Transcriptome analysis reveals differences in human placental metabolism, transport and endocrine function across the first-second trimester transition.

Biological Sciences – Physiology

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## Abstract

The human intraplacental environment undergoes major changes at the end of the first trimester associated with onset of the maternal arterial circulation. There is a three-fold rise in oxygen concentration and a switch from histotrophic to hemotrophic nutrition. To assess the impact of this transition on placental development and function, we performed RNA-Sequencing (RNA-Seq) on villi obtained at 7-8 (n=8) and 13-14 (n=6) weeks’ gestational age. To ascribe differentially expressed genes (DEG) to individual cell types the results were compared with a published single-cell RNA-Seq dataset. Most DEGs were associated with protein processing in the endoplasmic reticulum (ER), cellular metabolism and hormone secretion, transport, extracellular matrix, immune response, signalling pathways, vascular development, lipid metabolism, cell proliferation and reactive oxygen species metabolism. Transcripts upregulated in the first trimester were associated with synthesis and ER processing of peptide hormones (leptin, human chorionic gonadotropin (hCG), relaxin and insulin-like 4), along with glycolytic pathways. Transcripts encoding proteins mediating transport of oxygen, lipid, protein, glucose, and ions was significantly altered between the first and second trimesters. We also identified a novel hormone, spexin, in the syncytiotrophoblast that is upregulated in the second trimester and may modulate maternal lipid metabolism. Overall, the pattern is consistent with the need for a high level of cell proliferation and protein secretion by the early placenta, in particular hCG, to maintain the corpus luteum and prevent onset of the menses. Our results support the concept that the human placenta develops in a physiological low-oxygen, rather than a hypoxic, environment. (limit: 250 words)

## Significance Statement

The placenta is essential for pregnancy success. Failure of normal development leads to complications, including miscarriage and stillbirth. The human placenta undergoes a major challenge at 10-12 weeks of pregnancy with onset of the maternal arterial circulation, including a three-fold rise in oxygen concentration and increases in shear and other biomechanical forces. Transcriptome analysis of accurately dated placental samples collected from first and second trimesters revealed that paradoxically the placental tissues are highly proliferative and synthetic during the low-oxygen, glycolytic environment of the first trimester, secreting peptide hormones that signal pregnancy to the mother. Transport pathways appear to switch during the second trimester with more direct access to maternal blood, and 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 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 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 hemotrophic nutrition (4). This transition, which involves the same placental structure being supplied by different routes, 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, 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 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 villous regression to form the definitive placenta, 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, such as 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 performed RNA-Seq on placental villi obtained under optimal conditions using a chorionic villus sampling technique from pregnancies accurately dated as being 7-8 weeks and 13-14 weeks gestational age. We have focussed our analyses on transcripts encoding proteins involved in metabolism, hormonal activity, transport and cell proliferation.

**Results and Discussion**

Paired-end RNA sequencing was performed on 8 first trimester and 6 second trimester placentas. Placental samples separated clearly on the basis of pregnancy stage, using 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. Figs. 1A-C).

Differential expression analysis identified 3702 differentially expressed genes (DEG) with absolute fold change of at least 2 (Fig. 1B). Using Kegg pathway analysis and Gene set enrichment (GSE) we identified several classes of genes that simultaneously change between the first and second trimesters (Figs. 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 (Figs. 1C-F). Changes 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 (16), 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.

**Metabolism**

GSE 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.adj* = 2.34 x 10-2) (Suppl.Fig 2), ‘ER to cytosol transport’ and ‘ER unfolded protein response’ terms were enriched in first trimester in GSE analysis (*P.adj* = 2.9 x 10-2 and 3.5 x 10-2 respectively). These, and other related GSE terms, including ‘regulation of secretion’ and ‘response to oxidative stress’ (Figs. 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 of the early placental environment, there are no significant differences in the concentrations of the main energy metabolites (ATP/ADP, NAD+, glucose and lactate) across gestation (17). The transcript profile observed provides further evidence that the 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. 2D-E). In mammals, there are four hexokinase isoforms. HK1 and HK2 are unique in their ability to bind the outer mitochondrial membrane. Whilst absent or present at low levels in the majority of normal adult cells, HK2 is highly expressed in embryonic tissues, and is expressed at high levels in many cancer cells (18). Germ line deletion of *Hk2* in the mouse causes embryonic lethality (19). In our cohort, HK2 expression is significantly higher in the first trimester, but there is a switch to using the HK3 isoform in the second trimester. HK3 has a higher affinity for glucose than HK2, but it can be inhibited by glucose at high concentration, and its substrate binding is attenuated by intracellular ATP (20). HK3 is regulated by hypoxia where it exerts protective effects against oxidative stress, by increasing ATP levels, reducing oxidant-induced ROS production, preserving mitochondrial membrane potential, and increasing mitochondrial biogenesis (21). Our data thus support the notion that glycolysis is replaced by oxidative mitochondrial respiration as the primary method of energy generation. Indeed, this is further underpinned by the high expression of the transcriptional co-repressor, *CBFA2T3*, which contributes to inhibition of glycolysis and stimulation of mitochondrial respiration (22) in the second trimester, also consistent with an established utero-placental circulation. Equally, 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. 2D). These changes may serve to protect the placental tissues from excessive production of reactive oxygen species when oxygen availability is low (10, 23). There are also profound differences in lipid and cholesterol metabolism, which are discussed below.

The endoplasmic reticulum is a key intracellular organelle responsible for the synthesis and post-translational modification of secretory 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. This activates the ER stress response pathway, the unfolded protein response (UPR), which is mediated by three conserved signalling pathways: IRE1 (inositol-requiring transmembrane kinase-endoribonuclease 1), PERK (PKR-like endoplasmic reticulum kinase) and ATF6 (activating transcription factor 6), whose activities are inhibited in the physiological state by binding of the ER chaperone protein BiP/GRP78. The genes encoding the sensor IRE1, also known as *ERN1*, and its downstream X-box binding protein 1 (*XBP1*) were significantly higher in the first trimester (Figs. 2A, H), with no change in *ATF6* (Fig. 2H) or *GRP78* (Fig. 2F), and a modest increase in *PERK* (*EIF2AK3)* (Fig. 2A, and Suppl Fig. 2. Kegg ER)). 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 mechanisms in response to 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 IRE1 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 (24). Knock-out of the gene leads to abnormal vascularisation, secondary to reduced levels of VEGF (24). 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 (25). We confirmed activation of the IRE1/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 IRE1 and P-IRE1 (Fig. 2E).

In contrast, phosphorylation of eIF2α was increased in the second trimester (Fig. 2F). This kinase is downstream of the PERK pathway, but can also be activated by oxidative and pro-inflammatory stressors. 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. 2C, G), which is in agreement with our previous findings (6). Several GSE terms associated with metabolism of oxygen are also significantly different between the two placental groups, including ‘cellular responses to oxygen-containing compounds’ and ‘regulation of response to oxidative stress’ (see Suppl. Table 2).

**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* (26), 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 (27). EGF is a component of histotroph (uterine milk produced by the endometrial glands), and so this signalling loop may be part of the trophoblast-endometrial dialogue that stimulates early placental development (9). Also higher in the first trimester were transcripts encoding leptin (LEP) (fold change 3.16), relaxin (RLN1) (fold change 2.82) and insulin like 4 (INSL4) (fold change 3.15. Comparisons with the scRNA-Seq localised most of these hormones to the syncytiotrophoblast (Fig. 3A).

Of the transcripts higher in the second trimester, the greatest change was for spexin (*SPX*) (fold change 11.08) (Figs. 3A, C). Spexin has not previously been described in the human placenta, but is involved in the regulation of body weight and metabolism and inhibits the uptake of long-chain fatty acids by adipocytes and hepatocytes (28). Immunostaining showed it to be localised to the syncytiotrophoblast (Fig. 3C). Secretion of spexin may therefore play a novel role in regulating maternal lipid metabolism during pregnancy, possibly making more fatty acids available for transport to the fetus. Another pathway that regulates appetite and lipid metabolism is mediated by the hypocretin receptor 2 (HCRTR2), also known as orexin receptor 2 (reference) and its mRNA was increased fold change 8.55) (Fig. 3A). In contrast, the mRNA encoding the hunger and satiety-maintaining hormone leptin was higher in the first trimester placenta (Fig. 3A-B). Leptin concentrations are elevated during pregnancy due to an accumulation of body fat and placental production. However, in contrast to leptin’s effect on satiety, pregnancy requires increased food intake to meet the nutrient demands of the fetus. This apparent paradox is resolved by the development of central leptin resistance, which occurs in the second trimester of pregnancy (29). Leptin mRNA and protein were 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 (30). In addition, human fetal adipose tissue is capable of producing leptin at the beginning of lipidogenesis and differentiation (31).

Placental leptin is transported to both fetal and maternal interfaces, in both the apical-to-basolateral and basolateral-to-apical direction (32, 33). Leptin may play other roles at the maternal-fetal interface, however. The receptor is expressed on invading extravillous trophoblast cells, and addition of leptin to isolated cytotrophoblast cells increases the production of matrix metalloproteinases (34). Thus, the hormone may stimulate trophoblast invasion, particularly in 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 (35). Placental leptin might thus play a role in organogenesis during the first trimester.

Kegg pathway analysis revealed transcripts associated with ‘autoimmune thyroid disease’, ‘thyroid hormone synthesis’ (Fig. 2C) and ‘parathyroid hormone synthesis, secretion and action’ to be differentially expressed. 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 (36). The three major binding proteins, T4 binding globulin, transthyretin and albumin have all been identified in the mature placenta (37). 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 (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 raise the possibility of novel regulatory pathways for the transfer of thyroid hormones across the placenta and that these 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 production by the end of the first trimester (38). Transcripts for two enzymes that catalyse the conversion of cholesterol to progesterone (via pregnenolone), *CYP11A1* (P450scc) or *CYP17A1*, did not change significantly, whilst the gene for cholesterol 25-hydroxylase (*CH25H*), which converts cholesterol to 25-hydroxycholesterol (25OHC), was significantly upregulated in the second trimester (Figs. 2C, 3A) and seems to be methylation regulated (Fig. 5B-C). 25OHC and other oxysterols are substrates of P450scc, and 25OHC has been reported to enhance the production of steroids by the ovary and testis (39, 40). Direct exposure of human primary trophoblast cells to 25OHC had a biphasic effect; low 25OHC concentrations stimulated progesterone and hCG secretion (41).

**Transport**

Transcripts encoding proteins mediating oxygen, lipid, protein, glucose, and ion transport were significantly altered between the first and second trimester placentas (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). These chains make up embryonic hemoglobin that predominates during the first trimester, and has in the past been associated with erythropoiesis in the yolk sac (42). The yolk sac was not represented in our samples, and these transcripts most likely arise from the hemangioblastic clusters within the villous stromal core (43). 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. 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 haemoglobin and while HBG2 is a component of fetal haemoglobin it is present at birth.

The pattern of expression of lipid transporters and apolipoproteins was profoundly different between the first and second trimester. Transcripts highly abundant during the first trimester included *APOA2* and *ABCB11,* whilst there seemed to be a switch in the usage of transporters, many being upregulated in the second trimester, for example: *ABCA6, ABCC8, APOD* and *APOA1* (Figs. 3E-F, suppl fig lipids??). 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 coelomic fluid. From there it is taken up by the secondary yolk sac, which expresses abundant mRNAs encoding multiple apolipoproteins, the cholesterol efflux transporter ABCA1, and lipoprotein receptors, including megalin and cubilin (44). 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 coelomic fluid. The placental villi, the coelomic cavity, and the secondary yolk sac thus function together as a physiological equivalent of the choriovitelline placenta during early gestation. The coelomic cavity is obliterated by the start of the second trimester and, following onset of the maternal and fetal placental circulations, transport of cholesterol and lipids can occur across the placental membrane. Our data appear to reflect this change in the transport pathway.

Transcripts encoding transporters of metal ions important for antioxidant defences were also higher in the first trimester, for example *SLC30A10* and *SLC30A2* (fold changes 9.56 and 3.14, respectively) (Fig. 3E) that transport manganese and zinc ions that are essential cofactors for the superoxide dismutase enzymes. This may be in preparation of the oxidative stress that accompanies (or is caused by) the rapid rise in the local PO2 as maternal blood flows into the intervillous space (6). Genes involved in the transport of iron also changed, notably *LTF* that encodes lactotransferrin and *HEPH* that encodes hephaestin (fold changes 3.68 and 4.07 respectively). Hephaestin has previously been reported in the trophoblast-like cell line, BeWo (45), but the finding of *LTF* transcripts is novel. [need some comment about transferrin and its receptors – even if the levels don’t change, I would expect them to be high. Please check and add something]

Transcripts encoding ion channels, for example *SCN1A*, *SCN7A*, *TRPA1*, *KCNQ3*, *KCNA4*, *KCNK17*, *KCNJ16*, *KCNMA1*, and *TRP6* (Fig. 3E) were among the most significantly raised in the second trimester. These data suggest that ionic homeostasis within the placental tissues becomes more important with onset of hemotrophic nutrition. This may reflect in part a switch in the way in which amino acids are transported across the placenta. In the first trimester, they are transported by uptake and subsequent breakdown of maternal histotroph proteins (7); whereas in the second it is by active uptake of individual amino acids from the maternal circulation through accumulative and exchange transporters.

**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 regulated (7.55 fold change higher in the first trimester) (Fig. 4A), typical of embryonic tissues. HMGA2 plays a role in proliferation and differentiation, homozygous mutations in the *Hmga2* gene result in the *pygmy* phenotype (46), while haploinsufficiency of the *Hmga1* gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice (47). Expression of *HAND1*, a transcription factor which regulates differentiation of trophoblast sub-types in the mouse (48) was also upregulated in the first trimester (fold change 6.63, Fig. 4A), but its function 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), *SOX14,* *SOX18, LHX6, MEF2C, SOX7, HEYL*, *TFAP2E, MYT1* (CNS development), *BNC2,* and *STAT4* (differentiation of T helper cells) (Fig. 4A).

**Cell proliferation and differentiation**

WNT signalling plays an important role in cell proliferation, differentiation and motility under normal and malignant conditions (49), and the WNT signalling pathway has been identified as essential for the growth of trophoblast organoids (50). Recent evidence suggests that WNT signalling may also be implicated in the regulation of placental development and human trophoblast differentiation (51-53). *RSPO4*, *WNT10B*, as well as several other genes mediating the canonical WNT signalling, including *PORCN* (mediates WNT transport and secretion) (54), and *SDC1* (inhibitor role) were increased in the first trimester. In contrast, *WNT3A, WNT10A, WNT2, LRRK2, RYR2, LRP6, CCND1,* and *RSPO3* transcripts were significantly higher in the second trimester (Figs. 4B-C). Canonical WNT signalling has been shown to be critical for invasive trophoblast differentiation (55). 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 paracrine mechanisms to regulate trophoblast invasion in the second trimester. The majority of the WNT signalling transcripts upregulated 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**

Significant differences were observed between the first and second trimester in placental expression of transcripts regulating extracellular matrix (ECM) remodelling (Fig. 4D). 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, whilst *LAMB3, LAMA2, LAMC3* were increased in the second trimester. Laminins have an important role during implantation (56), maintenance of trophoblast stemness (57) and EVT migration (58). LAMA1, which is upregulated in the first 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 (56).

Shortly 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 (59, 60). We found an upregulation of the mRNAs for *MMP9* (fold change 3.14), *MMP1* (fold change 7.99) and *MMP28* (fold change 3.77) in the second trimester. 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 matrix digestion, or membrane-bound, where they may be involved in the extracellular cleavage of proteins (61). 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 (62-64). Meprins are abundantly expressed by epithelial cells of the intestine, kidney and skin, and we show for the first time 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. This is in keeping with our previous 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 (fold change 5.72 and 3.23, respectively). Angiogenin has been localised at the mRNA and protein levels to the trophoblast and endothelial cells of the fetal placental vessels (65). Markers of vascularisation such as *PECAM1 (CD31), VWF, ICAM1* were significantly upregulated during the second trimester (Figs. 4D, F), reflecting the fact that the highly vascularised terminal villi, the most important component of the villous tree for materno-fetal exchange, only develop 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 (66, 67). The transcription factor musculin (MSC) is critical for the development of induced Treg cells by repression of the T helper type 2 transcriptional programme (68). The transcript for MSC was significantly upregulated in the second trimester (Figs. 4D, G). This is the first report of this regulator in the placenta, where it localizes to the syncytiotrophoblast cells (Fig. 4G).

### Methylation

We investigated whether any changes in gene expression observed between the first and second trimester placental samples were associated with changes in DNA methylation. Since DNA methylation of gene promoters and gene bodies can effect gene expression, we determined whether the list of differentially methylated promotors (regions 2 kb upstream of transcription start site) and gene bodies overlapped with the list of differentially expressed genes (Fig. 5A). We identified 430 DMRs, with 372 overlapping at least one gene promoter/body, and 61 overlapping DEGs with fold change more than 1. In the majority of cases, we observed gene expression increase being associated with a corresponding decrease in methylation at the promoter (Fig. 5B-C). Genes affected by methylation clustered into several Kegg categories. Of particular interest was the differential regulation of the WNT signalling pathway and pluripotency by methylation (Fig. 5D). We report increased transcript levels and corresponding reduced promoter methylation of the activator of the canonical WNT signalling, *RSPO4*, in the first trimester, reflecting the important role of the canonical WNT signalling for invasive trophoblast differentiation (55), whilst *WNT2* increase was regulated by methylation in the second trimester (Fig. 5B-C). WNT2 is expressed by the syncytiotrophoblast and EVTs, and its expression is reduced in the third trimester pre-eclamptic placenta, which may influence the biological functions of trophoblast cells and the development of PE (69).

In the first trimester, suppressed methylation activated the transcript levels of the EGFR ligand, epiregulin (EREG). EGF is abundantly secreted from the endometrial glands in early gestation when it stimulates cytotrophoblast proliferation and maintains their stemness (8, 9). Epiregulin promotes the cytotrophoblast-EVT transition through O-fucosylation on urokinase-type plasminogen catalysed by protein O-fucosyltransferase 1 (poFUT1) (70). This role seems critical for the pregnancy viability as both epiregulin and poFUT1 were found downregulated in placentas of patients who had a spontaneous miscarriage in the first trimester (70).

Increased expression of the MHC class I molecules, HLA-F, and HLA-A, was increased in the second trimester and methylation levels were reduced. HLA-F is exclusively expressed by EVTs (71), whilst HLA-A is expressed by fetal endothelial cells, macrophages and other stromal cells, coinciding with placental maturation and vascularisation in the second trimester.

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 to bind its receptor, 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 (72), and vitamin A (retinoic acid) is essential for the development of heart, embryonal circulatory and central nervous systems and the regulation of heart asymmetry (73).  Also differentially regulated were *TLL1*, which, too, regulates heart development, and *SV2B*, which controls secretion in neural endocrine cells.

Methylation regulated the expression 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, whilst magnesium and zinc transporter *SLC39A8* was increased in the first trimester; methylation levels were differentially regulated in all these transcripts.

**Conclusion**

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, lipid, protein, glucose, and ions was significantly altered between the first and second trimesters. We also identified a novel hormone, spexin, in the syncytiotrophoblast that is upregulated in the second trimester and may modulate maternal lipid metabolism. Overall, the pattern is consistent with the need for a high level of cell proliferation and protein secretion by the early placenta, in particular hCG, to maintain the corpus luteum and prevent onset of the menses. Our results support the concept that the human placenta develops in a physiological low-oxygen, rather than a hypoxic, environment. In conclusion, it appears that although the first-trimester human placenta develops in a relatively low-oxygen environment and is heavily glycolytic, the tissues are highly proliferative and synthetic. 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 changes occurring at the transition between the first and second trimesters of pregnancy. Our study is very unique and rigorous because all placental samples had been collected using the CVS technique, and accurately dated by ultrasound.

## 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 7-8 wk (n=8) and 13-14 wk (n=6) uncomplicated pregnancies. Gestational age was confirmed by ultrasound measurement of the crown-rump length of the embryo. 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, fastq\_screen and Picard, summarised with MultiQC (v0.9dev 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). Sex of the samples was incorporated to the design as blocking factor. Read counts were normalised on the estimated size factors. Principal component analysis (PCA) was performed on rlog-transformed count data for all genes. Differentially expressed genes (DEGs) were defined by adjusted p value (padj) < 0.05 and absolute fold change > 2. Gene Ontology and Kegg pathway analysis was performed using clusterProfiler package (v.3.10.1). The data matrix for scRNA-seq data were obtained from the Wang lab (16) (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

**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 arrau 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)1,2. 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 SNPs3 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 BMIQ4 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.

EPIC methylation array data have been deposited in the ArrayExpress database at EMBL-EBI under accession number E-MTAB-XXXX (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTABXXXX>). Code used to analyse the EPIC array samples is available at [https://github.com/CTR-BFX/XXXX](https://github.com/CTR-BFX/)

#### 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: <https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6683/>

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (74) 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 (74), 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**. Performance of RNA-seq and significant terms and pathways distinct to first and second trimester placenta. A) PCA. B) Volcano plot of differentially expressed genes, with genes up regulated 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) Barplot 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) 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 metabolism genes associated with ER processing, secretion and oxidative stress. A-C) Heatmaps of top DEGs in the first *vs*. second trimester samples, compared to scRNA-Seq dataset with first and late second 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 DEGs related to oxygen-response. D) Heatmap of DEG involved in the glycolytic pathway. E) Immunostaining of first and second trimester sections with anti-pyruvate kinase antibody F) Western blots depicting GRP78 and phospho-eIF2α in first and second trimester placental lysates. Poncaeau S (Ponc S) staining was used to normalise the protein loading. G) Immunolocalisation of XBP1, P-IRE1 and IRE1 in first and second trimester placental sections, stained with anti-XBP1, -IRE1 or P-IRE1 antibodies. Arrows denote cell columns (CC). H) 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

**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. Poncaeau S (Ponc S) staining was used to normalise the protein loading. 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. F) Western blots depicting the expression of ApoA1 in first and second trimester placental lysates. Poncaeau S (Ponc S) staining was used to normalise the protein loading.

**Figure 4.** Overview of DEGs related to transcription factors, Wnt signalling and proliferation 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 DEGs related to cell cycle regulation. D) Heatmap of extracellular matrix related genes differentially expression in the first and second trimester. E-G) Imunolocalisation of meprin (E), CD31 (F) and musculin (MSC) (G) in first and second trimester placental sections.

**Figure 5.** DNA methylation changes in the first and second trimester. A) Scatterplot showing relation between expression change and methylation change in the first and second trimester placenta for each gene. Statistically significant DMRs are marked red (first trimester) and blue (second trimester). 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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