ESM Methods

Statistical power calculations The calculated sample size needed for clinical multiple reaction monitoring (MRM)–mass spectrometry (MS) measurements in order to detect >10% changes in serum biomarker levels with an expected standard deviation of 25% and a type I error rate $\alpha$ of 0.05 and type II error rate $\beta$ of 0.10 (90% statistical power), is 66 gestational diabetes mellitus (GDM) cases and controls respectively. This criterion was met for the obese group (GDM, $n=134$ and controls, $n=139$) even when only the 75% training set was analysed (GDM, $n=101$ and controls, $n=104$). For the non-obese group (GDM, $n=64$ and controls, $n=69$) however, the number of GDM cases was slightly lower than required and when considering the training set separately the discrepancy was even higher (GDM, $n=48$ and controls, $n=52$). This deficiency in sample numbers for non-obese GDM cases likely explains the difficulty of the MRM-MS assay to produce more significant results for this group.

Sample preparation and MRM-MS analysis In order to optimise manageability and robustness of the assay procedure, the 407 samples were divided at random into batches of 20 samples (10 controls and 10 GDM cases in each) within the obese and non-obese group. Each batch was prepared and analysed by MRM-MS, including 3 replicas of the same serum pool for CV calculations.

Sample preparation for MRM-MS analysis was performed mainly as described by Overgaard et al [1]. Serum samples were diluted 1:20 in 50 mmol/l ammonium bicarbonate and 15 $\mu$l were subjected to an equal volume of 100% trifluoroethanol for 1 h at 65°C. Dithiothreitol was added to a final concentration of 7 mmol/l for 30 min at 65°C and samples were alkylated by 20 mmol/l iodoacetamide for 30 minutes at room temperature in the dark. Following a 1:5 dilution of the samples in 25 mmol/l ammonium bicarbonate, digestion was done by incubation with 1:20 w/w of Porcine trypsin (a gift from Novo Nordisk A/S, Bagsvaerd, Denmark, www.novonordisk.com) for 19-20 h at 37°C. Digestion was terminated by addition of formic acid (FA) at a final concentration of 1%.

Individually adjusted amounts of isotopically heavy labeled standard peptides (+8 Da or +10 Da), SpikeTides™_L (JPT Peptide technologies, Berlin, Germany), aiming at a ratio 1:1 to endogenous light peptide, were added to each sample before purification on Oasis HLB 10 mg cartridges (Waters). Samples were dried down and reconstituted in 0.1% FA. For each
sample 1 µg was run on a Easy-nLC II nano LC system (Thermo Scientific) using a C18 trapping column, 2 cm, inner diameter 100 µm, 5 µm, 120 Å (Thermo Scientific) for desalting and a C18 analytical column, 10 cm, inner diameter 5 µm, 3 µm, 129 Å (Thermo Scientific) for separation. For peptide elution a 60 min gradient of acetonitrile (ACN) in 0.1% FA was applied in three steps (5→10% ACN in 5 min, 10→33% ACN in 43 min and 33→100% ACN in 4 min and 100% ACN for 8 minutes) at a flow rate of 300 nl /min. Peptides were ionised with a Nanospray Flex ion source (Thermo Scientific) and analysed on a TSQ Vantage triple quadruple mass spectrometer (Thermo Scientific) in selected reaction monitoring mode.

**MRM-MS assay development**  For each of the 46 proteins a maximum of 8 peptides, represented by all possible transition pairs, comprising precursors of charge states +2 or +3 and product ions of charge states +1 or +2, respectively (y-ions >y3; mass to charge ratio <1500), were tested in the MRM-MS assay development process. All peptides selected were tryptic (arginine /lysine cleavage) and where included if peptide length was in the range 7-25 amino acid residues. Peptides where excluded if containing any of the following: NXS/T motif (N-linked glycosylation site), missed cleavages, methionines (oxidation), histidines, cysteines or serine and threonine rich areas, proline following arginine or lysine). All selected peptides where finally subjected to BLASTP analysis for uniqueness in the human proteome and excluded if redundancy was observed. The MRM-MS assay was tested on a pool of first trimester serum and correct peptide identities were confirmed by addition of coeluting spike-in isotopically heavy labeled standard peptides. Collision energies were optimized and the MS signals of all transition pairs from heavy labeled spike-in peptides and light endogenous peptides were manually evaluated for best performance and robustness. The final assay comprised 25 proteins represented by 43 (86) peptides and a total of 182 (364) transitions. Numbers in parenthesis include heavy isotope peptide standards.

**Immunological assays**  Adiponectin and resistin levels were measured by ELISA kit DRP300 and DRSN00 (R&D systems) according to the manufactures manual with the following exceptions; for DP300 the kit washing buffer was substituted for DELFIA Wash Concentrate (PerkinElmer) and samples were diluted 1:140 or 1:280. Absorbance was measured using a Victor X5 (PerkinElmer, Waltham, MA, USA) at wavelength 450 nm. For CV calculations 4 replicas of the same serum pool were included on each plate. Interassay CVs for adiponectin and resistin measurements were 3.9% and 3.6% respectively.
Doubletest for serum PAPP-A and free β-hCG was performed as part of routine Downs screening with a 1235 AutoDELFIA analyser, using an automated time-resolved fluoro-immunoassay (AutoDELFIA; PerkinElmer, Turku, Finland).

For method comparison analysis 60 serum samples were selected, representing the range of the determined levels of SHBG, Apo B and fibronectin as determined by MRM-MS, respectively.

Serum SHBG was measured using the Immulite 2000 SHBG test, a solid-phase, two-site chemiluminescent immunoassay (L2SH2; Siemens Healthcare Diagnostics). Interassay CV was 5.1% at 4.55 nmol/l and 7.8% at 70.7 nmol/l. Apo B and fibronectin were quantified using Quantikine ELISA kit DAPB00 and DFBN10 (R&D systems) according to manufactures recommendations. Intraassay CV’s for Apo B and fibronectin were 9.7% and 1.7%, respectively.

**ESM references**