place during the first-second trimester transition, we have performed RNA-Seq on placental villi obtained under optimal conditions using a chorionic villus sampling technique from pregnancies accurately dated as being pre-and post-onset of the maternal circulation (7-8 weeks and 13-14 weeks gestational age, respectively). We have focussed our analyses on transcripts encoding proteins involved in metabolism, hormonal activity, transport and cell proliferation.

## Results and Discussion

To investigate differences in gene expression between the first and second trimester, paired-end RNA sequencing was performed. 14 samples were sequenced in total, with 8 first trimester vs 6 second trimester samples. Samples separated clearly on the basis of pregnancy stage, both using PCA and hierarchical clustering (Figure 1A, Suppl.Fig. 1A-C). Genes contributing to the PC1 separation on PCA plot include: SCN1A, MSC, IDO1, ELN, PRRX1, ALDH1A1, FGF10, ASB4, CCL13 and SPX. Changes were validated at the protein level, using Western blotting and immunohistochemistry.

Differential expression analysis identified 3702 differentially expressed (DE) genes with log fold change of at least 2 (Figure 1B). Using Kegg pathway analysis and Gene set enrichment (GSE), we identified several classes of genes that simultaneously change between the first and second trimester (Figure 1C-F). Notably, genes associated with protein processing in the endoplasmic reticulum (ER) were amongst the most differentially expressed, as were genes regulating cellular metabolism and hormone secretion, transport, and extracellular matrix (Fig. 1C-F). These are discussed below. To ascribe the DE genes to individual cell types the RNA-seq results were compared with a published scRNA-seq dataset (16), with first (8 weeks, comparable with 7-8 weeks first trimester of our RNA-seq) and late second trimester (24 weeks) samples. The scRNASeq identified several cell types in 8-week placentas, including cytotrophoblast (CTB), syncytiotrophoblast (STB), extravillous trophoblast (EVT) and stromal cells (STR); and EVT and for 24-week placentas. The dataset is particularly useful to determine the origin of DE genes that are upregulated in first trimester placenta.

### Metabolism

GO and Kegg enrichment pathway analyses both showed genes associated with protein processing in the endoplasmic reticulum (ER) to be amongst the most differentially expressed between the two groups. The term ‘Protein processing in ER’ was enriched in Kegg analysis (*P* = 1.29 x 10-3) (Suppl.Fig 2), while ‘ER lumen’, and ‘lumenal side of ER membrane’ were enriched in first-trimester samples on GO analysis (*P* =6.33 x 10-5 and 2.00 x 10-2 respectively). These, and other related GO terms, including regulation of secretion and response to oxidative stress (Fig. 2A-C), suggest that ER functional activity is greater during the first than the second trimester, despite the relatively low oxygen concentration. Such a pattern is consistent with the need for a high level of protein secretion by the syncytiotrophoblast during early pregnancy, in particular of the hormone human chorionic gonadotropin (hCG) that serves to maintain the corpus luteum and prevent onset of the menses. Maternal serum levels of hCG peak at around 10 weeks of pregnancy and subsequently decline as placental secretion of progesterone becomes dominant. Protein synthesis is energy demanding, yet, despite the low oxygen early placental environment, there are no significant differences in the concentration of the main energy metabolites (ATP/ADP, NAD+, glucose and lactate)(17). The transcript profile observed provides further evidence that the placental tissues are not energetically compromised during the first trimester. Glycolysis is the primary energy source, supported by the polyol pathways that preserve carbon skeletons for synthesis of purines and other molecules required for rapid cell proliferation (10). Consistent with this metabolic profile is the finding that *HK2* and *PKLR*, which encode hexokinase and pyruvate kinase, are among the most differentially expressed genes (Fig. 2G-H). Equally, β-oxidation of fatty acids appears to be suppressed for transcripts encoding long-chain acyl-CoA dehydrogenase (*ACADL*) rise in the second trimester. These changes may serve to protect the placental tissues from excessive production of reactive oxygen species when oxygen availability is low {Huang, 2014 #4444}(10). There are also profound differences in the lipid and cholesterol metabolism, which are discussed below.

Of the three conserved signalling pathways of the unfolded protein response, only the genes encoding the sensors PERK (PRKR-like endoplasmic reticulum kinase) and IRE1/XBP (inositol requiring transmembrane kinase-endoribonuclease 1/X-box binding protein) were significantly upregulated in first trimester, with no change in *ATF6* (Fig. 2 A/B/C, E, and SupplFig. 2.Kegg ER), or *GRP78* (Fig. 2D). These are likely to be homeostatic responses, as the PERK pathway upregulates anti-oxidant defences, in particular glutathione transport, which are indeed increased in the second trimester (Fig. 2F) as is the phosphorylation of eIF2α (Fig. 2D), while XBP is involved in the synthesis of lipoproteins essential for cell and organelle membranes. However, the actions of these pathways may be broader than just restoration of ER homeostasis and support of cell proliferation. Thus, activation of the IRE-1 pathway has been observed during the development of the labyrinth zone of the murine placenta using a transgenic reporter mouse, and knock-out of the gene leads to abnormal vascularisation, secondary to reduced levels of VEGF (18). Furthermore, ChIP-seq analysis has revealed that in skeletal muscle approximately 40% of the downstream XBP-1 transcription factor targets are unrelated to ER function, including genes associated with myogenic differentiation (19). We confirmed activation of the IRE1/XBP pathway in the first trimester by immunostaining, where XBP1 was strongly expressed by the villous, as well as extravillous trophoblast of the cell columns. XBP expression coincided with that of IRE1 and P-IRE1 (Fig. 2E).

Several GO terms associated with metabolism of oxygen are also significantly different between the two placental groups, including cellular response to oxygen-containing compound, reactive oxygen species metabolic process, positive regulation of reactive oxygen species metabolism and regulation of oxidoreductase activity (see Suppl. Table 2). As expected, there was upregulation of genes associated with antioxidant defences during the second trimester, most notably *SOD3, HIF3A, COX4I2, CYP1A1, CYP1A2* and *NOS1AP* (Fig. 2C, F).

### Hormonal activity

Transcripts encoding peptide hormones showed considerable differential expression, with some being higher in the first trimester and others in the second trimester (Fig. 3A-B). The former included sub-units of hCG; *CGA* showed a 2.66 log2 fold change while *CGB1*, *CGB2*, *CGB3*, *CGB5*, *CGB7* and *CGB8* showed log2 fold changes of 1.90, 2.89, 2.51, 2.54, 2.45 and 2.52, respectively. These results confirmed the findings of Bo and Boime that all six hCG genes are transcribed *in vivo*(20), and are consistent with secretion of hCG peaking at around 10 weeks of gestation and then declining. Recent evidence indicates that hCG secretion may be mediated in trophoblast-like cell lines by the epidermal growth factor receptor (EGFR) pathway {Wang, 2018 #4445}. EGF is a component of histotroph and so this signalling loop may be part of the trophoblast-endometrial dialogue that stimulates early placental development(21). Also higher in the first trimester were transcripts encoding leptin (*LEP*) (log2 fold change 2.97), relaxin (RLN1) (log2 fold change 1.51) and insulin like 4 (*INSL4*) (log2 fold change 1.74).

Of the transcripts higher in the second trimester, the greatest changes were seen for spexin (SPX) (log2 fold change 3.52)(Fig. 3A,C). Spexin is involved in the regulation of body weight and metabolism, and inhibits the uptake of long-chain fatty acids by adipocytes and hepatocytes (22). It has not previously been described in the human placenta, but IHC showed it to be localised to the syncytiotrophoblast (Fig. 3D). Secretion of spexin may therefore play a novel role in regulating maternal lipid metabolism during pregnancy, possibly by making more fatty acids available for transport to the fetus. Related to spexin, transcripts encoding hypocretin receptor 2 (*HCRTR2*), also known as orexin receptor 2, were upregulated (log2 fold change 3.08)(Fig. 3A). This G-coupled receptor binds the neuropeptide orexin that regulates appetite, but may also be involved in lipid metabolism. In contrast, the hunger and satiety-maintaining hormone leptin was upregulated in the first trimester placenta (Fig. 3A-C). Compared to non-pregnant women, the high energy demand of pregnancy is associated with elevated maternal leptin concentrations due to an accumulation of body fat and placental production. However, in contrast to leptin’s effect on satiety, human pregnancy requires increased nutrient delivery to the fetus and it thus associated with increased food intake. This is due to central leptin resistance, which occurs in the second trimester of pregnancy(23). Research in animal models has demonstrated that leptin is involved in the development and maturation of a number of organs, including the heart, brain, kidneys, and pancreas (24). Placental leptin might thus play a role in organogenesis during the first trimester.

Kegg pathway analysis revealed ‘autoimmune thyroid disease’, ‘thyroid hormone synthesis’ and ‘thyroid hormone signaling pathway’ to be enriched. Thyroid hormones are important for fetal development during the first trimester, in particular for the central nervous system, and must be transported across the placenta (25). The three major binding proteins, T4 binding globulin, transthyretin and albumin have all been identified in the mature placenta (26). Here we show for the first time that transcripts of *CRYM*, which encodes crystalline mu, a T3 binding protein, are present in the placenta and enriched in the first trimester (1.20 log2 fold change). By contrast, TTR encoding transthyretin is more highly expressed in the second trimester (1.44 log2 fold change)(Fig. 3A,B). These findings raise the possibility of novel regulatory pathways for the transfer of thyroid hormones across the placenta.

### Transport

Gene expression of transport proteins mediating oxygen, lipid, protein, glucose, and ions were significant altered between the first and second trimester placenta (Fig. 4A-B). Transcripts encoding the haemoglobin subunits epsilon 1 and zeta were within the top 3 differentially expressed genes, with log2 fold changes of 5.28 and 4.83, respectively (Fig. 3D,E). These chains make up embryonic haemoglobin that predominates during the first trimester, and has in the past been associated with erythropoiesis in the yolk sac (27). The yolk sac was not represented in our samples, and these transcripts most likely arise from the haemangioblastic clusters within the villous stromal core (28). Our findings suggest that a similar switch takes place within the villi, but whether this is driven by changes in the oxygen concentration or is ontogenetic is not known.

The pattern of expression of lipid transporters and apolipoproteins was profoundly different in the first and second trimester. Transcripts highly abundant during the first trimester included *APOA5*, *APOA2, APOL5* and *SLC27A6* whilst there seemed to be a switch in the usage of transporters, many being upregulated in the second trimester: *ABCA6, ABCA8, ABCA9, ABCA3, APOD, APOL1, APOLD1, APOLD3, APOE, APOA1, ATP8A1, ATP8B4, ATP9A, ATP8B3, PLTP, OSBPL7* (Fig. 3E, suppl fig lipids??). This is perhaps not surprising. Cholesterol is essential in early metabolism. The secondary yolk sac is likely to be essential for the survival of the embryo during the first weeks of development, expressing abundant mRNAs encoding multiple apolipoproteins, the cholesterol efflux transporter ABCA1, and lipoprotein receptors, including megalin and cubilin (29). The high abundance (i.e., top 0.5%) of transcripts encoding apolipoproteins present in lipoprotein particles and chylomicrons (ApoB, ApoA1, ApoA2, and ApoA4) was matched by the high levels of these proteins in the coelomic fluid. The placental villi, the exocoelomic cavity, and the secondary yolk sac thus function together as a physiological equivalent of the choriovitelline placenta in early gestation. This changes in the second trimester, following the onset of maternal blood flow.

Transcripts encoding transporters of metal ions important for antioxidant defences are also over-represented, for example *SLC30A10* and *SLC30A2* (log2 fold changes 3.35 and 1.78 respectively)(Fig. 3E) that transport manganese and zinc ions that are essential cofactors for the superoxide dismutase enzymes. Genes involved in the transport of iron are also abundantly expressed, notably *LTF* that encodes lactotransferrin and *HEPH* that encodes hephaestin (log2 fold changes 1.77 and 2.91 respectively). Hephaestin has previously been reported in the trophoblast-like cell line, BeWo (30), but the finding of *LTF* transcripts is novel.

During the second trimester the most significant changes are observed amongst transcripts encoding ion channels, for example *SCN1A*, *SCN7A*, *TRPA1*, *KCNQ3*, *KCNA4*, *KCNK17*, *KCNIP1*, *KCNJ16*, *KCNMA1*, and *MCOLN2* (Fig. 3E). These findings suggest that ionic homeostasis within the placental tissues becomes more important with onset of haemotrophic nutrition. This may reflect in part a switch in the way in which amino acids cross the placenta, being derived through uptake and subsequent breakdown of maternal histotroph proteins in the first trimester (7) to more active uptake of individual amino acids from the maternal circulation.

### Cell proliferation and differentiation

Rapid cell proliferation and differentiation occurs during the first trimester to establish the placenta. Transcripts encoding the transcriptional regulator high-mobility group AT-hook 2 protein (*HMGA2*) were highly differentially expressed (log2 fold change 3.33)(Fig. 4C), typical of embryonic tissues. HMGA2 plays a role in proliferation and differentiation, homozygous mutations in the *Hmga2* gene result in the *pygmy* phenotype(31), while haploinsufficiency of the *Hmga1* gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice (32). Expression of *HAND1*, which regulates differentiation of trophoblast sub-types in the mouse (33) was also upregulated in the first trimester (log2 fold change 2.61)(Fig. 4A), but the function of this transcription factor during human placental development is less certain. Many of the transcription factors upregulated in the second trimester regulate mammalian development and differentiation processes. These include *KLF2* (adipogenesis, embryonic erythropoiesis), *SOX18, PBX4, MEF2C, GPER1, SOX7, HEYL*, *TFAP2E, MYT1L* (CNS development), *BNC2, STAT4* (differentiation of T helper cells) etc. (Fig. 4A).

Wnt signalling plays an important role in cell proliferation, differentiation and motility under normal and cancer conditions (34). Recent evidence suggest that Wnt signalling may also be implicated in the regulation of placental development and human trophoblast differentiation (35). Indeed, *WNT1* was most active in the first trimester samples (fold change 1.39)(Fig. 4B), as has been previously described (14). In addition, *WNT10B*, as well as several other genes mediating the canonical Wnt signalling, including *PORCN* (mediates Wnt transport and secretion)(36), and *SDC1* (inhibitor role) were upregulated in the first trimester. In contrast, *WNT3A, WNT10A, WNT2, WNT9B, LRRK2, RYR2, LRP6, CCND1, NR4A2* and *RSPO3* transcripts were significantly upregulated in the second trimester (Fig. 4B). The canonical Wnt signalling has been shown to be critical for invasive trophoblast differentiation (37). In addition, several genes that regulate the non-canonical Wnt pathway are upregulated in the first trimester, *WNT7A*, and in the second trimester, including *WNT4, WNT5B and LEF1*. In addition, negative regulators of the Wnt signalling, NKD2 and DKK3, were also upregulated in the second trimester. DKK3 inhibits canonical Wnt signalling by disrupting binding of LRP-5/6 to the Wnt/FZD complex. This might suggest paracrine mechanisms to regulate trophoblast invasion in the second trimester. The majority of the Wnt signalling transcripts upregulalted in the second trimester localised to EVTs, whilst the first trimester transcripts that were upregulated localised to EVTs and the syncytiotrophoblast (Fig. 4B).

**Extracellular matrix and angiogenesis**

There were significant differences between the first and second trimester in the placental expression of transcripts regulating extracellular matrix (ECM) remodelling (Fig. 4D). Soon after implantation, one of the trophoblast sub-populations, the extravillous trophoblast, migrate from the placenta into the endometrium where they are involved in the remodelling of the maternal spiral arteries that ultimately supply the placenta. Invasive properties of these cells are widely attributed to the matrix metalloproteinases 2 and 9. MMP-2 mediates trophoblast invasion during the early implantation stage up to 7 to 8 weeks of gestation, whereas MMP-9 facilitates subsequent invasion (38, 39).  Indeed, we found an upregulation of the *MMP2* transcript in the first trimester (log2 fold change 1.21), whilst *MMP9* was upregulated in the second trimester (log2 fold change 1.60), as was *MMP1* (log2 fold change 2.79) and *MMP28* (log2 fold change 1.98). One of the most differentially expressed transcripts was *MEP1A* that encodes meprin, a member of the astacin family of metalloproteinases. These can be secreted, and so may assist in in matrix digestion, or membrane-bound, where they may be involved in the extracellular cleavage of proteins (40). Meprins can hydrolyse biologically active peptides, cytokines, chemokines, and ECM proteins, in particular the basal lamina proteins (e.g. collagen type IV, laminin-1, nidogen-1 and fibronectin), as well as proteins that are involved in interactions between cells (41-43). Meprins are abundantly expressed the epithelial cells of the intestine, kidney and skin, and we show they localize to the human villous trophoblast cells (Fig. 4E).

Vasculogenesis and angiogenesis are critical for successful placental exchange, and it is often cited that the low oxygen conditions during the first trimester stimulate these processes. However, we did not find classical hypoxia-regulated factors, such as *VEGF,* to be differentially expressed, in keeping with our demonstration that HIF protein is not stabilised during early pregnancy (17). By contrast, transcripts encoding angiogenin (*ANG*) and endoglin (*ENG*), powerful regulators of angiogenesis, were highly differentially expressed (log2 fold change 2.61 and 1.76, respectively). Angiogenin has been localised at the mRNA and protein levels to the trophoblast and endothelial cells of the fetal placental vessels (44). Markers of endothelial vascularisation such as *PECAM1, VWF, ICAM1* were significantly upregulated in the second trimester placenta (Fig. 4D), reflecting the fact that terminal villi, the most important component of the villous tree for materno-fetal exchange, only develop during the second half of pregnancy (45). Transcripts encoding the FSH receptor (*FSHR*) were also highly abundant in the first trimester (log2 fold change 1.67). FSHR is present on fetal endothelial cells, and knock-out in the mouse causes reduced vascularisation of the labyrinth zone of the placenta (46). Hence, placental angiogenesis may be driven predominantly by non-VEGF dependent pathways during the first trimester.

During pregnancy, the maternal immune system is modulated by signals from the placenta, there is a shift from a Th1 to Th2 profile with evidence of increased activation of innate cells in the systemic circulation. Regulatory CD4+CD25+Foxp3+ T cells (Tregs) expand during the second and third trimesters of pregnancy in the peripheral blood and in the decidua, believed to be induced by paternal antigens and contributing to the local control of fetus-specific maternal immune responses (47, 48). The transcription factor musculin (MSN) is critical for the development of induced Treg cells by repression of the T helper type 2 transcriptional programme (49). The transcript for *MSN* was significantly upregulated in the second trimester (Fig. 4D, G). This is the first report of this regulator in the placenta, where it localizes to the syncytiotrophoblast cells (Fig. 4G).

Overall, it appears that although the first-trimester placenta develops in a relatively low-oxygen environment, the tissues are highly proliferative and synthetic. The transcriptome can only provide an indication of the potential secretome due to differential translation and post-translational modifications. However, obtaining the secretomes for first and second trimester placentas is impossible due their inaccessibility and the short survival time of placental explants *in vitro*.

### Methylation

There were significant differences in gene expression observed between the first and second trimester, and we have shown that many of them were associated with oxidative stress, ER, hormones, transport and proliferation. We then investigated whether any changes in gene expression levels between first and second trimester placenta could be explained by changes in DNA methylation. The DNA and RNA samples were from the same individuals. Since DNA methylation levels at the gene promoters most likely affect their expression, we overlapped identified DMRs with gene promoters (regions of 2 kb upstream of transcription start site), and compared gene expression levels with changes in DNA methylation (Fig. 5A). We identified 430 DMRs, with 264 overlapping at least one gene promoter, and 126 DEGs (62 DEGs with fold change more than 2). With a more stringent cut-off for DNA methylation changes in DMRs (> 10%) and minimum fold change of 2 in DEGs, 22 genes were identified where the change in expression levels is likely to be driven by DNA methylation changes at their promoters. These genes are distributed across the genome and not clustered to specific genomic regions (Fig. 5B). Among these, 3 genes *FAS, CH25H* and *CYP2R1*, are involved in oxidative stress response; metalloproteinase *TLL1* and *RNF17* associated with proliferation and differentiation; and *ASTN1* and *CLDN10* involved in cell-cell adhesion (Fig. 5C).

**Conclusions**

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## Methods

### RNA extraction

### RNA Sequencing

### Bioinformatics

#### RNA-Seq Analysis

Paired-end sequencing was performed on Illumina NextSeq Direct High Output with read lengths of 100 bp. QC of sequencing was assessed using FastQC, fastq\_screen and Picard, summarised with MultiQC (VERSION). Reads were trimmed with TrimGalore! and aligned to the human genome (GRCh38) with STAR aligner, with 91.2% reads uniquely mapped and mean of 53.4M paired reads/sample. Gene quantification was determined with HTSeq-Counts (v0.6.1p1). Additional quality control was performed with rRNA and mtRNA counts script. Counts extracted with htseq-counts were used to perform differential gene analysis in R (version 3.5.2) using package DESeq2 (v.1.22.2). Read counts were normalised on the estimated size factors. Principal component analysis (PCA) was performed on rlog-transformed count data for all genes. Gene Ontology and pathway analysis was performed using clusterProfiler package (v.3.10.1).

The data matrix for scRNA-seq data was obtained from the WANG lab {ref, GEO accession number GSE89497},

Heatmaps were generated with ‘ComplexHeatmap' R package (v 1.20.0). Karyoplot was generated with karyoploteR (v1.8.8).

#### DNA Methylation Analysis

DNA methylation analysis was performed on EPIC array (Illumina). Samples were treated with oxBS (bisulfite treatment followed by oxidation) to investigate only 5mC signal, without 5hmC noise. DNA methylation analysis of EPIC dataset was performed using Champ (v.xxxxx).

 (window size xx CpGs),

#### Data and Code Availability

For reproducibility, all R scripts can be found on GitHub page: Link to GitHub site (<https://github.com/nmalwinka/2019_Prater_Cindrova>).

The RNA-sequencing data is accessible through the ArrayExpress series accession number:  E-MTAB-6683. The EPIC data can be accessed from: data repository link

## Figure legends

**Figure 1**. Performance of RNA-seq and significant terms and pathways distinct to first and second trimester placenta. a) PCA. b) Volcano plot of DE genes, with 1st trimester genes in red, and second trimester genes in blue. c) Selected significant Kegg pathways. d-f) Barplot showing selected significant GO terms in Biological Processes(d), Molecular Functions (e) and Cellular Components (f). Each barplot shows how many genes within each term are expressed more in 1st (red) or 2nd trimester (blue). Transparency is used to show the most significant (padj) terms as least transparent. Terms were ordered by qvalue.

**Figure 2.** Differentially expressed metabolism genes associated with ER processing, secretion and oxidative stress. a-c) Heatmaps of top DE genes in 1st vs 2nd trimester samples, compared to sc RNA-seq dataset with 1st and late 2nd trimester placenta samples (see methods).  a) Heatmap showing top DE genes involved in ER processing and stress. b) heatmap showing top DE genes involved in protein secretion. c) Heatmap of top DE oxygen-response genes. D-??) antibody stainings of pre/post-flow sections- Tereza? .  h) Heatmap of DE glycolytic genes.

**Figure 3**. Overview of hormonal activity and transport related genes differentially expressed between 1st and 2nd trimester. a) Heatmap showing top DE genes related to hormone activity, in comparison with sc-RNAseq data. b/c) validation and pre/post-flow sections?. d) Volcano plot of transport-related genes, with DE genes coloured red (enriched in 1st trimester) and blue (enriched in 2nd). e) Heatmap of top transport related genes, split by type of transport.  fg) antibody stainings of pre/post-flow sections?//Western/qPCR

**Figure 4.** Overview of TFs and proliferation- related genes differentially expressed between 1st and 2nd trimester. a) Heatmap of differentially expressed transcription factors. b) Heatmap of DE genes associated with WNT signaling. c) Heatmap of DE cell cycle genes. d) Heatmap of ECM- related DE genes. efg) Antibody staining of pre/ post-flow sections for Musculin- Tereza.

**Figure 5.** DNA methylation changes around genes between first and second trimester are very minor. a) Scatterplot showing relation between expression change and methylation change in 1st fs 2nd trimester placenta for each gene. Statistically significant DMRs are marked red (FT) and blue (ST). Genes with at least 2-fold change in expression and at least 10% methylation change are labelled.  b) Karyoplot showing chromosomal distribution of genes which differential expression was likely driven by change in methylation in promoter. c) Heatmap of genes likely regulated by DNA methylation in promoters.

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