

# Gestational diabetic transcriptomic profiling of microdissected human trophoblast

Muhammad Furqan Bari<sup>1,\*</sup>, Sherry Ngo<sup>2,\*</sup>, Claire C Bastie<sup>3</sup>, Allan M Sheppard<sup>2</sup> and Manu Vatish<sup>4</sup>

<sup>1</sup>Department of Pathology, Dow International Medical College, Karachi, Pakistan

<sup>2</sup>Liggins Institute, University of Auckland, Auckland, New Zealand

<sup>3</sup>Division of Biomedical Sciences, Warwick Medical School, Coventry, UK

<sup>4</sup>Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, UK

(\*M F Bari and S Ngo contributed equally to this work)

Correspondence should be addressed to A M Sheppard or M Vatish

**Emails**

[a.sheppard@auckland.ac.nz](mailto:a.sheppard@auckland.ac.nz) or [manu.vatish@obs-gyn.ox.ac.uk](mailto:manu.vatish@obs-gyn.ox.ac.uk)

## Abstract

Gestational diabetes mellitus (GDM), the most common metabolic complication of pregnancy, is influenced by the placenta, and its prevalence directly increases with obesity. Therefore, to define the aetiology of GDM requires that the confounding influence of obesity and the heterogeneous nature of the placenta impairing accurate quantitative studies be accounted for. Using laser capture microdissection (LCM), we optimized RNA extraction from human placental trophoblast, the metabolic cellular interface between mother and foetus. This allowed specific transcriptomic profiling of trophoblast isolated from GDM, and obese and normal human placentae. Genome-wide gene expression analysis was performed on the RNA extracted from the trophoblast of GDM and obese and normal placentae. Forty-five differentially expressed genes (DEGs) specifically discriminated GDM from matched obese subjects. Two genes previously linked with GDM, pregnancy specific beta-1 glycoprotein 6 (*PSG6*) and placental system A sodium-dependent transporter system (*SLC38A1*), were significantly increased in GDM. A number of these DEGs (8 ubiquitin-conjugating enzymes (UBE) splice variants (*UBE2D3* variants 1, 3, 4, 5, 6, 7, and 9) and *UBE2V1* variant 4)) were involved in RNA processing and splicing, and a significant number of the DEGs, including the UBE variants, were associated with increased maternal fasting plasma glucose. It is concluded that DEGs discriminating GDM from obese subjects were pinpointed. Our data indicate a biological link between genes involved in RNA processing and splicing, ubiquitination, and fasting plasma glucose in GDM taking into account obesity as the confounder.

## Key Words

- ▶ placenta
- ▶ obesity
- ▶ gestational diabetes
- ▶ laser capture microdissection
- ▶ RNA processing
- ▶ ubiquitination

*Journal of Endocrinology*  
(2016) **229**, 47–59

## Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance resulting in hyperglycaemia of variable severity during pregnancy, is the commonest metabolic complication of pregnancy (Virjee *et al.* 2001), with

increasing prevalence directly linked to increasing obesity in women of reproductive age (Dabelea *et al.* 2005). Women with GDM and obesity (and their offspring) have a higher prevalence of metabolic syndrome than those

without a history of GDM or obesity (Catalano *et al.* 2012; Vohr & Boney 2008) as well increased GDM recurrence in subsequent pregnancies (Bottalico 2007). Approximately 50 % of women with GDM are expected to develop type 2 diabetes within 5–10 years of delivery (Bellamy *et al.* 2009). Other risks of GDM include an increased likelihood of pregnancy complications, such as pre-eclampsia and offspring metabolic syndrome (Boney *et al.* 2005; Bryson *et al.* 2003).

GDM is linked with aberrant placental function and a number of placenta-derived molecules implicated in the manifestation of insulin resistance associated with GDM (Desoye & Hauguel-de Mouzon 2007). The key functioning unit of the placenta is the chorionic villus, in which a multi-nucleated trophoblast (comprising of syncytiotrophoblast and cytotrophoblast) lies in direct contact with maternal blood and remains the primary site of metabolic exchange at the maternal–foetus interface (Huppertz 2008). In addition to the trophoblast, however, there is an underlying stromal tissue and foetal capillaries as well as placental macrophages (known as Hofbauer cells) (Huppertz 2008). The heterogeneous nature of this tissue has rendered quantitative studies of the trophoblast difficult. While flow cytometry and cell culture of cellular fractions can be used to examine other cell types within the placenta, the trophoblast (part of which is a true syncytium) is not amenable to these techniques without contamination. This issue can be avoided by using LCM, a technique that allows isolation of distinct cell types (in our case, trophoblast) from frozen section or formalin-fixed samples of tissue. Studies have been hampered both by the difficulties of separating the effects of obesity from those of GDM and by extracting RNA of sufficient quality to perform meaningful downstream analysis. Thus our data allows the effects of obesity to be subtracted from GDM at the level of the trophoblast with high quality RNA subjected to microarray.

Our findings indicate that GDM placental trophoblast is fundamentally, biologically distinct from the states of normality and obesity at gene expression levels. Taking into account obesity as the confounder, genes that showed increased expression in GDM were largely involved in RNA processing and splicing. We also found increased expression of multiple splice variants of the ubiquitin-conjugating enzymes *UBE2D3*, and *UBE2V1* variant 4, in GDM. The expression of these genes was significantly associated with maternal fasting plasma glucose, suggesting an association of these genes with glucose intolerance, a hallmark of GDM.

## Materials and methods

### Samples

All study participants were pregnant women scheduled for elective Caesarean section at 39–40 weeks of gestation. The indication for Caesarean section was either breech presentation or repeat Caesarean section. Oral glucose (75 g) tolerance tests (OGTTs) were carried out at 26–28 weeks of gestation in all participants to diagnose or exclude GDM. All GDM patients were well controlled on insulin and only those with male offspring were recruited. The Coventry Local Research Ethics committee approved the study, and all patients gave written informed consent (Research Ethics Committees 07/H1210/118). Women with multiple pregnancies, as well as patients with cardiovascular disease, pre-eclampsia, or other relevant diseases were excluded. Placental biopsies were taken in the operating theatre and immediately snap-frozen in liquid nitrogen and transferred to a –80°C freezer until needed. Placental biopsies (by a random sampling method (Burton *et al.* 2014)) from four lean (normal; Norm), four obese (Ob), and four gestational diabetic (GDM) subjects were used.

### Laser microdissection (LCM): efficacy of staining and duration of LCM procedure on RNA Integrity Number (RIN)

The sections of 8 µm thickness were cut using a cryostat (Leica Microsystems, Milton Keynes, UK) at –25°C and adhered onto MembraneSlides (Molecular Machines & Industries AG, Glattbrugg, Switzerland) pre-cooled inside the cryostat and maintained dehydrated in –80°C freezer until needed. All slides, LCM caps, and solutions were obtained from Molecular Machines & Industries. The slides were dehydrated in graded alcohols and xylene and placed in a desiccator until laser capture. The sections were stained with (a) Acridine orange (Life Technologies), (b) Alcoholic cresyl violet (Life Technologies), (c) Alcoholic cresyl violet and alcoholic eosin Y (Sigma-Aldrich), (d) Arcturus Paradise stain (Life Technologies) in combination with RNase inhibitor, (e) Arcturus Paradise stain, (f) haematoxylin (Leica Microsystems, Milton Keynes, UK) and alcoholic eosin Y in combination with RNase inhibitor, and (g) haematoxylin and alcoholic eosin Y together with alcoholic eosin-Y. RIN values were assessed using Nano/Pico Chips on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) (Clement-Ziza *et al.* 2008). Having identified the optimal stain, LCM was performed in 0, 30 and 50 min to assess the effect of duration of LCM on RIN values.

## RNA extraction and gene expression profiling

The RNA was extracted using RNeasy Micro Kit (Qiagen Inc.) for the analysing RIN on the Bioanalyzer, while for gene expression profiling, microdissected samples were exposed to Prelude Direct Lysis Module (NuGEN, CA, USA) and hybridized to Affymetrix HuGene ST 1.0 arrays using manufacturer's protocols (Affymetrix Inc.). One chip was used for each clinical sample. Raw intensity data files (.CEL) were read using the publicly available oligo package in R (Carvalho *et al.* 2007). Background subtraction, quantile normalization and summarization via median-polish, and finally log<sub>2</sub> transformed were performed on the raw data using the R package RMA (Robust Multichip Average preprocessing methodology) (Irizarry *et al.* 2003). Analysis of differential gene expression using a linear models approach was performed using the R package Limma (Smyth 2004). Adjusted *P*-values <0.05 using FDR correction were considered statistically significant. Results were filtered using ranked two-fold change in gene expression from pairwise comparisons between the three subject groups (Norm, Ob and GDM). Heatmap illustration of sample hierarchical clustering was generated using the R package gplots (<http://www.cran.r-project.org/>).

## Validation of gene expression microarray data using qRT-PCR

The four genes that were upregulated by two-fold in GDM group compared with Ob and Norm groups were selected for validating the gene expression microarray data in the same four samples for each group as used for gene expression microarrays. All experiments were carried out in triplicate. The genes and primers sequences used in this study are UBE2V1 (Forward: CAAGAGCGACGCAAG ATG; Reverse: TCTGGGTATTTAGGTCCACATT); UBE2D3 transcript variant 6 (Forward: GCAGAAAGGATACTGT GTG; Reverse: CAGGATTGTCTCGTCTCA); UBE2D3 transcript variant 5 (Forward: CATTTGGAGGCGGA ACC; Reverse: AACTTCGTGGCTGGCTAA), and PSG6 Forward: CCTTACATCACCATCAACAA; Reverse: TCCGA CTCTTAGGTTAC). All the primers were designed as described previously (Bari *et al.* 2013). RNA from laser microdissected samples was extracted using the Arcturus PicoPure RNA Isolation Kit (Life Technologies) with on-column DNase digestion (Qiagen) and converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies) as recommended by the manufacturer. Genomic DNA was digested using

recombinant shrimp DNase (Affymetrix). The qRT-PCR was performed using SYBR green-based chemistry on an Applied Biosystems 7500 Fast PCR system using Fast SYBR green master mix (Life Technologies), following the manufacturer's recommendations. The qRT-PCR data were analysed using DataAssist software ver 3.01 (Life Technologies), 18S was taken as endogenous control, and *P*-values were adjusted using Benjamini-Hochberg false discovery rate.

## Statistical analysis

All statistical analysis was performed in R (<http://www.bioconductor.org/>). Assessment of statistically significant difference in clinical measures between subject groups was performed using Tukey method of multiple comparisons test. Linear regression analysis was performed to determine association of gene expression with maternal fasting plasma glucose using deltaGT. 'deltaGT' was calculated as [Glucose]<sub>2h</sub> - [Glucose]<sub>Baseline</sub> measured using oral glucose tolerance test. KEGG pathway data base is available at <http://www.genome.jp/kegg/pathway.html>

## Results

### Maternal and foetal clinical characteristics

The subjects used in this study averaged 39 weeks of gestation at delivery (Table 1). The BMI for Norm group (24.1 ± 1.0 kg/m<sup>2</sup>) was significantly lower than those in GDM (32.3 ± 0.8 kg; *P*=0.0001) or Ob (30.8 ± 0.3; *P*=0.0003) groups. Baseline glucose levels were similar between all subjects (GDM: 5.3 ± 2 mM; Norm: 5 ± 0.2 mM; Ob: 5.3 ± 0.1 mM). As expected, fasting plasma glucose following an oral glucose tolerance test (OGTT) (8.6 ± 0.3 mM) was significantly increased in GDM subjects compared with Norm (5.5 mM; *P*=3.2 × 10<sup>-6</sup>) or Ob subjects (5.6 ± 0.1 mM; *P*=3.2 × 10<sup>-6</sup>). Maternal insulin resistance has been recently reported to correlate with foetal insulin resistance (Wang *et al.* 2013). In our study, maternal insulin level was mildly elevated in GDM (25 ± 12.3 μIU/mL) and Ob (22 ± 3.5 μIU/mL) groups compared with Norm (14 ± 8.1 μIU/mL) group, albeit not significant. Maternal glucose was mildly elevated in the GDM (4.8 ± 0.3 mM) compared with Ob (4.3 ± 0.2 mM) or Norm (4.4 ± 0.1 mM) group. Foetal insulin level was lower in GDM (6.1 ± 0.7 μIU/mL) group compared with Norm (12.4 ± 1.2 μIU/mL; *P*=0.11) or Ob (15.8 ± 4.4 μIU/mL; *P*=0.07) group. Foetal glucose was mildly elevated in GDM (4.6 ± 0.3 mM) group compared with Norm

Table 1 Subjects characteristics.

Variable	GDM		Norm		Ob		P-value		GDM		Norm		Ob	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.			min	max	min	max	min	max
<b>Maternal characteristics</b>														
Age (years)	32.8	3.0	34.5	1.3	34.3	5.1	n.s.		27.0	41.0	32.0	38.0	22.0	47.0
Weeks of gestation	38.6	0.3	39.4	0.1	39.1	0.3	n.s.		38.1	39.4	39.3	39.6	38.3	39.6
Body mass index (kg/m <sup>2</sup> )	32.3	0.8	24.1	1.0	30.8	0.3	0.0001 (N vs GDM) 0.0003 (N vs Ob)		1.5	1.6	1.6	1.7	1.6	1.7
OGTT at baseline (mM)	5.3	0.2	5.0	0.2	5.3	0.1	n.s.		4.9	5.8	4.6	5.3	5.0	5.6
OGTT post glucose load (mM)	8.6	0.3	5.5	0.0	5.6	0.1	3.2x10 <sup>-6</sup> (N vs GDM) 3.9 x 10 <sup>-6</sup> (GDM vs Ob)		8.0	9.2	5.4	5.6	5.4	5.7
Maternal haemoglobin (g/dL)	11.3	0.3	12.5	0.3	10.8	0.9	n.s.		10.5	11.8	11.9	13.3	8.4	12.9
Maternal insulin (µIU/mL)	25.6	12.3	14.5	3.5	22.4	8.1	n.s.		5.6	61.2	8.8	24.7	7.7	42.3
Maternal glucose (mM)	4.8	0.3	4.4	0.1	4.3	0.2	n.s.		4.0	5.6	4.2	4.6	3.9	4.9
Maternal HOMA-IR	5.8	3.2	2.9	0.7	4.5	1.8	n.s.		1.1	15.2	1.7	4.9	1.4	9.2
Maternal leptin (pg/mL)	44,782.1	5407.3	18,554.2	1289.3	38,175.3	2647.7	0.01 (N vs Ob) 0.001 (N vs GDM)		33,287.2	59,049.3	15,427.0	21102.9	31,500.7	43,976.1
<b>Foetal characteristics<sup>a</sup></b>														
Foetal insulin (µIU/mL)	6.1	0.7	12.4	1.2	15.8	4.4	0.069 (GDM vs Ob)		4.8	8.1	10.3	15.8	3.0	22.8
Foetal glucose (mM)	4.6	0.3	3.9	0.3	4.0	0.1	n.s.		3.9	5.4	3.2	4.5	3.7	4.2
Foetal HOMA-IR	1.3	0.2	2.2	0.3	2.8	0.8	n.s.		0.9	1.9	1.5	2.8	0.6	4.3
Foetal leptin (pg/mL)	4352.7	400.5	1702.3	136.8	7354.7	1371.0	0.071 (GDM vs Ob) 0.002 (N vs Ob)		3765.8	5522.6	1429.3	2062.9	3954.3	9818.8
Foetal birthweight (kg)	3.70	0.05	3.42	0.09	3.81	0.06	0.005 (N vs Ob) n.s. (GDM vs Ob)		3.59	3.82	3.1	3.55	3.7	4.0
Foetal length	48	1.47	47.8	0.62	49.8	1.7	0.01 (GDM vs N) n.s.		45	52	46	49	45	53

Maternal and foetal clinical measures of the Norm, GDM, and Ob subjects expressed as mean ± standard error or the mean (S.E.M.), and minimum (min) and maximum (max) value. Statistical significance was performed using the Tukey's method of multiple comparisons test.

OGTT, oral glucose tolerance test. n.s., not significant ( $P > 0.05$ ).

<sup>a</sup>From cord blood collected at delivery.



( $3.9 \pm 0.3$  mM) or Ob ( $4.0 \pm 0.1$  mM) group, but was not statistically significant. We found maternal leptin was significantly elevated in GDM ( $44,782.1 \pm 5407.3$  pg/mL;  $P=0.001$ ) and Ob ( $38,175.3 \pm 2647.7$  pg/mL;  $P=0.01$ ) compared groups with Norm ( $18,554.2 \pm 1289.3$  pg/mL) group. Similarly, foetal leptin was significantly elevated in GDM ( $4352.7 \pm 400.5$  pg/mL) and Ob ( $7354.7 \pm 1371.0$  pg/mL) groups compared with Norm ( $1702.3 \pm 136.8$  pg/mL) group.

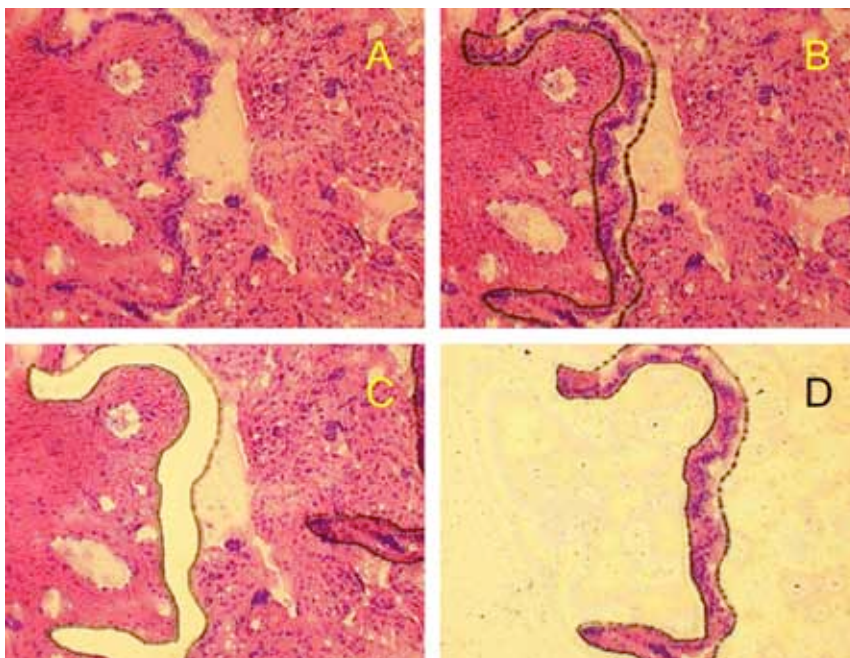
### Acridine orange and alcoholic cresyl violet stains are most effective in preserving human placental trophoblast RNA

LCM was performed as described above (Fig. 1). The tissues processed for RNA with all steps of hydration and dehydration without any stain yielded a RNA Integrity Number (RIN) of 8.6 (Supplementary Figure 1, see section on supplementary data given at the end of this article). Staining with Acridine orange gave the highest RIN value of 8.5 (a drop of only 0.1; Supplementary Figure 1). However as the filter for this fluorescent dye is not available in all instruments, we used other non-fluorescent dyes as well. Among the non-fluorescent dyes, the alcoholic cresyl violet was the most effective dye in preserving RIN and yielded a RIN of 8.0 (drop of 0.6; Supplementary Figure 1). As cytoplasmic staining was also needed in dissecting the trophoblast, it was combined with alcoholic eosin Y,

which dropped the RIN to 7.8 (drop of 0.8; Supplementary Figure 1). The Arcturus Paradise stain with the addition of RNase inhibitor at a concentration of 1 unit/ $\mu$ L of stain yielded a RIN of 6.7 (drop of 1.9; Supplementary Figure 1), whereas the use of Arcturus Paradise stain alone dropped the RIN to 6.0 (drop of 2.6; Supplementary Figure 1). In contrast, the use of routine haematoxylin in combination with alcoholic eosin Y together with RNase inhibitor yielded a RIN of 5.4 (drop of 3.2; Supplementary Figure 1) and finally haematoxylin with alcoholic eosin Y without the RNase inhibitor dropped the RIN to 3.0 (drop of 5.6; Supplementary Figure 1). Having identified alcoholic cresyl violet and alcoholic eosin-Y as the optimal non-fluorescent stain, we next assessed the effect of duration of LCM on RIN values.

### LCM within 30 min maintains RIN

Time points at 0, 30, and 50 min were selected and resulting RIN values 7.5, 6.9, and 6.2 respectively (Supplementary Figure 2) indicated that the degradation is proportional to the duration of dissection; with 30 min being optimal to allow laser-capture to be performed while maintaining RNA integrity. Tissue extraction for microarray experiments was thus performed under 30 min in tissues stained with alcoholic cresyl violet and alcoholic eosin-Y. The tissues destined for microarray were placed directly in the



**Figure 1**  
Representative image showing laser capture microdissection of human placental trophoblast. (A) Native placental tissue stained with haematoxylin and eosin. (B) Laser cut around trophoblast layer. Residual tissue (C) and trophoblast (D) capture.

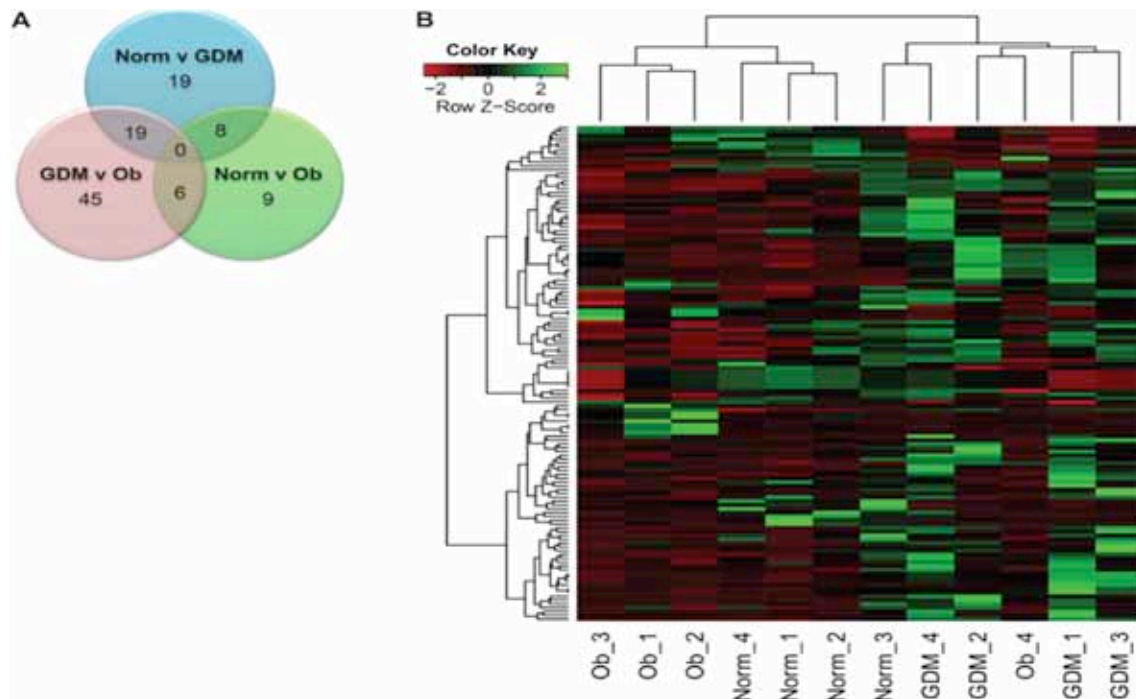
NuGEN Direct Lysis Module before hybridisation on Affymetrix HuGene ST 1.0 array.

### Differential gene expression profiles of Norm, Ob, and GDM subjects

Among the 28,869 transcript-level probes comprising the main design of the microarray, 329 probes exhibited ranked differential gene expression of at least two-fold change (up/down) between the subject groups. Of these, 130 transcripts were annotated with Entrez Gene ID (corresponding to 106 genes) and these were used for downstream functional enrichment analysis. We assessed the gene expression profile differences between Norm, Ob, and GDM subjects. We found 46 DEGs from Norm versus GDM comparison (Contrast 1); 23 genes from Norm versus Ob (Contrast 2); 70 genes from GDM vs Ob comparison (Contrast 3) (Fig. 2A). Of the DEGs from Contrast 1, 19 DEGs were unique to this pairwise comparison; these genes characterise GDM subjects who are also obese. We found that nine DEGs were unique to Contrast 2 i.e., Ob individuals compared

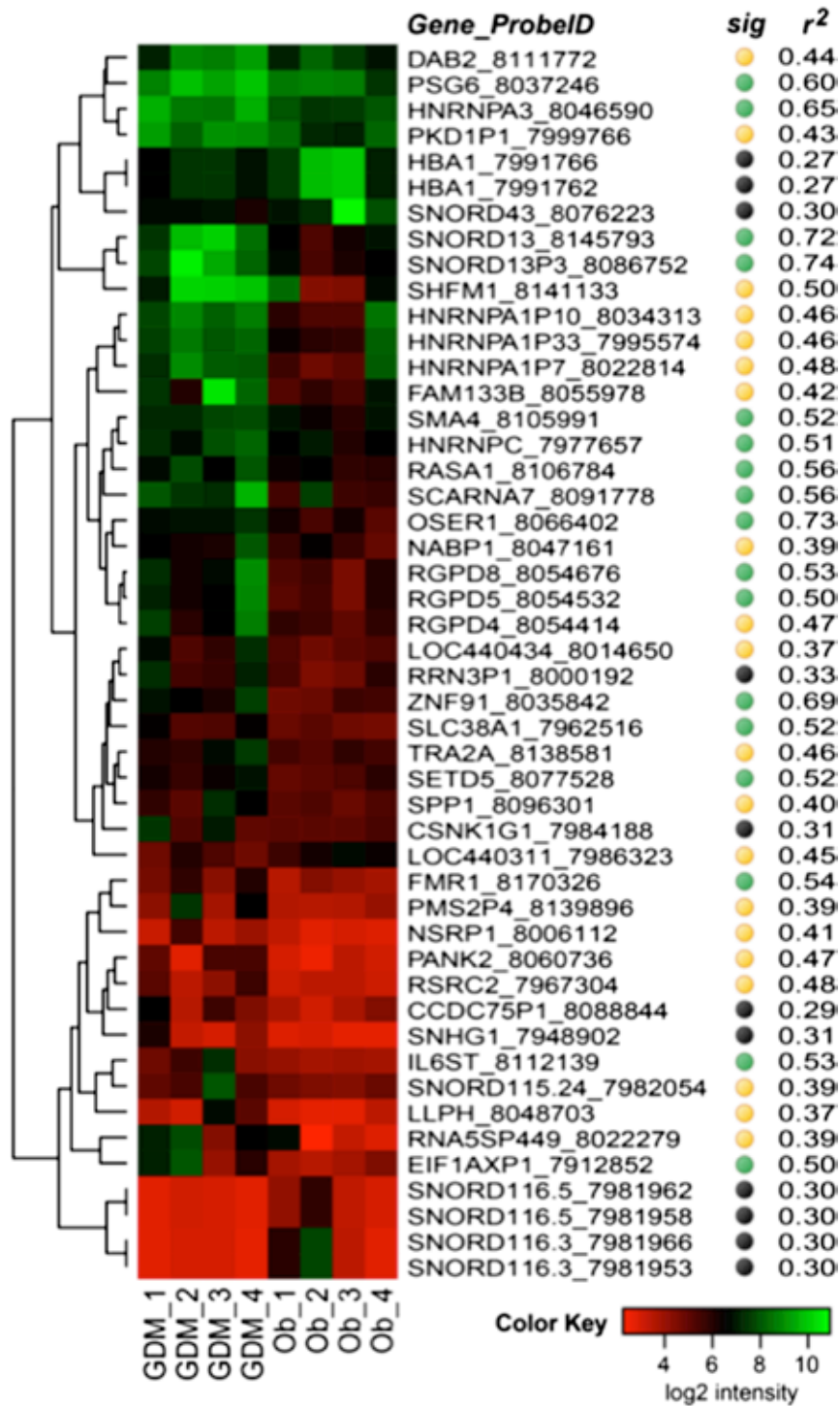
with normal individuals. In contrast, 45 DEGs were unique to Contrast 3 wherein GDM individuals were subject-matched to obese individuals. As such, genes of interest from Contrast 3 characterise a state of GDM taking into account obesity as a confounder (Fig. 2A). We also found that 8 DEGs were common between Contrast 1 and Contrast 2. In addition, 6 DEGs were common between Contrast 2 and Contrast 3, whereas 19 genes were common between Contrast 1 and 3 (Fig. 2A). The list of DEGs identified from each pairwise comparison (Contrast 1–3) is found in Supplementary Table 1 (see section on supplementary data given at the end of this article). Probe level information including annotated GeneID and fold change in gene expression between subject groups are listed in Supplementary Table 2.

Array intensities (after preprocessing and data normalization) of all probes for genes with at least two-fold change (i.e., 391 probes) were visualized using a heatmap (Fig. 2B). As illustrated in the dendrogram on the horizontal axis of the heatmap, hierarchical sample clustering generated two main spot clusters. The



**Figure 2**

Gene expression profile characterising Norm, Ob, and GDM subjects. (A) The number of differentially expressed genes that are unique or common between Norm, Ob, and GDM pairwise comparisons. Contrast 1: Norm vs GDM; Contrast 2: Norm vs Ob; Contrast 3: GDM vs Ob. Criterion:  $\geq 2$  ranked fold change (up/down) between any pairwise comparison. (B) Hierarchical clustering of subjects showing GDM as distinctly different from Norm and Ob individuals. Array intensities for probes with  $\geq 2$ -fold change (up/down) between any pairwise comparisons were centred and scaled (row z-score), and then used to generate the heatmap. Agglomeration method: complete linkage. Distance measure: Euclidean. Red-Black-Green color key denotes low to high gene expression. “\_Integer” on the horizontal axis denotes individual subjects in each group.

**Figure 3**

Genes differentially expressed in GDM are associated with maternal fasting plasma glucose. *Left*: Differentially expressed genes *unique* to GDM vs Ob comparison (Contrast 3) were clustered by expression level ( $\log_2$  intensity). Agglomeration method used was complete linkage. Distance measure used was Euclidean. Genes are illustrated at the transcript level for individual subjects. Colour key: red-black-green represents low to high expression. 'Gene\_ProbeID' denotes Gene Symbol and corresponding Transcript Cluster Identifier from the microarray. *Right*: Results of the linear regression analysis to determine association between gene expression and fasting plasma glucose level post OGTT for Ob and GDM subjects using 'deltaGT'. 'deltaGT' was calculated as  $[\text{Glucose}]_{2h} - [\text{Glucose}]_{\text{Baseline}}$  measured by OGTT. 'sig' denotes level of significance from the regression analysis; Green:  $P < 0.05$ , Yellow:  $0.05 < P < 0.1$ ; Black:  $P > 0.1$ .  $r^2$ : coefficient of determination.

GDM subjects comprise one spot cluster, whereas the Norm and Ob subjects comprise the second larger spot cluster. Overall, the heatmap illustrates GDM subjects as a distinct group of individuals relative to Norm and Ob subjects based on their gene expression profiles. In support of this result, functional Gene Ontology term

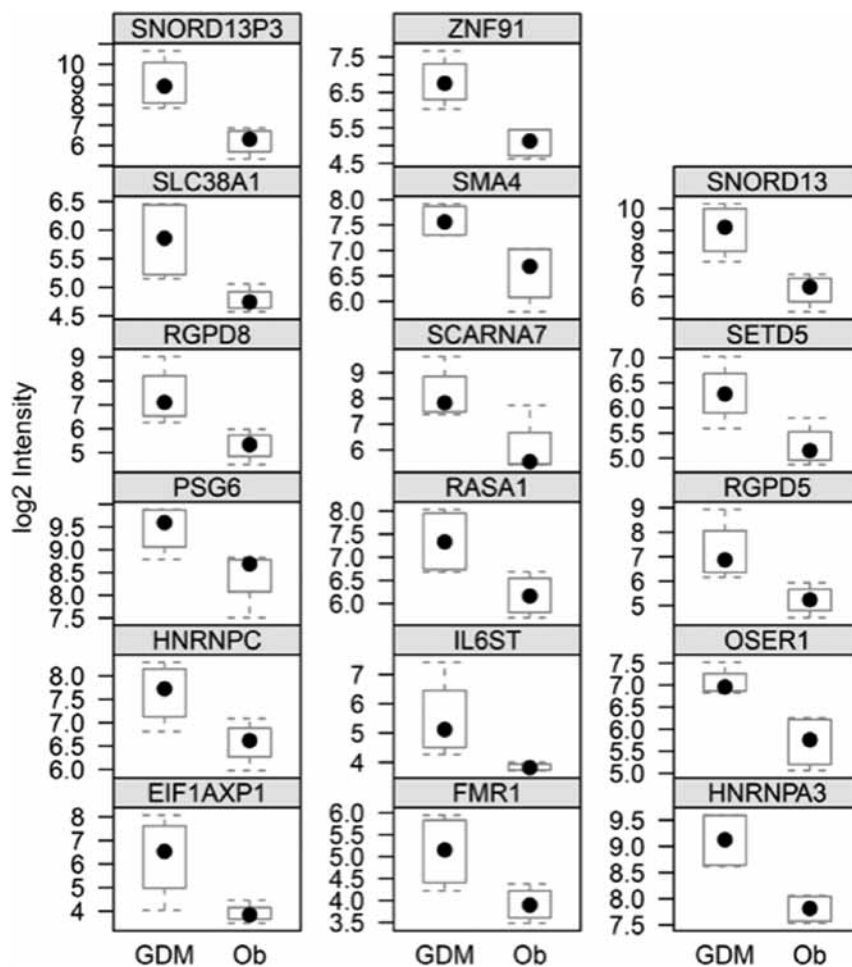
enrichment analysis showed that the significantly enriched GO terms for Contrast 1 and Contrast 2 were virtually identical and related to a wide range of processes including protein metabolic process, RNA processing, gene expression, and translational processes with the exception of GO:0022904 (respiratory electron



transport chain) which was associated with Contrast 1 but not Contrast 2. However, the significantly enriched GO terms for Contrast 3 are distinct from those of both Contrasts 1 and 2: enriched GO terms annotated to RNA splicing processes, intracellular transport of proteins to Golgi (Supplementary Table 3). Similarly, several KEGG pathways including oxidative phosphorylation (Kegg:00190) and ribosome (Kegg:03010) pathways were predicted as being significantly enriched in both Contrasts 1 and 2. However, only the spliceosome pathway (Kegg:03040) was significantly over-represented in Contrast 3 (Supplementary Table 4). Three miRNAs (hsa-miR-876-5p, hsa-miR-151-3p, hsa-miR-641) were predicted as being significantly over-represented in Contrast 3 (GDM), whilst no miRNA enrichment was found for Contrasts 1 and 2 (Supplementary Table 5), suggesting that these miRNAs may be the useful indicators of GDM taking into account obesity as the confounder.

### Genes uniquely differentially expressed between GDM and Ob subjects correlate with maternal fasting plasma glucose

Given that elevated fasting plasma glucose following an OGTT characterises a state of GDM, regression analyses were performed to determine correlations between the DGEs unique to Contrast 3 (GDM vs Ob) and maternal OGTT response. As illustrated in Fig. 3, among the DEGs, 17 genes were significantly associated with maternal fasting plasma glucose ( $P < 0.05$ ; Fig. 3). The expression of these genes (*EIF1AXP1*, *FMR1*, *HNRNPA3*, *HNRNPC*, *IL6ST*, *OSER1*, *PSG6*, *RASA1*, *RGPD5*, *RGPD8*, *SCARNA7*, *SETD5*, *SLC38A1*, *SMA4*, *SNORD13*, *SNORD13P3*, and *ZNF91*) was increased in GDM by two- to five-fold compared with Ob group (Fig. 4). Of these, several are known to be involved in RNA processing and splicing (*FMR1*, *HNRNPA3*, *HNRNPC*, *SCARNA7*, *SNORD13*); full gene names are found in Fig. 4 legend.



**Figure 4**

Increased expression of GDM-associated genes taking into account obesity as the confounder and which are significantly associated with maternal fasting plasma glucose. Expression levels of genes *unique* to Contrast 3 (GDM vs Ob). Box denotes 25th to 75th percentile, tails denote the range (maximum and minimum), and dot inside box denotes median expression. Gene names: *EIF1AXP1*: eukaryotic translation initiation factor 1A, X-linked pseudogene 1; *FMR1*: fragile X mental retardation 1; *HNRNPA3* and *HNRNPC*: heterogeneous nuclear ribonucleoprotein A3 and C respectively; *IL6ST*: interleukin 6 signal transducer; *OSER1*: Oxidative Stress Responsive Serine-rich 1; *PSG6*: Pregnancy Specific beta-1-Glycoprotein 6; *RASA1*: RAS p21 protein activator (GTPase activating protein) 1; *RGPD5* and *RGPD8*: RANBP2-like and GRIP domain containing 5 and 8 respectively; *SCARNA7*: small Cajal body-specific RNA 7; *SETD5*: SET domain containing 5; *SLC38A1*: solute carrier family 38, member 1; *SMA4*: glucuronidase, beta pseudogene; *SNORD13*: small nucleolar RNA, C/D box 13; *SNORD13P3*: small nucleolar RNA, C/D box 13 pseudogene 3; *ZNF91*: zinc finger protein 91.



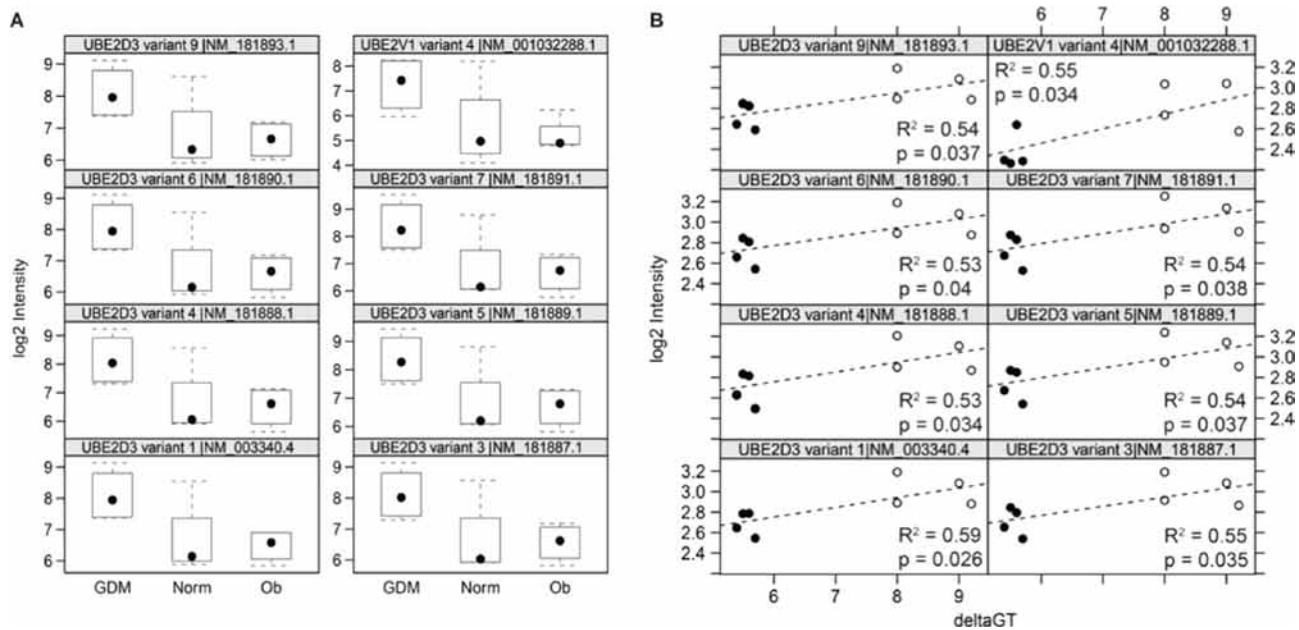
We also assessed the presence of differentially expressed splice variants between Norm, Ob, and GDM groups. We applied the two-fold change criterion to assess 'flmrna' probes (which are assigned to mRNA transcripts based on the Affymetrix annotation metafile). We found several differentially expressed transcript variants in Contrast 1 (Norm vs GDM), Contrast 2 (Norm vs Ob), and Contrast 3 (GDM vs Ob) (Table 2). Interestingly, transcript variants of ubiquitin-conjugating enzymes (*UBE2D3*) and (*UBE2V1*) were heavily represented in Contrasts 1 and 3. The levels of *UBE2D3* transcript variants were increased in GDM group by 2.5- to 3.0-fold relative to Norm, and 2.8- to 3.3-fold relative to Ob groups. Similarly, *UBE2V1*

transcript variant 4 level was increased in the GDM group by three-fold compared with Norm, and by four-fold compared with Ob group (Table 2). Distribution of the log2 intensities of these eight mRNA variants across the Norm, Ob, and GDM groups is shown in Fig. 5A. Microarray data were further confirmed by separate qRT-PCR (Supplementary Table 6). Furthermore, our regression analysis showed that increased maternal fasting plasma glucose (post OGTT) is significantly associated with increased levels of these eight UBE variants (Fig. 5B;  $P < 0.05$ ). These results suggest that increased *UBE2D3* and *UBE2V1* expression is associated with maternal glucose intolerance, which characterizes a state of GDM.

**Table 2** Differentially expressed mRNA transcripts in GDM compared with Norm or Ob subjects.

Group	FC	mRNA_AccNo	mRNA_Name
1 (Norm-v-GDM)	-2.68	NM_003340.4	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 1, mRNA
	-2.78	NM_181887.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 3, mRNA
	-2.84	NM_181888.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 4, mRNA
	-2.93	NM_181889.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 5, mRNA
	-2.63	NM_181890.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 6, mRNA
	-3.01	NM_181891.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 7, mRNA
	-2.47	NM_181893.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 9, mRNA
	-2.21	NM_001187.1	Homo sapiens B melanoma antigen ( <i>BAGE</i> ), mRNA
	2.07	NM_001029945.1	Homo sapiens chromosome 1 open reading frame 178 (C1orf178), transcript variant c, mRNA
	-3.26	NM_001032288.1	Homo sapiens ubiquitin-conjugating enzyme E2 variant 1 ( <i>UBE2V1</i> ), transcript variant 4, mRNA
2 (Norm-v-Ob)	2.10	NM_145326.1	Homo sapiens similar to hypothetical protein <i>FLJ13659</i> (LOC115648), mRNA
	2.09	NM_001014450.1	Homo sapiens small proline-rich protein 2F ( <i>SPRR2F</i> ), mRNA
3 (GDM-v-Ob)	3.01	NM_145326.1	Homo sapiens similar to hypothetical protein <i>FLJ13659</i> (LOC115648), mRNA
	3.08	NM_003340.4	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 1, mRNA
	2.94	NM_181887.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 3, mRNA
	3.15	NM_181888.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 4, mRNA
	3.23	NM_181889.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 5, mRNA
	2.84	NM_181890.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 6, mRNA
	3.30	NM_181891.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 7, mRNA
	2.77	NM_181893.1	Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast) ( <i>UBE2D3</i> ), transcript variant 9, mRNA
4.16	NM_001032288.1	Homo sapiens ubiquitin-conjugating enzyme E2 variant 1 ( <i>UBE2V1</i> ), transcript variant 4, mRNA	

FC denotes fold change in expression. Positive FC indicates increased expression in Norm for Contrasts 1 and 2, and increased expression in GDM for Contrast 3. mRNA\_AccNo denotes GenBank Accession Number.



**Figure 5**

Increased expression of mRNA variants of ubiquitin-conjugating enzymes which discriminates GDM phenotype from Ob and Norm is positively correlated with maternal OGTT. (A) Box denotes 25th to 75th percentile; tail denotes the range (maximum and minimum); dot inside box denotes median expression. Norm: Normal; Ob: Obese; GDM: Gestational diabetic. (B) Scatter plot of UBE variants (log<sub>2</sub> intensity) versus fasting plasma glucose in Ob and GDM subjects. 'deltaGT' was calculated as [Glucose]<sub>2h</sub> - [Glucose]<sub>baseline</sub> measured by OGTT. Closed circle: Ob; Open circle: GDM. Panel title: 'UBE variant name|Accession number'.

## Discussion

The impact of GDM on the progeny is made difficult to assess because of the confounding influence of maternal obesity in the manifestation of foetal phenotypes. The strength of the current study is the inclusion of obese subjects for comparison with GDM subjects with the aim of more accurately defining the molecular basis for disturbed placental function in GDM pregnancies. Indeed, our findings suggest that genes involved in RNA splicing processes and the intracellular transport of proteins to the Golgi apparatus may be differentially expressed in GDM subjects compared with normal or obese subjects, implying that GDM may be fundamentally distinct from states of normality or obesity at the molecular level.

Additional strengths include careful selection of patient to exclude other pathologies that might have impacted data analysis, optimization of RNA integrity using a variety of commonly used stains, and finally using patients who had undergone elective Caesarean section, which excluded potential stresses placed on the placenta by labour (Reddy *et al.* 2008).

A further strength is our approach of using LCM to investigate the changes in placental trophoblast gene

expression. As the placenta is a heterogeneous organ (Huppertz 2008), our approach of using LCM facilitates molecular analysis of trophoblast without contamination from other cell types. In this study, we present an optimized protocol for RNA extraction using LCM, which demonstrates that RNA integrity (RIN) is sustained when staining with alcoholic cresyl violet and alcoholic eosin-Y (Clement-Ziza *et al.* 2008). Moreover, we demonstrate that LCM can be performed with this stain for up to 30 min per slide with no significant degradation of RNA. We note that other publications using laser capture of placenta (Kim *et al.* 2013b) have used haematoxylin and eosin (which we have shown does not stabilize RNA) without demonstrating any formal assessment of RIN before hybridisation.

GDM is linked to placental function, and a number of placenta-derived molecules in particular from the trophoblast have been implicated in causing insulin resistance in GDM (Desoye and Hauguel-de Mouzon 2007). The expression of pregnancy-specific 1 glycoprotein (PSG), a common placental factor produced by trophoblast to allow for a viable pregnancy, has been reported to increase in preeclampsia (also known to be associated with increased insulin resistance (Catalano

*et al.* 2012) and GDM (Okazaki *et al.* 2007, Tamsen *et al.* 1983)). Increased PSG levels are associated with elevated carbohydrate levels in humans with GDM (Neufeld *et al.* 1984). Consistent with this, we found increased *PSG6* expression in GDM group compared with Ob group. In addition, increased *PSG6* expression in our GDM subjects significantly associated with increased maternal fasting plasma glucose as a result of impaired glucose tolerance. Moreover, *PSG6* and *PSG11* genotypes have recently been found to be located with a genomic region enriched in segmental duplication in preeclampsia patients (Zhao *et al.* 2012) (a disease, perhaps suggesting a linkage between *PSG6* and GDM and preeclampsia). Further studies into how glucose modulates placental *PSG6* function may improve our understanding of the pathogenesis of GDM.

The trophoblast cell layer, in direct contact with maternal blood, is the transporting epithelium of the human placenta integrating foetal and maternal signals to modulate placental growth in response to alterations in maternal nutrient supply (Jansson & Powell 2013). The placental system A sodium-dependent transporter system, comprising of the members *SNAT1* (*SLC38A1*), *SNAT2* (*SLC38A2*), and *SNAT4* (*SLC38A4*), is important for supplying neutral amino acids necessary for foetal growth (Jansson & Powell 2013). In addition, *SLC38A1* is a key contributor to System A activity in term placenta (Desforges *et al.* 2010). The molecular mechanisms by which diabetes in pregnancy affects placental nutrient transport is still unclear; however Jansson and coworkers reported that System A-mediated amino acid transport activity is significantly increased in the trophoblast microvillous plasma membrane (MVM) in GDM, and the authors suggested the increase to be related to increased amino acid catabolism in the placenta (Jansson *et al.* 2002). In another study, *SLC38A1* mRNA levels were found to be unchanged in placentae from preeclampsia, intrauterine growth-restricted, and small-for-gestational-age pregnancies i.e., models of intrauterine undernutrition. The authors concluded that regulation might occur at the transporter expression level or activity (Malina *et al.* 2005). Indeed, other studies found decreased System A transport activity in the MVM of intrauterine growth compared with the MVM of normal foetal growth (Glazier *et al.* 1997, Jansson *et al.* 2002). Our findings of increased *SLC38A1* expression in trophoblast (Fig. 3) are in agreement with Jansson and coworkers study who reported increased transport activity with this transporter in the MVM from GDM pregnancies.

The novel finding of increased expression of ubiquitin-conjugating enzyme *UBE2D3* and *UBE2V1*

transcript variants in GDM is intriguing. The ubiquitin-proteasome system is multi-functional (Lecker *et al.* 2006); it is involved in rapid removal of unwanted or misfolded proteins (Lecker *et al.* 2006) and tightly regulates gene transcription (Dhananjayan *et al.* 2005, Lecker *et al.* 2006) and immunological processes (Lecker *et al.* 2006). Ubiquitin-conjugating enzymes (*UBE2* variants facilitate the binding of ubiquitin molecules to ubiquitin ligases (*UBE3* variants) to target a protein for proteasome-dependent degradation (David *et al.* 2010). A recent study has reported the potential association of *UBE2E2* polymorphisms with fasting plasma glucose and GDM (Kim *et al.* 2013a). Furthermore, *UBE2* variants *D1* and *V2* have been recently found to be significantly differentially methylated in 8 to 12 year-old children exposed to maternal GDM *in utero* (West *et al.* 2013). Our finding and the emerging literature collectively suggest a link between *UBE* function and fasting plasma glucose in GDM.

The pattern of the DEGs we observed suggests that the ubiquitin-conjugating system is functionally more active in GDM compared with Ob, implying greater turnover rates of translation products. Interestingly, a recent proteomics study has reported accumulation of misfolded proteins in the urine of preeclampsia subjects, thus implying compromised proteasome function (Buhimschi *et al.* 2014). Given that ubiquitination and the proteasomal system are tightly coupled, it is conceivable that perturbed regulation of protein turnover may underlie the pathogenesis of common pregnancy disorders such as GDM and preeclampsia. That these phenotypes may share the elements of common aetiology are perhaps not entirely unexpected. A considerable literature describes a propensity for maternal GDM to be associated with postnatal occurrence of offspring insulin resistance (reviewed in (Borgono *et al.* 2012)). Similarly, mid-trimester maternal insulin resistance has been identified as a possible potentiating factor in the incidence of preeclampsia (Hauth *et al.* 2011). Furthermore, the incidence of preeclampsia has also been identified as an harbinger of infant type 2 diabetes (Libby *et al.* 2007). Collectively, these observations suggest that maternal insulin resistance in pregnancy increases the likelihood of preeclampsia complications at parturition, and that both clinical features are strongly associated with the subsequent emergence of metabolic syndrome in the infant. Our findings warrant expansion into larger studies using populations representing wide multi-cohort comparisons, and encourage the development of appropriate *in vitro* models to functionally validate the relevance of the emerging candidate genes in a GDM context.

**Supplementary data**

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-15-0424>.

**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**

This study was supported by the National Institutes of Health (NIH) DK81412 (B.C.C.).

**Author contribution statement**

M V and A M S originated the concept and designed the study. M B F and C C B optimized the laser capture methodology, and carried out the experiments. M B F, C C B and S N performed the microarray data analysis and participated in the drafting of the manuscript. All authors participated in the critical interpretation of the results, reviewed, and approved the final version of the manuscript.

**References**

- Bari MF, Brown H, Nicholson AG, Kerr KM, Gosney JR, Wallace WA, Soomro I, Muller S, Peat D, Moore JD *et al.* 2013 BAI3, CDX2 and VIL1: a panel of three antibodies to distinguish small cell from large cell neuroendocrine lung carcinomas. *Histopathology* **64** 547–556. (doi:10.1111/his.12278)
- Bellamy L, Casas JP, Hingorani AD & Williams D 2009 Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *Lancet* **373** 1773–1779. (doi:10.1016/S0140-6736(09)60731-5)
- Boney CM, Verma A, Tucker R & Vohr BR 2005 Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. *Pediatrics* **115** e290–e296.
- Borgono CA, Hamilton JK, Ye C, Hanley AJ, Connelly PW, Sermer M, Zinman B & Retnakaran R 2012 Determinants of insulin resistance in infants at age 1 year: impact of gestational diabetes mellitus. *Diabetes Care* **35** 1795–1797. (doi:10.2337/dc12-0173)
- Bottalico JN 2007 Recurrent gestational diabetes: risk factors, diagnosis, management, and implications. *Seminars in Perinatology* **31** 176–184.
- Bryson CL, Ioannou GN, Rulyak SJ & Critchlow C 2003 Association between gestational diabetes and pregnancy-induced hypertension. *American Journal of Epidemiology* **158** 1148–1153.
- Buhimschi IA, Nayeri UA, Zhao G, Shook LL, Pensalfini A, Funai EF, Bernstein IM, Glabe CG & Buhimschi CS 2014 Protein misfolding, congophilia, oligomerization, and defective amyloid processing in preeclampsia. *Science Translational Medicine* **6** 245ra292. (doi:10.1126/scitranslmed.3008808)
- Burton GJ, Sebire NJ, Myatt L, Tannetta D, Wang YL, Sadovsky Y, Staff AC & Redman CW 2014 Optimising sample collection for placental research. *Placenta* **35** 9–22. (doi:10.1016/j.placenta.2013.11.005)
- Carvalho B, Bengtsson H, Speed TP & Irizarry RA 2007 Exploration, normalization, and genotype calls of high-density oligonucleotide SNP array data. *Biostatistics* **8** 485–499.
- Catalano PM, McIntyre HD, Cruickshank JK, McCance DR, Dyer AR, Metzger BE, Lowe LP, Trimble ER, Coustan DR, Hadden DR *et al.* 2012 The hyperglycemia and adverse pregnancy outcome study: associations of GDM and obesity with pregnancy outcomes. *Diabetes Care* **35** 780–786. (doi:10.2337/dc11-1790)
- Clement-Ziza M, Munnich A, Lyonnet S, Jaubert F & Besmond C 2008 Stabilization of RNA during laser capture microdissection by performing experiments under argon atmosphere or using ethanol as a solvent in staining solutions. *RNA* **14** 2698–2704.
- Dabelea D, Snell-Bergeon JK, Hartsfield CL, Bischoff KJ, Hamman RF & McDuffie RS 2005 Increasing prevalence of gestational diabetes mellitus (GDM) over time and by birth cohort: Kaiser Permanente of Colorado GDM Screening Program. *Diabetes Care* **28** 579–584.
- David Y, Ziv T, Admon A & Navon A 2010 The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *Journal of Biological Chemistry* **285** 8595–8604. (doi:10.1074/jbc.M109.089003)
- Desforges M, Greenwood SL, Glazier JD, Westwood M & Sibley CP 2010 The contribution of SNAT1 to system A amino acid transporter activity in human placental trophoblast. *Biochemical and Biophysical Research Communications* **398** 130–134. (doi: 10.1016/j.bbrc.2010.06.051)
- Desoye G & Hauguel-de Mouzon S 2007 The human placenta in gestational diabetes mellitus. The insulin and cytokine network. *Diabetes Care* **30** (Supplement 2) S120–S126. (doi: 10.2337/dc07-s203)
- Dhananjayan SC, Ismail A & Nawaz Z 2005 Ubiquitin and control of transcription. *Essays in Biochemistry* **41** 69–80.
- Glazier JD, Cetin I, Perugino G, Ronzoni S, Grey AM, Mahendran D, Marconi AM, Pardi G & Sibley CP 1997 Association between the activity of the system A amino acid transporter in the microvillous plasma membrane of the human placenta and severity of fetal compromise in intrauterine growth restriction. *Pediatric Research* **42** 514–519.
- Hauth JC, Clifton RG, Roberts JM, Myatt L, Spong CY, Leveno KJ, Varner MW, Wapner RJ, Thorp JM, Jr., Mercer BM *et al.* 2011 Maternal insulin resistance and preeclampsia. *American Journal of Obstetrics & Gynecology* **204** 327 e321–e326.
- Huppertz B 2008 The anatomy of the normal placenta. *Journal of Clinical Pathology* **61** 1296–1302.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U & Speed TP 2003 Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4** 249–264.
- Jansson T & Powell TL 2013 Role of placental nutrient sensing in developmental programming. *Clinical Obstetrics and Gynecology* **56** 591–601. (doi:10.1097/GRE.0b013e3182993a2e)
- Jansson T, Ekstrand Y, Bjorn C, Wennergren M & Powell TL 2002 Alterations in the activity of placental amino acid transporters in pregnancies complicated by diabetes. *Diabetes* **51** 2214–2219.
- Kim JY, Cheong HS, Park BL, Baik SH, Park S, Kim S, Shin HD & Kim SH 2013a Putative association between UBE2E2 polymorphisms and the risk of gestational diabetes mellitus. *Gynecological Endocrinology* **29** 904–908. (doi:10.3109/09513590.2013.813465)
- Kim SH, Shim SH, Sung SR, Lee KA, Shim JY, Cha DH & Lee KJ 2013b Gene expression analysis of the microdissected trophoblast layer of human placenta after the spontaneous onset of labor. *PLoS One* **8** e77648. (doi:10.1371/journal.pone.0077648)
- Lecker SH, Goldberg AL & Mitch WE 2006 Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *Journal of the American Society of Nephrology* **17** 1807–1819.
- Libby G, Murphy DJ, McEwan NE, Greene SA, Forsyth JS, Chien PW, Morris AD & Collaboration DM 2007 Pre-eclampsia and the later development of type 2 diabetes in mothers and their children: an intergenerational study from the Walker cohort. *Diabetologia* **50** 523–530.



- Malina A, Daftary A, Crombleholme W, Markovic N & Roberts JM 2005 Placental system A transporter mRNA is not different in preeclampsia, normal pregnancy, or pregnancies with small-for-gestational-age infants. *Hypertens Pregnancy* **24** 65–74.
- Neufeld N, Braunstein G, Corbo LM, Gratacos J & Mestman J 1984 Insulin receptors and placental proteins in normal and gestational-diabetic pregnancies. *Biological Research in Pregnancy and Perinatology* **5** 84–89.
- Okazaki S, Sekizawa A, Purwosunu Y, Farina A, Wibowo N & Okai T 2007 Placenta-derived, cellular messenger RNA expression in the maternal blood of preeclamptic women. *Obstetrics & Gynecology* **110** 1130–1136.
- Reddy A, Zhong XY, Rusterholz C, Hahn S, Holzgreve W, Redman CW & Sargent IL 2008 The effect of labour and placental separation on the shedding of syncytiotrophoblast microparticles, cell-free DNA and mRNA in normal pregnancy and pre-eclampsia. *Placenta* **29** 942–949. (doi:10.1016/j.placenta.2008.08.018)
- Smyth GK 2004 Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3** Article3.
- Tamsen L, Axelsson O & Johansson SG 1983 Serum levels of pregnancy-specific beta 1-glycoprotein (SP1) in women with pregnancies at risk. *Gynecologic and Obstetric Investigation* **16** 253–260.
- Virjee S, Robinson S & Johnston DG 2001 Screening for diabetes in pregnancy. *Journal of the Royal Society of Medicine* **94** 502–509.
- Vohr BR & Boney CM 2008 Gestational diabetes: the forerunner for the development of maternal and childhood obesity and metabolic syndrome? *Journal of Maternal-Fetal and Neonatal Medicine* **21** 149–157. (doi:10.1080/14767050801929430)
- Wang Q, Huang R, Yu B, Cao F, Wang H, Zhang M, Wang X, Zhang B, Zhou H & Zhu Z 2013 Higher fetal insulin resistance in Chinese pregnant women with gestational diabetes mellitus and correlation with maternal insulin resistance. *PLoS One* **8** e59845. (doi:10.1371/journal.pone.0059845)
- West NA, Kechris K & Dabelea D 2013 Exposure to maternal diabetes in utero and DNA methylation patterns in the offspring. *Immunometabolism* **1** 1–9.
- Zhao L, Triche EW, Walsh KM, Bracken MB, Saftlas AF, Hoh J & Dewan AT 2012 Genome-wide association study identifies a maternal copy-number deletion in PSG11 enriched among preeclampsia patients. *BMC Pregnancy Childbirth* **12** 61.

Received in final form 4 February 2016

Accepted 11 February 2016

Accepted Preprint published online 11 February 2016