Appendix A

details of sample preparation protocol and LC-MS analysis

PERINATAL CHANGES IN PLASMA OF TYPE 2 DIABETES MELLITUS PREGNANCIES WHO DELIVERED NEWBORNS WITH CARDIOMYOPATHY, DEPRESSION OF THE CENTRAL NERVOUS SYSTEM, OR HEPATOMEGALY

Appendix A

Reagents used throughout the workflow

Urea (99%) and formic acid (98%+, pure) were obtained from Acros Organics (Geel, Belgium). Trifluoroacetic acid (99%, Reagent Plus®), triethylammonium bicarbonate (1 M solution), 4-vinylpyridine (95%), sodium deoxycholic acid (>97% titration) were from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade, filtered for 0.2 µm) was purchased from Fisher Chemical (Loughborough, UK). Acetic acid (EMSURE®, glacial, anhydrous for analysis) was from Merck (Darmstadt, Germany). TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) was purchased from Pierce™ (Thermo Fisher, Rockford, IL, USA). Trypsin (sequencing grade modified) was supplied by Promega (Madison, WI, USA). Water (TOC<3 ppb) was obtained from Milli-Q Integral 3 purification system, Millipore S.A.S (France).

Samples preparation protocol

Following overnight fasting peripheral venous blood samples in a volume up to 6 mL (approximately) were collected into EDTA-2K+ vacuum tubes (BD, USA). Blood samples were collected in the morning between 8 a.m. to 10 a.m. Plasma fractions were obtained according to the manufacturer's instructions: tubes were not chilled immediately after blood samples collections; tubes were centrifuged at 10^{o}C and acceleration of 2500 *g* for 10 min. Obtained plasma fractions were retrieved and a volume of 1 mL of each sample was filtered through 0.22-µm cellulose-acetate filters. Filtered plasma samples in a volume of 0.5 mL were transferred into the clean plastic tubes and stored at -80°C until use.
Before further proceeding, the total protein concentration was determined by the BCA (Smith assay) Protein Assay Kit ab102536 (Abcam, Cambridge, MA, USA).

For samples preparation, 100 µg of the estimated proteins fraction were taken and diluted to 20 µL in denaturation buffer solvent comprised 5M urea, 7.5 mM TCEP, 100 mM TEABC and 1% sodium deoxycholic acid. Diluted plasma samples were incubated at 40^{o}C for 30 minutes for completion of cysteins residues reduction. Then, a volume of 2 µL of alkylating solvent (2% solution of 4-vinylpyridine in 30% isopropanol) was added to diluted reduced samples and the reaction mixtures were incubated for 20 minutes at ambient temperature in darkness. After completion of alkylation reaction, samples were diluted to 200 µL finally by buffering solvent of 100 mM TEABC to reduce concentration of urea and sodium deoxycholic acid, and to achieve pH level of 8.5 amenable for enzymatic digestion. Digestion was performed with trypsin at a ratio of 1:100 (w/w) at 37^{o}C for 3 hours. Next step, additional amount of trypsin (500 ng, ratio 1:200, w/w) was added to each sample and the reaction was incubated for 3 hours at 37^{o}C.

The reaction was quenched by 10% formic acid added in a volume of 10 µL to 0.5% final concentration. Samples were centrifuged at 12 000 *g* at 10^{o}C to sediment undissolved deoxycholic acid. Supernatants were collected into the new clean tubes and dried under vacuum at 30^{o}C for about 90 minutes. Obtained pellets were re-dissolved in 100 µL of 0.5% formic acid for LC-MS analysis.

Liquid chromatography and high-resolution mass spectrometry analysis

High resolution LC-MS analysis was performed on an Orbitrap Fusion (Thermo Scientific, Waltham, MA, USA) mass spectrometer equipped with nano-flow NSI ion source and coupled with an Ultimate 3000 RSLC Nano (Thermo Scientific, Waltham, MA, USA) liquid chromatography system. Samples (in a volume of 1 µL) were loaded for 4 at a rate of 15 µL/min for enrichment onto an Acclaim PepMap® (5 mm x 0.3 mm, 300A pore size, 5 µm particle size) column. The enrichment column was continual washed in an isocratic mode in a mobile phase C comprised water with 3.5% acetonitrile supplied with 0.1% formic acid and 0.05% acetic acid (pH about 2.63 at t=20^{o}C). The retained peptides were washed out from the

enrichment onto analytical column Acclaim Pepmap® (75 µm x 150 mm, 1.8 µm particle size, 60A pore size) at a flow rate of 0.30 µL/min for chromatographic separation in a gradient of mobile phase A (water) and mobile phase B (90% acetonitrile and 10% methanol) both supplied with 0.1% formic acid and 0.03% acetic acid. The following gradient was applied: 0-7 minutes – 2.5%-5% of B; 7-22 minutes – 5%-18% of B; 22-37 minutes – 18%-25% of B; 37-44 minutes – 25%-33% of B; 44-47 minutes – 33%-80% of B; 47-48 minutes – 80%-95% of B; 48-58 minutes – 95% of B at a flow rate of 0.45 µL/min; 58-60 minutes – 95%-20% of B at a flow rate of 0.45 µL/min; 60-62 minutes – 20%-2.5% of B at a flow rate of 0.3 µL/min; 62-75 minutes – 2.5% of B for columns system pre-conditioning and equilibration before the next run.

Mass-spectrometer was operated in a positive ionization mode. Precursor ions were acquired at a resolution of R=60K in a range of 425 – 1250 m/z and were isolated using quadrupole mass filter with an isolation window of ±1.0 *Th* and offset of +0.25 *Th*. Precursor ions were collected for the maximum integration time of 15 ms or until AGC (acquisition gain control) reached 3e6 ions saturation. Only ions fit with charge states of $z=2^{+}$-6^{+} were collected and triggered for tandem (MS/MS) analysis.

Fragment ions were obtained in a collision cells carrying nitrogen (N_2) as a collision gas. Precursor ions were decomposed using HCD (high-energy collision dissociation) activation energy normalized at 27% and ramped within ±20%. The decomposed ions were accumulated for a maximum integration time of 47 ms, or until AGC reached saturation of 5e5 ions, and recorded in an ultra-high field orbital mass analyzer at a resolution of R=15K.

Raw data files obtained after data-dependent LC-MS/MS analysis were converted in a peak list format using MSConver (Proteowizard; http://proteowizard.sourceforge.net/download.html) and used for proteins identification. Peak lists obtained from spectra were identified using OMSSA version 2.1.9. The search was conducted using Search GUI version 2.3.17 **[S1]**. Protein identification was conducted against a concatenated target/decoy database of human proteins (Uniprot release December 2019). The decoy sequences were created by reversing the target sequences in Search GUI. The identification settings were as follows: trypsin as a specific protease, with a maximum of 1 missed cleavage, tolerance of ±5 ppm as MS1 level and tolerance of ±0.0025 Da as MS2 level tolerances; variable modifications: oxidation of M (+15.994915 *u*), deamination of N (+0.984016 *u*), deamination of Q (+0.984016 *u*). Peptides and proteins were inferred from the spectrum identification results using Peptide Shaker version 1.16.15 **[S2]**. Peptide Spectrum Matches (PSMs), peptides and proteins were validated at a 1.0% false discovery rate (FDR) estimated using the decoy hit distribution **[S3]**. Excluded from validation were proteins identified by site only, external contaminants, and reversed proteins.

- **[S1]**. Vaudel, M.; Barsnes, H.; Berven, F.S.; Sickmann, A.; Martens, L. SearchGUI: An open-source graphical user interface for simultaneous OMSSA and X!Tandem searches. Proteomics. 2011, 11, 996–999; DOI: 10.1002/pmic.201000595
- **[S2]**. Vaudel, M.; Burkhart, J.M.; Zahedi, R.P.; Oveland, E.; Berven, F.S.; Sickmann, A. et al. PeptideShaker enables reanalysis of MS-derived proteomics data sets. Nat Biotechnol. 2015, 33, 22–24; DOI: 10.1038/nbt.3109
- **[S3]**. Elias, J.E.; Gygi, S.P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods. 2007, 4, 207–214; DOI: 10.1038/nmeth1019