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Changes in Placental Adipocytokine Gene Expression Associated with Gestational Diabetes Mellitus

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Summary

Leptin and adiponectin, two adipocytokines, may work together in regulating energy homeostasis and insulin action. Leptin gene expression has been investigated in term placental tissue complicated by gestational diabetes mellitus (GDM), but never in conjunction with all isoforms of the leptin receptor (LEPR A-D), or with adiponectin receptors (ADIPOR1 and 2). In this study we examined the association between changes in expression of these genes in placental tissue and GDM risk. We assessed placental gene expression of leptin, LEPR A-D and ADIPOR1 and 2 by real time PCR using mRNA from maternal and fetal biopsies. Tissues were collected from uncomplicated pregnancies (n=28) and those complicated by GDM (n=19). Gene expression was normalized to three endogenous housekeeping genes. Relative gene expression values were reported as fold change between groups. Adiponectin gene expression was out of the sensitive range of our assay. There were increases in leptin mRNA expression in GDM cases compared with controls for maternal-side ($p=0.06$), and fetal-side ($p=0.09$) placental biopsies. No significant changes were seen in GDM cases compared with controls in LEPR A-D or ADIPOR1 and 2. mRNA derived from maternal-side tissue was positively correlated with tissue from the fetal side for all genes studied (all $p<0.01$). Finally, we noted that absence or presence of GDM was a major factor in leptin mRNA expression after adjusting for maternal age, mode of delivery, parity and smoking status. In conclusion, increases in leptin mRNA expression in term placenta, but not that of its receptors, are associated with the diagnosis of GDM. Changes seen in the ligand, but not the receptor, of the leptin pathway in GDM-complicated pregnancies may also apply to the adiponectin pathway, as the ADIPOR1 and 2 mRNAs do not change with GDM diagnosis.

Key words
Gestational diabetes mellitus (GDM) • Leptin • Adiponectin • Placenta • Gene expression

Introduction

Adipocytokines are peptide signals secreted by adipose tissue and required in a number of physiological and metabolic processes (Trayhurn and Wood 2004). Two notable adipocytokines, leptin and adiponectin, are

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involved in regulating energy homeostasis and insulin action (Havel 2002) and may function together in this capacity (Yamauchi *et al*. 2001). Leptin inhibits food intake, reduces body weight and regulates energy balance by influencing energy expenditure (Havel 2000). Adiponectin also reduces body weight and fat as well as stimulates thermogenesis (Jackson *et al*. 2005). Both proteins are also thought to play a role in other physiological processes such as pregnancy. Serum leptin concentrations are highly increased during the second and third trimesters of pregnancy (Margetic *et al*. 2002), whereas adiponectin concentrations in the blood are suppressed from mid-gestation through lactation in mice (Combs *et al*. 2003).

Leptin and its receptors were originally discovered in mice as mutations in the *ob* and *db* genes, respectively (Margetic *et al*. 2002). In humans there are four isoforms of the receptor due to alternative splicing during transcription (Li *et al.* 2004), LEPR A-D (see methods section for alternate names). The long form of the protein, LEPR B signals through a JAK/STAT pathway, which is thought to be the pathway associated with the role of leptin in energy balance and hormone concentrations in the brain (Jackson *et al.* 2005). In the placental cells, leptin signaling is thought to go through the MAPK cascade, by the short isoforms of the receptor (Cauzac *et al.* 2003). Expression of leptin and all four transcriptional isoforms of the receptor are found in the placenta (Li *et al.* 2004). Adiponectin mRNA is expressed almost exclusively in adipose tissue, but high concentrations of protein can be found in the blood (Jackson *et al.* 2005). The adiponectin receptors, ADIPOR 1 and 2, are expressed in multiple tissues including muscle and liver, and likely signal through phosphorylation of the protein kinase AMPK, among other pathways (Yamauchi *et al.* 2003).

Leptin has been implicated in diabetes associated with obesity, but results have been inconsistent, perhaps due to a complex relationship between the central and peripheral functions of the hormone (Ceddia *et al.* 2002). In animal models, diabetic indicators can be decreased in leptin-treated obese mice (Pelleymounter *et al*. 1995). Adiponectin is decreased in obese and type 2 diabetic humans and its administration in mice can increase insulin sensitivity and glucose tolerance (Wolf 2003). Mice with depleted white adipose tissue can be remedied for insulin resistance by treating with adiponectin and leptin, but each hormone alone was only moderately beneficial (Yamauchi *et al*. 2001).

In humans with pregnancies complicated by gestational diabetes mellitus (GDM), both leptin and adiponectin concentration in the maternal circulation is dysregulated compared to non-diabetic pregnancies. Conflicting reports show an increase in relative maternal leptin protein concentrations in the third trimester (Kautzky-Willer *et al.* 2001, Cseh *et al.* 2002, Radaelli *et al.* 2003) and during early pregnancy (Qiu *et al*. 2004) while others show a decrease during late pregnancy (Festa *et al.* 1999). mRNA of leptin and one of the short isoforms of the leptin receptor been shown to be overexpressed in the placentas of women with GDM, increasing by 2.3 and 4.3 fold, respectively (Radaelli *et al*. 2003). Finally, reduced adiponectin protein concentrations in maternal plasma were predictive of a 4.6-fold increased risk of GDM (Williams *et al*. 2004).

In this study we sought to determine changes in mRNA relative expression of leptin, leptin receptors and adiponectin receptors in pregnancies complicated with GDM compared with controls. To verify that there was no influence due to site of selection, both the maternal and fetal sides of the placenta were separately studied. Finally, we evaluated the correlation of relative expression values of these genes with each other and with maternal and fetal characteristics including the pre-pregnancy body mass index and infant birth weight.

Methods

Study population and placenta collection

The procedures used in this study followed the protocol approved by the Institutional Review Board of Swedish Medical Center and all participants provided written informed consent. Forty-seven placentas from 28 normoglycemic and 19 diabetic women were collected at the end of pregnancy. The 19 GDM cases included 10 patients who were diet controlled and 9 patients who required insulin therapy. All 47 patients had a 50-gram 1-hour glucose tolerance screening test, followed by a 100-gram 3-hour oral glucose tolerance diagnostic test among those who had a positive screening test result.

GDM was defined as two or more of the four plasma glucose concentrations exceeding ADA criteria (Report of the Expert Committee 1997): fasting glucose greater than 5.3 mmol/l, 1-hour post challenge greater than 10.0 mmol/l, 2-hour post challenge greater than 8.6 mmol/l, 3-hour post challenge greater than 7.8 mmol/l.

The placental tissue was collected immediately after delivery. The chorionic plate, including overlying

membranes, was removed. Tissue biopsies were taken from the fetal side, which consisted of the intervillous tissues and chorionic villi, and from the maternal side, which consisted mainly of fetal villous tissue, but also contains tissue of maternal origin in the deciduas basalis (basal plate) (Benirschke and Kaufmann 2000). Our long-term goal is to separate out cells of maternal and fetal origin to improve the test whether gene expression is based on tissue origin. However, in this pilot test we have examined whether there are any gene expression changes based on general biopsy selection. During sampling, each placenta was laid flat with the fetal side facing up and mapped based on a grid developed for this study into four quadrants, with two samples coming from each quadrant, one medial (about 2 cm from the center) and one lateral (about 2 cm from the margin). Four biopsies were used in this study from each side of the placenta, the two biopsies from the upper left hand quadrant and two from the lower right hand quadrant. The placenta was then turned over and four corresponding samples are taken from the maternal side. Biopsies of approximately 0.5 cm^3 were taken, placed in cryotubes and stored in RNAlater (Qiagen Inc., Valencia, CA) at 10 µl per 1 mg of tissue at –80 °C.

Clinical data collection

Information on maternal socio-demographic, medical and reproductive characteristics, labor and delivery characteristics, as well as anthropometric measurements (maternal height and pre-pregnancy weight) was abstracted from medical records. Gestational age was based on the last menstrual period and, when possible, was confirmed by ultrasound examination conducted prior to 20 weeks gestation. Pre-pregnancy body mass index (BMI), used as a measure of adiposity, was calculated as weight (kg) divided by square of height (m^2).

RNA extraction

From each of the four stored biopsies used to represent a placental side, 60 mg of tissue were cut, weighed, and pooled, totaling 240 mg per sample. The weighed samples were homogenized using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). Total RNA was extracted by using the RNeasy Fibrous Midi Kit (Qiagen Inc.), following the standard protocol including DNase. Total RNA was eluted from the columns in 300 µl of sterile water. Total RNA concentration was calculated by determining absorbance at 260 nm (Spectramax Plus 384 spectrophotometer, Molecular Devices, Sunnyvale, CA). Protein contamination was monitored by A260/A280 ratio. All samples ranged in concentration from 0.31 µg/µl to 1.30 µg/µl and all had an A260/A280 ratio of > 1.8. All samples were diluted to 0.25 µg/µl in sterile water and aliquoted for storage at –80 °C.

Reverse transcription and real time pcr

Leptin, four leptin receptor and two adiponectin receptor genes were tested in this study. We have previously analyzed placental samples for the most stable control genes in the placenta (Meller *et al.* 2005). We have shown that normalizing genes of interest to the geometric mean of succinate dehydrogenase complex, subunit A (SDHA), TATA box binding protein (TBP) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) allows for the best control of RNA loading in the placenta. First strand cDNA was synthesized by using the High Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's instructions. Amounts of 2.5 µg or less of total RNA were run in 25 µl reverse transcription reactions and the resulting cDNA was diluted in sterile water to 2.5 ng/µl. Real time PCR was performed in duplicate on 25 µl mixtures, containing 150 ng of template cDNA, 12.5 µl of 2X Taqman Universal Master Mix (Applied Biosystems), and 1.25 µl of Taqman Assay on Demand (Applied Biosystems), or 565 nM primer and 315 nM probe for leptin receptor genes. The following Taqman Gene Expression Assays were purchased from Applied Biosystems (Foster City, CA): leptin (Cat. # Hs00174877), ADIOPR1 (Cat. # Hs00360422), ADIPOR2 (Cat. # Hs00226105), SDHA (Cat # Hs00188166), TBP (Cat. # Hs99999910) and YWHAZ (Cat. # Hs00237047). The sequences of leptin receptor primers (Applied Biosystems) were:

5'-ATCACACCAAAGAATGAAAAAGCTATT-3'
(forward primer, common to all leptin receptor A-D isoforms),

5'-TGATTAGACTTCAAAGAATGTCCGTT-3'
(reverse primer specific to leptin receptor A (LEPR A), also called 6.4 and HuB219.3),

5'-TGTATGCTTGATAAAAAGATGCTCAA-3'
(reverse primer specific to leptin receptor B (LEPR B), also called the long isoform, 13.2' OB- and "long isoform"),

5'-GAGATTAGGGAGTGGTGATGACTCTTA-3'
(reverse primer specific to leptin receptor C (LEPR C),

also called 12.1 and HuB219.1),
5'-AGTTCCTTTGTGCCAGGCAT-3' (reverse primer specific to leptin receptor D (LEPR D), also called HuB219.2),
5'-TCCTCTTCCATCTTATTGCTTGG-3' (forward primer LEPR ALL which amplifies a sequence common to all four transciptional isoforms of the leptin receptor),
5'-TGCCCAGGAACAATTCTTGG-3' (reverse primer LEPR ALL which amplifies a sequence common to all four transciptional isoforms of the leptin receptor) and of the probes (Applied Biosystems):
6FAM–AAGATGTTCCGAACCC – MGBNFQ (used in all LEPR A-D assays) and
6FAM–TATTAATATCACACCAAAGAAT–MGBNFQ (used in LEPR ALL assays). A plate control calibrator of Human Universal total RNA (Clontech/Takara Bio, Palo Alto, CA) was run for every Taqman Gene Expression Assay or primer/probe set on every real-time PCR plate.

Reactions were run on an ABI PRISM 7000 Real Time PCR machine (Applied Biosystems) using the default cycling conditions of 50 °C, 2 min, 95 °C, 10 min, followed by 40 cycles of 95 °C, 15 s and 60 °C, 1 min. Four point standard curves of normal placental RNA were used for primer efficiency comparison of all Assays on Demand and primer/probe set based on the slope of each standard curve calculated by the ABI PRISM 7000 SDS Software, Version 1.1.

Results from the ABI PRISM 7000 SDS Software using the Absolute Quantitation method were exported into Excel (Microsoft Corporation, Redmond, WA) for data analysis. Threshold cycle (Ct) values of the duplicates differing by a >0.5 standard deviation were re-tested. Ct value duplicates differing by $\leq$0.5 standard deviations are averaged for analysis. The relative expression was determined based on previously reported methods (Pfaffl 2001, Vandespompele *et al*. 2002). Raw quantities were corrected by normalizing to the geometric mean of SDHA, TBP and YWHAZ genes of the same sample.

Statistical analysis

Our analysis included 28 normoglycemic and 19 diabetic women, the 19 GDM cases included 10 patients who were diet-controlled and 9 patients who required insulin therapy. However, when we separated these GDM case groups, the distribution of log-transferred leptin on the maternal or fetal side showed no significant difference compared to when they were grouped together, and the analysis was underpowered. The distribution of maternal socio-demographic, medical and clinical characteristics according to case-control status was examined. The distribution of continuous variables (e.g., body mass index and infant birth weight) were checked and found to be approximately normal, hence parametric statistical analytical procedures were used when assessing these variables. Relative expression values for leptin were found to be non-normally distributed. Hence, we used non-parametric statistical procedures when analyzing these data. Additionally, we transformed the data for leptin gene expression (using the log transformation) and repeated relevant analyses using parametric procedures. Normally distributed continuous variables are presented as mean ± standard error (S.E.M.). Unadjusted mean differences for each variable according to case and control status were assessed using Students' *t*-test statistics. Non-normally distributed data (e.g. gene expression values) were presented as median [inter-quartile range]. Unadjusted differences in medians for cases and controls were assessed using the Wilcoxon Rank-Sum test statistics. Comparisons of categorical variables were made between case and controls using chi-squared or Fisher's exact tests. The Spearman's correlation coefficient was used to measure the closeness of a liner relationship between relative gene expression values derived from tissue sampled from maternal and fetal sides of the placenta. Multivariate linear regression analyses were conducted in order to examine the independent relationship between placental leptin gene expression and GDM while controlling for potential confounding covariates such as maternal age, parity, smoking, mode of delivery, maternal pre-pregnancy BMI and fetal birth weight. To assess the confounding, we entered variables into a linear regression model one at a time and then compared coefficients. Final linear regression models included covariates that altered unadjusted coefficients by at least 10 %, as well as those covariates of *a priori* interest (e.g. maternal age). Based on these results, infant birth weight was not in the final model on either placental side.

Results

Characteristics of GDM cases and controls are summarized in Table 1. GDM cases and controls were similar with regards to maternal age, parity, pre-pregnancy adiposity, gestational age, and mode of delivery. Cases were more likely than controls to be non-Hispanic White, to be unmarried and to report smoking

Table 1. Socio-demographic characteristics of GDM cases and control, Seattle, Washington State, February 2003 – April 2004.

Characteristics	GDM Cases (n=19)		Controls (n=28)		p-value
	n	%	n	%	
Maternal age at delivery (years)					
< 35	12	63.2	16	57.1	0.68
≥ 35	7	36.8	12	42.9	
Maternal race/ethnicity					
Non-Hispanic White	9	47.4	6	21.4	0.06
Other	9	47.4	22	78.6	
Missing	1	5.2	0	0	
Single marital status	8	42.1	2	7.1	<0.01
Nulliparous	8	42.1	7	25.0	0.22
Smoked during pregnancy	5	26.3	2	7.1	0.01
Pre-pregnancy BMI (kg/m^2)	28.5±1.8		25.7±1.3		0.21
Gestational age at delivery (wks)	37.7±0.7		38.0±0.6		0.70
Infant birth weight (grams)	3484.2±240.7		3539.0±89.3		0.81
Mode of delivery					
Vaginal	7	36.8	13	46.4	0.62
C-section	12	63.2	15	53.6	

during pregnancy. The expression profiles of leptin, leptin receptors, and adiponectin receptors are summarized in Table 2. Separate analyses were performed for expression values derived from tissues dissected from maternal and fetal sides of the placenta, respectively. There was no evidence of case-control differences or maternal-fetal side difference in the expression of any of the leptin and adiponectin receptor isoforms (p values all >0.05). However, the levels of leptin mRNA expression in GDM cases were higher with marginal statistical significance as compared with controls when tissues from the maternal side of the placenta (305.10 vs. 116.8 relative units, for cases and controls, p=0.06) were evaluated. Similar differences were observed when leptin gene expression was determined from tissues dissected from the fetal side of the placental (805.80 vs. 455.21 relative units for cases vs. controls, p=0.09), however the difference did not reach statistical significance. Results were essentially the same when we transformed the expression values and repeated the comparisons (Table 2). The data for relative leptin gene expression for GDM cases and controls are also summarized in Figure 1. Similar case-control differences were seen regardless of the site of tissues collection (e.g., maternal versus fetal sides of the placenta).

We next examined the association between leptin, leptin receptors and adiponectin receptor gene expression values according to site of tissue collection. Analyses were stratified by case and control status. Among GDM cases, leptin mRNA values derived from the maternal side was strongly positively correlated with values derived from tissue dissected from the fetal side of the placenta (r=0.94, p<0.001) (Fig. 2). Correlations for the other genes ranged from 0.60 to 0.83 (all p<0.01) indicating strong agreement in gene expression regardless of site of tissue sampling. Notably, correlation coefficients estimated for controls were all statistically significant (p<0.01), though correlation coefficients were somewhat lower than those observed among cases. Among controls, leptin mRNA values derived from the maternal side was positively correlated with values derived from tissue dissected from the fetal side of the placenta (r=0.70, p<0.001)

We next sought to further explore the association between leptin gene expression and GDM after

Table 2. Comparison of mRNA expression of adiponectin receptors, leptin and leptin receptors according to GDM case and control status, Seattle, Washington, February 2003 - April 2004.

Measurement	GDM Cases (n=19)		Controls (n=28)		Rank-Sum Test
	Median	IQR	Median	IQR	p-value
MATERNAL SIDE					
ADIPOR1	1.81	1.67-1.96	1.85	1.73-2.04	0.96
ADIPOR2	0.79	0.73-0.94	0.83	0.76-0.88	0.65
Leptin	305.10	165.5-1286.01	116.8	61.1-636.6	0.06
Log-transformed leptin	5.72	5.11-7.16	4.76	4.11-6.46	0.06
LEPRA	0.89	0.63-1.08	0.94	0.80-1.02	0.50
LEPRB	0.40	0.33-0.59	0.44	0.36-0.52	0.75
LEPRC	1.60	1.20-1.71	1.52	1.36-1.67	0.94
LEPRD	1.23	1.05-1.64	1.22	1.05-1.51	0.90
LEPR ALL	0.74	0.58-0.98	0.79	0.65-0.87	0.68
FETAL SIDE					
ADIPOR1	1.81	1.63-2.07	1.90	1.75-2.02	0.83
ADIPOR2	0.75	0.69-0.86	0.77	0.70-0.85	0.99
Leptin	805.80	323.9-2206.0	455.21	128.3-717.1	0.09
Log-transformed leptin	6.69	5.78-7.70	6.11	4.85-6.58	0.09
LEPRA	0.93	0.86-0.99	0.90	0.76-1.02	0.95
LEPRB	0.48	0.38-0.55	0.41	0.37-0.56	0.86
LEPRC	1.56	1.30-1.66	1.52	1.33-1.70	0.98
LEPRD	1.26	1.08-1.57	1.41	1.16-1.78	0.24
LEPR ALL	0.84	0.66-1.02	0.77	0.66-0.93	0.63

controlling for potential confounding factors. Estimated coefficients and p-values for covariates included in the final regression model are presented in Table 3A. This table shows that the presence or absence of GDM was a major determinant of leptin gene expression (β-coefficient = 1.12, p=0.008) on the maternal side of the placenta after adjusting for maternal age, mode of delivery, parity, and smoking status. When the estimated coefficient was back-transformed, we noted that GDM diagnosis was associated with a 3.1-fold increase in placental leptin gene expression (p=0.008). Table 3B shows the results when expression values were derived from tissues dissected from the fetal side of the placenta (β-coefficient = 0.64, p=0.08). After back-transformation of mRNA expression values, we observed a 1.9-fold increase in placental leptin gene expression associated with GDM diagnosis. Hence, regardless of site of placental tissue collection, we noted an almost 2-3 fold increase in leptin gene expression in placentas delivered from mothers with GDM as compared with the controls.

Discussion

In this study, we observed an association between GDM diagnosis and leptin mRNA expression in placental tissues. After adjusting for maternal age, mode of delivery, parity and smoking status, a diagnosis of GDM was the major determinant of placental leptin gene expression. The association reached statistical significance in tissue sampled from the maternal side, but not the fetal side. The relationship between GDM and alterations in mRNA expression for leptin receptors or adiponectin receptors, however, was not evident.

Our results are generally consistent with published studies that assessed leptin mRNA expression in term placentas from women with pregnancies complicated by diabetes. Microarray analysis (Radaelli *et al.* 2003) and *in situ* hybridization (Lea *et al*. 2000) of placental mRNA from GDM and control women shows an increase in leptin expression compared to the controls. Additionally, women with pregnancies complicated by

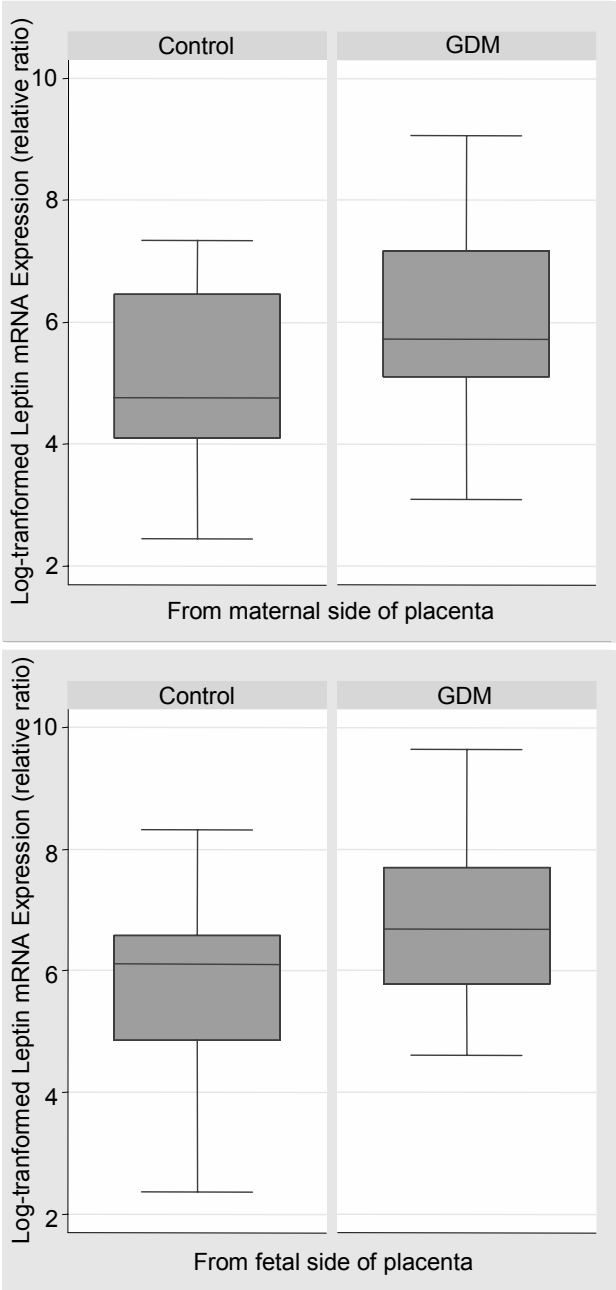

Fig. 1. Box plot of log-transformed leptin mRNA relative expression in 19 women having GDM and 28 women who remained normoglycemic throughout pregnancy. The p values were 0.06 (maternal side) and 0.09 (fetal side) when comparing the leptin mRNA expression between cases and controls from Wilcoxon Rank Sum test.

insulin-dependent diabetes mellitus (IDDM) also show increases in leptin mRNA expression by Northern blot analysis (Lepercq *et al.* 1998) and by *in situ* hybridization (Lea *et al.* 2000) compared with controls. Our results differ with published results on one of the short forms of the leptin receptor, which showed an increase in gene expression by both microarray and real time PCR analysis in GDM placentas compared to controls (Radaelli *et al.* 2003), whereas we saw no large differences in gene expression for any of the leptin receptors (Table 2). Differences in housekeeping genes used for control of gene expression and laboratory method used for mRNA quantification may account for the disparity seen between these studies. Additionally, severity of GDM may account for variations seen in the samples, as well as the method of glycemic control after GDM diagnosis. Interestingly, expression of leptin receptor protein for all isoforms was similar for diabetic and control placentas at term by immunohistochemical analysis (Lea *et al*. 2000). These data are consistent with our mRNA results. Finally, our analysis of gene expression comparing maternal and fetal compartment samples (Fig. 2) mostly corresponds with existing reports. Leptin mRNA and protein has been shown to be expressed in villous vascular endothelial cells and syncytiotrophoblasts (Lea *et al.* 2000), while leptin receptor protein (all isoforms) is expressed only in syncytiotrophoblasts (Lea *et al*. 2000, Challier *et al*. 2003). Although all of these cells are of fetal origin, syncytiotrophoblasts are in contact with the maternal circulation (Junqueira *et al*. 1998) and may have easily been included in our maternal side biopsies, in addition to the fetal, thus showing similar gene expression regardless of site of sampling.

Our study has a number of important advantages. Namely, we analyzed a relatively large sample population (controls, n=28, GDM, n=19) compared to previous analyses of leptin or leptin receptor mRNA in diabetic placentas (Lepercq *et al*. 1998, Lea *et al*. 2000, Radaelli *et al*. 2003). The size of our population allowed us to perform multivariate analysis, from which we can account for confounding factors and show that changes in leptin gene expression are determined by the absence or presence of GDM. This is also the first study of which we are aware that compares adiponectin receptor gene expression in GDM placentas to controls. Although previous studies have shown adiponectin receptor expression in the placenta (Caminos *et al*. 2005), no studies have compared gene expression levels in complicated pregnancies to those in uncomplicated pregnancies. Additionally, these real time PCR data were normalized to the geometric mean of three housekeeping genes identified as highly stable in term placental tissue (Meller *et al*. 2005), which has been substantiated as a more satisfactory normalization factor than a single endogenous control gene alone (Vandesompele *et al*. 2002).

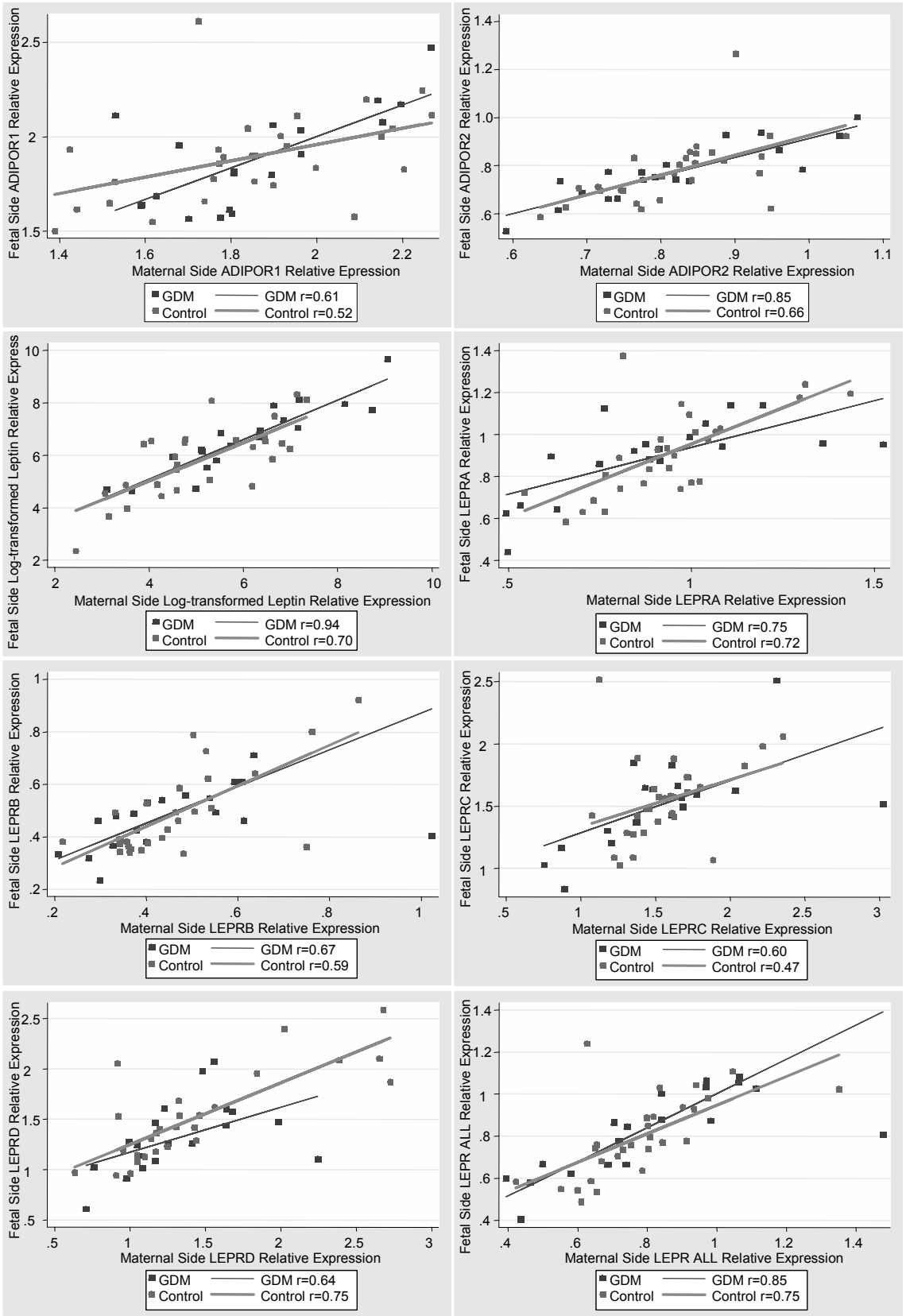

Fig. 2. Scatterplots and linear regression lines show the mRNA expression levels for adiponectin receptors, leptin and leptin receptors. Expression values are from tissues dissected from maternal or fetal sides of placenta. Values are reported according to GDM case-control status.

Table 3A. Relationship between leptin mRNA expression (tissue dissected from the maternal side of the placenta) and GDM after adjusting for confounders. Seattle, Washington, February 2003 - April 2004.

Parameter	Coefficient	P-value
GDM	1.12	0.008
Maternal age (years)	0.08	0.03
Mode of delivery (c-section/vaginal)	-1.22	0.003
Parity (nullipara/multipara)	-0.74	0.14
Smoked during pregnancy (yes/no)	-0.97	0.14
Constant	3.63	0.005

Leptin mRNA values expressed as relative units were log-transformed. 39 % (adjusted R^2 = 39 %) of the total variance in leptin gene expression was explained by this linear model.

Table 3B. Relationship between leptin mRNA expression (tissue dissected from the fetal side of the placenta) and GDM after adjusting for confounders. Seattle, Washington, February 2003 - April 2004.

Parameter	Coefficient	P-value
GDM	0.64	0.08
Maternal age (years)	0.08	0.08
Mode of delivery (c-section/vaginal)	-1.20	0.002
Parity (nullipara/multipara)	-0.85	0.06
Pre-pregnancy overweight status	-0.34	0.38
Constant	4.56	0.004

Leptin mRNA values expressed as relative units were log-transformed. 35 % (adjusted R^2 = 35 %) of the total variance in leptin gene expression was explained by this linear model.

However, some limitations also merit discussion. First, this cross-sectional study is unable to clearly elucidate whether increased leptin mRNA expression functions in GDM pathogenesis or is itself induced by disease-related metabolic changes. Second, we chose not to analyze adiponectin mRNA in the placenta due to low expression level. Our initial tests showed levels of adiponectin mRNA that were below the sensitive range of our assay. Published reports vary on adiponectin mRNA expression in the human placenta. By reverse transcription PCR, no adiponectin mRNA was detected in 25-week placental tissues and no adiponectin protein was found by immunohistochemistry in placental cells obtained from samples of gestational age ranging from 16-38 weeks (Corbetta *et al*. 2005). However, others saw expression of adiponectin mRNA in term placentas by reverse transcription PCR (Caminos *et al.* 2005), although at low levels of detection compared to the endogenous control. Comparison of gene expression in GDM versus control would be meaningless if detection was just at the level of detection for the assay. However, if advances in mRNA detection technology allow for a greater range of sensitivity, adiponectin studies comparing GDM to controls should be re-visited in this tissue. The final limitation of this study is the use of biopsies to represent maternal and fetal tissues in term placentas. During pregnancy cytotrophoblast cells, which invade from the fetal tissue, form anchoring villi and anchor into the maternal decidua basalis (Junqueira *et al.* 1998). Therefore samples taken from the maternal side in this study, although consisting physically of some maternal cells, are not exclusively comprised of these cell types. Future studies should include microdissection of cells from maternal and fetal origin for separate analysis to show conclusively whether there is any correlation in gene expression between these two compartments.

The correlation detected between increased leptin gene expression in the placenta and increased risk of GDM is biologically plausible. Previous studies of leptin protein show that it is released into both the

maternal and fetal circulation from the placenta (Masuzaki *et al*. 1997, Hoggard *et al*. 2001), with higher concentrations detected in the maternal circulation (Yura *et al*. 1998, Linnemann *et al*. 2000). Therefore, leptin protein produced in the placenta might be responsible for the increased risk of GDM. Several studies suggest that leptin protein in circulation may increase glucose uptake both *in vivo* (Kamohara *et al.* 1997, Wang *et al.* 1999) and *in vitro* (Ceddia *et al*. 1998, Harris 1998, Ceddia *et al.* 1999) in skeletal muscle, an important target for glucose-mediated uptake and metabolism, as well as other peripheral tissues (Ceddia *et al.* 2002). It is also plausible that GDM risk factors may regulate leptin gene expression. Glucose uptake, measured by decrease in glucose in the media of rat adipocyte cultures, stimulates leptin protein production in these cells, independent of absolute insulin concentration (Mueller *et al*. 1998) and likely involves the metabolism of glucose to oxidation or lipogenesis (Mueller *et al*. 2000).

In conclusion, we have shown that women with GDM are more likely to have higher leptin mRNA expression in maternal-side placental tissue than normal controls. In addition, all leptin and adiponectin receptor isoforms had similar gene expression in cases and controls. Taken together our results suggest that GDM may be associated with changes in ligand expression, but not receptor expression in the placenta. Studies on adiponectin levels in GDM placental tissue are critical to determine if this observation can also be applied to the adiponectin pathway. However, changes in adiponectin expression are currently out of the range of sensitivity for our real time PCR assay. Further expression analysis during placental development may help to identify the relationship between adipocytokine changes and the occurrence of GDM.

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