Transcript analysis reveals changes in placental metabolism, transport and endocrinology across the first-second trimester transition

Biological Sciences – Physiology

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Key words: early placenta, metabolism, transcriptome

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## Abstract

The human gestational sac undergoes major changes at the end of the first trimester associated with onset of the maternal intraplacental circulation. Onset leads to a three-fold rise in oxygen concentration, formation of the definitive placenta and membranes and a switch from histotrophic to hemotrophic nutrition. We evaluated the impact of this transition on trophoblastic development and function using RNA-Sequencing (RNA-Seq) and DNA methylation (EPIC array) on the same chorionic villous samples at 7-8 (n=8) and 13-14 (n=6) weeks of gestation. Differentially expressed genes (DEG) were ascribed to individual cell types by comparsion with a published single-cell RNA-Seq dataset. Most DEGs were associated with protein processing in the endoplasmic reticulum (ER), cellular metabolism, hormone secretion, transport, extracellular matrix, vasculogenesis, lipid metabolism, and reactive oxygen species metabolism. Transcripts upregulated in the first trimester were associated with synthesis and ER processing of peptide hormones, along with glycolytic pathways. Transcripts encoding proteins mediating transport of oxygen, lipids, protein, glucose, and ions were significantly altered between the first and second trimesters. We identified a novel hormone in the syncytiotrophoblast, spexin, which is upregulated in the second trimester and may modulate maternal lipid and glucose metabolism. Gene expression was correlated with promoter/gene body methylation. Overall, the pattern is consistent with the need for a high level of cell proliferation and hormone secretion by the early placenta, such as hCG to secure implantation and prevent menses. Our results support the concept that the human placenta develops in a physiological low-oxygen, rather than an hypoxic, environment.

## Significance Statement

Failure of normal placental development leads to complications, including miscarriage and fetal growth restriction. The human gestational sac undergoes major physiological changes at 10-12 weeks’ gestation with onset of the maternal intraplacental circulation, including a three-fold rise in oxygen concentration. Transcriptome and DNA methylation analyses of accurately dated placental samples collected across the transition revealed that, paradoxically, placental tissues are highly proliferative and synthetic during the first trimester low-oxygen, glycolytic environment, secreting peptide hormones that signal pregnancy. Transport pathways appear to switch during the second trimester with more direct access to maternal blood. Correlations between differentially expressed genes and promoter/gene body methylation were observed across the trimesters. Further, we identify a novel placental hormone, spexin, that may modulate maternal metabolism.

**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 miscarriage, 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 biomarker studies indicate that the pathophysiology of many non-communicable complications of pregnancy starts during early pregnancy (3). The human gestational sac from which the placenta and fetus develop undergoes a major transition towards the end of the first trimester with the switch from primarily histotrophic to hemotrophic nutrition (4). This transition, which involves the same placental structure being supplied with nutrients by contrasting pathways, is unique to the human and great apes, and may explain why conditions such as pre-eclampsia are virtually restricted to the human species.

During the first trimester, maternal arterial blood flow into the placental intervillous space is restricted by aggregates of endovascular trophoblast that migrate down the lumens 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 placental tissues develop 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, including epidermal growth factor (EGF) that stimulates proliferation of cytotrophoblast cells when applied to explant cultures (8, 9). Metabolism of the placental tissues is heavily glycolytic, and the phylogenetically ancient polyol pathways are highly active (10).

With full onset of the maternal intraplacental circulation towards the end of the first trimester there is a three-fold rise in local 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 villous regression to form the definitive placenta and the membranes, trophoblast proliferation and invasion, hormone production and transporter expression (11-13). The transition from histotrophic to hemotrophic nutrition involves other potential influences, such as the dilution of growth factor support and increased biomechanical forces, including shear stress at the villous surface. Previous studies have compared gene expression in the first trimester placenta as assessed by microarray analysis with that of term placentas (14, 15). In order to address the critical changes taking place during the first-second trimester transition, we performed RNA-Seq and array based DNA methylation profiling (matched samples) on placental villous tissue obtained from accurately dated narrow windows of gestation; 7-8 weeks and 13-14 weeks respectively. The samples were obtained under optimal conditions using an ultrasound guided chorionic villous sampling (CVS) technique that avoided the stress induced by curettage (16), and were frozen immediately. We focussed our analyses on transcripts encoding proteins involved in metabolism, hormonal activity, transport and cell proliferation. We also discuss transcripts with both differential expression and differentially methylated regions found in their promoters/gene-bodies.

**Results and Discussion**

Paired-end RNA sequencing was performed on eight first trimester (7-8 weeks) and six early second trimester (13-14 weeks) samples from uncomplicated pregnancies. DNA for EPIC array methylation was extracted from the same set of samples (matched samples). Villous samples separated clearly on the basis of gestational age with both principal component analysis (PCA) and hierarchical clustering. Genes contributing to the PC1 separation on PCA plot include: *SCN1A*, *MSC*, *IDO1*, *ELN*, *PRRX1*, *ALDH1A1*, *FGF10*, *ASB4*, *CCL13* and *SPX* (Fig. 1A, Suppl. Fig. 1).

Differential expression analysis identified 3702 differentially expressed genes (DEG) with absolute fold change of at least 2 (Fig. 1B, Suppl. Table 1). Using Kegg pathway analysis and Gene set enrichment (GSE) we identified several classes of genes that change simultaneously between the first and second trimesters (Figs. 1C-F, Suppl. Tables 2-5). 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 (Figs. 1C-F). Changes for selected transcripts were validated at the protein level, using either western blotting or immunohistochemistry. To ascribe the DEGs to individual cell types the RNA-Seq results were compared with a published scRNA-Seq dataset (17), with first (8 weeks) and late second trimester (24 weeks) samples. The scRNA-Seq identified several cell types in 8-week placentas, including cytotrophoblast (CTB), syncytiotrophoblast (STB), extravillous trophoblast (EVT) and stromal cells (STR), and EVT in 24-week placentas. Matching of DEGs with the scRNA data allowed the cell types expressing selected DEGs to be identified.

**Metabolism**

Analysis of GSE and Kegg pathway enrichment showed genes associated with protein processing in the endoplasmic reticulum (ER) to be amongst the most differentially expressed between the two time points. The Kegg pathway ‘Protein processing in ER’ was enriched in the first trimester (*P.adj* = 1.07 x 10-2) (Suppl. Fig. 2), as were the GSE terms ‘ER to cytosol transport’(*P.adj* = 3.00 x 10-2), ‘ER unfolded protein response’ (*P.adj* = 2.90 x 10-2), ‘negative regulation of response to ER stress’(*P.adj* = 3.05 x 10-2), and ‘IRE1-mediated unfolded protein response’(*P.adj* = 3.24 x 10-2)(Figs. 1C-F). These, and other related GSE terms, including ‘regulation of secretion’ and ‘response to oxidative stress’ (Figs. 2A-B), suggest that ER functional activity is greater during the first than the second trimester, despite the relatively low oxygen concentration. This is consistent with the need for a high level of peptide hormone secretion by the syncytiotrophoblast during early pregnancy to prevent onset of the next menses.

Protein synthesis is energy demanding, yet, despite the low-oxygen early environment, there are no significant differences in placental concentrations of the main energy metabolites (ATP/ADP, NAD+, glucose and lactate) across gestation (16). The transcript profile observed provides further evidence that placental tissues are not energetically compromised during the first trimester. Glycolysis is the primary route to energy generation, 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 the key regulators of glycolysis - hexokinase and pyruvate kinase, are among the most differentially expressed genes (Figs. 2C-D). In mammals, there are four hexokinase isoforms. HK1 and HK2 are unique in their ability to bind the outer mitochondrial membrane. Whilst present at low levels in most normal adult cells, HK2 is highly expressed in embryonic tissues and in many cancer cells (18). Germ line deletion of *Hk2* in the mouse causes embryonic lethality (19). We found HK2 expression to be significantly higher in the first trimester, with a switch to the HK3 isoform during the second trimester. HK3 is regulated by hypoxia, when it exerts protective effects against oxidative stress by increasing ATP levels, reducing oxidant-induced ROS production, preserving mitochondrial membrane potential, and increasing mitochondrial biogenesis (20). Our data thus support the notion that glycolysis is replaced by oxidative mitochondrial respiration as the primary method of energy generation. Indeed, this is underpinned by the upregulation of the transcriptional co-repressor, *CBFA2T3* in the second trimester, which contributes to inhibition of glycolysis and stimulation of mitochondrial respiration (21), once the utero-placental circulation is established. Conversely, β-oxidation of fatty acids appears to be suppressed in the first trimester. Transcripts encoding long-chain acyl-CoA dehydrogenase (*ACADL*) rise in the second trimester (Fig. 2B). These changes may serve to protect the placental tissues from excessive production of reactive oxygen species when oxygen availability is low (10, 22).

The endoplasmic reticulum is a key intracellular organelle responsible for the synthesis and post-translational modification of secreted and membrane proteins, and for intracellular Ca2+ storage. Perturbation of ER homeostasis occurs if the ER folding capacity is exceeded or if ER Ca2+ is depleted. Loss of homeostasis activates the ER stress response pathway, the unfolded protein response (UPR), which is mediated by three conserved signalling transducers: IRE-1 (inositol-requiring transmembrane kinase-endoribonuclease 1), PERK (PKR-like endoplasmic reticulum kinase) and ATF6 (activating transcription factor 6). Activity of these transducers is inhibited in the physiological state by binding of the ER chaperone protein BiP/GRP78. Transcripts encoding the sensor IRE1, known as *ERN1* (Fig. 2A), and its downstream X-box binding protein 1 (*XBP1*) were significantly higher in the first trimester (Figs. 2A, G-H), with no change in *ATF6* (Fig. 2H) or *GRP78* (Fig. 2E), and a modest increase in *PERK* (*EIF2AK3)* (Fig. 2A, and Suppl. Fig. 2). In addition, transcripts encoding several heat shock proteins (DNAJB9, DNAJB11, DNAJC3, HSPA5, HSP90B1), those involved in protein post-translational modifications (LMAN1, MAN1C1, MAN1A2, PDIA6, PDIA3, ERO1A) and protein quality control (ERP44, HERPUD1, EDEM2), were also higher in the first trimester (Fig. 2A). These are likely to be homeostatic responses to a high synthetic activity.

Heat shock proteins perform chaperone functions by stabilizing new polypeptides, while PDIAs, ERO1A and MAN1s assist in disulphide bond formation and glycosylation to ensure correct folding or refolding of proteins. Activation of IRE-1 and its downstream XBP1 are 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 as 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 (23). Knock-out of the gene leads to abnormal vascularisation, secondary to reduced levels of VEGF (23). Furthermore, ChIP-Seq analysis has revealed that in skeletal muscle approximately 40% of the downstream XBP1 transcription factor targets are unrelated to ER function, including genes associated with myogenic differentiation (24). We confirmed activation of the IRE-1/XBP1 pathway in the first trimester by immunostaining. XBP1 was strongly expressed by the villous and extravillous trophoblast of the cell columns and XBP1 expression coincided with that of IRE-1 and P-IRE-1 (Fig. 2H).

As expected, there was upregulation of genes associated with antioxidant defences during the second trimester, most notably *GPX1, CAT, SOD3, HIF3A, COX4I2, CYP1A1, CYP1A2* and *NOS1AP* (Figs. 2B, F), in agreement with our previous findings (6). Several GSE terms associated with metabolism of oxygen are also significantly different between the gestational ages, including ‘cellular responses to oxygen-containing compounds’ and ‘regulation of response to oxidative stress’ (see Suppl. Tables 3-5). Consistent with these terms, we observed increased phosphorylation of eIF2α by western blotting during the second trimester (Fig. 2E). Phosphorylation of eIF2α prevents ribosomal assembly and suppresses non-essential protein synthesis. While phosphorylation may reflect ER stress and activation of the PERK pathway, it can also result from kinases activated by oxidative and pro-inflammatory stressors.

**Hormonal activity**

Transcripts encoding peptide hormones showed considerable differential expression, with some being higher in the first trimester and others lower (Figs. 3A-B). The former included sub-units of hCG; *CGA* showed a 3.79-fold change while *CGB1*, *CGB2*, *CGB3*, *CGB5*, *CGB7* and *CGB8* showed fold changes of 3.68, 6.69, 3.84, 3.87, 4.94 and 3.59, respectively. These results confirmed previous findings that all six hCG genes are transcribed *in vivo* (25), 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 (26). EGF is produced by the endometrial glands (histotroph pathway), and so this signalling loop may be part of the trophoblast-endometrial dialogue that stimulates early placental development (27). Also higher in the first trimester were transcripts encoding leptin (LEP) (fold change 3.16, Fig. 3A-B), relaxin (RLN1) (fold change 2.82) and insulin like 4 (INSL4) (fold change 3.15). Comparisons with the scRNA-Seq data indicated the transcripts were enriched in the syncytiotrophoblast (Fig. 3A). This corresponds to the known site of expression of hCG, supporting our analytical approach (28).

Of the transcripts higher in the second trimester, the greatest change was observed for spexin (*SPX*) (fold change 11.08) (Figs. 3A, C). Spexin has not previously been described in the human placenta. It is known to be involved in the regulation of body weight and metabolism and inhibits the uptake of long-chain fatty acids by adipocytes and hepatocytes (29). Immunostaining showed it to be localised to the syncytiotrophoblast (Fig. 3C). Thus synthesis of spexin by the villous trophoblast suggest that this protein may play a role in regulating maternal lipid metabolism during pregnancy, possibly making more fatty acids available for transport to the fetus. A recent study measured circulating spexin (SPX) during the course of pregnancy in women with gestational diabetes mellitus (GDM) *vs*. healthy controls. The paper does not provide direct comparison with our study, as their starting measurements were taken at 10.3 (± 4.9) weeks and thus spanned both the first and second trimesters. However, the authors reported a significant increase in circulating levels of spexin in patients who developed GDM, with values correlating positively with glucose levels (30). Hence, the increase in placental spexin expression in the second trimester might influence maternal glucose utilisation and availability during pregnancy. The hypocretin receptor 2 (HCRTR2), also known as orexin receptor 2 is another pathway that regulates appetite and lipid metabolism (31) and we found that its mRNA increased 8.55 fold between the first and second trimester (Fig. 3A).

By contrast, the mRNA encoding the hunger and satiety-maintaining hormone leptin was higher in the first trimester placenta (Fig. 3A-B). Interestingly, an inverse correlation between circulating spexin and leptin levels was also reported in adolescents with obesity (32). Leptin mRNA and protein have been found to colocalize to the syncytiotrophoblast and fetal endothelial cells in human placentas, suggesting that the placenta is a source of both fetal and maternal leptin (33). In addition, human fetal adipose tissue is capable of producing leptin at the beginning of lipogenesis and differentiation (34). Placental leptin is transported at both fetal and maternal interfaces, in both the apical-to-basolateral and basolateral-to-apical directions (35, 36). However, leptin may play other roles at the maternal-fetal interface. Its receptor is expressed on invading extravillous trophoblast cells, and the addition of leptin to isolated cytotrophoblast cells in culture increases the production of matrix metalloproteinases (37). These findings suggest that this hormone may stimulate trophoblast invasion, particularly during the first trimester. In addition, 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 (38). Placental leptin may, therefore, also play a role in organogenesis during the first trimester.

Kegg pathway analysis showed that transcripts associated with ‘autoimmune thyroid disease’, ‘thyroid hormone synthesis’ (Fig. 1C, Suppl. Table 2) and ‘parathyroid hormone synthesis, secretion and action’ were differentially expressed across the transition. 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 (39). The three major binding proteins, T4 binding globulin, transthyretin and albumin have all been identified in the mature placenta (40). 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 during the first trimester (2.97-fold change). By contrast, *TTR* encoding transthyretin is more highly expressed in the second trimester (2.9-fold change) (Figs. 3A-B). These findings suggest novel regulatory pathways for the transfer of thyroid hormones across the placenta, which may change as pregnancy progresses.

The corpus luteum, under the trophic stimulation by hCG, is the source of progesterone and estrogen during the first trimester. Luteal production of progesterone declines after 6-8 weeks of pregnancy and the placental syncytiotrophoblast takes over as the main source of progesterone by the end of the first trimester (41). We found that the transcripts for two enzymes that catalyse the conversion of cholesterol to progesterone (via pregnenolone), *CYP11A1* (P450scc) or *CYP17A1*, did not change significantly between the first and second trimesters. By contrast, the gene encoding cholesterol 25-hydroxylase (*CH25H*), which converts cholesterol to 25-hydroxycholesterol (25OHC), was significantly upregulated in the second trimester (Figs. 2B, 3A), and seems to be methylation regulated (Fig. 5D). 25OHC and other oxysterols are substrates of P450scc, and 25OHC has been reported to enhance the production of steroids by the ovary and testis (42, 43). The increase in *CH25H* may contribute to the increase in progesterone synthesis by the placenta at the end of the first trimester, and which is pivotal to maintain the pregnancy.

**Transport**

Our data show that the transcripts encoding proteins mediating oxygen, lipid, protein, glucose, and ion transport changed significantly between the first and second trimesters (Figs. 3D-E). Transcripts encoding the hemoglobin subunits epsilon 1 and zeta (HBE1 and HBZ) were within the top 3 differentially expressed genes, and were 43.16 and 42.47 -fold lower in the second trimester samples (Figs. 3D-E). Conversely, the mRNA for hemoglobin G2 and hemoglobin beta (HBG2 and HBB) were 2.98 and 3.49 -fold higher in the later samples. HBB is a component of adult hemoglobin and while HBG2 is a component of fetal hemoglobin it is present at birth. HBE1 and HBZ make up embryonic hemoglobin that predominates during the first trimester, and has in the past been associated with erythropoiesis in the secondary yolk sac (44). The yolk sac was not included in our CVS samples, and thus these transcripts are likely to arise from the hemangioblastic clusters within the villous stromal core (45). However, previous transcriptome analysis of purified CTB and EVT cells has also shown high expression of both embryonic and fetal hemoglobin transcripts within villous trophoblast cells (46). The role this expression serves in trophoblast cells is still unclear, but expression of hemoglobins in non-erythroid lineages, including lung alveolar and mesangial cells, has been associated with protection against oxidative stress (47, 48). Together, these findings show hemoglobin transcripts are expressed within villous stroma and trophoblast cells and subunit expression changes with the onset of blood flow. Whether this is determined by changes in the oxygen concentration or is ontogenetic remains unknown.

We found that the pattern of expression of lipid transporters and apolipoproteins in villous samples was profoundly different between the first and second trimesters. Transcripts highly abundant during the first trimester included *APOA2* and *ABCB11,* whereas there seemed to be a switch in the expression of transporters, with many being upregulated in the second trimester, in particular *ABCA6, ABCC8* and *APOD* (Figs. 3E, Suppl. Fig. 3). ApoD has been shown to protect against ischemia-reperfusion injury in myocardial infarcts and has potent antioxidant activity, which may buffer the placenta once maternal blood flow is established (49). Cholesterol is essential in early embryonic metabolism, cell signalling and elaboration of cell and organelle membranes, and must be transported across the placenta into the chorionic cavity (extra-embryonic coelom) where the secondary yolk sac lies. From there it is transported into the embryonic circulation via the secondary yolk sac, which expresses mRNAs encoding multiple apolipoproteins, the cholesterol efflux transporter ABCA1, and lipoprotein receptors, including megalin and cubilin (50). The high abundance (i.e., top 0.5%) of transcripts encoding apolipoproteins present in lipoprotein particles and chylomicrons (ApoB, ApoA1, ApoA2, and ApoA4) is matched by high levels of these proteins in the chorionic cavity. The placental villi, the chorionic cavity, and the secondary yolk sac thus function together as a physiological equivalent of the choriovitelline placenta during early gestation. At the end of the first trimester, the chorionic cavity becomes obliterated by the growing amniotic cavity and the secondary yolk sac degenerates. At the same time, onset of the maternal and fetal placental circulations permits transport of cholesterol and lipids across the villous membrane. Our data may reflect the upregulation of this transport pathway.

Transcripts encoding transporters of metal ions important for antioxidant defences were also higher in the first compared to the early second trimester, for example *SLC30A10* and *SLC30A2* (fold changes 9.56 and 3.14, respectively). This differential might reflect higher transport of manganese and zinc ions that are essential cofactors for the superoxide dismutase enzymes during early pregnancy (Fig. 3E) in preparation for the rapid rise in local PO2 when a continuous maternal arterial flow enters the intervillous space (6). Genes involved in the transport of iron also changed, in particular *LTF* that encodes lactotransferrin and *HEPH* that encodes hephaestin (fold changes 3.68 and 4.07 respectively). Hephaestin has previously been reported in trophoblast-like cell line, BeWo (51), but the finding of *LTF* transcripts is novel to our study.

Transcripts encoding ion channels, for example *SCN1A* (fold change 31.37), *MCOLN2* (fold change 8.83), *TRPA1* (fold change 7.38) and *SCN7A* (fold change 3.42)(Fig. 3E) were among the most significantly raised in the early second trimester. These data suggest that ionic homeostasis within the placental tissues becomes more important following the onset of hemotrophic nutrition. This may reflect in part a switch in the way amino acids are transported across the placenta. In the first trimester, they are transported by uptake and subsequent breakdown of proteins in maternal histotroph (7) whereas in the second, there is active uptake of individual amino acids from the maternal circulation through accumulative and exchange transporters. Activity of the latter needs to be balanced by other ionic fluxes in order for them to function.

**Chromatin remodelling and transcription factors**

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 7.55-fold higher in the first compared to the early second trimester (Fig. 4A). HMGA2 is known to play a role in proliferation and differentiation, homozygous mutations in the *Hmga2* gene result in the *pygmy* phenotype in mice (52). Furthermore haploinsufficiency of the *Hmga1* gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders (53). We found that expression of *HAND1*, a transcription factor which regulates differentiation of trophoblast sub-types in the mouse (54) was also upregulated in the first trimester (6.63-fold) compared to early second trimester (Fig. 4A), but its function during human placental development is unknown. Many of the transcription factors upregulated in the second trimester regulate mammalian development and differentiation processes. These include *KLF2* (adipogenesis, embryonic erythropoiesis), *SOX14,* *SOX18, LHX6, MEF2C, SOX7, HEYL*, *TFAP2E, MYT1* (CNS development), *BNC2,* and *STAT4* (differentiation of T helper cells) (Fig. 4A).

**Cell proliferation, differentiation and WNT signalling**

WNT signalling plays an important role in cell proliferation, differentiation and motility under normal and malignant conditions (55), and the WNT signalling pathway has been identified as essential for the growth of trophoblast organoids (56). Recent evidence suggests that WNT signalling may also be implicated in the regulation of placental development and human trophoblast differentiation (57-59). Our data show that *RSPO4*, *WNT10B*, as well as several other genes mediating the canonical WNT signalling, including *PORCN* (mediates WNT transport and secretion) (60), and *SDC1* (inhibitor role) were increased in the first trimester compared to the early second trimester. By contrast, *WNT3A, WNT10A, WNT2, LRRK2, RYR2, LRP6, CCND1,* and *RSPO3* transcripts were significantly higher in the second than the first trimester (Figs. 4B). Canonical WNT signalling has been shown to be critical for invasive trophoblast differentiation (61). In addition, several genes that regulate the non-canonical WNT pathway were upregulated in the second trimester, including *WNT5B* and *LEF1*. 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 suggests that paracrine mechanisms play a role in the regulation of trophoblast invasion in the second trimester. The majority of the WNT signalling transcripts upregulated in the second trimester were localised to EVT, whilst the first trimester transcripts that were upregulated were localised to both EVT and villous syncytiotrophoblast (Fig. 4B).

**Extracellular matrix and angiogenesis**

Significant differences were found between the first and second trimester in placental expression of transcripts regulating the extracellular matrix (ECM) remodelling (Fig. 4C). The expression of laminins, ECM components of the basal lamina, and of cell-matrix adhesion molecules was differentially regulated in the first and second trimester, in particular laminin *LAMA1* was highly expressed in the first trimester whereas *LAMB3, LAMA2, LAMC3* were increased in the second trimester. Laminins have an important role during implantation (62), maintenance of trophoblast stemness (63) and EVT migration (64). LAMA1, which is upregulated in the first compared to the second trimester, was also found to be overexpressed in the trophectoderm of the early blastocyst during the time of implantation and its interaction with adhesion molecules is likely to facilitate the trophectoderm-endometrium dialogue (62).

Shortly after implantation, the extravillous trophoblast migrate from the placenta into the endometrium where they are involved in the remodeling of the maternal spiral arteries that ultimately supply the placenta. The 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 7-8 weeks of gestation, whereas MMP-9 facilitates subsequent invasion (65, 66). We found an upregulation of the mRNAs for *MMP9* (3.14-fold), *MMP1* (7.99-fold) and *MMP28* (3.77-fold) in the second trimester. One of the most differentially expressed transcripts was *MEP1A* (2.46-fold)that encodes for meprin, a member of the astacin family of metalloproteinases. Meprins can be secreted, and thus may assist in matrix digestion, or membrane-bound, where they may be involved in the extracellular cleavage of proteins (67). 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 involved in interactions between cells (68-70). Meprins are abundantly expressed by epithelial cells of the intestine, kidney and skin, and we show for the first time that are located in the villous syncytiotrophoblast (Fig. 4D). We speculate they might be involved in remodeling the basement membrane during villous growth.

Vasculogenesis and angiogenesis are critical for successful placental exchange, and it is suggested that low oxygen conditions during the first trimester stimulate these processes. However, we did not find the classical hypoxia-regulated factors, such as *VEGF,* to be differentially expressed. This supports our previous findings showing that HIF protein is not stabilised and signaling during early pregnancy (16). By contrast, transcripts encoding other potent regulators of angiogenesis angiogenin (*ANG*) and endoglin (*ENG*), were found to be higher during the first trimester (fold change 5.72 and 3.23, respectively). Angiogenin mRNA and protein have been localised in the trophoblast and endothelial cells of the fetal placental vessels (71). Markers of vascularisation such as *PECAM1 (CD31), VWF, ICAM1* were also significantly upregulated during the second trimester compared to the first trimester (Figs. 4C, E), indicating that the highly vascularised terminal villi, the most important component of the villous tree for materno-fetal exchange, develop rapidly during the second half of pregnancy.

During pregnancy, the maternal immune system is modulated by signals from the placenta, 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 (72, 73). The transcription factor musculin (MSC) is critical for the development of induced Treg cells by repression of the T helper type 2 transcriptional programme (74). The transcript for MSC was significantly upregulated in the second trimester (Figs. 4C, F). This is the first demonstration of the presence of this immune-regulator in the syncytiotrophoblast. It is likely to have a different function in the placenta to its role in Tregs (Fig. 4F).

### DNA Methylation

Placental DNA methylation increases over gestation, and *in utero* exposures alter methylation and impact placental function and fetal health (75). Previous studies have compared first and second trimester methylation; however these studies were not designed specifically to study the impact of onset of the maternal circulation inside the placenta. Thus, Novakovic *et al*. compared 8-12 week *vs*. 17-24 samples using a 27K methylation array focusing on gene promoters, but the first trimester samples overlap the first-second trimester transition (76). Nordor *et al.* compared 8-10 week to 12-14 week samples using a 450K methylation array, with the later time points overlapping the onset of maternal blood flow (77). In both studies, gene expression data were not taken from matched samples, and standard bisulfite-treatment was used, which also included a confounding 5-hydroxymethylcytosine (hmC) signal. In the present study we have used an oxidative bisulfite treatment on matched samples assaying only 5-methylcytosine (mC). We also performed the methylation analysis using an EPIC array assaying over 850K CpG sites with higher coverage to previous studies. Our study is therefore uniquely placed to study the gene expression and methylation differences immediately before and after the onset of maternal blood flow on matched samples (see Fig. 5A for a comparison of related datasets).

Samples clustered separately in a PCA by gestational age using the most variably methylated CpG positions (Figure 5B). Mean CpG methylation across all positions assayed on the EPIC arrays showed that there is a globally higher level of methylation during the second trimester (Figure 5C). Previous studies have indicated the importance of promoter methylation (76), and its positive correlation with gestational age. Our study compared the first and second trimester methylation levels across specific genomic features associated with gene regulation: promoters (2 kb upstream of TSS), gene bodies and CpG Islands. We found the largest difference in methylation within gene bodies (Figure 5D). We performed differential methylation analysis between the first and second trimester samples and found 430 DMRs, with 372 overlapping at least one gene promoter/body. We then correlated the differentially expressed genes from the RNA-Seq with differentially methylated CpGs in the matched first and second trimester samples. After applying minimum thresholds of a fold change of 2 (log2 fold change 1) for gene expression and a 0.2 (20% difference) methylation difference for DMRs, we found 75 DMRs overlapped DEGs. In the majority, increased gene expression was associated with a corresponding decrease in methylation at the promoter/gene body (Figure 5E, Suppl. Table 6). GO terms associated with these 56 genes suggest a role for specific organelles such as the nucleus, mitochondria, endoplasmic reticulum and the Golgi apparatus. Cellular components were associated with structural components, such as cytoskeleton, extracellular matrix, microtubule organizing centers and the nuclear envelope (Suppl. Table 7).

An example of increased transcript levels with corresponding reduced promoter methylation is the activator of the canonical WNT signalling, *RSPO4*. Increased *WNT2* expression correlated with reduced methylation in the second trimester (Fig. 5C-D), and may reflect the important role of canonical WNT signalling for the differentiation of invasive trophoblast (61).

### In the first trimester samples, reduced methylation also correlated with higher expression of transcripts encoding the EGFR ligand, epiregulin (EREG), and EREG expression decreased after the onset of maternal blood flow. EGF is abundantly secreted from the endometrial glands in early gestation when it stimulates cytotrophoblast proliferation and maintains their stemness (8, 27). Epiregulin promotes the cytotrophoblast-EVT transition through O-fucosylation on urokinase-type plasminogen catalysed by protein O-fucosyltransferase 1 (poFUT1) (78). This role seems critical for the pregnancy viability as both epiregulin and poFUT1 were found downregulated in placentas of patients suffering early pregnancy failure (78).

### By contrast, the expression of *CYP2R1* and *RBP7* was increased in the second trimester, with a corresponding decrease in methylation. These genes regulate vitamin D and A metabolism; CYP2R1 converts vitamin D into its active form, whilst RBP7 affects vitamin A stability and metabolism. Adequate vitamin D function is essential for fetal skeletal development, tooth enamel formation and general fetal growth and development (79), and vitamin A (retinoic acid) is essential for the development of heart, embryonal circulatory and central nervous systems and the regulation of heart asymmetry (80).

### Decreased methylation levels were also correlated with the upregulation of genes activated in response to oxidative stress in the second trimester (*FAS, WFDC1, AOX1, CH25H*). Transcripts encoding several transporters mediating the uptake of sodium/potassium (*SLC24A4*), choline and thiamine pyrophosphate (B1 homeostasis; *SLC44A4*), organic anions and bile acids (*ABCC3*), and drugs (*ABCC9*) were increased in the second trimester, whereas magnesium and zinc transporter *SLC39A8* were increased in the first trimester. Methylation levels were differentially regulated in all these transcripts.

The inverse correlation of DNA methylation with expression suggests an association for a subset of genes in response to the onset of blood flow. GO pathways in this subset of genes include signal transduction, anatomical structure development, cellular protein modification process, cell differentiation, small molecule metabolic process, response to stress, transport and immune system process (Suppl. Table 8).

**Conclusion**

Onset of the maternal arterial circulation into the intervillous space towards the end of the first trimester constitutes an environmental stress to which the placenta must successfully adapt. It is associated with major changes in proliferation, differentiation, and metabolism in both the villous trophoblast and stroma. **In the present study we have shown** that villous samples taken before the establishment of the maternal circulation are characterised by high expression of transcripts involved in the synthesis and ER processing of peptide hormones, along with glycolytic pathways. The transcripts patterns encoding proteins mediating transport of oxygen, lipid, protein, glucose, and ions changed significantly between the first and early second trimesters, reflecting increased oxidative stress and the onset of exchange between maternal and fetal blood. We also identified a novel hormone, spexin, in the syncytiotrophoblast that is upregulated in the second trimester and may modulate maternal lipid and glucose metabolism.

Key strengths of this study were that placental tissue was sampled directly using a CVS technique, that the gestational age was accurately dated with ultrasound, and that the RNA-Seq and methylation analyses were performed on matched sample sets. Rapid changes in the transcriptome have been documented in villous tissue collected using other commonly used methods, such as curettage (16), potentially confounding the analyses. The transcriptome can only provide an indication of potential protein levels and function due to differential translation and post-translational modifications. Nonetheless, we have identified major differences in transcript levels, some novel and others consistent with current physiological paradigms, that provide new insights into how the placenta adapts to physiological changes occurring at the end of the first trimester of pregnancy. There are several possible explanations for the changes. For example, they may be purely ontological, they may be responsive to the change in oxygenation, they may be due to the loss of growth factor support from the histotroph, or they may reflect increases in biomechanical stimuli such as increased shear stress. All these factors can affect methylation and transcription, and further research is required to dissect out the pathways involved.

Nonetheless, it is clear that onset of the maternal circulation is associated with major physiological and morphological changes in the placenta. Villous regression over the superficial pole of the gestational sac leads to formation of the membranes and the definitive placenta (4), and there is a loss of stemness and proliferation of the villous cytotrophoblast cells (9). Equally, premature onset of blood flow occurs in many patients with first trimester miscarriage and fetal growth restriction, suggesting that a failure to adapt successfully may trigger subsequent pregnancy complications (81). **Our findings suggest that DNA methylation changes are part of the placental response to these major environmental changes and may explain the altered DNA methylation profiles seen in placentas from pregnancies complicated by fetal growth restriction (82).**

**The unique nature of the first-second trimester transition during human pregnancy may explain the high incidence of complications of pregnancy in our species. In other mammals histotrophic and hemotrophic exchange occur in separate areas of the extra-embryonic membranes, such as the inverted yolk sac and the chorioallantoic placenta of the mouse respectively, often in parallel. The situation in the human may reflect our invasive interstitial form of implantation, which is restricted to the great apes and for which the evolutionary advantage has yet to be identified.**

## Methods

### Human Tissue Collection

First and second trimester tissue samples were collected with informed written patient consent and approval of the Joint University College London/University College London Hospital Committees on the Ethics of Human Research (05/Q0505/82) from uncomplicated pregnancies at 7-8 (n=8) and 13-14 weeks of gestation (n=6). Gestational age was confirmed by ultrasound measurement of the crown-rump length (CRL) immediately before the procedure. All samples were collected from patients undergoing surgical pregnancy termination under general anaesthesia for psycho-social reasons. Villous samples were obtained under transabdominal ultrasound guidance from the central region of the placenta using a chorionic villus sampling (CVS) technique. All samples were snap-frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

### RNA extraction and RNA-Seq

RNA was extracted from human first-trimester placental villi using the RNeasy Plus Universal Mini Kit (catalog no. 73404; Qiagen). Libraries were made using the Illumina TruSeq Stranded mRNA Library Kit according to the manufacturer’s instructions. Libraries were quantified (kappa qPCR), and equimolar pools were sequenced (paired end 100 base reads, PE100) in several lanes of the Illumina NextSeq.

### 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 (v 0.11.5), fastq\_screen (v0.9.3) and Picard Tools (v 2.9.0) and summarised with MultiQC (v1.8dev). Reads were trimmed to remove adapters and low quality bases with TrimGalore! (v0.6.4) and aligned to the human genome (GRCh38) with STAR aligner (v2.5.1b)), with a mean of 90.4% reads uniquely mapping and mean of 56M 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 estimated size factors. Principal component analysis (PCA) was performed on rlog-transformed count data for all genes. Gene Set Enrichment (GSE) and Kegg pathway analysis were performed using clusterProfiler package (v.3.10.1). For GSE, DEGs with absolute log2 fold change > 1 and adjusted p.value < 0.05 were used, for Kegg pathway analysis, DEGs with absolute log2 fold change > 0.6 and adjusted p.value < 0.05 were used. The data matrix for scRNA-seq data were obtained from the Wang lab (16) (GEO accession number GSE89497). Regularised log transformation function (from DESeq2 package) was applied to counts for heatmaps. Heatmaps were generated with ‘ComplexHeatmap' R package (v 1.20.0). Selection of differentially expressed genes on heatmaps was based on highest significance (lowest adjusted p.value) and highest absolute log2 fold changes.

#### DNA Methylation Analysis

**Infinium MethylationEPIC array**

Genomic DNA was isolated by QIAamp DNA mini kit (Qiagen, cat. no. 51304) following manufacturer’s instructions. Buffer AL (200 μl) was added to the sample, mixed by pulse-vortexing for 15 sec, before incubating at 70oC for 10 min. Absolute Ethanol (200 μl) was then added to the sample, and mixed by pulse-vortexing for 15 sec before transferring to the QIAamp Mini spin column and centrifuged at 6000 g for 1 min. The Mini spin column was washed once with Buffer AW1 (500 μl) following by Buffer AW2 (500 μl) before centrifuging at full speed for 1 min. For elution of genomic DNA, DNase-free water (100 μl) was added and incubated for 1 min before centrifuging at 6000 g for 1 min. The step repeated one more time with another 100 μl DNase-free water. DNA concentration of the samples were quantified by NanoDrop and the DNA quality was checked by resolving in 0.8% agarose gel, in which there was a major band visualized at around 10 kbp without obvious smear below, indicating good quality DNA.

Genomic DNA oxidative bisulfite (oxBS) conversion was performed using the CEGX TrueMethyl kit (Cambridge Epigenetix/NuGEN, cat. no. CEGXTMS) and used for microarray-based DNA methylation analysis, performed at GenomeScan (GenomeScan B.V., Leiden, The Netherlands) on the HumanMethylation850 BeadChip (Illumina, Inc., San Diego, CA, U.S.A). The EPIC array interrogates approximately 865,000 CpG sites representing about 99% of the RefSeq genes. The resulting iDAT files were imported and analysed using ChAMP (v2.9.10) (83, 84). Samples were processed filtering for a probe detection p-value <= 0.01, probes with a beadcount <3 in at least 5% of samples, no CpG and known SNPs (85) at probe starts, probes aligning to multiple locations, and QC using the on array control probes. In total, 750150 probes on the array passed the filtering and QC steps. The BMIQ (86) method was used to normalise the two probe types (I and II) present on the array. Beta methylation values from the EPIC array range from 0 (unmethylated) to 1 (methylated) and are equivalent of percentage methylation. GO analysis on genes with a correlation with increased methylation and decreased expression from first to second trimester was performed with GOnet (87).

#### Data and Code Availability

RNA-Seq

For reproducibility, scripts can be found on GitHub (<https://github.com/CTR-BFX/2020_Prater_Cindrova>). RNA-sequencing data is accessible through the EMBL-EBI ArrayExpress accession number: E-MTAB-9203. http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9203.

DNA Methylation (EPIC Array)

Code used to analyse the EPIC array samples is available on GitHub (<https://github.com/CTR-BFX/First-Second-Trimester-Methylation>). EPIC methylation array data have been deposited at EMBL-EBI ArrayExpress under accession number E-MTAB-9312 (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9312>).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (88) using the following primary antibodies: anti-XBP1 (ab109621, Abcam), anti-phospho-IRE1 (ab48187, Abcam), anti-IRE1 (ab37073, Abcam), anti-ATF6 (ab37149, Abcam), anti-pyruvate kinase L/R (PB9499, Boster), anti-spexin (H-023-81, Phoenix Pharmaceuticals), anti-MEP1α (ab232892, Abcam), anti-CD31 (M0823, Dako), anti-musculin (ab64954, Abcam).

**Western Blotting**

Placental lysates were processed and run on western blots, as previously described (89), using the following antibodies: anti-TTR (PA5-80197, Thermo Fisher Scientific), anti-leptin (ab2125, Abcam), anti-ApoA1 (PA5-78798, Thermo Fisher Scientific), anti-eIF2α (#3398, Cell Signaling), anti-GRP78 (610978, Transduction Laboratories), anti-GPX1 (ab167989, Abcam).

## Figure legends

**Figure 1**. RNA-seq identifies differentially expressed genes (DEGs), functions and pathways distinct to first and second trimester placenta. A) PCA clearly separates first and second trimester samples. B) Volcano plot of DEGs, with genes upregulated in the first trimester in red, and those up regulated in the second trimester in blue. C) Selected Kegg pathways, which were differentially regulated during the first and second trimester. D-F) Barplots showing selected differentially regulated GSE terms related to Biological Processes (D), Molecular Functions (E) and Cellular Components (F). Each barplot shows how many genes within each term are expressed more in the first (red) or second trimester (blue). Transparency is used to show the most significant (padj) terms as least transparent. Terms were ordered by qvalue.

**Figure 2.** Differentially expressed genes associated with ER processing, and oxidative stress and glycolytic processes. A-C) Heatmaps of top DEGs in the first *vs*. second trimester samples, were compared to a previously published scRNA-Seq dataset with first and late second trimester placenta samples (see methods). A) Heatmap showing top DEGs involved in ER processing and stress. B) Heatmap of top DEGs related to oxidative stress. C) Heatmap of DEGs involved in the glycolytic pathway. D) Immunostaining of first and second trimester sections with anti-pyruvate kinase antibody E) Western blots depicting GRP78 and phospho-eIF2α in first and second trimester placental lysates. Ponceau S (Ponc S) staining was used to normalise the protein loading. F) Western blots depicting catalase (CAT) and glutathione peroxidate (GPX) in first and second trimester placental lysates. Poncaeau S (Ponc S) staining was used to normalise the protein loading. Scale bars in E and H are 50 µm

G-H) Immunolocalisation of XBP1, ATF6, P-IRE1 and IRE1 in first and second trimester placental sections, stained with anti-XBP1, -ATF6, -IRE1 or P-IRE1 antibodies. Arrows denote cell columns (CC).

**Figure 3**. Overview of hormonal activity and transport related placental genes differentially expressed in the first and second trimester of pregnancy. A) Heatmap showing top DEGs related to hormone activity and compared to the scRNA-Seq data. B) Western blots depicting the expression of catalase transthyretin (TTR) and leptin in first and second trimester placental lysates. Ponceau S (Ponc S) staining was used to normalise the protein loading in panels 3B. C) Immunostaining of first and second trimester sections with anti-spexin antibody. D) Volcano plot of transport-related genes, with DEGs coloured red were enriched in the first trimester, and those in blue enriched in the second trimester. E) Heatmap of top transport related genes, split by the type of transport.

**Figure 4.** Overview of DEGs related to transcription factors, WNT signalling and extracellular matrix related genes, differentially expressed between the first and second trimester. A) Heatmap of differentially expressed transcription factors. B) Heatmap of DEGs associated with WNT signalling. C) Heatmap of extracellular matrix related genes differentially expression in the first and second trimester. D-F) Imunolocalisation of meprin (D), CD31 (E) and musculin (MSC) (F) in first and second trimester placental sections (n=4).

**Figure 5.** DNA methylation increases from first to second trimester. A. Comparison of sample gestational ages between this and related studies. B. PCA shows a clear separation between first and second trimesters. C. Global methylation levels are higher in the second trimester than the first as indicated by a shift in points towards the top left. D. At specific genomic features, such as promoters, gene bodies and CpG islands there is the largest difference at gene bodies with higher methylation during the second trimester. E. A scatter plot comparing RNA expression and DNA methylation at promoters and gene bodies shows a correlation of reduced expression with increased methylation from first to second trimester for 61 genes (purple).

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