Original Article

MOLECULAR METABOLISM

Insulin increases placental triglyceride as a potential mechanism for fetal adiposity in maternal obesity

Anika K. Anam [1], Katherine M. Cooke [2], Milana Bochkur Dratver [2], Jane V. O'Bryan [2], Lauren E. Perley [2], Seth M. Guller [2], Janice J. Hwang [1], Hugh S. Taylor [2], Leigh Goedeke [1], Harvey J. Kliman [2], Daniel F. Vatner [1], Clare A. Flannery [1,2,*]

ABSTRACT

Objective: Maternal obesity increases the incidence of excess adiposity in newborns, resulting in lifelong diabetes risk. Elevated intrauterine fetal adiposity has been attributed to maternal hyperglycemia; however, this hypothesis does not account for the increased adiposity seen in newborns of mothers with obesity who have euglycemia. We aimed to explore the placental response to maternal hyperinsulinemia and the effect of insulin-like growth factor 2 (IGF-2) in promoting fetal adiposity by increasing storage and availability of nutrients to the fetus.

Methods: We used placental villous explants and isolated trophoblasts from normal weight and obese women to assess the effect of insulin and IGF-2 on triglyceride content and insulin receptor signaling. Stable isotope tracer methods were used *ex vivo* to determine effect of hormone treatment on *de novo* lipogenesis (DNL), fatty acid uptake, fatty acid oxidation, and esterification in the placenta.

Results: Here we show that placentae from euglycemic women with normal weight and obesity both have abundant insulin receptor. Placental depth and triglyceride were greater in women with obesity compared with normal weight women. In syncytialized placental trophoblasts and villous explants, insulin and IGF-2 activate insulin receptor, induce expression of lipogenic transcription factor SREBP-1 (sterol regulatory element-binding protein 1), and stimulate triglyceride accumulation. We demonstrate elevated triglyceride is attributable to increased esterification of fatty acids, without contribution from DNL and without an acceleration of fatty acid uptake.

Conclusions: Our work reveals that obesity-driven aberrations in maternal metabolism, such as hyperinsulinemia, alter placental metabolism in euglycemic conditions, and may explain the higher prevalence of excess adiposity in the newborns of obese women.

Keywords Esterification; Fatty acids; Insulin; Macrosomia; Obesity

1. INTRODUCTION

Maternal obesity is common. Almost 40% of reproductive-age women in the United States have obesity and more than 20% of reproductive-age women will have obesity globally by 2025 [1]. Maternal obesity is associated with excess adiposity in newborns [2–6]. Excess neonatal adiposity is a risk factor for the development of obesity and type 2 diabetes (T2DM) in childhood and adulthood [7–12]. While there is a clear relationship between higher maternal pre-pregnancy weight and obesity in offspring, the links between a dysfunctional intrauterine environment and excess neonatal adiposity remain poorly understood. Studying how maternal obesity alters the intrauterine environment may be key to understanding the mechanisms underlying the intergenerational transmission of obesity and diabetes.

Insulin is an anabolic hormone that drives the storage of excess energy, mainly triglyceride (TG), in white adipose tissue. Six decades ago, large newborns were documented in women with insulin deficiency or type 1 diabetes (T1DM). To explain this phenomenon, Pedersen hypothesized that high levels of maternal glucose pass through the placenta to the fetus and stimulate the fetal pancreas to secrete more insulin [13]. With the influx of glucose and fetal insulin, the fetal liver converts glucose to TG by *de novo* lipogenesis (DNL), and TG accumulates in fetal adipose. This glucose-centric model is successfully applied in pregnant women with T1DM, T2DM and gestational diabetes, in whom glucose-lowering techniques prevent neonatal overgrowth and excess adiposity [14]. However, this model has several weaknesses and does not adequately address excess neonatal adiposity in offspring of obese women who have normoglycemia or only intermittent mild hyperglycemia. It discounts any role of maternal insulin, which increases in the third trimester of pregnancy and is further elevated in obesity [15]. Additionally, DNL is minimal in the fetal liver, and fetal mitochondrial capacity for fatty acid oxidation is limited [16,17]. Thus, lipids are more likely to provide substrate for fetal fat accretion than glucose. TG levels increase with each trimester of

[1]Section of Endocrinology, Department of Internal Medicine, Yale School of Medicine, New Haven, CT, USA [2]Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT, USA

*Corresponding author. Yale School of Medicine, Department of Obstetrics, Gynecology, and Reproductive Sciences, 333 Cedar Street, PO Box 208063, New Haven, CT 06520, USA. E-mail: clare.flannery@yale.edu (C.A. Flannery).

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pregnancy, with normative ranges of 40–159 mg/dL in the first trimester, 75–382 mg/dL in the second trimester, and 131–453 mg/dL in the third trimester [18]. By the third trimester when fetal fat accretion is at its highest, maternal TG levels increase by two-fold [19]. Finally, the Pedersen hypothesis interprets the placenta as a passive player in nutrient flux, ignoring the placenta's capability to regulate nutrient metabolism and to respond to maternal and fetal signals.

A myriad of maternal and placental metabolic signals is present throughout pregnancy, including insulin, insulin-like growth factor 2 (IGF-2), and insulin-like growth factor 1 (IGF-1). The effect of maternal insulin on the placenta is poorly defined since most glucose uptake is through insulin-independent glucose transporters (GLUT) and there are conflicting reports on the location of insulin receptor in the placenta [20]. Maternal IGF-1 has been shown to stimulate fetal growth by increasing the transfer of nutrients to the fetus acting through placental IGF-1 receptor, and is secreted by the cytotrophoblast and syncytiotrophoblast at all stages in gestation [21,22]. IGF-2 is a paracrine hormone secreted by placental syncytiotrophoblasts and encoded by an imprinted gene of paternal origin [23]. Since IGF-2 regulates placenta size and fetal growth, in part by controlling nutrient flux to the fetus, and activates placental IGF-1 receptor and the mitogenic insulin receptor A isoform (IR-A) [24–26], it is an ideal hormone to study alongside insulin and IGF-1.

Placental metabolism remains poorly understood, due to challenges posed by different placentation in animal models. Here we use placental villous explants and isolated trophoblasts from normal weight and obese women as models to investigate how the placental response to maternal hyperinsulinemia may promote fetal adiposity by increasing storage and availability of nutrients to the fetus. We examined the effect of insulin and IGF-2 on triglyceride content *ex vivo* in conjunction with stable isotope and radioisotope techniques to investigate placental lipid metabolism. Our study reveals that insulin and IGF-2 stimulate triglyceride accumulation *ex vivo* via esterification, and not DNL. These results favor a role of maternal fatty acids and triglyceride over glucose for energy storage in the intrauterine environment.

2. METHODS

2.1. Study design

We studied two cohorts of pregnant women. In the first cohort, we studied de-identified clinical data and placenta tissue from women enrolled in a pregnancy study, through the Yale University Reproductive Sciences (YURS) Biobank. Collection was approved by the Yale University Human Investigations Committee. We enrolled women into two groups: normal weight (NW, BMI 18.5–24.9 kg/m^2) and obese (defined as class 1 obesity, OB, BMI 30–35 kg/m^2), as determined from pre-pregnancy BMI. Gestational diabetes (GDM) screening was determined using the two-step approach, in which a 1-h oral glucose tolerance test with 50 g glucose was performed, and if glucose was above the threshold ($\geq$135 mg/dL), diagnosis was made per Carpenter and Coustan criteria using a 3-h oral glucose tolerance test (100 g oral glucose solution) [27]. All women included in the study had a negative screening test. Inclusion criteria included delivery by scheduled cesarean section (CS) and gestational age of 38.5–39.9 weeks. Exclusion criteria included any trial of labor prior to CS, history of pre-pregnancy Type 1 or Type 2 diabetes mellitus, use of glucose-lowering or lipid-lowering therapy, hypertension, or pre-eclampsia during pregnancy. The study population for this cohort comprised of 28 NW women of mean age 33.8 $\pm$ 0.8 years and mean BMI 22.0 $\pm$ 0.3 kg/m^2 and 19 OB women of mean age 32.1 $\pm$ 1 years and mean BMI 31.9 $\pm$ 0.4 kg/m^2. In a second cohort, we evaluated maternal levels of hormones and nutrients in NW (n = 9) and OB women (n = 5). Fasting maternal serum was obtained at the third trimester of pregnancy and was drawn on the day of scheduled CS. Clinical data were collected on maternal age, BMI, race/ethnicity, gravidity/parity, and pregnancy complications. Neonatal data included gestational age, gender, birth weight, neonatal hypoglycemia, congenital abnormalities, and medical conditions diagnosed in first 28 days after birth.

2.2. Isolation and culture of placental explants and primary trophoblasts

Placentae were processed within 30 min of delivery and placed on ice. Placentae were weighed with the umbilical cord and membranes attached using a digital scale. Placental weight was recorded in grams. Placental thickness was recorded in units of 0.1 cm by piercing the chorionic plate with a wooden toothpick and measuring against a ruler. Chorionic membranes and maternal decidua layers were removed. Placenta were placed in RNAlater® (RL) or flash frozen (FF), then stored at −80 °C. For *ex vivo* experiments including RNA expression analysis, full thickness placental samples from multiple cotyledons were obtained while avoiding calcified or hypoperfused cotyledons. Large villous tissue samples were dissected into small 50 mg (5 mm) pieces and rinsed in cold phosphate buffered saline (PBS). Then, ~400 mg of placental villous explants was transferred to a six-well plate containing Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies, Carlsbad, CA) with 5.5 mM glucose, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% amphotericin B, and incubated at 37 °C with 5% CO_2–95% O_2, as modified from prior studies [28,29]. For *ex vivo* and *in vitro* experiments, the glucose level in the culture media was 5.5 mM (equivalent to 99 mg/dL), which was intentionally selected to reflect normoglycemic conditions in pregnancy. For *in vitro* experiments, cytotrophoblasts were isolated using the protocol described by Lee et al. [30]. Syncytiotrophoblasts were generated by culturing cytotrophoblasts for 72 h, leading to spontaneous differentiation as initially described by Kliman et al. [31].

2.3. Hormone treatment

After 24-h incubation, placental villous explants were starved in serum-free, phenol-free DMEM (with 5.5 mM glucose) for 4 h at 37 °C. Placental explants were treated with insulin (100 nM), IGF-2 (100 nM), IGF-1 (100 nM), or vehicle in phenol-free DMEM containing 10% charcoal-stripped serum and 5.5 mM glucose. As determined by mass spectrometry, the treatment medium contained 3 mg/dL triglyceride and 10 μmol/L nonesterified fatty acids. After 30 min, 6 h, or 48 h of hormone stimulation, placental villous explants were collected for triglyceride, protein, or mRNA quantification and analysis. In addition, placental explants from NW women (n = 8–9) were cultured in media containing different levels of glucose (5.5 mM [99 mg/dL], 10 mM [180 mg/dL] or 25 mM [450 mg/dL]) for 24 h, starved, and treated with insulin (100 nM), IGF-2 (100 nM), or vehicle. Separately, a group of placental explants from NW women (n = 7) and OB women (n = 6) were cultured for 24 h, starved, and then treated for 48 h with 0.1 nM, 1 nM, 10 nM, or 100 nM of insulin, or vehicle to assess tissue response to insulin stimulation.

2.4. RNA isolation and qRT-PCR analysis

For whole placenta tissues, tissue was homogenized in TRIzol® (Life Technologies). RNA was isolated with chloroform, precipitated with isopropanol, washed twice with 75% ethanol, and dissolved in RNase-free water. RNA was treated with RNase-free DNase I and purified via

RNeasy spin columns. For *in vitro* studies, RNA was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA). The purity and concentration of the isolated RNA was assessed using a Nanodrop™ 2000 Spectrophotometer (Thermo Scientific). RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). For each sample, 12.5 ng of cDNA template was amplified in triplicate using iQ™ SYBR® Green Supermix on the MyiQ™ Real-Time PCR Detection System (Bio-Rad). PCR primers were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast, NCBI, Bethesda, MD), and synthesized at W.M. Keck Foundation Oligo Synthesis Resource (Yale University, New Haven, CT). Optimal primer concentrations were determined by calculation of primer efficiency using Human XpressRef™ Universal Total RNA (Qiagen), and primer specificity was assessed by melting curve analysis. Supplementary Table 1 summarizes the primer sequences, concentrations, and efficiency for each assay. Each qRT-PCR assay included non-amplification and water controls. Gene expression levels were normalized to β-actin and β-actin levels remained stable across treatment groups.

2.5. Protein extraction

Protein was isolated from TRIzol®-chloroform fraction following RNA isolation. Protein was isolated with ethanol, precipitated with isopropanol, and washed with 0.3 M guanidine in 95% ethanol. The protein pellets were re-dissolved by sonication in 1:1 solution of 1% SDS and 8 M urea, containing PIC and PMSF. Insoluble material was separated from soluble protein by centrifugation. The protein concentration in each lysate was quantified using the Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) and the Bio-Rad iMark Microplate Absorbance Reader.

2.6. Western blot analysis

Proteins were separated on 4–12% SDS-PAGE gels (Bio-Rad), transferred onto Amersham™ Hybond™ polyvinylidene fluoride membranes (GE Healthcare), incubated with antibodies, and developed using Amersham™ ECL Advance (GE Healthcare). Immunoprecipitation for Western blot analysis of phosphorylated IR was performed using Dynabeads Protein A (Invitrogen, Thermo Fisher Scientific, Waltham, MA) for cell lysate pre-clearing, IR total for formation of immunocomplex, and then isolation of the immunocomplex with magnetic bead slurry. Antibodies to phosphorylated and/or total forms of AKT, β-Actin, and GAPDH were from Cell Signaling Technology (Danvers, MA), as previously cited [32]. Antibodies to phosphorylated and total forms of S6, were obtained from Cell Signaling Technology (Danvers, MA). Antibodies to the total form of IR-β C-terminal, SREBP-1, PPARγ, and GPAM (GPAT1) were obtained from Santa Cruz Biotechnology (Dallas, TX). Antibodies to DGAT1 and DGAT2 were obtained from Abcam (Waltham, MA). Antibodies to GPAT3 were obtained from Invitrogen – Thermo Fisher Scientific (Waltham, MA) (Supplementary Table 2). Densitometry was performed for immunoblots using ImageJ software, version 1.44 (National Institutes of Health, Bethesda, Maryland). Densitometry was performed for immunoblots using ImageJ software, version 1.44 (National Institutes of Health, Bethesda, Maryland).

2.7. Immunohistochemistry

Placenta tissue specimens were fixed in 10% neutral buffered formalin for at least 1 day and embedded in paraffin, after which 5-μm sections were placed on coated glass slides designed for immunohistochemistry (IHC) processing. Hematoxylin and eosin-stained slides were also produced for histological examination of each placenta. Normal rabbit serum (R9133, used at 1 μg IgG/mL; Sigma–Aldrich) was used as a negative control. Placenta tissue specimens from 11 NW women and 6 OB women were blindly screened with anti-insulin receptor antibody, and subsequently, one representative from each group was selected for performing additional markers. Antibodies and concentrations used are listed in Supplementary Table 3. Serial sections (5 microns each) were immunohistochemically stained using EnVision+ horseradish peroxidase Rabbit DAB+ (K4011; Dako Agilent, Santa Clara, CA) to mark the sites of antibody binding with a brown deposit as previously described by Kliman et al. [33]. Antibody concentrations were chosen to produce strong staining in the positive cellular structures without background staining.

2.8. Triglyceride assay

Total tissue lipid was extracted from 150 to 200 mg placental explant tissue with chloroform:methanol [2:1 volume-to-volume ratio (v/v)] using a modified Bligh and Dyer method [34]. Following a 48-h hormone treatment, placental villous tissue was homogenized in chloroform:methanol using a bead mill homogenizer (Tissue Lyser, Qiagen, Germantown, MD). Phase separation was achieved by adding sulfuric acid, and the organic layer was retrieved and dried under a nitrogen stream. Triglyceride content was quantified using the Sekisui Triglyceride-SL Kit (Burlington, MA) and a spectrophotometer (Bio-Rad Smartspec™ Plus, Hercules, CA) in duplicate. Explant triglyceride content was normalized to explant weight.

De novo lipogenesis measurement. Placental villous explants (~400 mg) were transferred to a six-well plate and incubated in DMEM with 10% FBS, 5.5 mM glucose, 1% penicillin/streptomycin, and 1% amphotericin B, and incubated at 37 °C with 5% CO_2—95% O_2 for 24 h. Next, placental villous explants were starved in serum-free, phenol-free DMEM supplemented with 5.5 mM glucose for 4 h at 37 °C. Placental explants were treated with insulin (100 nM), IGF-2 (100 nM), IGF-1 (100 nM), or vehicle in phenol-free DMEM supplemented with 5.5 mM glucose and enriched with 5% deuterium (^2H_2O, 99.9%, Cambridge Isotope Laboratories, Tewksbury, MA) and 10% charcoal-stripped serum. The 5% ^2H_2O-enriched media was changed every 12 h for the duration of the 48-h hormone treatments. Placental explants were harvested on dry ice and then stored at −80 °C. Aliquots of media were saved from the final time point for all samples. Triglycerides were extracted into chloroform as described above.

Fatty acid stable isotope enrichment by GC–MS. Explant lipid samples were spotted onto Silica gel 60 plates, and thin layer chromatography (TLC) was performed with a mobile phase of hexane:diethyl ether:-acetic acid (80:20:1). Plates were developed with 0.005% primuline in acetone:water (80:20). Purified samples were collected while absorbed to Silica and eluted with diethyl ester. Triglyceride-fatty acids were analyzed by GC–MS (5975CI, Agilent Technologies, Santa Clara, CA, USA) as fatty acid methyl esters following derivatization with methanolic boron trifluoride.

2.9. Plasma deuterium enrichment

The plasma ^2H_2O pool was assessed by exchange of hydrogens from plasma to acetone in the presence of sodium hydroxide; acetone deuterium enrichment was analyzed by GC–MS.

2.9.1. Calculation of % DNL

The calculation of *de novo* palmitate synthesis from isotopic data has been previously described [35–37]. The fraction of the placental explant palmitate pool newly synthesized during the experiment (F) was calculated as $F = ME \div (N \times p)$, where molar enrichment (ME) equals $m1 + (2 \times m2)$, where m1 and m2 are the atom percent enrichments (APEs) of singly and doubly deuterium-labeled palmitate; N is the number of exchangeable hydrogen atoms in a palmitate molecule, and p is the plasma APE of deuterium in water.

2.9.2. Measurement of triglyceride synthesis by fatty acid esterification

Triglyceride biosynthesis by esterification of preformed fatty acids was assessed by incorporation of ^{13}C-16 palmitate into placental villous explant triglyceride. Potassium U-^{13}C-16 palmitate (Cambridge Isotope Laboratories, Tewksbury, MA) was conjugated to bovine serum albumin (BSA), in a 7% BSA/5 mM ^{13}C-16 palmitate (~5:1 molar ratio) solution following a previously published protocol [38]. Placental explants were incubated in 5.5 mM glucose DMEM with 10% FBS for 24 h, then starved in serum-free, phenol-free 5.5 mM glucose DMEM for 4 h at 37 °C. The explants were placed in phenol-free DMEM (5.5 mM glucose) labeled with 0.1 mM palmitate and treated with insulin (100 nM), IGF-2 (100 nM), or vehicle for 0 (no tracer), 1, 2, 4, and 6 h. Aliquots of media were saved at the end of each time point, and explant tissue was weighed at the end of each experiment and flash frozen. Triglycerides were extracted into chloroform as described above and explant lipid samples were analyzed as indicated in 'Fatty acid stable isotope enrichment by GC–MS.'

2.10. Fatty acid uptake assay in placental explants

Fatty acid uptake in placental explants was performed as previously described with the following modifications [39,40]. Briefly, six-well plates containing placental explants were incubated in 5.5 mM glucose DMEM. Then, 1 mL assay buffer containing 200 μM palmitate, 20 μM BSA, 0.5 μCi/mL 1-^{14}C palmitic acid (Perkin Elmer) and hormone treatment (100 nM vehicle, insulin, or IGF-2) were added to each well and the explants were incubated for nine different timepoints, ranging from 5 min to 24 h at 37 °C. Assays were stopped by adding ice cold stop buffer (500 μM phloretin and 0.1% BSA in PBS) and washing the placental explants three times. The villous explants were suspended in water, kept on ice, and homogenized using a bead mill homogenizer (Tissue Lyser, Qiagen, Germantown, MD). Then, tissue homogenate was transferred into scintillation vials and analyzed by scintillation counting. All assays were corrected for the tissue weight per assay.

2.11. Fatty acid oxidation assay in placental explants

Ex vivo placental fatty acid oxidation experiments were performed as previously described with minor modifications [41,42]. For each placenta, there were 3–4 experimental replicates which were representative of at least 2 technical replicates per condition. Briefly, fresh placental villous explants were transferred to glass vials containing 2 mL oxygenated Krebs–Ringer bicarbonate buffer (KRBB) supplemented with 2.6% $NaHCO_3$ (w/v), 100 mM $CaCl_2$ (w/v), and 0.5% fatty acid free BSA (w/v), pH 7.4 for 1 h at 35 °C, 70 RPM. Following incubation, explants were transferred to vials containing 1.5 mL of the above media supplemented with 100 μM sodium palmitate, 200 μM carnitine, and 0.2 μCi/mL [1-^{14}C] palmitic acid [Perkin Elmer, Waltham, MA]. Explants were then treated with 100 μM Etomoxir, 100 nM insulin, 100 nM IGF2 or vehicle control and incubated for 2 h at 35 °C, 70 RPM. Etomoxir is a commonly used inhibitor of fatty acid oxidation through its irreversible inhibition of carnitine palmitoyl-transferase 1a (CPT1a) and was used as a positive control, since fatty acid oxidation is expected to decrease with etomoxir. Following incubation, placental tissue was removed, blotted (to remove excess media), weighed and snap frozen in liquid nitrogen. Vials were subsequently tightly capped and media was acidified with 0.5 mL of 2 N HCl. $^{14}CO_2$ was trapped in Eppendorf tubes containing 0.3 mL 2 N NaOH for 2 h at 40 °C, 120 RPM. The $^{14}CO_2$ absorbed in the NaOH solution was quantified by liquid scintillation counting. Rates of fatty acid oxidation were determined as a function of $^{14}CO_2$ produced over time and normalized to explant weight.

2.12. Statistical analysis

Triglyceride content was normalized to tissue/explant weight and analyzed by log transformation and mixed effects analysis with Dunnett's multiple comparisons test, using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Protein densitometry data was normalized to total protein, β-actin, or GAPDH. Gene expression was normalized to β-actin. Data was log-transformed, analyzed by one-way ANOVA or by two-tailed Student's t-test, and represented as fold-change per vehicle. Significance was defined as $P < 0.05$.

2.13. Study approval

De-identified clinical data and placenta tissue from women was collected through the Yale University Reproductive Sciences (YURS) Biobank. Collection was approved by the Yale University Human Investigations Committee (approval number 2000023300). For the second cohort, collection of maternal serum and cord blood was approved by the Yale University Human Investigations Committee (approval number 1301011387). Written informed consent was obtained from all women prior to participation.

3. RESULTS

3.1. Obesity increases placental depth independently of changes in placental weight

To evaluate the effect of insulin on placental lipid metabolism, we recruited a cohort of normal weight (NW, $n = 28$) and obese (OB, $n = 19$) women without history of diabetes, undergoing elective cesarean section. Mean maternal age, maternal race/ethnicity, parity, and gestational age at delivery were similar between the two groups (Table 1). Per study design, the pre-pregnancy BMI and BMI at delivery were significantly higher in the OB group compared to the NW group ($P < 0.0001$ for both, Table 1). All glucose levels at 1-h during oral glucose tolerance testing were in euglycemic range, and mean glucose was not significantly different between NW and OB women (Table 1). In

Table 1 – Maternal and neonatal characteristics of study population.

	Normal weight	Obese	*P* value
Maternal characteristics			
n	28	19	
Maternal age (years)	33.8 ± 0.8	32.1 ± 1	0.17
Race, n (%)			0.23
Asian	2 (7)	0 (0)	
Black	2 (7)	3 (16)	
Caucasian	21 (75)	13 (68)	
Hispanic	0 (0)	2 (11)	
Other/Unspecified	3 (11)	1 (5)	
Parity	1.3 ± 0.2	1.6 ± 0.3	0.37
BMI, pre-pregnancy (kg/m^2)	22.0 ± 0.3	31.9 ± 0.4	<0.0001
BMI, at delivery (kg/m^2)	28.0 ± 0.5	36.0 ± 0.5	<0.0001
Glucose, 1 h OGTT (mg/dL)	116 ± 5	124 ± 11	0.37
Placenta weight (g)	691 ± 29	739 ± 44	0.36
Placenta depth (cm)	2.3 ± 0.1	2.6 ± 0.1	<0.05
Neonatal characteristics			
n	28	19	
Sex (M/F)	16/12	10/9	0.77
Gestational age at delivery (weeks)	38.9 ± 0.1	39.2 ± 0.2	0.33
Birthweight (kg)	3.5 ± 0.1	3.6 ± 0.1	0.67
Occurrence of hypoglycemia, n (%)	0 (0)	2 (11)	0.15
Large for gestational age, n (%)	1 (4)	2 (11)	0.56

Data are mean ± SEM.
The maternal and neonatal characteristics reflect the cohort of women whose de-identified clinical data and placenta tissue were obtained (total $n = 47$).
Abbreviations: OGTT, oral glucose tolerance test. Large for gestational age defined by weight >90%ile for gestational age.

a second cohort, fasting serum glucose, fasting insulin, and HOMA-IR were significantly higher in OB women (74 ± 2 vs 87 ± 4 mg/dL, 7.4 ± 1 vs 23.8 ± 6 mIU/L, 1.4 ± 0.3 vs 4.5 ± 1.4, $P<0.01$, <0.01, and <0.02 respectively). Neither fasting maternal nonesterified fatty acids (NEFA) nor triglyceride (TG) levels were different between the groups (Figure 1). All women in the NW and OB groups had serum TG levels within the expected laboratory range for the 3rd trimester. Finally, birthweight and frequency of neonatal hypoglycemia did not differ in the offspring of NW and OB women (3.5 ± 0.1 vs 3.6 ± 0.1 kg, 0% vs 11%, P = NS) (Table 1). Demographic information about the study cohort is summarized in Table 1.

Placenta depth was significantly higher in the OB group (2.2 ± 0.1 vs 2.6 ± 0.1 cm, $P < 0.05$), but there were no differences in placenta weight between the groups (691 ± 29 vs 739 ± 44 g, P = NS, respectively) (Figure 2A–B). Since IGF-2 directly affects placental size and fetal growth by controlling placental supply of, and genetically driven demand for maternal nutrients [24–26], we examined placental mRNA expression of IGF-2 and IGF2-R. Although we found that placenta depth was significantly increased in the OB group, this was not associated with differences in IGF-2 or IGF-2R in the placentae of NW and OB women (0.675 ± 0.07 vs 0.651 ± 0.07, 0.029 ± 0.009 vs 0.034 ± 0.009, respectively, P = NS) (Figure 2C–D).

3.2. Insulin receptor abundance did not differ between NW and OB placentae

To assess placental receptor content, we evaluated it at a morphological level by immunohistochemistry, and quantitated its abundance by qPCR and immunoblotting. Immunohistochemistry was performed in a subset of whole placental tissue from NW ($n = 11$) and OB ($n = 6$) women, and the slides were evaluated in a blinded fashion. The trophoblast villous tree is the functional unit of the placenta and is covered by multinucleated syncytiotrophoblasts, which are derived from fused cytotrophoblasts. The hormone-producing epithelium of the placenta, the syncytiotrophoblast, is the main barrier for movement of nutrients from the maternal to fetal circulation. Staining of term placentae from NW and OB women with anti-insulin receptor antibody revealed uniform and abundant syncytiotrophoblast surface staining (Figure 3A–D). Notably, the trophoblast layer appeared considerably thicker in placentae from OB women compared to those from NW women. To distinguish which trophoblast cell type was enlarged, placentae were stained for syncytiotrophoblast and cytotrophoblast specific markers. Staining with human placental lactogen (hPL), a hormone made only in syncytiotrophoblasts, demonstrated a markedly thickened syncytiotrophoblast layer in the placenta from OB women, compared to that from NW women (Figure 3E–F). Conversely, anti-vestigial like family member 1 (VGLL1), a marker specific for cytotrophoblasts, showed a similar amount of staining in placentae from both groups, confirming that the syncytiotrophoblast layer is hypertrophied in placentae from obese women.

Western blot analysis for total insulin receptor (IR) protein in NW and OB placentae did not reveal differences in IR protein levels (Figure 3I). The two splice variant isoforms of IR are the mitogenic IR-A and the metabolic IR-B, which are expressed with variable abundance in tissues. To evaluate the relative abundance of IR isoforms, we quantified mRNA expression and found a 60:40 ratio of IR-A to IR-B in placenta. We did not find significant differences in the mean mRNA expression of mitogenic IR-A, metabolic IR-B, and insulin-like growth factor 1 receptor (IGF1-R) in term placentae between NW and OB women (Figure 3J–L). In placentae from fasting NW and OB women, phosphorylated IR was not detected.

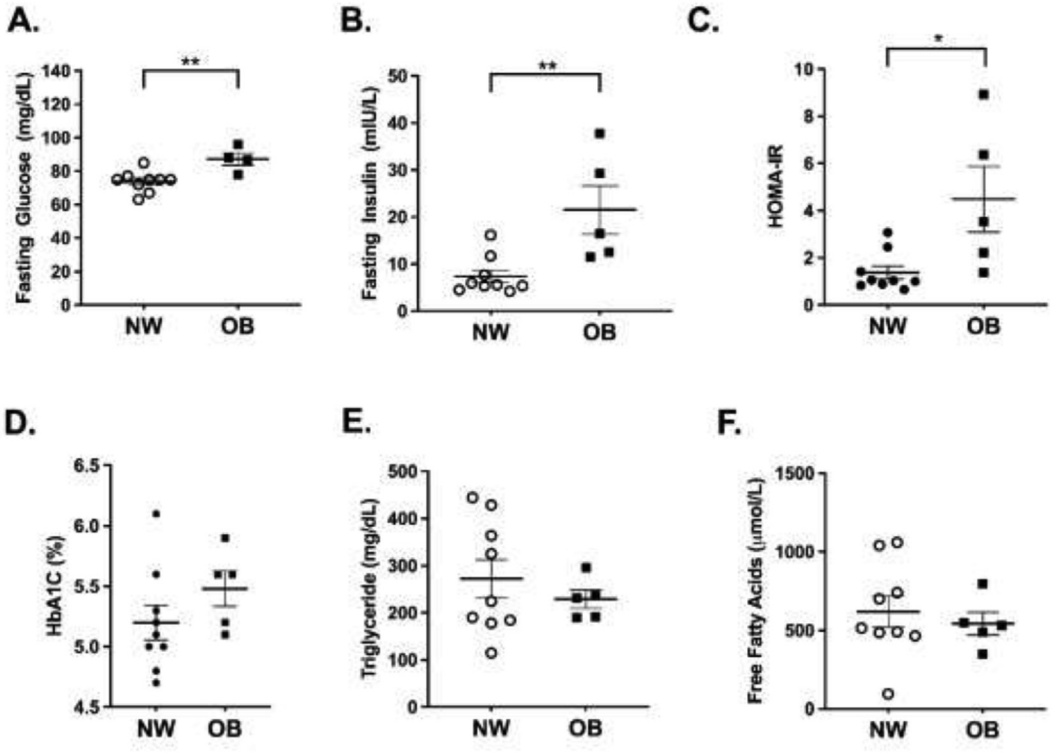

Figure 1: A second cohort of pregnant women with obesity had higher fasting glucose, insulin, and HOMA-IR relative to pregnant normal weight women. (**A**–**F**) Fasting plasma glucose (**A**), fasting plasma insulin (**B**), HOMA-IR (**C**), HbA1C (%) (**D**), fasting plasma triglycerides (**E**) and fasting free fatty acid levels (**F**) in normal weight (NW) and obese (OB) women. All women had negative screening for GDM and fasting maternal serum was obtained in the 3rd trimester of pregnancy, on the day of scheduled cesarean section. Data are mean ± SEM. The maternal serum values reflect data from a second cohort of women, $n = 4$–9 per group. $^{**}P < 0.01$, $^{*}P < 0.05$ by student's unpaired t-test.

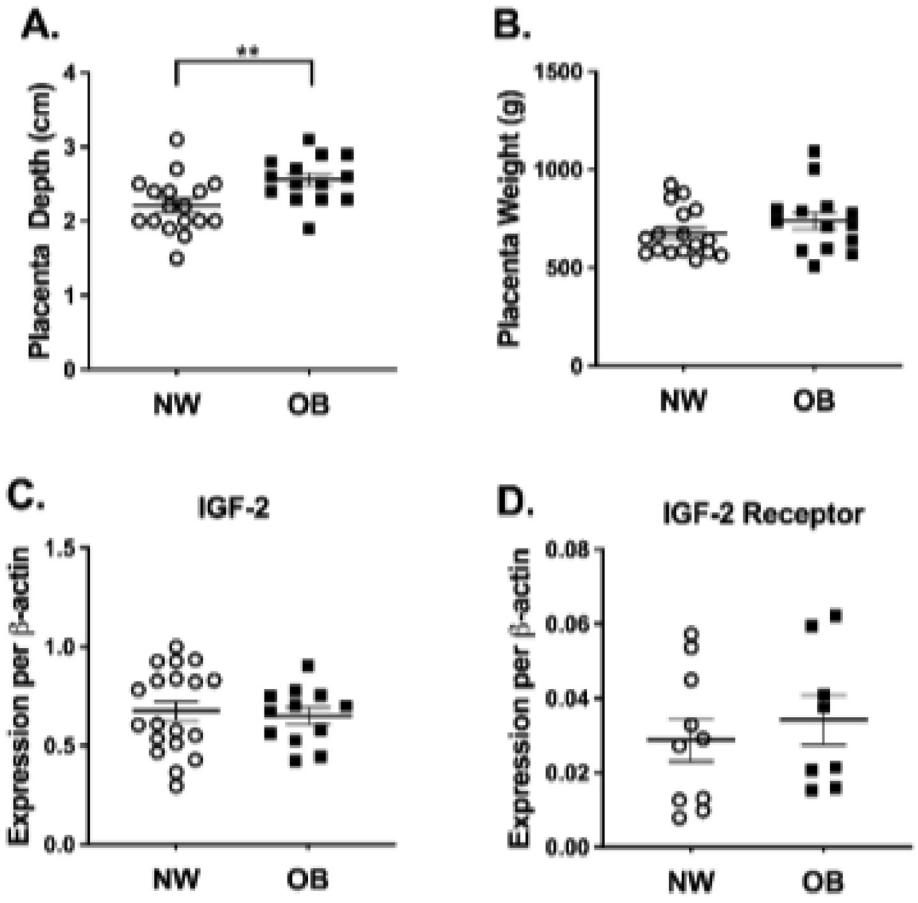

Figure 2: Placenta thickness increased in women with obesity. (**A**–**B**) Placenta depth (**A**) and placenta weight (**B**) in term placentas from normal weight (NW) and obese (OB) women. (**C**–**D**) *IGF2* mRNA expression (**C**) and *IGF2* receptor mRNA (**D**) in term placentas from NW and OB women. Data are mean ± SEM. $n = 14–17$ (**A**–**B**), 12–19 (**C**), or 8–10 (**D**) per group. $^{**}P < 0.01$ by student's unpaired *t*-test.

3.3. Women with obesity have higher placental triglyceride content

We observed that mean TG levels were 1.6-fold higher in placentae from OB women, compared with NW women (9.4 ± 0.6 vs 5.7 ± 0.5 μg/mg tissue, $P = 0.0001$, Figure 4A). Furthermore, we found a strong positive correlation between maternal BMI (kg/m^2) and placental TG content ($r^2 = 0.53$, $P = 0.0002$) (Supplementary Fig. 1). As such, placental TG content is a potential link between maternal obesity and dysfunctional placental metabolism.

3.4. Insulin and IGF-2 stimulate triglyceride accumulation in placental villous explants

We investigated the effect of different glucose levels on placental TG content in NW women *ex vivo*. We found that 25 mM glucose (equivalent of 450 mg/dL) was associated with increased TG content in both vehicle and insulin-treated placental explants (Figure 4B). Since OB women had significantly higher serum insulin levels and placental TG content compared to NW women, we sought to determine whether insulin regulates placental TG metabolism. While insulin has equal affinity for IR-A and IR-B isoforms, we also assessed the effect of IGF-2 because of its known physiological role in the placenta and its high affinity for IR-A and minimal binding to IR-B [43]. After 48-h hormone treatment in normoglycemic conditions (5.5 mM glucose), we found that insulin increased TG content by 2.5-fold in villous explants from NW women, relative to vehicle (20.6 ± 1.6 vs 8.1 ± 0.7 μg/mg tissue, $P < 0.0001$), indicating that maternal insulin regulates placental TG accumulation (Figure 4C). Similarly, in OB women, insulin stimulated a 2-fold increase in placental TG content, relative to vehicle (17.9 ± 2.2 vs 9.1 ± 1.3 μg/mg tissue, $P < 0.0001$) (Figure 4D). Interestingly, IGF-2 significantly increased placental TG content by 3.4-fold in NW women (25.3 ± 1.5 vs 8.1 ± 0.7 μg/mg tissue, $P < 0.0001$) and by 2.7-fold in OB women, relative to vehicle (24.1 ± 3.0 vs 9.1 ± 1.3 μg/mg tissue, $P < 0.0001$). IGF-1 treatment was also associated with increased TG content in explants from NW (15.2 ± 2.1 vs 8.1 ± 0.7 μg/mg tissue, $P < 0.0001$) and OB women, relative to vehicle (14.6 ± 1.9 vs 9.1 ± 1.3 μg/mg tissue, $P < 0.01$).

3.5. Placental sensitivity to insulin is preserved in OB women

During pregnancy, the placenta acts as an endocrine organ and reprograms maternal physiology to an insulin-resistant state to provide adequate nutrients for fetal growth [44]. Insulin resistance occurs in maternal liver, muscle and adipose tissue as a result of placental hormone action, and shunts metabolic substrates toward the placenta and fetus [45]. In women with obesity, insulin resistance is present prior to conception and may compound pregnancy-induced insulin resistance. Importantly, it is unclear whether the placenta becomes resistant to the action of maternal insulin in women with obesity-induced insulin resistance. To assess tissue response to insulin stimulation, we treated placental explants from NW and OB women with a dose curve of insulin (0.1 nM–100 nM) or control for 48 h. We found that TG content peaked with 1 nM insulin treatment of *ex vivo*

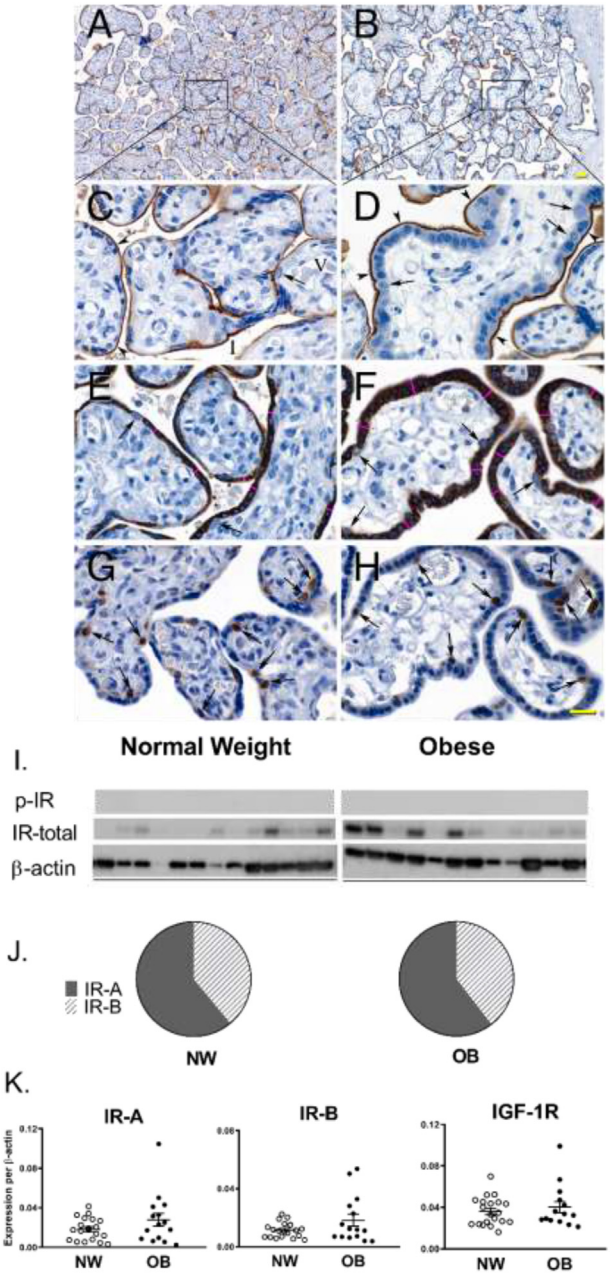

Figure 3: Placental insulin receptor expression does not differ between normal weight and obese women. (**A**—**D**) Representative insulin receptor (IR) expression as assessed by immunohistochemistry in placentae from normal weight (**A**) and obese women (**B**). Magnified fields in insets are shown in panels (**C**) and (**D**), respectively. Arrowheads denote IR staining of syncytiotrophoblast microvillus apical surface. I, intervillous space; V, villous vessel; arrows, cytotrophoblast. (**E**—**F**) Serial sections of panels (**C**) and (**D**), respectively, stained for human placental lactogen (hPL), a syncytiotrophoblast specific marker. Arrows denote cytotrophoblasts. (**G**—**H**) Serial section of fields shown in panels (**C**—**E**) and (**D**—**F**), respectively, stained with anti-vestigial like family member 1 (VGLL1) (arrows), a marker specific for cytotrophoblasts. Magnification bar = 50 μM (**A**—**B**) or 25 μM (**C**—**H**). (**I**) Western blot analysis of phosphorylated IR and total IR in placentae from NW and OB women. B-actin was used as a loading control. $n = 12$—13 per group. (**J**) Relative abundance of IR isoforms in term placentae from NW and OB women. (**K**) mRNA expression of *IR-A*, *IR-B*, and *IGF-1R* in term placentas from NW and OB women. $n = 15$—20 per group. Data are mean ± SEM.

placental explants from NW women (20.2 ± 3.3 mcg/mg tissue), while explants from OB women required the highest insulin dose of 100 nM (22.1 ± 1.7 mcg/mg tissue). In NW women, placental response to insulin plateaued at the higher doses of 10 nM (18.6 ± 3.3 mcg/mg) and 100 nM (22.8 ± 2.8 mcg/mg, P = NS respectively). The differences in TG content in placentae from both NW and OB women were variable at 100 nM insulin, but, on average, were approximately 2-fold higher compared to vehicle-treated explants from each group (22.8 ± 2.8 vs 9.6 ± 1.1 mcg/mg, 22.1 ± 1.7 vs 10.8 ± 1.7 mcg/mg, $P = 0.002$, $P < 0.001$, respectively). Overall, however, among both NW and OB women, there was heterogeneity in insulin sensitivity. On average, the placentae from OB women did not demonstrate remarkable insulin resistance (Figure 4E).

3.6. Insulin receptor signaling and mRNA expression in syncytialized trophoblasts

We next sought to identify whether insulin or IGF-2 (as a paracrine factor that activates IR) increases TG content in the placenta by activating insulin signaling pathways in primary, syncytialized trophoblasts isolated from the placenta of NW women. We found that acute stimulation with insulin significantly increased phosphorylation of IR ($P < 0.01$) and AKT ($P = 0.0005$), relative to vehicle, but no changes were seen in phosphorylated S6 in the mTOR pathway. Similarly, acute stimulation with IGF-2 resulted in increased phosphorylation of IR ($P < 0.05$) and AKT ($P < 0.01$), relative to vehicle, albeit less robustly compared to insulin stimulation (Figure 5A—B). SREBP-1 (sterol regulatory element-binding protein 1), an insulin responsive transcription factor, induces the expression of genes involved in glucose utilization and fatty acid synthesis. Chronic stimulation of placental trophoblasts with either insulin ($P < 0.05$) or IGF-2 ($P < 0.005$) significantly increased levels of the active, cleaved form of SREBP-1 protein. In addition, while chronic insulin stimulation resulted in increased PPARγ (peroxisome proliferator-activated receptor γ) protein levels ($P < 0.05$), IGF-2 had no significant effect (Figure 5C—D). Also, at 48-h, mRNA expression of *SREBF1*, the gene encoding SREBP-1, was increased by insulin ($P < 0.05$), but no significant differences were seen in the genes encoding enzymes downstream of SREBP-1, such as acetyl Co-A carboxylase (*ACACA*) and fatty acid synthase (*FASN*) (Figure 5E).

3.7. Insulin does not alter fatty acid uptake or fatty acid oxidation in placental explants

Maternal TG carried in lipoproteins are hydrolyzed to non-esterified free fatty acids (NEFA) at the placental microvillous membrane by endothelial lipase (EL) or lipoprotein lipase (LPL) [46]. NEFA are thought to enter cells by multiple mechanisms, including passive diffusion into the placental syncytium, and facilitated transport by fatty acid transporter proteins (FATP1-6) or the fatty acid translocase CD36 [47,48]. We evaluated the effect of insulin and IGF-2 on fatty acid uptake in placental villous explants. Notably, neither insulin nor IGF-2 treatment resulted in significant increases in palmitate uptake, compared with vehicle treated controls (Figure 6A). Fatty acid (FA) oxidation in the placenta may also affect lipid delivery to the fetus. In FA oxidation assays of fresh placental villous explants, we observed similar amounts of FA oxidation with vehicle, insulin, or IGF-2 treatment (Figure 6B).

3.8. Insulin and IGF-2 stimulate esterification, but not *de novo* lipogenesis in the placenta

Glucose and other small molecules are converted to fatty acids (*de novo* lipogenesis) in the postabsorptive state, and the relationship

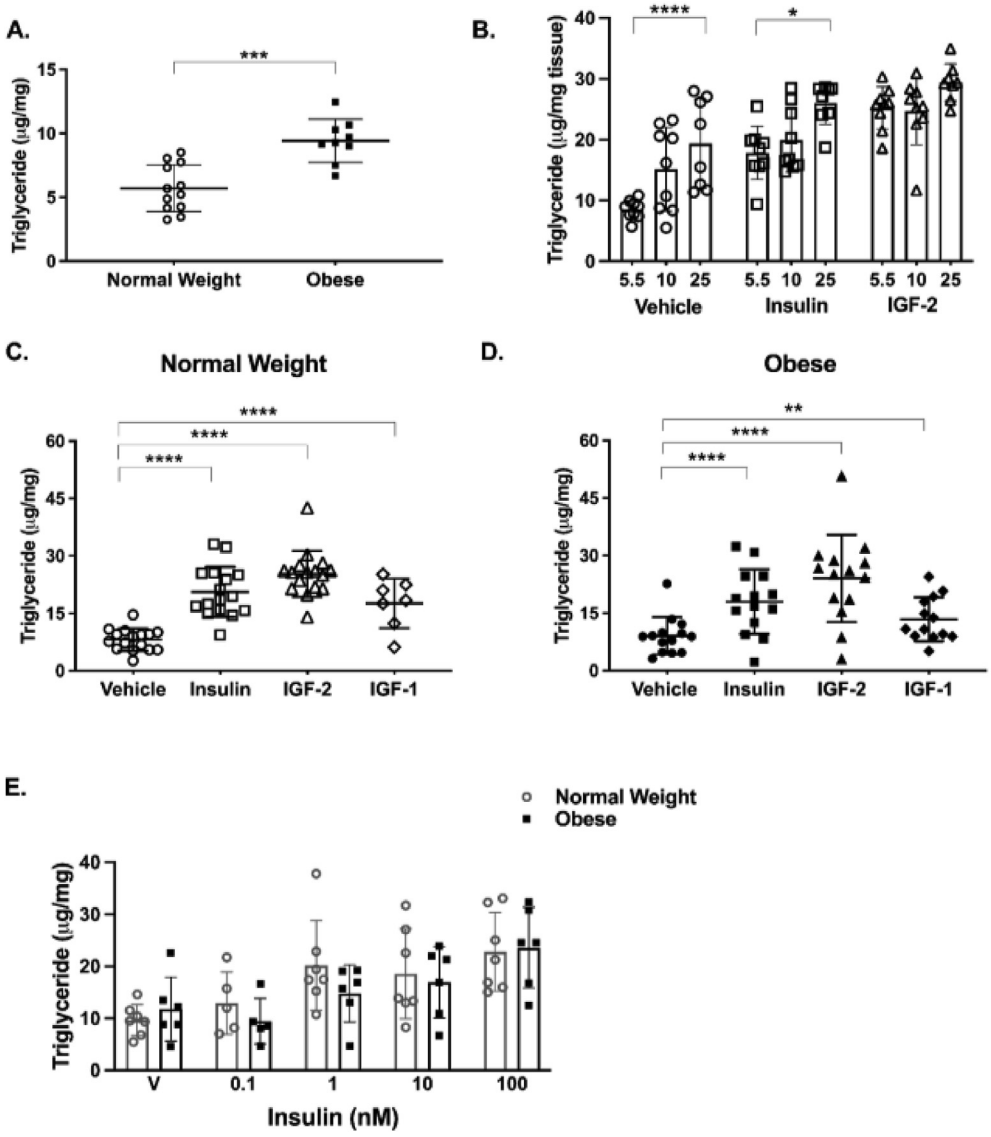

Figure 4: Insulin stimulates triglyceride accumulation in placenta villous explants. (**A**) Basal triglyceride (TG) content in placentae from normal weight (NW) and obese (OB) women. $n = 9$—12 per group. (**B**) TG content in placental villous explants from NW women cultured in media with 5.5 mM (99 mg/dL), 10 mM(180 mg/dL) or 25 mM (450 mg/dL) glucose and 100 nM insulin, 100 nM IGF-2, or vehicle control for 48 h. $n = 8$—9 per treatment group. (**C**—**D**) TG content in placental villous explants from NW (**C**) and OB women (**D**) treated with 100 nM insulin, 100 nM IGF-2, 100 nM IGF-1 or vehicle control for 48 h. $n = 14$—16 per treatment group. (**E**) TG content in NW and OB placentae treated with the indicated doses of insulin for 48 h, $n = 6$—7 per group. Data are mean ± SEM. $^{****}P < 0.0001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared to vehicle by student's *t*-test or one-way ANOVA with Dunnett's multiple comparisons test.

between lipogenic gene expression and insulin is well-established to be mediated by the transcription factor SREBP-1c. On the other hand, esterification of fatty acids to form triglycerides is primarily substrate-dependent in the liver, with insulin's role in transcription of lipogenic enzymes [49]. To gain insight into the mechanism by which insulin and IGF-2 increase TG content in placental explants, we assessed insulin's effect on rates of DNL and esterification under normoglycemic conditions in explants from NW women. DNL was assessed by incorporation of deuterium from deuterated water-enriched media into palmitate from triglyceride. Over the course of a 48-h study, essentially no deuterium was incorporated into palmitate, and calculated rates of lipogenesis were effectively zero, ranging from 0 to 0.6% (Figure 6C). In the same placental villous explants used to measure DNL, TG significantly increased with insulin, IGF-2, and IGF-1 treatment, relative to vehicle, confirming that TG accumulated over the same period of time despite negligible rates of lipogenesis (Figure 6D). In preliminary dose range studies with small groups of *ex vivo* placental explants, we did not find any increase in DNL in explants treated with insulin (100 nM), IGF-2 (100 nM) or vehicle in the presence of varying glucose levels (10 mM or 25 mM). In addition, we did not detect DNL with additional substrates, such as fructose, glycerol, lactate, or pyruvate in our *ex vivo* model (data not shown).

Next, we assessed whether insulin stimulates esterification in placental villous explants. We found that over time, insulin ($P < 0.0005$) or IGF-2 ($P < 0.0001$) treatment of *ex vivo* placental explants led to significant increases in TG accumulation, relative to vehicle (Figure 6E). The accelerated rate of TG accumulation in *ex vivo* placental explants treated with insulin or IGF-2 was

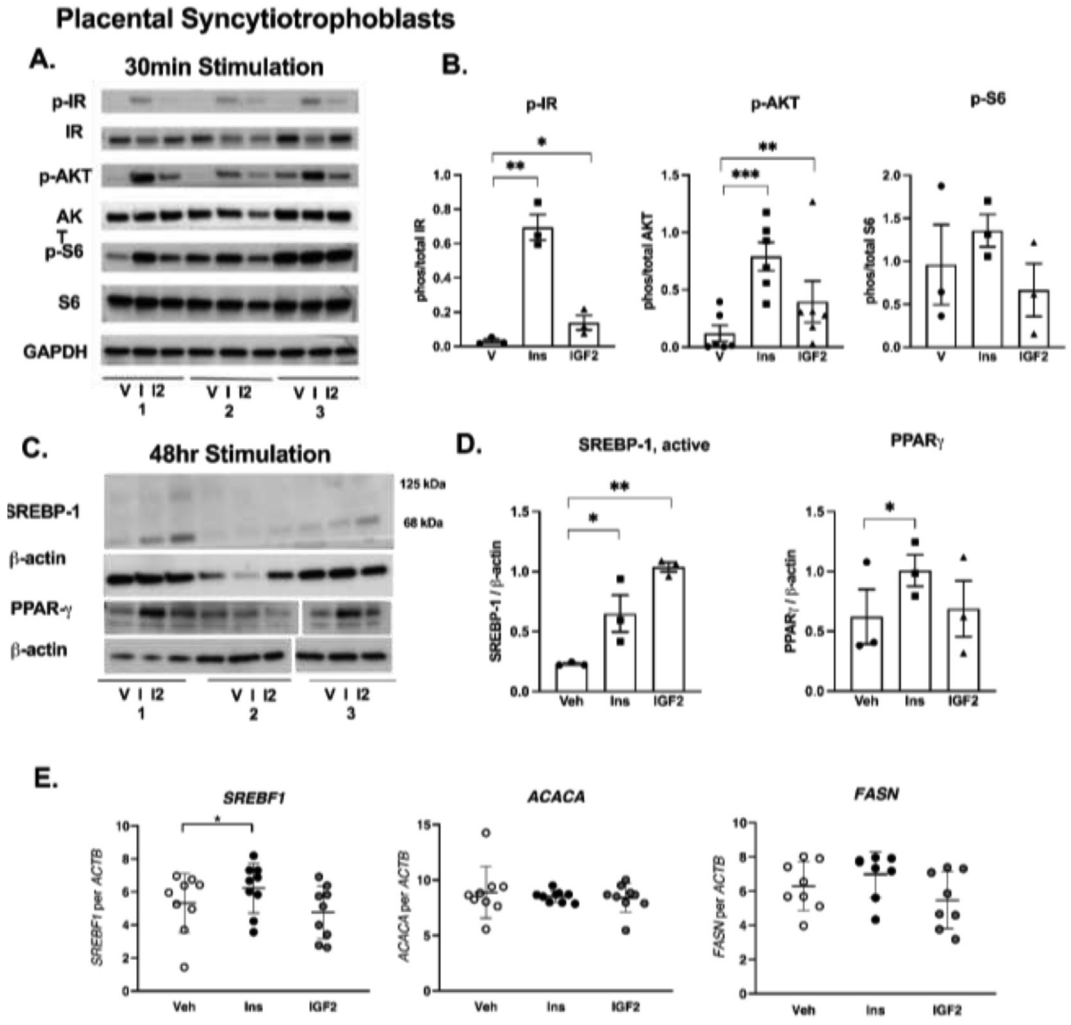

Figure 5: Insulin signaling in syncytialized trophoblasts after 30-minute, 48-hour, and 6-hour hormone stimulation. (**A**) Western blot analysis of pIR (pY1150/1151), pAKT (pS473), and pS6 (pS235/236) in primary placental syncytialized trophoblasts isolated from placenta of normal weight (NW) women treated with vehicle (Veh), 100 nM insulin (I), or 100 nM IGF2 (I2) *in vitro* for 30 min. GAPDH was used as a loading control. Quantification relative to IR, AKT and S6 shown in panel (**B**). $n = 3$ per treatment group. (**C**) Western blot analysis of SREBP1 and PPARγ following 48 h stimulation with 100 nM insulin or IGF2. $n = 3$ per treatment group. β-actin was used as a loading control. Quantification shown in panel (**D**). (**E**) *SREBF1*, *ACACA* and *FASN* mRNA expression in primary trophoblasts isolated from NW women ($n = 9$). Data are mean ± SEM. $^{***}P = 0.005$, $^{**}P < 0.01$, $^{*}P < 0.05$, relative to vehicle by one-way ANOVA.

proportional to increased rates of TG production by esterification of fatty acids taken from the media free fatty acid pool. ($r^2 = 0.6$; $P = 0.0005$) (Figure 6F).

3.9. Abundance of esterification-catalyzing enzymes is not affected by insulin or IGF-2 *in vitro*

Since insulin appears to drive TG synthesis via esterification of free fatty acids from the culture media in placental villous explants, we sought to determine whether insulin increased the quantity of enzymes catalyzing the process of fatty acid esterification to glycerol-3-phosphate. We did not detect differences in the ubiquitous or mitochondrial enzymes for glycerolipid synthesis, such as GPAT and GPAM (glycerol-3-phosphate acyltransferase) in syncytialized trophoblasts treated with insulin or IGF-2 for 48-h (Supplementary Fig. 4). DGAT1 and DGAT2 (diacylglycerol O-acyltransferase) enzymes were also not significantly different between treatment groups.

4. DISCUSSION

Evidence is accumulating that the intrauterine environment is a source of transgenerational programming of metabolic disorders, including obesity and T2DM [3,4,6,10]. Indeed, the placenta may mediate the adverse effects of maternal obesity on fetal development and neonatal adiposity. Our study demonstrates that insulin regulates lipid metabolism and storage in the placenta, thus raising the potential that maternal hyperinsulinemia may contribute to excess neonatal adiposity (Figure 7). We report that insulin and IGF-2 increase placental TG content via esterification, without contribution from FA uptake or *de novo* lipogenesis, or changes in FA oxidation under euglycemic conditions in an *ex vivo* model. From our findings, we propose that the placenta has a much larger role than previously thought in determining substrate flux and nutrient availability for the fetus. Importantly, maternal glucose does not need to be elevated for hyperinsulinemia to sequester lipid in the placenta.

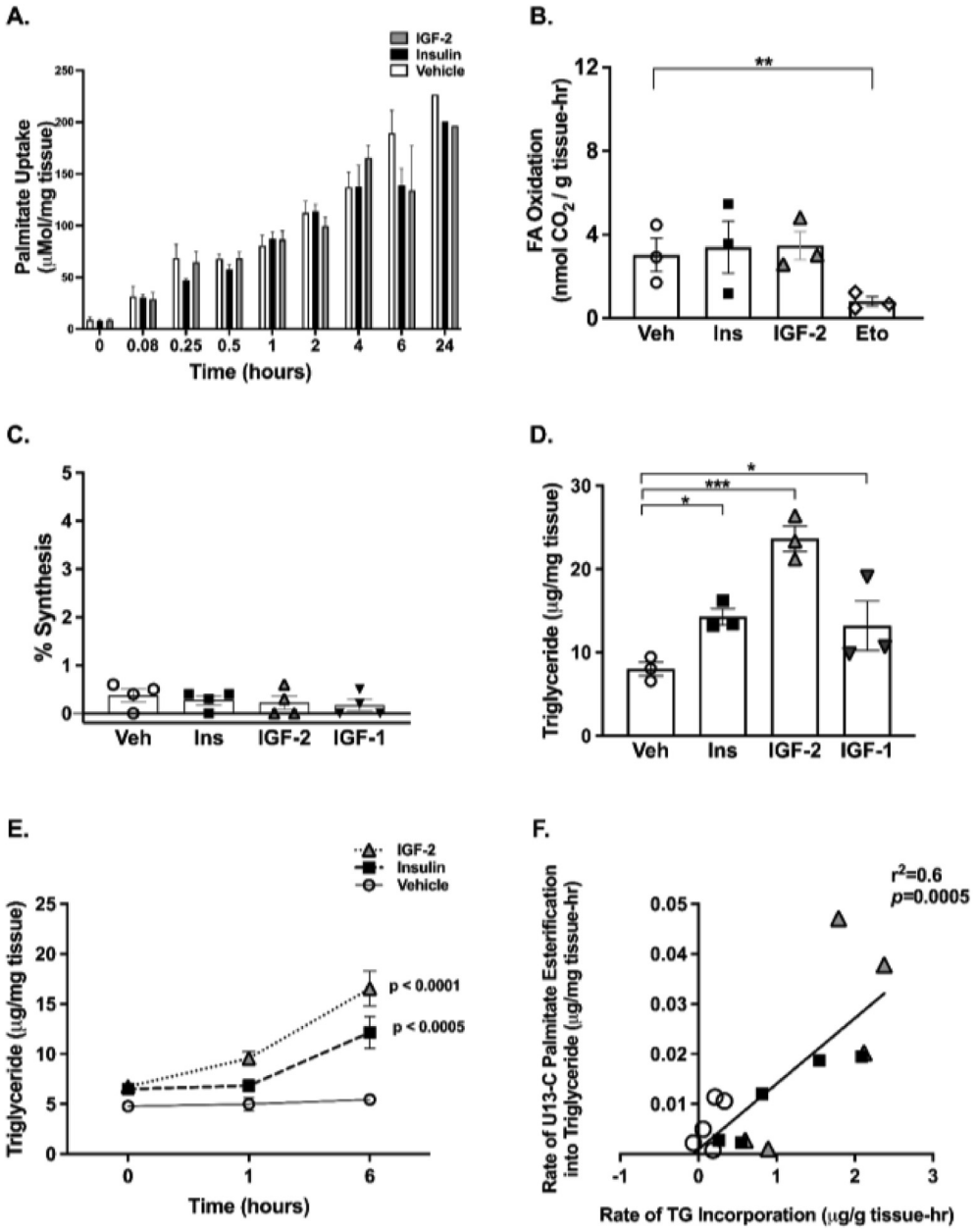

Figure 6: The effect of insulin or IGF2 on placental lipid metabolism in villous explants. (**A**) Fatty acid uptake in NW placentae treated with 100 nM insulin, 100 nM IGF-2 or vehicle control for 5 min to 24 h. $n = 5$ per treatment group. (**B**) Fatty acid oxidation rates in villous explants treated with 100 nM insulin, IGF2 or IGF1. Etomoxir, an inhibitor of FA oxidation was used as a control. For each placenta, there were 3–4 experimental replicates for each treatment condition. $n = 3$ per treatment group. (**C**) % *de novo* lipogenesis (DNL) in explants treated with 100 nM insulin, IGF2 or IGF1 for 48 h. $n = 4$ per treatment group. (**D**) TG content from DNL assay in panel (**C**). (**E**) TG accumulation in placental explants treated with 100 nM insulin, IGF2 or vehicle control for up to 6 h. $n = 4$–5 per treatment group. (**F**) Rate of U-^{13}C palmitate esterification in explants treated with 100 nM insulin, IGF2 or vehicle over 6 h. $n = 5$ per group. $r^2 = 0.6$; $P = 0.0005$. Data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relative to vehicle by one-way ANOVA.

We found that pathophysiological levels of glucose results in higher placenta triglyceride, in the presence or absence of insulin, consistent with the Pedersen Hypothesis. However, women with gestational diabetes or even T2DM, rarely have glucose levels approaching 25 nM (450 ng/dL). Instead, clinical studies have found that maternal obesity and lipid levels correlate with excess neonatal fat deposition, particularly liver fat [11,50]. At this time, *in vivo* studies have not been done to determine whether placenta triglyceride is used directly for fetal nutrient supply and storage. Future studies are needed to assess placental ability to hydrolyze stored triglyceride and provide non-esterified fatty acids (NEFA) to the fetal circulation. Multiple prior investigations suggest this may be an important mechanism by which fetal NEFA supply is regulated. Perazzolo et al. conducted a modeling study that suggests the slow delivery of fatty acids from the maternal circulation to the fetal circulation can be explained at the level of placenta triglyceride assembly [51]. It has been reasoned for years that one explanation for the preferential transfer of polyunsaturated fatty acids to the fetal circulation is due to a placenta esterification step [52]. Our finding that the insulin receptor abundantly lines the microvillous membrane of the syncytiotrophoblast in term placenta is critical to

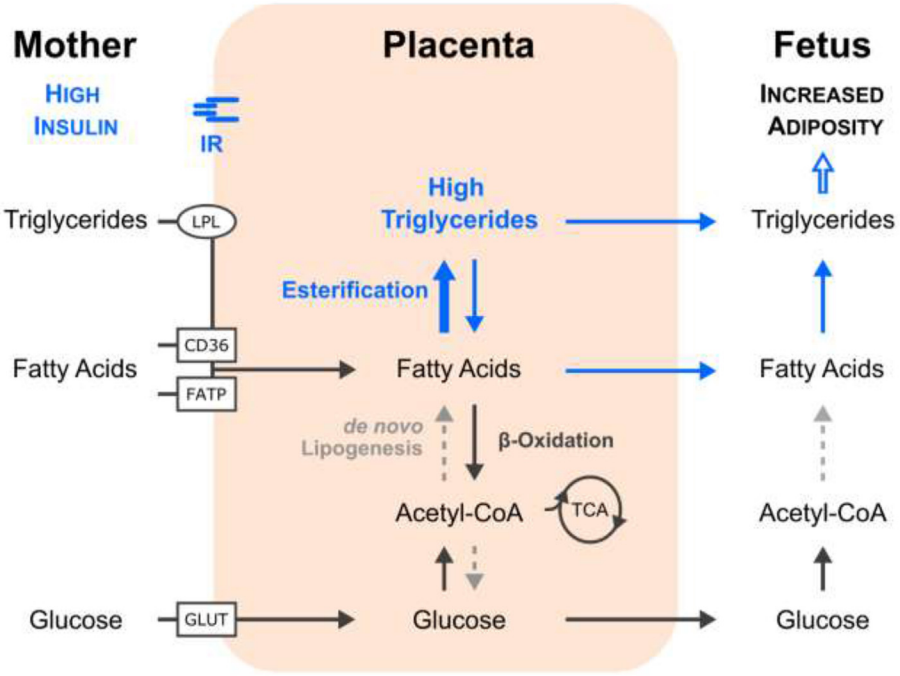

Figure 7: Proposed model of how maternal hyperinsulinemia leads to excess fat accumulation in the fetus. Insulin activates insulin receptor (IR), which is abundant on the maternal-facing side of the syncytiotrophoblasts, and signals through AKT. Maternal nonesterified fatty acids (NEFA) derived from lipoprotein lipase (LPL) mediated lipolysis of maternal triglyceride (TG) enter directly through fatty acid transport proteins (FATP) or CD36, the fatty acid translocase. Insulin induces esterification of FFAs into TG. These TG are stored in the trophoblasts until FFAs are needed as energy for placenta metabolism or to supply the fetus. Although glucose is known to be the preferred substrate for placenta metabolism, fatty acids may undergo β-oxidation to form acetyl-CoA and enter the tricarboxylic acid (TCA) cycle. It is suggested, but still unknown whether the placenta is capable of either gluconeogenesis or *de novo* lipogenesis, for the biosynthesis of glucose or fatty acids, respectively. Importantly, in this model, maternal hyperinsulinemia induces large stores of placenta TG, even with normal levels of maternal glucose and FFAs. Thus, placenta regulation of TG metabolism may explain why euglycemic mothers with obesity have an increased risk of neonatal macrosomia. Black arrows indicate known pathways of substrate flux. Dotted gray lines indicate limited evidence to verify certain pathways. Blue arrows show proposed flux and storage of TG.

understanding a physiological role for maternal insulin. Since insulin does not cross the placenta [53,54], our study of insulin action using a villous explant model, in which tissue architecture is maintained, further supports receptor localization to the microvillus membrane. Prior findings that insulin receptor expression at term localizes only to placental endothelial cells and fetoplacental vessels [55,56] may be attributable to the use of different antibodies and variations in immunohistochemistry techniques. In our study, the severity of maternal hyperinsulinemia and degree of excess TG accumulation in placentae from women with obesity are consistent with reports by other investigators [15,29,57]. All women in the obese and normal-weight groups had serum TG levels within the expected laboratory range for the 3rd trimester, supporting our assertion that insulin's role in placental TG accumulation is not dependent on pathophysiological levels of maternal triglyceride. Importantly, we show that placental sensitivity to insulin is intact in women with obesity with respect to insulin-induced TG synthesis. Placental insulin sensitivity is preserved despite a peak of systemic insulin resistance in the third trimester of pregnancy and background of obesity-induced insulin resistance in peripheral organs. Other investigators also found that the placenta maintains insulin-responsiveness as characterized by AKT signaling activity and amino acid transport [58]. In sum, these experimental findings suggest maternal obesity does not protect the placenta from the effects of hyperinsulinemia, and this may have implications for placental metabolism and function earlier in pregnancy as well.

Notably, we found that IGF-2 also robustly increased placental TG content in our *ex vivo* model. Placental IGF-2 plays a role in placental size, nutrient delivery, and fetal growth [24,25]. In our study, placental IGF-2 and IGF-2R expression were similar between women with normal weight and obesity, and placental TG correlated significantly with maternal body mass index. In animal models, diet-induced increases in maternal adiposity were associated with changes in placental *IGF2* expression independently of methylation, suggesting the role of maternal obesity in transgenerational metabolic programming [59].

Our key findings suggest that insulin and IGF-2 drive TG assembly by shifting intracellular fatty acid flux toward esterification and increasing levels of transcription factors involved in esterification pathways, such as SREBP-1 and PPARγ. Our results are consistent with an *in vitro* study showing placental prioritization of esterification over β-oxidation for fatty acid metabolism, leading to greater lipid droplet formation in trophoblasts [60]. Another study localized lipid droplets, using perilipin-2, within the syncytiotrophoblast layer of term placentae from women with obesity [29]. In a study tracking the movement of a fluorescently labeled long-chain fatty acid analog in placental explants, all cell layers took up long-chain fatty acids [61]. However, in the absence of insulin, investigators found that these fatty acids were rapidly esterified and incorporated into lipid droplets primarily in the cytotrophoblast layer. Insulin may alter the relative metabolic activities of syncytiotrophoblasts and cytotrophoblasts. Nutrient status may also alter differentiation of cytotrophoblasts to syncytiotrophoblasts [60]. On

a functional level, the re-esterification of maternal fatty acids into placental TG storage could be either protective or detrimental for the fetus in the setting of maternal obesity. To determine the balance between buffer and reservoir in the setting of obesity, more work is needed to trace placental TG export and lipolysis for fatty acid transport to the fetal circulation.

DNL occurred at negligible rates in our euglycemic *ex vivo* model, a finding which favors a role of maternal fatty acids and triglycerides over glucose for energy storage in the intrauterine environment. However, the placenta expresses enzymes for DNL, such as FASN. FASN and related enzymes may play a role in placental lipid biosynthesis under different conditions or toward a different cellular function. For example, fatty acids synthesized *de novo* may be funneled toward the synthesis of other molecules containing fatty acid moieties, such as those specialized for signaling, rather than large quantities of energy-storing TG.

We also found that neither insulin nor IGF-2 significantly affected fatty acid uptake *ex vivo*, and other reports also suggest that fatty acid transporters in the placenta may not be insulin sensitive [62,63]. In addition, a study using orally ingested stable isotope-labeled fatty acids and computational modeling, materno-fetal NEFA transfer was equal between normal weight and obese women, yet slow in the fasting state [64], indicating that NEFA uptake is highly regulated and not a process of passive diffusion as once thought [65]. Another study provided some evidence that regulation of specific fatty acids may differ, when they found equal enrichment of ^{13}C-palmitic acid in placentae from women with GDM and women with normoglycemia, yet impaired uptake of ^{13}C-docosahexaenoic acid in women with GDM [66].

In an unexpected finding, we observed thicker placenta depth and a hypertrophied syncytiotrophoblast layer in term placentae from women with obesity. This finding suggests that obesity may induce changes in placental structure, which in turn, affects placental function. Further investigation is needed to define structural markers of obesity-driven pathophysiology, since gross metrics such as placenta weight are affected by collection techniques and may lack specificity. Strengths of our study include the use of multiple molecular and physiological methods to investigate placental lipid metabolism, and the use of placental tissue from a well-characterized cohort of women. Our conclusions are confined by the limitations of an *ex vivo* placenta model and a smaller number of biological replicates for assessing the abundance of esterification-catalyzing enzymes. Despite its limitations, the *ex vivo* placental perfusion model maintains placental function and allows direct assessment of physiology [67], and can be used to support future *in vivo* studies of maternal–fetal placental lipid metabolism in pregnant women.

4.1. Conclusions

In conclusion, we propose that elevated maternal insulin and an abundance of placental insulin receptor have consequences for placental lipid metabolism, even in the setting of normoglycemia. Fatty acids are an efficient substrate for TG synthesis and storage in fetal adipose tissue. Our findings suggest that the obesogenic intrauterine environment is characterized by hyperinsulinemia and may impact the transgenerational programming of metabolic disease.

AUTHOR CONTRIBUTIONS

AKA, KMC, DFV, and CAF conceived and designed the studies, interpreted results, and wrote the manuscript. SMG and HST contributed with key insights to study design. JVO and LEP recruited participants and collected clinical data and placentae. AKA, KMC, MBD performed experiments, analyzed data, and interpreted results. JJH contributed clinical data and samples. LG and HJK contributed to interpreting results. All authors edited and approved the manuscript.

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DATA AVAILABILITY

Data will be made available on request.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101574.

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