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Technical note

Optimization of label-free nano LC-MS/MS analysis of the placental proteome

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ABSTRACT

The placenta can be regarded as a mirror of the events to which the fetus is exposed during development. The placental proteome has been studied with several methodologies differing in sample handling, protein extraction, and processing. We optimized a protocol to analyze the placental proteome by means of label-free nano-LC-MS/MS mass spectrometry with regard to sample treatment, protein extraction, and protein digestion, in order to obtain a high protein concentration for identification of a specific protein signature according to the conditions studied. We recommend mechanical tissue disruption, blood removal prior to protein extraction, and FASP-based or in-gel digestion.

1. Introduction

The placenta is a complex organ that can be used postnatally to examine the morphological and molecular effects of environmental exposures or disease processes during pregnancy. The placental proteome has been studied for numerous adverse pregnancy conditions, such as preeclampsia [1,2] and in the context of environmental stressors, such as maternal smoking during pregnancy [3]. Several techniques are currently used in an attempt to identify the entire placental proteome or a placental sub-proteome [4,5]. However, the placenta is a complex tissue with various cell types and it has proven to be very challenging to study the low molecular weight, low abundance placental proteins [5]. Hence, optimization of protein processing prior to the final analysis, for example with label-free mass spectrometry, is needed to improve identification. Protocols applied in different research groups vary in the size of the placental biopsies and in the methods to process the protein extracts into peptides. Therefore, more attention is needed to optimize and unify how to handle placental tissue samples before proteins are extracted, the technique to separate and concentrate placental proteins, and the digestion of the protein mixture into peptides, prior to peptide injection in the mass spectrometer.

Two important aspects of proteomics are sensitivity (the ability to detect the protein if it is present) and specificity (the ability to distinguish a specific target protein from other proteins in the sample) [6], with a high risk of different outcomes for the same research question when different protocols are applied. In addition, since protein material can be lost in each step of the label-free nano LC-MS/MS analysis process, from protein extraction until the final injection of the peptide mixture, a generalized protocol should entail a high number of proteins identified. In this study we optimized an approach to study the proteome of the placenta through label-free nano LC-MS/MS, since this technique allows the untargeted identification of a substantial part of the protein entity, with high accuracy and precision. We paid specific attention to sample treatment regarding blood removal, protein extraction, and post-extraction sample processing.

2. Methods

2.1. Sample collection

Placental tissue samples were collected from 10 randomly selected participants of the ongoing ENVIR*ON*AGE (ENVIRonmental influence *ON* AGEing in early life) birth cohort. The study protocol was approved by the ethical committees of Hasselt University and East-Limburg Hospital (EudraCT B37120107805). Before delivery, an informed consent form was signed by the mothers, and placentas were collected within 10 min after birth [7]. The samples were taken at the fetal side of the placenta, as described by Janssen et al. [7] and were either directly frozen in liquid nitrogen or rinsed shortly in phosphate-buffered saline (PBS) before freezing. The biopsies were kept at $-80\ ^\circ C$ until protein extraction.

2.2. Protein extraction

Proteins were extracted from approximately 200 mg placental tissue in 1.5 ml lysis buffer containing 2 M thiourea, 7 M urea, 2% CHAPS, and 2% dithiothreitol (DTT). Four tissue disruption methods were tested: by using a syringe (VWR, Pennsylvanian USA), by three subsequent 10-sec sonication bursts (UP100H, Hielscher, Teltow, Germany), by mechanical disruption with an Ultra Turrax T8 mixer (IKA, Staufen, Germany) three times during 10 s, or by crushing the tissue in liquid nitrogen. The lysates were incubated on a shaking plate for 30 min at 1400 rpm and 15 °C and subsequently centrifuged for 5 min at 16.000 g. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Massachusetts, USA).

2.3. Trypsin digestion and detergent removal

2.3.1. Protein precipitation and liquid digestion

Protein extracts were treated with the 2-D Clean-up kit (GE Healthcare Life Sciences, Illinois, USA) according to the manufacturers' instructions. The resulting pellet was re-suspended in 0.2% Rapigest (Waters, Massachusetts, USA), incubated at 15 °C for 30 min, centrifuged for 5 min at 13,000 g, heated for 5 min at 100 °C and finally kept at $-80\ ^\circ C$. Samples were reduced with 10 mM dithiothreitol (DTT) and shaken at 500 rpm for 45 min at 37 °C, then, alkylated with 40 mM iodoacetamide (IAA) in the dark under the same conditions. After adding 1 mM $CaCl_2$ and trypsin in a ratio of 1/50 w/w, protein digestion occurred overnight, shaking at 300 rpm and 37 °C. The reaction was stopped by adding 2% trifluoroacetic acid (TFA; Biosolve) and incubating for 45 min at 300 rpm and 37 °C. After 10 min of centrifugation at 13,000 rpm, the supernatant was used for nano LC-MS/MS analysis.

2.3.2. Liquid digestion with the FASP kit

Samples were digested using filter-aided sample preparation (FASP) digestion, based on modified protocols of the FASP Protein Digestion kit (Expedeon, San Diego, USA) [8,9]. We used 10 µg of protein input, and protein digestion was performed with either 1/20 or 1/50 w/w trypsin, for either 2 h, 5 h, or overnight at 37 °C and 300 rpm. Following final centrifugation during 10 min at 13,000 g, the digestion was stopped with 2% TFA, after which the peptide mixture was directly analyzed with nano LC-MS/MS.

2.3.3. In-gel digestion

Five µl of loading buffer was added to 20 µg of protein sample. This mixture was heated at 100 °C for 5 min, centrifuged during 2 min at 13,000 rpm and subsequently loaded on a 10% polyacrylamide gel (Biorad, California, USA). Following a run of 5 min at 200 V and 400 mA, the gel containing proteins was cut into pieces of $\pm$ 5–6 mm^3 and kept in H_2O at $-20\ ^\circ C$ prior to trypsin digestion.

Gel fragments were dehydrated with acetonitrile (ACN), shaking 10 min at 900 rpm. After ACN removal, reduction and alkylation of the in-gel protein samples were performed similar to that of the liquid digestion protocol, by incubation with 10 mM DTT and 55 mM IAA. Following two incubation steps with ACN and one with 100 mM NH_4HCO_3, the in-gel protein mixtures were digested with trypsin at a final concentration of 6.25 ng/µl, overnight at 37 °C and 300 rpm.

2.4. Nano LC-MS/MS

We analyzed the peptide samples with a nano-liquid chromatography Ultimate 3000 system (Thermo Scientific), connected to a maXis Impact electrospray Ultra-High resolution Q-TOF mass spectrometer (Bruker, Massachusetts, USA). Initial peptide separation occurred by reverse-phase liquid chromatography on a 75 µm by 250 mm C18 column (Acclaim™ PepMap™ 100 C18 LC Column, Thermo Scientific). Mobile phase A in this column was composed of 0.1% formic acid and 2% acetonitrile, mobile phase B contained 0.1% formic acid in 80% acetonitrile. For each run, 2 µg of sample was injected, and the organic concentration of the mobile phase was increased linearly from 4% to 35%. We used a 90 min gradient for in-gel digestion and one of 215 min for other strategies. Subsequently, the resulting flow-through was ionized in the electrospray ionization (ESI) CaptiveSpray (Bruker), which was directly coupled to the C18 column. In survey scan, MS spectra were acquired for 0.5 s in a 50–2200 *m/z* range.

Peak lists were created using the DataAnalysis 4.0 software (Bruker) and uploaded to the ProteinScape 3.1 software (Bruker) which uses Mascot 2.4 (Matrix Science) as protein identifier search engine, through the UniProt database (version September 2019, Uniprot-HomoIsoform 190904). Finally, we used the Scaffold 4.8 software (Proteome Software Inc., Portland, Oregon, USA) to visualize the protein identification lists. For the total number of identified proteins, we set the protein and peptide threshold at 1.0% false discovery rate (FDR), and the minimum number of peptides for identification at two. We used the total spectral count method, counting the total number of analyzed spectra associated to a single protein group, including those shared with other proteins.

2.5. Data analyses and publication

We used the built-in statistical package of the Scaffold 4.8 software (Proteome Software Inc.) for initial protein identification data analyses. Relative protein abundance was determined using the spectral counts method. We determined the difference in total protein numbers between liquid digestion and FASP-based digestion, and between one and two PBS washing steps, by a two-sided *t*-test with SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE [10] partner repository with the dataset identifiers PXD020438 and 10.6019/PXD020438.

3. Results

3.1. Mechanical tissue disruption and extra washing improves protein identification

We tested placental tissue disruption with a syringe, sonication, liquid nitrogen, or mechanical disruption. The total number of identified proteins was the highest after mechanical separation (n = 1458) (Fig. 1A), although the number for sonication did not seem to differ greatly (n = 1330). Because of the presence of more uniquely identified proteins and higher unique peptide counts for important functional proteins (such as ATP synthase and superoxide dismutase) in mechanically disrupted samples compared to those treated with sonication, for all further optimization tests on pre-extraction blood removal and on protein digestion methods, tissue samples were mechanically disrupted.

Using the same biological sample, we performed either no sample treatment, one PBS washing step after tissue collection, or a washing step with PBS both after tissue collection and after thawing to determine
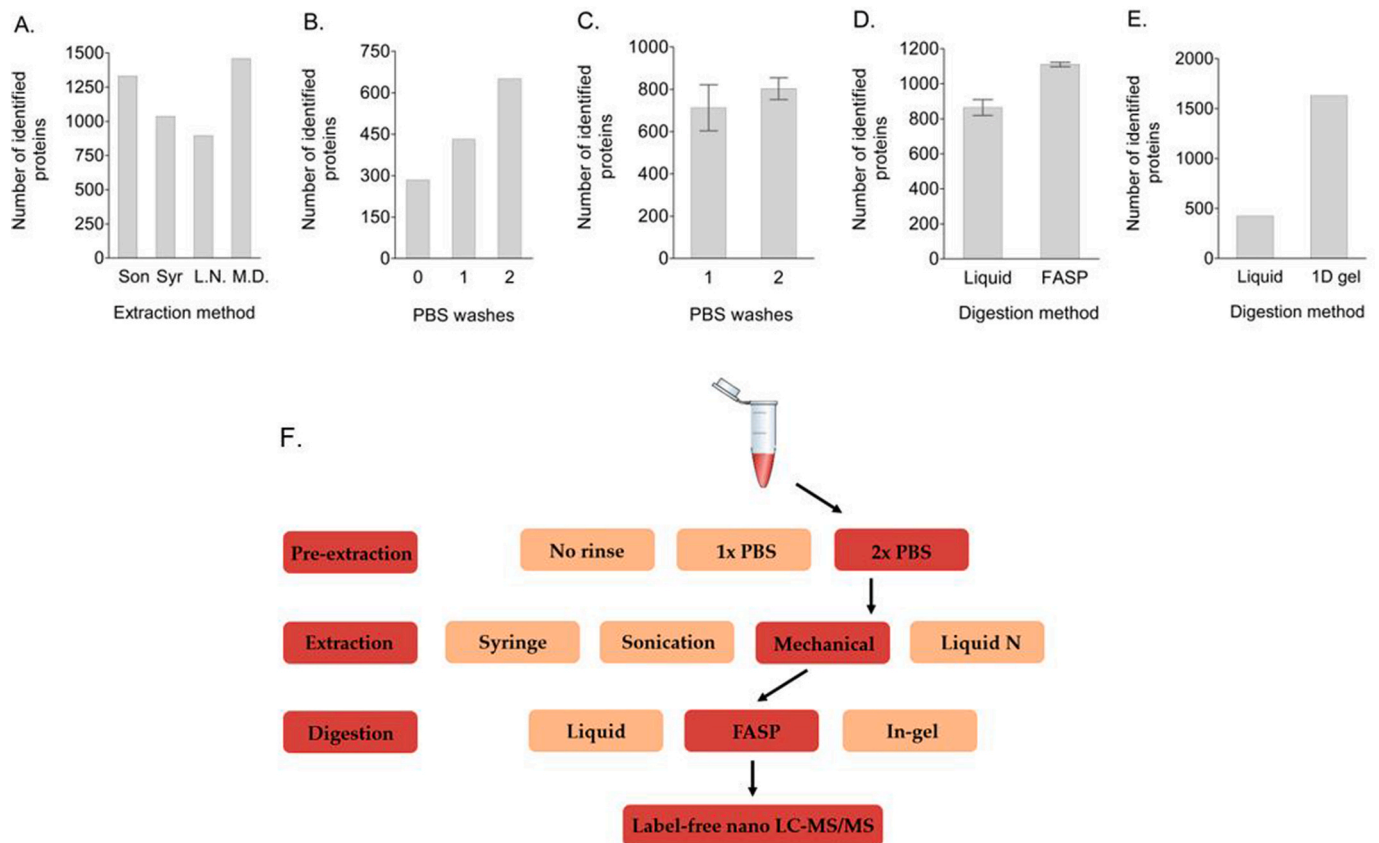
Fig. 1. Increase in the total number of identified proteins after protein extraction through mechanical tissue disruption, after a PBS washing step both before freezing and after thawing the samples, and either FASP-system-based or gel-based protein digestion. Figure A: Tested methods of sample tissue disruption are depicted in the x-axis. Mechanical disruption of the placental tissue resulted in the highest number of identified proteins. Figures B and C: The x-axis indicates the number of times that the samples had been washed before protein extraction. An extra wash of the placental biopsies after thawing increased the total number of identified proteins. Figure D: Protein digestion with the FASP system increased the total number of identified proteins, compared to the numbers obtained with liquid protein digestion. Figure E: In-gel digestion following 1D gel sample separation increased the total number of identified proteins, compared to the numbers obtained with liquid protein digestion. Figure F: To increase the total number of proteins identified by label-free nano LC-MS/MS, we advise to wash placental tissue samples two times (once directly after taking the biopsies, and a second time before protein extraction) with a neutral substance such as PBS for excess blood removal prior to tissue disruption (line "Pre-extraction"), mechanically disrupt the washed tissue samples (line "Extraction") for protein extraction, and perform FASP-based protein digestion (line "Digestion") for 5 h with 1/20 w/w trypsin to minimize the number of miscleavages and non-specific cleavages.
Abbreviations: FASP, filter-aided sample preparation; L. N, crushing tissue that was snap-frozen in liquid nitrogen; M. D., mechanical tissue disruption; PBS, phosphate-buffered saline; Son, sonication bursts; Syr, disruption of placental tissue through a syringe.

the effect on protein identification. PBS washing increased the number of protein identifications (Fig. 1B). Repeating this experiment, with the conditions of one and two PBS washes in triplicate, showed that after two PBS washes the average total amount of identified proteins increased from 713 to 803, although the difference was not statistically significant ($p = 0.27$) (Fig. 1C) and that the numbers of several blood-specific proteins decreased (data not shown).

3.2. Increased protein identification with gel- and FASP-based separation and digestion compared to liquid digestion

Firstly, we tested FASP digestion as an alternative for protein precipitation and liquid digestion. We used one biological sample and tested each technique in triplicate. At this optimization stage, all tissue samples were washed both before freezing and after thawing, and were mechanically disrupted for protein extraction. FASP digestion resulted in a higher number of protein identifications (n = 1110) compared to liquid digestion (n = 864) ($p = 0.003$) (Fig. 1D). Furthermore, we compared the percentage of non-specific cleavages (i.e. peptides were cut at amino acids other than lysine or arginine) and miscleavages (i.e. trypsin failed to cut the carboxyl end of lysine or arginine). The percentage of miscleavages was approximately the same for liquid- (9.8%)

and FASP-based digestion (10.2%), while the percentage of non-specific cleavages was higher for liquid digestion (9.1% vs 3.7%).

Secondly, we compared liquid-based digestion with in-gel digestion of the same biological replicate. Gel-based digestion increased the number of protein identifications (n = 1640) compared to liquid digestion (n = 462) (Fig. 1E). Although the number of non-specific cleavages was comparable (7.3% for liquid digestion and 5.6% for gel-based digestion), the percentage of miscleavages was higher for gel-based digestion (5.1% vs 17.1%).

In a third test, we compared three digestion periods, i.e. 2 h, 5 h, or overnight, in combination with either 1/20 or 1/50 w/w of trypsin. All samples, derived from one biological replicate, were digested by the FASP method. Although the total number of proteins did not differ substantially, the number of miscleavages was higher for the lower trypsin concentrations during each of the three digestions periods (Table 1). Overnight digestion increased the number of non-specific cleavages, especially for the digestion with 1/20 w/w trypsin. Therefore, a 5-h digestion with 1/20 w/w trypsin appears to be most favorable.

Table 1

Total number of protein identifications and percentage of miscleavages and non-specific cleavages for each of six conditions combining three different digestion durations and two trypsin concentrations.

Incubation conditions (duration - w/w trypsin)	Total number of identified proteins	Miscleavages (%)	Non-specific cleavages (%)
2 h - 1/50	392	23.0	0.3
2 h - 1/20	352	16.0	2.7
5 h - 1/50	382	19.6	1.4
5 h - 1/20	349	11.9	1.5
ON - 1/50	374	11.3	2.2
ON - 1/20	359	7.2	7.3

Abbreviations: ON, overnight.

4. Discussion

We have evaluated a protocol to analyze the placental proteome through label-free nano-LC-MS/MS. Our key findings are: 1) Blood removal prior to protein extraction and mechanical tissue disruption increase the total amount of identifications; 2) In-gel and FASP-based protein digestion result in a higher number of identified proteins, compared to liquid-based digestion and protein precipitation, and 3) To reduce the number of miscleavages and non-specific cleavages during placental protein digestion, a concentration of 1/50 w/w trypsin and overnight digestion are not advised (Fig. 1F). Depending on the research question and number of samples, research groups can choose between FASP-based digestion or in-gel protein digestion, of which the latter is more labor-intensive and time consuming (due to all gel fragments for one sample being analyzed separately), but results in higher protein identification compared to FASP-based digestion.

One of the challenges of using placental tissue in proteomic research is its complexity and heterogeneity. Concerning cellular compartment distribution, our placental protein extracts contained a higher percentage of mitochondrial proteins, and a similar number of cytosol- and membrane-related proteins for all extraction techniques in comparison with the human proteome [11–13]. Nuclear proteins could be identified to a lower level compared with the human proteome for each technique, apart from the technique without blood removal before protein extraction, for which no nucleus-related protein was found (Supplemental Table 1).

Recent research suggests that the placenta contains different subproteomes, rather than one proteome entity [14]. Therefore, tissue sampling is an essential initial step in placental proteomic research. Burton and colleagues have optimized a protocol regarding the placental storage and sampling process for various purposes [15]. If these steps proceeding sample treatment and protein digestion would be combined and unified for proteomic research purposes in placental tissue, this would aid reproducibility and reliability in the search for protein biomarkers related to the impact of environmental exposure or disease development during pregnancy.

Declaration of competing interestCOI

Acknowledgements

Appendix A. Supplementary data

References

[1] W. Zhang, X. Chen, Z. Yan, Y. Chen, Y. Cui, B. Chen, C. Huang, W. Zhang, X. Yin, Q.-Y. He, T. Wang, Detergent-insoluble proteome analysis revealed aberrantly aggregated proteins in human preeclampsia placentas, J. Proteome Res. 16 (2017) 4468–4480, https://doi.org/10.1021/acs.jproteome.7b00352.

[2] F. Wang, Z. Shi, P. Wang, W. You, G. Liang, Comparative proteome profile of human placenta from normal and preeclamptic pregnancies, PLoS One 8 (2013), e78025, https://doi.org/10.1371/journal.pone.0078025.

[3] P. Huuskonen, M.R. Amezaga, M. Bellingham, L.H. Jones, M. Storvik, M. Häkkinen, L. Keski-Nisula, S. Heinonen, P.J. O'Shaughnessy, P.A. Fowler, M. Pasanen, The human placental proteome is affected by maternal smoking, Reprod, Toxicol 63 (2016) 22–31, https://doi.org/10.1016/j.reprotox.2016.05.009.

[4] D.D. Vandré, W.E.I. Ackerman, A. Tewari, D.A. Kniss, J.M. Robinson, A placental sub-proteome: the apical plasma membrane of the syncytiotrophoblast, Placenta 33 (2012) 207–213, https://doi.org/10.1016/j.placenta.2011.12.010.

[5] K. Kedia, C.A. Nichols, C.D. Thulin, S.W. Graves, Novel "omics" approach for study of low-abundance , low-molecular-weight components of a complex biological tissue: regional differences between chorionic and basal plates of the human placenta, Anal. Bioanal. Chem. 407 (2015) 8543–8556, https://doi.org/10.1007/s00216-015-9009-3.

[6] R. Wilson, Sensitivity and specificity: twin goals of proteomics assays. Can they be combined? Expert Rev. Proteomics 10 (2013) 135–149, https://doi.org/10.1586/epr.13.7.

[7] B.G. Janssen, N. Madhloum, W. Gyselaers, E. Bijnens, D.B. Clemente, B. Cox, J. Hogervorst, L.J. Luyten, D.S. Martens, M. Peusens, M. Plusquin, E.B. Provost, H.A. Roels, N.D. Saenen, M. Tsamou, A. Vriens, E. Winckelmans, K. Vrijens, T.S. Nawrot, Cohort Profile: the ENVIRonmental influence ON early AGEing (ENVIRONAGE): a birth cohort study, Int. J. Epidemiol. 46 (2017) 1386–1387m, https://doi.org/10.1093/ije/dyx033.

[8] J.R. Wiśniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359–362, https://doi.org/10.1038/nmeth.1322.

[9] U. Distler, J. Kuharev, P. Navarro, S. Tenzer, Label-free quantification in ion mobility–enhanced data-independent acquisition proteomics, Nat. Protoc. 11 (2016) 795–812, https://doi.org/10.1038/nprot.2016.042.

[10] Y. Perez-Riverol, A. Csordas, J. Bai, M. Bernal-Llinares, S. Hewapathirana, D. Kundu, A. Inuganti, J. Griss, G. Mayer, M. Eisenacher, E. Pérez, J. Uszkoreit, J. Pfeuffer, T. Sachsenberg, S. Yilmaz, S. Tiwary, J. Cox, E. Audain, M. Walzer, A. Jarnuczak, T. Ternent, A. Brazma, J. Vizcaíno, The PRIDE database and related tools and resources in 2019: improving support for quantification data, Nucleic Acids Res. 47 (2019) D442–D450, https://doi.org/10.1093/nar/gky1106.

[11] A. Pandey, S. Chakraborty, N. Chakraborty, Nuclear proteome: Isolation of Intact nuclei, extraction of nuclear proteins, and 2-DE analysis, Methods Mol. Biol. 1696 (2018) 41–55, https://doi.org/10.1007/978-1-4939-7411-5_3.

[12] S.M. Smith, Strategies for the purification of membrane proteins, Methods Mol. Biol. 1485 (2017) 389–400, https://doi.org/10.1007/978-1-4939-6412-3_21.

[13] P.J. Thul, L. Åkesson, M. Wiking, D. Mahdessian, A. Geladaki, H.A. Blal, T. Alm, A. Asplund, L. Björk, L.M. Breckels, A. Bäckström, F. Danielsson, L. Fagerberg, J. Fall, L. Gatto, C. Gnann, S. Hober, M. Hjelmare, F. Johansson, S. Lee, C. Lindskog, J. Mulder, C.M. Mulvey, P. Nilsson, P. Oksvold, J. Rockberg, R. Schutten, J.M. Schwenk, Å. Sivertsson, E. Sjöstedt, M. Skogs, C. Stadler, D.P. Sullivan, H. Tegel, C. Winsnes, C. Zhang, M. Zwahlen, A. Mardinoglu, F. Pontén, K. von Feilitzen, K.S. Lilley, M. Uhlén, E. Lundberg, A subcellular map of the human proteome, Science 356 (2017), https://doi.org/10.1126/science.aal3321 eaal3321.

[14] W.E. Heywood, R. Preece, J. Pryce, J. Hallqvist, R. Clayton, A. Virasami, K. Mills, N.J. Sebire, Proteomic profiling reveals sub proteomes of the human placenta, Placenta 59 (2017) 69–72, https://doi.org/10.1016/j.placenta.2017.09.014.

[15] G.J. Burton, N.J. Sebire, L. Myatt, D. Tannetta, Y.-L. Wang, Y. Sadovsky, A.C. Staff, C.W. Redman, Optimising sample collection for placental research, Placenta 35 (2014) 9–22, https://doi.org/10.1016/j.placenta.2013.11.005.