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Feto-placental adaptations to maternal obesity in the baboon

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Abstract

Maternal obesity is present in 20–34% of pregnant women and has been associated with both intrauterine growth restriction and large-for-gestational age fetuses. While fetal and placental functions have been extensively studied in the baboon, no data are available on the effect of maternal obesity on placental structure and function in this species. We hypothesize that maternal obesity in the baboon is associated with a maternal inflammatory state and induces structural and functional changes in the placenta. The major findings of this study were 1) decreased placental syncytiotrophoblast amplification factor, intact syncytiotrophoblast endoplasmic reticulum structure and decreased system A placental amino acid transport in obese animals; 2) fetal serum amino acid composition and mononuclear cells (PBMC) transcriptome were different in fetuses from obese compared with non-obese animals 3) maternal obesity in humans and baboons is similar in regard of increased placental and adipose tissue macrophage infiltration, increased CD14 expression in maternal PBMC and maternal hyperleptinemia. In summary, these data demonstrate that in obese baboons in the absence of increased fetal weight, placental and fetal phenotype are consistent with those described for large- for-gestational age human fetuses.

Introduction

Obesity is a serious condition that affects approximately 310 million people worldwide and represents a major health problem [1]. Maternal obesity (prior to and during pregnancy) is present in 20–34% of all pregnant women [2,3] and has been associated with both intrauterine growth restriction and large-for-gestational age human fetuses [4,5]. Both conditions are characterized by altered insulin secretion and are connected to adiposity and

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diabetes in later life [6–8]. Elevated maternal cholesterol level is correlated with fetal hypercholesterolemia and fetal atherosclerotic lesions in the aorta [9].

Pre-pregnancy obesity is associated with increased risk of placental dysfunction and fetal death as gestation advances [4,10]. The biologic mechanisms underlying various risks of maternal obesity in pregnancy are unknown. Maternal inflammatory status has been linked to placental changes in obesity [11]. It has been hypothesized that maternal obesity results in increased placental nutrient transport to the fetus [12]. Growing evidence in human and animal models of maternal obesity indicates several placental changes: increased idiopathic villitis [13], macrophage infiltration [14], and increased placental vascularity [15].

Non-human primates are closely related to the human species and have been used extensively in reproductive studies. One of the well-characterized non-human primate model used to study maternal obesity is Rhesus macaques. However, Rhesus macaques have certain disadvantages including a bidiscoid placenta and an immune system with only three IgG subclasses [16,17]. Another non-human primate species, the baboon, has been extensively studied as a model for obesity [18,19]. Increased weight in this species has been associated with insulin resistance [20] and stillbirth [21]. These data are in agreement with the published human data. Baboon placental structure and development are also very similar to humans [22].

The effect of maternal obesity on placental structure and function has never been reported in baboons. The goals of our study were 1) to characterize the baboon model of maternal obesity, 2) to examine maternal and feto-placental inflammatory status and 3) to evaluate the effect of maternal obesity on placental structure and amino acid transport at the end of gestation. We hypothesize that inflammatory status associated with maternal obesity induces placental structural and functional changes and increases nutrient transport to the fetus.

Material and Methods

Animal care and maintenance

All animals were maintained in a social group environment with partly controlled climate conditions, fed and given water *ad libitum* (LEO5, Purina). Two groups of pregnant female baboons (*Papio* spp.) were selected based on weight and obesity index at delivery by cesarean section at 165 days gestation – G, (0.9 G; term 185 days gestation; Table (1A) [23, 24]). Obesity index (Rh index) was defined as body weight divided by the square of the crown-rump length in another non-human primate species – Rhesus monkeys [25]. We aimed to compare animals that had around a 1.3 incremental increase in the index. This degree of incremental increase was described in obese vs. non-obese Rhesus monkeys [25]. Rh index was used rather than Body mass index (BMI) since baboons, like Rhesus monkeys are quadruped animals and have a central type of obesity [20]. In the fetal morphometric studies ponderal index was calculated as [weight (in g) × 100] ÷ [length (in cm)]^3 and BMI as [weight (in kg)] ÷ [length (in m)]^2. All procedures were approved by the Animal Care and Use Committee of the Southwest Foundation for Biomedical Research.

Cesarean section, blood and tissue processing

Maternal femoral vein blood samples were collected with the mother in the fasted state(12 hours fasting). This was done immediately prior to cesarean section under general anesthesia as previously described [26]. Fetal umbilical vein blood samples were collected while the fetus was still *in utero*. Blood samples were drawn under sterile conditions: into a 1 mL potassium EDTA BD Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ, USA) to determine hematological values, into 4 mL a BD Vacutainer clot tube without additives (Becton Dickinson, Franklin Lakes, NJ, USA) for serum separation and biochemical,

cytokine, and amino acid analyses. For peripheral blood mononuclear cells (PBMC) separation, blood was collected into cell preparation tubes (BD Vacutainer CPT mononuclear cell preparation, BD Biosciences, San Jose, CA) containing a density gradient polymer gel and sodium citrate (Becton Dickinson, Franklin Lakes, NJ). Cells were collected by centrifugation in cell preparation tubes (BD Biosciences), washed twice with 10 mL sterile phosphate-buffered saline, and frozen at −80°C for subsequent RNA extraction. The fetus was euthanized by exsanguinations while under general anesthesia; the placenta was removed manually and immediately processed as described previously [26]. Additionally, samples of central placenta, fetal visceral fat and maternal visceral fat were flash frozen in liquid nitrogen and stored at −80°C. Kidneys from pregnant obese and control animals were removed and processed for paraffin embedding. 4μm thick sections were stained with hematoxylin & eosin (H&E) to assess morphology and Masson trichrome to identify collagen fibrils within the interstitium. Interstitial inflammation within the cortex was graded from 0 to 3 on three histological sections for each animal according to standard criteria [27].

Hematological and biochemical parameters

Maternal and fetal blood biochemical and hematological parameters were evaluated as described previously using Coulter MAXM autoloader instrument and Beckman Synchron CX5CE (Beckman Coulter, Inc., Fullerton, CA) [28]. White and red blood cell counts, serum concentrations of glucose, cholesterol and bilirubin, and serum transaminase activities were determined in fetus and the mother.

Serum measurements of leptin, adiponectin, VEGF (vascular endothelial growth factor), IL-6 (interleukin), MCP-1 (monocyte chemoattractant protein), TNF-α (tumor necrosis factor) and insulin

Serum inflammatory cytokines (IL-6, MCP-1 and TNF-α), adipocytokines (leptin and adiponectin), angiogenic factor (VEGF) and insulin concentrations were estimated in fetal and maternal serum. All these factors are playing an important role in human obesity as well as in materno-feto-placental adaptation to pregnancy and obesity. The leptin and adiponectin were measured by radioimmunoassay in a single assay according to the manufacturer's instructions (LINCO Research, Inc., St. Charles, MO) as described previously [29,30]. The intra-assay coefficients of variation (%CV) were 3.0% at a leptin concentration of 4.9 ng/mL and 3.6% at adiponectin concentration of 1.5 μg/mL. Insulin concentration was measured with ELISA (LINCO Research, Inc., St. Charles, MO) [31], the intra-assay %CV was 5.3% at insulin concentration 95 μU/ mL. Multiplex assays (Lincoplex, Millipore, Inc., St. Charles, MO) were used for MCP-1 and VEGF evaluation (# HCVD3-67CK-02) with intra-assay %CV of 11.3% and 8.2%, respectively. For IL-6 and TNF-α (high sensitivity triplex assay, #HSCYTO-60SK) the intra-assay %CV were 3.5% and 3.5%, respectively.

PBMC isolation, RNA extraction and Illumina

RNA extractions—Obesity-related PBMC changes are central to the chronic inflammation associated with excess of body fat [32,33]. Maternal obesity in pregnancy is associated with maternal PBMC activation and placental macrophage infiltration [14]. Maternal and fetal PBMC RNA was extracted using Trizol reagent according to manufacturer's instructions. Samples were incubated for 5 min at 15–30°C in Trizol reagent to permit complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL per 1 mL of Trizol reagent) was added to each sample. Tubes were shaken vigorously by hand for 15 sec and incubated at 15 – 30°C for 2 to 3 min and then centrifuged at 12,000 × g for 15 min at 4°C. Following centrifugation, the aqueous phase was transferred to a fresh tube. The RNA was then precipitated in 0.5 ml of isopropyl alcohol per 1 mL of Trizol reagent and

incubated at 15 – 30°C for 10 min with centrifugation at 12,000 × g for 10 min at 4°C. The supernatant was decanted and the pellet washed with 75% ethanol. The samples were mixed by vortexing and centrifuged at 7,500 × g for 5 min at 4°C. After air-drying at room temperature, the RNA pellet was dissolved in RNAse-free water and quantified spectrophometrically and stored at −140°C.

Whole genome expression profiling of PBMC RNA

Human BeadChip (Illumina Inc., San Diego, CA) was used to interrogate baboon RNA. Complementary RNA probes were synthesized from total RNA by first synthesizing cDNA and then using the cDNA to synthesize fluorescently labeled cRNA by including biotinylated UTP in the *in vitro* transcription reaction.

Complementary RNA synthesis by *in vitro* transcription was performed using the TotalPrepTM RNA Labeling Kit (Ambion, Austin, TX) by adding 7μl nuclease free water to the cDNA pellet and then adding: 2μl 10x Transcription Buffer, 1μl 10mM NTP mix containing biotinylated UTP, 1μl 10mM GTP, 1μl 10mM CTP, 1μl 250uM UTP (12.5uM) and T7 Enzyme Mix. The reaction was incubated for 1 hour at 37°C and the cDNA template removed by adding 1μl DNase I, incubating 15 min. at 37°C and inactivating with 1μl 0.5 M EDTA. Complementary RNA (cRNA) was cleaned using a Sephadex G-50 column (Roche mini Quick Spin RNA Columns) according to manufacturer's instructions. An aliquot of cRNA was counted in a scintillation counter to determine synthesis efficiency.

Gene expression data was acquired using BeadScan software (Illumina Inc., San Diego, CA) and basic data cleaning was performed using BeadStudio software (Illumina Inc., San Diego, CA). Array data were all-mean normalized and log2 transformed using BeadStudio software (Illumina Inc., San Diego, CA). Statistical analyses of array data were performed by t-test using BeadStudio software for pair wise comparisons. Signaling pathway impact analysis (SPIA) was used to calculate impact factor (IF) and global pathway p value (http://vortex.cs.wayne.edu/projects.htm) [34].

Q-RT PCR

The target genes for Q-RT-PCR were selected according to the major PBMC and serum cytokine changes affected by maternal obesity: angiogenic (VEGF, Notch-1), inflammatory (IL-6), monocyte activation (CD14, MCP-1) and adipogenesis (adiponectin) pathways. Q-RT PCR (fetal and maternal PBMC, fetal and maternal visceral fat and placenta) was performed as previously described [35]. The specific primers and probes set were used for VEGF (#Rh02621759_m1), CD14 (#Hs02621496_s1), NOTCH1 (# Hs00413187), MCP-1 (#NT_007819), IL-6 and Adiponectin (Assays on demand, Applied Biosystems). The number of mRNA copies in each sample was calculated using a standard curve for each gene. Ribosomal 18S RNA and the rRNA Ambiprobe (Applied Biosystems) were used as an endogenous control using primers from the universal 18S system (Ambion, Austin, TX) and rRNA Ambiprobe (Applied Biosystems) in a 4:6 ratio.

Placental studies

Based on systemic (PBMC, serum and fat tissues) inflammatory changes associated with obesity we evaluated placental structural and inflammatory response. This included placental macrophage infiltration, capillarization, endoplasmic reticulum (ER) and microvillous changes. We additionally evaluated placental amminoacid transport.

Placental Immunohistochemistry

5 μm sections were cut for sodium-dependent amino acid transporter (SNAT 1, 2, and 4). 7 μm sections were cut for CD68 staining. CD68 immunostaining and quantification were

performed as previously described [36. SNAT 1, 2, and 4 immunohistochemistry was performed following manufacturers instructions. Briefly, endogenous peroxidase activity was stopped by immersing sections in 0.3% hydrogen peroxide in methanol. Antigen was retrieved by microwaving at full power for 10 minutes in DAKO target retrieval solution (pH 6.20) (DAKO Corporation, Carpinteria, CA). All sections were blocked for 30 minutes with 10% normal goat (or rabbit, depending on the primary Ab) serum in phosphate-buffered saline (PBS) and then incubated for 1 h at room temperature with the primary rabbit (or goat) polyclonal anti-human SNAT 1, 2, or 4 Ab (Santa Cruz Corporation; Cat#: SNAT1: sc-33441; SNAT2: sc-33444 and SNAT4; sc-33448) and used at various concentrations (SNAT 1, 2, 1:100, SNAT 4, 1:40) in DAKO Ab diluents with background-reducing components. Sections were washed in PBS and then further incubated with the second Ab (biotinylated goat anti-rabbit IgG or vice versa; Vector Laboratories) diluted 1:200 in 2% goat serum in PBS for 30 minutes at room temperature. Antigens were localized using 3,3′-diaminobenzidine in PBS for 8 minutes. Finally, tissues were stained with hematoxylin/methylene green and mounted. Positive and negative controls included slides incubated with and without the primary Ab and sections incubated with pre-immune rabbit serum instead of primary Ab [37]. Quantification was performed at 20× magnification, using Image J (NIH program). Six pictures were taken of each specimen (the photographer was blinded to the group) and quantification was represented as percentage of the total area of the photo stained.

Placental stereology

Placental sampling was performed as described elsewhere [26]. Briefly, at cesarean section the umbilical cord was tied in two places close to the placenta and sectioned between the ties. The placenta was removed manually followed by intravenous administration of 10 IU oxytocin. The placenta was trimmed of fetal membranes and umbilical cord and weighed. Placental volume was calculated by dividing the placental weight by specific gravity (1.03 kg/m^3). Placental samples were taken in a systematic, random manner using a transparent sheet with quadratic patterns (grid system), fixed in formalin (10% buffered formalin), and embedded in paraffin. Randomly chosen 5-μm sections were cut from each block and processed for H&E staining.

The Computer Assisted Stereology Toolbox (CAST) 2.0 system from Visiopharm (Ballerup, Denmark) was used to perform all microscopical measurements. Absolute volumes, star volumes of terminal and intermediate villi, fetal capillaries, and intervillous space were determined.

Placental transmission electron microscopy (TEM)

Tissue slices were randomly selected as described elsewhere [37]. Two slices were processed by fixation in 4% glutaraldehyde, 0.5% formaldehyde in 0.1 M Pepers buffer. Postfixation in 1% Zetterqvist's buffered osmium tetroxide occurred for 30 min followed by alcohol dehydration (70–100%). Resin embedding was performed with 1:1 propylene oxide/resin (30 min) and 100% resin (30 min) under 25 psi vacuum. TEM was performed on a JEM 1230 (MFG.JEOL) microscope. Fields of view were selected randomly and systematically. A total of 30 to 50 images per placenta were taken for microvilli (MV) and ER evaluations at 30,000× magnification. The images were analyzed using the CAST 2.0 system software. Surface densities for MV were estimated by superimposing a series of test lines in a random orientation on the histological images and counting intersects with the surface of villi and MV. Density of ER to the syncytiotrophoblast (ST) was calculated by superimposing a series of test points (9 per field of view) in a random orientation on the histological images and counting number of points in ST and ER. The villious amplification factor was calculated as a ratio of number of intersections with MV divided by number of

intersections with villous surface [37]. Additionally, diameter and length of MV [39] and ER were calculated. ER diameter was measured in 60 randomly selected ER cross sections, and the length was calculated as the average length of the five longest ER per image [38].

Placental system A amino acid transporter activity (SAA)

System A amino acid uptake was measured using the fragment method developed by Jansson and colleagues as described elsewhere [40]. Dulbecco's Modified Eagle's Medium (DMEM) containing 5.6 millimolar (mM) glucose, $NaHCO_3$, amino acids, vitamins, and minerals was used. To achieve more physiological concentrations of amino acids, DMEM/Tyrode's (Tyrode's buffer contained 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES, and 5.6 mM sucrose) medium (mixed 1:3) was used for pre-incubation and incubation during the amino acid uptake step. The pH of the Tyrode's solution was adjusted to 7.4 at room temperature, stored at −20°C, and heated to 37°C for the amino acid uptake procedure. In sodium-free Tyrode's medium, 135 mM choline chloride was replaced with NaCl. All chemicals were purchased from Sigma (St. Louis, MO). 14-C-methyl-amino-isobutyric acid (14-C-MeAIB) was supplied from University of Texas Health Science Center Radioactive Laboratory (San Antonio, Texas). The final MeAIB concentration was 10nM in both the Na and Na-free incubation media. To quantify System A amino acid transporter activity, two uptake curves were performed for each placenta. One curve was generated in Na-containing Tyrode's solution and one in Na-free Tyrode's solution. For calculation of the area under the curve, the following time points were studied, 5, 20, 40, 80, 120, and 160 minutes. The area for sodium dependent uptake was calculated from the area between the two curves. Sections of placenta were randomly taken at the time of placental processing immediately after delivery as described under "Placental Stereology". Due to the time-sensitive nature of this experiment, the sections were placed in Tyrode's/DMEM 3:1 medium during the 20 minute transport back to the laboratory at which point the caps were removed to minimize oxygen deprivation while keeping the tissue in as physiologic-similar conditions as possible. Sodium-dependent amino acid transporter activity (SAA) was evaluated by radio-labeled MeAIB uptake (picomoles per milligram villous protein per minute). The uptake aspect of the experiment was accomplished within 7 hours of placental delivery and the tissue was always kept in solution as stated. SAA was quantified as the area under the curve between the uptake curve generated by the fragments in sodium-containing solution and the uptake curve generated by the fragments in the sodium-free solution analyzed over time (5, 20, 40, 80, 120, and 160 minutes). The AUC (area under the curve) approach was used instead of a linear expression of the activity because there was much less transporter activity in the baboon placenta as compared to the human placenta (data not shown).

Serum amino acid analyses

0.1 mL of 1.5 M $HClO_4$ solution was used to remove protein from serum samples. Samples were then neutralized with 0.05 mL of 2 M K_2CO_3. The solution was centrifuged at 12,000×*g* at 4°C for 1 minute, and the supernatant was used for amino acid analysis. Amino acids were determined by HPLC (high pressure liquid chromatography) methods involving pre-column derivatization with o-phthaldialdehyde, as previously described. [41]. All amino acids in samples were quantified on the basis of authentic standards (Sigma Chemicals, St. Louis, MO) using Millenium-32 Software (Waters, Milford, MA).

Statistical analysis

Comparisons between obese and non-obese groups were made with one-tailed Student's t-tests for fetal, placental and maternal morphometry and two-tailed Student's t-tests for all other parameters. Leptin and adiponectin data were normalized using a natural logarithmic transformation before analysis. Correlation of certain variables was performed with linear

regression analysis and calculation of a Pearson's correlation coefficient. Data throughout are presented as mean ± SEM and n=4 for all data in each group. Significance was set at $p < 0.05$.

Results

Maternal morphometry and kidney pathology

The Rh index was elevated in the baboons in the obese group by 31% prior to pregnancy and 25% after cesarean section. Baboons in the obese group had seven times the perirenal fat (13.9 ± 4.6 g obese vs. 1.9 ± 0.4 g control) and nearly twice the amount of omental fat (112.1 ± 32.6 g obese vs. 58.3 ± 29.0 g control) ($p<0.05$). There was a positive correlation of Rh index to maternal leptin levels ($r= 0.87$, $p = 0.004$). However, maternal Rh index was not significantly correlated with maternal omental fat weight or fetal leptin concentrations (data not shown).

Combined kidney weight was lower (65.7 ± 2 g vs. 72 ± 2 g, respectively) in obese vs. non-obese animals ($p<0.05$). Histological preparations of the kidneys showed normocellular glomeruli in both obese and non-obese pregnant baboons. The mesangium was not significantly expanded, the basement membranes were not thickened and the capillary lumina were patent. In both groups of animals, there was minimal to mild patchy interstitial inflammation, with inflammation scores that were not significantly different between groups.

Fetal morphometry and placental measurements

Placental size and weight did not differ between the two groups with the exception of the placental thickness, which was greater in obese animals (Table 1B). Fetal BMI and ponderal index ($p < 0.05$) were higher in the obese group, while fetal weight (810 ± 20 g obese vs 770 ± 70 g control), and organ weights did not differ between the two groups (Table 1C).

Maternal and fetal biochemical parameters

There were no differences in maternal and fetal white and red blood cell counts (RBC), serum bilirubin concentrations, and transaminase activities between obese and non-obese mothers. Serum glucose (58.5 ± 7.0 vs 74.0 ± 17.0 g/dL) and cholesterol (61.8 ±3.4 vs 67.3 ± 6.6 mg/dL) in obese and non-obese mothers respectively were not different.

Maternal and fetal serum leptin, adiponectin, insulin, VEGF, IL-6, MCP-1 and TNF-α

Maternal leptin concentration was higher in obese than non-obese mothers (Fig.1Aa). Maternal insulin in obese (34.8 ± 7.5 μU/ml) and non-obese (42.4 ± 16.4 μU/ml) and IL-6 and VEGF concentrations did not differ between the two groups (Fig. 1A b,c). There was no difference in maternal MCP-1 and TNF-α concentrations between groups (data not presented). There was no difference in fetal leptin (Fig. 1Bc), insulin, VEGF, IL-6, MCP-1, or TNF-α concentrations (data not presented).

Maternal and fetal PBMC transcriptome analyses

The most significant pathways (total affected = 29) affected in fetal PBMCs were cell adhesion molecules, phosphatidylinositol, m-TOR, and VEGF (Table 2A). Changes in maternal PBMCs involved ubiquitin-mediated proteolysis, leukocyte transendothelial migration, insulin signaling with total pathways number 77 (Table 2B).

CD14, adiponectin, VEGF, MCP1, IL-6, NOTCH1 mRNA transcripts in maternal PBMCs, maternal and fetal visceral fat and placenta

CD14 expression was significantly higher in maternal visceral fat and PBMC from obese animals (Fig. 2A). NOTCH1, VEGF, IL-6 and adiponectin expressions were not different in placenta, fetal and maternal visceral fat, and maternal PBMC.

Placental SNAT 1, 2, 4 and CD 68 expression

No differences were seen in distribution and expression of SNAT 1, 2, and 4 protein in the placental villi. The number of macrophages (CD68) per cross-section of intermediate villi was significantly higher in obese than the non-obese animals (Fig. 2B).

Placental stereology

Star volumes of intervillous space ($7.85\pm 4\times10^6$ μm^3 vs. $8.59\pm 3\times10^6$ μm^3) and villous star volume ($1.58 \pm 0.7\times10^6$ μm^3 vs. $1.05 \pm 0.3\times10^6$ μm^3) were not different between non-obese and obese animals. There were no differences in absolute volume of villi (81.8 ± 6.5 vs. 79.3 ± 10.1 cm^3), capillaries (18.43 ± 3 vs. 22.05 ± 7 cm^3), or ST (50.94 ± 6 vs. 54.56 ± 5 cm^3) between the two groups.

Placental transmission electron microscopy

Microvilli were thicker and the microvillous amplification factor was less in the placenta of obese mothers compared with non-obese mothers (Fig. 3A and B). ER width (264.9 ± 46 nm vs. 266.2 ± 28 nm), length (1.5 ± 0.09 μm vs. 1.8 ± 0.2 μm), and density (to ST) (0.2 ± 0.02 vs. 0.2 ± 0.02) were not different between obese and non-obese groups.

Placental system A amino acid transporter activity

Mean System A activity was higher in the non-obese than obese mothers (73 ± 9.3 vs. 36 ± 7.8 pmol. mg $protein^{-1}$ min^{-1}) ($p<0.05$). Linear regression was used to compare maternal leptin levels and SAA in the entire group of baboons. System A activity correlated negatively with maternal leptin concentration ($r=-0.70$, $p=0.08$).

Maternal and fetal amino acid concentrations

The only significant changes in the plasma concentrations of neutral amino acids were lower maternal and fetal alanine concentrations in obese compared with non-obese pregnancies (Table 3). However, the average fetal to maternal (F:M) concentrations ratio fell from 1.62 + 0.06 in the control pregnancies to 1.51 + 0.05 in the obese pregnancies ($p<0.05$). The F:M ratio fell in all but two of the neutral amino acids – B-alanine and taurine. Among the basic and acidic amino acids the only change in either maternal or fetal blood was a rise in fetal glutamate, this rise was higher in obese baboons than in non-obese baboons (76.3±7.9 vs. 42.0±4.8 μM/L, $p=0.01$) (Fig. 1F). There were no differences between two groups in other amino acid concentrations (Table 3).

Discussion

Overweight is generally defined as weight that exceeds the threshold of a criterion standard or reference value. In the human population reference values are generally based on observed population distributions of measured weight, whereas criterion standards are based on the relation of weight to morbidity or mortality outcomes [42,43]. The pathological conditions associated with obesity in the human population are insulin resistance, hyperlipidemia, and risk of stillbirth. The same associations have been also found in baboons [20,21,44]. The incremental differences of body fat content between obese and non-obese baboons are close to those seen in humans [18]. We used an indirect index (Rh index)

of body composition developed for nonhuman primates [25] instead of BMI, since the trunk is the major place of fat deposition in baboons [20] and visceral fat deposition is a major risk factor for insulin resistance in humans [45].

Maternal systemic and local changes associated with obesity

During pregnancy the major sites of leptin and TNFα production are placenta and fat depots. The significant elevation of maternal leptin levels in the obese animals in our study is in agreement with published human data. Increased maternal leptin and inflammatory cytokines (IL-6, MCP-1, TNFα) are well described features of human obesity that could be corrected by the weight loss [46,47]. The discrepancy between elevated leptin and unchanged TNFα levels in our study is quite interesting. Kirwan et al. [48] found that despite the similarities of the sites of the synthesis and increased production of TNFα and leptin during pregnancy, these cytokines differ in the prediction of insulin sensitivity. TNFα had inverse direct correlation with insulin sensitivity. Leptin concentration did not show any predictive value [48]. Authors concluded that such differences might be due to leptin resistance in late gestation. In the context of our study one might speculate, that placental leptin and TNFα production are intact in obese animals. The unchanged MCP-1 serum concentration in obese baboons in our study is in agreement with the finding in obese human subjects [49].

The circulatory changes in obese baboons are associated with increased CD14+ transcriptional expression in maternal visceral adipose tissue in obese animals. These data are consistent with the findings in obese humans and rodents [50,51]. Increased CD14+ expression in PBMC, which we observed in our study, has been also described in maternal obesity in humans [13]. Maternal PBMC transcriptome changes in the baboons parallel changes seen in obese humans that include ubiquitin-mediated proteolysis and leukocyte transendothelial migration [52].

The kidneys in pregnant obese animals showed a mild but significant decrease in weight compared to controls. The kidney in one pregnant obese animal showed mild patchy interstitial fibrosis that most likely contributed to the decreased weight. In the other pregnant obese animals, interstitial fibrosis was not identified. In humans, obesity may contribute to the development and progression of chronic renal disease that includes glomerulosclerosis and interstitial fibrosis [53]. Our findings suggest that decreased kidney weight in pregnant obese animals may be an early indicator of renal injury and, as in humans, a subgroup of obese animals may be predisposed to developing renal lesions such as interstitial fibrosis. Interestingly, increased glomerulosclerosis was not observed when obesity was superimposed on pregnancy.

Our data demonstrate for the first time the striking similarities between the typical features of maternal obesity in humans and the baboon model of maternal obesity.

Fetal systemic and local changes associated with obesity

The fetal consequences of maternal obesity are controversial. Maternal obesity has been associated with both intrauterine growth restriction and large-for-gestational age human fetuses. In our study we did not find any changes in fetal weight but we observed an increased ponderal index in fetuses from obese mothers. These minimal morphological changes have been associated with significant fetal PBMC response to maternal obesity in our study. Urashima et al. [54] showed differences in gene expression between adult and fetal PBMC. In agreement with that data, we found that the response of fetal PBMC included 27 significantly changed pathways while in mothers 10 pathways differed. The major pathway affected in fetus was cell adhesion molecule pathway, while in mother the

most significant pathway affected by maternal obesity was ubiquitin mediated proteolysis. VEGF signaling pathways were significantly up-regulated in fetuses from obese animals in our study. VEGF-A is a potent regulator of the integrity, permeability, and proliferation of blood vessels [55]. Increased expression of this pathway might be associated with increased fetal organs capillarization and function. The vascularization of pancreatic islets for example is essential for pancreatic function and development [56]. Fetal hyperglutamatemia is another interesting finding in fetuses from obese animals. Hyperglutamatemia is a key factor in obesity development in human population and animal models of obesity [57]. Moreover, chronic fetal glutamate intoxication (either as a result of fetal hypoxia or maternal dietary overload) has been linked to the subsequent development of metabolic syndrome in later life [58]. We did not observe any correlation between maternal and fetal glutamate levels.

Placental morphological changes

While placental weight is related to maternal BMI [59], data on placental changes in obesity in human pregnancy are limited and placental weights are not always provided in publications examining the effects of obesity on human pregnancy. One report on obesity in human pregnancy observed a 12% increase in placental weight [13], while in our study although placental thickness was significantly increased by 69%, placental weight was decreased by 16%. In the baboon placenta at term, 1 g of placenta supports 4.5 g of fetal tissue, compared to 6 g in humans [60]. Placenta efficiency was not different between the groups in our study.

The placental microvillous amplification factor (Fig 3 A and B) in the pregnant baboon was at the higher end of the human range [38]. The significant shortage of the microvilli accompanied by the absence of ER changes is quite intriguing. Shorter microvilli and oxidative stress (increased ER diameter) have been described in pre-eclamptic placentas [61]. Pre-eclamsia is a pregnancy complication that is also associated with maternal obesity [62]. The normal appearance of the ER in the obese placentas indicates the absence of placental oxidative stress damage. The other mechanisms of microvillous shortening are insulin receptor activation and hypertonicity [63]. In our study the insulin level did not differ in fetuses from obese vs. non-obese animals, however, the possible increase in insulin receptors sensitivity in obese placentas needs to be elucidated.

Increased intravillous macrophage accumulation in our study is consistent with placental changes observed in humans [13,64]. The intravillous CD68+ cells are of mostly fetal origin in cases of villitis of unknown etiology [65]. The source of macrophage infiltration in maternal obesity still remains to be elucidated.

Placental functional changes

Fetal nutrient delivery depends on the complex interaction of maternal uterine and fetal umbilical blood flow, nutrient supply, placental microstructure and transport capacity. The concept of the placenta as a nutrient sensor has been recently developed by Jansson and Powell [66]. Godfrey et al. [67] showed that neutral amino acid transport in the placenta inversely correlated to size at birth. Kuruvilla et al. confirmed that placental SAA is decreased in diabetic pregnancies associated with fetal macrosomia [68].

To our knowledge this is the first report of *in vitro* placental amino acid uptake in any nonhuman primate species. We observed that the absolute amino acid uptake per gram by the baboon placenta is only 14% of that we have observed in human placental villi with the same methods (unpublished data). A potential explanation is that the baboon placenta has a smaller amount of transporter per unit placental weight than the human placenta. We did find an inverse correlation between maternal serum leptin to SAA activity. In a rodent model

of obesity mice fed a high fat diet before mating and throughout gestation become obese with increased maternal leptin and decreased serum adiponectin concentrations accompanied by fetal macrosomia. In this model transport of glucose and neutral amino acids was increased in microvillous plasma membranes [69]. There are several differences between this study and ours: the mouse placenta does not express leptin and the mouse placental structure differs from primates. It is difficult in studies of obesity, whether in animals or humans, to determine whether the observed changes are due to the obesity or the dietary composition (e.g.higher fat or carbohydrates content). Diet induced obesity exposes the fetus both to maternal obesity and an altered diet. One unique value of our study is that the dietary composition was not different between the groups.

The major findings of this study were: 1) maternal obesity in baboons and humans are similar in regard to the following obesity-induced changes: increased placental macrophage infiltration, maternal PBMC transcriptome changes, increased CD14 expression in maternal PBMC and visceral fat, maternal hyperleptinemia and kidney changes. 2) maternal obesity in baboons is accompanied by decreased placental ST amplification factor, intact ST ER structure, and decreased system A placental amino acid transport in obese animals 3) fetal serum amino acid composition (hyperglutamatemia) and PBMCs transcriptome were different in fetuses from obese compared to non-obese animals.

In summary, these data demonstrate that in obese baboons, in the absence of increased fetal weight maternal, placental and fetal phenotypes are consistent with those described for large-for-gestational age human fetuses. The decrease in system A transport is probably due to structural impairment of the ST microvilli surface in obesity. Further research is needed to understand the implications of impaired microvillous structure in obesity as well as unaffected fetal growth in the baboon model.

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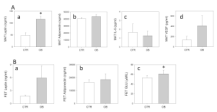

Fig. 1.

A. Maternal serum leptin (a), adiponectin (b), IL-6 (c), and VEGF (d) concentrations in non-obese (CTR, open bars, *n*=4) and obese (OB, gray, *n*=4) pregnant baboons at term (mean ±SEM, **p*<0.05 compared to non-obese group). **B.** Serum leptin (a), adiponectin (b), and glutamate (GLU) (c) concentrations in fetuses from non-obese (open bars, *n*=4) and obese (gray bars, *n*=4) mothers (Mean ± SEM, **p*< 0.05 compared to non-obese group).

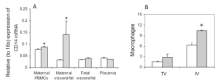

Fig. 2.

A. Relative CD14 expression in maternal PBMCs, fetal and maternal visceral fat and placenta from non-obese (open bars, *n*=4) and obese (OB, gray bars, *n*=4) pregnant baboons at term (mean±SEM, **p*<0.05 compared to non-obese group). **B.** Macrophage number in the intermediate villi (IV) and terminal villi (TV) of placentas from obese (filled bars, *n*=3) and non-obese (white bars, *n*=3) animals. CD68 immunostaining quantified by stereology (number of macrophages per villi section) (mean ± SEM, **p*<0.05 compared to non-obese group).

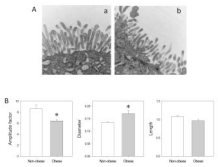

Fig. 3.

A. Representative photomicrographs of placental villi from (a) non-obese and (b) obese baboon placentas at 0.9 gestation. Magnification is 30,000×. **B.** Surface area amplification factor, microvilli length and diameter in placentas from non-obese (open bars; *n*=4) and obese (gray bars; *n*=4) mothers (**p*<0.05 compared to non-obese group).

Table 1

Morphometric characteristics of baboons enrolled in the study, placental and fetal morphometry from obese and non-obese animals (mean±SEM).

A. Maternal characteristics	**Non-Obese *n*=4**	**Obese *n*=4**
Pre-pregnancy weight (kg)	15.7 ± 1.1	18.3 ± 1.6
Pre-pregnancy Rh index (kg/m^2)	40.6 ± 3.7	53.3 ± 3.3 ^{A}
Post cesarean weight (kg)	15.2 ± 0.7	16.7 ± 1.1
Post cesarean Rh index (kg/m^2)	39.1 ± 3.2	48.7 ±1.0 ^{A}
Body length (cm)	103.9 ± 2.1	99.0 ± 3.5
Abdominal distance (cm)	20 ± 1.3	22.7 ± 1.5
Femur length (cm)	22 ± 0.9	21.5 ± 1.0
B. Placenta		
Placenta wt (g)	203.0 ± 12.5	174.9 ± 20.5
Placenta/fetal wt	0.25±0.01	0.23 ±0.02
Minimum diameter (cm)	12.1 ± 0.5	10.9 ± 1.1
Maximum diameter (cm)	13.8 ± 0.5	12.9 ± 1.8
Thickness of mid-placenta (cm)	1.3 ± 0.2	2.2 ± 0.2^A
Umbilical cord length (cm)	21.4 ± 1.1	17.9 ± 1.5
Placental efficiency	4.0 ± 0.2	3.5 ± 1.0
C. Fetus		
Heart (g)	4.4 ± 0.6	4.5 ± 0.5
Lungs (g)	23.01 ± 2.94	22.28 ± 3.63
Thymus (g)	3.42 ± 0.34	3.78 ± 0.44
Thyroids (Combined) (g)	0.29 ± 0.09	0.31 ± 0.04
Liver (g)	24.93 ± 1.57	25.36 ± 4.85
Spleen (g)	1.61 ± 0.10	1.54 ± 0.15
Pancreas (g)	0.66 ± 0.10	0.56 ± 0.22
Right Kidney (g)	2.58 ± 0.17	2.02 ± 0.23
Left Kidney (g)	2.53 ± 0.15	1.95 ± 0.23
Brain (g)	80.22 ± 1.54	76.1 ± 6.88
Right Adrenal (g)	0.15 ± 0.02	0.14 ± 0.02
Left Adrenal (g)	0.19 ± 0.02	0.18 ± 0.02
Pericardial Fat (g)	0.35 ± 0.09	0.2 ± 0.07
Right Perirenal Fat (g)	1.03 ± 0.71	0.31 ± 0.14
Omental Fat (g)	1.34 ± 0.64	1.43 ± 1.32
Gall Bladder (g)	0.49 ± 0.1	0.3 ± 0.05

^{A} $p<0.05$ compared to non-obese group.

Table 2

Pathways affected by maternal obesity in fetal and maternal PBMC in the baboons at the end of gestation.

Pathway name (KEGG)	Impact factor	p value
A. Fetal PBMC		
Antigen processing and presentation	42.5	0.000E0
Phosphatidylinositol signaling system	22.6	3.6E-9
Pathogenic Escherichia coli infection	10.3	3.7E-4
mTOR signaling pathway	10.1	14.7E-4
Epithelial cell signaling in Helicobacter pylori infection	8.2	12.6E-3
Ribosome	8.0	3.44E-3
Focal adhesion	7.6	4.4E-3
Compliment and coagulation cascades	7.2	6.0E-3
Leukocyte transendothelial migration	6.4	1.2E-2
Adherent junction	6.4	1.2E-2
Regulation of autophagy	6.3	1.3E-2
Thyroid cancer	6.1	1.6E-2
Vibrio cholerae infection	6.0	1.8E-2
Pancreatic cancer	5.8	2.0E-2
Proteasome	5.8	2.1E-2
VEGF signaling pathway	5.7	2.3E-2
Chronic myeloid leukemia	5.3	3.2E-2
B cell receptor signaling pathway	5.2	3.3E-2
Regulation of actin cytoskeleton	5.2	3.56E-2
Non-homologous end-joining	5.1	3.6E-2
Tight junction	5.1	3.7E-2
ErbB signaling pathway	5.1	3.9E-2
Ubiquitin mediated proteolysis	5.1	3.9E-2
MAPK signaling pathway	5.0	4.1E-2
Non-small lung cancer	5.0	4.1E-2
B. Maternal PBMC		
Ubiquitin mediated proteolysis	8.3	2.4E-3
Adherent Junction	7.3	5.6E-3
Ribosome	6.5	1.1E-2
Basal cell carcinoma	6.5	1.2E-2
Long-term potentiation	6.1	1.5E-2
Gap junction	5.5	1.8E-2
Bladder cancer	5.8	2.1E-2
Amyotrophic lateral sclerosis (ALS)	5.5	2.6E-2
Renal cell carcinoma	5.5	2.7E-2
Leukocyte transendothelial migration	4.9	4.7E-2

Table 3

Maternal and fetal amino acid serum concentrations from obese and non-obese animals (mean±SEM).

	Mother		**Fetus**		**Fetus/Mother**	
Amino acid	***n*=4**		***n*=4**		***n*=4**	
(μM/L)	**Non-Obese**	**Obese**	**Non-Obese**	**Obese**	**Non-Obese**	**Obese**
ASP	6.0±1.51	5.9±0.95	4.3±0.34	9.8±3.74	0.8 ± 0.17	1.5 ± 0.34
GLU	57.5±9.07	74.3±15.68	42.0±4.79	76.3±7.86^{A}	0.8 ± 0.16	1.1 ± 0.14
ASN	36.7±2.77	39.9±2.42	53.7±3.03	54.7±2.62	1.5 ± 0.14	1.4 ± 0.11
SER	108.2±13.89	104.1±17.53	176.1±28.51	142.7±19.84	1.6 ± 0.18	1.4 ± 0.15
GLN	370.1±15.49	348.8±28.88	717±32.73	625±73.43	2.0 ± 0.15	1.8 ± 0.16
HIS	73.8 ± 8.74	83.6±2.97	141.6±13.47	132.4±10.35	1.6 ± 0.09	1.4 ± 0.13
GLY	283.6±8.38	319.9±61.79	451.4±29.19	440.1±51.90	1.6 ± 0.06	1.6 ± 0.18
THR	94.3±8.84	113.8±3.10	151.1±14.87	180.2±24.92	1.6 ± 0.11	1.5 ± 0.14
CTT	22.2±3.46	24.9±5.39	33.8±2.79	34.7±4.82	1.0 ± 0.11	1.5 ± 0.25
ARG	62.3±10.94	68.3±5.01	114.9±10.6	140.3±11.99	1.6 ± 0.30	1.8 ± 0.19
β-ALA	7.5±0.58	6.5±1.59	7.7±0.34	8.4±0.64	1.7 ± 0.09	1.6 ± 0.17
TAU	120.0±17.27	113±17.43	177.8±20.44	189.5±7.09	1.6 ± 0.23	1.4 ± 0.09
ALA	304.6±5.96	240.3±8.46^{A}	524.4±23.83	387.3±42.73^{A}	2.2 ± 0.20	1.9 ± 0.18
TYR	33.8±2.21	29.2±2.49	55.9±9.82	40.3±5.09	1.8 ± 0.13	1.6 ± 0.15
TRP	19.3±2.6	21.4±0.86	43.5±8.73	40.1±3.61	1.6 ± 0.13	1.4 ± 0.12
MET	23.7±2.18	24.0±1.03	42.7±4.68	37.7±2.75	1.6 ± 0.19	1.5 ± 0.04
VAL	123.4±3.66	126.1±10.24	196.6±17	176.6±10.6	1.6 ± 0.21	1.3 ± 0.11
PHE	47.3±3.76	43.3±2.60	76.7±15.28	66.8±1.60	1.3 ± 0.28	1.1 ± 0.06
ILE	56.5±2.55	56.4±4.94	87.5±9.59	70.7±3.10	1.6 ± 0.21	1.3 ± 0.11
LEU	72.9±6.51	82.6±6.56	95.0±16.95	91.2 ±5.80	1.3 ± 0.28	1.1 ± 0.06
ORN	8.0±1.85	10.1±1.44	29.84±6.15	41±12.93	1.9 ± 0.26	2.1 ± 0.25
LYS	142.7±12.01	131.2±10.92	419.1±23.31	314.7±37.82	1.9 ± 0.04	1.6 ± 0.14

^{A} $p<0.05$ compared to non-obese group.