A preliminary study on human placental tissue impaired by gestational diabetes: a comparison of gel-based vs. gel-free proteomics approaches

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KEYWORDS: gel-based proteomics, label-free proteomics, human placenta, gestational diabetes, mass spectrometry.

Abstract

Gestational diabetes (GDM) is the most common complication of pregnancy and it is associated with maternal and fetal short- and long-term consequences. GDM modifies placental structure and function, but many of the underlying mechanisms are still unclear. The aim of this study is to develop and compare two different methods, based respectively on gel-based and gel-free proteomics, in order to investigate the placental proteome in the absence or in the presence of GDM and to identify, through a comparative approach, possible changes in protein expression due to GDM condition.

Placenta homogenates obtained by pooling 6 control samples and 6 samples from GDM pregnant women were analyzed by 2D electrophoresis coupled with mass spectrometry (nLC-MS/MS and MALDI-MS) and by a label-free mass spectrometry method based on LC-MS^E.

The gel-based approach highlights 13 over-expressed proteins and 16 under-expressed proteins, while the label-free method shows the over-expression of 10 proteins and the under-expression of 9 proteins. As regards 2D-gel electrophoresis, a comparison between two different protein identification methods, based respectively on nLC-ESI-MS/MS and MALDI-MS/MS, was performed taking into consideration the Sequence Coverage, the MASCOT Score and the emPAI index.

The analysis of complex proteome through an integrated strategy revealed that the quantitative gel-free and label-free MS approach might be suitable to identify candidate markers of GDM.

Introduction

Gestational diabetes mellitus (GDM), defined as any degree of glucose intolerance with onset or first recognition during pregnancy, that is not clearly overt diabetes, affects from 5–6% to 15–20% of pregnancies worldwide, depending on population demographics, screening methodology, diagnostic criteria in use and maternal lifestyle [1]. Advanced maternal age, ethnicity, obesity and family history of type 2 diabetes are associated with an increased risk of maternal complications, such as hypertensive disorders, preterm delivery, operative delivery and subsequent development of diabetes mellitus type 2 in adult life [2-4]. Fetal and neonatal complications include macrosomia, neonatal hypoglycemia, respiratory distress syndrome, jaundice and long-term consequences such as childhood obesity and metabolic syndrome in adults [5]. An early identification of GDM is critical to providing an opportunity for the application of primary prevention strategies during pregnancy and early in life [6].

Moreover, human placenta in presence of GDM undergoes a number of functional and structural histomorphometric abnormalities compared to women with normoglycemia; in particular an higher incidence of placental immaturity, fibrinoid necrosis, chorangiosis, villous ischemia and immaturity have been described [7,8]. These alterations seem to lead to an increase in placenta weight, diameter and in central thickness compared to control placentas [9].

Proteomics, i.e. the systematic analysis of all the proteins expressed by the genome of a cell, tissue or organism in a specific moment and in relation with well-defined environmental and pathological conditions [10], is one of the –omics approaches underlying system medicine, together with metabolomics, genomics and metallomics. Proteomics has a great potential in the clarification of the etiology and pathogenesis of many diseases and in the development of new methods for diagnosis, prognosis and evaluation of medical treatments. In particular, the changes caused by a specific disease, drug or physiological activity towards the various biochemical pathways characteristic of an organism, induce modifications or alterations in protein expression, both from a quantitative and qualitative point of view. The determination of these modifications permits the identification of novel

biomarkers, which in principle might be useful in the management of diseases in clinical practise [11,12]. Recent advances in the field of liquid chromatography/capillary electrophoresis and mass spectrometry, such as nLC-MS^E [13], made it possible to work out new highly sensitive and efficient methods for biomarkers discovery [14].

Obstetrics is one of the branches of medicine that has benefits from these techniques. Numerous proteomics studies regarding pre-eclampsia, perinatal infections, preterm birth and intrauterine growth restriction [15,16] are reported in scientific literature [12].

Considering GDM, both gel-based and gel-free proteomic approaches [17-19] have been used to identify differentially expressed proteins between GDM and healthy pregnants. Most of these studies, reviewed by Singh et al. [20], have been limited to the analysis of serum/plasma, urine or amniotic fluid samples.

Altered protein expression in placenta tissue from GDM women has also been demonstrated by Liu et al. [21]. In particular, placenta villi homogenate was analysed by 2D gel electrophoresis followed by MALDI-TOF/TOF mass spectrometry. The study reported twenty-one protein spots differentially expressed in placental tissue from GDM women and the identified proteins are involved in insulin resistance, transportation of glucose, coagulation and fibrinolysis.

The aim of this study is the development of a rapid and simple mass spectrometry-based method for the analysis of the placental proteome. Both gel-based and gel-free proteomics approaches were compared and applied in a preliminary investigation, in order to identify possible alterations of the placental proteome in pregnancy complicated by GDM compared to normal.

Material and methods

Tissue collection and protein extraction

Human placenta villi from 6 healthy pregnant women and 6 patients affected by GDM were collected after birth. To remove excess of blood, placental samples were extensively washed with saline solution, until a clear solution was obtained. Samples were stored at -80 °C until treatment.

GDM was diagnosed according to IADPSG criteria (OGTT 75 gr), in particular between the 24th-28th gestational week (g.w.) [22]. Cases with high risk for GDM, i.e. pregnant women with BMI (Body Mass Index) > 30, previous GDM, impaired glucose tolerance or overt diabetes, were excluded. [23]. A dietician prescribed all GDM patients with individualized medical nutrition therapy, taking into consideration their gestational age, pre-pregnancy BMI, and physical activity levels. None of GDM patients needed insulin therapy during pregnancy. Clinical data of the women under study are reported in Table 1.

The study protocol complied with the Helsinki Declaration and was approved by the local Ethical Committee.

On the day of the analysis, for each subject under investigation, placental tissue fragments were subjected to protein extraction. Tissue samples were chopped, solubilized in a buffer containing 100 mmol/L Tris, 0.1%w/v SDS pH 7 (1g in 1ml buffer) and sonicated for 15 sec on ice. A second extraction was performed diluting the sample with 9 volume of urea buffer (8 mol/L urea, 2 mol/L thiourea, 4% w/v CHAPS, 0.8% v/v carrier ampholytes, pH 3-10, 20 mmol/L Tris, 55 mmol/L DTT, and bromophenol blue). After 1 hour on a rotary shaker at room temperature, samples were centrifuged for 20 minutes at 13000 x g at room temperature.

Placental extracts from control subjects and GDM patients were pooled in two groups obtaining the control sample and the GDM sample.

Protein concentration was evaluated by means of the Bradford protein assay (Bio-Rad, Milan, Italy) [24].

Two-dimensional electrophoresis (2-DE) and protein identification

2-DE was carried out in accordance with the manufacturer's protocol (Protean IEF cell, Bio-Rad, Milan, Italy) as previously described [25]. Briefly, 2-DE was performed on control and GDM samples, analysing each sample in triplicate. Isoelectrofocusing was performed on IPG ready strips, 17 cm, pH3-10 non linear gradient (Biorad, Milan, Italy), actively rehydrated at 50 V for 24 h with a final concentration of carrier ampholytes of 1.5%, and focused for a total of 50 KVh.

Progenesis SameSpot software (v 4.5, TotalLab Ltd, Newcastle upon Tyne, UK) was used for gel alignment, spot detection, spot quantification, and normalisation for total spot volume in each gel, and the data were statistically analysed using the incorporated statistical package, that included statistical analysis calculations such as Anova p-value [25]. A p value <0.05 was considered as statistically significant with a fold change cut-off of 1.2.

The protein spots selected for mass spectrometry analysis by LC-MS/MS were in-gel digested with trypsin as previously described [26]. Peptides analysis was performed by means of LC-ESI-MS/MS, with a hybrid quadrupole orthogonal acceleration time-of-flight Q-Tof mass spectrometer, Synapt-MS (Waters corporation, Manchester, UK) connected to a Nano-Acquity UPLC system. The samples, dissolved in 0.1% formic acid, were injected onto a TRIZAIC nanoTile (Waters corporation, Manchester, UK) and the elution was performed at a flow rate of 450 nL/min by increasing the concentration of solvent B (0.1% formic acid in acetonitrile) from 3 to 40% in 30 min, using 0.1% formic acid in water as reversed phase solvent A. Calibration and lockmass correction was performed as previously described [27]. The capillary voltage was set to 3800 V. A survey scan over the m/z range of 350–1990 was used to identify protonated peptides with charge states of 2, 3 or 4, which were automatically selected for data-dependent MS/MS analysis (Mass links v4.1 SCN639, Waters). All raw MS data were processed with PLGS software (version 2.5.3, Waters corporation, Manchester, UK) and the proteins were identified by correlating the uninterpreted spectra with entries in UniProt as previously described [27]. A UniProt database (release 2014-8; number of human sequence entries, 20195) was used for database searches of each run.

MALDI-MS and MALDI-MS/MS measurements

MALDI-MS measurements were performed using an UltrafleXtreme MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany), equipped with 1 kHz smartbeam II laser (λ= 355 nm) operating in reflectron positive ion mode. The instrumental conditions were: IS1= 25.00 kV; IS2= 22.40 kV; lens= 8.00 kV, reflectron potential= 26.45 kV; delay time= 120 ns. The matrix was α-cyano-4-hydroxycinnamic acid (saturated solution in H_2O/Acetonitrile (50:50; v/v) containing 0.1% TFA). Five microliters of sample and 5 μL of matrix solution were mixed and 1 μL of the resulting mixture was deposited on a stainless steel sample holder and allowed to dry before introduction into the mass spectrometer. External mass calibration (Peptide Calibration Standard, Bruker Daltonics, Bremen, Germany) was based on monoisotopic values of $[M+H]^+$ of Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip (1–17), ACTH clip (18–39), Somatostatin 28 at m/z 1046.5420, 1296.6853, 1347.7361, 1619.8230, 2093.0868, 2465.1990 and 3147.4714, respectively. MS/MS experiments were performed using the LIFT device [28] in the following experimental conditions: IS1: 7.5 kV; IS2: 6.75 kV; Lift1: 19 kV; Lift2: 3.7 kV; Reflector1: 29.5 kV; delay time: 70 ns.

Label free LC-MS^E analysis

On the day of analysis, placenta extracts from the two different groups, prepared as described above, were subjected to protein precipitation with the Protein precipitation kit (Calbiochem, MerckMillipore, Milan, Italy) according to manifacturer's instruction. Protein pellets were then dissolved in 25 mmol/L NH_4HCO_3 containing 0.1% RapiGest (Waters Corporation, Milford, MA, USA) and digested as previously described [27].

The control and GDM samples (0.5 μg) were then mixed with 50 fmol yeast alcohol dehydrogenase (ADH) digest as an internal standard for molar amount estimation [29] and quality control.

Tryptic peptides separation was conducted with a TRIZAIC nanoTile using a nanoACQUITY UPLC System coupled to a SYNAPT-MS Mass Spectrometer equipped with a TRIZAIC source. The TRIZAIC nanoTile used for this study, Aquity HSS T3, integrates a trapping column (5 μm, 180 μm x 20 mm) for desalting and an analytical column (1.8 μm, 85 μm x 100 mm) for peptide separation

with an high level of reproducibility of retention time. Elution was performed at a flow rate of 550 nL/min by increasing the concentration of solvent B (0.1% formic acid in acetonitrile) from 3 to 40% in 90 min, using 0.1% formic acid in water as reversed phase solvent A. Calibration and lockmass correction was performed as previously described [30]. Precursor ion masses and their fragmentation spectra were acquired in MS^E mode as previously described [30] in order to obtain a qualitative and quantitative analysis of placenta tissues.

The software Progenesis QI for proteomics (Version 1.0, http://www.nonlinear.com) was used for the quantitative analysis of peptide features and protein identification. Analysis of the data by Progenesis QI included retention time alignment to a reference sample, feature filtering (based on retention time and charge (>2)), normalisation considering all proteins, peptide search and multivariate statistical analysis. The principle of the search algorithm has been previously described in detail [31]. The following criteria were used for protein identification:1 missed cleavage, Carbamidomethyl cysteine fixed and methionine oxidation as variable modifications. A UniProt database (release 2014-8; number of human sequence entries, 20195) was used for database searches. The entire data set of differentially expressed proteins was further filtered by considering only the identifications from data with identified peptides that replicated at least two out of three technical instrument replicates.

Fold changes in the quantitative expression, p-value and Q-value were calculated with the statistical package included in Progenesis QI for proteomics, using only unique/proteotypic peptides to quantify proteins that were part of a group. A p-value <0.05 was considered significant. The significance of the regulation level was determined at a 20% fold change, but only proteins identified with at least 2 peptides were considered. The data set was then subjected to unsupervised PCA analysis.

Statistics

Values are expressed as median and interquartile range. Student's t-test was used for the multiple comparison of continuous variables. Differences were deemed statistically significant when $p<0.05$.

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Results

As showed in Table 1, GDM women and controls did not differ in terms of metabolic characteristics, maternal and fetal outcomes.

Proteomic analysis of the effect of gestational diabetes (GDM) on placenta tissues by means of two-dimensional electrophoresis (2-DE)

In a previous investigation [32], it was reported that the excess of blood, intrinsic of placenta samples, negatively affected the results achievable from the analysis of the placenta protein profile. In the present study, in order to prevent blood contamination, placenta tissues have been subjected, as described in the method section, to extensive washes with an isotonic saline solution. Furthermore, placenta samples were subjected to a double extraction protocols in order to maximize the yield of extracted proteins (3.84±1.02 mg proteins extracted from 100 mg tissue).

Considering the large biological variability among samples from subjects of the same group, it was retained that an investigation on pooled samples was to be preferred in this first stage concerning the development and optimization of the analytical method.

Placenta proteins were resolved on a 12% polyacrylamide gel after isoelectrofocusing on 3-10 non linear pH gradient IPG strips. Around 1000 spots were visualised by Coomassie staining and aligned with Progenesis SameSpot for spot quantitation. A total of 26 protein spots were found to vary significantly in GDM placenta tissues in comparison with healthy controls placenta tissues (Figure 1). For example, the protein species contained in spot n° 824 has shown a 28% decrease of its abundance in case of GDM samples, while the protein contained in spot n° 283 has shown a 61% increase of its abundance in case of GDM samples compared to control (details for other spots are reported in Table 2). Spots of interest were in-gel digested with trypsin and underwent LC-MS/MS for protein identification. Peptide mass fingerprints (PMF) from MALDI-MS and MALDI-MS/MS experiments, performed for some of the digested spots (Supplementary Table S1), confirmed the identification obtained by the LC-MS/MS method (spots 824, 910, 904, 1150, 1697, 283, 1483, 1584)

although for some of the species of interest it was not possible to obtain significant information (spots 1260, 1342, 1031, 284,1695).

In particular 17 spots were more abundant in placenta from healthy subjects and 16 protein were identified (Figure 1, Table 2 and Supplementary Table S2), showing that these spots were attributable to different isoforms of specifically altered proteins, such as fibrinogen alpha, beta and gamma chains or Tubulointerstitial nephritis antigen like protein.

Figure 2 specifically shows the results related to different spots that were all identified as Fibrinogen beta chain.

On the other hand, 9 spots were more abundant in GDM placenta tissues (Figure 1) and were attributed to 13 proteins (Table 3 and Supplementary Table S2) among which the Chorionic somatomammotropin hormone (CSH). Figure 3 specifically shows the behavior of Chorionic somatomammotropin hormone spot.

Proteomic analysis of the effect of gestational diabetes (GDM) on placenta tissues by means of LC/MS^E

To investigate on the proteome profiles of healthy and GDM placenta tissues with this MS-based approach we pooled 6 controls and 6 GDM placenta protein extracts. After protein precipitation, to eliminate the high amount of salts used for protein extraction, we digested them with trypsin and we compared the peptides mixtures by means of a label-free MS-based proteomic approach, LC-MS^E.

By this method, differences can be evidenced. The corresponding ion intensity maps generated by Progenesis QI, reported in Figure 4 (4A for samples from healthy subjects and 4B for samples from GDM patients), summarize these results.

Quality controls of the data were made in order to determine the analytical reproducibility using PLGS 2.5 software (Waters, Manchester, UK) and the Expression^E clustering algorithm and were confirmed by the good alignment of the ion intensity maps (score 98±0.3 %) and the similarity of the normalisation factors (0.93±0.08) obtained with Progenesis QI for proteomics. The mass precision of

the extracted peptides was calculated as 1.5 ppm and 1.3 ppm median levels for control and GDM, respectively. The variability of the intensity measurements between the technical replicates showed a coefficient of variation below 4.4% for both samples and the reproducibility of the retention time was also very high, with CV of only 0.3% (median level for both samples).

Quantification was carried out by means of Progenesis QI for proteomics comparing a total of 2849 peptides corresponding to 159 proteins and revealed that 10 proteins were more abundant in GDM placenta tissue and 9 were less abundant in GDM placenta tissues.Supplementary Tables S3 and S4 show the complete list of the proteins and peptides identified in placenta tissue extract by means of LC/MS^E. Table 4 and 5 shows the complete list of proteins with higher abundance in control and GDM placenta tissues, respectively.

Among proteins with different abundance in healthy and GDM tissue it is noteworthy the increased level of Chorionic somatomammotropin hormone and the decreased levels of Tubulointerstitial nephritis antigen-like and Fibrinogen alpha, beta and gamma chains, as demonstrated also by means of 2-DE analysis.

Discussion

Proteomics, i.e. the study of the entire protein content of a cell or a tissue in relation to a well-defined pathological state, would allow the detection of disease-related biomarkers that might be useful for diagnosis, therapeutic monitoring or for the identification of new protein targets for the development of improved intervention therapies.

In the first part of the study, placental tissue homogenates from GDM and healthy pregnant women were analyzed by means of 2D electrophoresis: 13 up-regulated and 16 down-regulated proteins were identified in placenta samples from GDM women compared to healthy subjects. The proteins contained in these spots were enzymatically digested and analyzed by LC-MS/MS and MALDI-MS/MS in order to obtain their ID. In a previous study Liu et al [21] demonstrated, by means of MALDI-MS/MS mass spectrometry, that fibrinogen beta chain and fibrinogen alpha chain are under-expressed in GDM placenta. As expected, our data confirmed these previous results.

However, the LC-MS/MS approach revealed that many of the electrophoretic spots contained more than one protein and consequently it was not possible to establish for a single spot which was the protein differently regulated. Partly emPAI index (reported in Table 2 and Table 3), which is proportional to the protein content in a complex protein mixture [33], helps to estimate which is the protein species contained in a particular spot with the highest concentration, that in principle mostly affect the altered abundance of the spot itself. However, considering emPAI index some hypotheses might be formulated, but it is difficult to obtain accurate and quantitative data.

Using these techniques, significant results were obtained only for Fibrinogen beta chain that is down-regulated in GDM placenta and for Collagen alpha 2 VI chain and Chorionic somatomammotropin hormone that show an higher abundance in the case of GDM. Furthermore, Fibrinogen beta chain is located in various spots and this is probably due to the presence of different isoforms or to possible modifications of the amino acidic residues (mass-spectrometric data has not confirm this last hypothesis).

In order to compare and better analyze our results with the ones obtained by Liu et al. [21], PMF spectra of the digestion mixture resulting from some of the electrophoretic spot of interest were acquired by means of MALDI-MS and some of the peptides detected were subjected to MS/MS analysis. The interpretation of the PMF spectra with MASCOT (http://www.matrixscience.com) confirmed the identity for the majority of the proteins. Interestingly, MALDI-MS analysis allowed to detect only one proteins for each spot, instead of multiple protein species as in the case of LC-MS/MS. Similar observations are deducible from the work by Liu et al. [21]. It is noteworthy that the % Coverage values obtained by MALDI-MS and LC-MS/MS approaches are comparable, while the Mascot Score values are usually lower in the case of PMF analysis. In fact, LC-MS/MS allows to obtain a greater number of MS/MS data, instead of MALDI-MS/MS in which the quality of MS/MS spectra is negatively affect by the low abundance of some of the ions present in the PMF (due to ion suppression effects) and by the limited efficiency in parent ions isolation in the MS/MS experiments. Since MASCOT score also depends on this factor, LC-MS/MS analysis turns out in higher score values if compared with MALDI-MS/MS approach (Table 2 and Table 3).

Also the concentration of the protein in the electrophoretic spot affects the possibility to obtain the correct identification by mean of MALDI-MS analysis of the digestion mixture. Considering emPAI index as an estimate of the protein content in a spot, it is important to note that a correct and statistically significant identification species is possible only for the proteins that show an emPAI index greater than 0.9.

In conclusion, our data demonstrate that the analysis of the placenta tissue homogenate by 2D-gel electrophoresis followed by LC-MS/MS and MALDI-MS/MS, despite the potential advantage in evaluating PTMs, presents some weaknesses. In particular, the technological improvements in protein identification using nanoLC-MS/MS demonstrate that electrophoretic spots contain more than one protein, while MALDI-MS/MS allows the identification of only one protein per spot, thus complicating the interpretation of the results.

To overcome the limitations of the gel based method, a gel-free approach was applied to placenta tissues. The quantification and identification of the proteins were based on proteolytic digestion of the whole placental tissue homogenate, followed by a label-free LC-MS^E method. LC-MS^E consists of a reproducible chromatographic separation system hyphenated with an high resolution orthogonal time-of-flight mass spectrometer. In this approach, the mass spectrometric analysis is carried out by alternating the acquisition of spectra at lower and higher collision energy, in order to generate accurate peptide fragmentation data. The product ions are then correlated to precursor ions, in their turn used to identify the proteins, while the data originating from the integration of the chromatographic peak areas are employed for the determination of the differences in protein abundances. [17]. This approach allowed the detection of 159 proteins, of which 10 over-expressed in GDM placental tissue and 9 under-expressed compared to normal placenta. Some of these proteins, such as Fibrinogen beta chain and chorionic somatomammotropin hormone confirm the data obtained with the previous described gel-based approach.

In order to provide a clinical significance to the collected data, the specific cellular component and the relative biological processes of the different expressed proteins in GDM placenta were determined by Gene Ontology (GO) terms. Considering only cellular component distribution, it was demonstrated that the identified proteins were present mainly in the cell compartment (cytosol, organelle and membrane), but also in the extracellular region. On the other hand, in terms of biological process, the pattern is more complex revealing the presence of proteins involved in cellular processes (i.e. cell cycle, cell communication, cellular component movement), metabolic processes (i.e. byosinthetic processes, catabolic processes), cellular component organisation and localisation, but, most importantly, developmental processes (i.e. cell differentiation, system development, ectoderm and mesoderm development).

In addition, this last approach point out a series of statistically significant alterations in the abundances of some of the proteins expressed by the placental tissue in presence of GDM. In our opinion, based on the study of placental tissue, the gel-free approach provides more reliable and

complete information than the gel-based method, which also turns out to be more expensive, time-consuming and poorly automatable.

One possible limitation of this study is the low number of identified proteins. However, it is clear that technological improvements obtained with more recent high resolution mass spectrometers could increase the number of proteins identified through a gel free approach, but could not improve the performance of the 2-DE approach.

Furthermore having as purpose the optimization of the analytical method, the present investigation was carried out on two pooled samples, composed of placenta tissues from 6 healthy pregnant women and 6 GDM patients respectively. Aware of this limit and also to better characterized the molecular pathway of GDM, further studies concerning an higher amount of samples, individually analyzed, are in progress.

Acknowledgement

This study was developed within the project “A clinical, metabolomics and proteomics approach to the study of gestational diabetes and the investigation on potential markers for early prediction and diagnosis” (CPDA144008) with funding from University of Padova, Italy

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Table 1. Clinical characteristics of the pregnant women under investigation**.**

	CASES (5)	CONTROLS (6)	p
Age (years)	37 (32-39)	34 (30-38)	0.38
Para (nulliparous)	50%	50%	NS
Pre-pregnancy BMI	23 (23-32)	21 (19-25)	0.17
BMI at term	32 (27-33)	27 (22-28)	0.12
Weeks at delivery	38 (37-40)	39 (38-40)	0.64
Modality of delivery (CS n°)	60%	50%	NS
Weight at birth (gr)	3610 (3435-3960)	3550 (3035-3880)	0.95
Percentile of weight at birth	(79-86)	80 (70-85)	0.31
Lenght at birth (cm)	49 (49-51)	49 (48-53)	0.39
Fetus Sex [Male (%)]	60%	50%	NS
Apgar at 5’	1**0 (9-10)**	**10 (10-10)**	0.42

Data are espressed as median and interquartile range

Table 2. List of the proteins identified by LC-MS/MS from spots with lower abundance in GDM placenta tissues as assessed by 2-DE electrophoresis

#	Anova (p)	Fold	Accession[a]	Protein Description	Protein MW	Protein pI	Mascot Score LC-MS/MS	emPAI	% Coverage LC-MS/MS	% Coverage MALDI-MS	Mascot Score MALDI-MS
824	0.045	1.28	P02675	Fibrinogen beta chain	55892	8.3	1033	1.66	46.84	49.7	140
886	0.013	1.40	P02675	Fibrinogen beta chain	55892	8.3	861	2.81	40.94	-	-
			Q9GZM7	Tubulointerstitial nephritis antigen like	52352	6.5	95	0.14	10.92		
			Q9NVA2	Septin 11	49367	6.4	42	0.34	4.43		
910	0.032	1.31	P02675	Fibrinogen beta chain	55892	8.3	827	1.93	43.18	31.6	86
			Q9GZM7	Tubulointerstitial nephritis antigen like	52352	6.5	264	0.61	33.19	NR	NR
904	0.048	1.26	P02675	Fibrinogen beta chain	55892	8.3	448	0.92	26.07	32.4	120
			Q9GZM7	Tubulointerstitial nephritis antigen like	52352	6.5	173	0.26	9.85	NR	NR
919	0.033	1.23	P02675	Fibrinogen beta chain	55892	8.3	1212	2.88	43.18	-	-
			Q9GZM7	Tubulointerstitial nephritis antigen like	52352	6.5	411	1.04	33.19		
			P36957	Dihydrolipoyllysine residue succinyltransferase component of 2 oxoglutarate dehydrogenase complex	48724	9.3	149	0.3	8.17		
			Q13228	Selenium binding protein 1	52357	5.9	35	0.2	7.84		
1010	0.020	1.26	P02675	Fibrinogen beta chain	55892	8.3	803	2.58	42.97	-	-
			P02768	Serum albumin	69321	5.9	68	0.32	9.52		
			P02679	Fibrinogen gamma chain	51478	5.2	64	0.16	7.50		
1143	0.002	1.46	P02675	Fibrinogen beta chain	55892	8.3	934	2.28	37.88	-	-
1150	0.041	1.35	P02675	Fibrinogen beta chain	55892	8.3	541	1.02	25.66	24.4	60
1253	0.016	1.49	P02671	Fibrinogen alpha chain	94914	5.6	419	0.7	9.24	-	-
			P04083	Annexin A1	38689	6.6	149	0.44	15.61		
1256	0.001	1.71	P02675	Fibrinogen beta chain	55892	8.3	406	0.97	24.24	-	-
1260	0.005	1.44	P02675	Fibrinogen beta chain	55892	8.3	257	0.66	9.36	NR	NR
			Q15006	Tetratricopeptide repeat protein 35	34811	6.1	154	0.36	11.11	NR	NR

1342	0.018	1.34	P02675	Fibrinogen beta chain	55892	8.3	71	0.12	7.54	NR	NR
			P02768	Serum albumin	69321	5.9	49	0.19	7.72	NR	NR
1693	0.009	1.38	P02675	Fibrinogen beta chain	55892	8.3	363	0.66	25.25	-	-
			P60709	Actin cytoplasmic 1	41709	5.1	286	0.57	23.47		
			P62736	Actin aortic smooth muscle	41981	5.1	282	0.7	21.75		
1697	0.029	1.33	P02675	Fibrinogen beta chain	55892	8.3	508	1.08	27.09	31.4	83
1700	0.015	1.44	P37802	Transgelin 2	22377	8.4	123	1.3	23.12	-	-
			Q01995	Transgelin	22596	9.3	61	0.32	16.92		
			P02671	Fibrinogen alpha chain	94914	5.6	266	0.4	6.93		
			P02675	Fibrinogen beta chain	55892	8.3	158	0.25	7.13		
			P30086	Phosphatidylethanolamine binding protein 1	21043	7.4	82	0.55	12.30		
1031	0.011	1.31	P02675	Fibrinogen beta chain	55892	8.3	330	0.47	16.09	NR	NR
1701	0.018	1.42	P02671	Fibrinogen alpha chain	94914	5.6	174	0.4	4.85		
			P02675	Fibrinogen beta chain	55892	8.3	125	0.18	9.78		
			P30086	Phosphatidylethanolamine binding protein 1	21043	7.4	307	1.8	40.11		

[a] Accession code in UniProt.

NR: not recognised (no significant ID was obtained for this protein)

Table 3. List of the proteins identified by LC-MS/MS from spots with higher abundance in GDM placenta tissue as assessed by 2-DE electrophoresis.

#	Anova (p)	Fold	Accession[a]	Protein Description	Protein MW	Protein pI	Mascot Score LC-MS/MS	emPAI	% Coverage LC-MS/MS	% Coverage MALDI-MS	Mascot Score MALDI-MS
283	0.034	1.61	P12110	Collagen alpha 2 VI chain	108511	5.7876	418	0.42	12.46	21.0	61
282	0.048	1.43	P12110	Collagen alpha 2 VI chain	108511	5.7876	403	0.42	10.89	-	-
			P02768	Serum albumin	69321	5.8608	245	0.7	20.53		
284	0.039	1.50	P12110	Collagen alpha 2 VI chain	108511	5.7876	394	0.5	11.68	NR	NR
			Q9Y6N6	Laminin subunit gamma 3	171116	6.1187	84	0.09	3.68	NR	NR
1228	0.017	1.49	P07195	L lactate dehydrogenase B chain	36615	5.6396	359	1.36	30.53	-	-
			O00764	Pyridoxal kinase	35079	5.6953	177	0.57	13.46		
1283	0.014	1.46	O15144	Actin related protein 2 3 complex subunit 2	34311	6.9785	42	0.1	6.67	-	-
1483	0.037	1.20	P28070	Proteasome subunit beta type 4	29185	5.6001	230	1.08	23.48	28.8	38
			Q9UKL6	Phosphatidylcholine transfer protein	24827	5.4668	20	0.15	8.88	NR	NR
1695	0.049	1.22	P0C0L4	Complement C4 A	192649	6.6445	149	0.09	2.98	NR	NR
			P45880	Voltage dependent anion selective channel protein 2 2	31546	7.4678	108	0.48	18.71	NR	NR
1712	0.011	1.33	P40939	Trifunctional enzyme subunit alpha mitochondrial	82946	9.3413	94	0.3	6.42	-	-
			Q9UJS0	Calcium binding mitochondrial carrier protein Aralar2	74128	8.77	55	0.1	6.96		
1584	0.037	1.39	P01243	Chorionic somatomammotropin hormone	25004	5.2178	477	2.45	38.25	44.2	90

[a] Accession code in UniProt.

NR: not recognised (no significant ID was obtained for this protein)

Table 4. List of proteins with lower abundance in GDM placenta tissue in respect to control tissue identified by LC–MS^E. Data were obtained from pools of 6 healthy subjects or 5 GDM patients .

Description	Accession	Score[a]	Fold change	Anova (p)	Peptide count	Peptides used for quantitation	% Coverage
Alpha-1-antitrypsin	P01009	215.7	1.26	4.E-06	22	21	49.49
Alpha-2-antiplasmin	P08697	11.5	1.35	3.2E-02	2	2	7.97
Apolipoprotein A-I	P02647	116.8	1.13	9.4E-04	13	13	54.22
Clusterin	P10909	25	1.35	5.2E-0.4	4	4	14.29
Fibrinogen alpha chain	P02671	384.4	1.39	2.4E-04	41	41	37.30
Fibrinogen beta chain	P02675	506	1.32	9.0E-06	47	47	76.29
Fibrinogen gamma chain	P02679	442.7	1.37	1.91E-04	40	40	73.77
Ig gamma-1 chain C region	P01857	115.1	1.21	9.1E-04	10	4	43.64
Tubulointerstitial nephritis antigen-like	Q9GZM7	13.2	1.21	9.8E-04	2	2	6.50
Vitronectin	P04004	48.6	1.20	5.3E-03	7	7	18.09

[a] confidence score from Progenesis QI.

Table 5. List of proteins with higher abundance in GDM placenta tissue in respect to control tissue identified by LC–MS^E. Data were obtained from pools of 6 healthy subjects or 5 GDM patients.

Description	Accession	Score[a]	Fold change	Anova (p)	Peptide count	Peptides used for quantitation	% Coverage
Periostin	Q15063	22.0807	1.369	8.80E-03	4	4	9.20
Ig gamma-2 chain C region	P01859	68.6956	1.342	3.29E-04	7	7	31.60
Chorionic somatomammotropin hormone	P01243	83.8715	1.290	2.23E-04	8	8	49.74
Moesin	P26038	33.9726	1.266	1.10E-03	5	5	8.51
Heat shock-related 70 kDa protein 2	P54652	78.1285	1.245	3.20E-02	12	12	21.75
Triosephosphate isomerase	P60174	44.3923	1.235	2.50E-05	6	6	36.01
Protein disulfide-isomerase	P07237	122.5778	1.231	1.70E-03	15	15	35.03
Galectin-1	P09382	60.7725	1.225	3.10E-03	7	7	58.96
Vimentin	P08670	351.3324	1.218	1.21E-04	34	34	64.09
14-3-3 protein beta/alpha	P31946	66.0787	1.218	1.38E-02	6	6	29.39

[a] confidence score from Progenesis QI.

Captions for figures

Figure 1: Differentially expressed proteins in GDM placenta tissue identified by 2-DE analysis. Representative image of 2DE analysis of placenta proteins in which protein spots resulted to be significantly more abundant and less abundant in GDM tissue in comparison with healthy placenta tissue are highlighted in green or red , respectively.

Figure 2: Fibrinogen beta chain downregulation in GDM placenta tissue revealed by 2-DE. Representative images of 4 different spots attributed to Fibrinogen beta chain (A) and their corresponding densitometric analysis (B).

Figure 3: Chorionic somatomammotropin hormone upregulation in GDM placenta tissue revealed by 2-DE. Representative images of the spot attributed to Chorionic somatomammotropin hormone (A) and its corresponding densitometric analysis (B).

Figure 4: Label free LC-MS^E analysis of placenta tissue extracts. Representative ion intensity maps of control (A) and GDM (B) placenta extracts showing a two dimensional view of the ions separated by LC-MS^E

Figure 1: Differentially expressed proteins in GDM placenta tissue identified by 2-DE analysis. Representative image of 2DE analysis of placenta proteins in which protein spots resulted to be significantly more abundant and less abundant in GDM tissue in comparison with healthy placenta tissue are highlighted in green or red , respectively.

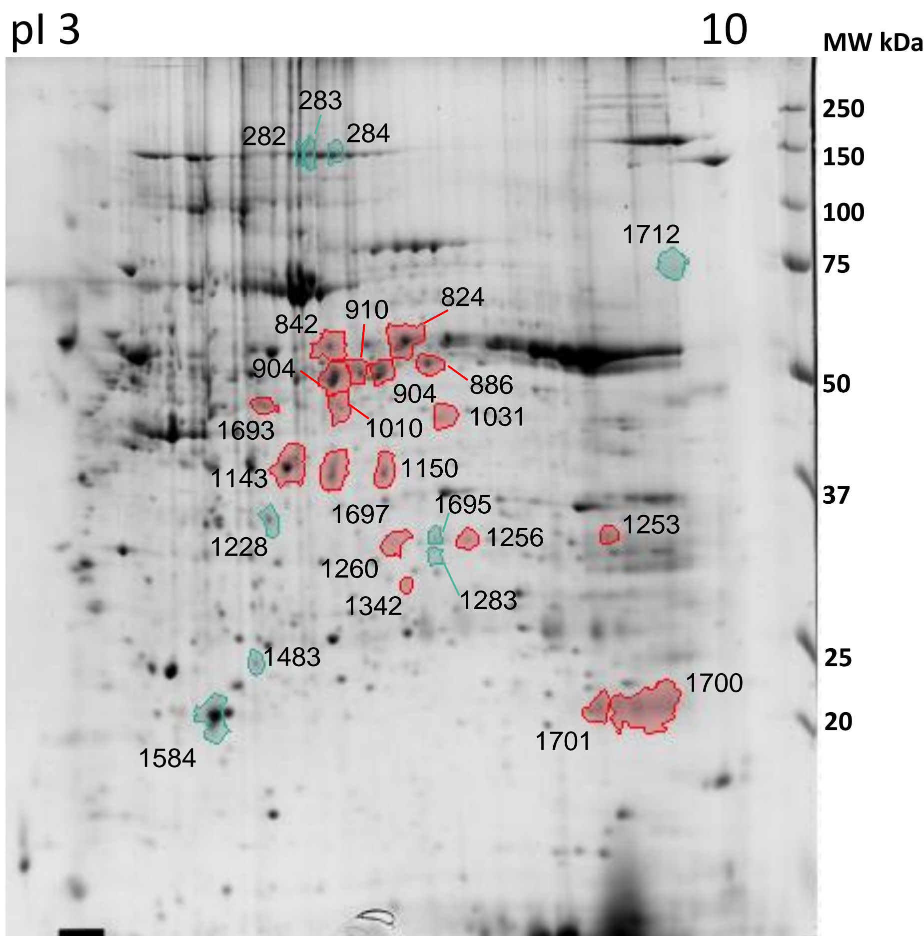
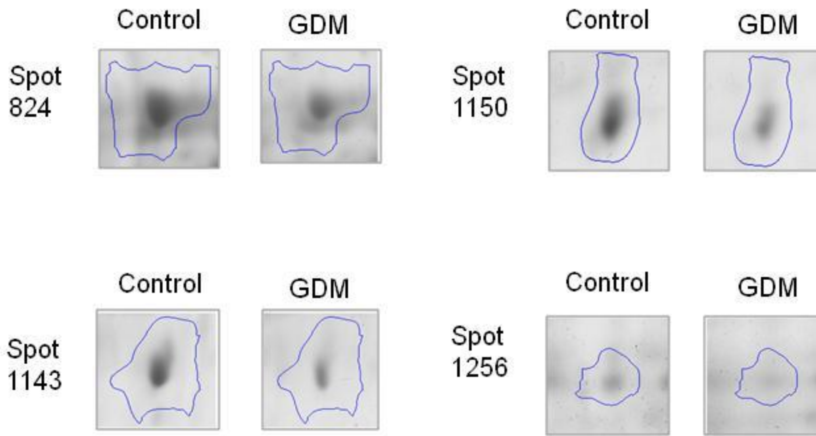


Figure 2: Fibrinogen beta chain downregulation in GDM placenta tissue revealed by 2-DE. Representative images of 4 different spots attributed to Fibrinogen beta chain (A) and their corresponding densitometric analysis (B).



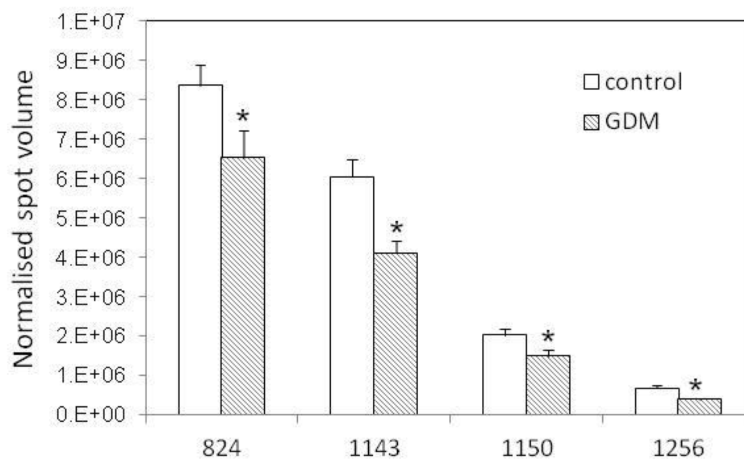


Figure 3: Chorionic somatomammotropin hormone upregulation in GDM placenta tissue revealed by 2-DE. Representative images of the spot attributed to Chorionic somatomammotropin hormone (A) and its corresponding densitometric analysis (B).

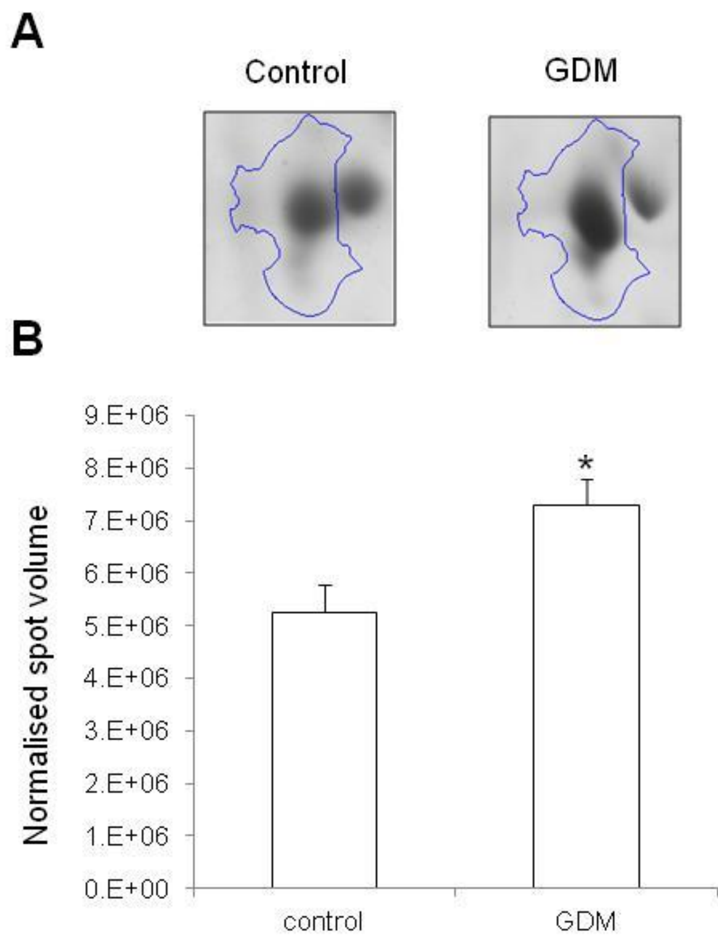


Figure 4: Label free LC-MS^E analysis of placenta tissue extracts. Representative ion intensity maps of control (A) and GDM (B) placenta extracts showing a two dimensional view of the ions separated by LC-MS^E