Proteomic Characterisation of Placenta Tissue for Biomarker Discovery – Workflow Development and Application

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***Abstract*—Preeclampsia (PE) occurs in up to 5% of worldwide pregnancies. It is a complex disease characterised by sudden onset hypertension, and it is harmful to the mother as well as the growing baby, accounting for 40% of fetal deaths worldwide. The disease can be divided into early and late-onset PE, defined as diagnosis earlier or later than 34 weeks of gestation. Despite decades of research, the cause of PE is still poorly defined, but the placenta is central to pathogenesis. Currently, no biomarkers for early detection, progression, and estimation of severity exist, especially for late-onset PE. In this study, two sample preparation strategies were compared in terms of protein extraction and digestion for processing placenta tissue samples. Urea protein extraction followed by in-solution digestion was compared to sodium dodecyl sulfate (SDS) extraction combined with suspension trapping (S-Trap). The Urea protocol showed lower experimental variation and offered a reproducible and high-throughput workflow for proteomic analysis of placenta tissue. In the second part of the study, the importance of meticulous sample collection and storage on relative protein abundances was investigated. Proteins and pathways mainly related to the mitochondria and the ribosome were identified and shown to be strongly affected by long sample processing times. The optimised workflow was successfully applied to a cohort of 19 placenta samples, identifying 6,700–7,000 proteins per sample, which, to the best of our knowledge, is far more than previously published studies. In the last part of the study, analysis of differentially expressed proteins between the sample groups revealed that placentas of women with early-onset PE differ more from late-onset PE and controls.**

I. Introduction

PREECLAMPSIA (PE) is a severe pregnancy complication characterised by high blood pressure and damage to organs such as the liver and kidneys [1]. Alongside its grave prognosis, PE currently lacks biomarkers for early detection and progression. The disease develops after 20 weeks of gestation and can appear in two main forms: early-onset (EPE) and late-onset (LPE). EPE occurs before 34 weeks of gestation. It tends to be more severe and is often associated with complications such as fetal growth restriction, placenta abruption, and premature birth. LPE occurs after 34 weeks of gestation, and while it still poses risks to the mother and child, the complications are usually less severe [2] [3]. Both forms require medical attention and management by healthcare professionals. Treatment may involve close monitoring of blood pressure, blood pressure regulating medication, and preventive measures for seizures. Additionally, it is not uncommon for a necessary implementation of premature delivery of the baby to avoid further complications in the mother [3] [4]. The disease is believed to involve an excessive inflammatory response triggered by the unique conditions of pregnancy. During pregnancy, the placenta plays a crucial role in regulating maternal-fetal interactions. In PE, abnormalities in placenta development and function can lead to the release of factors that stimulate an inflammatory response in the mother [5].

A two-step placenta model can be used to define the development of the disease. It suggests that PE begins with abnormal placenta development in early pregnancy, followed by a second stage characterised by systemic inflammation alongside endothelial dysfunction [6]. In the first stage, abnormal placenta development occurs in early pregnancy, typically during the first trimester. More explicitly, it affects the formation and function of the maternal-fetal interface, including inadequate invasion of trophoblast cells, abnormal spiral artery remodelling, and imbalances in angiogenic factors. As a result, the placenta may become hypoxic and release factors that trigger an inflammatory response in the maternal circulation [6]. In the second stage, abnormal placenta development triggers the maternal inflammatory response, leading to widespread systemic inflammation and endothelial dysfunction. Pro-inflammatory cytokines, damage-associated molecular patterns (DAMPs), and other inflammatory mediators released by the placenta circulate in the maternal bloodstream, causing damage to blood vessels and impairing endothelial function. This endothelial dysfunction contributes to typical features of PE, such as high blood pressure, proteinuria, and organ dysfunction. Additionally, the inflammatory response may trigger activation of the coagulation system, further complicating the condition and increasing the risk of maternal as well as fetal complications [6].

Proteomics has become pivotal in uncovering PE-related disease mechanisms by scrutinising the proteomes from placenta tissues, maternal blood, and urine samples. The focus of future research should, therefore, include extensive patient cohorts where samples have been meticulously collected, stored, and documented, aiming to unveil clinically relevant biomarkers [7] [8] [9].

This study aimed to develop a reproducible and high-throughput sample preparation protocol for the upcoming analysis of a large cohort of placenta tissue samples. The method development included optimising protein extraction

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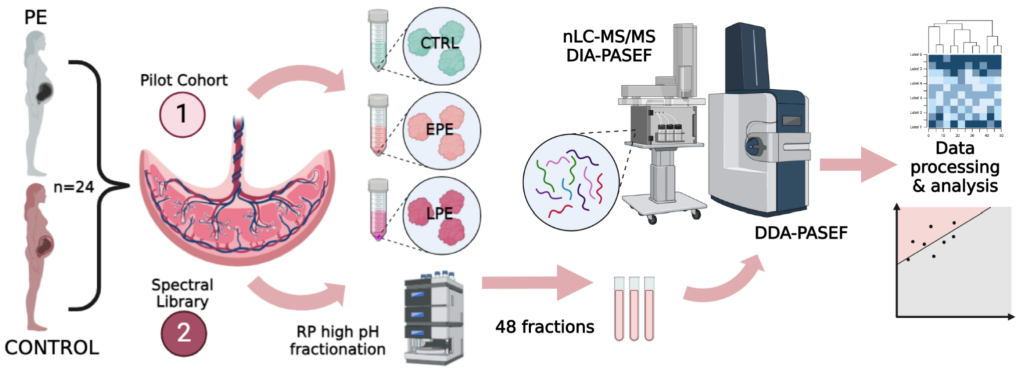

Figure 1. Overview of the workflow process.

from placenta tissue and efficient protein digestion (Figure 1). Subsequently, a thorough investigation of sample integrity was performed, revealing the importance of standardised sample collection in proteomic studies to obtain reliable quantification [10]. Lastly, the study included the analysis of a pilot cohort of placenta samples from women with PE and controls. Notably, the technologies used uncovered novel biological pathways not previously described in mass-spectrometry-based studies, underscoring its utility in unravelling the complexities of PE at the tissue level [11] [12].

II. Method

Table 1. Clinical data of the cohort.

	Control	LPE	EPE
Age (Mean±SD)	29±7.17	32±3.70	32±8.76
BMI (Mean±SD)	22.70±1.30	23.17±3.92	24.25±0.58 25.25*±4.60

*If samples from 2010 were taken into consideration.

A. Ethical Approval and Description of Cohort

The study was approved by the Ethics Committee Review Board for studies on human subjects at Lund University and Skåne University Hospital, Lund, Sweden (Dnr 243/2005, Dnr 2014/191 and Dnr 2016/178). After receiving written informed consent from the patients, placentas from normal and PE pregnancies were collected following both cesarean section and vaginal deliveries at Skåne University Hospital. A summary of the clinical demographics of the patients is listed in Table 1. Two protocols were used when processing the placenta samples; one was standardised, whilst the other was not. The non-standardised protocol encompassed longer processing times and a non-specified collection site. Non-standardised sampling was performed before 2010, while standardised sampling was conducted on samples collected after 2014. The latter were stored at 4°C and processed within 4 h after delivery. One part of the sample was frozen and stored at -80°C until further analysis, where the remnants were preserved using formalin-fixation and paraffin-embedding (FFPE) based on standard protocols. The study cohort consisted of early- and late-onset PE, defined by whether delivery occurred earlier or later than 34 weeks of gestation, respectively, and a control group. The FFPE tissues were sectioned into 4 µm thick sections and stained with hematoxylin and eosin according to a previously described protocol [13].

B. Protein Extraction and Digestion

Pools of frozen placentas from women diagnosed with PE were used for method evaluation. This was followed by the analysis of 24 individual placenta tissue samples. Tissue homogenisation and protein extraction were performed using two lysis buffers: 4 M Urea in 100 mM triethylammonium bicarbonate (TEAB) and 5% sodium dodecyl sulfate (SDS) with 25 mM dithiothreitol (DTT) in 100 mM TEAB. 600 µl of respective lysis buffer was added to each sample, followed by sonication, which sheared DNA and reduced viscosity of the sample, enabling protein extraction, in the Bioruptor Plus (Diagenode) for 20 cycles (30 s on and 30 s off) at 4°C. The lysates were cleared by centrifugation at 14,000 g at 4°C for 10 min. Centrifugation allowed separation of soluble and insoluble components. These steps also enabled protein determination to be performed in the NanoDrop (Implen N120). After which all replicates would be equalised to contain the same amount of proteins for fair comparisons between replicates. For digestion, 50 µg of protein was used, and three experimental replicates were digested using each method.

After the proteins had been successfully extracted, digestion of the urea-containing samples commenced with a reduction using 10 mM DTT followed by a 1 h incubation at 37°C, breaking the disulfide bonds. Alkylation was performed by adding iodoacetamide (IAA) to a final concentration of 20 mM, this was done to impede the reforming of disulfide bonds. Incubation was done at room temperature in the dark for 30 minutes, due to IAA being sensitive to light. To enable the digestion process, which is the cleaving of proteins to peptides, the urea concentration was gradually diluted. The samples were diluted with 50 mM TEAB to a urea concentration of 1 M and digested with endoproteinase Lys-C in a 1:50 w/w ratio (enzyme/protein) followed by incubation at 37°C for 3 h. The samples were diluted with 50 mM TEAB to a final urea concentration of 0.5 M. Subsequently, trypsin was added in a 1:50 w/w (enzyme/protein) ratio and incubated overnight at

37°C. The following day, digestion was terminated by adding 20% trifluoroacetic acid (TFA) to a final pH of ∼3. Cleaning of the samples using solid phase extraction was performed in the Assay MAP Bravo robot. Cleaning is a crucial step for the removal of components such as cell debris and salts, that could interfere with the mass spectrometry analysis. The cleaning of the samples using a robot was unique for the Urea protocol. The procedure can be described as follows: C18 cartridges (Agilent, 5 µL bead volume) were primed with 100 µL of 80% acetonitrile and equilibrated with 70 µL of 0.1% TFA. The samples were loaded at 5 µL/min, followed by an internal cartridge wash alongside a cup wash with 0.1% TFA. To release the peptides from the C18 columns the peptides were eluted with 35 µL 80% acetonitrile (ACN) with 0.1% TFA at 3.5 µL/min. Upon elution, a pre-existing volume of 50 µL of 0.1% TFA was present in the collection plate. The elution buffers were introduced to the instrument deck prior to elution. The fractions were dried in a centrifugal evaporator and stored at -80°C until further analysis. The samples now contained peptides that were ready for analysis. This protocol was repeated for the pilot cohort, including 24 placenta tissue samples in single replicates.

The SDS and DTT-containing samples were reduced by a 1h incubation at 37°C followed by alkylation with 50 mM IAA at room temperature in the dark for 30 minutes. 12% phosphoric acid was added to the SDS lysate in a 1:10 acid-to-sample ratio (v/v) for a final concentration of ∼1.2%. Due to SDS being a detergent, it had to be removed before samples could be digested, due to possible interference between the detergent and enzymes. The removal process commenced with 7x of suspension trapping (S-Trap) binding buffer (90% methanol, 100 mM TEAB, pH ∼7.3) being added to each sample. The lysate was loaded onto the S-Trap Mini Spin Column (ProtiFi). The captured proteins were washed 3 times with 400 µL of S-Trap binding buffer, followed by centrifugation for 2 min. The spin columns were moved to new collection tubes. 100 µL of digestion buffer (50 mM TEAB) containing LysC in a 1:50 w/w (enzyme/protein) ratio was put on top of the columns. The solution was spun at low speed and added to the top of the columns. The spin columns were lightly capped and incubated for 2 h at 37°C. Following incubation, 25 µL of digestion buffer (50 mM TEAB) was added, containing trypsin in a 1:50 w/w (enzyme/protein) ratio. The columns were lightly capped and incubated overnight at 37°C. To release the peptides from the columns, the peptides were the following day first eluted using a digestion buffer, followed by elution with 0.2% formic acid (FA), and lastly with 50% ACN containing 0.2% FA. This promoted the recovery of hydrophobic peptides. The eluded peptides were acidified with TFA to a final pH of ∼3, dried in a centrifugal evaporator, and stored at -80°C until further analysis. Peptide concentrations were measured for all samples using Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific). After extraction and digestion, the recovery of peptides differed between samples, therefore the peptide concentrations were measured and samples were equalised so that they contained equal amounts before analysis. This ensured fair comparison between samples.

C. Spectral Library Generation

A spectral library was generated from a pool of peptides. In total, 150 µg of peptides were separated with high pH RP fractionation using a Waters XBridge BH130 C18 3.5 µm, 2.1 × 150 mm column on an Ultimate 3000 RS HPLC (Thermo Scientific, USA) operating at 200 µL/min. The mobile phases of the chromatography were solvent A: 10 mM ammonium formate pH 10, and solvent B: 90% ACN and 10% water containing 10 mM ammonium formate pH 10. Peptides were separated using the following gradient: 0 min 0% B; 3 min 0% B, 97 min 35% B; 98 min 80% B; 108 min 80% B. As the gradient increased the total mobile phase solution became more polar, allowing for separation of peptides to occur gradually, according to their inherent polarity. Chromatography is a crucial separation step before ionisation and mass spectrometry, allowing easier identification of peptides. The column was operated at room temperature, and the detection wavelength was 214 nm. 96 fractions were collected at 1-minute intervals and further concatenated to 48 fractions. The fractions were dried in a centrifugal evaporator and stored at -80°C until further analysis. Generating a spectral library helped facilitate the search for low abundance peptides in the mass spectrometry analysis of our cohort.

D. nLC-MS/MS Analysis

The nLC-MS/MS analysis of the fractions and the 24 individual placenta samples was performed with an Evosep One LC system (Evosep Biosystems) coupled to a timsTOF HT mass spectrometer equipped with a CaptiveSpray 2 source (Bruker). The CaptiveSpray 2 source would later serve as the interface between chromatography and mass spectrometry, ionising the separated peptides to ions, so that they could be measured by the mass spectrometer. The peptides were first reconstituted in 2% ACN with 0.1% TFA. Approximately 600 ng of peptides were loaded on Evotips according to the manufacturer's instructions and separated using a 15 cm x 150 µm PepSep column with 1.9 µm C18-beads at 40 °C (Bruker). All samples were analysed using the 30 samples per day (SPD) method (gradient length 44 min, vendor standard settings). For the chromatography the mobile phases consisted of a solvent A: 0.1% FA, and solvent B: 0.1% FA, and 99.9% ACN. The default data-dependent acquisition (DDA) method using parallel accumulation-serial fragmentation (PASEF) and data-independent acquisition PASEF (diaPASEF) methods were used for spectral library generation and for individual samples, respectively. The DDA method only selects the most abundant ions for the second mass spectrometry step, whilst the DIA method allows for a more in depth analysis, of all ions, in the second mass spectrometry step, therefore, the latter method was chosen for the analysis of our cohort. The spectral library for DIA-NN analyses was generated with FragPipe v. 20.0, which served as a search engine for our generated spectral library [14]. Default settings were kept. The data was filtered for a 1% false discovery rate (FDR) using Percolator [15] and ProteinProphet [16], which served as databases for controlling FDR. The DIA data was processed with DIA-NN v1.8.1. The search was performed with default

parameters using our spectral library, which consists of 9,917 protein groups and 66,693 peptides. The mass accuracy was automatically calculated based on the first raw file. The search implemented carbamidomethylation of cysteine residues as a fixed modification, oxidation of methionine residues, and N-terminal protein acetylation as dynamic modifications. Up to two missed cleavages were allowed, "Match between run" was enabled and "robust LC (high precision)" was used as the quantification strategy. The output tables were filtered for 1% FDR at both peptide and protein levels. These modifications and settings were arranged and describe what was done to maximise the extractable information regarding the peptides and proteins generated at each step of the nLC-MS/MS process.

E. nLC-MS/MS - Method Optimisation

The nLC-MS/MS analysis of the six samples from the method optimisation was performed using another mass spectrometer than previously, an Orbitrap Exploris 480 MS coupled to a Dionex Ultimate Vanquish Neo UHPLC system (Thermo Scientific), with an EASY-Spray ion source, that would later ionise the separated peptides to ions before mass spectrometry. The peptides were reconstituted in 2% ACN with 0.1% TFA and loaded onto an Acclaim PepMap 100 C18 (75 µm × 2 cm, 3 µm, 100 Å, nanoViper) trap column. They were then separated by chromatography on an Acclaim PepMap RSLC C18 column (75 µm × 25 cm, 2 µm, 100 Å) (Thermo Scientific) using a flow rate of 300 nL/min and a column temperature of 45 °C.A 90 min gradient was applied for separation, using solvents A (0.1% formic acid) and B (0.1% formic acid in 80% ACN), increasing solvent B from 5 to 25% in 75 min, then to 32% in the next 9 min, and to 45% in 6 min. Finally, the gradient increased to 95% solvent B in 2 min, continuing for another 8 min.

For the DIA analysis, a complete acquisition cycle consisted of three MS1 full scans, each followed by 18 MS2 DIA scans with variable isolation windows. MS1 full scans were acquired at m/z 375-1,455, with a resolution of 120,000 (at 200 m/z), normalised target AGC value of 300% and maximum injection time of 45 ms. The MS2 scans were acquired with a resolution of 30,000 (at 200 m/z), fragmentation with stepped NCE of 27, 30 and 32, normalised target AGC value of 1,000%, automatic maximum injection time and fixed first mass of 200 m/z. The isolation windows were overlapped with 1 m/z, and the variable windows were set to 12.0, 15.0, 25.0 and 60.0 m/z (with 27, 13, 8, and 6 loop counts, respectively). The use of variable windows involved the division of mass ranges depending on the abundance of ions. Regions that had more ions had narrow windows, thus minimising inclusion of non-relevant ions. Regions with less ions had large windows, allowing increased ion collection and sensitivity, all in all, resulting in easier detection of low abundance peptides.

The DIA data files were searched with direct DIA using the Biognosys (BGS) factory default settings in Spectronaut 16.0.220606.5300 (Biognosys, Schlieren, Switzerland), which is a software used for peptide determination. The number of identifications was filtered at an FDR of 1% at both peptide and protein levels, allowing our generated data to be reliable.

F. Data Preprocessing and Statistical Analyses

Data preprocessing, including log2 transformations and median subtraction, was performed in Perseus v1.6.10.43 [17]. Statistical analyses were performed using Perseus, RStudio and GraphPad Prism. Statistical tests, such as ANOVA, Tukey post hoc test, and t-test, were performed to find statistically significant differences between the different groups. Reactome, Gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG) and MSigDB Hallmark databases were used to analyse differentially expressed proteins.

III. RESULTS & DISCUSSION

A. Sample Preparation Workflow for Placenta Tissue

Two methods were evaluated to develop a protocol for efficient, reproducible and robust sample preparation of placenta tissue. A urea-based protein extraction followed by in-solution digestion and C18 clean-up, was compared to an SDS-based protein extraction followed by digestion utilising suspension trapping (S-Trap). Both methods yielded similar numbers of identified peptides (urea: 53,714 and SDS: 57,256) and proteins (urea: 4,898 and SDS: 4,715) (Figure 2a). The digestion efficiency, as measured by the proportion of miss-cleaved peptides, was also comparable (Figure 2b).

Pearson correlation analysis was performed to assess the quantitative reproducibility between experimental replicates. High correlations were evident within all pairwise comparisons of protein abundances within and between methods, with correlation coefficients exceeding 0.87 (Figure 2c). However, the urea method displayed better experimental reproducibility, which was apparent by the slightly lower median coefficient of variation (urea: 4.2% and SDS: 4.5%) and by principal component analysis (PCA) (Figure 2d and Figure 2e). In the urea method, a pipetting robot based on a 96-well plate format (Bravo AssayMAP platform from Agilent Technologies) can be used to eliminate errors induced by human factors, further enhancing the reproducibility and throughput of the method. These findings provided a clear rationale for selecting the urea method for the in-depth analysis of 24 individual placenta samples.

B. Investigation of Sample Integrity

Maintaining sample integrity in clinical studies is essential for obtaining reliable and reproducible data to advance knowledge and improve patient care. Conducting research on mishandled samples might provide unreliable results. In our cohort, certain samples were collected using a non-standardised protocol. Therefore, we explored the impact of mishandling on the relative protein abundances in the placenta samples. The samples collected in 2010 were processed using a non-standardised protocol with unspecified collection sites, unlike those collected between 2014 and 2016, which were processed according to a standardised protocol. The samples from 2010 came from women in the EPE group and were therefore compared to the other EPE samples. A comparison of the two groups using Student's t-test (FDR < 0.05) yielded 737 significantly differentially expressed proteins (Figure 3a).

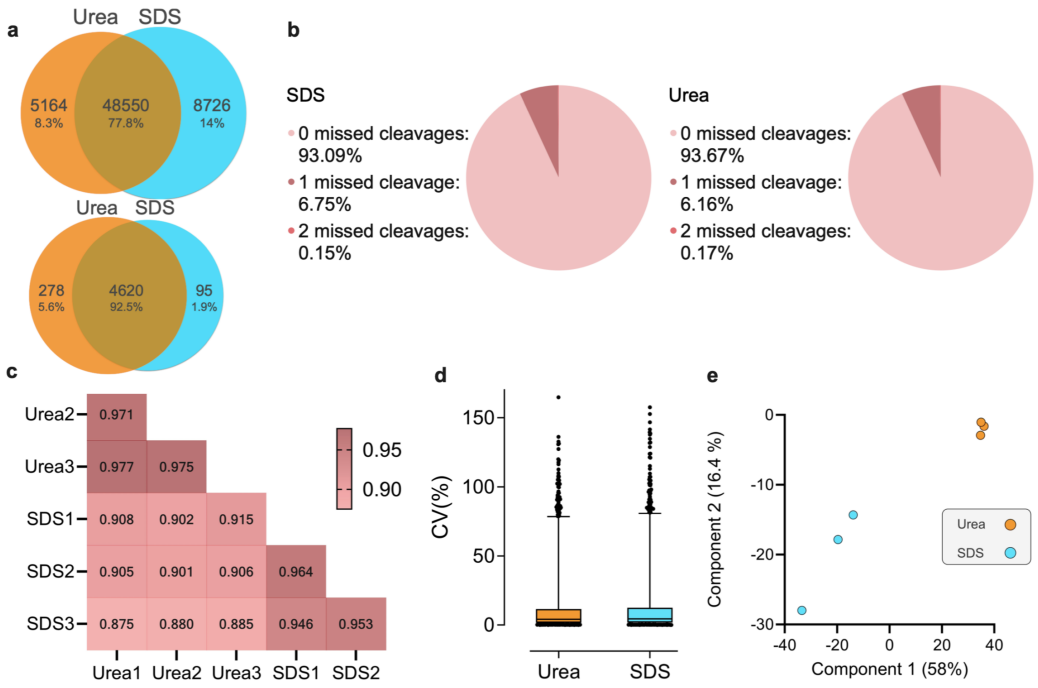

Figure 2. (a) Overlap of identified peptides (top) and proteins (bottom) in each extraction method, based on three experimental replicates, represented by Venn diagrams. (b) Digestion efficiency is defined by the missed cleavage distribution in the urea and SDS methods. The percentage of 0, 1 and 2 missed cleavage sites are shown, based on three experimental replicates. (c) Pearson correlation heatmap of log2 transformed protein intensities showing pairwise correlations within and between each method comparison and replicate. (d) Boxplot representing the coefficients of variation (CVs) for the quantified proteins in three experimental replicates for the urea and SDS methods, respectively. (e) Principal component analysis (PCA) shows three urea and SDS experimental replicates, indicating a greater variance among the SDS samples than the urea samples.

In particular, one of the mishandled samples had fewer protein identifications and lower relative abundances compared to the other samples, underscoring the importance of standardised protocols to avoid protein degradation. Subsequently, the identified proteins were subjected to 1D annotation enrichment analysis using the Gene Ontology (GO), Reactome and KEGG databases (Figure 3b). Most affected pathways were associated with mitochondrial, ribosomal and immune system-related functions.

Individual differentially expressed proteins were further explored using a volcano plot (Student's t-test, p-value < 0.05, log2 fold change > |1|) (Figure 3c). Proteins upregulated in the samples collected in a standardised manner, including BUB3, NDUFAB1, NECTIN2, PLPP3 and SLC37A4, among others, are involved in mitochondrial and ribosomal function, which are processes that depend on the presence of oxygen. The proteins upregulated in the samples collected in a non-standardised way included the haemoglobin protein chains HBA1, HBB, and HBG2. This might be an indication of oxidative stress [18]. During ischemic-like conditions, the overproduction of reactive oxygen species is one of many mechanisms used by the body to compensate for the lack of oxygen [19]. This suggests that if the samples are not frozen immediately, the preservation of the tissue is partly reduced due to ischemia, which occurs shortly after the placenta is separated from the uterine wall and the mother's body. The mishandling of samples can also result in compromised cellular function, mainly affecting metabolic processes, which underlines the importance of proper sample handling according to standardised protocols [20] [21]. Therefore, the samples from 2010 were not included in further biological analyses, reducing the cohort to 20 samples.

C. Differentially Expressed Proteins and Pathways Among EPE, LPE and Controls

Among the 20 placenta tissues, one sample generated a significantly lower protein yield and fewer protein identifications. Due to the unknown nature of this occurrence, the sample was excluded from further biological analyses. In the 19 placenta samples, a total of 7,202 proteins were identified, with 6,700–7,000 proteins being identified per sample. To uncover biological differences between the three groups, EPE, LPE and controls, ANOVA (p-value < 0.05) and Tukey post hoc test (FDR < 0.05) were performed, identifying 553 significant proteins (Figure 4a). As suspected, the highest number of differentially expressed proteins were found in the EPE group compared to LPE and controls. Following pathway enrichment using the Reactome, GO and MSigDB Hallmark databases, we found upregulation of pathways involving the mitotic spindle, UV-response, adipogenesis, glycolysis, MTORC1-signalling, hypoxia and unfolded protein response in the EPE group (Figure 4b). Pathways being significantly downregulated in EPE compared to the other two groups were, amongst others, collagen-containing extracellular matrix, regulation of cell

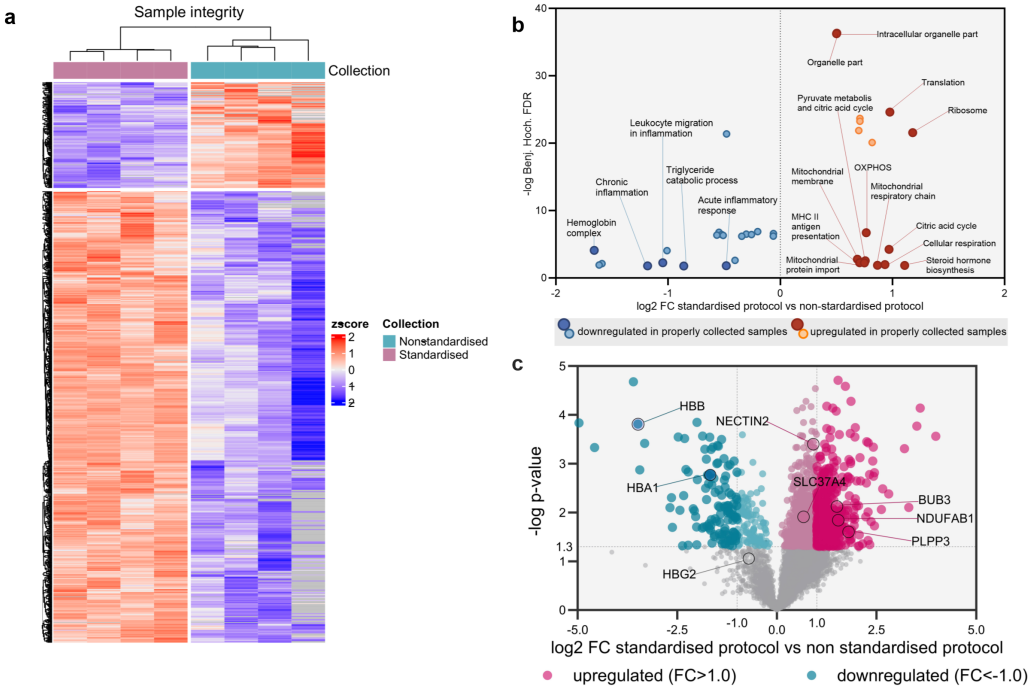

Figure 3. (a) Heatmap displaying the significantly dysregulated proteins between the two collection procedures in eight EPE patient samples (Student's t-test, FDR < 0.05). (b) 1D annotation enrichment illustrating representative significant KEGG, GO and Reactome signalling pathways (FDR < 0.02) up-regulated (orange to red) in the EPE samples acquired using a standardised collection procedure or upregulated (light to dark blue) in the samples acquired in a non-standardised way. (c) Volcano Plot showing the upregulated (pink) and downregulated (turquoise) proteins in the samples collected with the standardised protocol compared to the non-standardised (p-value < 0.05, log2 fold change > |1|), resulting in 1,457 upregulated and 230 downregulated proteins.

migration, cytoskeletal protein binding, actin filament bundle, actomyosin and stress fibre. Different types of pathways regulating junctions were also seen, such as focal adhesion and adherens junction. Other downregulated pathways in EPE were the ERBB signalling pathway, epidermal growth factor receptor (EGFR) signalling pathway and cellular response to insulin stimulus. From the HE-images of the placenta, it is apparent that the villi, shown by a representative EPE sample (Figure 4c), have been subjected to the degradation of collagen, compared to the LPE sample (Figure 4d) and the control sample (Figure 4e). An environment of oxidative stress can also be seen in the EPE images, which supports the upregulation of hypoxia in EPE. Oxidative stress damages the cristae of the mitochondria and can be enhanced by hypoxic conditions.

D. Upregulation of Hypoxia in EPE

Hypoxia is crucial for both placenta and fetal development. Hypoxia was found to be upregulated in EPE compared to LPE and controls. Proteins overexpressed in placentas from EPE, promoting hypoxic conditions, include enzymes such as NAGK, GAPDH, and the cytokine MIF, which all are proinflammatory mediators [22]. MIF is a pro-inflammatory cytokine regulating the function of macrophages in host defence and is involved in the innate immune response towards bacterial pathogens. The enzyme NAGK is involved in the innate immune response, acting as a pro-inflammatory mediator, and the GAPDH enzyme is partly responsible for initiating apoptosis and glycolysis [23].

Hypoxia in the placenta might interfere with critical stages of placenta development, such as blastocyst implantation, cytotrophoblast invasion, and spiral artery remodelling initiation [24]. The dysregulated oxygen levels may also affect maternal immune cell function [25]. Additionally, an overexpression of the hypoxia pathway can negatively affect the syncytiotrophoblast and early placenta development and is, therefore, mostly correlated with EPE. However, hypoxia can also be present in LPE to some extent [24]. Genes involved in glycolysis, such as the enzymes ALDOC, TPI1 and the transporter SLC37A4, were also upregulated in EPE [22]. An upregulation of these genes indicates an increased metabolism of glucose in the placenta, either by glycogenolysis, gluconeogenesis or glycolysis, where the latter is a metabolic pathway induced under the influence of hypoxic conditions [26]. The increase in hypoxia might result from impaired placentation due to an inadequate blood supply to the developing fetoplacental unit, resulting in chronic hypoxia, which is predominantly associated with EPE [27].

E. Upregulation of Adipogenesis and Inflammation in EPE

Adipogenesis is the process by which fat cells, adipocytes, develop as adipose tissue in the body. Adipocytes regulates autocrine and paracrine functions, which regulate insulin sensitivity, inflammation, cell growth and cardiovascular function [28]. Therefore, it is important for placenta development

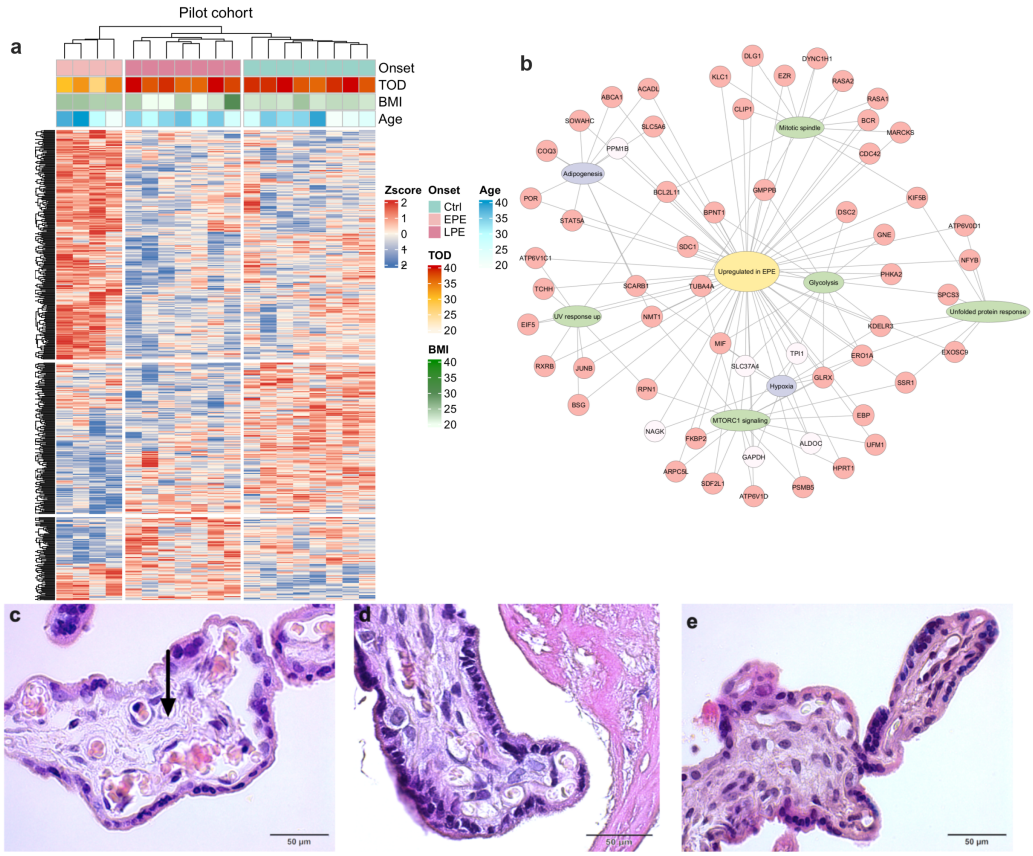

Figure 4. (a) Heatmap visualising the significantly dysregulated proteins between EPE, LPE and controls (ANOVA, p-value < 0.05). (b) Pathway enrichment of the proteins from cluster one in the heatmap using the MSigDB Hallmark database. Essential proteins and pathways related to PE are highlighted in light pink and blue, respectively. Representative histological images of placental tissues show (c) degraded ECM in EPE (black arrow), (d) non-degraded ECM in LPE, and (e) control.

and growth. Being overweight involves chronic inflammation, leading to an increased accumulation of immune cells and the production of pro-inflammatory molecules [28]. The PPM1B gene affects the cyclin-dependent kinases, which directly regulate the cell cycle by coding for the enzyme protein phosphatase 1B. An overexpression of the PPM1B protein, which could be observed in the EPE group, can induce necrosis and upregulate inflammation [29]. Additionally, a higher BMI, a known risk factor for PE, can be considered a contributing factor leading to the overexpression of this protein, promoting a proinflammatory response that might stress the placenta, resulting in hypoxia and disrupted transportation of nutrients. As a result, this metabolic inflammatory response may lead to an increased risk of PE [28]. Additionally, we found an upregulation of the SR-B1 protein, an adipokine receptor encoded by the gene SCARB1, in the EPE group. The placenta is sometimes compared to a tumour due to the great amount of growth it can achieve during a short period of time, making the adipokine receptors expressed on tumours interesting to look at in PE alongside an upregulation of adipogenesis [30]. The proteins and pathways upregulated in EPE show an intricate interplay between hypoxia, adipogenesis, and inflammation. They need to be further investigated in a large cohort of properly collected and characterised placenta tissue samples.

F. Ethics and Sustainability

As mentioned in the method section, the placenta samples were collected after written informed consent from the patients. The placentas were stored in a Biobank at Lund University, and all samples were anonymous. With respect to the goals for sustainable development established by the United Nations, our project aids the promotion of goal 3: “Ensure healthy lives and promote well-being for all at all ages”. This study investigates protein and macro-level differences of PE to improve our understanding of disease aetiology. The project also creates foundations for innovations by promoting biomarker discovery and thereby improving healthcare and health, which can be seen in goal 9: “Build resilient infrastructure, promote inclusive and sustainable industrialisation and foster innovation”.

IV. Conlusions

The study consisted of three main parts: workflow development for proteomic analysis of placenta samples, investigation of tissue integrity related to sample collection, and biological analysis of a pilot cohort. A high throughput and reproducible workflow able to identify more proteins from placenta tissue than in previous studies was proposed. The study also highlights the essence of sample integrity related to

standardised sample collection procedures for reliable protein quantification. Analysis of a pilot cohort showed that placenta tissues from EPE differed the most from LPE and controls, where EPE was mainly characterised by the upregulation of pathways related to hypoxia and metabolism. This study sheds light on the importance of deep proteomic characterisation of placenta tissues and provides insight into the biological processes involved in PE. It might serve as a basis for future studies, including large cohorts with properly collected and stored placenta samples.

V. Limitations of the Study

A small cohort size of 24 samples poses significant limitations to the study by compromising statistical power, increasing variability, increasing vulnerability to confounding factors, and impeding the ability to draw biological conclusions. Therefore, a study based on a larger cohort should be analysed to confirm our findings. One must also consider the discussed aspects of sample integrity when assessing the study's results.

VI. Acknowledgements

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