

## CLINICAL STUDY

# Defective insulin signaling in placenta from pregnancies complicated by gestational diabetes mellitus

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## Abstract

**Objective:** Studies in adipose tissue and skeletal muscle suggest that impaired insulin action is due to defects in the insulin signaling pathway and may play a role in the pathophysiology of insulin resistance associated with gestational diabetes mellitus (GDM) and obesity. The present study tested the hypothesis that endogenous expression levels in the human term placenta of insulin signaling components are altered in placental tissue from GDM women in comparison with normal controls and maternal obesity.

**Design and methods:** Placental tissue was collected from normal, diet-controlled GDM, and insulin-controlled GDM in both non-obese and obese women ( $n=6-7$  per group). Western blotting and quantitative RT-PCR was performed to determine the level of expression in the insulin signaling pathway.

**Results:** There was a significant increase in insulin receptor (IR) substrate (IRS)-1 protein expression with a concurrent decrease in IRS-2 protein expression in non-obese women with insulin-controlled GDM compared with diet-controlled GDM and normal controls. Furthermore, a decrease in both protein and mRNA expression of phosphatidylinositol-3-kinase (PI3-K) p85 $\alpha$  and glucose transporter (GLUT)-4 was observed in non-obese and obese women with insulin controlled GDM compared with normal controls. When comparing non-obese to obese patients, significant decreases in mRNA expression of IR- $\beta$ , PI3K p85 $\alpha$  and GLUT-4 was found in obese patients.

**Conclusion:** Our results suggest that post receptor defects are present in the insulin signaling pathway in placenta of women with pregnancies complicated by diabetes and obesity. In addition, expression studies demonstrate post receptor alterations in insulin signaling possibly under selective maternal regulation and not fetal regulation.

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## Introduction

The second half of human pregnancy is an insulin resistant state in which glucose tolerance is weakened. When the accompanying increase in insulin levels in maternal serum is inadequate to accommodate this, overt gestational diabetes mellitus (GDM) arises. GDM is a maternal complication defined as any degree of glucose intolerance with onset or first recognition during pregnancy (1). Women born in Asian countries such as China, Vietnam, and India display the highest prevalence of GDM with up to 17% of women likely to acquire GDM (2) in comparison with North Europeans and white Americans which have a prevalence at around 4% (2, 3). In Australia, 5–8% of pregnant women are affected by GDM (3). Although it is characterised by greater insulin resistance, little is known of the cellular mechanisms underlying GDM.

GDM is a fundamental source for several maternal and fetal complications. Although most women with GDM return to normal glucose tolerance after delivery, some individuals have an increased risk of developing type II diabetes mellitus (T2DM) later in life (4) and consequently, GDM is classified as a pre-diabetic state. The incidence of T2DM later in life is even greater if obesity is present (5–7). The offspring of women with GDM are prone to adverse side-effects, primarily undue fat accumulation, a manifestation strongly linked with fetal death, prematurity, birth trauma, and respiratory distress syndrome. Moreover, offspring have a higher risk of developing obesity, impaired glucose tolerance, and T2DM later in their adult life (8, 9).

Insulin signaling is critical for the regulation of intracellular and blood glucose levels. Activation of the insulin signaling pathway involves insulin binding to the insulin receptor (IR), which results in receptor auto-phosphorylation on cytoplasmic tyrosine residues and

the tyrosine phosphorylation of IR substrates (IRS). This allows association of IRS with downstream effectors such as phosphatidylinositol-3-kinase (PI3-K). PI3-K proteins are a family of heterodimeric lipid kinases that consist of a regulatory (p85) and a catalytic unit (p110). They have the ability to regulate, among others, glucose uptake by inducing end point events such as glucose transporter (GLUT)-4 translocation. Consequently, their functionality is critical for the intracellular regulation of glucose levels (10).

While alterations in the number of insulin binding sites reflecting IR expression in the placenta have been known in various forms of maternal diabetes mellitus (11), a paucity of data is available on insulin signaling proteins and their expression in placental tissue. A recent study has investigated changes in protein expression of IR, IRS-1, and PI3-K p85 $\alpha$  in both normal and GDM pregnancies and found differential expression present between maternal side and fetal side of the placenta (12), implying varied regulation within the placenta between mother and fetus (13, 14). Previous studies in insulin-sensitive maternal peripheral tissues have demonstrated altered expression in the focal proteins involved in insulin signaling in GDM (15). In skeletal muscle, IRS-1 protein expression decreases while IRS-2 and PI3-K p85 $\alpha$  increases in GDM patients in comparison with controls (16). Similar results have been found in adipose tissue and adipocytes extracted from GDM patients associated with a decrease in IRS-1 protein expression (15).

The insulin signaling cascade results in the uptake of glucose by the cell, a vital step for maintaining euglycaemia within individuals. This involves the translocation of GLUTs from the cytoplasm to the cell membrane, allowing glucose uptake by the cell. The ubiquitously expressed GLUT-1 isoform is inversely related to extracellular glucose concentration in placenta (17) whereas GLUT-4, which is found to a lesser extent in the placenta, are insulin sensitive GLUTs (18). Studies have demonstrated diminished glucose uptake in placental tissue from individuals with GDM (19–22) and furthermore, increased expression of GLUT-1 in placenta basal membranes from GDM women in comparison with normal controls (23, 24). Studies are yet to determine the role of GLUT-4 in GDM.

Therefore, the aim of this study was to determine whether mRNA and protein expression of insulin signaling and downstream glucose transport are altered in placental tissue from women with GDM. Furthermore, as obesity is a risk factor for T2DM, and GDM women have increased risk T2DM later in life, we investigated insulin signaling components in normal obese patients in comparison with normal non-obese controls to determine the sole affects of obesity on insulin signaling. Increased knowledge of the possible defects present in the insulin signaling pathway may lead to identification of molecular targets critical for maintaining optimal glucose regulation.

## Methods

### Reagents

Rabbit polyclonal anti-IR- $\beta$  (C-19), rabbit polyclonal anti-IRS-1 (A-19), rabbit polyclonal anti-IRS-2 (H-205), goat polyclonal anti-PI3-K p85 $\alpha$  (N-18), goat polyclonal anti-PI3-K p110 $\alpha$  (I-19), goat polyclonal anti-actin (C-11), goat anti-rabbit immunoglobulin (IgG) horse-radish peroxidase (HRP) conjugated antibody, mouse anti-goat IgG HRP antibody, and luminol reagent were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Criterion 7.5% Tris-HCl gels, immunoblot PVDF membrane (0.45  $\mu$ m pore size), 10 $\times$  Tris/glycine/sodium dodecylsulfate running buffer, iScript cDNA synthesis kit and kaleidoscope protein standard were all purchased from Bio-Rad Laboratories. TRI Reagent was purchased from Sigma-Aldrich. Sensimix Plus SYBR Green was purchased from Quantace (Alexandria, NSW, Australia). Pre-validated primers for IR (QT00082810), IRS-1 (QT00074144), IRS-2 (QT00064036), PI3-K p85 $\alpha$  (QT01005984) PI3-K p110 $\alpha$  (QT00014861), PI3-K p110 $\beta$  (QT00029148), GLUT-1 (QT00068957), GLUT-4 (QT00097902), and  $\beta$ -actin (QT00095431) were purchased from Qiagen.

### Patients and sample collection

The study was approved by the Mercy Hospital for Women's Research and Ethics Committee (Mercy Health & Aged Care Human Research Ethics committee # R06-06), Heidelberg, Victoria, Australia. Human placentae were obtained from a total of 42 pregnant women who delivered healthy, singleton infants at term (> 37 weeks' gestation) undergoing elective Caesarean section (indications for Caesarean section were breech presentation and/or previous Caesarean section). Women with any underlying medical conditions such as asthma, polycystic ovarian syndrome, pre-eclampsia, and macrovascular complications were excluded from the study. Normal women were categorised as a body mass index (BMI; weight (kg)/height<sup>2</sup> (m<sup>2</sup>)) of < 25 kg/m<sup>2</sup> and obese patients with a BMI of > 30 kg/m<sup>2</sup>. Homeostasis model assessment for insulin resistance is defined as fasting insulin  $\times$  fasting glucose/22.5 (25). Rohrer's Ponderal Index is defined as birth weight (g)/birth length<sup>3</sup> (cm<sup>3</sup>)  $\times$  100 (26).

Tissue was collected as previously described (27–29). Briefly, placenta was obtained within 10 min of delivery. A placental lobule (cotyledon) was removed from the central region of the placenta. The basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross-section. Placental tissue was bluntly dissected to remove visible connective tissue and calcium deposits, blotted dry on filter paper, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis. Small sections of dissected placental tissue were also placed in

embedding cassettes and submerged in a neutral, phosphate buffered 4% formaldehyde solution for 24 h and then paraffin-embedded for immunohistochemistry studies.

Women with GDM were diagnosed according to the criteria set by the Australian Diabetes in Pregnancy Society (ADIPS) by either a fasting venous plasma glucose level of 5.5 mmol/l glucose or more and/or 8.0 mmol/l glucose or higher 2 h after a 75 g oral glucose load. Women were controlled by diet, if their fasting glucose readings were maintained below 5.5 mmol/l over a 2-week period post diagnosis. Women with fasting glucose readings greater than 5.5 mmol/l were placed on insulin for optimal glucose control (30, 31). Glycemic control was monitored by measurements of maternal HbA1c and was within the normal range ( $\leq 6\%$ ) for all study subjects.

### **Insulin and C-peptide ELISA**

Maternal and cord plasma was assayed for insulin and C-peptide. The insulin and C-peptide sandwich ELISA was conducted according to the manufacturer's instructions (Biosource International Camarillo, CA, USA) with a limit of detection as defined by two s.d.s from the zero standards of 0.2 and 0.6 ng/ml respectively.

### **Immunohistochemistry**

Immunohistochemical analysis was performed on placental tissue as previously described (32–34). Briefly, paraffin-embedded tissues were cut to 4  $\mu\text{m}$  thickness, mounted onto Superfrost slides and incubated at 65 °C for 30 min. Sections were deparaffinised with xylene and rehydrated with a graded series of ethanol washes. Antigen retrieval was performed by heating slides in citrate buffer (100 mmol/l, pH 6.0) for 10 min and further incubating in buffer for 20 min. Endogenous peroxidases were inactivated using 3% hydrogen peroxide in methanol. The sections were incubated for 1 h in 1:100 dilution of primary antibody (IRS-1, IRS-2, PI3-K p85 $\alpha$  and PI3-K p110 $\alpha$ ) diluted in 1% BSA/Tris buffered saline. Secondary staining at a dilution of 1:2500 was then added for a period of 30 min at room temperature. DAB (Dakocytomation, Glostrup Denmark) was added for 10 min at room temperature. Sections were finally stained with Mayer's hematoxylin, mounted and dried ready for viewing. IgG controls (negative controls) were used for all species of antibody. Skeletal muscle sections were used as positive controls.

### **Whole cell lysate preparation**

Whole protein lysate was prepared as previously described with minor amendments (35, 36). Placental tissue (100 mg) was homogenised (2  $\times$  20 s bursts) in 500  $\mu\text{m}$  of radioimmunoprecipitation assay buffer

(50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.25% Na deoxycholate, 1 mM EDTA, pH 7.4, 1 mM AEBSE, 10  $\mu\text{g}/\text{ml}$  aprotinin, 5  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM activated  $\text{Na}_3\text{VO}_4$ , 1 mM NaF) with a metal blade tissue homogeniser (Ultraturrax, S25N 8G dispersing tool; Jenke and Kunkel GMBH and Co., Staufen, Germany). Tissue homogenates were incubated at 4 °C for 1 h on a rotator. Homogenates were centrifuged at 15 000 **g** for 20 min. The supernatant was collected and re-spun. Whole protein lysates were assayed for protein concentration using BCA protein assay (Pierce Chemical Co., Rockford, IL, USA) with BSA as the reference standard (37).

### **Membrane fraction extraction**

GLUTs are capable of facilitating glucose entry into cells when they reside in the plasma membrane. After prolonged hyperglycaemia (GLUT-1) (24) or in unstimulated cells (GLUT-4) a proportion of GLUTs are located in intracellular pools, where they do not contribute to cellular glucose uptake. In order to semi-quantitatively determine the amount of GLUTs promoting glucose uptake, placental plasma membrane fraction was prepared according to previously validated and published methods (18). Briefly, 500 mg placental tissue was homogenised in 2 ml HEPES-Sucrose buffer (25 mM HEPES, 250 mM sucrose, 1 mM AEBSE, 10  $\mu\text{g}/\text{ml}$  aprotinin, 5  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM activated  $\text{Na}_3\text{VO}_4$ , 1 mM NaF). Tissue homogenates were incubated at 4 °C for 1 h on a rotator. Homogenates were then centrifuged for 10 min at 1000 **g**. The supernatant was collected and was centrifuged for 20 min at 10 000 **g**. The supernatant was collected and centrifuged for 1 h at 100 000 **g**. The supernatant was then removed and the crude membrane pellet was homogenised in HEPES-Sucrose buffer, and stored at  $-80$  °C until further analysis. Membrane fraction protein concentrations were determined using Coomassie Protein Assay according to the manufacturer's instructions (Pierce Chemical Co). Coomassie Protein assay was used rather than BCA protein assay due to buffer incompatibility.

### **SDS-PAGE and western blot**

SDS-PAGE and western blot was performed by the methods described previously (32, 38). Briefly, 80  $\mu\text{g}$  of placental whole cell lysate or 50  $\mu\text{g}$  membrane fraction (for GLUT-1 and GLUT-4 analysis) was separated on 7.5% Criterion gels and resolved proteins were transferred to PVDF membrane. Molecular weights were identified by comparison with the motility of a protein standard (Kaleidoscope protein standard, Bio-Rad Laboratories). The blots were probed with antibodies as listed above at the following dilutions (IR- $\beta$  1:500, IRS-1 1:250, IRS-2 1:250, PI3-K p85 $\alpha$  1:250, PI3-K p110 $\alpha$  1:250, GLUT-1 1:500, and GLUT-4 1:500). Blots were stripped and re-probed with  $\beta$ -actin

(1:500) for loading control. Proteins were detected using a chemiluminescence kit according to manufacturer's instructions (Luminol, Santa Cruz, CA, USA) and membranes were developed using the Chemidot (Bio-Rad). Data were corrected for  $\beta$ -actin expression. Densitometry was performed on all blots to determine the density of the bands ( $\text{OD}/\text{mm}^2$ ), using Quantity One computer program (Bio-Rad Laboratories).

### RNA extraction and real time PCR

Total RNA was extracted from  $\sim 100$  mg of placenta using Tri Reagent according to manufacturer's instructions (Sigma–Aldrich). RNA concentrations were quantified using a spectrophotometer (Smart Spec, Bio-Rad). RNA quality and integrity was determined via the  $A_{260}/A_{280}$  ratio and agarose gel electrophoresis. We converted 1  $\mu\text{g}$  of RNA to cDNA using the iScript cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad). RT-PCR was performed using 1 of  $\mu\text{l}$  cDNA and Sensimix Plus SYBR green and primers as listed above.  $\beta$ -actin was used to normalise the mRNA expression. Additionally, the specificity of the product was assessed from melting curve analysis. All samples were run in duplicate with positive and negative (RNA without reverse transcriptase) controls run on each plate to ensure quality of run and to confirm the absence of contaminations. The cycling conditions for RT-PCR were as follows: 95 °C for 3 min, 95 °C for 15 s (denaturation), 55 °C for 45 s (annealing), 72 °C for 45 s (extension), cycle 39 times, 95 °C for 1 min, 55 °C for 1 min, a melt curve analysis was programmed for 55 °C to 95 °C (1 °C intervals) and held for 20 s.

### Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics, STSC, Rockville, MD, USA). The homogeneity of the data was assessed by Bartlett's test and when significant, data were logarithmically transformed before additional analysis. Unless otherwise stated, sample comparisons were analysed either by Student's *t*-test or one-way ANOVA (Duncan *post hoc* test). Statistical difference was accepted with *P* values  $< 0.05$ . Data are expressed as the mean  $\pm$  s.e.m.

Linear regression analysis was used to evaluate the relationship among the variables of interest. Statistically, significant weak relationships were indicated with a *P* value  $< 0.1$ . A moderate to strong relationship was denoted with a *P* value of  $< 0.05$ .

## Results

### Participants

Demographic data of all participants involved in this study are summarised in Table 1. In both the non-obese and obese groups, 1 and 2 h plasma glucose concentrations during the OGTT were significantly greater ( $P < 0.05$ ) in GDM (both diet- and insulin-controlled) than in normal pregnant women. When comparing non-obese with obese healthy pregnant women all demographic parameters were similar with the exception of BMI at 12 weeks and at term ( $P < 0.05$ ). Birth weight was lower ( $P < 0.05$ ) among diet-controlled GDM women in comparison with healthy pregnant

**Table 1** Patient characteristics of study group.

	Non-obese			Obese		
	Normal ( <i>n</i> =7)	GDM diet ( <i>n</i> =7)	GDM insulin ( <i>n</i> =7)	Normal ( <i>n</i> =7)	GDM diet ( <i>n</i> =7)	GDM insulin ( <i>n</i> =7)
Age (years)	33.3 $\pm$ 1.2	36.0 $\pm$ 1.8	33.8 $\pm$ 1.8	33.8 $\pm$ 1.9	36.6 $\pm$ 2.0	35.1 $\pm$ 2.0
BMI, 12 weeks ( $\text{kg}/\text{m}^2$ )	21.3 $\pm$ 0.5	21.8 $\pm$ 1.5	22.0 $\pm$ 0.7	35.2 $\pm$ 1.2 <sup>§</sup>	38.8 $\pm$ 0.8	41.2 $\pm$ 2.6
BMI, term ( $\text{kg}/\text{m}^2$ )	25.9 $\pm$ 0.7	25.9 $\pm$ 0.5	26.5 $\pm$ 0.6	38.8 $\pm$ 0.7 <sup>§</sup>	39.9 $\pm$ 5.2	42.8 $\pm$ 2.6
Fasting plasma glucose (mmol/l)	4.4 $\pm$ 0.2	4.7 $\pm$ 0.2	4.5 $\pm$ 0.2	4.6 $\pm$ 0.1	4.6 $\pm$ 0.1	5.6 $\pm$ 0.4
1-h plasma glucose (mmol/l)	5.9 $\pm$ 0.3	9.3 $\pm$ 0.8*	10.3 $\pm$ 0.5 <sup>†</sup>	7.3 $\pm$ 0.6	8.9 $\pm$ 0.3	11.8 $\pm$ 0.8 <sup>†,‡</sup>
2-h plasma glucose (mmol/l)	5.1 $\pm$ 0.3	8.4 $\pm$ 0.1*	9.3 $\pm$ 0.2 <sup>†,‡</sup>	5.8 $\pm$ 0.2	8.5 $\pm$ 0.2*	9.5 $\pm$ 0.3 <sup>†,‡</sup>
Birth weight (g)	3438 $\pm$ 128	3486 $\pm$ 153	3378 $\pm$ 109	3677 $\pm$ 81	3204 $\pm$ 107*	3600 $\pm$ 95 <sup>‡</sup>
Gestation (weeks)	38.9 $\pm$ 0.2	38.8 $\pm$ 0.2	38.8 $\pm$ 0.2	38.7 $\pm$ 0.2	38.4 $\pm$ 0.1	38.0 $\pm$ 0.2 <sup>†</sup>
Gravida	2.1 $\pm$ 0.4	2.5 $\pm$ 0.3	2.5 $\pm$ 0.5	2.5 $\pm$ 0.3	2.3 $\pm$ 0.3	3.1 $\pm$ 0.7
Parity	2.0 $\pm$ 0.3	2.0 $\pm$ 0.3	1.9 $\pm$ 0.3	1.8 $\pm$ 0.2	2.1 $\pm$ 0.2	2.5 $\pm$ 0.5
Insulin ( $\mu\text{IU}/\text{ml}$ )						
Maternal blood	14.3 $\pm$ 2.9	12.2 $\pm$ 2.0	18.9 $\pm$ 2.5	31.5 $\pm$ 4.7 <sup>§</sup>	35.5 $\pm$ 4.7	51.7 $\pm$ 6.3 <sup>†</sup>
Cord blood	7.9 $\pm$ 1.1	9.1 $\pm$ 1.6	13.1 $\pm$ 4.5	12.4 $\pm$ 2.9	10.3 $\pm$ 1.8	16.1 $\pm$ 1.7
C-peptide (ng/ml)						
Maternal blood	1.1 $\pm$ 0.2	1.1 $\pm$ 0.3	0.6 $\pm$ 0.1	2.5 $\pm$ 0.4 <sup>§</sup>	2.1 $\pm$ 0.2	3.1 $\pm$ 0.4
Cord blood	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1	0.5 $\pm$ 0.2	0.5 $\pm$ 0.1
HOMA-IR	3.1 $\pm$ 0.3	2.7 $\pm$ 0.4	4.0 $\pm$ 0.5	6.4 $\pm$ 1.0 <sup>§</sup>	7.3 $\pm$ 1.3	13.2 $\pm$ 1.5 <sup>†</sup>
Rohrer's ponderal index ( $\text{g}/\text{cm}^3$ )	2.59 $\pm$ 0.13	2.57 $\pm$ 0.17	2.64 $\pm$ 0.15	2.96 $\pm$ 0.11	2.61 $\pm$ 0.13	2.79 $\pm$ 0.10

Values represent the mean  $\pm$  s.e.m. BMI, body mass index; NS, not significant. \* $P < 0.05$  normal versus diet-controlled GDM. <sup>†</sup> $P < 0.05$  normal versus insulin-controlled GDM. <sup>‡</sup> $P < 0.05$  diet-controlled GDM versus insulin-controlled GDM. <sup>§</sup> $P < 0.05$  normal (non-obese) versus normal (obese).

women. Furthermore, birth weight was significantly higher ( $P < 0.05$ ) among insulin-controlled GDM women as compared with diet-controlled GDM patients. Moreover, gestation was shorter ( $P < 0.05$ ) among insulin-controlled GDM women in comparison with healthy pregnant women.

### **Endogenous expression of insulin signaling proteins in placental tissue collected from non-obese patients**

Differential basal expression of insulin signaling protein and GLUT transporters from non-obese patients were seen in placental tissue at both protein (Fig. 1) and mRNA level (Table 2). GDM did not alter mRNA or protein expression (Fig. 1a) of IR- $\beta$ . IRS-1 protein expression (Fig. 1b), but not mRNA expression, was lower in insulin-controlled GDM compared with normal pregnant women. IRS-2 mRNA expression was higher in insulin-controlled GDM patients compared with diet-controlled GDM women and normal controls and protein expression (Fig. 1c) was higher in both diet- and insulin-controlled GDM patients in comparison with normal controls. Both mRNA and protein expression (Fig. 1d) levels of the PI3-K p85 $\alpha$  subunit were lower; while PI3-K p110 $\alpha$  protein expression was higher (Fig. 1e) in insulin-controlled GDM as compared with normal pregnant women. Subunits for PI3-K p110 mRNA expression remained unchanged between normal and GDM placentae. GLUT-1 mRNA and protein expression (Fig. 1f) were elevated in insulin-treated GDM patients compared with controls. When compared with normal patients, GLUT-4 mRNA expression and protein expression (Fig. 1g) was decreased significantly in insulin-controlled patients compared with normal controls.

### **Endogenous expression of insulin signaling proteins in placental tissue collected from obese patients**

Differences in basal expression of insulin signaling proteins and GLUT transporters from obese patients were noted in placental tissue at both protein (Fig. 2) and mRNA level (Table 2). IR- $\beta$  mRNA expression (Table 2) was decreased in insulin-controlled women compared with normal controls. Furthermore, IR- $\beta$  protein expression (Fig. 2a), was increased in women with diet-controlled GDM compared with normal pregnant women. IRS-1 protein expression (Fig. 2b) was decreased in insulin-controlled GDM women compared with both diet-controlled GDM women and normal controls, whereas IRS-1 mRNA and both mRNA and protein expression of IRS-2 (Fig. 2c) were unchanged by GDM. Again, no changes were found in mRNA expression of PI3-K p110 subunits, however, protein expression of PI3-K p110 $\alpha$  decreased in insulin-controlled women compared with normal controls

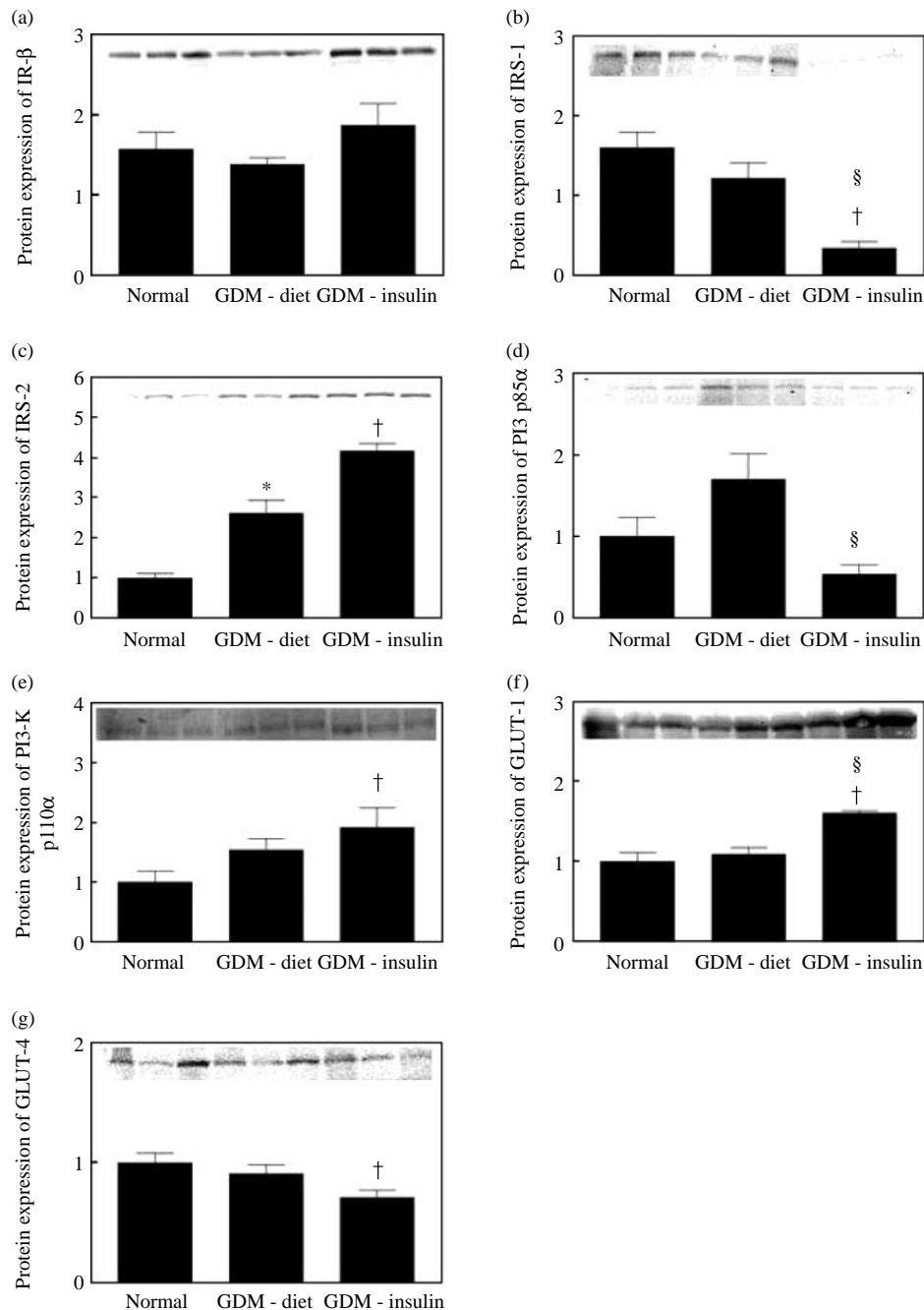
(Fig. 2e). By contrast, there was an increased PI3-K p85 $\alpha$  protein expression in diet-treated GDM (Fig. 2d) and decreased mRNA expression of PI3-K p85 $\alpha$  in insulin-controlled GDM women compared with normal controls (Fig. 2d). GLUT-1 mRNA and protein expression were unchanged (Fig. 2f), whereas GLUT-4 mRNA expression decreased in both diet- and insulin-treated GDM patients in comparison with controls with no changes in protein expression (Fig. 2g).

### **Endogenous expression of insulin signaling proteins in placental tissue from normal non-obese compared with normal obese patients**

Normal non-obese ( $n=6$ ) and normal obese groups ( $n=6$ ) were compared to determine whether the expression of any relationship exists between the expression of insulin signaling component and obesity. No changes in protein (Fig. 3a and b) and mRNA expression (Table 3) of IR- $\beta$  and IRS-1 were noted. An increase in IRS-2 protein expression (Fig. 3c) and a decrease in PI3-K p85 $\alpha$  mRNA and protein expression (Fig. 3d) was demonstrated in normal obese patients compared with normal non-obese controls. Although there was no change in GLUT-1 mRNA (Table 3) or protein expression (Fig. 3f), GLUT-4 mRNA expression significantly decreased (Table 3) in normal obese patients with no change in protein expression (Fig. 3g).

### **Localisation of IRS-1, IRS-2, PI3-K p85 $\alpha$ , and PI3-K p110 $\alpha$ in placental tissue**

Previous studies of human placenta have shown that IR are under fetal control while GLUTs are under maternal control (11, 17), where studies are yet to determine regulation of IRS and PI3K proteins. Thus, immunohistochemistry studies were performed in normal (non-obese) placentae ( $n=4$ ) to determine the location of insulin signaling proteins IRS-1, IRS-2, PI3-K p85 $\alpha$  and PI3-K p110 $\alpha$  in placental tissue. IRS-1 (Fig. 4a) displayed intense staining uniformly among placental tissue. Staining was predominant in the syncytiotrophoblast layer of the placenta, with staining also seen in the stroma of the villi. Intense staining was also noted around the fetal blood vessels indicating the staining of IRS-1 in endothelial cells and/or associated pericytes. IRS-2 (Fig. 4b) staining was predominant in the syncytiotrophoblast and the endothelial cells of the fetal blood vessels. Minimal staining was observed in the stromal villi. PI3-K p85 $\alpha$  also demonstrated intense staining in the syncytiotrophoblast layer with minimal staining around fetal vessels and no eminent staining in the stromal villi in placental tissue (Fig. 4c). PI3-K p110 $\alpha$  staining was localised in the syncytiotrophoblast layer (Fig. 4d).



**Figure 1** Protein expression ( $n=6$ ) of (a) IR- $\beta$ , 95 kDa, (b) IRS-1, 170 kDa, (c) IRS-2, 175 kDa, (d) PI3K p85 $\alpha$ , 85 kDa (e) PI3K p110 $\alpha$ , 110 kDa, (f) GLUT-1, 45 kDa, and (g) GLUT-4, 40 kDa in placental tissue collected from non-obese women. Each bar represents the mean ratio of the insulin signaling component in comparison with normal control, normalised against  $\beta$ -actin. \* $P < 0.05$  normal versus diet-controlled GDM, † $P < 0.05$  normal versus insulin-controlled GDM, § $P < 0.05$  diet-controlled GDM versus insulin-controlled GDM.

### **Correlation of IRS-1 and IRS-2 protein expression with maternal plasma insulin levels**

Correlation studies were performed in order to demonstrate whether insulin signaling intermediates are under maternal or fetal regulation. Correlation studies

demonstrated a significantly weak relationship between IRS-1 protein expression and maternal insulin levels ( $P < 0.1$ ,  $R^2 = 0.1768$ ). Furthermore, a moderate relationship was established between IRS-2 protein expression and maternal insulin levels ( $P < 0.05$ ,  $R^2 = 0.3157$ ; Fig. 5).

**Table 2** mRNA expression of insulin signalling intermediates in placental tissue in non-obese and obese cohorts.

	Non-obese			Obese		
	Normal (n=7)	GDM-diet (n=6)	GDM-insulin (n=6)	Normal (n=7)	GDM-diet (n=6)	GDM-insulin (n=6)
IR- $\beta$	1.04 $\pm$ 0.11	1.16 $\pm$ 0.14	1.50 $\pm$ 0.38	1.09 $\pm$ 0.13	0.98 $\pm$ 0.20	0.59 $\pm$ 0.09 <sup>†</sup>
IRS-1	1.10 $\pm$ 0.24	1.76 $\pm$ 0.28	1.42 $\pm$ 0.24	1.07 $\pm$ 0.14	1.10 $\pm$ 0.17	1.26 $\pm$ 0.19
IRS-2	1.02 $\pm$ 0.16	1.46 $\pm$ 0.29	3.00 $\pm$ 0.49 <sup>†,‡</sup>	1.08 $\pm$ 0.15	1.44 $\pm$ 0.22	1.05 $\pm$ 0.27
PI3-K p85 $\alpha$	1.21 $\pm$ 0.23	1.92 $\pm$ 0.46	0.41 $\pm$ 0.14 <sup>†,‡</sup>	1.26 $\pm$ 0.37	1.23 $\pm$ 0.43	0.51 $\pm$ 0.19 <sup>†,‡</sup>
PI3-K p110 $\alpha$	1.06 $\pm$ 0.16	0.80 $\pm$ 0.15	0.95 $\pm$ 0.12	1.04 $\pm$ 0.13	0.76 $\pm$ 0.16	0.87 $\pm$ 0.10
PI3-K p110 $\beta$	1.16 $\pm$ 0.33	1.38 $\pm$ 0.20	1.03 $\pm$ 0.15	1.15 $\pm$ 0.25	1.10 $\pm$ 0.22	0.89 $\pm$ 0.14
GLUT-1	1.04 $\pm$ 0.12	0.61 $\pm$ 0.09	1.50 $\pm$ 0.40 <sup>‡</sup>	1.11 $\pm$ 0.22	1.88 $\pm$ 0.34	1.26 $\pm$ 0.20
GLUT-4	1.32 $\pm$ 0.32	1.04 $\pm$ 0.23	0.29 $\pm$ 0.10 <sup>†,‡</sup>	1.10 $\pm$ 0.20	0.61 $\pm$ 0.15 <sup>*</sup>	0.34 $\pm$ 0.07 <sup>†</sup>

Values represent mean  $\pm$  s.e.m. \* $P$ <0.05 normal versus diet-controlled GDM. <sup>†</sup> $P$ <0.05 normal versus insulin-controlled GDM. <sup>‡</sup> $P$ <0.05 diet-controlled GDM versus insulin-controlled GDM.

## Discussion

This study is the first to systematically compare the effect of both GDM and obesity on intermediates of the insulin signaling pathway and the machinery of glucose uptake in human term placentae. The data presented here demonstrate altered expression of insulin signaling components particularly in insulin-controlled GDM women in comparison with normal pregnant controls in both non-obese and obese cohorts. In addition to defective insulin signaling found in GDM, changes were prominent in normal obese patients in comparison with normal non-obese controls.

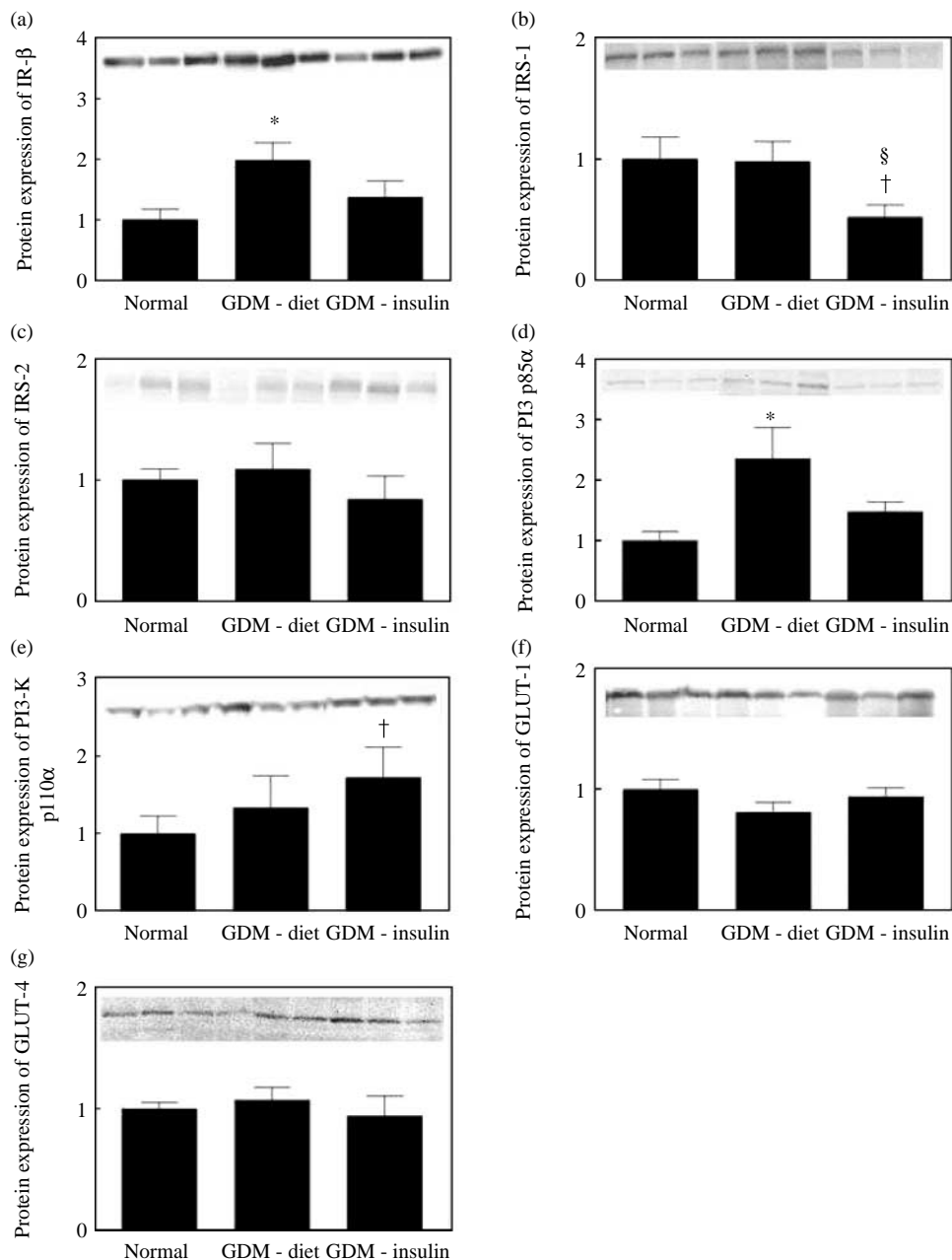
The IR is a transmembrane glycoprotein that binds insulin to cause activation to intrinsic receptor tyrosine kinase in the  $\beta$ -subunit of the IR, a critical component of the signaling pathway. The human placenta contains a large number of IRs, and previous studies have shown that at term, the IR is more abundantly expressed in endothelial cells and macrophages (fetal regulation) rather than the microvillous membrane of the syncytiotrophoblast (maternal regulation), (11) implying metabolic action of IR is via fetal control. Our study shows no changes in the expression of IR- $\beta$  in placental tissue when comparing GDM with controls regardless of maternal treatment. By contrast, when the receptor numbers were determined on trophoblast plasma membranes more IR was found in insulin-treated and fewer IRs in diet-controlled GDMs in comparison with normal controls (11). Furthermore, past studies have demonstrated that IR protein expression is higher in diet-controlled GDM women in comparison with normal controls (12). In this study, a decrease was demonstrated in the placental IR mRNA expression in obese patients when compared with non-obese controls. IR expression may thus be an attributing factor involved in obesity rather than a defect present in GDM.

IRS family members act as docking proteins between the IR and downstream intracellular signaling. This study demonstrates a decrease in IRS-1 protein expression in non-obese insulin-controlled GDM women in comparison with pregnant controls. Similar

trends are exhibited in obese insulin-controlled GDM women in comparison with pregnant controls. Furthermore, a significant reduction of IRS-1 protein expression was found in normal obese patients when compared with normal non-obese patients. Although no data are available for placenta, this is in agreement with previous studies in adipose tissue and skeletal muscle from women with GDM (15, 16, 39). The effects of GDM on IRS-1 appear generalised and not tissue-specific. Furthermore, our study demonstrated a concomitant increase in IRS-2 mRNA and protein expression in non-obese insulin-controlled GDM patients when compared with normal non-obese pregnant controls. Similarly, increased IRS-2 protein expression has been observed in skeletal muscle from obese GDM women compared with obese pregnant controls (16).

To determine whether these proteins are under maternal or fetal regulation, localisation studies were performed. Immunohistochemistry data demonstrated that IRS-1 and IRS-2 are expressed in the placenta at both the syncytiotrophoblast layer and the cells around the fetal blood vessels. These data are not enough to suggest regulation by both fetus and mother, so correlation studies comparing fetal and maternal insulin to the expression of IRS-1 and IRS-2 proteins were performed to understand regulation of these substrates. Both IRS-1 ( $P$ <0.1 weak) and IRS-2 ( $P$ <0.05 moderate) demonstrated correlation to maternal insulin levels. By contrast, fetal insulin demonstrated no correlation to IRS-1 expression and weak correlation to IRS-2 ( $P$ <0.1) expression, suggesting both IRS-1 and IRS-2 may be under maternal regulation within this fetal tissue.

Overall, the data suggest that IRS-1 and IRS-2 are under maternal control in GDM. It is believed that a compensatory relationship exists between IRS-1 and IRS-2, although molecular mechanisms underlying these effects remain elusive. In diabetic states, it can be postulated that an increase in IRS-2 expression may in fact be a compensatory mechanism in order to counterbalance defective insulin signaling found



**Figure 2** Protein expression ( $n=6$ ) of (a) IR- $\beta$ , 95 kDa, (b) IRS-1, 170 kDa, (c) IRS-2, 175 kDa, (d) PI3K p85 $\alpha$ , 85 kDa (e) PI3K p110 $\alpha$ , 110 kDa, (f) GLUT-1, 45 kDa and (g) GLUT-4, 40 kDa in placental tissue collected from obese women. Each bar represents the mean ratio the insulin signaling component in comparison with normal control, normalised against  $\beta$ -actin. \* $P < 0.05$  normal versus diet-controlled GDM, † $P < 0.05$  normal versus insulin-controlled GDM, § $P < 0.05$  diet-controlled GDM versus insulin-controlled GDM.

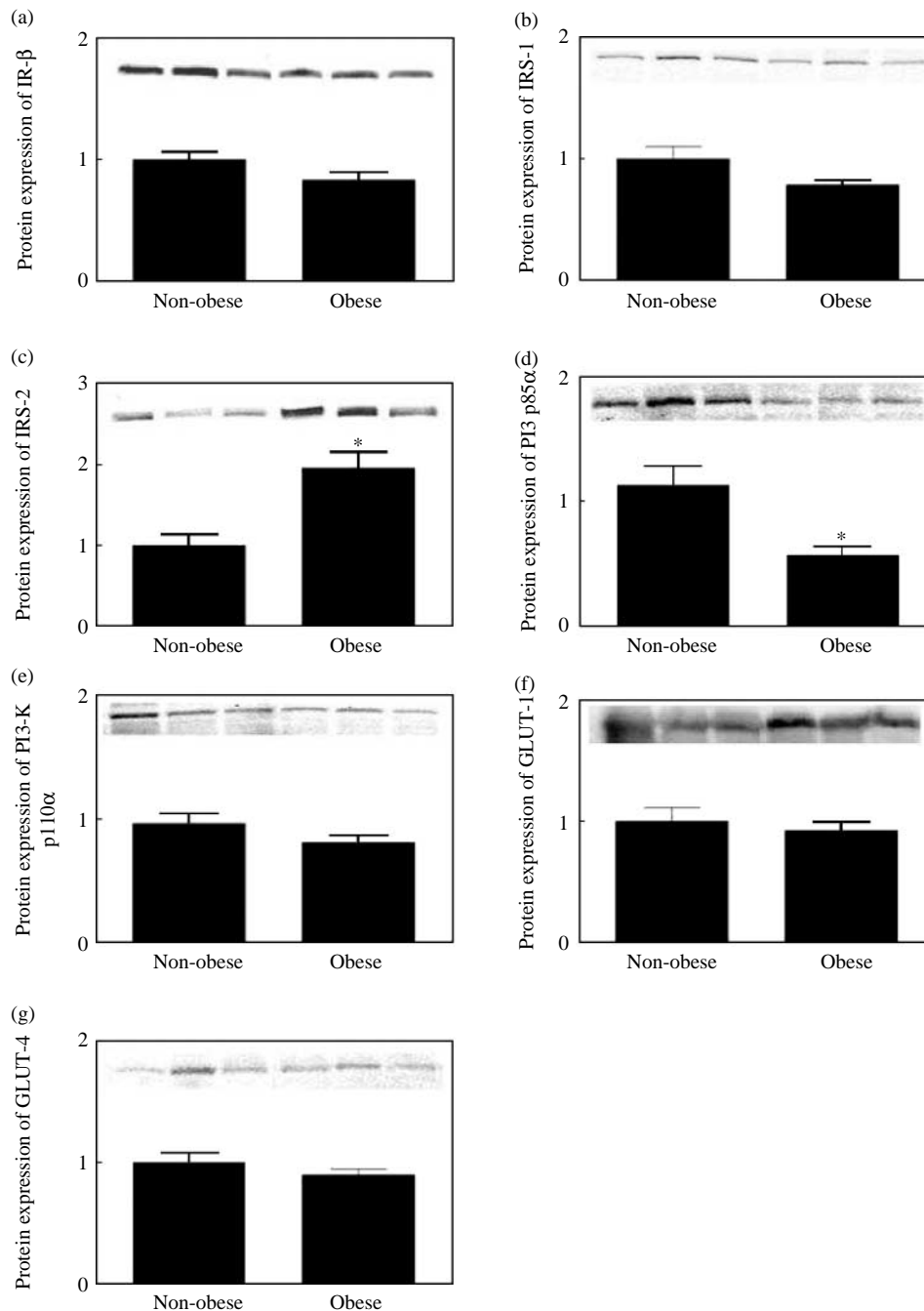
via the IRS-1 protein as previously described in T2DM (40).

Both subunits of PI3-K were examined, but only the regulatory p85 $\alpha$  showed distinct changes associated with GDM and obesity. In both obese and non-obese patients, PI3-K p85 $\alpha$  protein and mRNA expression decreased in insulin-controlled GDM in comparison with normal controls. Furthermore, a significant decrease in p85 $\alpha$  mRNA and protein expression was

found in obese patients when comparing with normal controls. Immunohistochemistry demonstrated strong syncytiotrophoblast staining only, implying maternal regulation of the protein, although regulation of this protein is still quite obscure.

There are no data available on PI3-K protein in placenta, but previous studies in peripheral tissues show higher PI3-K p85 $\alpha$  protein expression in skeletal muscle from obese GDM women in comparison with





**Figure 3** Protein expression ( $n=6$ ) of (a) IR- $\beta$ , 95 kDa, (b) IRS-1, 170 kDa, (c) IRS-2, 175 kDa, (d) PI3K p85 $\alpha$ , 85 kDa (e) PI3K p110 $\alpha$ , 110 kDa, (f) GLUT-1, 45 kDa, and (g) GLUT-4, 40 kDa in placental tissue collected from non-obese versus obese women. Each bar represents the mean ratio the insulin signaling component in comparison to normal control, normalised against  $\beta$ -actin. \* $P < 0.05$  non-obese versus obese.

pregnant controls (16). A similar increase in PI3K p85 $\alpha$  expression in skeletal muscle was also found in insulin resistant transgenic mice in comparison with controls (41, 42). The results presented in this study demonstrate inverse expression, possibly due to differences in tissue origin. Varied mechanisms of response in the mother in comparison with offspring

could be the underlying reason as previously hypothesised (14).

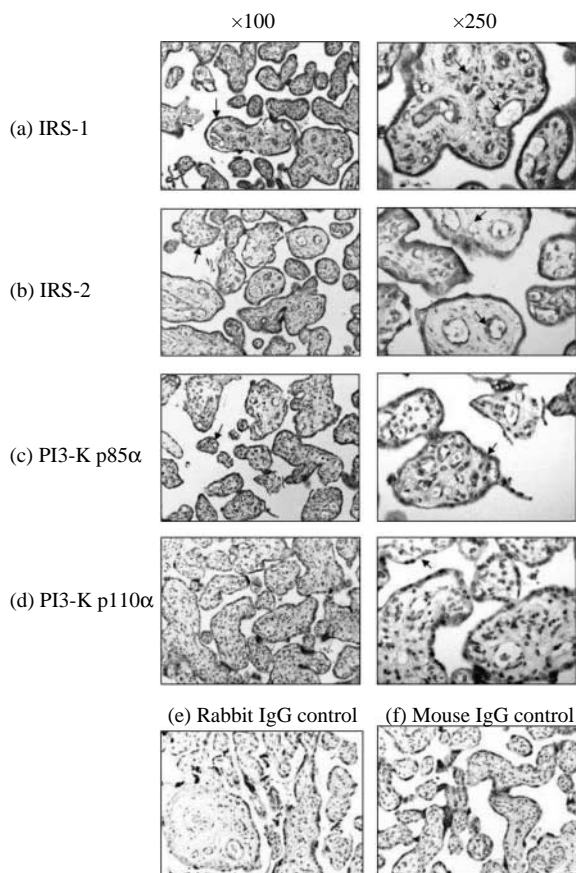
GLUT transporters are integral membrane proteins that transport glucose into the cell. Our study demonstrated in non-obese patients an increase in GLUT-1 mRNA and protein expression and a decrease in GLUT-4 mRNA and protein expression in insulin-controlled

**Table 3** mRNA expression of insulin signalling intermediates in placental tissue comparing non-obese and obese patients.

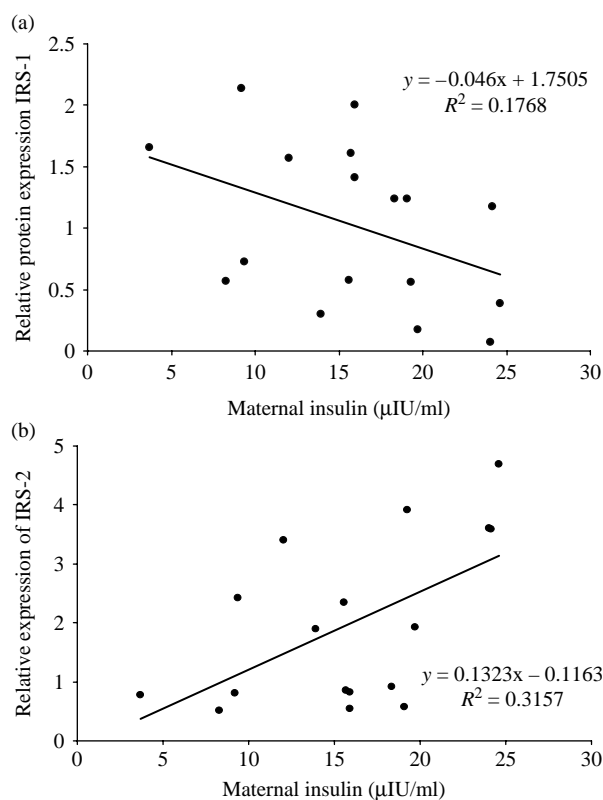
	Non-obese (n=7)	Obese (n=7)	Significance
IR-β	1.04 ± 0.11	1.06 ± 0.21	NS
IRS-1	1.15 ± 0.26	1.41 ± 0.19	NS
IRS-2	1.02 ± 0.16	1.15 ± 0.16	NS
PI3-K p85α	2.94 ± 0.84	0.89 ± 0.32	P < 0.05
PI3-K p110α	1.06 ± 0.16	1.02 ± 0.13	NS
PI3-K p110β	1.16 ± 0.33	0.89 ± 0.19	NS
GLUT-1	1.06 ± 0.15	0.95 ± 0.19	NS
GLUT-4	1.32 ± 0.32	0.44 ± 0.08	P < 0.05

Values represent mean ± S.E.M.

GDM in comparison with normal controls. Furthermore, GLUT-4 mRNA expression was lower in obese insulin-controlled GDM women compared with obese pregnant controls. In addition, GLUT-4 mRNA expression was lower in obese controls compared with non-obese controls. Similarly, previous studies performed in basal membrane extracted from placental



**Figure 4** Localisation of (a) IRS-1, (b) IRS-2, (c) PI3-K p85α and (d) PI3-K p110 in placental tissue. These sections are a representative of one patient. The arrowheads point to positive staining of protein. (e) IgG controls for both rabbit and (f) mouse species are also demonstrated to confirm the lack of non-specific binding. Magnification of each image is as displayed in figure.



**Figure 5** Correlation between maternal plasma insulin levels and relative protein expression of (a) IRS-1 ( $P < 0.1$ ) and (b) IRS-2 ( $P < 0.05$ ) from placental tissue. Data are shown for 16 pregnant women, six normal pregnant women, and 10 women with GDM.

tissue demonstrated a significant increase in GLUT-1 protein expression in insulin-dependent diabetics during pregnancy (23) and a decrease in GLUT-4 protein and mRNA expression in placental samples complicated by diabetes (18). Defective glucose uptake contributes to insulin resistance in peripheral tissues and is generally linked with a decrease in GLUT-4 transporter in diabetic states (43). Here, we see a compensatory increase in GLUT-1 mRNA and protein expression in GDM placentae in comparison with normal controls. This basal GLUT isoform responsible for cellular metabolism and glucose transport may in fact be what is primarily transporting glucose into the cell in placental tissue rather than GLUT-4, which is affected by defective insulin signaling upstream.

The human placenta is a vital organ required for adequate nutrient transport between mother and fetus. Glucose is the principal energy substrate for the placenta and the fetus and is essential for normal fetal metabolism and growth. Under diabetic conditions, the placenta undergoes structural and functional changes (44). With the addition of lifestyle factors such as obesity, a spectrum of perinatal implications is eminent (45). Maternal and fetal influences can discordantly alter placental function in women with GDM and

consequently, hinder transport of nutrients vital for normal fetal growth. It can be postulated that defects present in placental tissue which are shown to hinder insulin signaling and subsequent glucose transport may be a contributing factor to long-term risks for both mother and fetus and may be under the regulation of the mother despite the fetal origin of the tissue. With an increase in GLUT-1, the basal GLUT, in GDM patients it can be hypothesised that GLUT-1 is the main GLUT utilised in the presence of defective insulin signaling. Although a detailed and comprehensive understanding of insulin effects on the placenta is lacking (14), the changes reported here are likely to contribute to some of the changes in the placenta associated with GDM and/or obesity.

### 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.

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