



## Maternal insulin therapy does not restore foetoplacental endothelial dysfunction in gestational diabetes mellitus



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### ABSTRACT

Pregnant women diagnosed with gestational diabetes mellitus subjected to diet (GDMd) that do not reach normal glycaemia are passed to insulin therapy (GDMi). GDMd associates with increased human cationic amino acid transporter 1 (hCAT-1)-mediated transport of L-arginine and nitric oxide synthase (NOS) activity in foetoplacental vasculature, a phenomenon reversed by exogenous insulin. Whether insulin therapy results in reversal of the GDMd effect on the foetoplacental vasculature is unknown. We assayed whether insulin therapy normalizes GDMd-associated foetoplacental endothelial dysfunction. Primary cultures of human umbilical vein endothelial cells (HUVECs) from GDMi pregnancies were used to assay L-arginine transport kinetics, NOS activity, p44/42<sup>mapk</sup> and protein kinase B/Akt activation, and umbilical vein rings reactivity. HUVECs from GDMi or GDMd show increased hCAT-1 expression and maximal transport capacity, NOS activity, and eNOS, and p44/42<sup>mapk</sup>, but not Akt activator phosphorylation. Dilution in response to insulin or calcitonin-gene related peptide was impaired in umbilical vein rings from GDMi and GDMd pregnancies. Incubation of HUVECs *in vitro* with insulin (1 nmol/L) restored hCAT-1 and eNOS expression and activity, and eNOS and p44/42<sup>mapk</sup> activator phosphorylation. Thus, maternal insulin therapy does not seem to reverse GDMd-associated alterations in human foetoplacental vasculature.

### 1. Introduction

Gestational diabetes mellitus (GDM) is a disease of pregnancy that characterizes by abnormal maternal D-glucose metabolism and altered insulin signalling in the foetoplacental circulation [1–5]. GDM-associated alterations lead to foetal hyperglycaemia and hyperinsulinemia resulting in endothelial dysfunction, and abnormal placental development and foetal growth [5,6]. Pregnant women that develop GDM are subjected to controlled diet (GDMd pregnancies) to reduce glycaemia to

the recommended goals [1,4,7]. However, a fraction of these women does not reach normal glycaemia with diet and are passed to treatment with insulin (i.e., insulin therapy, GDMi pregnancies) until delivery [3,4,8–10]. Both the mother and newborns in GDMd and GDMi pregnancies show with normal glycaemia at term [11–13]. However, the foetoplacental endothelial cell function and vascular reactivity are altered in GDMd [3,5,13].

Increased expression and activity of the endothelial nitric oxide synthase (eNOS) and uptake of the amino acid L-arginine via the human

**Abbreviations:** GDM, Gestational diabetes mellitus; GDMd, Gestational diabetes mellitus treated with diet; GDMi, Gestational diabetes mellitus treated with diet and insulin; eNOS, Endothelial nitric oxide synthase; hCAT-1, Human cationic amino transporter 1; HUVECs, Human umbilical vein endothelial cells; NO, Nitric oxide; NOS, Nitric oxide synthase; eNOS, Endothelial nitric oxide synthase

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cationic amino acid transporter 1 (hCAT-1), an isoform of human cationic amino acid transporters (hCATs) family [14,15], is reported in human umbilical vein endothelial cells (HUVECs) from GDMd pregnancies [13,16]. Exposure of HUVECs *in vitro* to physiological concentrations of insulin (up to 1 nmol/L) reverses the effects of GDMd on L-arginine transport [13] and NO synthesis [16]. Interestingly, high extracellular D-glucose (~25 mmol/L) also increases L-arginine transport via hCAT-1 and NO synthesis in HUVECs from normal pregnancies suggesting that hyperglycaemia could cause these changes in endothelial function [17]. Since insulin therapy results in normalization of the glycaemia in the mother and newborn at birth [4,9,18,19], a reversal of the effects of GDMd in the foetoplacental endothelial function is likely. In this study, we assayed whether maternal insulin therapy reverses the effects seen in GDMd on HUVECs function at birth. The results suggest that insulin therapy is not enough to reverse the foetoplacental endothelial dysfunction seen at birth in normoglycaemic, diet-controlled women with GDM.

## 2. Material and methods

### 2.1. Antibodies and materials

Primary monoclonal mouse *anti*-hCAT-1 and *anti*- $\beta$ -actin antibodies were from Sigma Aldrich (St Louis, MO, USA). Primary monoclonal mouse *anti*-eNOS phosphorylated at serine<sup>1177</sup>, polyclonal rabbit *anti*-total p44/42<sup>mapk</sup>, *anti*-phosphorylated p44/42<sup>mapk</sup>, *anti*-total Akt and *anti*-phosphorylated Akt antibodies were from Cell Signaling Technology (Danvers, MA, USA). Primary monoclonal mouse *anti*-eNOS phosphorylated at threonine<sup>495</sup> antibody was from BD Transduction Laboratories (San Jose, CA, USA). Primary monoclonal mouse *anti*-total eNOS antibody and secondary horseradish peroxidase-conjugated goat *anti*-rabbit or *anti*-mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For isolation of HUVECs from umbilical cords Collagenase Type II from *Clostridium histolyticum* (Boehringer, Mannheim, FRG) was used. Medium M199, new born (NBCS) and fetal calf (FCS) sera, L-glutamine, and penicillin-streptomycin were from Gibco Life Technologies (Carlsbad, CA, USA). L-[<sup>3</sup>H]Arginine and D-[<sup>3</sup>H]mannitol were from NEN (Dreieich, FRG). N<sup>G</sup>-Nitro-L-arginine methyl ester (L-NAME) and calcitonine gene related peptide (CGRP) were from Sigma Aldrich, and Akt Inhibitor IV (Inh-IV) and PD-98059 from Calbiochem (La Jolla, CA, USA). Immobilon-P polyvinylidene difluoride membranes was from BioRad Laboratories (Hertfordshire, UK). Qiagen RNAeasy kit was from Qiagen (Crawley, UK) and brilliant SYBR green qPCR Master Mix from Stratagene (La Jolla, CA, USA). The fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) was from Molecular Probes (Leiden, The Netherlands).

### 2.2. Study groups

This study included samples collected from 13 full-term normal or 32 full-term GDM pregnancies from the Hospital Clínico UC-CHRISTUS (HCUC-C) and Hospital San Juan de Dios (HSJD) in Santiago de Chile. Pregnant women included in this study did not smoke or consume drugs or alcohol, and had no intrauterine infection or any other medical or obstetrical complications. Ethnicity of patients included in this study was Hispanic. The investigation conforms to the principles outlined in the Declaration of Helsinki. Ethics Committee approvals from the Faculty of Medicine of the Pontificia Universidad Católica de Chile and HSJD, and informed written consent of patients were obtained. All pregnant women with a normal glycaemia at the first trimester of pregnancy were evaluated by an oral glucose tolerance test (OGTT) with a unique glucose load (75 g fasting) at 24–28 weeks of gestation. Patients with basal glycaemia  $\geq 5.56$  ( $\geq 100$  mg/dL, 8–9 h from last feeding in at least two different days) and with  $\geq 7.9$  mmol/L ( $\geq 140$  mg/dL) at 2 h after glucose load at the second or third trimester

of pregnancy were diagnosed with GDM (according to the Perinatal Guide 2015 report from the Health Ministry of Chile) [20]. Weight and body mass index (BMI) were recorded before pregnancy (i.e., pre-gestational) and for each trimester of pregnancy and at term.

Three groups of pregnant women were included in this study, i.e., normal pregnancies ( $n = 13$ ), GDMd pregnancies ( $n = 14$ ), or GDMd pregnancies where the mother was treated with insulin (GDMi, i.e., on insulin therapy) ( $n = 18$ ). Qualified personnel of the HCUC-C and HSJD selected the groups of pregnant women according to their clinical characteristics following established protocols. Women coursing with normal pregnancies followed standard self-controlled diet and physical activity. Women with GDMd were subjected to dietary treatment with 1500 kcal/day and a maximum of 200 g per day carbohydrates. Plasma glucose was weekly measured at fasting and post-breakfast, before and 1 h after the midday and evening meals. Women with GDMd that did not reach normal glycaemia after two weeks of diet were passed into insulin therapy following protocols adopted at the Division of Obstetrics and Gynaecology at the HCUC-C and HSJD as recommended in the Perinatal Guide 2015 Chile [20]. In brief, women with GDMd not responsive to diet were treated for 8–10 weeks until delivery with two injections (one before breakfast and one at bedtime) of neutral protamine Hagerdon human insulin (Humulin-N or Insuman-N, 0.5–0.65 units/kg of pre-pregnancy body weight). Additional three injections of regular (rapid-acting) insulin (Humulin-R or Insuman-R) depending on pre-prandial self-controlled capillary glycaemia (0.1 to 6 units/kg per meal for 81–120 to  $> 250$  mg/dL glucose, respectively) were administered to women with GDMd as described for insulin therapy in pregnant women with pre-gestational diabetes mellitus type 1 or 2 [11,21].

### 2.3. Human placenta and umbilical cords

Placentas were collected at delivery on ice and transferred to the laboratory until use 15–30 min later. Middle sections of umbilical cords (100–120 mm length) were dissected into 200 mL phosphate-buffered saline (PBS) solution (mmol/L: 130 NaCl, 2.7 KCl, 0.8 Na<sub>2</sub>HPO<sub>4</sub>, 1.4 KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 4 °C) until use 6–12 h later for isolation of endothelial cells.

### 2.4. Cell culture

HUVECs were isolated by collagenase digestion (0.25 mg/mL collagenase) from umbilical cords obtained at birth from normal, GDMd, or GDMi pregnancies and cultured (37 °C, 5% CO<sub>2</sub>) in 1% gelatin-coated petri dishes (100 mm diameter) up to passage 3 in primary culture medium (PCM; M199 containing 5 mmol/L D-glucose, 10% NBCS, 10% FCS, 3.2 mmol/L L-glutamine and 100 U/mL penicillin-streptomycin). 16 h prior experiments the incubation medium was changed to M199 medium containing 0.25% NBCS and 0.25% FCS. Experiments were performed in the absence (hereafter referred as ‘without insulin’) or presence (8 h, hereafter referred as ‘with insulin’) of insulin (1 nmol/L), L-NAME (100  $\mu$ mol/L, NOS inhibitor) [22], Inh-IV (1  $\mu$ mol/L, protein kinase B/Akt inhibitor) [23], and/or PD-98059 (10  $\mu$ mol/L, MAP kinase kinases 1/2 inhibitor) [24].

### 2.5. L-Arginine transport

Overall 0–1000  $\mu$ mol/L L-arginine transport (3  $\mu$ Ci/mL L-[<sup>3</sup>H]arginine, 1 min incubation, 37 °C) was measured in Krebs solution (mmol/L: 131 NaCl, 5.6 KCl, 25 NaHCO<sub>3</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 20 Hepes, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, pH 7.4, 37 °C) as described [13]. Overall transport of L-arginine was defined as the result of the sum of a saturable component plus a nonsaturable, linear component of transport in the range of L-arginine concentrations used in this study (hereafter referred as a  $K_D$  value defined by  $m \cdot [Arg]$ , where  $m$  corresponds to slopes of lineal phases of transport at a given L-arginine concentration  $[Arg]$ ) [13]. Cell

monolayers were rinsed with ice-cold Krebs to terminate tracer uptake.

Initial rate for transport (i.e., linear uptake up to 1 min) was derived from the slope of the linear phases of L-arginine transport. Values for transport were adjusted to the one phase exponential association equation considering the least squares fit:

$$v_i = V_m \cdot (1 - e^{-(k \cdot t)})$$

where  $v_i$  is initial velocity,  $V_m$  is mayor velocity at a given time ( $t$ ) and L-arginine concentration, and  $e$  and  $k$  are constants. Overall L-arginine transport at initial rates was adjusted to the Michaelis-Menten hyperbola plus a nonsaturable, linear component ( $K_D$ ) as described [13]. The saturable transport of L-arginine was derived by subtracting the  $m \cdot [Arg]$  components from overall transport, and the transport kinetic parameters maximal velocity ( $V_{max}$ ) and apparent Michaelis-Menten constant ( $K_m$ ) of transport were calculated [13]. The relative contribution of GDMd, GDMi, insulin, PD-98059, or Inh-IV to the saturable L-arginine transport kinetic parameters was estimated from the maximal transport capacity ( $V_{max}/K_m$ ) values for L-arginine transport by:

$$\frac{1}{C/X_F} = \frac{C K_m \cdot X V_{max}}{C V_{max} \cdot X K_m}$$

where  $C V_{max}$  and  $C K_m$  are the kinetics parameters for L-arginine transport in control conditions (i.e., in the absence of insulin, PD-98059, or Inh-IV) in cells from normal, GDMd, or GDMi pregnancies, and  $X V_{max}$  and  $X K_m$  are kinetics parameters of L-arginine transport in HUVECs from normal, GDMd, or GDMi pregnancies exposed to different experimental conditions [13,25]. Each transport assay was run in duplicate with transport activity expressed as pmol/ $\mu$ g protein/minute. Radioactivity in 0.5 N KCl cell digests was determined by liquid scintillation counting, and uptake was corrected for D-[<sup>3</sup>H]mannitol disintegrations per minute (d.p.m.) in the extracellular space [13].

## 2.6. Western blotting

Total protein was obtained from confluent cells washed twice with ice-cold PBS and harvested in 100  $\mu$ L of lysis buffer composed by 63.7 mmol/L Tris/HCl (pH 6.8), 10% glycerol, 2% sodium dodecylsulphate (SDS), 1 mmol/L sodium orthovanadate, 50 mg/mL leupeptin, and 5% 2-mercaptoethanol, as described [13]. Cells were sonicated (6 cycles, 5 s, 100 W, 4 °C), and total protein was separated by centrifugation (14,000g, 15 min, 4 °C). Proteins (70  $\mu$ g) were separated by polyacrylamide gel (10%) electrophoresis and transferred onto Immobilon-P polyvinylidene difluoride membranes. The proteins were then probed against hCAT-1 (1:500 dilution, 12 h, 4 °C), total eNOS (1:500 dilution, 12 h, 4 °C), eNOS phosphorylated at serine<sup>1177</sup> (P~Ser<sup>1177</sup>-eNOS, 1:1000 dilution, 12 h, 4 °C), eNOS phosphorylated at threonine<sup>495</sup> (P~Thr<sup>495</sup>-eNOS, 1:1000 dilution, 12 h, 4 °C), total p44/42<sup>mapk</sup> (1:1000 dilution, 3 h, room temperature), phosphorylated p44/42<sup>mapk</sup> (1:1000 dilution, 3 h, room temperature), total Akt (1:1000 dilution, 3 h, room temperature), phosphorylated Akt (1:1000 dilution, 3 h, room temperature), and  $\beta$ -actin (1:3000, 1 h, room temperature). Membranes were rinsed in Tris buffer saline Tween and incubated (1 h) in TBS-T/0.2% BSA containing secondary horseradish peroxidase-conjugated antibodies. Proteins were detected by enhanced chemiluminescence (film exposure time was 1 min) in a ChemiDoc-It 510 Imagen System (UVP, LCC Upland, CA, USA) and quantified by densitometry [13].

## 2.7. Isolation of total RNA and reverse transcription

Total RNA was isolated using the Qiagen RNeasy kit. RNA quality and integrity were insured by gel visualization and spectrophotometric analysis (OD<sub>260/280</sub>), quantified at 260 nm. Aliquots (2  $\mu$ g) of total RNA were reversed transcribed into cDNA as described [13].

## 2.8. Quantitative RT-PCR

Experiments were performed using a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) in a reaction mix containing 0.5  $\mu$ mol/L primers, and master mix provided in the brilliant SYBR green qPCR Master Mix as described [13]. SecureStartTaq DNA polymerase was activated (15 min, 95 °C), and the PCR cycling profile included a 95 °C denaturation (15 s), annealing (20 s) at 54 °C (hCAT-1 and 28S) or 60 °C (eNOS, insulin receptor A (IR-A), insulin receptor B (IR-B)), and extension at 72 °C for variable times (hCAT-1, 10 sec; eNOS, 17 sec; IR-A and IR-B, 20 sec; 28S, 10 s). Product melting temperature values were 79.1 °C (hCAT-1), 86.43 °C (eNOS), 87.2 °C (IR-A), 87.6 °C (IR-B), and 86.7 °C (28S). The oligonucleotide primers used in this study were: hCAT-1 sense: 5'-GAGTTAGATCCAGCAGACCA-3', hCAT-1 anti-sense: 5'-TGTTACAATTAGCCAGAG-3', eNOS sense: 5'-CCAGCTAGCCAAAGTACCAT-3', eNOS anti-sense: 5'-GTCTCGGAGC-CATACAGGATT-3', IR-A sense: 5'-GCTGAAGCTGCCCTCGAGGA-3', IR-A anti-sense: 5'-CGAGATGGCCTGGGGACGAA-3', IR-B sense: 5'-GCTG-AAGTCCCTCGAGGA-3', IR-B anti-sense 5'-AGATGGCCTAGGGTCC-TCGG-3', 28S sense: 5'-TTGAAAATCCGGGGAGAG-3', 28S anti-sense: 5'-ACATTGTTCCAACATGCCAG-3'. Expected size products were hCAT-1 148 bp, eNOS 354 bp, IR-A 210 bp, IR-B 244 bp, and 28S 100 bp.

## 2.9. Intracellular NO determination

Intracellular NO was determined using the fluorescent dye DAF-FM. Confluent cells grown on 100 mm<sup>2</sup> culture plate were incubated in the absence or presence of insulin (1 nmol/L, 8 h) and exposed (45 min, 37 °C) to 10  $\mu$ mol/L DAF-FM in PBS (37 °C, pH 7.4). The fluorescence ( $\lambda_{exc}/\lambda_{em}$ : 495/510 nm) was determined in a Sinergy 2 microplate reader (Biotek, Winooski, VT, USA) [17].

## 2.10. NOS activity

NOS activity was assayed by quantification of the intracellular content of L-citrulline by high performance liquid chromatography in confluent HUVECs in the absence or presence of 100  $\mu$ mol/L L-NAME, as reported [17].

## 2.11. Human umbilical vein reactivity

Ring segments of 2-4 mm in length were dissected from human umbilical veins in ice-cold PBS (pH 7.4, 4 °C). Vein rings were mounted in a myograph (610M Multiwire Myograph System, Danish Myo Technology A/S, Denmark) for isometric force measurements in a Krebs physiological solution (mmol/L: 118.5 NaCl, 4.7 KCl, 25 NaHCO<sub>3</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, 5.5 D-glucose, 0.3 L-arginine, 37 °C, pH 7.4). Vein rings were constantly bubbled with a mixture of 95% O<sub>2</sub>/5% CO<sub>2</sub>. The optimal diameter for each vessel was adjusted through the determination of the maximal active response evoked by 65 mmol/L KCl [13]. Endothelium-dependent relaxation was evaluated as the concentration-dependent response to CGRP (0.01 – 100 nmol/L, 5 min) and insulin (0.01 – 1000 nmol/L, 5 min) in KCl-precontracted vessels. Experiments were performed in the absence or presence of 100  $\mu$ mol/L L-NAME. Changes in isometric tension were recorded using the software LabChart (LabChart 7 for Windows, ADInstruments, Australia) coupled to a PowerLab (PowerLab 8/30 Data Acquisition System, ADInstruments, Australia).

## 2.12. Statistical analysis

The sample size was estimated considering a power of 80% to detect a difference between groups (on the basis of a two-sided alpha level of 0.05). Values for clinical parameters are given as mean  $\pm$  S.D. For *in vitro* assays the values were mean  $\pm$  S.E.M., where *n* indicates number of different biological samples and corresponding cell cultures with 3–4

**Table 1**  
Clinical characteristics of pregnant women and newborns.

Variable	Normal (n = 13)	GDMd (n = 14)	GDMi (n = 18)
<b>Mother</b>			
Age (years)	32.1 ± 3.8	33.7 ± 5.2	30.5 ± 4.5
Height (cm)	162.5 ± 4.3	157.6 ± 6.1	160.2 ± 4.4
<b>Weight (kg)</b>			
Pre-pregnancy	62.8 ± 7.7	69.2 ± 9.3	72.2 ± 10.9*
1st trimester	63.3 ± 8.2	70.1 ± 9.6	72.5 ± 10.8*
2nd trimester	67.4 ± 6.2	74.3 ± 7.9	79.2 ± 9.5*
3rd trimester	73.7 ± 4.6	76.1 ± 8.8	81.0 ± 10.6
Term	73.8 ± 4.9	78.1 ± 4.1	82.1 ± 9.4*
<b>BMI (kg/m<sup>2</sup>)</b>			
Pre-pregnancy	23.8 ± 2.5	26.9 ± 3.2	28.3 ± 3.9*
1st trimester	23.6 ± 2.7	27.8 ± 3.2*	28.4 ± 4.1*
2nd trimester	25.4 ± 1.9	28.9 ± 3.1*	31.1 ± 2.9*
3rd trimester	28.1 ± 0.8	33.1 ± 2.8*	31.4 ± 3.6*
Term	28.1 ± 1.4	33.4 ± 4.4*	32.5 ± 3.1*
<b>Systolic blood pressure (mm Hg)</b>			
Pre-pregnancy	101.4 ± 6.9	113.9 ± 9.9	104.6 ± 6.3
1st trimester	106.9 ± 5.4	110.1 ± 11.1	106.7 ± 7.7
2nd trimester	105.7 ± 9.8	109.2 ± 9.2	105.4 ± 9.7
3rd trimester	108.1 ± 7.5	109.6 ± 15.6	109.5 ± 11.7
Term	113.8 ± 5.2	110.4 ± 7.1	110.2 ± 10.6
<b>Diastolic blood pressure (mm Hg)</b>			
Pre-pregnancy	62.9 ± 4.9	70.7 ± 6.2	68.6 ± 6.3
1st trimester	67.7 ± 6.4	65.2 ± 9.1	66.4 ± 6.1
2nd trimester	67.1 ± 7.6	66.3 ± 8.5	65.7 ± 5.8
3rd trimester	66.3 ± 5.8	69.2 ± 10.1	69.5 ± 7.7
Term	67.5 ± 8.9	72.2 ± 8.8	71.5 ± 5.1
<b>Mean arterial pressure (mm Hg)</b>			
Pre-pregnancy	75.7 ± 4.6	70.7 ± 6.2	80.6 ± 5.2
1st trimester	80.8 ± 3.9	81.8 ± 8.6	80.1 ± 5.6
2nd trimester	80.1 ± 7.2	81.9 ± 9.2	78.9 ± 6.7
3rd trimester	80.2 ± 6.2	82.7 ± 11.8	82.8 ± 8.2
Term	82.9 ± 7.1	84.3 ± 7.1	84.4 ± 5.7
<b>Glycemia fasting (mg/dL)</b>			
Pre-pregnancy	82.9 ± 7.1	85.1 ± 4.9	87.8 ± 8.1
<b>OGTT (mg/dL)</b>			
Glycemia basal	78.3 ± 5.1	85.8 ± 8.8	84.4 ± 15.3
Glycemia 2 h after glucose	82.9 ± 7.1	155.5 ± 13.9*	165.9 ± 20.1*
<b>Newborn</b>			
Sex (female/male)	7/6	8/6	10/8
Gestational age (weeks)	38.5 ± 1.3	38.8 ± 0.6	38.4 ± 0.6
Birth weight (grams)	3374 ± 411	3441 ± 447	3552 ± 517
Height (cm)	50.2 ± 1.3	50.1 ± 1.5	50.1 ± 1.4
Ponderal index (grams/cm <sup>3</sup> × 100)	2.7 ± 0.3	2.7 ± 0.2	2.8 ± 0.3

Women that coursed with normal pregnancies (*Normal*), GDM under treatment with diet (GDMd) or GDMd passed onto insulin therapy (GDMi) were included in this study. Weight, body mass index (BMI), and blood pressure were determined before pregnancy (pre-pregnancy) and at 1st (0–14 weeks of gestation (wg)), 2nd (14–28 wg) or 3rd (28–40 wg) trimesters of pregnancy and at term (38–41 wg). Values for pre-pregnancy weight and blood pressure correspond to those autoreferred by patients. In Normal pregnancies, 11 women were with pre-pregnancy normal weight (BMI < 25 kg/m<sup>2</sup>) and two with pre-pregnancy overweight (BMI ≥ 25 and < 30 kg/m<sup>2</sup>). In GDMd, 11 women were with pre-pregnancy normal weight, two pre-pregnancy overweight, and one pre-pregnancy obese (BMI ≥ 30 kg/m<sup>2</sup>). In GDMi, 14 women were with pre-pregnancy normal weight, two pre-pregnancy overweight, and two pre-pregnancy obese. BMI was calculated by weight in kilograms divided by the square of the height in meters. Ponderal index was calculated by weight in grams divided by the cube of height in centimeters multiplied by 100. Oral glucose tolerance test (OGTT) was measured at 24–28 weeks of gestation in all women with a normal glycaemia (see [Material and methods](#)). Values are mean ± S.D.

\*  $P < 0.05$  versus corresponding values in *Normal*.

replicates per experiment. Comparisons between two groups were performed by means of Student's unpaired *t*-test and between more than two groups by analysis of variance (ANOVA, two-ways). If the ANOVA demonstrated a significant interaction between variables, *post hoc*

analyses were performed by the multiple-comparison Bonferroni test. The statistical software GraphPad InStat 3.1 and GraphPad Prism 7.0c (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis.  $P < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Study groups

Pregnancies were singleton, and pregnant women showed with similar age and height, were normotensive, and with normal fasting glycaemia at term ([Table 1](#)). The total gestational weight gain between pre-pregnancy and term was  $10 \pm 1$  kg in normal pregnancies,  $8.9 \pm 0.9$  kg in GDMd pregnancies, and  $9.9 \pm 1.1$  kg in GDMi pregnancies. Clinical parameters of newborns from the three study groups did not show significant differences.

#### 3.2. L-Arginine transport kinetics

The  $v_i$  for overall 100  $\mu\text{mol/L}$  L-arginine transport was lineal up to 60 s in cells from normal, GDMd, or GDMi pregnancies without insulin ([Fig. 1A](#)). Values for  $v_i$  were higher in cells from GDMd and GDMi compared with normal pregnancies ([Table 2](#)). Overall L-arginine transport was semisaturable in cells without insulin from normal, GDMd, or GDMi pregnancies ([Fig. 1B](#)), with  $K_D$  values higher in GDMd and GDMi compared with normal pregnancies ([Table 2](#)). After subtracting the lineal, non-saturable transport component from overall transport, the remaining L-arginine transport was saturable, best fitted by a single Michaelis-Menten equation ([Fig. 1C](#)), and lineal in an Eadie-Hofstee plot ([Fig. 1D](#)). Saturable transport showed with higher  $V_{\text{max}}$  in cells from GDMd and GDMi compared with normal pregnancies. The apparent  $K_m$  values were not significantly altered in cells from these three study groups. However, the maximal transport capacity ( $V_{\text{max}}/K_m$ ) was higher in GDMd and GDMi when compared with normal pregnancies ([Table 2](#)).

Insulin increased the  $v_i$  and  $K_D$  in cells from normal pregnancies, but restored this parameter in GDMd and GDMi ([Fig. 1E](#)). Overall transport in the presence of insulin was also semisaturable ([Fig. 1F](#)), and saturable transport showed higher  $V_{\text{max}}$  and  $V_{\text{max}}/K_m$ , but unaltered  $K_m$  values in cells from normal pregnancies ([Fig. 1G](#)). GDMd and GDMi-increased L-arginine transport was blocked by insulin. Eadie-Hofstee plot of saturable data was lineal in cells treated with insulin from all study groups ([Fig. 1H](#)).

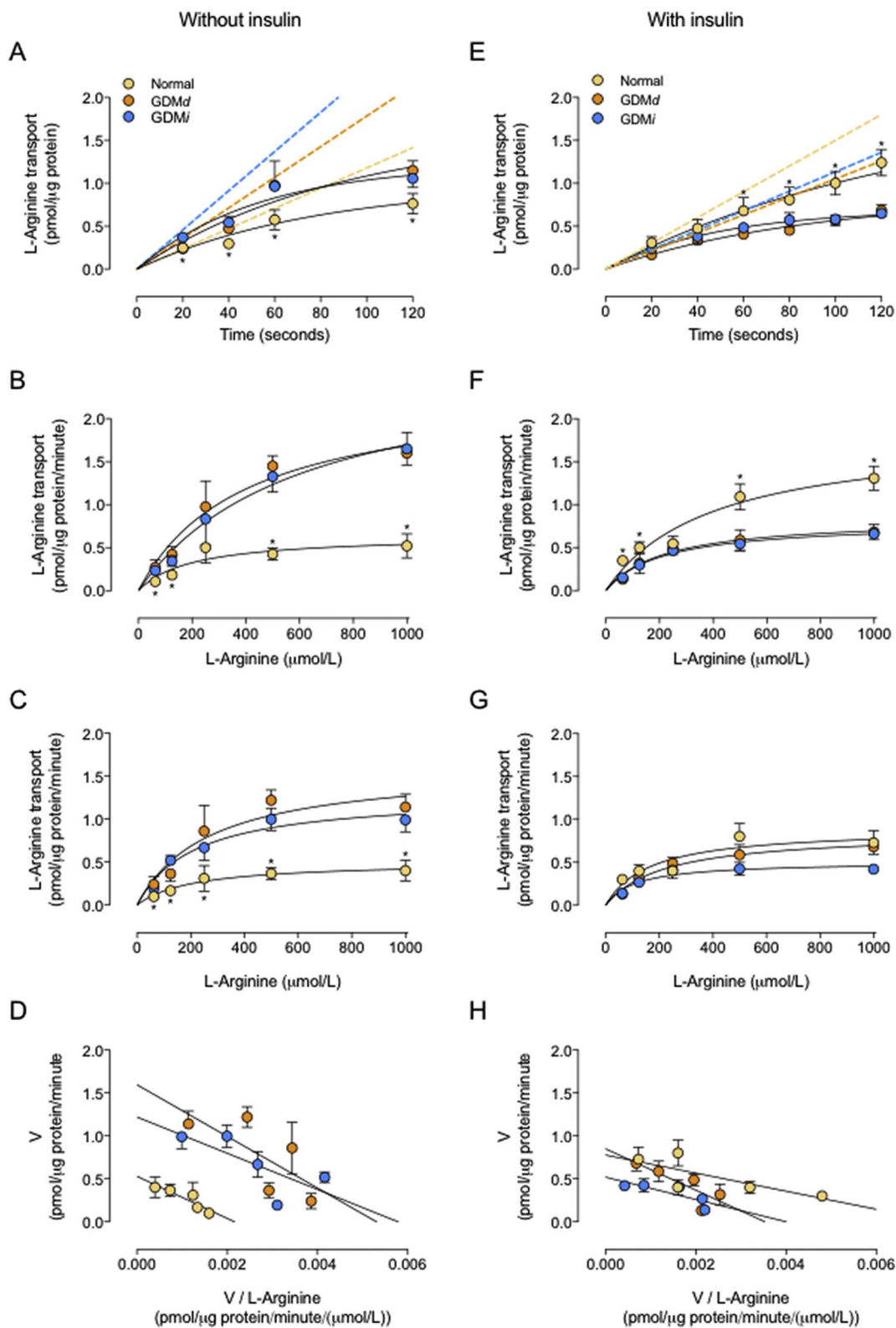
#### 3.3. hCAT-1 protein abundance and mRNA expression

In cells without insulin the hCAT-1 protein abundance was higher in GDMd and GDMi compared with normal pregnancies ([Fig. 2A](#)). Incubation of cells with insulin, increased hCAT-1 protein abundance in normal pregnancies, but partially reduced its abundance in GDMd and GDMi. Similar results were obtained for hCAT-1 mRNA expression without or with insulin; however, insulin fully reversed GDMd effect ([Fig. 2B](#)).

#### 3.4. Involvement of p44/42<sup>mapk</sup> and Akt on L-arginine transport

Cells from GDMd and GDMi without insulin show increased p44/42<sup>mapk</sup> phosphorylation compared with normal pregnancies ([Fig. 3A](#)). Insulin blocked the GDMd and GDMi effect, but increased p44/p42<sup>mapk</sup> phosphorylation in cells from normal pregnancies. Phosphorylated Akt protein abundance was unaltered by GDMd or GDMi, or insulin in cells from normal or GDM pregnancies. IR-A mRNA expression was higher in cells from GDMd and GDMi compared with normal pregnancies ([Fig. 3B](#)). Insulin blocked GDM effect on IR-A mRNA expression, but unaltered its expression in cells from normal pregnancies. However, IR-B mRNA expression was unaltered by GDM or insulin.





**Fig. 1.** L-Arginine transport in HUVECs from GDMi pregnancies. A. Initial velocity of 100  $\mu\text{mol/L}$  L-arginine uptake at various times in HUVECs from normal, gestational diabetes on diet (GDMd), and GDMd into insulin therapy (GDMi) without exogenous insulin *in vitro*. Straight dashed lines represent the lineal phase of uptake estimated for 0.5 s incubation (see Material and methods). B. Overall L-arginine transport measured at initial velocity fitted to a single Michaelis-Menten equation increased in a lineal component (see Material and methods). C. Saturable L-arginine transport derived from data in B adjusted to a single Michaelis-Menten equation. D. Eadie-Hofstee representation of data in C. E to H are cells in the presence (8 h) of 1 nmol/L insulin (with insulin). \* $P < 0.05$  versus corresponding values in GDMi or GDMd. For some data points the error bars were smaller than the symbols. Values are mean  $\pm$  S.E.M. ( $n = 7-11$ ).

**Table 2**  
Kinetic parameters for L-arginine transport in HUVECs from normal or GDM pregnancies.

	Saturable transport			Overall transport	
	$V_{max}$ (pmol/ $\mu$ g protein/ minute)	$K_m$ ( $\mu$ mol/L)	$V_{max}/K_m$ (pmol/ $\mu$ g protein/minute/ ( $\mu$ mol/L))	$K_D$ (pmol/ $\mu$ g protein/minute/( $\mu$ mol/ L))	$v_i$ (pmol/ $\mu$ g protein/0.5 s)
Normal pregnancies ( $n = 13$ )					
<i>Without insulin</i>					
Control	0.50 $\pm$ 0.06	144 $\pm$ 55	0.0035 $\pm$ 0.0009	0.00012 $\pm$ 0.0001	0.00171 $\pm$ 0.00021
PD-98059	0.60 $\pm$ 0.07	197 $\pm$ 59	0.0030 $\pm$ 0.0006	0.00014 $\pm$ 0.0001	0.00168 $\pm$ 0.00017
Inh-IV	0.45 $\pm$ 0.07	123 $\pm$ 71	0.0037 $\pm$ 0.0013	0.00015 $\pm$ 0.0003	0.00168 $\pm$ 0.00031
PD-98059 + Inh-IV	0.53 $\pm$ 0.05	274 $\pm$ 58	0.0019 $\pm$ 0.0003	0.00012 $\pm$ 0.0001	0.00118 $\pm$ 0.00009
<i>With insulin</i>					
Control	0.89 $\pm$ 0.07*	164 $\pm$ 42	0.0054 $\pm$ 0.0009*	0.00044 $\pm$ 0.0001*	0.00281 $\pm$ 0.00023*
PD-98059	0.62 $\pm$ 0.09**	223 $\pm$ 90	0.0028 $\pm$ 0.0008**	0.00012 $\pm$ 0.0001**	0.00159 $\pm$ 0.00021**
Inh-IV	0.63 $\pm$ 0.03**	123 $\pm$ 15	0.0051 $\pm$ 0.0004*	0.00040 $\pm$ 0.0001*	0.00235 $\pm$ 0.00010*
PD-98059 + Inh-IV	0.46 $\pm$ 0.03**	258 $\pm$ 40	0.0030 $\pm$ 0.0009**	0.00024 $\pm$ 0.0001**	0.00107 $\pm$ 0.00005**
GDMd pregnancies ( $n = 14$ )					
<i>Without insulin</i>					
Control	1.61 $\pm$ 0.14*	269 $\pm$ 61	0.0060 $\pm$ 0.0009*	0.00046 $\pm$ 0.0001*	0.00363 $\pm$ 0.00028*
PD-98059	0.65 $\pm$ 0.07**	143 $\pm$ 51	0.0045 $\pm$ 0.0010**	0.00092 $\pm$ 0.0003****	0.00223 $\pm$ 0.00025**
Inh-IV	1.44 $\pm$ 0.20*	295 $\pm$ 96	0.0049 $\pm$ 0.0012*	0.00040 $\pm$ 0.0001*	0.00303 $\pm$ 0.00035*
PD-98059 + Inh-IV	0.93 $\pm$ 0.09**	211 $\pm$ 53	0.0044 $\pm$ 0.0005**	0.00080 $\pm$ 0.0002****	0.00249 $\pm$ 0.00021**
<i>With insulin</i>					
Control	0.61 $\pm$ 0.03***	141 $\pm$ 24	0.0042 $\pm$ 0.0005‡	0.00019 $\pm$ 0.0001‡	0.00210 $\pm$ 0.00028‡
PD-98059	0.83 $\pm$ 0.04***	210 $\pm$ 27	0.0039 $\pm$ 0.0004***	0.00096 $\pm$ 0.0001****	0.00227 $\pm$ 0.00023***
Inh-IV	0.49 $\pm$ 0.03***	159 $\pm$ 23	0.0031 $\pm$ 0.0003***	0.00040 $\pm$ 0.0001****	0.00298 $\pm$ 0.00025**
PD-98059 + Inh-IV	0.73 $\pm$ 0.07***	144 $\pm$ 45	0.0051 $\pm$ 0.0010***	0.00114 $\pm$ 0.0003****	0.00208 $\pm$ 0.00026***
GDMi pregnancies ( $n = 18$ )					
<i>Without insulin</i>					
Control	1.28 $\pm$ 0.07*	213 $\pm$ 32	0.0060 $\pm$ 0.0006*	0.00066 $\pm$ 0.0003*	0.00340 $\pm$ 0.00017*
PD-98059	0.92 $\pm$ 0.14**	223 $\pm$ 75	0.0041 $\pm$ 0.0009**	0.00056 $\pm$ 0.0001*	0.00237 $\pm$ 0.00028**
Inh-IV	1.48 $\pm$ 0.09*	254 $\pm$ 40	0.0058 $\pm$ 0.0007*	0.00022 $\pm$ 0.0001****	0.00348 $\pm$ 0.00018*
PD-98059 + Inh-IV	0.63 $\pm$ 0.08**	164 $\pm$ 60	0.0038 $\pm$ 0.0009**	0.00102 $\pm$ 0.0010****	0.00198 $\pm$ 0.00024**
<i>With insulin</i>					
Control	0.51 $\pm$ 0.03***	111 $\pm$ 17	0.0046 $\pm$ 0.0005***	0.00024 $\pm$ 0.0003***	0.00201 $\pm$ 0.00010***
PD-98059	0.46 $\pm$ 0.03***	108 $\pm$ 24	0.0043 $\pm$ 0.0006***	0.00120 $\pm$ 0.0010****	0.00184 $\pm$ 0.00013***
Inh-IV	0.54 $\pm$ 0.03***	193 $\pm$ 29	0.0028 $\pm$ 0.0003‡	0.00036 $\pm$ 0.0001****	0.00153 $\pm$ 0.00007***
PD-98059 + Inh-IV	0.62 $\pm$ 0.09***	218 $\pm$ 93	0.0028 $\pm$ 0.0014***	0.00130 $\pm$ 0.0010****	0.00162 $\pm$ 0.00023***

L-Arginine transport (0–1000  $\mu$ mol/L, 1 min, 37 °C) was measured in HUVECs from normal pregnancies (Normal), GDM under treatment with diet (GDMd) or GDMd passed onto insulin therapy (GDMi) exposed (8 h) to culture medium without (*Without insulin*) or with (*With insulin*) insulin (1 nmol/L). Transport assays were done in the absence (Control) or presence of the PD-98059 (10  $\mu$ mol/L, MEK1/2 inhibitor) and Akt Inhibitor IV (Inh-IV, 1  $\mu$ mol/L) or both (see Material and methods). Maximal velocity ( $V_{max}$ ) and apparent Michaelis-Menten constant ( $K_m$ ) of saturable transport were calculated assuming a single Michaelis-Menten hyperbola.  $V_{max}/K_m$  represents maximal L-arginine transport capacity. The lineal phase of overall transport of L-arginine ( $K_D$ ) was obtained from transport data fitted to a Michaelis-Menten equation increased in a lineal component. Initial velocity ( $v_i$ ) was calculated for 0.5 s with 100  $\mu$ mol/L L-arginine transport.

Values are mean  $\pm$  S.E.M.

\*  $P < 0.05$  versus corresponding Control values in Normal *Without insulin*.

\*\*  $P < 0.05$  versus corresponding Control values.

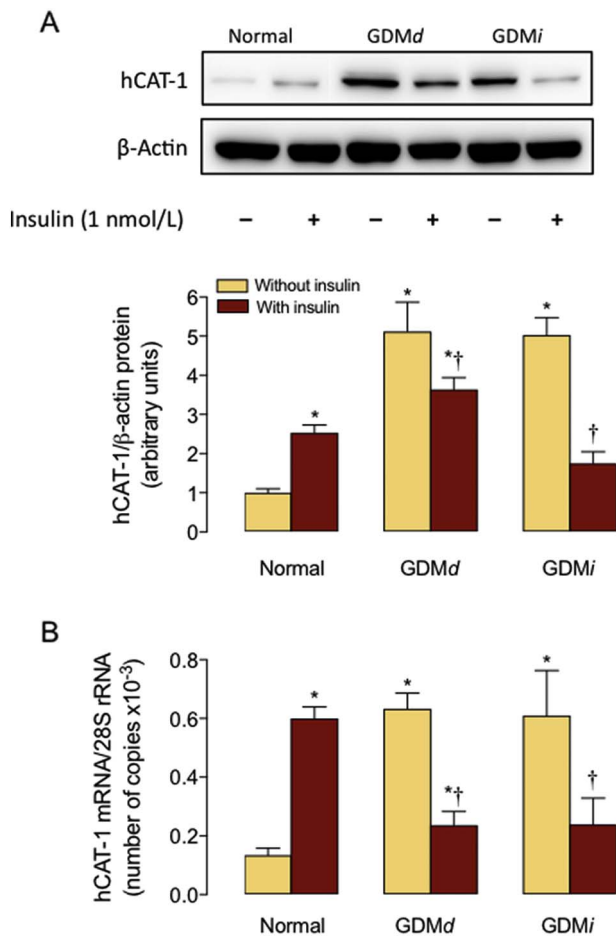
\*\*\*  $P < 0.05$  versus corresponding Control values *Without insulin*.

Overall L-arginine transport in the presence of PD-98059 and Inh-IV was semisaturable as in the absence of these inhibitors (not shown). Incubation of cells with PD-98059, Inh-IV, or both, did not alter the  $v_i$  or  $K_D$  values in cells from normal pregnancies without insulin (Table 2). However, insulin-stimulatory effect on these parameters was blocked by PD-98059 or PD-98059 + Inh-IV, but unaltered by Inh-IV alone. Insulin-increased  $V_{max}$  and  $V_{max}/K_m$  for saturable transport was blocked by PD-98059 or PD-98059 + Inh-IV; however, Inh-IV alone blocked the increase caused by insulin in the  $V_{max}/K_m$ , but not in  $V_{max}$  (Fig. 3C, Table 2). In cells without insulin the GDMd-increased  $v_i$  and  $V_{max}$  were blocked only by PD-98059 or PD-98059 + Inh-IV. The GDMd-increased  $V_{max}/K_m$  was blocked by PD-98059, Inh-IV, or PD-98059 + Inh-IV. However, incubation of cells with PD-98059 or PD-98059 + Inh-IV, but not with Inh-IV alone, resulted in higher  $K_D$  values compared with cells from GDMd in the absence of these inhibitors (Table 2). Insulin reversed the increase caused by GDMd in all the kinetic parameters in the absence or presence of inhibitors. However,  $K_D$  values in cells with insulin and inhibitors were unaltered compared with cells without insulin in the presence of the inhibitors. The inhibitors caused a similar pattern of

inhibition in cells from GDMi without insulin, except for cells incubated with PD-98059 where  $K_D$  value was unaltered compared with cells in the absence of this inhibitor (Table 2). In cells with insulin the pattern of effects of the inhibitors in the kinetic parameters was similar compared to those in cells from GDMd, except for Inh-IV which inhibits the increase in  $v_i$  caused by GDMi.

### 3.5. NOS activity

The intracellular content of L-citrulline generated by NOS activity (i.e., fraction inhibited by L-NAME) was higher reaching similar values in cells from GDMi and GDMd, compared with normal pregnancies (Fig. 4A). Insulin caused a substantial reduction in GDM-increased L-citrulline content, but increased intracellular content of this amino acid in cells from normal pregnancies. In addition, the NO level detected in cells from GDMd and GDMi was higher than normal pregnancies (Fig. 4B). Insulin increased NO level in cells from normal pregnancies, and partially reversed the effect of GDM.



**Fig. 2.** hCAT-1 expression in HUVECs from GDMi pregnancies. A. Western blot for hCAT-1 and  $\beta$ -actin (internal reference) protein abundance in HUVECs from normal, gestational diabetes on diet (GDMd), and GDMd into insulin therapy (GDMi) without or with (8 h) exogenous insulin *in vitro*. Lower panel: hCAT-1/ $\beta$ -actin ratio densitometries normalized to 1 in Normal. B. hCAT-1 mRNA expression as in A. \* $P < 0.05$  versus Normal Without insulin. † $P < 0.05$  versus corresponding values Without insulin. Values are mean  $\pm$  S.E.M. ( $n = 5$ ).

### 3.6. eNOS expression and phosphorylation

Cells from GDMd and GDMi without insulin show comparable higher eNOS protein abundance compared with normal pregnancies (Fig. 4C). Insulin partially reduced GDM effect on eNOS, but increased its protein abundance in cells from normal pregnancies. In cells without insulin the P-Ser<sup>1177</sup> eNOS protein abundance was higher in GDM pregnancies reaching similar values, an effect blocked by insulin in cells from GDMd, but partially reversed by this hormone in GDMi (Fig. 4D). Insulin increased P-Ser<sup>1177</sup> eNOS protein abundance in cells from normal pregnancies. However, neither GDM nor insulin altered P-Thr<sup>496</sup> eNOS protein abundance (Fig. 4E). GDMd and GDMi associated with higher and comparable eNOS mRNA expression (Fig. 4F) compared with cells from normal pregnancies. Insulin reversed the GDM effect on eNOS mRNA, but increased mRNA expression in cells from normal pregnancies.

### 3.7. Umbilical vein reactivity

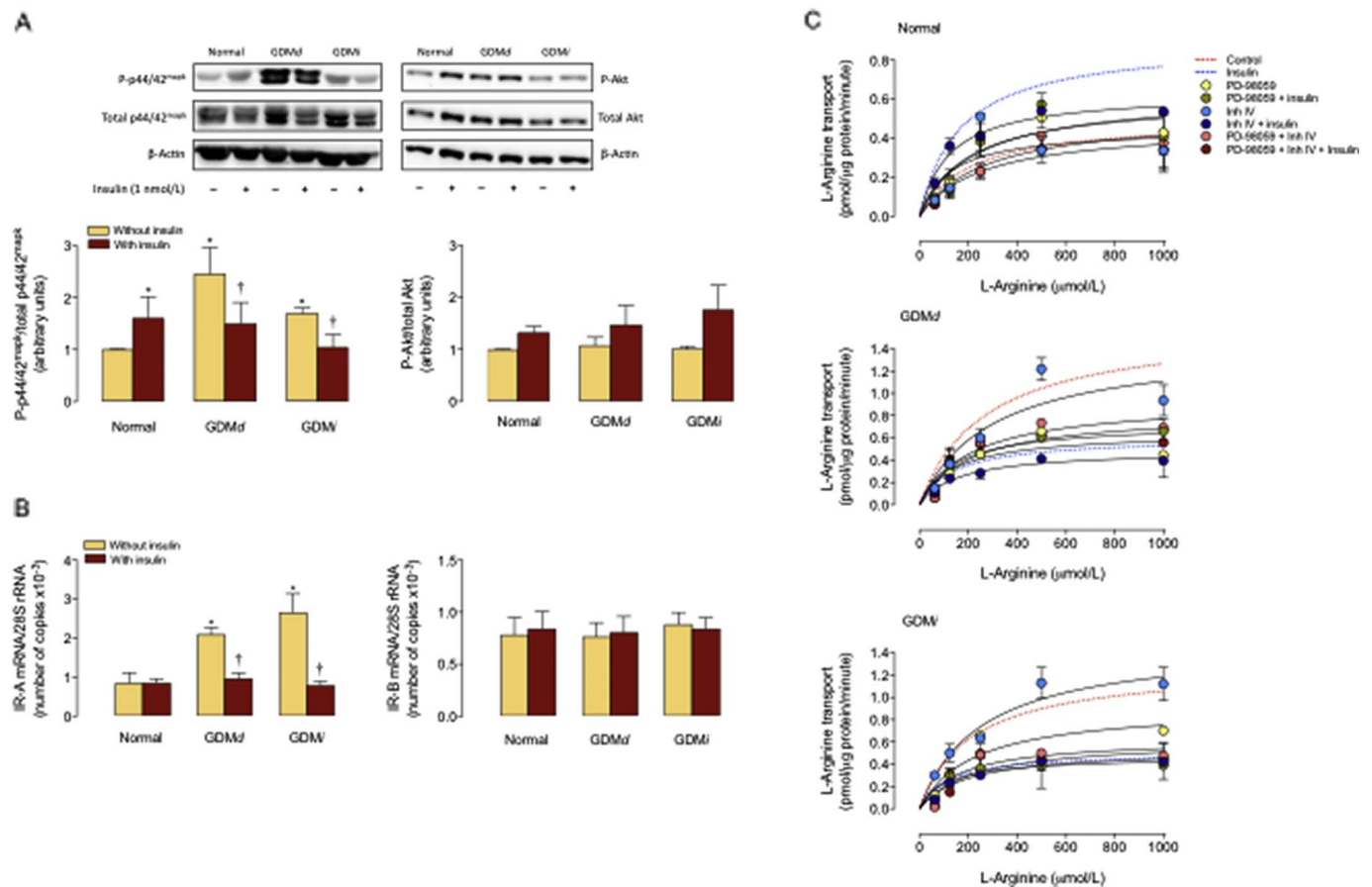
Insulin caused concentration, NOS-dependent relaxation in umbilical vein rings (Fig. 5A). Maximal relaxation ( $R_{max}$ ) in response to insulin was lower, but half-maximal effect ( $EC_{50}$ ) was higher in vein rings from GDMd and GDMi compared with normal pregnancies (Table 3). The required insulin concentration to cause a similar  $R_{max}$  (i.e.,  $EC_{50}/$

$R_{max}$ ) was higher in GDMd and GDMi compared with normal pregnancies. The  $EC_{50}/R_{max}$  was higher in GDMi compared with vessel rings from GDMd. CGRP also caused concentration-dependent relaxation of vein rings (Fig. 5B) with an  $EC_{50}$  values lower than those for insulin effect in vessel rings from all conditions (Table 3). The  $EC_{50}$  in vein rings from GDMd was lower than vessel rings from normal or GDMi pregnancies, but higher in samples from GDMi (Table 3). The  $R_{max}$  in response to CGRP was similar than in response to insulin in normal pregnancies. The  $EC_{50}/R_{max}$  values for CGRP were higher in GDMd and GDMi compared with normal pregnancies, higher in GDMi compared with GDMd pregnancies. In addition, the  $EC_{50}/R_{max}$  values for CGRP were higher for vessel rings from all pregnancies.

## 4. Discussion

This study shows for the first time that HUVECs from pregnancies where the mother with GDMd was subjected to insulin therapy (GDMi) exhibit increased L-arginine transport and NO synthesis, which was comparable to cells from GDMd without insulin therapy or from normal pregnancies exposed to insulin *in vitro*. Cells from GDMi show increased p44/42<sup>mapk</sup> activation and IR-A mRNA expression, but unaltered Akt activation and IR-B expression. Insulin *in vitro* reversed GDMi effect on L-arginine transport, NO synthesis and p44/42<sup>mapk</sup> activation. NO and endothelium-dependent dilation of umbilical vein rings from GDMi was reduced in response to insulin compared with GDMd or normal pregnancies. Mothers and newborns from GDMi showed with normoglycaemia at term, but insulin therapy seems not enough to restore associated foetoplacental vascular dysfunction.

GDM associates with reduced foetoplacental vascular reactivity due to microvascular [26] and macrovascular [12,13,16] endothelial dysfunction [5,6]. HUVECs from women with GDMd (i.e., without insulin therapy) show increased L-arginine transport mediated by hCAT-1 [13] and NO synthesis due to eNOS activation [16] compared with normal pregnancies. Incubation of HUVECs from GDMd with insulin *in vitro* reversed the increased transport of L-arginine (half-maximal effective dose ( $ED_{50}$ )  $\sim 0.3$  nmol/L [27] and NO synthesis ( $ED_{50}$   $\sim 0.4$  nmol/L [12] seen in this condition. Our results show that HUVECs from GDMi pregnancies exhibit similar changes to those in GDMd pregnancies. Thus, insulin therapy does not restore GDMd-associated human umbilical vein endothelial dysfunction. L-Arginine transport is mainly mediated by hCAT-1 with an apparent  $K_m$   $\sim 100$ – $200$   $\mu$ mol/L in HUVECs from normal pregnancies [28–30], a value close to the concentration of this amino acid in the human umbilical vein blood ( $\sim 80$   $\mu$ mol/L) [31,32]. Thus, hCAT-1 plays a crucial role in the uptake of L-arginine in HUVECs. Saturable L-arginine transport in HUVECs from GDMi, and GDMd pregnancies shows apparent  $K_m$  within hCAT-1 range. Furthermore, hCAT-1 protein and mRNA expression were higher in HUVECs from GDMi and GDMd compared with normal pregnancies. Thus, hCAT-1 is likely involved in the removal of L-arginine from the extracellular medium, and GDMi or GDMd unaltered this membrane transporter's intrinsic properties. hCAT-2B, another member of hCATs family [14,15], is coexpressed with hCAT-1 in HUVECs [33] mediating L-arginine transport with  $K_m$   $\sim 300$ – $400$   $\mu$ mol/L [14,15]. Therefore, hCAT-2B may also mediate L-arginine transport in HUVECs from GDMi pregnancies. However, since Eadie-Hofstee plot of saturable transport and overall transport up to 250  $\mu$ mol/L L-arginine were lineal, it is likely that a single membrane transport system in the range of 100–200  $\mu$ mol/L (likely hCAT-1), or more than one transport system with  $K_m$  within this range, accounted for uptake of L-arginine into HUVECs. hCAT-1 involvement on GDMi and GDMd-increased transport is further supported by the results showing that hCAT-1 protein abundance and mRNA expression was higher ( $\sim 5$  fold) in cells from GDMi and GDMd compared with normal pregnancies. Thus, increased L-arginine transport may result from higher hCAT-1 availability in HUVECs. Since the  $V_{max}$  increase in GDMi and GDMd versus normal pregnancies was  $\sim 2.6$  and  $\sim 3.2$  fold, respectively, and because these conditions



**Fig. 3.** Involvement of p44/42<sup>mapk</sup> and Akt on L-arginine transport in HUVECs from GDMi pregnancies. **A.** Western blot for total (Total p44/42<sup>mapk</sup>) and phosphorylated (P-p44/42<sup>mapk</sup>) p44/42<sup>mapk</sup>, and total (Total Akt) and phosphorylated (P-Akt) Akt, and  $\beta$ -actin (internal reference) protein abundance in HUVECs from normal, gestational diabetes on diet (GDMd), and GDMd into insulin therapy (GDMi) without or with (8 h) exogenous insulin *in vitro*. **Lower panels:** P-p44/42<sup>mapk</sup>/total p44/42<sup>mapk</sup> or P-Akt/total Akt ratio densitometries normalized to 1 in Normal Without insulin. **B.** Insulin receptor A (IR-A) and B (IR-B) mRNA expression in HUVECs as in **A.** **C.** Saturable L-arginine transport measured at initial velocity adjusted to a single Michaelis-Menten equation in cells incubated in the presence of PD-98059 (10  $\mu$ mol/L), Akt Inhibitor IV (Inh-IV, 1  $\mu$ mol/L), or both, without or with insulin (1 nmol/L). Saturable L-arginine transport in cells in the absence of insulin and inhibitors (Controls, red dotted lines) or presence of insulin (Insulin, 1 nmol/L, blue dotted lines) from Fig. 1C and G are shown. \* $P < 0.05$  versus Normal Without insulin. † $P < 0.05$  versus corresponding values Without insulin. Values are mean  $\pm$  S.E.M. ( $n = 5$ ).

caused similar increase in hCAT-1 expression, a fraction not larger than 36–50% may be due to a change in hCAT-1 expression or availability at the plasma membrane in HUVECs from GDMi and GDMd.

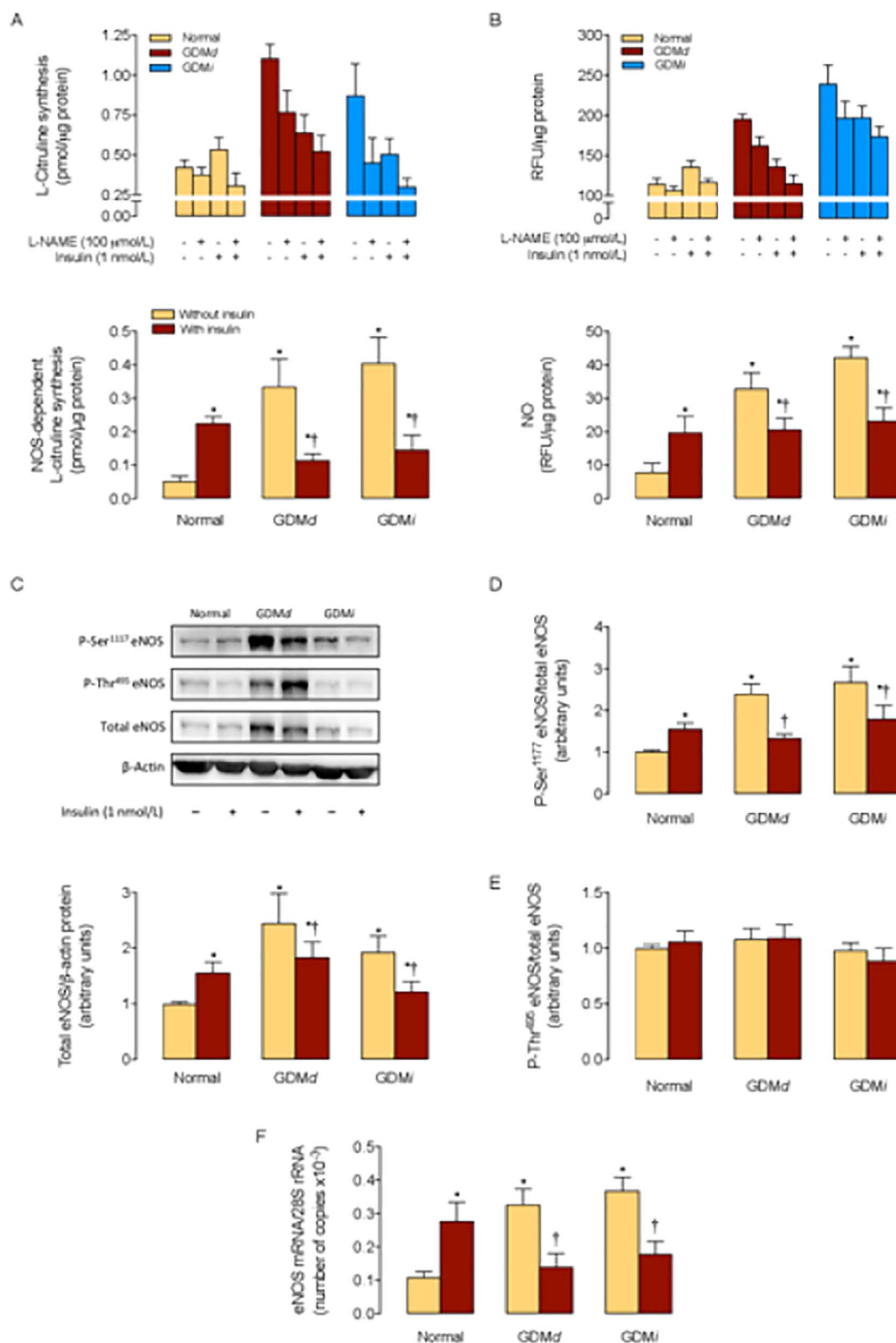
The  $V_{max}$  and  $V_{max}/K_m$  values in GDMi were similar to those in GDMd pregnancies, but higher compared with normal pregnancies. Thus, insulin therapy does not result in reversal of hCAT-1-mediated maximal transport capacity in HUVECs from GDM pregnancies. Since the increase in  $V_{max}/K_m$  was similar in GDMi and GDMd, but GDMi caused lower increase in  $V_{max}$  compared with GDMd ( $V_{max}^{GDMi}/V_{max}^{GDMd} \sim 0.7$ ), different component(s) to those in GDMd may modulate L-arginine transport in cells from GDMi pregnancies. Interestingly, overall L-arginine transport for values  $> 250 \mu$ mol/L showed higher  $K_D$  for GDMi compared with GDMd ( $\sim 1.43$  fold) and normal pregnancies ( $\sim 5.5$  fold). Thus, along with hCAT-1, L-arginine uptake seems mediated via a transport system(s) that does not get saturated up to 1000  $\mu$ mol/L in cells from GDMi and GDMd pregnancies. Since apparent  $K_m$  for hCAT-3, member of hCATs family [14,15], is  $> 500 \mu$ mol/L [34], these transporters may be involved in overall L-arginine transport playing a minor contribution at  $< 250 \mu$ mol/L L-arginine in HUVECs.

Phosphorylation of p44/42<sup>mapk</sup> was increased, but Akt phosphorylation was unaltered in cells from GDMi compared with normal pregnancies or in HUVECs from women with GDM not separated by diet only versus diet plus insulin therapy (GDMpool) [28]. Since GDMi and GDMd-increased L-arginine transport was reversed by inhibiting p44/42<sup>mapk</sup>, but unaltered by Akt inhibition, increased transport may result

from p44/42<sup>mapk</sup> activation. This is likely in cells from GDMi, but may only partially explain the GDMd increase in transport (increased p44/42<sup>mapk</sup> phosphorylation/increased transport  $\sim 0.53$ ). It is likely that increased IR-A expression accounts for GDMi effects since activation or overexpression of this type of insulin receptors results in preferential activation of p44/42<sup>mapk</sup> (i.e., p44/42<sup>mapk</sup> activation/Akt activation  $> 1$ ) in HUVECs [5,12,16]. All together these findings highlight potential differences on L-arginine transport modulation in HUVECs from these two conditions of pregnancy. Previous studies show increased eNOS activity in HUVECs from GDMpool [28,35] or GDMd [16] pregnancies compared with normal pregnancies. We extended these observations to GDMi pregnancies where higher NO level and NOS activity was found, a phenomenon likely due to higher activator Ser<sup>1177</sup> phosphorylation of eNOS [36,37]. Since eNOS protein abundance and mRNA expression were also increased in cells from these conditions, increased eNOS expression, reduced degradation or increased stability of eNOS protein or mRNA are alternative mechanisms that may be involved in this phenomenon.

Insulin restored the  $V_{max}/K_m$  of transport by reducing the  $V_{max}$  in cells from GDMi to values in normal pregnancies, confirming previous results in cells from GDMpool [28] or GDMd [13] pregnancies. Since the  $K_m$  was not changed by insulin, it is likely hCAT-1 affinity for L-arginine uptake is unaltered by this hormone in cells from GDMi pregnancies. However, since GDMi-increased  $K_D$  and  $v_i$  was reversed by insulin, this hormone may restore the functionality of an alternative mechanism, potentially hCAT-3, for L-arginine transport. Since insulin also restored





**Fig. 4.** Nitric oxide synthesis in HUVECs from GDMi pregnancies. **A.** L-citrulline synthesis was measured by h.p.l.c. (see Material and methods) in HUVECs from normal, gestational diabetes on diet (GDMd), and GDMd into insulin therapy (GDMi) incubated without or with exogenous insulin (1 nmol/L, 8 h) *in vitro* in the absence or presence of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L). **Lower panel:** L-citrulline synthesis via nitric oxide synthase (NOS) activity derived from data in A. **B.** Relative fluorescence units (RFU) were measured in cells preloaded with the fluorescent dye 4-amino-5-methylamino-2',7'-difluorescein (DAF-FM, 10 μmol/L) as in A. **Lower panel:** nitric oxide (NO) generation via NOS activity derived from data in B. **C.** Western blot for total eNOS (Total eNOS), eNOS phosphorylated at serine<sup>1177</sup> (P-Ser<sup>1177</sup>-eNOS) or threonine<sup>495</sup> (P-Thr<sup>495</sup>-eNOS) and β-actin (internal reference) protein abundance as in A. The graph shows Total eNOS/β-actin ratio densitometries normalized to 1 in Normal Without insulin. **D.** P-Ser<sup>1177</sup>-eNOS/total eNOS ratio densitometries normalized to 1 in Normal Without insulin from data in C. **E.** P-Thr<sup>495</sup>-eNOS/total eNOS ratio densitometries normalized to 1 in Normal Without insulin from data in C. **F.** eNOS mRNA expression as in A. \**P* < 0.05 versus Normal Without insulin. †*P* < 0.05 versus corresponding values Without insulin. Values are mean ± S.E.M. (*n* = 5).

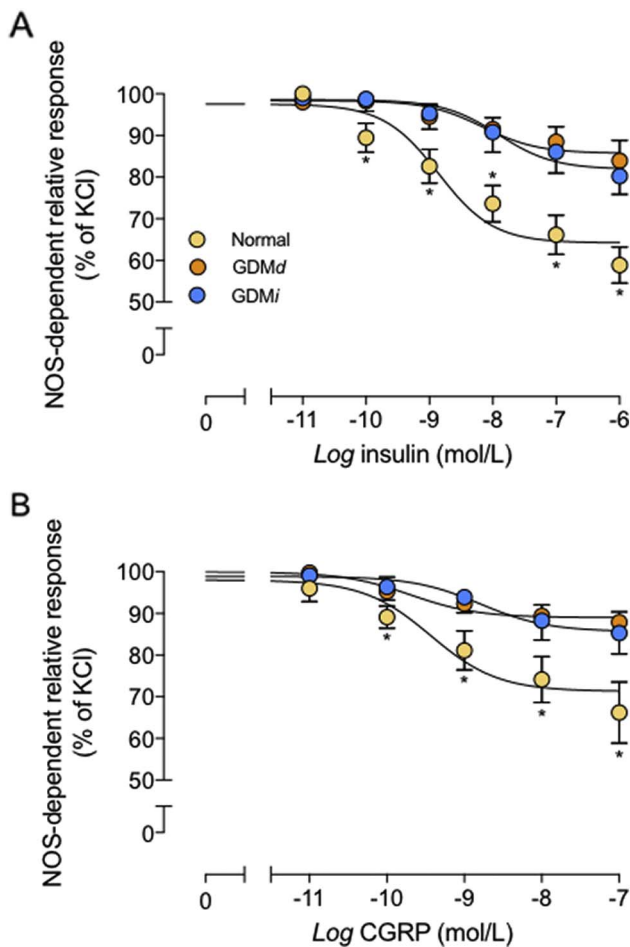


Fig. 5. Human umbilical vein dilation in GDMi pregnancies. A. Human umbilical vein rings isolated from normal, gestational diabetes on diet (GDMd), and GDMd into insulin therapy (GDMi) were incubated without (0) or with exogenous insulin in the absence or presence of *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L). B. Vein rings response to calcitonin gene related protein (CGRP) as in A. \**P* < 0.05 versus corresponding values in GDMi or GDMd. Values are mean ± S.E.M. (*n* = 3–6).

GDMi-increased hCAT-1 expression, this hormone may regulate hCAT-1 bioavailability in HUVECs from GDMi pregnancies. The contribution of a reduced hCAT-1 protein abundance to the lower  $V_{max}/K_m$  for transport caused by insulin in GDMi is partial since not more than two-thirds reduction in transport could be explained by changes in hCAT-1 protein abundance [(reduced  $V_{max}/K_m$ ) / (reduced hCAT-1) – 1 is ~0.65]. However, in GDMd the reduced  $V_{max}/K_m$  could be explained by similar reduction in hCAT-1 protein abundance. Insulin-reduced hCAT-1 mRNA expression (~60%) was similar to this transporter's protein abundance in GDMi pregnancies. Since insulin-restoration of GDMd-increased hCAT-1 mediated transport in HUVECs resulted from lower protein abundance due to lower transcription of *SLC7A1* gene (for hCAT-1) [13], insulin restoration of GDMi-increased hCAT-1 mediated transport may result from a similar phenomenon.

Inhibition of p44/42<sup>mapk</sup> restores GDMi- and GDMd-increased L-arginine transport in the same proportion as insulin. Thus, p44/42<sup>mapk</sup> over-activation could be responsible for these pathological conditions's associated increased transport, complementing similar results in cells from normal pregnancies in response to insulin [28,33]. Insulin also activates insulin receptor A (IR-A) that mediates preferential activation of p44/42<sup>mapk</sup> compared with Akt (p44/42<sup>mapk</sup>/Akt > 1), and insulin receptor B (IR-B) mediating preferential Akt activation compared with p44/42<sup>mapk</sup> (p44/42<sup>mapk</sup>/Akt < 1) in HUVECs [16]. Since HUVECs from GDMi in the absence of exogenous insulin show p44/42<sup>mapk</sup>/Akt ~1.7, a value close to the increase in  $V_{max}/K_m$ , IR-A signalling is likely

Table 3  
Vasodilation parameters in human umbilical vein rings.

	Normal ( <i>n</i> = 13)	GDMd ( <i>n</i> = 14)	GDMi ( <i>n</i> = 18)
<b>Insulin</b>			
$EC_{50}$ (nmol/L)	1.39 ± 0.05	6.95 ± 0.31*	12.3 ± 0.71***
$R_{max}$ (%)	38 ± 6	14 ± 2*	18 ± 3*
$EC_{50}/R_{max}$ (a.u.)	0.037 ± 0.005	0.496 ± 0.005*	0.682 ± 0.007***
<b>CGRP</b>			
$EC_{50}$ (nmol/L)	0.37 ± 0.02***	0.20 ± 0.03****	1.62 ± 0.31*****
$R_{max}$ (%)	29 ± 5	12 ± 4*	14 ± 3*
$EC_{50}/R_{max}$ (a.u.)	0.013 ± 0.001***	0.017 ± 0.003****	0.121 ± 0.002*****

Human umbilical vein rings were obtained from cords from normal pregnancies (Normal), GDM under treatment with diet (GDMd) or GDMd passed onto insulin therapy (GDMi). Vein rings were preconstricted with 65 mmol/L KCl and incubated with increasing concentrations of insulin (0.01–1000 nmol/L) or calcitonin gene related protein (CGRP) (0.01–100 nmol/L) in the absence or presence of *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L). Half-maximal effective concentration ( $EC_{50}$ ) and maximal relaxation ( $R_{max}$ ) were calculated for the difference in insulin and CGRP response in the absence or presence of L-NAME (i.e., nitric oxide synthase (NOS)-dependent relative response).

Values are mean ± S.E.M.

a.u., arbitrary units.

\* *P* < 0.05 versus corresponding values in Normal.

\*\* *P* < 0.05 versus corresponding values in GDMd.

\*\*\* *P* < 0.05 versus corresponding values in the presence of *Insulin*.

increased in GDMi. Indeed, IR-A, but not IR-B mRNA expression is higher in cells from GDMi compared with normal pregnancies, and is similar to the increase seen in GDMd, as reported [12,16]. Since insulin restored p44/42<sup>mapk</sup>/Akt in cells from GDMi, and GDMi-increased IR-A mRNA is reversed to values in normal pregnancies, insulin beneficial effect on L-arginine transport, NO synthesis and eNOS expression and activation in HUVECs from GDMi was likely due to restoring IR-A expression and its associated cell signalling. A likely functional consequence of the described alterations caused by GDM at a cellular level, is a lower response of umbilical vessels to insulin. The results show that required insulin to generate a NO-dependent dilation of these vessels [( $^{Normal}EC_{50}/R_{max}$ ) / ( $^{GDMi}EC_{50}/R_{max}$ )] was higher in GDMi (~18 fold) and GDMd (~13 fold), compared with vessel rings from normal pregnancies. This phenomenon could result from a lack of endothelial function since the response of vessels to CGRP was also reduced in GDMi (~10 fold). However, GDMi would be a stronger condition than GDMd generating endothelial dysfunction since CGRP response to insulin was ~7 fold higher than in GDMd.

In conclusion, HUVECs from GDMi pregnancies show increased L-arginine transport and NO synthesis, and reduced vascular reactivity compared with normal pregnancies. These findings are comparable to those in GDMd, suggesting that insulin therapy in pregnancy does not resolve the foetoplacental endothelial dysfunction associated with GDM pregnancies. However, since we used primary cultures of HUVECs and umbilical vein rings *in vitro*, our conclusions should not be taken as categorical. Insulin therapy normalizes maternal and foetal glycaemia [9,18,19], but nothing is reported regarding the potential effect of this intervention on foetus wellbeing [3,5]. Insulin therapy associates with higher frequency of complications in neonates [38], including larger chance to be born large for gestational age [39], larger number (~25%) of infants with one or more episodes with neonatal morbidity (neonatal asymptomatic hypoglycaemia) [40], and polyhydramnios [41], compared with infants born to mothers with GDMd. Since maternal insulin therapy effect in foetus development, newborn, and postnatal alterations are still unclear [3,5,9,42], maternal and neonatal complications are comparable in GDMi and GDMd [19,39], and insulin therapy seems not to revert GDM-associated foetoplacental endothelial dysfunction,

subjecting pregnant women with GDM to insulin therapy should be taken with caution since the risk of foetal health alterations.

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### Disclosures

None.

### Transparency document

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