Low abundance plasma proteins in labour

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Abstract

Every year, millions of births worldwide are complicated by prematurity or difficult post-term deliveries, resulting in a high incidence of perinatal mortality and morbidity. Our poor understanding of human parturition is a key reason for our inability to improve the management of preterm and post-term birth. In this study, we used proteomic techniques to look into protein changes in placental blood plasma obtained from women before or after spontaneous or induced labour, with vaginal or caesarean section deliveries. Our aim was to understand the basic mechanisms of human parturition regardless of whether the signals that trigger labour are of maternal and/or fetal origin. We found proteins from 33 genes with significantly altered expression profiles in relation to mode of labour and delivery. Most changes in labour occurred in proteins associated with 'immune and defence responses'. Although the signal transduction and regulation of these pathways varied among modes of delivery, hepatocyte nuclear factor 1 homeobox A emerged as a shared protein in the mechanism of labour. Moreover, several apolipoproteins such as apolipoprotein A-IV and APOE were found to change with labour, and these changes were also confirmed in maternal plasma. This study has identified significant protein changes in placental intervillous plasma with labour and has revealed several pathways related to human parturition.

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Introduction

Deliveries at or around term are crucial, not only for the well-being of women and newborns but also to reduce the cost to health services from medical complications arising from alterations in the timing of birth. However, about 8% of pregnant women have an indicated or spontaneous preterm delivery (before 37 weeks of gestation), which is a major cause of perinatal mortality and morbidity throughout the world (Beck *et al.* 2010). On the other hand, ~4% of women deliver post-term (42 weeks of gestation or more), and this is associated with complications such as placental insufficiency, stillbirth or neonatal death; more than half of them require induction of labour (IoL), with increased risk of difficult instrumental delivery or caesarean section (Smith 2001, Olesen *et al.* 2006).

Increased understanding of human parturition will help control the onset and management of labour and help reduce the incidence of preterm and post-term deliveries (Slattery & Morrison 2002, Iams *et al.* 2008). However, despite advances in clinical and laboratory

research, the mechanism of human parturition is not well known (López Bernal 2003, Catalano *et al.* 2010). Excellent studies in pregnant sheep have demonstrated the involvement of the fetal pituitary–adrenal axis in the onset of labour (Liggins 1979) and the role of cortisol in the induction of placental enzymes regulating progesterone metabolism before delivery (Anderson *et al.* 1975). In rodents, parturition depends on activation of intrauterine prostaglandin release and the demise of the corpus luteum in the ovary. However, these animal mechanisms are not applicable to women because of differences in placental, uterine and ovarian function between primates and other mammals (Mitchell & Taggart 2009). Evidence from studies in rhesus monkeys suggests that activation of the fetal pituitary–adrenal axis, as demonstrated by increased levels of DHEAS (which is a precursor for placental oestrogens in the placenta) in the fetal circulation, is associated with parturition (Mecenas *et al.* 1996, Giussani *et al.* 1998). While efforts to understand human parturition will continue to involve research using animal models, progress will benefit from direct studies in pregnant women.

The onset of labour in women could be due to loss of uterine quiescence, the induction of uterine contractility or a combination of both (Price & Lopez Bernal 2001, López Bernal 2003). Multiple related pathways have been studied: one of the pathways involves corticotrophin-releasing hormone (CRH; McLean *et al.* 1995). CRH levels in the maternal circulation increase exponentially from the second trimester with advancing gestation and reach a peak at delivery (Smith 2007). At a molecular level, CRH binds to specific receptors in myometrium and functions through the cAMP pathway to keep the uterus quiescent during pregnancy. At term, a change in CRH receptors in myometrium reduces CRH function (Smith 2007), and at the same time, increased oxytocin receptor activity, gap junctions and prostaglandin release promote labour (Grammatopoulos & Hillhouse 1999, Maul *et al.* 2003). Inflammatory pathways have also been linked with changes in prostaglandin release and initiation of labour (López Bernal *et al.* 1989, Keelan *et al.* 1997, 2003). During pregnancy, progesterone maintains uterine quiescence by blocking myometrial contractions, and it is thought that labour is initiated by withdrawal of the progesterone blockade, which allows the muscle to enter the labouring state (Csapo 1956). However, while this may be true of some animal species (e.g. sheep, mice and rats), the role of progesterone withdrawal in human parturition remains controversial (Mitchell & Taggart 2009). On the other hand, changes in the sensitivity to uterotonic agents such as oxytocin, prostaglandins or endothelin may trigger the onset of labour (López Bernal 2003, 2007). Other agents such as epidermal growth factor and uterine relaxants, such as β_2 adrenoceptor agonists and phosphodiesterase inhibitors, may be involved in maintaining uterine quiescence through transactivation of uterine receptors and modulating cyclic nucleotide pathways (Yuan & Lopez Bernal 2007). Surfactant protein A (SP-A) levels increase exponentially towards term in human amniotic fluid (Miyamura *et al.* 1994), and a role for SP-A in regulating pro-inflammatory cytokine (IL1 β , TNF- α) production by intrauterine tissues has been proposed, but its role in human parturition remains controversial (Lee *et al.* 2010). It is possible that different pathways interact with each other and that other as yet unknown factors are involved. The advent of reliable proteomics methodology has opened a new window of opportunity in the study of human parturition by allowing the simultaneous study of multiple proteins in complex biological samples (Pereira *et al.* 2007, Scholl *et al.* 2012). The need for clinically useful biomarkers to predict preterm labour remains (Conde-Agudelo *et al.* 2011).

In this paper, we have used proteomic techniques based on differential in-gel electrophoresis (DiGE) for a global analysis of changes in low abundance plasma proteins in relation to mode of onset of labour and

delivery. This approach has disclosed novel pathways that widen our understanding of the process of spontaneous and induced labour in women.

Results

DiGE analysis of intervillous plasma samples

Normalized DiGE data sets of 21 unique placental intervillous plasma samples were used to study the proteomic patterns among the five labour/delivery groups. As shown in Fig. 1, an unsupervised k-mean cluster based on all protein spots grouped the samples into five categories. Most of the women delivered by emergency caesarean section clustered together, except one patient who had a caesarean due to failure to progress after spontaneous onset labour (ID: 57892). Three of four women from the spontaneous vaginal delivery (SVD) group had similar proteomic patterns except one sample (ID: 57776) whose pattern was more comparable to women having elective caesarean section (NiL). There was strong heterogeneity in the IoL vaginal delivery group as one sample (ID: 57846) clustered with women having elective caesarean section (NiL) and another sample (ID: 57841) had no common pattern with any other groups. Heterogeneity was also revealed in the NiL group as one sample (ID: 57774) appeared similar with women having induction of labour and the other sample (ID: 57800) was classified as SVD.

Protein expression differences in intervillous plasma were estimated by unpaired comparison of all the in-labour cases (SVD, IoL, spontaneous labour followed by emergency caesarean section (SLEC) and induced labour followed by emergency caesarean section (ILEC)) with the NiL group. The number of protein spot changes for each group is presented in Table 1. A total of 92 protein spots were selected for analysis by mass spectrometry, and for 59 of them (64%), we obtained confident identification. These proteins were the products of 33 genes, shown in Table 2. There were 25 protein spots from 14 unique genes with significant expression differences in SVD compared with NiL. In the IoL group, 15 significantly different protein spots were identified representing 12 genes. In both emergency section groups, 17 differentially expressed protein spots were identified, corresponding to 11 genes in the SLEC group, and 10 genes in the ILEC group. The expressions of the protein isoforms from each gene were consistent in both change of direction and fold change.

The majority of the gene products identified which changed significantly in more than one mode of delivery compared with NiL showed a similar change. However, amyloid P component of serum (APCS) and apolipoprotein A-IV (APOA4) were found to be down-regulated in the SVD group (fold change for APCS was -1.5 and for APOA4 -1.6) but had increased expression in the ILEC

Figure 1 Unsupervised k-mean cluster of 21 unique placental intervillous plasma samples. The abbreviations describe the original group and the colour of the spots indicates the cluster each sample belongs to after k-mean analysis. The position of each spot is generated by cluster software based on its actual biological group, which is not expression related. The numbers refer to sample ID.

group (APCS fold change 1.5) and IoL group (APOA4 fold change 1.4), respectively.

DiGE analysis of pre- and in-labour maternal plasma samples

In order to confirm the effect of labour on the maternal plasma proteome, a further DiGE analysis was carried out on serial blood samples obtained from six women before and after the onset of spontaneous labour (SVD group). In this analysis, 17 proteins were found to be down-regulated (median fold change 1.2, change range 1.1–1.4), while 33 proteins were up-regulated (median fold change 1.1, change range 1.1–1.6) by labour. The largest fold change was found with C4B, which decreased 1.56-fold in spontaneous labour compared with the pre-labour status in the same women, while other proteins from inflammatory pathway genes (CFI and complement component 1, r subcomponent (C1R)) also decreased (Table 3). A similar decreasing trend in the levels of inflammatory gene-related proteins was found in placental intervillous plasma.

In silico analysis of parturition pathways: gene ontology

Gene ontology of the 33 identified protein products was performed in order to find out the possible signalling pathways involved in the different modes of delivery. The majority of the proteins found to change significantly in the four labour/delivery-mode comparisons are from the

extracellular region (GO: 0005576, GO: 0005615 and GO: 0044421), which is reassuring because it demonstrates low contamination from intracellular proteins. The molecular function analysis showed that the proteins that changed significantly in SVD are associated with oxygen binding and transport (GO: 0019825 and GO: 0005344), with defence responses (GO: 0006952) and with innate immunity (GO: 0045087). Proteins obtained by comparing SLEC to NiL have a role in fatty acid binding (GO: 0005504), and they are known to be involved in positive regulation of the immune response. Proteins found differentially expressed in the IoL group belong to enzyme inhibitors (GO: 0004857, GO: 0004867, GO: 0030234, GO: 0004866 and GO: 0030414) and signal transporters (GO: 0060228, GO: 0017127 and GO: 0015248). In contrast to SLEC, the gene products found to change in IoL are involved in negative regulation of the immune and defence responses.

The functions of the proteins that changed significantly in the ILEC group fall into four categories. One of the

Table 1 Differentially regulated protein spots (in relation to NiL) identified in placental intervillous plasma by DiGE.

Group	Number of up-regulated protein spots	Median (range) of fold change	Number of down-regulated protein spots	Median (range) of fold change
SVD	53	2.6 (1.4–7.1)	38	1.6 (1.4–2.7)
IoL	49	1.9 (1.3–3.4)	28	1.7 (1.4–2.8)
SLEC	56	2.0 (1.4–4.5)	48	1.8 (1.4–4.0)
ILEC	63	2.0 (1.4–3.8)	39	1.7 (1.3–7.3)

Table 2 Mean fold change of proteins identified by DiGE and MS in placental intervillous plasma in relation to mode of onset of labour and delivery. Negative values indicate higher expression in NiL, and positive values indicate lower expression in NiL.

Gene symbol	ILEC – NiL	SLEC – NiL	SVD – NiL	IoL – NiL
AZGP1	–1.6	–1.7	–1.6	–
A2M	1.9	1.9	–	1.8
C1R	–	–1.5	–1.7	–1.4
ALB	–2.2	–2.2	–	–
APOE	–1.4	–	–	–1.4
APCS	1.5	–	–1.5	–
ITIH4	1.5	1.5	–	–
HPX	–	–1.4	–1.6	–
APOA4	–	–	–1.6	1.4
SERPINF1	–	–	–1.5	–1.5
CFH	–	–	1.4	1.6
TPI1	–1.8	–	–	–
ZC3H13	–1.6	–	–	–
CFI	–1.4	–	–	–
APOC3	2.2	–	–	–
C8G	–	–1.9	–	–
CFD	–	–1.7	–	–
LRG1	–	–1.6	–	–
F2	–	1.4	–	–
C7	–	1.5	–	–
AFP	–	–	–2.1	–
GSN	–	–	–1.6	–
ITIH1	–	–	1.6	–
CST3	–	–	1.9	–
HBB	–	–	4.3	–
HBC2	–	–	4.6	–
HBA1	–	–	4.7	–
A1BG	–	–	–	1.6
AHSG	–	–	–	1.3
AMBP	–	–	–	–1.7
ANXA1	–	–	–	–2.1
IGHG1	–	–	–	2.3
ITIH3	–	–	–	1.5

categories corresponds to the pathway found in SLEC, that is fatty acid binding (GO: 0005504). Another function is represented in IoL as enzyme inhibitor (GO: 004857). The other two functions are unique to the ILEC group: lipoprotein receptor (GO: 0070325) and antioxidant (GO: 0016209). As regards the various molecular functions of ILEC proteins, the biological processes involved show a wide range including inflammatory response, negative regulation of defence response, signal transduction and lipid metabolism.

In summary, immune and defence response are the major pathways found differentially expressed in labour, although changes in signal transduction and regulation were also found among the four modes of delivery. However, some of the pathways found in the different modes of delivery may reflect differences in the management of the labour process in these groups. For example, changes in oxygen transporters have been found in only SVD, while several small molecule transporters have been found in induction-related deliveries (IoL and ILEC).

In the maternal circulation, 14 out of the 50 gene products that changed with labour come from the innate immune response pathway (GO: 0045087).

In comparison with placental plasma, more genes involved in cholesterol transport (GO: 0030301) and blood coagulation pathway (GO: 0007596) were found to be changed during labour.

In silico analysis of labour pathways: Ingenuity Pathway Analysis database

The Ingenuity Pathway Analysis (IPA) database contains the extended database of gene ontology, and the results we obtained from IPA analysis reflected the results from

Table 3 Mean fold change of proteins identified by DiGE and MS in maternal plasma from spontaneous vaginal deliveries. Negative values indicate higher expression in-labour, and positive values indicate lower expression in-labour.

Gene symbol	Pre- to in-spontaneous labour
ALB	–1.33
AMBP	–1.29
AZGP1	–1.15
ATRN	–1.1
CNTRL	–1.1
CLU	–1.37
F2	–1.07
C1R	–1.09
C4B	–1.56
CFI	–1.1
ERC1	–1.1
FETUB	–1.07
FHAD1	–1.1
GSN	–1.12
GPLD1	–1.16
GC	–1.11
HPX	–1.07
DDX39B	–1.23
ITIH1	–1.06
ITIH4	–1.18
KIAA1328	–1.1
KIF4A	–1.1
KNG1	–1.1
LRG1	–1.15
PON1	–1.35
PRDX2	–1.63
PLEC	–1.14
PSG1	–1.11
PSG11	–1.15
PSG7	–1.11
PIBF1	–1.14
PPFIA4	–1.1
SMC1A	–1.1
ACTB	1.44
A2M	1.15
APOA4	1.18
APOC2	1.32
APOC3	1.22
APOH	1.19
C1S	1.15
C6	1.05
C8A	1.07
CFB	1.14
CFH	1.05
ERC1	1.37
FGB	1.34
FCN3	1.29
POTEE	1.44
RAI14	1.07
TBK1	1.07

GoMiner but with detailed pathway networks. As with GoMiner, IPA located the majority of proteins in the extracellular space ($n=26$). The rest of the proteins were cytoplasmic (HBA1, HBB, HBG2, TPI1 and C7), plasma membrane proteins (annexin A1 (ANXA1)) or unknown (ZC3H13). In order to minimize the potential effect from the cytoplasmic pathways, the network analysis was focused on the 26 extracellular proteins plus ANXA1 (situated on the plasma membrane). The core network pathways involved in labour were metabolic and inflammatory. As shown in Fig. 2, the results indicate that different modes of delivery use different signal processes to regulate the core pathways. Fewer signal transduction steps were detected in SVD compared with the other three modes of delivery. In addition, the protein network involved in the ILEC group overlapped with networks in the SLEC and IoL groups. However, only two proteins appeared in both IoL and SLEC networks (hepatocyte nuclear factor 1 homeobox A (HNF1A) and A2M, Fig. 2). Remarkably, only HNF1A appeared in all four modes of delivery.

IPA analysis was also performed to identify pathway alterations in spontaneous labour using serial maternal plasma samples. Inflammatory response pathways centred in TNF and IL8 were identified (Supplementary Figure 1, see section on supplementary data given at the end of this article). Moreover, as with placental plasma, the HNF1A-related pathway was involved (Supplementary Figure 2, see section on supplementary data given at the end of this article; Yuan 2011).

Protein biomarker validation

In the DiGE experiment, APOA4, APOE and APOC3 were associated with the onset of labour.

Apolipoproteins can also work as enzyme cofactors to influence signal transduction. Therefore, it is possible that apolipoproteins both promote the onset of labour and have the potential to be biomarkers for labour. APOA4 and APOE were selected for further validation based on the availability of reliable commercial antibodies.

Apolipoprotein A-IV

The presence of APOA4 in placental intervillous plasma was confirmed using western blotting in nine samples each of SVD and NiL, matched as closely as possible for maternal age and gestational age at birth (Fig. 3). The result of the immunoblots agreed with those of the DiGE experiment (Table 2) in that the level of APOA4 was lower in SVD compared with NiL; the difference in the APOA4/IgG ratios for these groups was statistically significant ($P=0.0078$).

Further validation of APOA4 was carried out in serial samples of maternal peripheral plasma taken before and during labour/at caesarean section, in women from the SVD ($n=6$), IoL ($n=6$) and NiL ($n=6$) groups (Fig. 4). In the SVD group, the protein change confirmed the trend found in the DiGE experiment; however, it did not reach statistical significance probably due to small sample number.

APOE

The level of APOE in placental intervillous plasma was measured by western blotting in nine samples each of SVD and NiL, as for APOA4 (Fig. 3). There was no significant difference in this comparison, which agrees with the DiGE results. APOE levels were also measured

Figure 2 Network analysis of the proteins found in the DiGE experiments using IPA. Red colour indicates protein changes in more than one group (given in the grey rectangles). Light green colour means protein changes in IOL only, blue colour means protein changes in SLEC only. The box explains the functional allocation of the proteins. Note the central role of HNF1A.

Figure 3 Levels of APOA4 and APOE proteins in placental intervillous plasma. Plasma was collected following spontaneous vaginal delivery (SVD) or elective caesarean section (NiL) and analysed by western blotting, as described in Materials and Methods section. (A) Representative blots of IgG, APOA4 and APOE in SVD and NiL plasma ($n=6$). (B) APOA4 and APOE levels, normalized to levels of IgG (individual and mean levels shown). $n=9$ in each group. For APOA4, there was a significant difference between the means of the SVD and NiL groups (* $P<0.01$).

in serial samples of maternal peripheral plasma, as for APOA4 (Fig. 4). There were no differences in APOE/IgG levels between the pre- and in-labour groups in SVD, NiL or IoL women. Likewise, with DiGE, no significant difference was seen between SVD pre- and in-labour levels of APOE (Table 3). No differences between the SVD, NiL and IoL groups were identified by ANOVA analysis of the western blotting results.

RIAs

In order to obtain more information about lipoprotein changes with labour, APOE, lipoprotein A (LPOA), APOA1 and APOB estimations were carried out in serial maternal plasma samples by established RIAs. This allowed the determination of absolute levels of these proteins in the different groups. As seen in DiGE, the level of APOE was higher in NiL ($\text{mean} \pm \text{s.d.} = 92.3 \pm 23.0 \text{ mg/l}$) than in IoL ($63.7 \pm 23.9 \text{ mg/l}$; $n=10$, $P=0.08$) or in SVD ($64.8 \pm 13.9 \text{ mg/l}$; $n=10$, $P=0.02$). In contrast to APOE, the levels of LPOA, APOA1 and APOB did not change at the time of delivery.

Discussion

HNF1A is a key mediator of human parturition

Parturition involves complex interactions across multiple signalling pathways that converge in functional uterine changes, notably cervical ripening and dilatation and myometrial contractions. In the DiGE-MS approach, we have identified 59 proteins from 33 genes involved in

human parturition. Some of the proteins have a consistent expression pattern across different modes of delivery. For example, the decrease of C1R in SVD, SLEC and IoL suggests the existence of a common pathway in the process of labour. However, there was no single protein that changed in all modes of delivery. The analysis using IPA indicates that genes that regulate HNF1A and genes that are regulated by HNF1A are involved in all modes of labour and delivery, which suggests that HNF1A may be a part of the common human parturition signal network. Here, we name it the HNF1A pathway (Fig. 2, red colour molecules).

In this signalling pathway, HNF1A acts as a key protein that regulates several other proteins directly, including albumin (ALB) and APCS. HNF1A is a transcription factor expressed in human placenta, liver, kidney, pancreatic islets and intestine but has not been described in myometrium (Mendel *et al.* 1991, Gautier-Stein *et al.* 2006). Physiological studies of HNF1A have found that it controls insulin-signalling transduction and that it is essential in linking metabolic and inflammatory pathways (Eeckhoutte *et al.* 2004). HNF1A is among several factors implicated in the pathogenesis of gestational diabetes (Shaath & Groop 2007). The range of proteins regulated by HNF1A is very broad, including acute phase proteins such as ALB, alpha fetoprotein (AFP), CRP, fibrinogen, insulin-like growth factor and APCS; proteins involved in cholesterol/lipoprotein metabolism such as APOB, APOA2, APOA1, APOC3,

Figure 4 Levels of APOA4 and APOE proteins in maternal peripheral plasma. Plasma was collected from women undergoing spontaneous vaginal delivery (SVD), elective caesarean section (NiL) or induction of labour (IoL), with paired pre- and in-labour samples taken from each subject (for the NiL group, in-labour samples were taken at the time of caesarean section). Samples were analysed by western blotting, as described in Materials and Methods section. (A) Representative blots of IgG, APOA4 and APOE in SVD, NiL and IoL plasma ($n=2$ pairs). (B) APOA4 and APOE levels, normalized to levels of IgG (individual and mean levels shown). $n=6$ pairs in each group. No significant differences were observed between normalized protein expression levels in pre- and in-labour groups.

APOM, lipoprotein lipase and acyl-coenzyme cholesterol acyltransferase 2; as well as proteins in glucose/fatty acid metabolism such as glucokinase, glucokinase regulator glucose transporter and liver fatty acid-binding protein (Armendariz & Krauss 2009); and others such as interalpha globulin inhibitor H4 (ITIH4) and insulin-like growth factor binding protein 1 (IGFBP1). HNF1A may also be involved in metabolic programming of the fetus (Rodriguez-Trejo *et al.* 2012). An interesting finding from this study is the change with parturition in various proteins involved in triglyceride transport and metabolism. It is known that maternal triglyceride levels gradually rise in the first trimester of normal pregnancy and reach a peak during the second trimester (Choi & Pai 2000, McGladdery & Frohlich 2001). After delivery, triglyceride levels decrease rapidly, and the fall is faster in breastfeeding women compared with women without lactation (Qureshi *et al.* 1999). In placental intervillous plasma, the triglycerides transporter APOA4 was found to decrease in SVD and to increase in IoL; APOE decreased in both IoL and ILEC and APOC3 increased in ILEC. This does not necessarily mean that changes happened on the ability to transport triglycerides, because one apolipoprotein can be replaced by another, for example APOA4, which binds chylomicrons, can be displaced by APOC3 (Green *et al.* 1979, Hayashi *et al.* 1990). Our findings suggest a complex balance among triglyceride transporters and chylomicron metabolism during human parturition, and this balance could be adjusted according to delivery mode and/or onset of labour.

Spontaneous labour and vaginal delivery

We propose that the HNF1A pathway is central to human parturition; however, there may be characteristic changes in this and associated pathways for each mode of labour and delivery. For example, in the SVD group, AFP in placental and maternal peripheral blood decreased after delivery; this may be a consequence of down-regulation by retinoids mediated by HNF1A (Magee *et al.* 1998). This suggests that in SVD, there is a distinct mechanism within the HNF1A pathway to decrease AFP levels.

APCS, a glycoprotein, is another important link to the HNF1A pathway. APCS is produced in blood vessel walls and it can bind to fibronectin (FN1) to regulate human inflammatory pathways (de Beer *et al.* 1981) by depositing APCS-FN1 into blood plasma in response to inflammatory/immune stimuli (Breathnach *et al.* 1983). Although there is no direct evidence connecting APCS with the onset of labour, abnormal FN1 levels in the maternal circulation have long been associated with high risk of preterm birth (Pepys & Baltz 1983). APCS is involved in modulating immune responses by promoting macrophage differentiation (Zhang *et al.* 2011), and there is strong interest in the role of decidual

macrophages in pregnancy maintenance (Houser *et al.* 2011). APCS levels in maternal plasma decrease with delivery in SVD and increase in ILEC, which indicates that different modes of delivery may promote different inflammatory responses. Interestingly, HNF1A is involved in a new mechanism of inflammation-induced transcriptional repression by competing with pro-inflammatory JUN proteins on the same regulatory regions of human apolipoprotein promoters (Mosialou *et al.* 2011). The anti-inflammatory effect of HNF1A may be beneficial in promoting uterine quiescence.

Apart from the HNF1A pathway, this study has shown that there are other signalling networks involved in SVD. These include several proteins from inflammatory pathways: HPX, complement factor H (CFH), C1R, cystatin 3 (CST3), APOA4 and AZGP1. In contrast to HNF1A, CFH and C1R do not respond to high levels of IL6 but react to other cytokines such as interferon-gamma (Goldberg *et al.* 2007). This suggests that part of the SVD signals may be independent from and/or complementary to the HNF1A network. Furthermore, high levels of CFH in SVD could cause the inhibition of both the classical and alternative pathways of complement activation, which emphasizes that a balance in inflammatory pathways must be maintained during human parturition (Whaley & Ruddy 1976, DiScipio 1981, 1992, Goldberg *et al.* 2007). Several proteins associated with TNF were found to change in maternal peripheral plasma with labour. A possible involvement of TNF in the mechanism of human term and preterm parturition has been proposed (Casey *et al.* 1989). TNF stimulates prostaglandin production by the amnion, decidua and myometrium (Hertelendy *et al.* 1993), and recent reports suggest that the uterus becomes very sensitive to TNF with advancing gestation and labour (Alexander *et al.* 2012).

In the SVD group, APOA4 decreased in both placental and maternal plasma with delivery. Few studies of APOA4 levels in human pregnancy or parturition have been carried out, but it is known that APOA4 activates the enzyme lecithin:cholesterol acyl transferase and modulates lipoprotein lipase activity. Moreover, APOA4 mediates the metabolism of chylomicrons and may be important in regulating plasma lipid levels. Collectively, these actions could promote the transport of cholesterol from extra-hepatic tissue and cells to the liver (Wong *et al.* 2007). In addition, APOA4 possesses anti-atherogenic (Warner *et al.* 2001) as well as anti-oxidant properties (Wong *et al.* 2007). In mice, a reduction of APOA4 levels exacerbated inflammatory responses (Vowinkel *et al.* 2004) and it is tempting to speculate that a decrease in maternal APOA4 levels may favour an inflammatory pathway in labour.

Beside the proteins involved in inflammatory pathways, there were several other proteins that changed in SVD in comparison to the NiL group. These included CST3, an endopeptidase inhibitor, which increased

exclusively in the SVD group. CST3 is produced by virtually all nucleated cells at a constant production rate, unaltered in inflammatory conditions but closely correlated to kidney function in pregnant and non-pregnant women. High levels of CST3 during pregnancy are associated with a high risk of developing pre-eclampsia (Kristensen *et al.* 2007). CST3 participates in matrix metalloproteinase regulation in vaginal tissues during the peripartum period (Wieslander *et al.* 2008). Interestingly, in SVD, another two endopeptidase inhibitors, ITIH1 and serpin peptidase inhibitor F1 (SERPINF1), were also found increased and decreased respectively. These protein inhibitors might be involved in remodeling of placental tissues, and a low level of SERPINF1 in placenta has been reported to be significantly associated with increased risk of stillbirth (Plunkett *et al.* 2008). SERPINF1 may participate in the process of cervical ripening through its collagen binding and proteoglycan interactions (Sekiya *et al.* 2011). In addition, a study in pregnant rats has demonstrated that endopeptidase inhibitors enhance myometrial contractility compared with non-pregnant rats, which were not affected by endopeptidase inhibitors (Shintani *et al.* 2000).

Another key finding in the SVD group is the exclusive presence of proteins belonging to the haemoglobin family, such as HBA1, HBG2 and HBB. This gene family is known to be related to oxygen transport. During pregnancy and parturition, oxygen levels and its transport have to be constantly adjusted. At the time of implantation and early pregnancy, the oxygen level in the uterus is low (Jauniaux *et al.* 2000). This early pregnancy hypoxic environment is optimal for embryo development, because it keeps a low level of metabolism and thereby minimizes the production of reactive oxygen species, as well as providing a beneficial effect on early placental development (Charnock-Jones 2002). By the end of the first trimester, following the developments in the fetoplacental interface, the placenta is exposed to higher oxygen levels and faces major oxidative challenges. Oxygen tension in the placenta continues to rise during the second trimester, in order to support the rapid fetal growth (Carter 2000) and then it decreases until term (Soothill *et al.* 1986). The increased expression of key antioxidant enzymes and improvement of their activities helps the placenta adapt its defences to suit the hyperoxic conditions (Jauniaux *et al.* 2000).

During normal term labour, the placenta's maternal arterial supply is cut off during uterine contractions, which can result in ischaemia-reperfusion stress (Grace & Mathie 1999). This condition, similar to the cause of pre-eclampsia, would increase the haemoglobin expression level in placenta, which would be detectable in placental intervillous plasma. This could be the explanation for the increased expression of haemoglobin family proteins that we have found in the SVD group.

Emergency caesarean section

Our study has revealed that the SLEC and ILEC groups share very similar protein expression patterns and stand out from the non-emergency groups. This would suggest that emergency caesarean section is associated with a distinct pathway, which is different from the normal human parturition network.

Proteins found significantly and consistently changed in both SLEC and ILEC could represent a conserved emergency pathway. For instance, ITIH4, was increased 1.5-fold in both SLEC and ILEC. This is an acute phase protein that can be regulated by HNF1A and has been functionally involved in cervical ripening and parturition in mice (Ruscheinsky *et al.* 2008) and in recurrent pregnancy loss in women (Kim *et al.* 2011). IGFBP1 was also increased in both emergency caesarean section groups, and its expression is regulated by cytokines (IL1 β) and hCG through HNF1A (Tang & Gurpide 1993). Epidemiological studies have shown that elevated maternal serum IGFBP1 levels in the second and early third trimesters significantly associate with pre-eclampsia, intrauterine fetal growth restriction and other pregnancy complications (Bujold *et al.* 2008). At term, IGFBP1 levels are higher in spontaneous labour than in elective caesarean section; this may reflect the higher incidence of fetal hypoxia and/or fetal distress in active labour (Hills *et al.* 1996) and is supported by the results of elevated IGFBP1 in both ILEC and SLEC, as a possible consequence of fetal distress (Marchini *et al.* 2005). The decidua secretes phosphorylated isoforms of IGFBP1, which have been measured in cervical secretions as a predictor of preterm delivery with some success (Kekki *et al.* 2001).

Apart from the similarities, the differences in protein patterns between SLEC and ILEC may underlie the clinical indications for emergency caesarean sections. For example, various complement factors (C1R, C7, C8G and CFD) were found altered in SLEC but not in ILEC. The decrease in C1R, C8G and CFD, and the increase in C7, may represent a balancing of inflammatory responses.

An interesting protein found to change in the SLEC group is CRP, which is also regulated by HNF1A. CRP is a sensitive biomarker of systemic inflammation and is primarily synthesized in hepatocytes in response to infection and tissue injury (Pepys & Baltz 1983). Previous studies have shown that elevated levels of CRP in amniotic fluid and maternal plasma in the second trimester are strongly associated with spontaneous preterm birth (Wei *et al.* 2010). CRP levels in maternal blood of women at the onset of labour were significantly higher than in women who were not in labour (Törnblom *et al.* 2005); this agrees with the results of our DiGE analysis.

In the ILEC group, APOC3 and APOE were found to increase and decrease respectively. APOE is essential for

the normal catabolism of triglyceride-rich lipoproteins and is involved in corpus luteum function and metabolic adaptations in the peripartum period in lactating animals (Ndikum-Moffor *et al.* 1997). Besides the well-known function of removal of excess cholesterol from the circulation via regulation of hepatic uptake (Belo *et al.* 2004), its function also involves modification of inflammatory responses, including suppressing T cell proliferation, macrophage regulation, lipid antigen presentation facilitation (by CD1) to natural killer T cells, as well as modulation of inflammation and oxidation (Zhang *et al.* 2010). Epidemiological studies have reported that increased levels of APOE are associated with an increased risk of pre-eclampsia, whereas genetic association studies suggested that polymorphisms in APOE are also associated with pre-eclampsia (Atkinson *et al.* 2009) and spontaneous abortions in patients with endometriosis (Collazo *et al.* 2012). In addition, APOE is involved in inflammatory pathways. APOC3 is another apolipoprotein found in many triglyceride-rich lipoproteins (Zheng *et al.* 2010). The increase of APOC3 and decrease of APOE in the ILEC group suggests that hypertriglyceridaemia happens during the emergency section procedure following the IoL.

IoL and vaginal delivery

Involvement of the HNF1A pathway (Fig. 2) in the IoL group was characterized by the appearance of several inflammation-related proteins, such as alpha-2HS-glycoprotein (AHSG), one of the HNF1A target genes, as well as the complement proteins C1R and CFH (Odom *et al.* 2004). Moreover, both A2M and APOE were found to change in IoL, which supports the hypothesis that the formation of the A2M–APOE complex in human parturition is a downstream target of HNF1A (Hussain *et al.* 1991). There is no previous information on potential roles for AHSG in human parturition; however, maternal AHSG levels are low in pre-eclampsia (Molvarec *et al.* 2009) and a decrease in sialic acid content of AHSG has been described in plasma from neonates with intrauterine growth restriction (Karamessinis *et al.* 2008).

Interestingly, APOA4 decreased in SVD but increased in IoL, which suggests that although women in both SVD and IoL groups had successful vaginal deliveries, the different modes of onset of labour, spontaneous vs induced, required alternative pathways. This suggestion is further supported by the fact that several proteins changed uniquely in IoL. For example, ANXA1 decreased exclusively in IoL. ANXA1 is involved in the regulation of phospholipase A₂ activity and is a mediator of the anti-inflammatory actions of glucocorticoids. Administration of ANXA1 to mice results in anti-inflammatory effects by potent inhibition of neutrophil trafficking (Lim & Pervaiz 2007). Therefore,

the decrease of ANXA1 in the plasma of IoL supports the idea that medical IoL may provoke excess activation of inflammatory pathways. In addition, a decrease of ANXA1 expression can result in liberation of phospholipase A₂ activity, making more arachidonic acid available for prostaglandin generation (Herbert *et al.* 2007), which is crucial in uterine contraction.

Moreover, alpha-1-B glycoprotein (A1BG) and immunoglobulin heavy constant gamma 1 (IGHG1) proteins increased whereas alpha-1-microglobulin precursor (AMBP) decreased exclusively in IoL. AMBP is reported to be increased in the amniotic fluid of women delivering at term compared with preterm deliveries (Bujold *et al.* 2008). Further studies are required to understand the involvement of this group of proteins in pregnancy and parturition.

Strengths and limitations

The initiation of labour is a complex physiological process and different modes of delivery apparently result in specific modifications of the parturition pathway. These variations could be one of the reasons why it has been difficult to develop drugs to delay safely and effectively the onset of preterm labour. Preterm labour may have a multifactorial aetiology and there are complications such as premature rupture of the membranes or vaginal bleeding that may underlie pathological pathways different from those involved in labour at term. In this study, we have considered two modes of onset of labour, spontaneous and induced, as well as three modes of delivery, vaginal, elective caesarean section and emergency caesarean section. The overlap in proteomic changes of the different modes of labour onset and delivery suggests a common pathway for human parturition involving HNF1A. This finding has been reinforced by comparing serial pre- and in-labour maternal plasma samples and highlights the importance of the HNF1A pathway and the need to investigate the functional involvement of HNF1A-regulated proteins in uterine function. This approach may discover specific drug targets for clinical applications. However, this study cannot differentiate between changes that are related to the cause of labour and changes that are the result of activation of the labour mechanism. For instance, the potential effects of the procedures for the IoL (intravaginal prostaglandins, artificial rupture of the membranes and intravenous oxytocin) alone or in combinations on the levels of plasma proteins are not known and may have contributed to heterogeneity in the IoL groups. The reasons for emergency caesarean section included failure to progress, fetal distress and failed induction. The different physiopathological mechanisms underlying these situations, e.g. protracted uterine activity vs acute fetal hypoxia and stress responses, may have provoked distinct alterations in plasma proteins levels. Finally, heterogeneity in the elective caesarean section group

may have arisen because at the time of the section some women may have been much closer to the spontaneous onset of labour than others. In theory, some of this heterogeneity could be determined by obtaining a Bishop Score, but this is a subjective and poorly reproducible score and vaginal examination is not routinely performed before elective caesarean section.

Given that the number of samples that can be analysed by DiGE is relatively small, differences in the clinical presentation of women or in the management of labour and delivery may have influenced the results. Moreover, the protein changes detected could be relatively small due to dilution in a large volume of plasma and the influence of many organs in the composition of the maternal plasma proteome. Changes at the foeto-maternal interface may be greater but would be more difficult to sample in a non-invasive manner. Future studies will be facilitated by more efficient and rapid proteomic techniques that allow the processing of larger sample numbers. This would allow better control of clinical heterogeneity. The answers to these challenges need further studies with methods that allow frequent serial estimations of maternal plasma proteins in women before and after labour, combined with *in vitro* and *ex vivo* physiological experiments using fresh intra-uterine tissues.

Materials and Methods

Clinical groups

We obtained placental intervillous blood from five groups of women according to their onset of labour and mode of delivery: elective caesarean section (not in labour, NiL), SVD, IoL, SLEC and ILEC (Table 4). We also obtained venous blood from women who were delivered within 2 days of recruitment and who went into spontaneous labour and had a vaginal delivery (serial-SVD), from women whose labour was induced and had a vaginal delivery (serial-IoL) and from women who were delivered by elective caesarean section with no signs of labour (serial-NiL). We obtained two blood samples from each woman. The first serial blood sample was taken at the time of the initial assessment, when there were no signs of labour and

the second sample at the time of established labour or elective caesarean section. Thus, we were able to compare peripheral blood from the same women before and after the onset of labour and from women who did not go into labour within the same time interval. Each patient was given an anonymized ID number. Clinical details are given in Table 4. Labour was induced with intravaginal prostaglandins, artificial rupture of the membranes and/or intravenous oxytocin. The length of labour in women with spontaneous onset was 5 h (1 h 40 min–21 h 45 min) (median and range) and in women with induced labour 8 h 30 min (8 h 30 min–21 h 30 min). Thus, the length of labour in spontaneous and induction groups was similar; however, there was a wide range of values in both groups, which may have given rise to heterogeneity in plasma protein levels. The reasons for emergency caesarean section included failure to progress, malposition, fetal distress, breech presentation and failed induction. All women had active management of the third stage of labour with an oxytocin agent (either oxytocin 5 IU alone or combined with ergometrine 250 µg administered intramuscularly or intravenously). In most cases including caesarean sections, delivery of the placenta was by controlled cord traction. All women delivered in St Michael's Hospital, Bristol, UK, and were of British White origin with good general health and had singleton pregnancies without complications such as infection, antepartum haemorrhage, hypertension, pre-eclampsia, diabetes or intrauterine growth restriction. The study was approved by the Central and South Bristol Research Ethics Committee and all women gave informed written consent.

Placental intervillous plasma

Human placental intervillous plasma was collected as soon as possible after delivery. The maternal side of the placenta was cut open, and 4 ml whole blood was collected by syringe from the placental intervillous space. Blood was transferred immediately into a pre-prepared K₂EDTA collection tube containing leupeptin hydrochloride (1 µg/ml), aprotinin (1 µg/ml) and pepstatin A (1 µg/ml) and mixed well. Plasma was separated by centrifugation at 2000 g for 10 min at room temperature. Clear plasma was given a further spin at 12 000 g for 5 min. Supernatant was taken and divided into 6–8 aliquots, then frozen in liquid nitrogen and stored at –80 °C.

Table 4 Clinical details of women according to onset of labour and mode of delivery.

	Number of women	Median maternal age (range; years)	Median gestational age (range; weeks)	Median newborn weight (range; kg)	Fetal sex male/female
Placental intervillous blood					
Elective caesarean section (NiL)	5	35 (34–39)	39 (39–40)	3.5 (3.2–3.9)	3/2
Spontaneous vaginal delivery (SVD)	4	29 (19–36)	41 (39–41)	3.4 (3.2–4.0)	3/1
Induction of labour vaginal delivery (IoL)	4	31 (27–42)	42 (40–42)	3.7 (3.4–4.5)	3/1
Spontaneous labour, emergency caesarean section (SLEC)	4	27 (21–35)	41 (37–41)	3.7 (3.4–3.9)	3/1
Induction of labour, emergency caesarean section (ILEC)	4	28 (25–44)	40 (38–41)	3.5 (3.4–4.2)	3/1
Maternal peripheral blood					
Serial-SVD	10	36 (21–46)	41 (39–42)	3.9 (3.1–4.3)	5/5
Serial-IoL	5	29 (19–34)	42 (41–42)	4.0 (2.9–4.4)	3/2
Serial-NiL	5	35 (28–46)	40 (38–42)	3.6 (3.0–4.1)	3/2

Maternal plasma

Maternal peripheral blood samples were collected from women before delivery and from the same women at the onset of labour or just before delivery by caesarean section. The blood samples were treated and the plasma stored in the same way as described earlier for placental intervillous blood.

Differential in-gel electrophoresis

Two DiGE experiments were carried out: in the first experiment, we compared placental intervillous blood from five groups of women, NiL, SVD, IoL, SLEC and ILEC (Tables 1 and 2); the second used peripheral vein blood from women recruited in the serial-SVD group (Table 3). Placental intervillous plasma samples from 21 women were used for DiGE. In all samples, the 20 most abundant proteins (including ALB, IgG and transferrin) were removed from the plasma using the ProteoPrep20 immunodepletion kit from Sigma. The immunodepleted samples were then resuspended in 7 M urea, 2 M thiourea, 30 mM Tris-HCl and 4% (w/v) CHAPS, pH 8.5, at a concentration of 5–10 mg/ml and labelled for DiGE analysis using fluorescent cyanine dyes according to the manufacturer's guidelines (GE Healthcare, Uppsala, Sweden). In brief, samples were labelled using Cy3 or Cy5 *N*-hydroxysuccinamide ester DiGE dyes freshly dissolved in anhydrous dimethylformamide by mixing 50 µg protein with 1 µl CyDye (400 pmol/µl). In addition, an internal standard generated by pooling all samples in the experiment was labelled with a third dye, Cy2. In each case, the labelling reaction was allowed to proceed on ice in the dark for 30 min. The reaction was terminated by the addition of 10 nmol lysine and subsequent incubation on ice in the dark for an additional 10 min. Each Cy3- and Cy5-labelled sample to be compared was then mixed with an aliquot of the Cy2-labelled internal standard, separated by 2D-PAGE as described below and scanned at three different wavelengths using a Typhoon 9400 variable mode imager (GE Healthcare) to generate Cy3-, Cy5- and Cy2 images.

All gel images were imported into SameSpots software and passed an image QC test. Images corresponding to Cy2, Cy3 and Cy5 labelling were grouped and the best quality Cy2 gel image was used as a reference image. Automatic gel images alignment was applied first and adjusted by manual check. For each gel, 30 alignment vectors were applied to ensure the best match of candidate and reference images. Every single spot detected by the software was reviewed, and artefacts were excluded. The data generated by SameSpots was extracted as text file for further analysis (Yuan 2011).

The extracted data from the software package was normalized using the 'Subtract: Global loess: scale' approach in the *Limma* package (BioConductors-R <http://www.r-project.org/>). Data analysis followed the general protocol of the *Limma* package with some modifications adapted to this proteomic study.

Protein identification

Selected protein spots were cut from the gel, using the Investigator ProPic Automated 2D spot picker and digested

with trypsin using the ProGest automated digestion unit (both from Perkin Elmer Life Sciences (UK) Ltd., Beaconsfield, England). The resulting peptides were analysed by mass spectrometry using a 4700 MALDI-ToF mass spectrometer (Applied Biosystems, Foster City, CA, USA) to give a peptide mass fingerprint and peptide sequence information, which was searched against various databases using the Mascot search program (www.matrixscience.com) to identify the protein present in the gel spot.

In silico analysis of parturition pathways

To identify the biological pathways involved in human parturition, the set of proteins that changed during labour was mapped by GoMiner, using the whole genome as reference and limiting the analysis to the human database (Zeeberg *et al.* 2005). A further pathway analysis was carried out through the use of IPA (Ingenuity Systems, www.ingenuity.com). Four data sets (for each comparison) containing gene identifiers (from the DiGE analysis) and the corresponding expression values (fold change) were uploaded into the application. Each identifier was mapped to its corresponding object, providing a series of Network Eligible molecules. In the functional analysis, the biological functions that were most significant to the data sets were identified. Right-tailed Fisher's exact test was used to calculate a *P* value determining the probability that each biological function assigned to that data set was due to chance alone.

We identified the canonical pathways that were most significant to the data set from the IPA library. The significance of the association between the data set and the canonical pathway was measured in two ways: i) by displaying a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway. ii) Fisher's exact test was used to calculate a *P* value determining the probability that the association between the genes in the data set and the canonical pathway could be explained by chance alone.

After function and canonical pathways analyses of all four data sets (SVD-NiL, IoL-NiL, SLEC-NiL and ILEC-NiL), we compared the pathway results in order to check any differences on the signalling process among the different modes of delivery.

Western blotting

Protein concentration was determined by the BCA assay. Plasma was diluted to give 50 µg protein in 30 µl 1 × Laemmli buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 2.5% (v/v) β-mercaptoethanol, pH 6.8), heated to 100 °C for 5 min and proteins separated on 12% acrylamide Tris-glycine SDS-PAGE gels. Proteins were transferred to PVDF membranes by semi-dry blotting. Blocking, washing and antibody dilution buffers were from the WesternBreeze kit (Invitrogen). Membranes were blocked and then exposed to primary antibody overnight at 4 °C. After washing, membranes were incubated with secondary antibodies for 2 h at room temperature, then washed and covered for 5 min with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

Excess substrate was removed with blotting paper; membranes were enclosed in SaranWrap and exposed to Hyperfilm ECL (GE Healthcare) for between 1 s and 10 min before developing. Developed films were scanned on an Epson Perfection 3490 PHOTO flat-bed scanner and protein levels estimated by densitometry using ImageJ v1.37 software (NIH).

Primary antibodies and dilutions used were APOA4 (1:20 000, mouse monoclonal ab81616 (Abcam, Cambridge, UK)), APOE (1:10 000, mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA)) and IgG γ chain (1:5000, rabbit polyclonal ORCM 15 (Behring Diagnostics, Marburg, Germany)). Secondary (HRP conjugated) antibodies were goat anti-mouse (1:10 000, sc-2055 (Santa Cruz)) and goat anti-rabbit (1:5000, sc-2054 (Santa Cruz)).

Each plasma protein sample was run in duplicate, APO and IgG (as a loading control) proteins were quantitated and values for different blots were normalized to each other using the values of three samples that were loaded on each gel run. For individual lanes, APO/IgG ratios were calculated, then average ratios for each sample were determined and plotted. For intervillous plasma, SVD and NiL groups were compared using two-tailed, unpaired *t*-tests. For maternal plasma, pre- and in-labour groups were compared using two-tailed, paired *t*-tests.

RIA

In order to obtain more quantitative measurements, selected proteins (AFP, A1BG, APOE, LPOA, APOA1, APOB and hCG) were measured in maternal plasma by established RIA methods using specific antibodies (Doctors Laboratory, London <http://www.tdlpathology.com/>).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0114>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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