2D-DIGE to identify proteins associated with gestational diabetes in omental adipose tissue

Karen Oliva^{1,2}, Gillian Barker^{1,3}, Gregory E Rice², Mark J Bailey² and Martha Lappas^{1,3}

¹Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Victoria, Australia

²University of Queensland Centre for Clinical Research, Brisbane, Queensland, Australia

³Department of Obstetrics and Gynaecology, Mercy Hospital for Women, University of Melbourne, Level 4/163 Studley Road, Heidelberg 3084, Victoria, Australia

Correspondence
should be addressed
to M Lappas
Email
mlappas@unimelb.edu.au

Abstract

Gestational diabetes mellitus (GDM) is a significant risk factor for the type 2 diabetes epidemic in many populations. Maternal adipose tissue plays a central role in the pathophysiology of GDM. Thus, the aim of this study was to determine the effect of GDM on the proteome of adipose tissue. Omental adipose tissue was obtained at the time of term Caesarean section from women with normal glucose tolerance (NGT) or GDM. 2D-difference gel electrophoresis (DIGE), followed by mass spectrometry, was used to identify protein spots ($n=6$ patients per group). Western blotting was used for confirmation of six of the spot differences ($n=6$ patients per group). We found 14 proteins that were differentially expressed between NGT and GDM adipose tissue (≥ 1.4 -fold, $P<0.05$). GDM was associated with an up-regulation of four proteins: collagen alpha-2(VI) chain (CO6A2 (COL6A2)), fibrinogen beta chain (FIBB (FGB)), lumican (LUM) and S100A9. On the other hand, a total of ten proteins were found to be down-regulated in adipose tissue from GDM women. These were alpha-1-antitrypsin (AIAT (SERPINA1)), annexin A5 (ANXA5), fatty acid-binding protein, adipocyte (FABP4), glutathione S-transferase P (GSTP (GSTP1)), heat-shock protein beta-1 (HSP27 (HSPB1)), lactate dehydrogenase B chain (LDHB), perilipin-1 (PLIN1), peroxiredoxin-6 (PRX6 (PRDX6)), selenium-binding protein 1 (SBP1) and vinculin (VINC (VCL)). In conclusion, proteomic analysis of omental fat reveals differential expression of several proteins in GDM patients and NGT pregnant women. This study revealed differences in expression of proteins that are involved in inflammation, lipid and glucose metabolism and oxidative stress and added further evidence to support the role of visceral adiposity in the pathogenesis of GDM.

Key Words

- ▶ adipose tissue
- ▶ gestational diabetes
- ▶ proteome
- ▶ inflammation

Journal of Endocrinology
(2013) **218**, 165–178

Introduction

The prevalence of type 2 diabetes is increasing rapidly. Alarming, it is predicted that ~350 million people worldwide will be diagnosed with type 2 diabetes by the year 2030, double the current number (Wild *et al.* 2004). Gestational diabetes mellitus (GDM), defined as any degree of glucose intolerance with onset or first recognition during pregnancy (Kuhl 1998), affects up to 18% of

all pregnancies (Pregnancy 2010). Like type 2 diabetes, the prevalence rate of GDM has also increased dramatically in the last decade (Darios *et al.* 2008, Laws & Sullivan 2009). Importantly, it is considered an important driver for the type 2 diabetes epidemic in many populations. In a recently published retrospective cohort study, the risk of developing diabetes was 9.6 times greater for patients with

GDM, with the cumulative risk of developing type 2 diabetes for GDM patients being 25% at 15 years post-diagnosis (Lee *et al.* 2007). Thus, increasing our understanding of the pathophysiology of GDM is essential in order to provide the best opportunity for early treatment to prevent onset or progression of the disease.

During human pregnancy, a number of metabolic changes occur in maternal adipose tissue that are essential for fetal growth and development. Early pregnancy is associated with adipose tissue accretion. By contrast, the later part of normal human pregnancy is characterised by maternal hyperinsulinaemia and insulin resistance, an adaptation that is required to meet the needs of the growing fetus (Buchanan & Xiang 2005). In women with GDM, however, peripheral insulin resistance is even more pronounced (Colomiere *et al.* 2010), leading to increases in the circulating concentrations of fatty acids and lipids (Metzger *et al.* 1980). Importantly, infants of women with GDM often display dyslipidaemia and have increased fat deposition even when they are average weight for gestational age (Catalano *et al.* 2003). This is clinically significant as these infants are at increased risk of later metabolic disease, including obesity, diabetes, cardiovascular disease and certain cancers (Catalano *et al.* 2003). The impact that these maternal metabolic disturbances have on community health, health economics and human capital is substantial (Chu *et al.* 2008). Thus, effective control of the current diabetes and obesity epidemics may well have to begin with the appropriate management of the intrauterine environment.

Human adipose tissue is divided in two depots, the subcutaneous and the omental fat. In addition to differences in the cellular composition, human omental and subcutaneous adipose tissues also display distinct differences in metabolic and biochemical properties. It has been shown that omental adipose tissue is strongly associated with an adverse metabolic risk profile such as insulin resistance and type 2 diabetes (Fox *et al.* 2007). It is thought that omental adipose tissue may contribute to these diseases because of its direct access to the liver through the portal vein. For example, excess free fatty acid release from the omental adipose tissue interferes with liver metabolism and contributes to the development of hyperinsulinaemia and hypertriglyceridaemia. In addition, subcutaneous and omental adipose tissues are characterised by differences in the expression of genes and proteins related to glucose and lipid metabolism, lipid transport and cellular stress and inflammation (Linder *et al.* 2004, Vohl *et al.* 2004, Perez-Perez *et al.* 2009, Peinado *et al.* 2012, Montes-Nieto *et al.* 2013, Perrini *et al.* 2013).

GDM is considered a pre-diabetic state, offering the opportunity to study abnormalities that may appear very early on in type 2 diabetes. Given that omental adipose tissue plays a central role in insulin resistance and type 2 diabetes, we considered it of interest to determine the effect of GDM on the protein expression profiles of omental adipose tissue. In this study, we used 2D-difference gel electrophoresis (2D-DIGE) to identify novel proteins associated with GDM in omental adipose tissues.

Subjects and methods

Tissue collection

Approval for this study was obtained from the Mercy Hospital for Women's Research and Ethics Committee and informed consent was obtained from all participating subjects. Human omental adipose tissue was obtained from 12 pregnant women with normal glucose tolerance (NGT) and from 12 women with insulin-treated GDM. To negate the putative effects of labour-induced changes in the expression of analytes, all samples were collected at the time of term Caesarean section before the onset of labour. Indications for Caesarean section included repeat Caesarean section or breech presentation. Women with any adverse underlying medical condition (i.e. including asthma, pre-existing diabetes and pre-eclampsia) were excluded. Women with GDM were diagnosed according to the criteria of the Australasian Diabetes in Pregnancy Society (ADIPS) by a fasting venous plasma glucose concentration of ≥ 5.5 mmol/l glucose and/or ≥ 8.0 mmol/l glucose 2 h after a 75 g oral glucose load at ~ 28 weeks of gestation. All women with GDM were prescribed insulin in addition to dietary management. All pregnant women were screened for GDM, and women participating in the normal group had a negative screen. The baseline characteristics for all patients used in this study are outlined in Table 1. Adipose tissue was obtained within 10 min of delivery and thoroughly washed in ice-cold PBS to remove blood. Dissected fragments were stored at -80°C until assayed as detailed below.

Sample preparation and DIGE labelling

For the 2D-DIGE studies, omental adipose tissue was obtained from six NGT women and six women with GDM. Baseline characteristics for these populations are outlined in Table 1. Samples were homogenised into DIGE lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea and 4% CHAPS). Proteins were extracted during 1 h at 4°C

Table 1 Characteristics of the study group used for the 2D-DIGE analysis. Values represent mean $\pm$ S.E.M.

	NGT (n=6)	GDM (n=6)	P value
Maternal age (years)	31.5 $\pm$ 1.2	32.0 $\pm$ 1.8	NS
Maternal BMI at ~12 weeks (kg/m ²)	34.8 $\pm$ 1.0	34.9 $\pm$ 1.4	NS
Maternal BMI at delivery (kg/m ²)	38.8 $\pm$ 2.0	38.0 $\pm$ 2.2	NS
Gestational age at birth (weeks)	38.8 $\pm$ 0.2	38.0 $\pm$ 0.4	NS
Maternal insulin at delivery (IU/ml)	22.0 $\pm$ 2.1	25.7 $\pm$ 4.2	NS
Fetal birth weight (g)	3399 $\pm$ 63	3193 $\pm$ 303	NS
Fetal gender	3 F; 3 M	3 F; 3 M	
Gravida	3.3 $\pm$ 0.6	4.2 $\pm$ 0.7	NS
Parity	2.5 $\pm$ 0.3	2.6 $\pm$ 0.2	NS
Maternal OGTT at ~28 weeks of gestation			
Fasting plasma OGTT (mmol/l)	4.4 $\pm$ 0.1	5.5 $\pm$ 0.2	<0.05
1-h plasma OGTT (mmol/l)	8.3 $\pm$ 0.3	10.4 $\pm$ 0.9	<0.05
2-h plasma OGTT (mmol/l)	6.3 $\pm$ 0.5	8.8 $\pm$ 0.4	<0.05
Maternal OGTT at ~6 weeks <i>postpartum</i>			
Fasting plasma OGTT (mmol/l)	ND	5.0 $\pm$ 0.2	
1-h plasma OGTT (mmol/l)	ND	6.8 $\pm$ 0.7	
2-h plasma OGTT (mmol/l)	ND	5.5 $\pm$ 0.7	

NS, not significant; ND, not done; OGTT, oral glucose tolerance test; F, females; M, males.

and lysates clarified by centrifugation at 25 000 g at 4 °C for 20 min. The supernatant was precipitated with acetone, and protein extracts were then prepared following general guidelines recommended for subsequent DIGE labelling (Oliva *et al.* 2012). Briefly, proteins were precipitated using the 2D clean-up kit (GE Healthcare, Uppsala, Sweden) and then buffer exchanged against DIGE lysis buffer using Vivaspin 2, 3 kDa concentrators (Sartorius Stedim, Goettingen, Germany) until conductivity could be reduced to below 200 μ S/cm and the pH was adjusted to ~8.5.

For 2D-gel electrophoresis, 25 μ g of each sample was labelled with 200 pmol Cy3 (NGT samples) or Cy5 (GDM samples) as detailed earlier. Additionally, an internal standard consisting of equal amounts of each omental sample was also labelled with Cy2 (Alban *et al.* 2003). Three labelled protein samples (one Cy2-labelled pooled internal control, one Cy3-labelled NGT sample and one Cy5-labelled GDM sample; NGT and GDM samples were paired randomly) were then combined and used for 2D gel electrophoresis analysis as detailed below. To avoid labelling bias arising from the varying fluorescence properties of gels at different wavelengths, samples from the same group were run twice in different gels but with the opposite dye-labelling pattern.

2D gel electrophoresis and imaging

Immobiline Dry-Strips (GE Healthcare, Piscataway, NJ, USA; 13 cm, pH 4–7 NL) were rehydrated overnight at room temperature in DeStreak solution (GE Healthcare) and 0.05% IPG buffer non-linear (4–7 NL). Isoelectric

focusing (IEF) was performed for a total of 25 000 Vh at 20 °C. IPG strips were then equilibrated in equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% glycerol and 2% SDS) supplemented with 1% dithiothreitol to maintain the fully reduced state of proteins followed by alkylation with 2.5% iodoacetamide. Proteins were separated on 10.5–14% Criterion Tris-HCl gels (Bio-Rad Laboratories) at 15 mA/gel for 60 min, 30 mA/gel for 2 h and 45 mA/gel for 1 h at room temperature until the bromophenol blue dye-front had run off the bottom of the gels.

CyDye DIGE Fluor-labelled protein gels were scanned at 100 μ m using a Typhoon Trio 9100 (GE Healthcare). The emission filters were Green 532 nm (Cy3) and Red 633 nm (Cy5). Gels were automatically aligned and relative protein quantification across lean and obese samples was performed using the Progenesis SameSpots software 3.3.3420.25059 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Normalisation was performed using the software algorithm. The Cy2-labelled pooled internal standard on every gel allowed accurate relative quantitation of protein spot features across different gels. Student's *t*-test was used to calculate significant differences in relative abundances of protein spot features in omental adipose tissue obtained from GDM and NGT pregnant women.

The 2D gels were fixed in 50% methanol and 3% phosphoric acid and then stained with Colloidal Coomassie stain and gel spots of interest excised using a gel pen. Gel spots were then transferred into water for short-term storage at 4 °C. The water was subsequently removed and discarded from each gel plug. Gel pieces were destained by four sequential washes in destaining solution

(10 mM ammonium bicarbonate (AmBic) and 50% acetonitrile (ACN)), followed by two washes in 100% ACN and then air dried at room temperature. The gel pieces were rehydrated by adding 10 µl of sequencing grade trypsin (0.02 µg/µl (Promega) in 10 mM AmBic) and left to incubate overnight at 37 °C. Digestion was quenched by addition of 2 µl of 2% formic acid (FA).

MALDI-mass spectrometry and protein identification

Digest supernatants (3 µl) were applied to a Bruker Biosciences (Bremen, Germany) Anchorchip MALDI target, prepared with α -cyano-4-hydroxycinnamic acid (CHCA) (see the thin layer affinity method for CHCA in the Bruker Anchorchip manual). After 3 min, this solution was removed and the spots were air dried overnight at room temperature. MALDI-mass spectrometry (MS) was performed on a Bruker Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Peak lists were generated using Flexanalysis (Bruker) and were calibrated by utilising trypsin autolytic peptides. BiTools software (Bruker) and the Mascot search engine were used to interrogate the SwissProt database (Release: July 2010, 517802 sequences; 182492287 residues) and proteins were identified by peptide mass fingerprinting (PMF). Search parameters were as follows: Taxonomy: Human; MS Tolerance: 100 ppm, Missing Cleavages: ≤ 1 ; Enzyme: Trypsin; Fixed Modifications: Carbamidomethylation; Variable Modifications: Oxidation (M). Identifications with Mascot expect probability values <0.05 were then manually verified by examination of spectra and/or

resubmission of peak lists to Mascot. A conservative approach to protein identification was implemented and based on a number of criteria other than these scores. These included theoretical and experimental M_r and pI being in accord, experimental peptide mass accuracy variation across the mass range and repeatability of identification across different gels. If multiple members of a protein family were identified, those with the highest ranked hit were selected.

Western blotting

For validation studies, omental adipose tissue was obtained from six NGT and six GDM women other than the six patients whose samples were used in the 2D-DIGE. Baseline characteristics for these populations are outlined in Table 2. For protein detection in western blot analysis, adipose tissue was homogenised in radioimmuno precipitation assay buffer (1% SDS, 0.25% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl and 50 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors (1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and 5 µg/ml leupeptin). Cellular debris and lipids were eliminated by centrifugation of the solubilised samples at 25 000 g for 30 min at 4 °C. Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA).

Assessment of protein expression was analysed by western blotting as described previously (Colomiere *et al.* 2009). Whole cell lysate blots were incubated with 1 µg/ml rabbit polyclonal anti-lumican (LUM; SAB1401232; Sigma),

Table 2 Characteristics of the study group used for the western blot analysis. Values represent mean $\pm$ s.e.m.

	NGT (n=6)	GDM (n=6)	P value
Maternal age (years)	35.5 $\pm$ 1.6	36.0 $\pm$ 2.4	NS
Maternal BMI at ~12 weeks (kg/m ²)	35.3 $\pm$ 1.8	34.0 $\pm$ 1.5	NS
Maternal BMI at delivery (kg/m ²)	40.6 $\pm$ 3.0	37.0 $\pm$ 1.6	NS
Maternal insulin at delivery (IU/ml)	24.7 $\pm$ 2.2	23.2 $\pm$ 3.3	NS
Gestational age at birth (weeks)	38.5 $\pm$ 0.2	38.6 $\pm$ 0.2	NS
Fetal birth weight (g)	3602 $\pm$ 265	3498 $\pm$ 218	NS
Fetal gender	4 F; 2 M	4 F; 3 M	
Gravida	3.8 $\pm$ 0.8	3.2 $\pm$ 0.3	NS
Parity	3.0 $\pm$ 0.7	2.2 $\pm$ 0.2	NS
Maternal OGTT at ~28 weeks gestation			
Fasting plasma OGTT (mmol/l)	4.7 $\pm$ 0.2	5.3 $\pm$ 0.3	<0.05
1-h plasma OGTT (mmol/l)	7.8 $\pm$ 0.3	10.3 $\pm$ 0.7	<0.05
2-h plasma OGTT (mmol/l)	6.2 $\pm$ 0.4	8.8 $\pm$ 0.7	<0.05
Maternal OGTT at ~6 weeks <i>postpartum</i>			
Fasting plasma OGTT (mmol/l)	ND	5.0 $\pm$ 0.2	
1-h plasma OGTT (mmol/l)	ND	6.5 $\pm$ 0.6	
2-h plasma OGTT (mmol/l)	ND	5.5 $\pm$ 0.6	

NS, not significant; ND, not done; OGTT, oral glucose tolerance test; F, females; M, males.

1 µg/ml rabbit polyclonal anti-PRX6 (P0058; Sigma), 1 µg/ml rabbit polyclonal anti-S100A9 (HPA004193; Sigma), 1 µg/ml rabbit polyclonal anti-HSP27 (SAB 4501457; Sigma), 1 µg/ml mouse monoclonal anti-ANXA5 (WH0000308M1; Sigma), 0.1 µg/ml rabbit polyclonal anti-fibrinogen β -chain (HPA001900; Sigma) and mouse monoclonal anti- β -actin (A5316; Sigma) diluted in blocking buffer (5% skim milk/TBS-T (0.05%)) for 24 h at 4 °C. Membranes were viewed and analysed using the Chemi-Doc system (Bio-Rad Laboratories). Quantitative analysis of the relative density of the bands in western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories). Data were corrected for background and expressed as optical density (OD/mm²).

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, MD, USA). The student's *t*-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann-Whitney *U* (Wilcoxon) test was used. Statistical difference was indicated by a *P* value of <0.05. Data are expressed as mean $\pm$ S.E.M.

Results

Identification of differentially expressed proteins using 2D-DIGE

For the 2D-DIGE studies, omental adipose tissue was obtained from six NGT women and six women with GDM. Baseline characteristics for these populations are outlined in Table 1. Of note, there was no difference in maternal age or BMI between the two groups. Proteins isolated from omental adipose tissue obtained from women with NGT (*n*=6) and GDM (*n*=6) were displayed and compared using 2D-DIGE. An internal standard consisting of a pool of the different samples was Cy2 labelled and included in each gel. After 2D-DIGE, the Cy2, Cy3 and Cy5 channels were individually imaged from each of the six gels using mutually exclusive excitation and emission wavelengths, and image analysis was performed with Progenesis Same-Spots software. A master map was created and ~840 spots common to all gels were detected. See Fig. 1A for a master map. In this image, the NGT samples are labelled with Cy3 (green) and the GDM samples are labelled with Cy5 (red).

The gels were stained with Coomassie Blue stain, and all visible protein spots were trypsin digested and

Figure 1

2D-DIGE analysis of omental adipose tissue obtained from women with NGT (Cy3, green) and GDM (Cy5, red). (A) Spot map corresponding to the mixed internal standard, which is common to all the gels analysed. pH 4–7 immobilised pH gradient strips were used for isoelectric focusing and 10.5–14% SDS-PAGE for the second dimension. (B) Numbered spots indicate proteins identified by MALDI-TOF MS. See Table 3 for a detailed listing of all proteins identified.

submitted to MS/MS for identification: MALDI-TOF MS. A total of 116 spots were positively identified, corresponding to 61 distinct proteins. Figure 1B shows the position of these spots in the 2D-DIGE gel. Table 3 displays detailed information about the corresponding proteins identified. This table summarises the protein accession number of identified muscle proteins, Mascot scores, the number of matched peptide sequences, the percentage sequence coverage, the theoretical molecular mass, the theoretical *pI* value, average normalised values and fold change of individual proteins affected by GDM.

Statistical analyses were performed by setting the threshold for differentially expressed proteins at >1.4-fold and *P*<0.05. Four proteins were significantly higher in adipose tissue obtained from GDM patients. These were

Table 3 Human adipose tissue proteins identified by 2D-DIGE and MALDI-TOF MS

Spot no.	Protein name (abbreviation)	Accession number	Mascot score	% MS coverage	MW (kDa)	pI value	No. of matched peptides	Average		Ratio	P value
								NGT	GDM		
1	14-3-3 protein beta/alpha (1433B)	P31946	78	35	28.2	4.6	13	1.20	0.95	-1.3	0.009
2	14-3-3 protein epsilon (1433E)	P62258	151	58	29.3	4.5	17	1.08	1.01	-1.1	0.167
3	14-3-3 protein zeta/delta (1433Z)	P63104	159	55	27.9	4.6	16	1.13	0.93	-1.2	0.013
4	Alpha-1-antitrypsin (AIAT)	P01009	263	59	46.9	5.3	25	1.17	0.83	-1.4	0.015
5	Actin, aortic smooth muscle (ACTA)	P62736	137	47	42.4	5.1	17	1.15	0.86	-1.3	0.167
6	Actin, cytoplasmic 1 (ACTB)	P60709	189	55	42.1	5.2	22	0.98	1.08	1.1	0.161
7	Actin, cytoplasmic 1 (ACTB)	P60709	165	54	42.1	5.2	21	1.11	1.02	-1.1	0.369
8	Actin, cytoplasmic 1 (ACTB)	P60709	174	52	42.1	5.2	21	1.15	1.02	-1.1	0.155
9	Actin, cytoplasmic 1 (ACTB)	P60709	169	54	42.1	5.2	23	0.98	1.12	1.1	0.192
10	Actin, cytoplasmic 1 (ACTB)	P60709	126	37	42.1	5.2	15	1.00	1.10	1.1	0.176
11	Actin, cytoplasmic 1 (ACTB)	P60709	188	55	42.1	5.2	21	0.86	0.75	-1.2	0.144
12	Serum albumin (ALBU)	P02768	363	57	71.3	5.9	34	1.16	1.12	1.0	0.508
13	Serum albumin (ALBU)	P02768	309	50	71.3	5.9	27	0.84	0.74	-1.1	0.175
14	Serum albumin (ALBU)	P02768	32	10	71.3	5.9	28	1.16	1.13	1.0	0.572
15	Serum albumin (ALBU)	P02768	318	40	71.3	5.9	25	1.02	0.86	-1.2	0.125
16	Serum albumin (ALBU)	P02768	443	68	71.3	5.9	47	1.27	1.03	-1.2	0.082
17	Aldehyde dehydrogenase, mitochondrial (ALDH2)	P05091	158	35	56.9	6.8	16	1.03	0.91	-1.1	0.108
18	Aldehyde dehydrogenase, mitochondrial (ALDH2)	P05091	185	43	56.9	6.8	22	1.21	0.89	-1.4	0.006
19	Annexin A2 (ANXA2)	P07355	139	40	38.8	8.5	13	1.16	1.03	-1.1	0.302
20	Annexin A5 (ANXA5)	P08758	153	58	36.0	4.8	18	1.30	0.86	-1.5	0.042
21	Annexin A5 (ANXA5)	P08758	194	46	36.0	4.8	14	1.11	0.74	-1.5	0.010
22	Annexin A5 (ANXA5)	P08758	265	67	36.0	4.8	24	1.15	0.84	-1.4	0.031
23	Annexin A6 (ANXA6)	P08133	173	34	76.2	5.3	22	0.97	0.84	-1.2	0.043
24	Annexin A6 (ANXA6)	P08133	255	39	76.2	5.3	28	1.17	0.84	-1.4	0.004
25	Apolipoprotein A-I (APOA1)	P02647	236	67	30.8	5.5	21	1.31	0.94	-1.4	0.002
26	Apolipoprotein A-I (APOA1)	P02647	217	63	30.8	5.5	19	1.02	1.27	1.2	0.122
27	Apolipoprotein A-I (APOA1)	P02647	247	72	30.8	5.5	25	1.17	1.19	1.0	0.811
28	Calreticulin (CALR)	P27797	196	44	48.3	4.1	16	0.94	1.08	1.2	0.074
29	60 kDa heat-shock protein, mitochondria (CH60)	P10809	196	45	61.2	5.6	22	1.34	1.04	-1.3	0.013
30	Chloride intracellular channel protein (CLIC1)	O00299	101	46	27.2	4.9	9	1.17	0.99	-1.2	0.035
31	Collagen alpha-1(VI) chain (CO6A1)	P12109	207	28	109.6	5.1	24	1.02	0.88	-1.2	0.305
32	Collagen alpha-1(VI) chain (CO6A1)	P12109	201	27	109.6	5.1	23	1.07	0.92	-1.2	0.348
33	Collagen alpha-1(VI) chain (CO6A1)	P12109	223	30	109.6	5.1	25	1.05	0.88	-1.2	0.376
34	Collagen alpha-1(VI) chain (CO6A1)	P12109	118	14	109.6	5.1	14	1.08	0.89	-1.2	0.314
35	Collagen alpha-1(VI) chain (CO6A1)	P12109	58	8	109.6	5.1	8	0.70	1.45	2.1	0.008
36	Collagen alpha-1(VI) chain (CO6A1)	P12109	42	5	109.6	5.1	5	0.61	1.42	2.3	0.001
37	Collagen alpha-1(VI) chain (CO6A1)	P12109	75	8	109.6	5.1	9	0.61	1.37	2.3	0.001
38	Collagen alpha-2(VI) chain (CO6A2)	P12110	176	18	109.7	5.8	18	0.83	1.17	1.4	0.048
39	Collagen alpha-2(VI) chain (CO6A2)	P12110	179	21	109.7	5.8	21	0.90	1.18	1.3	0.078
40	Collagen alpha-2(VI) chain (CO6A2)	P12110	121	12	109.7	5.8	13	0.78	1.16	1.5	0.040
41	Collagen alpha-2(VI) chain (CO6A2)	P12110	48	4	109.7	5.8	5	0.76	1.19	1.6	0.018
42	Collagen alpha-2(VI) chain (CO6A2)	P12110	118	13	109.7	5.8	14	0.78	1.23	1.6	0.019
43	Cytochrome b5 (CYB5)	P00167	81	49	15.3	4.7	5	1.15	1.08	-1.1	0.245
44	Dermatopontin (DERM)	Q07507	75	37	24.6	4.5	7	0.79	1.05	1.3	0.038
45	Enoyl-CoA hydratase, mitochondrial (ECHM)	P30084	118	43	31.8	9.4	12	1.23	0.92	-1.3	0.018

Table 3 Continued

Spot no.	Protein name (abbreviation)	Accession number	Mascot score	% MS coverage	MW (kDa)	pI value	No. of matched peptides	Average		Ratio	P value
								NGT	GDM		
46	EH domain-containing protein 2 (EHD2)	Q9NZN4	103	24	61.3	6.0	12	1.20	1.19	1.0	0.894
47	Endoplasmic (ENPL)	P14625	134	16	92.7	4.6	16	1.01	0.87	-1.2	0.078
48	Endoplasmic (ENPL)	P14625	79	11	92.7	4.6	9	1.01	0.92	-1.1	0.224
49	Fatty acid-binding protein, adipocyte (FABP4)	P15090	78	43	14.8	7.5	6	0.99	0.79	-1.3	0.009
50	Fatty acid-binding protein, adipocyte (FABP4)	P15090	104	59	14.8	7.5	8	1.30	0.80	-1.6	0.000
51	Fatty acid-binding protein, epidermal (FABP5)	Q01469	106	65	15.5	7.5	10	1.02	1.02	1.0	0.936
52	Fibrinogen beta chain (FIBB)	P02675	71	20	56.6	9.3	9	0.84	1.07	1.3	0.000
53	Fibrinogen beta chain (FIBB)	P02675	163	28	56.6	9.3	14	0.63	1.55	2.5	0.003
54	Fibrinogen beta chain (FIBB)	P02675	79	17	56.6	9.3	9	0.63	1.47	2.3	0.011
55	Fibrinogen gamma chain (FIBG)	P02679	44	8	52.1	5.3	4	0.78	1.31	1.7	0.097
56	Fibrinogen gamma chain (FIBG)	P02679	210	47	52.1	5.3	19	0.93	1.00	1.1	0.275
57	Fibrinogen gamma chain (FIBG)	P02679	190	43	52.1	5.3	19	0.95	1.12	1.2	0.074
58	Fibrinogen gamma chain (FIBG)	P02679	77	21	52.1	5.3	8	0.76	1.35	1.8	0.070
59	Ferritin light chain (FTL)	P02792	165	58	20.1	5.4	13	1.29	0.98	-1.3	0.181
60	Ferritin light chain (FTL)	P02792	114	35	20.1	5.4	7	1.65	0.53	-3.1	0.001
61	Neutral alpha-glucosidase AB (GANAB)	Q14697	84	12	107.3	5.7	10	0.99	0.92	-1.1	0.435
62	Neutral alpha-glucosidase AB (GANAB)	Q14697	89	13	107.3	5.7	12	1.00	0.84	-1.2	0.057
63	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 (GBB2)	P62879	134	47	38.0	5.6	15	1.02	1.00	1.0	0.637
64	Rho GDP-dissociation inhibitor 1 (GDIR1)	P52565	69	29	23.3	4.9	7	0.96	1.25	1.3	0.117
65	Glucosidase 2 subunit beta (GLU2B)	P14314	121	25	60.4	4.2	15	1.05	1.03	1.0	0.768
66	Glycerol-3-phosphate dehydrogenase [NAD +], cytoplasmic (GPDA)	P21695	236	77	38.2	5.8	25	1.22	0.92	-1.3	0.475
67	Glutathione S-transferase P (GSTP)	P09211	105	57	23.6	5.3	10	1.21	0.89	-1.4	0.004
68	Haptoglobin (HPT)	P00738	121	29	45.9	6.1	13	0.98	0.99	1.0	0.866
69	Heat-shock cognate 71 kDa protein (HSP7C)	P11142	121	22	71.1	5.2	12	1.05	0.84	-1.3	0.013
70	Heat-shock protein 27 (HSP27)	P04792	131	41	22.8	6.0	9	1.10	0.75	-1.5	0.000
71	Heat-shock protein beta 20 (HSP20)	O14558	106	60	17.2	6.0	7	1.17	0.96	-1.2	0.258
72	Keratin, type I cytoskeletal 10 (K1C10)	P13645	92	17	59.0	5.0	11	1.00	0.85	-1.2	0.220
73	Keratin, type I cytoskeletal 19 (K1C19)	P08727	163	44	44.1	4.9	21	1.00	0.96	1.0	0.770
74	Keratin, type I cytoskeletal 19 (K1C19)	P08727	270	75	44.1	4.9	30	0.86	1.07	1.2	0.313
75	Lactate dehydrogenase B chain (LDHB)	P07195	86	19	36.9	5.7	7	1.34	0.89	-1.5	0.001
76	Galectin-1 (LEG1)	P09382	149	77	15.0	5.2	10	0.99	1.14	1.2	0.023
77	Galectin-1 (LEG1)	P09382	130	58	15.0	5.2	8	0.89	1.12	1.3	0.001
78	Lumican (LUM)	P51884	165	38	38.7	6.2	11	0.86	1.28	1.5	0.023
79	Lumican (LUM)	P51884	140	37	38.7	6.2	11	0.87	1.30	1.5	0.033
80	Microfibril-associated glycoprotein 4 (MFAP4)	P50883	69	25	29.0	5.3	7	0.95	1.03	1.1	0.573
81	Myosin light polypeptide 6 (MYL6)	P60660	163	76	17.1	4.4	14	1.10	1.05	1.0	0.740
82	Protein disulfide-isomerase A1 (PDIA1)	P07237	252	42	57.5	4.6	22	0.94	0.89	1.0	0.321
83	Protein disulfide-isomerase A1 (PDIA1)	P07237	219	34	57.5	4.6	18	1.11	1.07	1.0	0.439
84	Protein disulfide-isomerase A1 (PDIA1)	P07237	114	16	57.5	4.6	9	1.04	0.99	1.0	0.352
85	Protein disulfide-isomerase A3 (PDIA3)	P30101	190	40	57.1	5.9	20	1.11	1.04	-1.1	0.129
86	Perilipin-1 (PLIN1)	O60240	267	50	56.2	6.0	25	1.37	0.80	-1.7	0.002
87	Peroxisomal-2 (PRX2)	P32119	107	39	22.0	5.6	7	1.00	1.12	1.1	0.143
88	Peroxisomal-2 (PRX2)	P32119	166	53	22.0	5.6	11	1.10	1.08	1.0	0.805

Table 3 Continued

Spot no.	Protein name (abbreviation)	Accession number	Mascot score	% MS coverage	MW (kDa)	pI value	No. of matched peptides	Average		Ratio	P value
								NGT	GDM		
89	Peroxiredoxin-6 (PRX6)	P30041	207	73	25.1	6.0	17	1.28	0.92	-1.4	0.000
90	S100A9	P06702	82	43	13.3	5.7	6	0.95	1.37	1.4	0.012
91	Selenium-binding protein 1 (SBP1)	Q13228	137	22	52.9	5.9	9	1.16	0.83	-1.4	0.004
92	Superoxide dismutase [Cu-Zn] (SODC)	P00441	80	42	16.2	5.7	5	1.23	0.97	-1.3	0.008
93	T-complex protein 1 subunit beta (TCPB)	P78371	97	25	57.8	6.0	12	0.86	0.87	1.0	0.832
94	Transitional endoplasmic reticulum ATPase (TERA)	P55072	65	16	90.0	5	11	1.20	0.98	-1.2	0.016
95	Protein-glutamine gamma-glutamyltransferase (TGM2)	P21980	273	43	78.4	5	28	0.79	0.94	1.2	0.198
96	Protein-glutamine gamma-glutamyltransferase (TGM2)	P21980	252	38	78.4	5	23	0.88	1.08	1.2	0.226
97	Protein-glutamine gamma-glutamyltransferase (TGM2)	P21980	143	23	78.4	5	14	0.77	1.13	1.5	0.031
98	Tropomyosin alpha-3 chain (TPM3)	P06753	74	34	32.9	4.5	13	1.11	1.23	1.1	0.223
99	Tropomyosin alpha-4 chain (TPM4)	P67936	146	38	28.6	4.5	14	1.07	1.15	1.1	0.403
100	Serotransferrin (TRFE)	P02787	254	29	79.3	7	22	1.06	0.88	-1.2	0.198
101	Serotransferrin (TRFE)	P02787	361	44	79.3	7	32	1.13	0.96	-1.2	0.252
102	Vimentin (VIME)	P08670	318	67	53.7	4.9	35	1.10	1.05	-1.1	0.705
103	Vimentin (VIME)	P08670	204	37	53.7	4.9	19	1.15	0.97	-1.2	0.110
104	Vimentin (VIME)	P08670	68	18	53.7	4.9	7	1.11	1.03	-1.1	0.282
105	Vimentin (VIME)	P08670	289	62	53.7	4.9	34	0.98	1.16	1.2	0.342
106	Vimentin (VIME)	P08670	307	65	53.7	4.9	31	0.82	1.33	1.6	0.069
107	Vimentin (VIME)	P08670	275	48	53.7	4.9	25	0.66	1.43	2.2	0.000
108	Vimentin (VIME)	P08670	238	39	53.7	4.9	17	1.00	1.44	1.4	0.265
109	Vimentin (VIME)	P08670	217	62	53.7	4.9	32	1.09	0.88	-1.2	0.030
110	Vimentin (VIME)	P08670	285	56	53.7	4.9	32	0.95	1.19	1.3	0.131
111	Vimentin (VIME)	P08670	287	70	53.7	4.9	38	1.12	0.97	-1.2	0.164
112	Vimentin (VIME)	P08670	223	66	53.7	4.9	34	0.92	1.12	1.2	0.022
113	Vimentin (VIME)	P08670	269	68	53.7	4.9	36	0.97	1.12	1.2	0.128
114	Vinculin (VINC)	P18206	147	23	124.3	5.4	24	1.24	0.92	-1.4	0.002
115	Vinculin (VINC)	P18206	167	22	124.3	5.4	21	1.16	0.86	-1.3	0.004
116	Vitamin D-binding protein (VTDB)	P02774	142	23	54.5	5.3	12	1.18	1.13	1.0	0.588

collagen alpha-2(VI) chain (CO6A2 (COL6A2)), fibrinogen beta chain (FIBB), LUM and S100A9. Of note, collagen alpha-1(VI) chain (CO6A1) appeared as two separate chains (spots 31–34 and 35–37); however, only one of the chains of CO6A1 (spots 35–37) was significantly different between NGT and GDM.

Ten proteins were significantly lower in adipose tissue obtained from GDM patients. These were alpha-1-antitrypsin (AIAT (SERPINA1)), annexin A5 (ANXA5), fatty acid-binding protein, adipocyte (FABP4), glutathione S-transferase P (GSTP (GSTP1)), heat-shock protein 27 (HSP27 (HSPB1)), lactate dehydrogenase B chain (LDHB), perilipin-1 (PLIN1), peroxiredoxin-6 (PRX6), selenium-binding protein 1 (SBP1) and vinculin (VINC).

Immunoblot analysis of GDM-related changes in the omental adipose tissue proteome

Western blot analysis was used to validate representative findings from the 2D-DIGE analysis. We chose to validate six proteins: three that were up-regulated in GDM adipose tissue (FIBB, LUM and S100A9) and three that were down-regulated in GDM adipose tissue (ANXA5, PRX6 and HSP27). For these studies, we analysed an independent set of omental tissue samples ($n=6$ per group). Western blotting validating the overexpression of FIBB, LUM and S100A9 in GDM omental samples is shown in Fig. 2A, B, and C. Western blotting validating the down-regulation of ANXA5, PRX6 and HSP27 in GDM omental samples are shown in Fig. 2D, E, and F.

Figure 2

Western blot and quantitation for (A) FIBB, (B) LUM, (C) S100A9, (D) ANXA5, (E) PRX6 and (F) HSP27 in adipose tissue ($n=6$ patients per group). Protein expression was normalised to β -actin expression. Data are

displayed as the mean $\pm$ S.E.M. * $P < 0.05$ vs GDM (Student's t -test).

Representative western blot from eight patients (four GDM and four NGT patients) is also shown.

Discussion

In this study, 2D-DIGE was used to identify GDM-associated changes in the expression of proteins in human adipose tissue. Using MALDI-TOF MS, 116 protein spots were identified, which corresponded to 61 distinct proteins. GDM was associated with an up-regulation of four proteins: CO6A2, FIBB, LUM and S100A9. On the other hand, a total of ten proteins were found to be down-regulated in adipose tissue from GDM women. These were AIAT, ANXA5, FABP4, GSTP, HSP27, LDHB, PLIN1, PRX6, SBP1 and VINC. Western blot analysis of six proteins (FIBB, LUM, S100A9, ANXA5, HSP27 and PRX6) confirmed the 2D-DIGE data. The possible roles of these proteins in the pathophysiology of GDM are discussed below.

There have been a number of studies that have also used 2DE to assess proteins that are increased and/or decreased in adipose tissue from subjects with obesity, insulin resistance and type 2 diabetes. For example, proteomic analysis revealed altered expression levels of several proteins that are involved in lipid and glucose metabolism, inflammation and oxidative stress processes in polycystic ovary syndrome (PCOS) omental adipose tissue in comparison with those of non-hyperandrogenic obese women (Corton *et al.* 2008). Differences regarding these studies and our studies are also discussed below.

Inflammation

S100A9, which belongs to the family of low-molecular weight S100 proteins, has emerged as an important pro-inflammatory mediator in acute and chronic inflammation (Roth *et al.* 2001, Gebhardt *et al.* 2006). It has been shown to have cytokine-like activities, activating receptor for advanced glycation end products (RAGE) and Toll-like receptor 4-dependent signalling cascades. S100A9 expression is increased in adipose tissue from subjects with impaired glucose tolerance (Ortega *et al.* 2012). Likewise, in this study, we found increased S100A9 expression in adipose tissue from women with GDM.

Fibrinogen, a 340 kDa glycoprotein consisting of three pairs of identical chains (α , β and γ), is a powerful and independent risk factor for cardiovascular diseases (Heinrich *et al.* 1994). It is also a positive acute-phase protein, playing a vital role in a number of physiopathological processes in the body, including inflammation (Kamath & Lip 2003). In keeping with this, we found increased expression of FIBB in adipose tissue of women with GDM.

A1AT and ANXA5 have also been implicated in inflammation. A1AT is a 52 kDa protease inhibitor

belonging to the serpin superfamily (Gettins 2002). It protects tissues from enzymes of inflammatory cells, especially neutrophil elastase, and as such its circulating levels rise upon acute inflammation. ANXA5 has anti-inflammatory, anti-thrombotic and anti-apoptotic properties (Leon *et al.* 2006, Ewing *et al.* 2011); it reduces local vascular and systemic inflammation, reduces vascular remodelling and improves vascular function. In this study, 2D-DIGE revealed significantly lower A1AT and ANXA5 in adipose tissue from GDM subjects, suggesting increased inflammation in this tissue. However, in contrast to our studies, ANXA5 was found to be overexpressed in PCOS omental adipose tissue compared with those of non-hyperandrogenic women (Corton *et al.* 2008).

Although we did not have the samples available, it would have been of interest to assess the infiltration of M1/M2 macrophages in omental adipose tissue. Nevertheless, and in keeping with our previous studies (Darios *et al.* 2008), our proteomics data are suggestive of increased inflammation in omental adipose tissue GDM.

Structural proteins

The collagens are a superfamily of proteins that play a role in maintaining the integrity of various tissues. Collagens are extracellular matrix (ECM) proteins and have a triple-helical domain as their common structural element. The ECM of adipose tissue, mainly represented by collagen VI (Scherer *et al.* 1998), is dysfunctional in obesity and contributes to the development of the metabolic syndrome. Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis (Spencer *et al.* 2010), and CO6A3 is higher in adipose tissue of obese women (Pasarica *et al.* 2009). Increased collagen expression is also evident in the mesangium of diabetic rats (Abrass *et al.* 1988). Furthermore, in *db/db* obese mice, various types of collagens are overexpressed in epididymal WAT (Khan *et al.* 2009). Of note, obese *ob/ob* mice lacking the *Col6a3* gene have a better metabolic profile compared with wild-type mice. They also gain less weight when fed a high-fat diet (Khan *et al.* 2009). These mice also have lower macrophage content in adipose tissue and decreased expression of inflammatory molecules, suggesting a role for collagen in adipose tissue inflammation. In this study, CO6A1 appeared as two separate chains (spots 31–34 and 35–37); however, only one of the chains of CO6A1 (spots 35–37) was significantly higher in GDM subjects. We also found that CO6A2 was significantly higher in GDM subjects. LUM is a leucine-rich ECM glycoprotein that belongs to the small leucine-rich

proteoglycan family of proteins. It interacts with collagen to maintain the integrity and function of connective tissues and has been implicated in various processes, such as cell migration, proliferation, wound healing and inflammation (Chakravarti 2003, Nikitovic *et al.* 2008). There is increased transcription of LUM in glomeruli from human diabetic kidneys at different stages of nephropathy (Schaefer *et al.* 2001). Similarly, in this study, we found increased LUM in omental adipose tissue from GDM subjects. In summary, an increase in ECM proteins in adipose tissue from women with GDM would result in an increase in the overall rigidity of adipose tissue, likely contributing to an increase in its mechanical strength, and thus fibrosis. In addition, increased collagen and LUM expression in adipose tissue may also play a role in inflammation observed in GDM.

VINC is a structural protein involved in linkage of integrin adhesion molecules to the actin cytoskeleton. It has been implicated in a number of processes including cell adhesion, cell shape control and motility (Ziegler *et al.* 2006, Peng *et al.* 2011). In this study, we observed lower VINC expression in adipose tissue of women with GDM, suggesting that it may play a role in differentiation and survival of these cells. Other studies have reported similar decreases in VINC in the presence of diabetes (Yohannes *et al.* 2008).

Oxidative stress

Oxidative damage caused by reactive oxygen species (ROS) has been implicated in a number of metabolic diseases, including GDM (Lappas *et al.* 2011). It has been suggested that oxidative stress mediates many of the pathological consequences of diabetes in pregnancy and is importantly associated with poor fetal outcome. Removal of ROS by antioxidant enzymes plays an important part in limiting this damage, and indeed, antioxidants can prevent diabetic embryopathy in rat models of diabetes in pregnancy (Higa *et al.* 2012). PRX6 is a cytosolic peroxidase (Fisher 2011) that has been demonstrated to protect various tissues, such as skin, lung and cardiac muscle, against acute oxidative insults, and that has been shown to be down-regulated in diabetes (Johnson *et al.* 2009). HSPs are molecular chaperones that are classified into families according to their molecular weight (Ellis 1999, Hwang *et al.* 2009). A growing body of literature has linked chaperone-like molecules to adipogenesis, obesity and diabetes (Cherian & Abraham 1995, Kurucz *et al.* 2002, Young *et al.* 2004). HSP27 is a chaperone of the small HSP group, which has been implicated in multiple functions

such as inflammation, cytoprotective stress proteins, inhibition of apoptosis, cell development, cell differentiation and signal transduction (Arrigo 2007, Salari *et al.* 2013). Indeed, overexpression of HSP27 mitigates cytokine-induced islet apoptosis and streptozotocin-induced diabetes (Dai *et al.* 2009). GSTs are also a family of antioxidant enzymes; they metabolise electrophilic aldehydes by catalysing their conjugation with glutathione. Direct antioxidant activity has also been postulated for SBP1 in analogy to other selenocysteine-containing enzymes such as thioredoxin reductase and glutathione peroxidase. Thus, the decrease in PRX6, HSP27, GSTP and SBP1 in adipose tissue from women with GDM compared with normal tissue is suggestive of increased oxidative stress. In support of our studies, 2D-DIGE also revealed that the antioxidant enzyme PRX2 was found to be down-regulated in PCOS omental fat (Corton *et al.* 2008).

Energy and lipid metabolism

Lactate dehydrogenase (LDH) is involved in glycolysis; it catalyses the conversion of lactate to pyruvate. In this study, we found that the β chain of LDH was decreased in adipose tissue of women with GDM. It is possibly that a deficiency in LDHB protein might contribute to impaired net lactate release from adipose tissue in response to insulin and may explain the lower plasma lactate levels that are seen in the obese women during hyperinsulinaemia (Qvisth *et al.* 2007).

Pregnancy is characterised by changes in maternal adiposity and thus changes in lipid metabolism. During late pregnancy, there is enhanced fat mobilisation (i.e. increased lipolysis) via hormone-sensitive lipase (HSL) to ensure fetal growth and development. PLIN1 is an important regulator of lipid storage; it acts as a protective coating from HSL, thereby blocking fat hydrolysis. Lower expression of PLIN1 in adipose tissue of women with GDM observed in this study is in keeping with studies showing that lipolysis is increased in women with GDM (Butte 2000).

We also report a decrease in FABP4 in adipose tissue from GDM women, which is consistent with others showing decreased FABP2 levels in GDM patients (Catalano *et al.* 2002). These data suggest the shunting of fatty acids away from adipose tissue where they are stored as fat and towards placenta where they would be oxidised as fuel or transported to fetus. Collectively, lower adipose tissue PLIN1 and FABP4 suggests the transition from lipid storage to increased lipolysis in GDM pregnancies. This may contribute to the dyslipidaemia and increased fat

deposition observed in infants of women with GDM (Catalano *et al.* 2003).

Study limitations

There are a number of caveats that must be considered when interpreting the data obtained in this study. First, intact adipose tissue samples were used instead of studying adipocytes and stromal vascular fraction separately. Previous studies have shown that the proteome of these two fractions is different (Peinado *et al.* 2012). Secondly, all pregnant women diagnosed with GDM were prescribed insulin, which may be responsible for some of the changes described. Thirdly, the present results were obtained from pregnant women who were obese and thus cannot be extrapolated to non-obese pregnant women with GDM. In addition, 2DE itself is associated with several limitations; for example, only a portion of all the proteins that are present in a sample can be displayed. In this study, we only analysed proteins in the 4–7 pH range, avoiding proteins with a more alkaline or acidic pI. Nevertheless, to our knowledge, this is the first study performed with humans in which 2D-DIGE has been used to identify protein expression changes, induced by GDM, in omental adipose tissue.

Conclusions

GDM is a significant risk factor for the type 2 diabetes epidemic in many populations (Rice *et al.* 2012). Long-term women and infants are prone to obesity, diabetes and cardiovascular disease (Catalano *et al.* 2003, Lee *et al.* 2007). Thus, increasing our understanding of the aetiology of GDM will provide the best opportunity for early treatment to prevent onset or progression of the disease. The data obtained in this study confirm that adipose tissue from women with GDM exhibits a different expression profile from that of NGT pregnant women. We have identified a number of novel proteins in visceral adipose tissue that may be involved in the pathophysiology of GDM. This study revealed differences in expression of proteins that are involved in inflammation, lipid and glucose metabolism and oxidative stress. Additionally, a number of structural proteins were also found to be differentially regulated in adipose tissue from NGT and GDM women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Dr M L was a recipient of Career Development Fellowships from National Health and Medical Research Council (NHMRC) (grant no. 454777 and 1047025). Prof. G E R was in receipt of an NHMRC Principal Research Fellowship. The work described in this manuscript was funded by project grants from NHMRC (grant no. 454310) and Diabetes Australia Research Trust (DART). Proteomic data analysis described in this work was supported by the use of the Australian Proteomics Computational Facility funded by the Australian NHMRC (grant no. 381413). Funding for the ChemoDoc system was, in part, provided by the Medical Research Foundation for Women and Babies.

Acknowledgements

The authors gratefully acknowledge the assistance of the Clinical Research Midwives Gabrielle Fleming and Astrid Tiefholz and the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their co-operation.

References

- Abbrass CK, Peterson CV & Raugi GJ 1988 Phenotypic expression of collagen types in mesangial matrix of diabetic and nondiabetic rats. *Diabetes* **37** 1695–1702. (doi:10.2337/diabetes.37.12.1695)
- Alban A, David SO, Bjorksten L, Andersson C, Sloge E, Lewis S & Currie I 2003 A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* **3** 36–44. (doi:10.1002/pmic.200390006)
- AP Arrigo 2007 The cellular "networking" of mammalian Hsp27 and its functions in the control of protein folding, redox state and apoptosis. In: Csermely, P., Vigh, L. (Eds.) *Molecular aspects of the stress response: Chaperones, membranes and networks*. Springer New York, pp. 14–26.
- Buchanan TA & Xiang AH 2005 Gestational diabetes mellitus. *Journal of Clinical Investigation* **115** 485–491. (doi:10.1172/JCI24531)
- Butte NF 2000 Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. *American Journal of Clinical Nutrition* **71** 1256S–1261S.
- Catalano PM, Nizielski SE, Shao J, Preston L, Qiao L & Friedman JE 2002 Downregulated IRS-1 and PPARgamma in obese women with gestational diabetes: relationship to FFA during pregnancy. *American Journal of Physiology. Endocrinology and Metabolism* **282** E522–E533. (doi:10.1152/ajpendo.00124.2001)
- Catalano PM, Kirwan JP, Haugel-de Mouzon S & King J 2003 Gestational diabetes and insulin resistance: role in short- and long-term implications for mother and fetus. *Journal of Nutrition* **133** 1674S–1683S.
- Chakravarti S 2003 Functions of lumican and fibromodulin: lessons from knockout mice. *Glycoconjugate Journal* **19** 287–293. (doi:10.1023/A:1025348417078)
- Cherian M & Abraham EC 1995 Diabetes affects α -crystallin chaperone function. *Biochemical and Biophysical Research Communications* **212** 184–189. (doi:10.1006/bbrc.1995.1954)
- Chu SY, Bachman DJ, Callaghan WM, Whitlock EP, Dietz PM, Berg CJ, O'Keeffe-Rosetti M, Bruce FC & Hornbrook MC 2008 Association between obesity during pregnancy and increased use of health care. *New England Journal of Medicine* **358** 1444–1453. (doi:10.1056/NEJMoa0706786)
- Colomiere M, Permezel M, Riley C, Desoye G & Lappas M 2009 Defective insulin signaling in placenta from pregnancies complicated by gestational diabetes mellitus. *European Journal of Endocrinology* **160** 567–578. (doi:10.1530/EJE-09-0031)
- Colomiere M, Permezel M & Lappas M 2010 Diabetes and obesity during pregnancy alter insulin signalling and glucose transporter expression in

- maternal skeletal muscle and subcutaneous adipose tissue. *Journal of Molecular Endocrinology* **44** 213–223. (doi:10.1677/JME-09-0091)
- Corton M, Botella-Carretero JI, Lopez JA, Camafeita E, Millan JLS, Escobar-Morreale HF & Peral B 2008 Proteomic analysis of human omental adipose tissue in the polycystic ovary syndrome using two-dimensional difference gel electrophoresis and mass spectrometry. *Human Reproduction* **23** 651–661. (doi:10.1093/humrep/dem380)
- Dai T, Patel-Chamberlin M, Natarajan R, Todorov I, Ma J, LaPage J, Phillips L, Nast CC, Becerra D, Chuang P *et al.* 2009 Heat shock protein 27 overexpression mitigates cytokine-induced islet apoptosis and streptozotocin-induced diabetes. *Endocrinology* **150** 3031–3039. (doi:10.1210/en.2008-0732)
- Darios G, Carl N, Cande VA, Martin RC & John CS 2008 Gestational diabetes in the United States: temporal trends 1989 through 2004. *American Journal of Obstetrics and Gynecology* **198** 525.e521–525.e525. (doi:10.1016/j.ajog.2007.11.017)
- Ellis RJ 1999 Molecular chaperones: pathways and networks. *Current Biology* **9** R137–R139. (doi:10.1016/S0960-9822(99)80082-7)
- Ewing MM, de Vries MR, Nordzell M, Pettersson K, de Boer HC, van Zonneveld AJ, Frostegard J, Jukema JW & Quax PHA 2011 Annexin A5 therapy attenuates vascular inflammation and remodeling and improves endothelial function in mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **31** 95–101. (doi:10.1161/ATVBAHA.110.216747)
- Fisher AB 2011 Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A(2) activities. *Antioxidants & Redox Signaling* **15** 831–844. (doi:10.1089/ars.2010.3412)
- Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, Vasan RS, Murabito JM, Meigs JB, Cupples LA *et al.* 2007 Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation* **116** 39–48. (doi:10.1161/CIRCULATIONAHA.106.675355)
- Gebhardt C, Nemeth J, Angel P & Hess J 2006 S100A8 and S100A9 in inflammation and cancer. *Biochemical Pharmacology* **72** 1622–1631. (doi:10.1016/j.bcp.2006.05.017)
- Gettins PGW 2002 Serpin structure, mechanism, and function. *Chemical Reviews* **102** 4751–4804. (doi:10.1021/cr010170+)
- Heinrich J, Balleisen L, Schulte H, Assmann G & van de Loo J 1994 Fibrinogen and factor VII in the prediction of coronary risk. Results from the PROCAM study in healthy men. *Arteriosclerosis, Thrombosis, and Vascular Biology* **14** 54–59. (doi:10.1161/01.ATV.14.1.54)
- Higa R, Kurtz M, Mazzucco MB, Musikant D, White V & Jaberbaum A 2012 Folic acid and safflower oil supplementation interacts and protects embryos from maternal diabetes-induced damage. *Molecular Human Reproduction* **18** 253–264. (doi:10.1093/molehr/gar080)
- Hwang YJ, Lee SP, Kim SY, Choi YH, Kim MJ, Lee CH, Lee JY & Kim DY 2009 Expression of heat shock protein 60 kDa is upregulated in cervical cancer. *Yonsei Medical Journal* **50** 399–406. (doi:10.3349/ymj.2009.50.3.399)
- Johnson DT, Harris RA, French S, Aponte A & Balaban RS 2009 Proteomic changes associated with diabetes in the BB-DP rat. *American Journal of Physiology. Endocrinology and Metabolism* **296** E422–E432. (doi:10.1152/ajpendo.90352.2008)
- Kamath S & Lip GYH 2003 Fibrinogen: biochemistry, epidemiology and determinants. *QJM: Monthly Journal of the Association of Physicians* **96** 711–729. (doi:10.1093/qjmed/hcg129)
- Khan T, Muise ES, Iyengar P, Wang ZV, Chandalia M, Abate N, Zhang BB, Bonaldo P, Chua S & Scherer PE 2009 Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Molecular and Cellular Biology* **29** 1575–1591. (doi:10.1128/MCB.01300-08)
- Kuhl C 1998 Etiology and pathogenesis of gestational diabetes. *Diabetes Care* **21**(Suppl 2) B19–B26.
- Kurucz I, Morva A, Vaag A, Eriksson KF, Huang X, Groop L & Koranyi L 2002 Decreased expression of heat shock protein 72 in skeletal muscle of patients with type 2 diabetes correlates with insulin resistance. *Diabetes* **51** 1102–1109. (doi:10.2337/diabetes.51.4.1102)
- Lappas M, Hiden U, Desoye G, Froehlich J, Hauguel-de Mouzon S & Jaberbaum A 2011 The role of oxidative stress in the pathophysiology of gestational diabetes mellitus. *Antioxidants & Redox Signaling* **15** 3061–3100. (doi:10.1089/ars.2010.3765)
- Laws P & Sullivan EA 2009 Australia's mothers and babies 2007. *Perinatal statistics series no. 23. Cat. no. PER 48. Sydney: AIHW National Perinatal Statistics Unit.*
- Lee AJ, Hiscock RJ, Wein P, Walker SP & Permezel M 2007 Gestational diabetes mellitus: clinical predictors and long-term risk of developing type 2 diabetes: a retrospective cohort study using survival analysis. *Diabetes Care* **30** 878–883. (doi:10.2337/dc06-1816)
- Leon C, Nandan D, Lopez M, Moenrezakhanlou A & Reiner NE 2006 Annexin V associates with the IFN-gamma receptor and regulates IFN-gamma signaling. *Journal of Immunology* **176** 5934–5942.
- Linder K, Arner P, Flores-Morales A, Tollet-Egnell P & Norstedt G 2004 Differentially expressed genes in visceral or subcutaneous adipose tissue of obese men and women. *Journal of Lipid Research* **45** 148–154. (doi:10.1194/jlr.M300256-JLR200)
- Metzger BE, Phelps RL, Freinkel N & Navickas IA 1980 Effects of gestational diabetes on diurnal profiles of plasma glucose, lipids, and individual amino acids. *Diabetes Care* **3** 402–409.
- Montes-Nieto R, Insenser M, Martinez-Garcia MA & Escobar-Morreale HF 2013 A nontargeted proteomic study of the influence of androgen excess on human visceral and subcutaneous adipose tissue proteomes. *Journal of Clinical Endocrinology and Metabolism* **98** E576–E585. (doi:10.1210/jc.2012-3438)
- Nikitovic D, Katonis P, Tsatsakis A, Karamanos NK & Tzanakakis GN 2008 Lumican, a small leucine-rich proteoglycan. *IUBMB Life* **60** 818–823. (doi:10.1002/iub.131)
- Oliva K, Barker G, Riley C, Bailey MJ, Permezel M, Rice GE & Lappas M 2012 The effect of pre-existing maternal obesity on the placental proteome: two-dimensional difference gel electrophoresis coupled with mass spectrometry. *Journal of Molecular Endocrinology* **48** 139–149. (doi:10.1530/JME-11-0123)
- Ortega FJ, Mercader JM, Moreno-Navarrete JM, Sabater M, Pueyo N, Valdes S, Ruiz B, Luche E, Naon D, Ricart W *et al.* 2012 Targeting the association of calgranulin B (S100A9) with insulin resistance and type 2 diabetes. *Journal of Molecular Medicine* **91** 523–534. (doi:10.1007/s00109-012-0979-8)
- Pasarica M, Gowronska-Kozak B, Burk D, Remedios I, Hymel D, Gimble J, Ravussin E, Bray GA & Smith SR 2009 Adipose tissue collagen VI in obesity. *Journal of Clinical Endocrinology and Metabolism* **94** 5155–5162. (doi:10.1210/jc.2009-0947)
- Peinado JR, Pardo M, de la Rosa O & Malagon MM 2012 Proteomic characterization of adipose tissue constituents, a necessary step for understanding adipose tissue complexity. *Proteomics* **12** 607–620. (doi:10.1002/pmic.201100355)
- Peng XA, Nelson ES, Maier JL & DeMali KA 2011 New insights into vinculin function and regulation. In *International Review of Cell and Molecular Biology*, vol 287, pp 191–231. Ed KW Jeon. San Diego: Elsevier Academic Press, Inc.
- Perez-Perez R, Ortega-Delgado FJ, Garcia-Santos E, Lopez JA, Camafeita E, Ricart W, Fernandez-Real JM & Peral B 2009 Differential proteomics of omental and subcutaneous adipose tissue reflects their unlike biochemical and metabolic properties. *Journal of Proteome Research* **8** 1682–1693. (doi:10.1021/pr800942k)
- Perrini S, Ficarella R, Picardi E, Cignarelli A, Barbaro M, Nigro P, Peschiera A, Palumbo O, Carella M, De Fazio M *et al.* 2013 Differences in gene expression and cytokine release profiles highlight the heterogeneity of distinct subsets of adipose tissue-derived stem cells in the subcutaneous and visceral adipose tissue in humans. *PLoS ONE* **8** e57892. (doi:10.1371/journal.pone.0057892)
- Pregnancy IAD 2010 International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. *Diabetes Care* **33** 676–682. (doi:10.2337/dc09-1848)

- Qvisth V, Hagström-Toft E, Moberg E, Sjöberg S & Bolinder J 2007 Lactate release from adipose tissue and skeletal muscle *in vivo*: defective insulin regulation in insulin-resistant obese women. *American Journal of Physiology. Endocrinology and Metabolism* **292** E709–E714. (doi:10.1152/ajpendo.00104.2006)
- Rice GE, Illanes SE & Mitchell MD 2012 Gestational diabetes mellitus: a positive predictor of type 2 diabetes? *International Journal of Endocrinology* **2012** 721653. (doi:10.1155/2012/721653)
- Roth J, Goebeler M & Sorg C 2001 S100A8 and S100A9 in inflammatory diseases. *Lancet* **357** 1041. (doi:10.1016/S0140-6736(05)71610-X)
- Salari S, Seibert T, Chen YX, Hu TQ, Shi CH, Zhao XL, Cuerrier CM, Raizman JE & O'Brien ER 2013 Extracellular HSP27 acts as a signaling molecule to activate NF-kappa B in macrophages. *Cell Stress & Chaperones* **18** 53–63. (doi:10.1007/s12192-012-0356-0)
- Schaefer L, Raslik I, Grone HJ, Schonherr E, Macakova K, Ugorcakova J, Budny S, Schaefer RM & Kresse H 2001 Small proteoglycans in human diabetic nephropathy: discrepancy between glomerular expression and protein accumulation of decorin, biglycan, lumican, and fibromodulin. *FASEB Journal* **15** 559–561. (doi:10.1096/fj.00-0493fje)
- Scherer PE, Bickel PE, Kotler M & Lodish HF 1998 Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. *Nature Biotechnology* **16** 581–586. (doi:10.1038/nbt0698-581)
- Spencer M, Yao-Borengasser A, Unal R, Rasouli N, Gurley CM, Zhu B, Peterson CA & Kern PA 2010 Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis and demonstrate alternative activation. *American Journal of Physiology. Endocrinology and Metabolism* **299** E1016–E1027. (doi:10.1152/ajpendo.00329.2010)
- Vohl MC, Sladek R, Robitaille J, Gurd S, Marceau P, Richard D, Hudson TJ & Tchernof A 2004 A survey of genes differentially expressed in subcutaneous and visceral adipose tissue in men. *Obesity Research* **12** 1217–1222. (doi:10.1038/oby.2004.153)
- Wild S, Roglic G, Green A, Sicree R & King H 2004 Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* **27** 1047–1053. (doi:10.2337/diacare.27.5.1047)
- Yohannes E, Chang J, Christ GJ, Davies KP & Chance MR 2008 Proteomics analysis identifies molecular targets related to diabetes mellitus-associated bladder dysfunction. *Molecular & Cellular Proteomics* **7** 1270–1285. (doi:10.1074/mcp.M700563-MCP200)
- Young JC, Agashe VR, Siegers K & Hartl FU 2004 Pathways of chaperone-mediated protein folding in the cytosol. *Nature Reviews. Molecular Cell Biology* **5** 781–791. (doi:10.1038/nrm1492)
- Ziegler WH, Liddington RC & Critchley DR 2006 The structure and regulation of vinculin. *Trends in Cell Biology* **16** 453–460. (doi:10.1016/j.tcb.2006.07.004)

Received in final form 5 May 2013

Accepted 24 May 2013

Accepted Preprint published online 24 May 2013