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Human maternal plasma proteomic changes with parturition

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ABSTRACT

The powerful proteomic technique of Tandem Mass Tag labelling with Orbitrap mass spectrometry was applied to the quantification of relative levels of proteins in serial plasma samples from 15 women prior to and during labour. Quantitative data were obtained for 1038 proteins, with 217 quantified in ≥10 sample pairs. Most proteins were unaffected by labour, 32 had lower levels in labour, and two were increased in labour, and the *t* test had sufficient power to determine a significant difference in expression in 14 proteins. ELISA confirmed the significant increase of acute phase response components C-reactive protein and serum amyloid A.

Significance: Proteomic technology is constantly advancing, and the latest techniques enable gel-free analysis of minimally preprocessed, complex biological samples, enabling simultaneous identification and quantification of many hundreds of proteins. The technique of TMT labelling and Orbitrap mass spectrometry is applicable to the analysis of serial maternal plasma samples in order to identify potential markers of the onset of labour.

1. Introduction

Human pregnancy has a nominal duration of 40 weeks, at the end of which a coordinated process of uterine connective tissue remodelling and smooth muscle activation should result in labour and the vaginal delivery of the fetus. Spontaneous labour occurring prior to 37 weeks of pregnancy (preterm), when fetal development is incomplete, is associated with perinatal mortality and morbidity, while continuation of pregnancy post-term is associated with risks and maternal requests at 42 weeks to artificially induce labour. Even when labour is underway, problems with fetal positioning, maternal blood loss, fetal distress and failure of the labour to progress can require delivery by emergency Caesarean section.

It is presumed that specific biomarkers will be associated with changes in each stage of pregnancy, from conception through growth of the fetus and uterus to uterine activation and labour. Unfortunately, only limited progress has been made in identifying such markers in amniotic fluid (AF), cervico-vaginal fluid (CVF), and maternal serum and

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plasma. Although factors such as chorionic gonadotropin and corticotropin-releasing hormone are known to increase in maternal circulation in pregnancy, there are currently no reliable biochemical measures for the onset of preterm labour in humans [1], with prediction largely relying on known risk factors (previous preterm delivery, smoking, stress, low BMI) and the observation of early symptoms of the onset of labour. Prediction may be improved by combining biochemical and clinical indicators such as cervical length, fetal fibronectin levels in CVF and maternal serum levels of α-fetoprotein, granulocyte-colony stimulating factor and defensins or alkaline phosphatase [2]. Inflammation associated with bacterial infection may be linked to the onset of labour in a subset of women, but this association is confounded by the occurrence of infection with no associated inflammation [3], and of inflammation in the absence of infection [4], and by the limited effectiveness of antibiotics and anti-inflammatory medication in the prevention of labour.

Historically, the search for biomarkers has focused on investigations of one or more targets selected on the basis of known physiological roles, molecular interactions or direct observations of specific localisation and/or expression changes. The development of powerful "omics" technologies is now fundamentally changing the nature of these investigations. Following the elaboration of a research hypothesis and selection of appropriate test samples, it is possible to screen for the presence and/or alteration of large numbers of biomolecules such as mRNAs, proteins or metabolites. In proteomics, liquid chromatography with tandem mass spectrometry (LC–MS/MS) has enabled identification of proteins in unlabelled samples, while Difference Gel Electrophoresis (DiGE) enabled protein identification and relative quantification in two samples by differential fluorescent-tag labelling, separation by two-dimensional gel electrophoresis, visualisation, spot-picking and MS. These approaches have led to the identification of proteins differentially expressed in AF, CVF and maternal serum [5–8].

Labelling of samples with isobaric (equal mass) tags, first demonstrated in 2003 [9], ensures that differentially labelled peptides comigrate in all processing and chromatographic separations. Quantification is achieved through collision-induced-dissociation in MS/MS, which releases the mass reporter regions of the mass tags. Each mass tag has a unique, identifiable isotopic label in the mass reporter region. Both the 'Isobaric Tag for Relative and Absolute Quantitation' (iTRAQ) and 'Tandem Mass Tag' (TMT) isobaric mass tagging approaches offer quantification without the need for gel electrophoresis. Instead, two or more protein samples are digested to peptides, differentially labelled and directly analysed by MS. These techniques enable identification and quantification of hundreds of proteins (from counts of thousands of peptides) per sample.

Maternal peripheral blood plasma is a readily accessible source of biomarkers that could potentially be used to determine the proximity of the onset of labour, susceptibility to induction of labour and the presence of pathological complications of pregnancy. The purpose of this research was to establish a proof of concept that the TMT proteomics method is capable of detecting changes in maternal plasma proteins during human labour. We have applied the relative quantification proteomic methodology of TMT MS/MS to comparison of serial samples of maternal plasma taken prior to and during active labour, to explore changes in the plasma proteome associated with the process of labour. We identified a number of differentially regulated proteins, and used absolute quantification by ELISA on an independent set of samples to attempt to validate the MS quantification of three selected proteins, and to compare the expression in similar serial plasma samples from women delivering in the absence of labour. As expected, most maternal proteins remained stable, but the TMT MS methods detected changes with labour in more than 30 proteins, although the study only had sufficient power to determine statistical significance in differential expression of 14 proteins, owing to the limited number of samples used for MS analysis. Strong correlation between the MS and ELISA results for two of the proteins suggests that TMT MS can be used to accurately determine changes in expression of potential biomarkers. However, the poor correlation seen with the third protein highlights the need for independent validation of biomarkers identified by MS.

2. Materials and methods

2.1. *Plasma samples*

This study had the approval of the National Research Ethics Service (NRES Committee South West – Central Bristol) and all women gave informed written consent. Venous blood was taken from 15 women who went into spontaneous labour (SL) and had vaginal deliveries, 13 women whose labour was induced (IOL; 10 with vaginal deliveries, three with emergency Caesarean sections) and 13 women who did not go into labour prior to delivery (NL; eight with elective C-sections, two with emergency C-sections). Medians of clinical attributes in these groups were: maternal age, SL 31, IOL 32, NL 32; gestational age, SL 41, IOL 41, NL 38; parity, SL 1.0, IOL 0.0, NL 1.5; birth weight (kg), SL 3.7, IOL 3.7, NL 3.5; male to female offspring; SL 5:10, IOL 6:7, NL 5:5; duration of labour (h:mm), SL 5:00, IOL 5:23. Two blood samples were obtained from each woman; the first at the initial clinical assessment, when there were no signs of labour and the second during established labour or at C-section. Proteomic analysis was conducted on a subset of 11 SL and four IOL sample pairs, with clinical details given in Table 1. All women delivered in St Michael's Hospital, Bristol, UK, and were of good general health with singleton pregnancies without complications such as antepartum haemorrhage, hypertension, pre-eclampsia or diabetes.

Blood was aspirated into K_2EDTA vacutainers (Becton Dickinson, Oxford, UK) and transferred into tubes containing protease inhibitors (leupeptin hydrochloride (1 μg/ml), aprotinin (1 μg/ml) and pepstatin A (1 μg/ml); Sigma–Aldrich, Gillingham, UK) and mixed well. Plasma was separated from cells by centrifugation at 2000 × *g* for 10 min at room temperature. Clear plasma was then spun at 12,000 × *g* for 5 min. Supernatant was divided into aliquots and stored at −80 °C.

Table 1 – Data for 15 women giving plasma samples for analysis of protein biomarkers.

Maternal age (years)	Parity	G. age (weeks + days)	Mode of onset	Delivery	Birth-weight (g)	Neonatal gender	Sampling interval[a] (hh:mm)	Duration of labour (hh:mm)	In-labour sample timing[b] (hh:mm)	Number of proteins quantified
46	1	41+4	Spont	SVD	3058	Female	44:55	2:08	0:15	293
20	1	41+3	Spont	SVD	3280	Female	13:40	2:49	1:50	283
37	0	41+5	Spont	SVD	4035	Female	49:49	5:01	3:59	337
25	0	41+5	Spont	For	4115	Female	29:04	16:44	0:20	362
27	1	41+3	Spont	SVD	3620	Female	12:34	2:55	0:20	304
32	0	42+3	Spont	For	4180	Female	106:05	6:05	0:40	383
35	1	41+6	Spont	SVD	3800	Male	75:45	1:40	0:45	316
31	0	42+0	Spont	SVD	3700	Female	79:44	12:15	2:00	339
23	1	39+6	Spont	SVD	4090	Male	144:34	1:54	1:40	331
22	1	41+4	Spont	SVD	3470	Male	9:05	8:06	1:19	305
35	1	41+5	Spont	SVD	3060	Male	49:09	4:47	0:59	299
38	2	41+5	Ind	SVD	3640	Female	38:49	4:05	6:10	338
39	0	42+0	Ind	For	4355	Male	95:39	5:23	2:04	373[c]
20	0	41+2	Ind	SVD	4110	Male	45:50	3:20	0:40	373[c]
36	1	42+1	Ind	SVD	3730	Female	62:19	6:35	1:59	373[c]

Abbreviations: For, forceps; G. age, gestational age at delivery; Ind, induced; Spont, spontaneous; SVD, spontaneous vaginal delivery.

[a] Two plasma samples were taken (pre-labour and in-labour), separated by the sampling interval.

[b] Time between onset of labour and collection of in-labour sample.

[c] Three sample pairs were labelled using sixplex TMT (the other 12 using duplex TMT).

2.2. *Proteomic analysis*

2.2.1. *TMT labelling and cation exchange chromatography*

Aliquots of 100 μg of two or six plasma samples per experiment were digested with trypsin and labelled with Tandem Mass Tag (TMT) duplex or sixplex reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, UK). Sixplex labelling produced comparable results to duplex labelling, with reduced expense owing to analysis of three sample pairs per MS run. In brief, 100 μg of each sample was resuspended in 50 mM TEAB containing 10 mM TCEP and incubated at 55 °C for 1 h. The samples were cooled and iodoacetamide was then added to a final concentration of 17 mM and the samples incubated for a further 30 min at room temperature protected from light. The reduced and alkylated proteins were then precipitated overnight using six volumes of pre-chilled acetone (−20 °C) and then spun at 8000 × *g* for 10 min at 4 °C to pellet the precipitated material. Following removal of the acetone, the protein pellets were resuspended in 100 mM TEAB containing 2.5 μg sequencing grade trypsin (Promega) and digested overnight at 37 °C. Each of the six TMT reagents (0.8 mg) were resuspended in anhydrous acetonitrile and added to one of the six plasma samples. The labelling reaction was allowed to proceed for 1 h at room temperature and then quenched by the addition of 0.25% hydroxylamine (final concentration) and incubation for a further 15 min. After labelling, samples were combined in equal amounts, and a 50 μg aliquot of this pooled sample evaporated to dryness and resuspended in buffer A (10 mM KH_2PO_4, 25% MeCN, pH3) prior to fractionation by strong cation exchange using an Ettan LC system (GE Healthcare, Little Chalfont, UK). The sample was loaded onto a polysulphoethyl A column (100 mm long × 2.1 mm internal diameter, 5 μm particle size, 200 Å pore size; PolyLC Inc, Columbia, MD, USA) in buffer A and peptides eluted with an increasing gradient of buffer B (10 mM KH_2PO_4, 25%MeCN, 1 M KCl, pH3) from 0 to 100% over 30 min. Five fractions were collected, evaporated to dryness, resuspended in 5% (v/v) formic acid and desalted using SepPak cartridges according to the manufacturer's instructions (Waters, Milford, MA, USA). Eluate from the SepPak cartridge was evaporated to dryness and resuspended in 1% (v/v) formic acid.

2.2.2. *Nano-LC mass spectrometry*

Cation exchange fractions were further fractionated using a Dionex UltiMate 3000 Nano LC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 Nano-Trap column (Dionex, Thermo Fisher Scientific). After washing with 0.5% (v/v) acetonitrile, 0.1% (v/v) formic acid, peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Dionex) over a 150 min organic gradient, using seven gradient segments of different dilutions of 80% (v/v) acetonitrile, 0.1% (v/v) formic acid (1–6% over 1 min, 6–15% over 58 min, 15–32% over 58 min, 32–40% over 3 min, 40–90% over 1 min, held at 90% for 6 min and then reduced to 1% over 1 min) with a flow rate of 300 nl/min. Peptides were ionised by nano-electrospray ionisation at 2.0 kV using a stainless steel emitter with an internal diameter of 30 μm (Thermo Fisher Scientific) and a capillary temperature of 250 °C. Tandem mass spectra were acquired

using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software and operated in data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at 60,000 resolution (at *m/z* 400) in the mass range *m/z* 300 to 1800 and the top ten multiply charged ions in each duty cycle selected for MS/MS fragmentation using higher-energy collisional dissociation (HCD) with normalised collision energy of 45%, activation time of 0.1 ms and at a resolution of 7500 within the Orbitrap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count 1; repeat duration 30 s; exclusion list size 500) were used.

2.2.3. *Data analysis*

The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Fisher Scientific) and searched against the UniProt/SwissProt Human database release version 57.3 (20,326 entries) using the SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da Search criteria included oxidation of methionine (+15.9949) as a variable modification and carbamidomethylation of cysteine (+57.0214) and the addition of the TMT duplex (+225.156) or sixplex (+229.163) mass tags to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of one missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy a peptide false discovery rate (FDR) of 5%. The Proteome Discoverer software generates a reverse "decoy" database from the same protein database and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identifications. The minimum cross-correlation factor (X_{corr}) filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5% based on the number of random false positive matches from the reverse decoy database. Thus each data set has its own passing parameters. Quantification used a peak integration window tolerance of 0.0075 Da with the integration method set as the most confident centroid. The data analysis parameters ensured that only quantitative data for peptides unambiguously assigned to a single protein were included. The data were not filtered to remove single peptide hits, so proteins were quantified on the basis of one or more unique peptides. For each plasma sample pair, the ratios of in-labour:pre-labour protein levels were calculated from medians of quantified peptide ratios.

The compatibility of peptide labelling using duplex and sixplex TMT was assessed by *t* test comparing ratios of protein levels determined with each type of label, which showed no significant differences between the labels for any protein (data not shown).

2.3. *ELISA*

Three proteins (CRP, SAA and APOA4) that were identified by proteomic analysis as being present at significantly different levels in pre-labour and in-labour samples were further analysed by quantitative ELISA. Total plasma protein concentration was determined for each sample by the BCA assay (Thermo Fisher Scientific), and was adjusted to 10 mg/ml. Further 1:40 sample dilutions were required to bring the samples within the linear ranges of the assays. ELISAs were performed according to manufacturers' instructions. Plasma samples were diluted as described above, and protein standards of known concentration were diluted serially seven times. Diluted samples, standards and protein-free blanks were pipetted into a 96-well plate pre-coated with capture antibody and incubated at room temperature to allow formation of antibody–antigen complexes. Unbound antigen was removed with five plate washes and then HRP-conjugated detection antibody was added and incubated at room temperature to allow formation of antibody–antigen–antibody complexes. Excess detection antibody was washed off, chromogenic TMB substrate was added and after 15 min colour development was stopped by addition of H_2SO_4. Absorbance at 450 nm was measured, and antigen concentration in each well determined by comparison of absorbance with the standard curve of known dilutions, correcting for the initial sample dilution. ELISA tests used were CRP (E-80CRP, ICL Inc., Portland, OR, USA), SAA (KHA0011, Life Technologies) and APOA4 (E91967Hu-USC, USCN Life Science, Houston, TX, USA).

3. Results

3.1. *Changes in the plasma proteome with labour*

Proteomic analysis was used to determine the ratios of in-labour:pre-labour protein levels in 15 women, with the number of proteins quantified in each sample pair shown in Table 1. This analysis provided quantitative data on a total of 1038 proteins, with 421 only counted in a single pair, 400 counted in two to nine of the 15 pairs, 66 counted in 10 to 14 pairs, and 151 quantified in all 15 sample pairs. For those 217 proteins quantified in 10 to 15 of the pairs, the individual in-labour:pre-labour ratios were $\log_2$-transformed prior to statistical analysis of the difference of the means by one-sample *t* tests. In these tests, the expected mean value was zero, corresponding to a null hypothesis that there was no difference between the pre-labour and in-labour ratios. A volcano plot (Fig. 1) shows the relationship between the means of the log-transformed ratios for each protein and the negative transformation of the $\log_{10}$-transformed *t* test *p*-values (so that lower *p*-values give a higher value on the Y-axis). This plot shows that there was a significant probability, according to the *t* test analysis, that 32 proteins were present at lower levels in labour and two were increased (Table 2), while 185 proteins were not significantly different in the pre-labour and in-labour samples (Table 3). Post hoc power analysis was performed on the results of the proteins that appeared to be differentially expressed. Using the values of sample means, standard deviations, an α (type I error rate) of 0.05, and the numbers of samples reporting a proteomic ratio for each protein, the power for a two-sided test was calculated with the online calculator at http://www.stat.ubc.ca/~rollin/stats/ssize/n1.html. Power is the probability of a test detecting a difference when one exists: it is determined using the type II error rate, and a power of $\geq$0.8 is a common target for statistical tests, indicating that

Table 2 – Proteins whose levels are changed in MS analysis of serial in-labour plasma relative to pre-labour plasma.

Uniprot	HUGO	Mean	S.D.	*p*	Power	v.m.d.	Protein name
P06727	APOA4	−0.34	0.25	0.00	1.00	0.25	Apolipoprotein A-IV
P02753	RBP4	−0.22	0.20	0.00	0.99	0.15	Retinol-binding protein 4
O75636	FCN3	−0.22	0.21	0.00	0.99	0.16	Ficolin-3
Q6PCB0	VWA1	−0.17	0.17	0.00	0.96	0.14	von Willebrand factor A domain-containing protein 1
O00391	QSOX1	−0.20	0.20	0.00	0.96	0.16	Sulfhydryl oxidase 1
P02749	APOH	−0.11	0.13	0.01	0.90	0.10	Beta-2-glycoprotein 1
P02741	**CRP**	**0.93**	**1.05**	**0.01**	0.89	0.85	**C-reactive protein**
P02765	AHSG	−0.13	0.16	0.01	0.86	0.12	Alpha-2-HS-glycoprotein
P04070	PROC	−0.16	0.20	0.01	0.86	0.15	Vitamin K-dependent protein C
P01243	CSH1	−0.16	0.17	0.01	0.88	0.15	Chorionic somatomammotropin hormone
P51884	LUM	−0.12	0.16	0.01	0.82	0.12	Lumican
P17936	IGFBP3	−0.15	0.21	0.01	0.82	0.16	Insulin-like growth factor-binding protein 3
P02735	**SAA2**	**1.11**	**1.52**	**0.01**	0.80	1.10	**Serum amyloid A protein**
Q8N5G2	TMEM57	−0.15	0.16	0.02	0.85	0.15	Macoilin
P02656	APOC3	−0.20	0.28	0.02	0.77	0.21	Apolipoprotein C-III
O43184	ADAM12	−0.11	0.14	0.02	0.78	0.11	Disintegrin, metalloproteinase domain-containing protein 12
Q01459	CTBS	−0.16	0.22	0.02	0.74	0.18	Di-N-acetylchitobiase
Q15582	TGFBI	−0.14	0.17	0.03	0.75	0.16	Transforming growth factor-beta-induced protein ig-h3
P10909	CLU	−0.12	0.20	0.03	0.69	0.15	Clusterin
P00740	F9	−0.09	0.13	0.03	0.68	0.10	Coagulation factor IX
P01019	AGT	−0.11	0.18	0.03	0.65	0.14	Angiotensinogen
P19823	ITIH2	−0.12	0.19	0.03	0.66	0.14	Inter-alpha-trypsin inhibitor heavy chain H2
P23142	FBLN1	−0.11	0.18	0.03	0.65	0.14	Fibulin-1
P29622	SERPINA4	−0.17	0.28	0.03	0.65	0.21	Kallistatin
P07477	PRSS1	−0.12	0.16	0.03	0.69	0.14	Trypsin-1
Q96PD5	PGLYRP2	−0.17	0.29	0.04	0.64	0.21	N-acetylmuramoyl-L-alanine amidase
Q96IY4	CPB2	−0.10	0.17	0.04	0.63	0.13	Carboxypeptidase B2
P00747	PLG	−0.12	0.21	0.04	0.63	0.16	Plasminogen
P49908	SEPP1	−0.17	0.26	0.04	0.63	0.21	Selenoprotein P
Q9UIQ6	LNPEP	−0.11	0.15	0.04	0.64	0.13	Leucyl-cystinyl aminopeptidase
Q9UGM5	FETUB	−0.20	0.35	0.05	0.59	0.26	Fetuin-B
Q9UHG3	PCYOX1	−0.19	0.27	0.05	0.61	0.24	Prenylcysteine oxidase 1
Q5SYB0	FRMPD1	−0.11	0.19	0.05	0.59	0.14	FERM and PDZ domain-containing protein 1
Q92954	PRG4	−0.10	0.19	0.05	0.58	0.14	Proteoglycan 4

Statistical significance was determined by *t* test. The power of the *t* test to determine significance, and the mean difference that can confidently lead to a declaration of significance, were calculated for each protein. The *t* test was only sufficiently powered to determine significance for the top 14 proteins in the table. *Abbreviations*: HUGO, approved symbol from the HUGO Gene Nomenclature Committee database; Mean, mean of $\log_2$[in-labour/pre-labour] ratios (positive values (**bold**) indicate increase in-labour, negative values indicate decrease with labour); MS, mass spectrometry; *p*, *p* values of one-sample *t* tests of log2[in-labour/pre-labour] ratios, test value = 0; S.D., standard deviation of $\log_2$[in-labour/pre-labour] ratios; Uniprot, Uniprot database accession number; v.m.d., valid mean difference, calculated with a power of 0.80.

a test has an 80% probability of detecting a difference when one exists. A related measure is the valid mean difference, and it was calculated using the numbers of samples reporting a proteomic ratio, the standard deviation and the test mean (0), with an α of 0.05 and power of 0.80, with the online calculator at http://www.biomath.info/power/ttest1gp.htm. The valid mean difference is the minimum difference from the test mean that can be confidently reported as significant, for a given power. Both of these test statistics are reported in Table 3.

3.1.1. *Quantification of selected proteins by ELISA*

The TMT proteomic analysis provides relative quantification of levels of a large number of proteins in samples compared directly with each other. ELISA was used to investigate the relationship between this relative quantification and the absolute protein concentrations. The three proteins chosen for ELISA analysis were C-reactive protein (CRP) and serum amyloid A (SAA), which were the proteins with the highest fold-change in expression according to the MS analysis, and apolipoprotein A4 (APOA4), the protein associated with the lowest *p*-value for comparison of pre-labour and in-labour expression, which we had previously identified as being differentially expressed in in-labour compared with not-in-labour plasma samples [10]. We measured the levels of these three proteins by ELISA in the 15 plasma sample pairs included in the MS analysis. The Pearson product-moment correlation coefficients (*r*) and associated *p*-values for the correlation between the ratios of pre-labour and in-labour samples by MS and ELISA were: CRP, $r = 0.99$, $p < 0.001$; SAA, $r = 0.636$, $p = 0.008$; APOA4, $r = 0.134$, $p = 0.620$, suggesting good correlation between the methods for CRP and SAA, but poor correlation for APOA4. Coefficients of variance (standard deviation/mean) for each method were calculated using the nontransformed ratios and were: CRP, MS = 1.06, ELISA = 1.03; SAA, MS = 1.36, ELISA = 1.92; APOA4, MS = 0.19, ELISA = 0.09.

We also measured the expression of these proteins in further paired, serial plasma samples from four spontaneous (SL) and nine induced (IOL) deliveries, and in serial samples from 13 deliveries by caesarean section in the absence of

Table 3 – Plasma proteins not significantly affected by labour.

Uniprot	Mean	S.D.	Uniprot	Mean	S.D.	Uniprot	Mean	S.D.	Uniprot	Mean	S.D.
A6NJ16	−0.079	0.216	P01833	−0.068	0.171	P04278	−0.148	0.361	P35858	−0.094	0.217
O14791	−0.049	0.200	P01834	0.001	0.282	P04433	−0.026	0.172	P35908	−0.373	1.349
O43423	0.134	0.547	P01842	−0.044	0.178	P05090	−0.109	0.324	P36955	−0.087	0.183
O43866	−0.027	0.260	P01857	0.004	0.264	P05155	0.051	0.282	P36980	0.019	0.227
O75443	−0.072	0.234	P01859	−0.025	0.202	P05156	−0.064	0.231	P43251	−0.026	0.413
O75533	−0.030	0.227	P01860	−0.086	0.242	P05160	−0.073	0.213	P43652	−0.071	0.200
O75882	−0.062	0.236	P01861	−0.146	0.557	P05452	−0.106	0.216	P48730	−0.001	0.253
O95445	−0.099	0.265	P01871	−0.003	0.235	P05543	−0.004	0.178	P48740	−0.141	0.245
P00450	−0.047	0.243	P01876	−0.093	0.270	P05546	−0.076	0.178	P51811	−0.029	0.225
P00734	−0.046	0.185	P01877	−0.001	0.223	P06310	0.271	0.902	P52848	−0.156	0.378
P00736	−0.047	0.189	P01880	0.046	0.426	P06396	−0.088	0.218	P54108	−0.091	0.228
P00738	0.075	0.302	P02042	0.071	0.278	P06681	−0.090	0.287	P55056	−0.038	0.292
P00739	−0.018	0.199	P02647	−0.095	0.206	P06702	−0.103	1.961	P55058	−0.051	0.274
P00740	−0.085	0.135	P02649	−0.154	0.318	P07225	−0.062	0.191	P59665	0.044	0.657
P00742	−0.084	0.210	P02652	−0.136	0.306	P07357	−0.034	0.234	P60709	−0.200	0.748
P00748	−0.129	0.243	P02654	−0.063	0.310	P07358	−0.053	0.174	P61626	0.092	0.311
P00751	0.007	0.228	P02655	−0.110	0.204	P07360	−0.027	0.177	P68871	0.111	0.315
P00915	0.072	0.320	P02671	−0.058	0.244	P08185	−0.153	0.282	P69905	0.103	0.339
P01008	−0.067	0.196	P02675	−0.052	0.241	P08519	−0.267	0.832	P78563	0.010	0.196
P01009	−0.025	0.217	P02679	−0.049	0.247	P08571	0.051	0.366	P80108	−0.080	0.167
P01011	0.081	0.258	P02743	−0.044	0.213	P08603	−0.056	0.200	P80748	−0.080	0.188
P01019	−0.112	0.181	P02745	−0.056	0.224	P08697	−0.035	0.308	Q00887	−0.234	0.418
P01023	−0.038	0.234	P02746	−0.055	0.198	P09871	−0.071	0.190	Q00888	−0.028	0.318
P01024	−0.051	0.194	P02747	−0.045	0.173	P0C0L4	0.086	0.274	Q03591	0.013	0.392
P01031	−0.014	0.231	P02748	0.048	0.226	P10643	−0.080	0.240	Q06033	−0.040	0.214
P01034	−0.014	0.183	P02750	0.137	0.285	P12259	−0.059	0.178	Q08380	−0.027	0.273
P01042	−0.091	0.203	P02751	−0.265	0.573	P13645	−0.149	1.222	Q13046	−0.165	1.185
P01591	−0.071	0.153	P02760	−0.077	0.234	P13671	−0.061	0.220	Q13219	−0.116	0.415
P01598	−0.016	0.184	P02763	0.050	0.255	P13727	−0.106	0.494	Q14520	−0.047	0.312
P01608	−0.032	0.244	P02766	−0.026	0.271	P14151	−0.020	0.189	Q14624	−0.038	0.177
P01609	−0.068	0.270	P02768	−0.044	0.208	P15169	−0.056	0.207	Q14964	−0.077	0.158
P01611	−0.054	0.217	P02774	−0.094	0.241	P18428	0.283	0.558	Q16610	−0.043	0.290
P01613	0.039	0.196	P02775	0.169	0.821	P19652	−0.012	0.216	Q4LDE5	−0.114	0.173
P01625	0.016	0.233	P02787	−0.060	0.205	P19827	−0.074	0.198	Q58FF6	0.129	0.371
P01700	−0.052	0.173	P02790	−0.051	0.192	P20742	−0.250	0.893	Q5T619	0.142	0.325
P01702	−0.053	0.465	P03952	−0.085	0.203	P20851	−0.040	0.175	Q6NUM9	−0.104	0.196
P01714	−0.041	0.256	P04003	−0.049	0.275	P22352	−0.082	0.262	Q7RTZ1	−0.010	0.116
P01715	−0.015	0.181	P04004	−0.062	0.206	P22792	−0.034	0.223	Q8TAQ9	−0.056	0.283
P01717	−0.088	0.197	P04114	−0.096	0.207	P22891	−0.053	0.222	Q96KN2	−0.108	0.258
P01743	0.008	0.361	P04180	−0.045	0.229	P23083	−0.080	0.310	Q9NZP8	−0.060	0.188
P01764	−0.058	0.160	P04196	−0.094	0.173	P25311	−0.084	0.351	Q9UK55	0.010	0.285
P01766	0.013	0.186	P04208	−0.042	0.183	P27169	−0.060	0.219	Q9UKX3	−0.024	0.204
P01767	−0.044	0.324	P04211	−0.082	0.217	P27918	−0.038	0.162	Q9UNH7	−0.095	0.257
P01773	−0.122	0.362	P04217	−0.057	0.217	P32119	0.112	0.277	Q9Y6R7	−0.157	0.299
P01779	−0.035	0.209	P04220	−0.038	0.246	P33151	−0.136	0.231			
P01780	−0.032	0.255	P04264	−0.282	1.019	P35527	−0.562	1.212			
P01781	−0.058	0.266	P04275	0.027	0.547	P35542	0.076	0.299			

Abbreviations: Mean, mean of $\log_2$[in-labour/pre-labour] ratios; S.D., standard deviation of $\log_2$[in-labour/pre-labour] ratios; Uniprot, Uniprot database accession number.

labour (NL). Fig. 2A shows the spread of the log-transformed ratios of protein concentrations in the combined in-labour (IL = SL + IOL) groups, compared with NL. Fig. 2B shows the relationships of the samples in each of the serial pairs.

To compare the levels of protein in the paired serial samples determined by ELISA, differences between the paired samples were assessed using the nonparametric Wilcoxon signed-rank test, with a null hypothesis that the medians of the groups of paired samples were identical. For the IL samples measured by both MS and ELISA, results of the Wilcoxon test were: CRP, $z = -2.783$, $p = 0.005$; SAA, $z = -3.294$, $p = 0.001$; APOA4, $z = -1.136$, $p = 0.254$. For the IL samples measured only by ELISA, the results were: CRP, $z = -1.782$, $p = 0.075$; SAA, $z = -2.271$, $p = 0.023$; APOA4, $z = -0.078$, $p = 0.936$. For the NL samples, the results were: CRP, $z = -0.734$, $p = 0.465$; SAA, $z = -0.357$, $p = 0.719$; APOA4, $z = -1.070$, $p = 0.285$. These results demonstrate higher levels of expression in labour for CRP and SAA. The changes in these proteins were similar in the SL and IOL groups.

4. Discussion

To the best of our knowledge, this study is the first to use TMT labelling and Orbitrap Velos mass spectrometry to

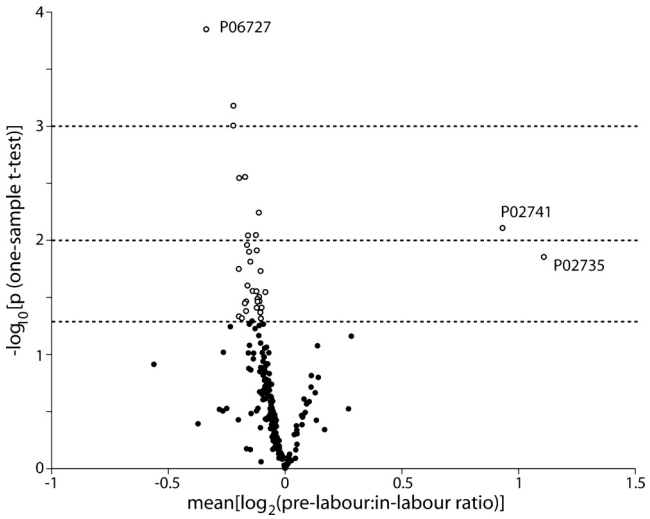

Fig. 1 – Volcano plot comparing protein levels in paired pre-labour and in-labour samples. The X-axis displays a measure of fold-change of expression, with higher levels in-labour shown to the right, and higher levels pre-labour to the left of zero. The Y-axis indicates the probability that the observed changes in protein level did not occur by chance alone; statistically significant changes according to t test are shown with open circles, and three open circles corresponding to proteins measured by ELISA are labelled.

describe proteomic changes in maternal plasma associated with human parturition. The results demonstrate the value of this approach to identify proteins relevant to spontaneous and induced labour. This technique enabled measurement of a broad spectrum of plasma proteins in serial samples from the same women before and during labour, without the additional sample-processing step of depletion of high-abundance proteins. We have previously demonstrated changes in maternal plasma proteins with labour in a cross-sectional study [10]: however, the results were limited to low-abundance proteins (after removal of the 20 most abundant proteins such as albumin, IgG, and transferrin, by immunodepletion) and to proteins that could be visualised after 2D-DIGE. Although immunodepletion is a useful step in MS workflows for enriching low-abundance proteins, it results in an artificial modification of the protein profile. Low-abundance proteins, such as apolipoproteins, can bind to high-abundance albumin and so can be affected by depletion. We therefore exploited the ability of the Orbitrap TMT MS approach to quantify low-abundance proteins without immunodepletion to obtain an unmodified profile of the plasma proteome. This is an important advantage, although it is possible that some low-abundance proteins were not identified by our approach.

It is notoriously difficult to predict the onset of labour, so we focused our study on women attending the post-term, pre-induction Day Assessment Unit at St Michael's Hospital, who we were able to monitor relatively closely. This study presents a quantitative record of the maternal plasma proteome in late pregnancy. These proteins are associated with many different physiological pathways, such as plasminogen activation, blood coagulation, integrin pathway signalling and the renin–angiotensin system. A similar range of maternal plasma proteins has been described in proteomic studies of potential biomarkers for pre-eclampsia [11]. This functional complexity is to be expected [12], given the essential homeostatic role of plasma, providing metabolic, hormonal and regulatory links to most maternal organs and to the placenta.

The proteins that we have identified as being differentially expressed in-labour compared with pre-labour have multiple functions. For instance, apolipoprotein A-IV is a part of the protein–lipid transport system and is a major component of high-density lipoproteins (HDL) and chylomicrons. The levels of APOA4 decrease during the last weeks of pregnancy and during labour [10]. Retinol-binding protein (RBP4) transports retinol (vitamin A) in plasma, and is involved in regulating insulin and glucose homeostasis in pregnancy. Women with gestational diabetes tend to have low levels of RBP4 in the circulation, but previous studies have found no changes in RBP4 with parturition [13]. Ficolin-3 is part of a family of pattern recognition molecules of the innate immune system. Decreased levels of ficolin-2 and ficolin-3, resulting in impaired removal of trophoblast material, have been proposed as a factor in the pathogenesis of pre-eclampsia [14]. APOH might have a role in the pathogenesis of antiphospholipid syndrome in pregnancy [15].

The patterns of protein changes that we detected support the view that some elements of labour resemble an inflammatory reaction [10,16]. Analysis of changes in acute phase proteins during inflammatory responses showed that CRP (C-reactive protein), SAA (serum amyloid A) and haptoglobins increased, whereas apolipoproteins A-I, A-IV and E, together with transthyretin, several macroglobulins and glycoproteins, decreased [17]. A decrease in APOA1 with labour has been described [18]. CRP and SAA are classical acute-phase proteins whose plasma concentrations increase in response to diverse stimuli, including trauma, infection and hypoxia [19]. A major function of CRP is to bind polysaccharides in pathogens and phospholipids in damaged cells, thus contributing to innate immunity. CRP can activate the complement cascade and also promote phagocytosis and the release of proinflammatory cytokines by monocytes. An association between maternal stress and preterm birth has been described, and recent evidence shows that antenatal stress and preterm labour are associated with increased maternal levels of interleukin-6, TNF-α and CRP [20]. The changes in acute phase proteins after inflammatory stimuli are evident within a few hours but can be maintained over a period of several days or weeks [19]. A study in an African population showed that maternal CRP levels peak a few days postpartum [21]. CRP is elevated in pregnancy complications such as pre-eclampsia that may have an immunological, inflammatory pathogenesis [22]. SAA2 is an apolipoprotein of the HDL complex and may influence cholesterol metabolism during inflammation. Little is known about maternal SAA levels in women around parturition, although SAA levels are known to increase in the newborn in response to ischaemia and have been used to predict the severity of hypoxic encephalopathy in affected children [23].

We have detected proteins not previously associated with labour, but reported as potential biomarkers for pregnancy complications. AHSG, also known as fetuin-A, might be involved in the regulation of trophoblast invasion in early

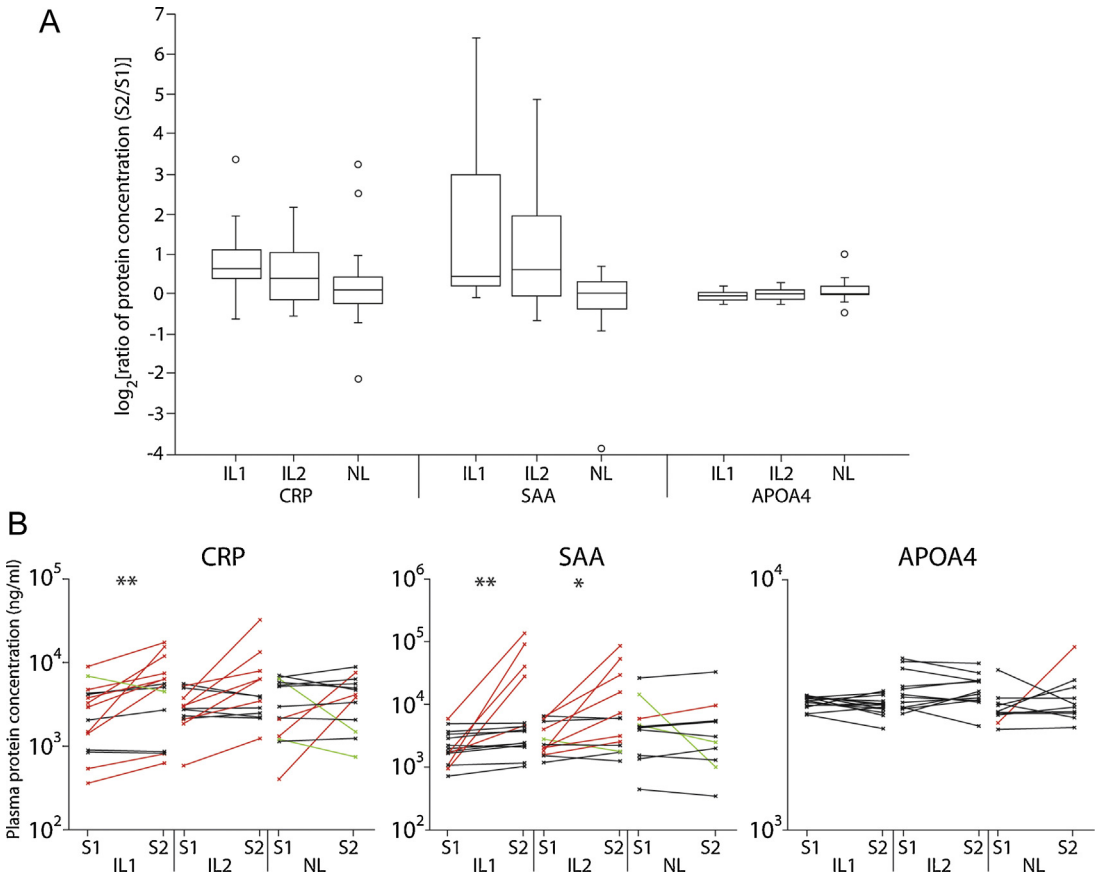

Fig. 2 – Measurement by ELISA of levels of selected proteins (CRP, SAA, APOA4) in serial plasma samples (S1 and S2) from women delivering in labour (IL). IL1, sample pairs from 11 spontaneous (SL) and four induced (IOL) deliveries, also measured by mass spectrometry. IL2, sample pairs from four SL and nine IOL deliveries. NL, sample pairs from 13 deliveries in the absence of labour (NL). (A) Box and whisker plot shows quartiles of log_2-transformed ratios of levels, with outliers indicated by open circles. Positive values indicate an increase in protein level with labour and/or advancing gestation. (B) Concentrations of proteins in individual serial plasma sample pairs. Substantially decreasing levels (S1 > 1.5*S2) are indicated with green colouring, increasing levels (S2 > 1.5*S1) with red. Medians of S1 and S2 were compared by Wilcoxon signed-rank test, identifying significant differences for CRP in IL1 ($p = 0.005$), and SAA in IL1 ($p = 0.001$) and IL2 ($p = 0.023$), as indicated by asterisks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

pregnancy, and there is controversy as to whether AHSG levels are elevated [24] or decreased [25] in women who develop pre-eclampsia. We also detected a significant decrease in fetuin-B in women in labour. This protein is a protease inhibitor that facilitates sperm penetration through the zona pellucida of the oocyte, but its potential role in parturition is not known.

Some proteins related to the blood coagulation cascade were significantly decreased in labour, including PROC, F9 and VWA1. The latter interacts with heparan sulphate proteoglycans that are essential components of basal membranes and play vital roles in vascularisation. It remains to be investigated whether these changes are related to parturition or to haemostatic changes to help prevent excessive postpartum bleeding.

Several of the proteins detected have enzyme activities related to oxidation and reduction reactions. For example, QSOX1 catalyzes the oxidation of sulphydryl groups in proteins and PCYOX1 cleaves thioether bonds in prenylated proteins. SERPINA4 (kallistatin) and SEPP1 have antioxidant properties. We observed reduced levels in labour of components of the renin-angiotensin system such as AGT (angiotensinogen) and LNPEP aminopeptidase, presumably reflecting their involvement in the regulation of maternal blood pressure and electrolyte homeostasis during the process of birth.

A cluster of proteins with growth and metabolic functions includes CSH1 (chorionic somatomammotropic hormone or 'placental lactogen'), which is only produced during pregnancy and is partly responsible for metabolic adaptation to pregnancy, fetal growth and lactation, and IGFBP3, which is involved in insulin-like growth factor (IGF)-dependent and IGF-independent regulation of growth and proliferation [26]. While more evidence is needed to relate these changes to the mechanism of parturition in women, the findings recall the ancient view that fetal demands for energy and growth near term determine the onset of labour [27].

The relative abundance of proteins related to the complement pathway, namely CRP, FCN3, CLU, and FBLN1, and the association between angiotensinogen and complement C3, and between plasminogen and C1 and C5 suggests a role for innate immune defence mechanisms at parturition and early puerperium, a time when the uterine cavity is vulnerable to microbial invasion. Our findings fit with the proposed tendency for classical (C1) complement activation during pregnancy [28]. Notably, some cases of spontaneous preterm labour are associated with activation of the alternative (C3) complement pathway [29]. Possible links between clusterin (CLU) and angiotensinogen (AGT) have been described in women with pre-eclampsia: both CLU and AGT modulate transcriptional activity and function of the labour-promoting NFκB pathway [30,31].

Most of the proteins detected did not show significant concentration changes with labour. The stability of these proteins in the circulation is reassuring and confirms that parturition is a physiological process that involves changes in a few selective pathways [32,33]. However, it should be noted that we do not know, for any of these proteins, what level of change is biologically significant, as opposed to statistically significant. Furthermore, with the limited number of samples used for MS analysis, the *t* test for differential expression was only sufficiently powered to confidently determine significance in the 14 proteins with the lowest *p* values, reflecting smaller standard deviations and/or larger effect sizes. We therefore attempted to validate the MS results using ELISA.

Three proteins were selected for further analysis by ELISA, on the basis of the high fold-change between serial samples (CRP and SAA) or very low *p*-value associated with the change (APOA4), rather than a particular physiological interest compared with other proteins. The increase in acute phase proteins CRP and SAA with labour that was observed by MS was confirmed by ELISA, with strong correlation between the two methods, demonstrating the value of the TMT mass spectrometry to identify selective protein changes in complex plasma mixtures. According to the proteomic analysis, the change in APOA4 with labour was smaller than the changes in CRP and SAA, although highly significant, but ELISA did not detect any alteration in APOA4, and the correlation between MS and ELISA values for APOA4 was poor. This difference might be due to more accurate measurement of small differences in protein levels by one or other of the methods. It is possible that, perhaps due to the presence of post-translational modifications or splice variants, the methods do not measure the same APOA4 moiety. Although the ELISA was validated by its manufacturer and contained purified APOA4 protein for calibration, and stringent criteria were followed for matching MS-quantified peptides to parent proteins, it is evident that more work is required to compare the performance of the two systems, to see if this discrepancy extends to other proteins with a small fold-change in expression. In this instance, our previous observation of similar changes in APOA4 using DiGE increases our confidence in these latest proteomics results, despite the inconsistency with the ELISA.

The ELISA analysis of these three proteins was extended from the 15 IL sample pairs also measured by MS to a further 13 IL and 13 NL sample pairs. Although Fig. 2 shows similarities between the two groups of SL samples, particularly compared with the NL samples, in the additional SL sample group only SAA showed a significant difference between pre-labour and in-labour samples in our analyses. Examination of the ratios of SAA and CRP levels in individual women indicates that the relative increase of these proteins is not uniform (Fig. 2B). Rather, there appear to be women with a substantial increase in these proteins with labour, and others with little or no change, or even a substantial decrease. Analysis of the known characteristics of the women in these groups did not reveal any obvious differences. Moreover, the individuals with substantial upregulation of these two proteins form overlapping, but nonidentical groups, suggesting that SAA and CRP, although both acute phase proteins, might not be acting in the same pathway at the time of labour.

The mechanism for the onset of labour in women remains elusive and we can only speculate as to whether the changes that we detected are causes or consequences of labour. Successful parturition involves connective tissue changes necessary for cervical ripening and dilatation, biochemical and electrophysiological activation of myometrial smooth muscle, metabolic changes in the uterus and placenta to cope with episodes of hypoxia during several hours of phasic contractions, and haemostatic responses as well as tonic contractions to prevent excessive uterine bleeding post-delivery. Other physiological changes occur in preparation for lactation and for the extensive uterine remodelling in the puerperium. Our data show that these complex events are reflected by changes in the relative concentrations of more than 30 proteins in plasma. Most of the proteins decrease with labour, although proteins of the acute phase reaction increase. Until we discover the physiological trigger(s) for parturition in women, it will be difficult to distinguish between primary pathways required to establish labour and secondary pathways that reflect a general or tissue-specific response to the progress of labour. TMT MS proteomics is a valid methodology for identification of such changes, if they are reflected in levels of maternal plasma proteins.

This study provides a basis for further studies measuring maternal plasma protein changes at more frequent intervals during pregnancy and parturition and analysing differences between spontaneous and induced labour at early stages of the labour process.

5. Conclusion

TMT labelling with Orbitrap mass spectrometry is ideally suited to analysis of changes in the maternal plasma proteome at the time of labour. The technique does not require depletion of high-abundance proteins or gel separation of peptides, and it provides quantitative and qualitative information on hundreds of proteins per run. We have used this technique to identify with confidence 14 plasma proteins whose levels change significantly with labour at term. Further research should address whether changes in these proteins occur prior to labour and whether they can be used to predict the onset of term and preterm labour.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.09.001.

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