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PII: S0303-7207(09)00405-5
DOI: doi:10.1016/j.mce.2009.08.002
Reference: MCE 7282

To appear in: *Molecular and Cellular Endocrinology*

Received date: 12-5-2009
Revised date: 27-7-2009
Accepted date: 5-8-2009

Please cite this article as: Kleiblova, P., Dostalova, I., Bartlova, M., Lacinova, Z., Ticha, I., Krejci, V., Springer, D., Kleibl, Z., Haluzik, M., Expression of adipokines and estrogen receptors in adipose tissue and placenta of patients with gestational diabetes mellitus, *Molecular and Cellular Endocrinology* (2008), doi:10.1016/j.mce.2009.08.002

Expression of adipokines and estrogen receptors in adipose tissue and placenta of patients with gestational diabetes mellitus

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Key words: gestational diabetes mellitus; subcutaneous adipose tissue; visceral adipose tissue; placenta; leptin; estrogen receptors.

SUMMARY

The purpose of this study was to assess the expression profile of genes with potential role in the development of insulin resistance (adipokines, cytokines/chemokines, estrogen receptors) in subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT) and placenta of pregnant women with gestational diabetes mellitus (GDM) and age-matched women with physiological pregnancy at the time of Caesarean section. qRT-PCR was used for expression analysis of the studied genes. Leptin gene expression in VAT of GDM group was significantly higher relative to control group. Gene expressions of interleukin-6 and interleukin-8 were significantly increased, whereas the expressions of genes for estrogen receptors α and β were significantly reduced in SAT of GDM group relative to controls, respectively. We found no significant differences in the expression of any genes of interest (*LEP*, *RETN*, *ADIPOR1*, *ADIPOR2*, *TNF-α*, *CD68*, *IL-6*, *IL-8*, *ERα*, *ERβ*) in placentas of women with GDM relative to controls. We conclude that increased expression of leptin in visceral adipose depot together with increased expressions of proinflammatory cytokines and reduced expressions of estrogen receptors in subcutaneous fat may play a role in the etiopathogenesis of GDM.

INTRODUCTION

Gestational diabetes mellitus (GDM) is the most frequent metabolic disorder in pregnancy, affecting 1-10% of all pregnancies (Beischer et al., 1996 and Gabbe, 1986). Although most of the women with GDM return to normal glucose tolerance after delivery, they still have significantly increased risk of developing diabetes later in life (Kim et al., 2002). GDM is considered a prediabetic state, therefore it may display many abnormalities that possibly appear in the very early stages of type 2 diabetes mellitus (T2DM) (Pendegrass et al., 1995). In addition to mechanisms similar to patients with T2DM (Friedman et al., 1999 and Catalano et al., 2002), the etiopathogenesis of GDM includes the combination of the disturbed endocrine function of adipose tissue and placenta (Coughlan et al., 2001; Cseh et al., 2002; Kirwan et al., 2002; Radaelli et al., 2003; Ranheim et al., 2004 and Meller et al., 2006), and the systemic hormonal changes affecting insulin sensitivity in peripheral tissues (Barros et al., 2008).

Adipokines [leptin (*LEP*; OMIM 164160), resistin (*RETN*; OMIM 605565), and adiponectin (*ADIPOQ*; OMIM 605441)], inflammatory cytokines [tumor necrosis factor-α (*TNF-α*; OMIM 191160), and interleukin-6 (*IL-6*; OMIM 147620)], and chemokines [interleukin-8 (*IL-8*; OMIM 146930)] have been implicated in the etiopathogenesis of insulin resistance and in the early defects of T2DM (Bastard et al., 2000; Moller, 2000; Wauters et al., 2000; Coppack, 2001; Kern et al., 2001; Steppan et al., 2001; Weyer et al., 2001; Greenberg and McDaniel, 2002; Havel, 2002; Housa et al., 2006; Kleiblova et al., 2006 and Lamounier-Zepter et al., 2008). Leptin, adiponectin and *TNF-α* have been suggested as much stronger predictors of pregnancy-associated insulin resistance than gestational hormones, including human placental lactogen and steroids (Lepercq et al., 1998; Kirwan et al., 2002 and Radaelli et al., 2003). In previously published studies, circulating levels of leptin, adiponectin

and *TNF-α* in the early pregnancy closely predicted the development of GDM (Qiu et al., 2004; Hernandez Valencia et al., 2005; Maghbooli et al., 2007 and Gao et al., 2008).

To our best knowledge, the contribution of different fat depots (subcutaneous and visceral) to altered levels of adipokines and cytokines in GDM is unknown. Studies of patients with obesity and/or T2DM have shown that metabolic and endocrine characteristics of adipose tissue significantly vary between different locations (Wajchenberg, 2000; Coppack, 2001; Wagenknecht et al., 2003 and Dolinkova et al., 2008). Placenta of patients with GDM typically displays structural and functional abnormalities (Alonso et al., 2006) suggesting possible alterations in its endocrine function including disturbed production of adipokines and/or proinflammatory factors (Masuzaki et al., 1997; Radaelli et al., 2003 and Yura et al., 2003). Previous reports confirmed that GDM elicits major changes in the expression profile of placental genes with a prominent increase in markers and mediators of inflammation (Radaelli et al., 2003).

We hypothesized that dysregulated endocrine function of adipose tissue and placenta during pregnancy may contribute to the development of insulin resistance and GDM. The aim of the present study was to characterize the changes in gene expression of adipokines [leptin, resistin, adiponectin and its receptors – adiponectin receptor 1 (*ADIPOR1*; OMIM 607945), and adiponectin receptor 2 (*ADIPOR2*; OMIM 607946)], proinflammatory cytokines (*IL-6*, *TNF-α*), chemokines (*IL-8*) and other receptors with potential role in the regulation of glucose and lipid homeostasis [genes for peroxisome proliferator-activated receptor-α (*PPAR-α*; OMIM 170998), estrogen receptor α (*ESR1*, *ERα;* OMIM 133430), and estrogen receptor β (*ESR2*, *ERβ*; OMIM 601663)] in subcutaneous and visceral adipose tissue and placenta of patients with gestational diabetes mellitus relative to those in healthy women with physiological pregnancy.

PATIENTS AND METHODS

Study subjects

Ten women with GDM (age 34.6 ± 2.50 years) and thirteen healthy age-matched women with physiological pregnancy (age 33.1 ± 1.25 years) were included in the study. Only women with single pregnancy who delivered by planned Cesarean section in the term (40 ± 2 weeks of pregnancy) were enrolled into the study. All GDM were treated by intensive insulin therapy. All women enrolled into the study underwent the 75-gram 2-hour oral glucose tolerance test (oGTT) between 24th and 28th week of their pregnancy. Physiological ranges of glycemia were set according to Recommendations of the Czech Diabetes Association as follows: fasting glycemia < 5.5 mmol/l, glycemia 1 hour after glucose ingestion < 8.8 mmol/l and glycemia 2 hours after glucose ingestion < 7.6 mmol/l. The positive oGTT and a need for insulin treatment were the inclusion criteria for GDM group. None of GDM patients had history of diabetes and impaired glucose tolerance before pregnancy. All women had normal fasting glucose levels at the beginning of the pregnancy. The study, performed in accordance with the quidelines proposed in the Declaration of Helsinki, was approved by the Ethical Commitee, First Faculty of Medicine, and General University Hospital, Prague, Czech Republic, and all participants signed written informed consent before being enrolled in the study.

Anthropometric examination and blood sampling

All subjects were measured and weighted and their body mass index (BMI) was calculated twice for the study need; at the beginning of pregnancy (these data as well as serum glucose concentrations were obtained retrospectively from maternity card) and 3rd day after delivery at the Department of Obstetrics and Gynecology.

Body fat content was estimated by bioimpedance measurement (Body Fat Monitor, BF 306, Omron) 3rd day after delivery at the same time as body weight and BMI were estimated.

All maternal and fetal blood and maternal tissue samples were collected at the time of delivery. At the beginning of Caesarean section blood drawings (13 ml of blood) were performed. Serum samples for insulin, adipokine and cytokine measurements as well as for all other examinant biochemical parameters were obtained by centrifugation and whole blood samples for glycated hemoglobin measurements were withdrawn. After manual removal of placenta, 5 ml of fetal umbilical blood was also obtained for serum separation. All serum samples were stored in aliquots at –80°C until further analysis.

Hormonal and biochemical assays

Serum adiponectin concentrations were measured by Human Adiponectin ELISA kit (Linco Research, USA) with intra- and inter-assay variability of (1.0 - 7.4%) and (2.4 - 8.4%), respectively. Serum leptin and resistin concentrations were measured by commercial ELISA kits (BioVendor, Brno, Czech Republic). Sensitivity was 0.2 ng/ml for resistin and 0.12 ng/ml for leptin, and the intra- and interassay variability were < 5% and < 8%, respectively. Serum insulin concentractions were measured by commercial RIA kit (Cis Bio International, Gif-sur-Yvette Cedex, France). Sensitivity was 2.0 μIU/ml and the intra- and interassay variability were 4.2% and 8.8%, respectively.

Biochemical parameters were measured in the Department of Clinical Biochemistry and Laboratory Medicine, General University Hospital and First Faculty of Medicine, Charles University in Prague, by standard laboratory methods. Serum glucose concentrations were measured by photometric enzyme assay GOD/POD (Pliva – Lachema), serum triglyceride concentrations were measured by photometric enzyme assay GPO/PAP (Human Gesellschaft für Biochemica und Diagnostica mbH) and serum C-peptide concentrations were measured by electrochemiluminiscence – all of them on the MODULAR System (ROCHE Diagnostics). Serum estradiol concentrations were measured by two-step immunoassays using Chemiluminiscent Microparticle Immunoassay (CMIA) technology with flexible assay protocols, referred to as ChemiflexTM. System: Architect i2000 (ABBOTT Laboratories).

Tissue samples

Subcutaneous adipose tissue (SAT) from the region of transverse Pfannenstiel incision and visceral (omental) adipose tissue (VAT) samples were obtained during surgery and placental tissue was sampled after manual removal of placenta from approximately 5 mm deep incision from fetal side of placenta in the central part of cotyledon. All samples were immediately immersed into RNAlater (Qiagen) and were stored according to manufacturer instructions until total RNA isolation.

Total RNA isolation and cDNA sample preparation

Total RNA from SAT and VAT were isolated using RNeasy Lipid Tissue Mini Kit (Qiagen) and placental total RNA was isolated by RNeasy Protect Mini Kit (Qiagen) according to manufacturer protocols. Quality of isolated RNA was controlled

spectrophotometrically (only the samples with 260/280 nm ratio >1.8 were proceed for further steps of analysis), the RNA integrity was controlled by agarose gel electrophoresis. RNA samples were stored at -80°C until cDNA preparation.

All RNA samples were treated by DNase (Fermentas) prior cDNA synthesis. Reverse transcription was performed in 40 µl reaction volume with 4 µl (placenta) and 2 µl (SAT and VAT) template total RNA, respectively, using SuperScript III reverse transcriptase (Invitrogen) and random hexanucleotides (Roche) according to manufacturer Synthetized cDNA was stored at –20°C until qPCR.

Real-time PCR (qPCR) and gene expression quantification

Gene expression was analyzed by quantitative real-time RT-PCR (qPCR) and quantified by REST (Relative Expression Software Tool) 2008 ver. 2.0.7 (Corbett Research Pty. Ltd) (Pfaffl, 2001 and Pfaffl et al., 2002) and/or by qGENE softwares.

Commercially designed primers were used for *CD68*, *ESR1*, *ESR2*, *IL-6*, *IL-8*, *RETN* and *TNF-α* genes (SuperArray, USA), whereas primers for amplification of other genes were designed by us and synthetized by Generi Biotech, Czech Republic: *B2M* forward (5'-ATGTCTCGCTCCGTGGCCTTA-3'), reverse (5'-TCGGATGGATGAAACCCAGACACA-3'); *GAPDH* forward (5'-GGTGAAGGTCGGAGTCAACGG-3'), reverse (5'-CGCTCCTGGAAGATGGTGATGG-3'); *PBGD* forward (5'-ATGTCTGGTAACGGCAATGCGG-3'), reverse (5'-TGTCCCCTGTGGTGGACATAGC-3'); *ADIPOQ* forward (5'-GTGATGGCAGAGATGGCACCC-3'), reverse (5'-AGGCACCTTCTCCAGGTTCTCC-3'); *ADIPOR1* forward (5'-TTGTGTACAAGGTCTGGGAGGGA-3'), reverse (5'-CAGCACGAAACCAAGCAGATGG-3'); *ADIPOR2* forward (5'-CTGATGGCCAGCCTCTACATCAC-3'), reverse (5'-CCCGCCGATCATGAAACGAAAC-

3') *LEP* forward (5'-CTATGTCCAAGCTGTGCCCATCC-3'), reverse (5'-CTGCCAGTGTCTGGTCCATCTTG-3'); *PPAR-α* forward (5'-CGCAATCCATCGGCGAGGATA-3'), reverse (5'-CTCCACTGGGAGACTCGTCCA-3'). The size of PCR amplicons of all tested genes ranged between 100 and 230bp. PCR product specificity for primers designed in our laboratory was confirmed by DNA sequencing (BigDye Terminator ver 3.1; Applied Biosystems) of PCR amplicons. The qPCR reaction containing 1 µl of cDNA and 4 pmol of each primer was carried out in 10 µl reaction volume using SybrGreen LightCycler FastStart DNA Master SYBR Green I (Roche) on LightCycler 2.0 (Roche) according to manufacturer instructions. The $MgCl_2$ concentrations (in range 1.75 – 4.0 $mmol.l^{-1}$) were optimized for each type of qPCR. qPCR was run in 45 cycles (95°C 10s; 60°C /66°C 10s; 72°C 10s) with initial denaturation (95°C for 10 min). Control of amplification specificity was routinely performed by implementation of melting analysis (72-95°C) at the end of each qPCR run and agarose gel electrophoresis of generated amplicons. All analyses were performed in doublets involving simultaneously analyzed negative controls for all types of analyzed amplicons.

The calibration curves using serial dilutions of control cDNA (200, 100, 50, 25, 10, 1, and 0.1 ng of cDNA) were prepared for each amplicon enabling assessment of qPCR efficiencies calculated by REST 2008 ver. 2.0.7 software.

The qGENE software (considering qPCR efficiency for each type of reaction) was used for relative quantification of analyzed genes against selected housekeeping gene (beta–2–microglobulin; *B2M*). This software allowed quantification of relative gene expression of each individual analyzed sample, but only to just one housekeeping gene.

Changes in expression of analyzed genes between tested cohorts (women with GDM and healthy pregnant women), as well as between tested tissue types (SAT, VAT, placenta) were compared using REST 2008 ver. 2.0.7 considering qPCR efficiency for each type of reaction.

The results from REST 2008 ver. 2.0.7 were obtained as mean expression ratios and 95% confidence intervals of the relative expressions of target genes. For relative quantification and normalization of gene expression analyses done by REST 2008 ver. 2.0.7, we used three housekeeping genes: *B2M*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; OMIM 138400), and porphobilinogen deaminase (*PBGD*; OMIM 609806), which minimized wrong results interpretation.

Statistical analysis

Pair Wise Fixed Reallocation Randomisation Test, a part of REST 2008 ver. 2.0.7 (Pfaffl et al., 2002), was used for the statistical analysis of gene expression changes (described in detail on http://www.gene-quantification.info/). Other data were analyzed by SigmaStat software for statistical analyses (SPSS Inc., Chicago, IL). Anthropometric, biochemical and hormonal results are expressed as means ± SEM. Differences between studied groups (GDM vs. controls) were evaluated by unpaired t-test or Mann-Whitney test as appropriate. The correlations between the values were estimated by Spearman correlation test. Statistical significance was set to $p < 0.05$.

RESULTS

Anthropometric characteristic of healthy women with physiological pregnancy and pregnant women with gestational diabetes mellitus (GDM) and their newborns

The study groups were age-matched. Pregnant women with GDM had significantly higher BMI before pregnancy, higher BMI third day after delivery, and higher percentage of body fat

third day after delivery relative to control group. Birth weight, birth length and birth wigth/length ratio of the newborns did not differ beween the groups (Table 1).

Maternal and fetal serum biochemical and hormonal parameters of GDM and control group

Maternal serum levels of adiponectin, resistin, C-peptide, triglycerides, and estradiol did not significantly differ in GDM group as compared with control group. Maternal serum leptin levels tended to be higher in GDM group, but the difference did not reach the statistical significance (p = 0.1). Serum glucose, insulin, and glycated hemoglobin levels were significantly higher in GDM relative to control group. Fetal serum adiponectin, resistin, and insulin levels did not significantly differ between GDM group and control group, whereas fetal serum leptin levels were markedly increased in GDM group as compared to control group. In both groups, fetal adiponectin concentrations were higher compared to maternal adiponectin concentrations (71.3 ± 8.9 vs. 22.7 ± 1.9 $mg.l^{-1}$; $p < 0.00001$). The opposite was true for leptin concentrations that were higher in maternal relative to fetal serum samples (15.0 ± 3.4 vs. 29.1 ± 4.1 $\mu g\ l^{-1}$; $p = 0.01$). Serum concentrations of resistin and insulin did not significantly differ between mothers and their newborns (Table 2).

Gene expression in subcutaneous adipose tissue, visceral adipose tissue, and placenta: comparison of women with GDM and healthy control pregnant women

Gene expression of resistin, adiponectin, *ADIPOR1*, *ADIPOR2*, *PPAR-α*, *TNF-α*, and *CD68* did not significatly differ between GDM and control group in any tissue studied. Leptin gene expression was 3.5-times higher in VAT of GDM group relative to control group. mRNA levels of *IL-6* and *IL-8* were 6.3-times and 4.8-times higher in SAT of GDM group as

compared to control group. On the contrary, the expressions of *ERα* and *ERβ* were significantly lower in SAT of GDM group as compared with control group (Figure 1, Table 3). Gene expression of adiponectin was undetectable in placenta (Table 3).

The comparison of the relative expression of the studied genes between subcutaneous adipose tissue, visceral adipose tissue and placenta

The proportion of relative gene expression (normalized to *B2M*) of all analyzed genes in all three tested tissues is summarized in Figure 2.

The expressions of genes for leptin, adiponectin, and ERβ were significantly higher in SAT than in VAT in both GDM and control groups. Gene expressions of resistin, *ADIPOR1* and *ADIPOR2* were significantly higher in SAT than in VAT in the control, but not in GDM group. Gene expression of *IL-8* was significantly higher in SAT than in VAT in GDM group only. Gene expression of *IL-6* was higher in VAT than in SAT in control group, whereas higher expression of *IL-6* in SAT relative to VAT was found in GDM. Gene expression of *ERα* was significantly higher in SAT than VAT in control group, whereas it did not significantly differ between the SAT and VAT depots in GDM (Figure 2, Table 4).

The expressions of genes for leptin, *ADIPOR2* and *CD68* were significantly higher in SAT than in placenta in control group, but not in GDM group. The expressions of *IL-8*, *ERα* and *ERβ* were markedly higher in SAT than in placenta in both control and GDM group. The expression of *IL-6* was significantly higher in SAT than in placenta in GDM group only. Inspite of undetectable adiponectin expression in placenta, the expression of *ADIPOR1* was 3-times higher in placenta than in SAT in both groups studied (Figure 2, Table 4).

DISCUSSION

The most important finding of this study is that visceral fat leptin gene expression and subcutaneous fat *IL-6* and *IL-8* gene expressions were significantly increased, whereas subcutaneous fat $ER\alpha$ and $ER\beta$ gene expressions were significantly decreased in women with GDM relative to healthy age-matched women with physiological pregnancy.

Inspite of previously suggested predictive role for leptin in the development of GDM (Qiu et al., 2004; Maghbooli et al., 2007 and Gao et al., 2008), there is still a controversy as far as the circulating levels of leptin in GDM are concerned (Festa et al., 1999; Kautzky-Willer et al., 2001 and Simmons et al., 2002). Such a discrepancy in published results could be attributed to the different gestational age at time of the maternal blood collection and the differences in severity of GDM and its compensation (Festa et al., 1999). Here we observed a strong, but non-significant, trend towards increased circulating leptin levels in the GDM group at the time of delivery. The lack of statistically significant difference between the groups could be at least partially attributable to a satisfactory diabetes compensation of GDM group at the time of blood collection as well as to relatively low number of subjects together with high interindividual circulating leptin concentrations variability.

Visceral fat has been shown to contribute to the development of impaired glucose metabolism in women with history of GDM (Lim et al., 2007). Markedly elevated expression of leptin in visceral adipose depot of women with GDM may thus play a role in the development of endocrine dysfunction of visceral fat and later on also in the development of GDM. The mechanism of leptin contribution to the development of insulin resistance may lie in its inhibitory effect on the insulin-signaling cascade; leptin promotes phosphorylation of Ser-318 in Irs1 by a janus kinase 2, Irs2, and PKC-dependent pathway (Hennige et al., 2006). Although the findings of majority (Lepercq et al., 1998; Lea et al., 2000; Radaelli et al., 2003 and Meller et al., 2006), but not all (Lappas et al., 2005), previous studies showed elevated leptin production in placenta from women with GDM, our results suggest that, at the time of

delivery visceral adipose tissue, rather than a placental tissue, appears to be a major contributor to increased leptin levels in GDM. We also suggest that high maternal visceral fat leptin expression and significantly increased fetal serum leptin levels at the time of delivery could play a role in later susceptibility to the potential post-pregnancy development of insulin resistance and T2DM in both mother and fetus (McNeely et al., 1999; Jansson et al., 2002 and Shahid et al., 2008).

It still remains to be determined what factors trigger increased expression of leptin in visceral fat of women with GDM and whether these factors precede the development of insulin resistance and diabetes or are rather secondary to these metabolic disturbances. One possible contributor to increased leptin expression in visceral fat in GDM might be altered estrogen receptor subtypes expression ratio in adipose depots of GDM in response to rising estrogen level with advancing gestation (Sivan et al., 1998; Brann et al., 1999; Tanaka et al., 2001 and Yi et al., 2008). Here we found similarly to Yi and colleagues (Yi et al., 2008) a significant positive relationship of visceral adipose tissue leptin gene expression with visceral fat *ERα* gene expression ($r = 0.68$, $p < 0.00001$), as well as with visceral fat *ERα*/*ERβ* mRNA expression ratio in a combined population of both study groups ($r = 0.46$, $p = 0.04$). Although we found no significant relationship between *LEP* and *ERβ* gene expressions in visceral fat depot, we found a significant positive relationship between *LEP* and *ERβ* gene expressions ($r = 0.56$, $p = 0.009$) in subcutaneous adipose depot. Leptin mRNA level in visceral fat was also inversely related to maternal estradiol serum concentrations ($r = -0.52$, $p = 0.02$). Furthermore, we found significantly different *ERα*/*ERβ* ratio in subcutaneous over visceral fat depot between GDM and controls (SAT/VAT expression ratio of *ERα*/*ERβ*: controls 1.34 vs. GDM 0.46, $p < 0.05$). Shin et al. (2007) previously showed that the subcutaneous *ERα*/*ERβ* over visceral *ERα*/*ERβ* ratio is significantly positively associated with leptin expression in visceral fat depot.

The second group of candidates possibly affecting leptin expression in visceral fat in pregnancy includes insulin and glucose (Boucher et al., 2005 and Salvatores et al., 2006). It has been previously documented that insulin increases leptin mRNA expression in human subcutaneous fat (Pratley et al., 2000). Mouse models of obesity, diabetes and insulin resistance have markedly increased adipose tissue leptin mRNA expression (Boucher et al., 2005). Here we showed that visceral fat leptin expression positively correlated with blood glucose (r = 0.52, p = 0.02).

Thirdly, previous reports postulated that the release of leptin from maternal adipose tissue positively correlates with maternal BMI (Lappas et al., 2005). Here we found that visceral fat leptin gene expression positively correlated with maternal BMI before pregnancy (r = 0.46, *p* = 0.04) and with maternal body weight 3rd day after delivery (r = 0.48, *p* = 0.03).

Increasing evidence suggests that GDM is a proinflammatory state similarly as T2DM (Radaelli et al., 2003). Increased expression of *IL-6* and *IL-8* in subcutaneous adipose depot of women with GDM is in agreement with such idea and with previously described increased circulating levels of IL-6 and IL-8 in GDM (Kuzmicki et al., 2008). Interestingly, although visceral adipose tissue is traditionally considered more proinflammatory fat depot (Wajchenberg, 2000 and Wagenknecht et al., 2003) here we found that in GDM patients mRNA expression of *IL-6* was higher in subcutaneous than in visceral fat. Even more strikingly, the opposite was true for healthy control group. We also observed significantly lower mRNA expression of *IL-6* in placenta than in subcutaneous fat of GDM patients, but not in controls, which further indicates altered inflammatory status regulation in patients with GDM. We have also showed that maternal adipose tissue *IL-6* expression positively correlates with fetal serum leptin (r = 0.52, *p* = 0.01) and that placental *IL-8* mRNA inversely correlates with fetal serum resistin (r = - 0.50, *p* = 0.03), suggesting that proinflammatory state of the

mother may influence the endocrine regulations of the fetus (Henson and Castracane, 2002 and Nathanielsz et al., 2007).

The results of our study underline a possible importance of estrogen receptors in the regulation of insulin resistance. In experimental studies, ERα activation appears to protect against insulin resistance while the opposite is generally true for ERβ activation (Naaz et al., 2002 and Foryst-Ludwig et al., 2008). The selective knock-out of ERα led to adipocyte hyperplasia and hypertrophy, insulin resistance, and glucose intolerance in mice (Heine et al., 2000). In mature adipocytes *ERα* mRNA expression was higher as compared to *ERβ* mRNA levels, but *ERβ* is quantitavely more expressed isoform in women than in men (Deroo and Korach, 2006). Here we showed that at the time of delivery the *ERβ* gene expression is in all three analyzed tissues higher than *ERα* (*ERα*/*ERβ* = 0.043 in SAT, 0.078 in VAT and 0.322 in placenta; data not shown). We also showed that GDM is associated with decreased expression of both types of estrogen receptors in subcutaneous fat depot suggesting another possible mechanism of modulation of insulin sensitivity in these patients. Since *ERα* appears to play a major role in adipose tissue (Naaz et al., 2002) its decreased expression in subcutaneous fat of patients with GDM may contibute to the development of insulin resistance within this adipose tissue depot.

We are aware, that our study has several limitations. The first one is the relatively low number of subjects included and the presence of significantly higher BMI of GDM group relative to control group before the pregnancy. Second, rather paradoxical limitation from the patients' point of view is that all tissue samples were collected at the time of delivery when diabetes of GDM patients was satisfactorily-compensated which may have partially blunted the differences between the groups.

In conclusion, our study has shown that GDM is accompanied by increased expression of leptin in visceral adipose depot together with increased expression of proinflammatory

cytokines and reduced expression of estrogen receptors in subcutaneous adipose depot. We suggest that these alterations may in concert contribute to some of metabolic disturbances seen in GDM patients not only during pregnancy but also after delivery.

Acknowledgements

Supported by IGA MHCR no. 8302-5 to MH and PK. We thank to obstetricians and the patients for their collaboration. The special thank belongs to Mrs. Hana Vágnerová for help with the majority of tissue sampling.

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Table 1. Anthropometric characteristics of healthy pregnant women, patients with gestational diabetes mellitus (GDM) (A) and their newborns (B).

A. MOTHERS	Controls (N = 13)	GDM (N = 10)	*P*-value
BMI before pregnancy [kg.m^{-2}]	**22.7** ± 0.9	**30.1** ± 2.3	**0.012**
BMI 3rd day after delivery [kg.m^{-2}]	**25.5** ± 1.0	**32.9** ± 2.5	**0.025**
% of body fat 3rd day after delivery	**32.4** ± 1.3	**39.6** ± 2.4	**0.023**
Age [years]	33.1 ± 1.3	34.6 ± 2.5	0.569
Gestational age [days]	276.9 ± 1.6	268.9 ± 4.2	0.055
B. NEWBORNS	Controls (N = 13)	GDM (N = 10)	*P*-value
Birth weight [kg]	3.42 ± 0.14	3.47 ± 0.27	0.838
Birth length [m]	0.51 ± 0.01	0.49 ± 0.01	0.051
Birth wigth/length [kg.m^{-1}]	6.75 ± 0.22	7.10 ± 0.45	0.456
Gender [N = girls / boys]	8/5	7/3	

Results are means ± SEM; N = number of subjects; $p<0.05$ denoted **statistical significant changes**.

Table 2. Maternal and fetal serum parameters in healthy pregnant women and patients with gestational diabetes mellitus (GDM) measured at the time of delivery.

MATERNAL SERUM	CONTROLS (N = 13)	GDM (N = 10)	*P*-value
Adiponectin [μg.ml^{-1}]	24.5 ± 2.33	20.1 ± 3.04	0.3
Leptin [ng.ml^{-1}]	21.8 ± 2.72	39.6 ± 8.11	0.1
Resistin [ng.ml^{-1}]	10.9 ± 0.58	12.6 ± 1.33	0.2
Glucose [mmol.l^{-1}]	**3.9** ± 0.12	**5.5** ± 0.82	**0.03**
Insulin [mIU.l^{-1}]	**23.4** ± 1.96	**144.8** ± 64.56	**0.04**
C-peptide [nmol.l^{-1}]	0.6 ± 0.05	1.0 ± 0.29	0.6
Glycated hemoglobin [%]	**3.4** ± 0.10	**4.1** ± 0.21	**0.002**
Triglycerides [mmol.l^{-1}]	3.9 ± 0.47	3.5 ± 0.27	0.5
Estradiol [nmol.l^{-1}]	83.4 ± 8.82	74.1 ± 11.93	0.5
FETAL SERUM	CONTROLS (N = 13)	GDM (N = 10)	*P*-value
Adiponectin [μg.ml^{-1}]	76.1 ± 12.99	64.9 ± 12.08	0.6
Leptin [ng.ml^{-1}]	**8.1** ± 1.76	**24.3** ± 6.44	**0.02**
Resistin [ng.ml^{-1}]	11.0 ± 1.46	10.9 ± 0.88	0.96
Insulin [mIU.l^{-1}]	18.4 ± 2.17	34.9 ± 12.99	0.2

N = number of subjects; Values are mean ± SEM; $p<0.05$ denoted **statistical significant changes**.

Table 3. Fold changes of relative gene expression levels in subcutaneous (SAT) and visceral (VAT) adipose tissue and in placenta from women with gestational diabetes mellitus (GDM) (N = 10) relative to healthy pregnant women (N = 13). Relative gene expression of the respective gene of the healthy pregnant group = 1.

GDM to control group expression	**SAT**		**VAT**		**PLACENTA**	
Gene	ExpR	*P*-value	ExpR	*P*-value	ExpR	*P*-value
Leptin (*LEP*)	1.18	0.4	**3.49**	**0.02**	1.56	0.6
Resistin (*RETN*)	0.79	0.6	1.13	0.7	1.09	0.8
Adiponectin (*ADIPOQ*)	0.74	0.07	0.81	0.6	ND	
Adiponectin receptor 1 (*ADIPOR1*)	1.11	0.5	1.01	0.96	1.07	0.7
Adiponectin receptor 2 (*ADIPOR2*)	1.04	0.8	1.18	0.4	1.21	0.2
PPAR-alpha (*PPAR-α*)	0.76	0.3	0.76	0.4	NE	
TNF-alpha (*TNF-α*)	1.51	0.4	0.97	0.9	1.32	0.4
CD68	1.18	0.6	0.89	0.3	1.10	0.6
Interleukin-6 (*IL-6*)	**6.32**	**0.01**	2.66	0.1	0.98	0.9
Interleukin-8 (*IL-8*)	**4.75**	**0.03**	1.26	0.6	0.95	0.9
Estrogen receptor alpha (*ERα*)	**0.52**	**0.045**	1.46	0.1	1.75	0.1
Estrogen receptor beta (*ERβ*)	**0.68**	**0.033**	1.21	0.4	1.07	0.8

ExpR expression ratio; ND not detected; NE not estimated; $p<0.05$ denoted **statistical significant changes**.

Table 4. The comparison of the relative gene expression (normalized to 3 housekeeping genes) of target genes in visceral adipose tissue (VAT) and placenta relative to subcutaneous adipose tissue (SAT) with (CONTROL and GDM subgroups) or without (ALL subjects together) the dependency of GDM status. Relative expression of respective gene in SAT = 1.

VAT	**ALL**		**CONTROLS**		**GDM**	
Gene	ExpR	*P*-value	ExpR	*P*-value	ExpR	*P*-value
Leptin (*LEP*)	**0.08**	**0.001**	**0.06**	**0.001**	**0.16**	**0.001**
Resistin (*RETN*)	**0.27**	**0.001**	**0.25**	**0.001**	0.35	0.1
Adiponectin (*ADIPOQ*)	**0.40**	**0.001**	**0.38**	**0.003**	**0.42**	**0.004**
Adiponectin receptor 1 (*ADIPOR1*)	**0.73**	**0.01**	**0.77**	**0.03**	0.71	0.2
Adiponectin receptor 2 (*ADIPOR2*)	**0.77**	**0.03**	**0.73**	**0.04**	0.83	0.3
PPAR-alpha (*PPAR-α*)	1.07	0.7	1.02	0.9	1.02	0.97
TNF-alpha (*TNF-α*)	0.62	0.1	0.73	0.3	0.47	0.1
CD68	0.88	0.4	0.97	0.9	0.74	0.2
Interleukin-6 (*IL-6*)	1.24	0.7	1.85	0.3	0.78	0.7
Interleukin-8 (*IL-8*)	**0.31**	**0.03**	0.55	0.4	**0.15**	**0.002**
Estrogen receptor alpha (*ERα*)	**0.59**	**0.01**	**0.38**	**0.001**	1.06	0.8
Estrogen receptor beta (*ERβ*)	**0.40**	**0.001**	**0.31**	**0.001**	**0.56**	**0.001**
PLACENTA	**ALL**		**CONTROLS**		**GDM**	
Gene	ExpR	*P*-value	ExpR	*P*-value	ExpR	*P*-value
Leptin (*LEP*)	**0.28**	**0.002**	**0.24**	**0.006**	0.32	0.1
Resistin (*RETN*)	1.12	0.7	1.01	0.98	1.40	0.5
Adiponectin (*ADIPOQ*)	ND		ND		ND	
Adiponectin receptor 1 (*ADIPOR1*)	**3.18**	**0.001**	**3.30**	**0.001**	**3.18**	**0.001**
Adiponectin receptor 2 (*ADIPOR2*)	**0.79**	**0.01**	**0.73**	**0.002**	0.86	0.4
PPAR-alpha (*PPAR-α*)	NE		NE		NE	
TNF-alpha (*TNF-α*)	1.10	0.7	1.15	0.7	1.01	0.98
CD68	**0.60**	**0.004**	**0.61**	**0.02**	0.57	0.1
Interleukin-6 (*IL-6*)	**0.38**	**0.02**	0.82	0.7	**0.13**	**0.001**
Interleukin-8 (*IL-8*)	**0.09**	**0.001**	**0.18**	**0.01**	**0.04**	**0.001**
Estrogen receptor alpha (*ERα*)	**0.12**	**0.001**	**0.07**	**0.001**	**0.24**	**0.005**
Estrogen receptor beta (*ERβ*)	**0.02**	**0.001**	**0.01**	**0.001**	**0.02**	**0.001**

ExpR expression ratio; NE not estimated; ND not detected; $p<0.05$ denoted **statistical significant changes.**

Figure Legends:

Figure 1. The whisker box plot of statistically significant ($p < 0.05$) changes of the relative gene expression ratio (normalized to 3 housekeeping genes) in GDM group compared to controls in VAT (black color) and SAT (grey color). The relative gene expression of the respective gene in control group = 1.

Figure 2. The log scale scatter plot of relative gene expression (compared to *B2M*) of all analyzed genes in SAT (squares), VAT (triangles) and placenta (circles) in GDM (white color) and control group (black color). The expression of *B2M* gene = 1.

Figure 1

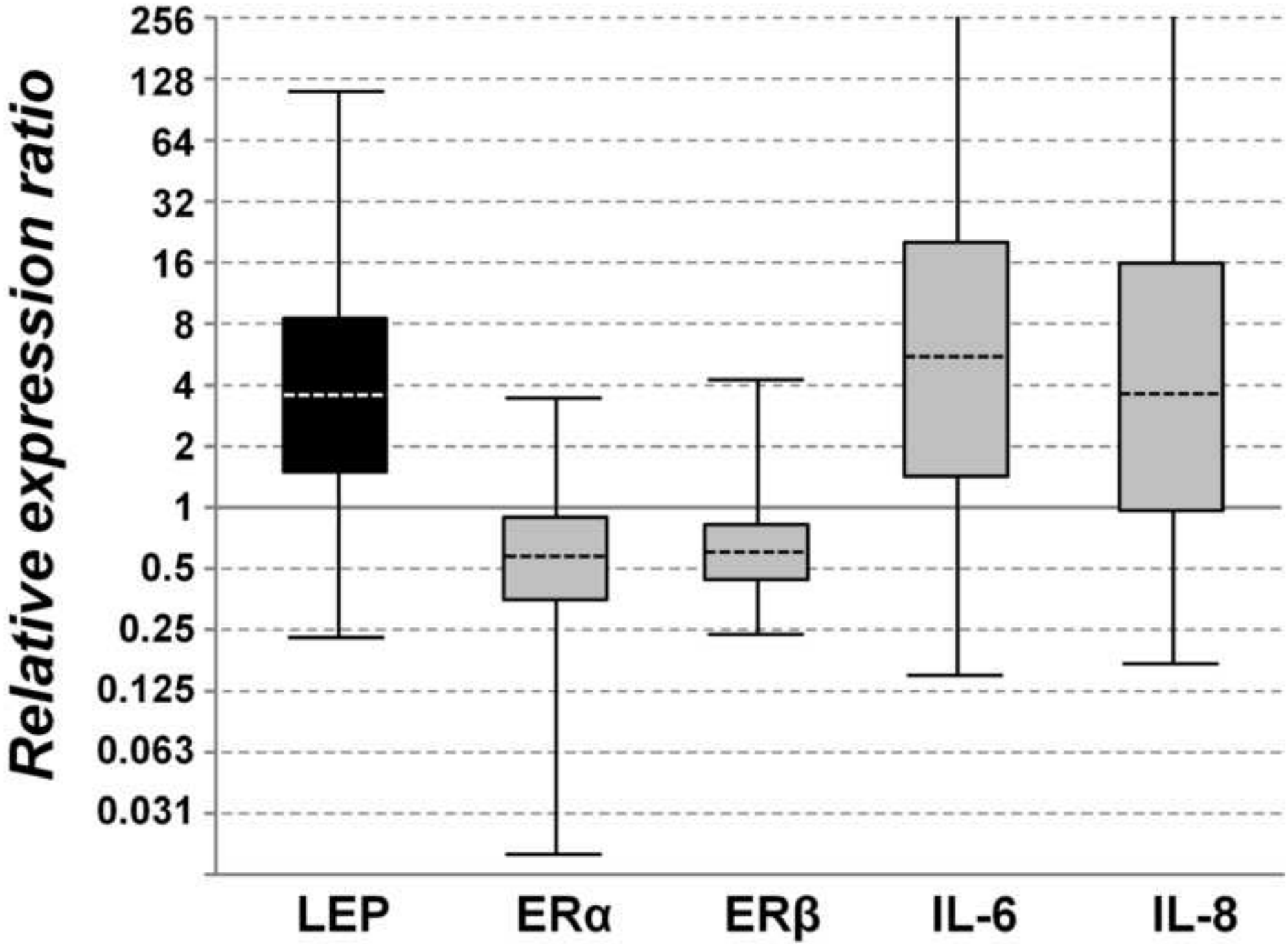
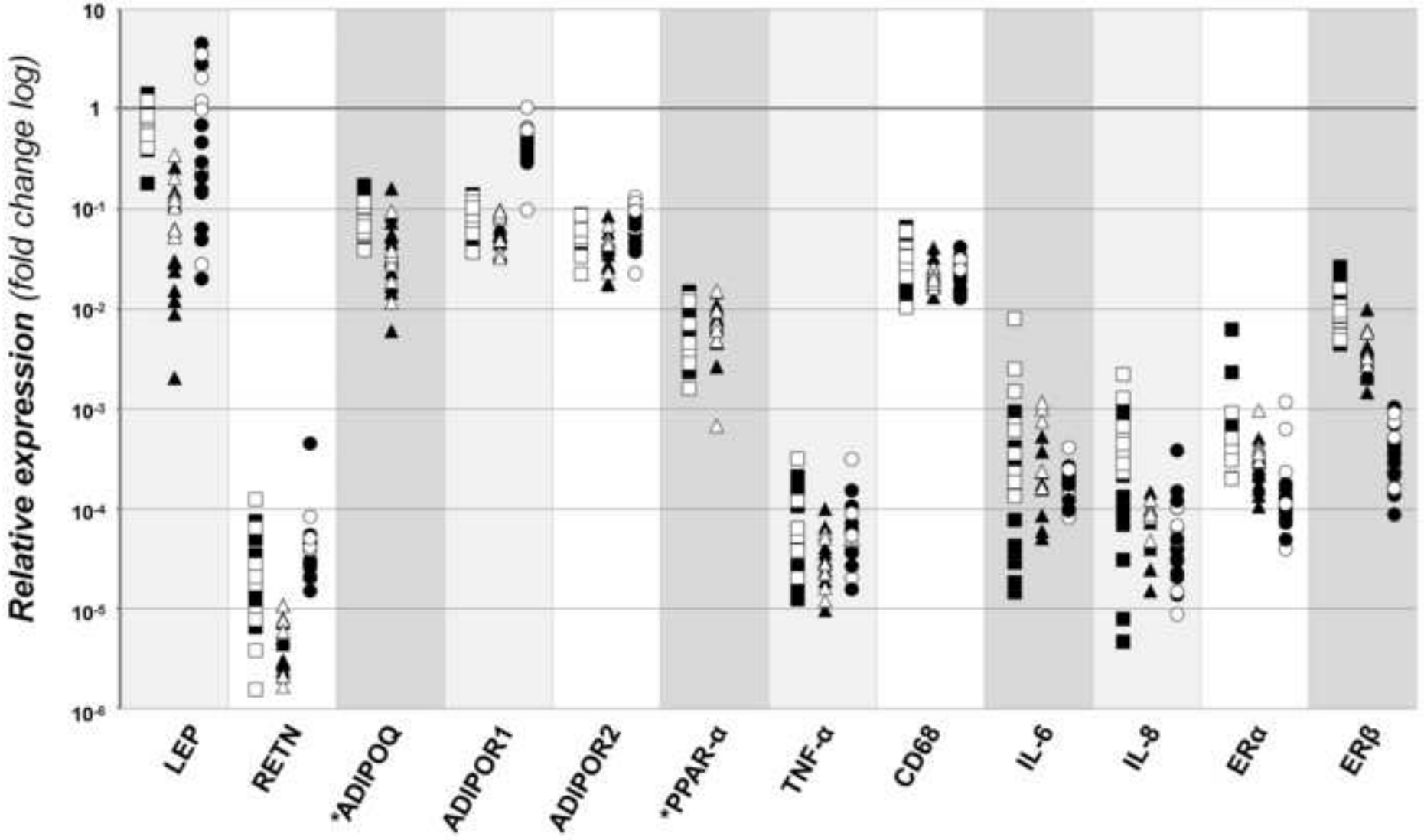

Relative expression ratio
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.063
0.031
LEP
ERα
ERβ
IL-6
IL-8

Figure 2


Relative expression (fold change log)
10
1
10-1
10-2
10-3
10-4
10-5
10-6
LEP
RETN
*ADIPOQ
ADIPOR1
ADIPOR2
*PPAR-α
TNF-α
CD68
IL-6
IL-8
ERα
ERβ