Hepatocyte growth factor is elevated in amniotic fluid from obese women and regulates placental glucose and fatty acid metabolism

Francisco Visiedo¹ PhD, Fernando Bugatto² MD-PhD, Cristina Carrasco-Fernández³ PhD, Ana Sáez-Benito³ PhD, Rosa María Mateos¹ PhD, Irene Cózar-Castellano¹,⁵ PhD, José L. Bartha²,⁴ MD-PhD and Germán Perdomo¹,⁶* PhD

¹Research Unit, Puerta del Mar University Hospital, Cádiz, Spain.
²Department of Obstetrics and Gynecology. Puerta del Mar University Hospital, Cádiz, Spain.
³Clinical Analysis Department, Puerta del Mar University Hospital, Cádiz, Spain.
⁴Department of Obstetrics and Gynecology, La Paz University Hospital, Madrid, Spain
⁵Instituto de Genética y Biología Molecular (Universidad de Valladolid-CSIC), Valladolid, Spain.
⁶University of Castilla-La Mancha, School of Environmental Sciences and Biochemistry, Toledo, Spain.

Running title: Hepatocyte Growth Factor and placental metabolism

* Author for correspondence:

Germán Perdomo, Ph.D.
University of Castilla-La Mancha, School of Environmental Sciences and Biochemistry.
Science-Technology Campus in the Old Weapons Factory.
Sabatini Building.
Avenue of Charles III, s/n
45071. Toledo. Spain.
Tel: (+34) 925268800 Extension 5480
e-mail: perdomogm@yahoo.com

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Abstract

Introduction: To evaluate the impact of the pro-inflammatory cytokine hepatocyte growth factor (HGF) on the regulation of glucose and lipid placental metabolism.

Methods: HGF levels were quantified in amniotic fluid and placenta from control and obese women. 2-deoxy-glucose (2-DOG) uptake, glycolysis, fatty acid oxidation (FAO), fatty acid esterification, de novo fatty acid synthesis, triglyceride levels and carnitine palmitoyltransferase activities (CPT) were measured in placental explants upon addition of pathophysiological HGF levels.

Results: In obese women, total- and -activated-HGF levels in amniotic fluid were elevated ~24%, and placental HGF levels were ~3-fold higher than in control women. At a similar dose to that present in amniotic fluid of obese women, HGF (30 ng/mL) increased Glut-1 levels and 2-DOG uptake by ~25-30% in placental explants. HGF-mediated effect on 2-DOG uptake was dependent on the activation of phosphatidylinositol 3-kinase signaling pathway. In addition, HGF decreased ~20% FAO, whereas esterification and de novo fatty acid synthesis increased ~15% and ~25% respectively, leading to 2-fold triglyceride accumulation in placental explants. In parallel, HGF reduced CPT-I activity ~70%.

Discussion: HGF is a cytokine elevated in amniotic fluid and placental tissue of obese women, which through its ability to stimulate 2-DOG uptake and metabolism impairs FAO and enhances esterification and de novo fatty acid synthesis, leading to accumulation of placental triglycerides.

Keywords: Placental inflammation, fatty acid metabolism, glucose metabolism, Hepatocyte Growth Factor.
Highlights

Maternal obesity is linked with elevated placental HGF.

The cytokine HGF is elevated in amniotic fluid and placenta of obese pregnant women.

HGF regulates placental fatty acid and glucose metabolism.

The mechanism relies on enhanced glucose uptake and inhibition of CPT-I activity.
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¹Research Unit, Puerta del Mar University Hospital, Cádiz, Spain.
²Department of Obstetrics and Gynecology. Puerta del Mar University Hospital, Cádiz, Spain.
³Clinical Analysis Department, Puerta del Mar University Hospital, Cádiz, Spain.
⁴Department of Obstetrics and Gynecology, La Paz University Hospital, Madrid, Spain
⁵Instituto de Genética y Biología Molecular (Universidad de Valladolid-CSIC), Valladolid, Spain.
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**Introduction**

Obesity is a growing health concern in women of reproductive age because is associated with a broad range of maternal and fetal complications, such as macrosomia, a condition characterized by excessive fetal fat accretion that predispose the newborn to suffer metabolic diseases later in life [1-4].

The mechanistic link between maternal obesity and fetal macrosomia is poorly understood. Maternal obesity is usually associated with hyperglycemia and hypertriglyceridemia, which may result in augmented transplacental nutrient transfer to the fetus. In the obesogenic-diabetogenic hypothesis proposed by Catalano et al. [2], changes in maternal availability of lipid surplus would facilitate non-esterified free fatty acids delivery to the adipocytes of the fetus, whereas maternal hyperglycemia and hyperinsulinemia would enhance lipogenesis leading to fetal adiposity. Hence, maternal hyperinsulinemia, and excessive circulating levels of glucose and lipids would play a direct role in the accumulation of fat in fetal adipose tissue. However, in pregnancies complicated with obesity, the expression of placental pro-inflammatory cytokines and certain immune cell populations are elevated leading to a chronic inflammatory milieu in which the fetus develops [5-7]. These observations have propelled the question whether excessive fetal adiposity can be explained solely as a result of higher circulating nutrients in maternal blood. Thus, it has been proposed that placental inflammation observed in obese women may modify the availability of nutrient supply at the maternal-fetal interface leading to augmented transplacental nutrient transfer to the fetus [2]. Unfortunately, this question remains to be addressed, and the contribution of intrauterine pro-inflammatory milieu on placental metabolism regulation has not been extensively investigated.
HGF is a pro-inflammatory cytokine elevated in serum of obese patients, which decline with weight loss and reduced body fat mass that occurs after gastroplasty [8-10]. The adipose tissue of these patients abnormally produces and secretes HGF, contributing to augmented serum HGF levels [9, 11]. HGF is activated by serine proteases, such as the HGF activator (HGFA), to exert its biological functions [12]. Although HGF was initially identified as a circulating factor that stimulates hepatocyte proliferation after liver injury [13], HGF exhibits pleiotropic biological functions in a broad range of cell types. Among them, HGF is a potent regulator of glucose and lipid metabolism in pancreatic β-cells, intestine epithelial cells, adipocytes and skeletal muscle cells [14].

HGF is also expressed in placental mesenchymal cells, syncytiotrophoblast cells of the chorionic villi and in amniotic epithelium cells [15]. The biological effects of HGF are mediated by its receptor (c-met) a transmembrane protein encoded by the MET proto-oncogene [16]. The receptor for HGF is primary localized in placental cytotrophoblast cells and to a lesser degree in syncytiotrophoblast cells [17]. In the literature, it can be found few and contradictory reports about plasma HGF levels during normal pregnancy and in pregnancies complicated with obesity. In normal pregnancy, HGF levels increased with gestational age until term [18, 19] or there was no change during pregnancy [20]. In pregnancies complicated with obesity, HGF levels were similar to normal women and remained unchanged with increasing BMI during the second trimester of pregnancy [21]. These results are in disagreement with the notion that in obese patients serum HGF associated with obesity [9, 11] and had a linear relationship with BMI [9]. Finally, total-HGF and activated-HGF blood levels in neonates are regulated in a time-dependent manner along fetal development [22].

In this study, we aimed to further understand the mechanistic link between maternal obesity, through its associated inflammatory uteroplacental environment, and the regulation of placental
metabolism. To this end, we tested the hypothesis that the pro-inflammatory cytokine HGF alters placental glucose and lipid metabolism leading to accumulation of placental triglycerides.

**Methods**

**Study subjects**

The study was performed on placentas and amniotic fluid from pregnant women recruited at the Department of Obstetrics and Gynecology, University Hospital “Puerta del Mar” (HUPM). Patient samples were obtained after written informed consent in accordance with the HUPM Ethics Committee requirements and the Declaration of Helsinki. Specific exclusion criteria included women under the age of 18, smokers, a history of long-chain 3-hydroxyacyl-CoA deficiency, hemolysis elevated liver function syndrome or acute fatty liver of pregnancy, preeclampsia, chronic hypertension, pregestational diabetes, GDM, other co-morbid disease, abnormal karyotype, fetal malformations and multiple pregnancy.

In the studies conducted using placental explants, pregnant women who planned to deliver by an elective Caesarean section due to clinical reasons such as breech presentation or prior Caesarean section were recruited. All Caesarean sections were performed at term. Placental samples and fasting maternal blood samples were obtained at the time of the elective Caesarean section. Neonatal anthropometric measurements were performed immediately at delivery as usual. In total, 26 women with no pregnancy complications (BMI 20-24.9) participated in the study for placental explants experiments. In addition, placentas from 10 obese women (pre-pregnancy BMI >30) were used in experiments showed in Figure 1. Randomly chosen subsets of placentas were used for the
experiments as indicated in the figure legends. In Table 1 are listed demographics and baseline data, as well as perinatal variables.

In the studies conducted using amniotic fluid, patients were eligible among pregnant women attending our antenatal clinic undergoing elective amniocentesis at 15-20 weeks of gestation for karyotype analysis, most of them due to advanced maternal age or combined screening showing high risk for trisomy 21. All fetuses were chromosomally and anatomically normal at delivery. Women were asked to give an extra amount of 3 mL of amniotic fluid for the study. Women were divided into two groups according to their pre-pregnancy BMI: the control group composed of 29 normal weight women (BMI 20-24.9); and the obese group, composed of 12 obese pregnant women (BMI >30). Demographics and baseline data, as well as perinatal variables, are shown in Table 2. Specific exclusion criteria included women under the age of 18, smokers, a history of long-chain 3-hydroxyacyl-CoA deficiency, hemolysis elevated liver function syndrome or acute fatty liver of pregnancy, preeclampsia, chronic hypertension, pregestational diabetes, GDM, other co-morbid disease, abnormal karyotype, fetal malformations and multiple pregnancy.

Materials

Cell culture reagents (RPMI-1640 medium without glucose) and fetal bovine serum were from Invitrogen/Gibco, California, USA. The [9,10-3H]-palmitic acid, [3H]-H2O, 2-[1,2-3H]-deoxy-D-glucose, [1-14C]-mannitol, [5-3H]-glucose, [1,2-14C]-acetic acid, L-[N-methyl-14C]-carnitine-HCl and liquid scintillation counting cocktail were from PerkinElmer, Massachusetts, USA. Methotrexate, Wortmannin, 2-deoxy-glucose (2-DOG) and cytochalasin B were purchased from Sigma-Aldrich (Madrid, Spain). Recombinant human HGF was purchased from Millipore Iberica (Madrid, Spain).
Biochemical parameters and HGF determination

All biochemical parameters were analyzed at the clinical laboratory, HUPM, using reagents and modular systems from Roche Diagnostics as described previously [23]. Low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald-Fredrickson formula.

Human total-HGF (t-HGF) and activated-HGF (a-HGF) were measured by ELISA using a commercially available kit supply by IBL International GmbH (Hamburg, Germany). Each assay required 100 µL of amniotic fluid to determine the levels of t-HGF and a-HGF.

Placental explants culture

Term placenta obtained from elective Caesarean section was placed on ice and arrived to the laboratory within 10-15 minutes of delivery. Afterwards, placental villous explants (~100 mg wet weight) were dissected and cultured in 6-well plate containing 2 mL of culture medium (RPMI-1640 supplemented with 5 mmol/l glucose, 10% FBS (vol/vol), 100 units/ml penicillin G, and 100 µg/ml streptomycin) as described previously [23].

Fatty acid oxidation assay

Stock of fatty acid solution was prepared by conjugating palmitate with essentially fatty acid-free bovine serum albumin (BSA) to generate a stock solution of 25% (wt/vol) BSA, 4 mmol/L palmitate in culture medium were prepared as described previously [23]. Mitochondrial fatty acid oxidation (FAO) assays were performed in placental explants as described previously [23-25]. Briefly, freshly isolated villous explants were incubated in culture media in the presence or absence of HGF, plus 1.25% BSA, 0.1 mmol/l cold palmitate, and 18500 Bq/ml [³H]-palmitate at 37°C for 18h. At the end of the incubation period, the medium was collected, and tritiated water determined by the vapor-
phase equilibration method of Hughes et al [26]. FAO was defined as nmol of palmitate per mg of tissue per hour.

**Esterification into total lipids assay**

Incorporation of $[^{3}H]$-palmitate into total lipids was determined as previously described [23, 24]. Briefly, after similar incubation conditions to those used for measurements of β-oxidation, in the presence or absence of HGF, plus 0.1 mmol/L cold palmitate and 18500 Bq/mL for 18h, explants were washed 3 times with 2 mL of ice-cold PBS and homogenized in 500 µL of PBS. An aliquot of 100 µL was used to extract the lipid content from samples according to Bligh and Dyer [27]. Afterwards, the radioactive content was determined by liquid scintillation counting. Esterification was plotted as % of control.

**De novo lipid synthesis assay**

De novo lipid synthesis was determined using $[^{14}C]$-acetate in placental explants according to the procedure described previously [23, 24]. Villous placental explants were incubated in RPMI-1640 culture media in the presence or absence of HGF plus 1850 Bq/mL $[^{14}C]$-acetate at 37ºC for 18h. At the end of the incubation period, culture media were discarded followed by tissue homogenization in 500 µL of PBS. After a total lipid extraction (as described for measurements of placental esterification rate), the radioactive content was determined.

**Glycolysis assay**

Glucose utilization (equivalent to glycolysis) was determined as the production of tritiated water (as for FAO experiments) after incubation of placental explants for 18h in the presence or absence of HGF, plus 240500 Bq/mL $[^{5-3}H]$-glucose [24, 28].
Glucose transport assay

Uptake of \(^{3}\)H-2-DOG was performed ex vivo in placental explants, as described previously [14], with the following modifications. Freshly isolated placental explants were preincubated for 18 h in culture medium in the presence or absence of HGF, plus cytochalasin B, a potent inhibitor of glucose transport mediated via facilitative glucose transporters. Afterwards, explants were washed in transport solution buffer (20 mmol/L Hepes-Na pH 7.4, 140 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L MgSO\(_4\), 1 mol/L CaCl\(_2\)) at room temperature, and immediately incubated in transport solution buffer plus 10 µmol/L 2-DOG (18500 Bq/mL \(^{3}\)H-2-DOG) and 39 mmol/L mannitol (11840 Bq/mL \(^{14}\)C-mannitol) with or without HGF for 1 min. Then, explants were removed rapidly, rinsed with cold 0.9% NaCl to stop reactions, blotted, digested in 1 mol/L NaOH and analyzed for \(^{14}\)C and \(^{3}\)H content. Glucose uptake was defined as nmol of 2-DOG per mg of protein per minute.

Quantification of triglyceride content

Placental triglyceride content was determined, as previously described by Perdomo et al. [29], in explants preincubated in the presence or absence of HGF for 18 h. Placental tissues (~100 mg) were homogenized in 400 µl HPLC-grade acetone, and aliquots of 5 µl of acetone-extracted lipid suspension were used to determined triglyceride concentrations using a triglyceride reagent kit (Biosystems, Barcelona, Spain). Proteins were quantified using the bicinchoninic acid method (Thermo Scientific, Madrid, Spain). Placental lipid content was defined as mg of triglyceride per mg of total placental proteins.
Western blot analysis

Placental explants from control and obese women were dissected and washed with ice-cold PBS, followed by homogenization in lysis buffer (20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% (v/v) Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na₃VO₄, 1 µg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride) plus protease inhibitors (Protease Inhibitor Cocktail, Sigma, St. Louis, MO). After 10 min. on ice, extracts were sonicated and centrifuged at 18,000 X g for 10 minutes at 4ºC. Pellets were discarded and solubilized proteins (40-60 µg/sample) were resolved by 10% SDS-PAGE for HGF and electrotransferred onto polyvinylidene difluoride (PVDF) filters for immunoblotting by conventional means. After probing with specific antibodies [glucose transporter 1 (Glut-1; 1:2000, Abcam, Cambridge, UK) HGF antibody (1:1000, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and protein kinase B (PKB; 1:1000, Cell Signaling, Barcelona, Spain)], the membranes were stripped and reprobed against phospho-PKB at Ser473 (p-PKB; 1:1000, Cell Signaling, Barcelona, Spain), or actin (1:3000, Sigma, Madrid, Spain). Signals were detected by chemiluminescence (Immun-Star western chemiluminescence kit, Bio-Rad, Madrid, Spain), and band densitometry was quantified with the ImageJ software (NIH, USA).

CPT assay

Activities of carnitine palmitoyltransferase I (CPT-I) and carnitine palmitoyltransferase II (CPT-II) were determined in the direction of acyl-carnitine formation, using [¹⁴C]-carnitine as substrate in placental explants as described previously [23, 25, 30]. Briefly, explants were preincubated in RPMI-1640 culture media in the presence or absence of HGF (30 ng/mL) at 37ºC for 18h. At the end of the incubation period, culture media were discarded, and explants were washed with ice-cold PBS prior
to homogenization in lysis buffer (5 mmol/l Tris-HCl, pH 7.2, 150 mmol/l KCl) with a glass homogenizer. For CPT-I assay, 100 µl of cell homogenate, in which the mitochondria remain largely intact, was incubated in the presence of 50 µmol/l palmitoyl-CoA, 500 µmol/l carnitine, and 9250Bq/ml [14C]-carnitine in a 30ºC shaking water bath for 10 min. For CPT-II assay, a portion of the homogenate was adjusted to 1 % (wt/v) of the detergent octylglucoside, which solubilizes the mitochondrial membranes, inactivating CPT-I and releasing CPT-II from the mitochondrial matrix in active form. Afterward, reactions were stopped by adding 500 µl of 1-butanol. Radioactive content was determined by liquid scintillation counting.

Statistical analysis

Statistical analysis of data was performed using the SPSS software (SPSS 20.0, Inc., Chicago, IL). Data were presented as mean ± SEM. Distributions were checked with a histogram and the Kolmogorov-Smirnov test. Comparisons between two groups were done by unpaired Student’s t-test, and comparisons between more than two groups were performed by ANOVA followed by Tukey’s Multiple Comparison Test. Differences were considered significant at p<0.05.”

Results

HGF is elevated in amniotic fluid and placenta from obese pregnant women

Obesity in pregnancy induces placental inflammation, which is associated with production of pro-inflammatory mediators [5]. To determine the presence of the pro-inflammatory cytokine HGF in the inflammatory milieu, in which the fetus develop, we measured t-HGF and a-HGF levels in amniotic fluid from control and obese pregnant women during early- mid pregnancy. As shown in Figure 1A-B, t-HGF and a-HGF are elevated up to ~30 ng/mL in amniotic fluid from obese women.
In addition, HGF levels in placentas from obese women were ~3-fold higher than in control women (Fig. 1C).

**HGF increases glucose transport and metabolism in human placental explants**

HGF is a potent stimulator of glucose transport and metabolism in different cell types [14]. Because HGF is elevated in placental explants and amniotic fluid from obese women, we hypothesized that HGF stimulates glucose uptake in human placenta. To test our hypothesis, we preincubated placental explants, from pregnancies without complications, in the presence or absence of HGF for 18h, and measured glucose transport and glycolysis. **HGF significantly increased 2-DOG uptake in human placental explants and the effect was maximal (~30%) at 30 ng/mL (Fig. 2A)**.

Interestingly, in the presence of 10µM cytochalasin B (CB; an inhibitor of facilitative glucose transport), 2-DOG uptake was suppressed by ~60%, whereas in the presence of 50µM CB B by ~90% (Fig. 2B). Likewise, CB completely abolished the effect of HGF on 2-DOG uptake, suggesting that HGF-mediated 2-DOG uptake in human placental explants was likely mediated by a facilitative glucose transport (Fig. 2B). Finally, HGF-mediated effect on 2-DOG uptake was accompanied by a significant augment (~20%) on glycolysis in placental explants (Fig. 2C). Collectively, these results demonstrate a novel role of HGF in the regulation of placental glucose metabolism.

**HGF-mediated glucose uptake in human placental explants is Glut-1 and phosphatidylinositol 3-kinase (PI3K) dependent**

Glucose transport is mainly regulated by Glut-1 in human placenta [31]. Consequently, we hypothesized that pathophysiological levels of HGF stimulates glucose uptake in human placental explants through up-regulation of Glut-1. To test this hypothesis, we analyzed Glut-1 levels in
protein extracts prepared from explants after chronic exposure to HGF for 18h. As shown in Figure 3A, treatment with HGF increased by ~25% Glut-1 levels.

To gain further insight into the molecular mechanism by which HGF regulates glucose transport and Glut-1 levels, we analyzed the possible signaling pathways downstream of c-met in placental explants. To this end, explants were preincubated in the presence or absence of HGF (30 ng/mL) in a time dependent manner (from 0 to 60 min), and PI3K signaling pathway was studied by immunoblot analysis. HGF potently and rapidly stimulated PKB phosphorylation in a time-dependent manner (Fig. 3B). To reveal whether the inhibition of this signaling pathway could reverse the effect of HGF on glucose transport, we preincubated placental explants with 100 nM wortmannin (WM; a PI3K inhibitor) for 30 min before and during chronic (18h) incubation with HGF (30ng/mL). At the end of the incubation period, the 2-DOG uptake was measured in these cells. As shown in Figure 3C, wortmannin completely abolish the effect of HGF on glucose transport. Taken together, these results indicate that PI3K signaling pathway is involved in HGF-mediated 2-DOG uptake in human placental explants.

**HGF alters the metabolic partitioning of fatty acids in human placental explants**

We showed above that HGF enhanced glucose transport and utilization in placental explants. Therefore, it is reasonable to hypothesize that HGF-mediated accelerated glucose metabolism would inhibit fatty acid oxidation leading to accumulation of placental triglycerides. To test this hypothesis we measured the effect of chronic exposure (18h) to pathophysiological levels of HGF (30 ng/mL) on FAO in placental explants. As shown in Figure 4A, HGF significantly reduced the FAO rate by ~20% in placental explants. In parallel, HGF enhanced fatty acid esterification by ~15% (Fig. 4B). Surprisingly, HGF also augmented *de novo* fatty acid synthesis by ~25% (Fig. 4C). Finally,
consistently with the expectation that fatty acids are preferentially directed towards esterification, and that HGF stimulated de novo fatty acid synthesis, placental triglyceride content significantly increased 2-fold in placental explants (Fig. 4D). Taken together, these data indicate that HGF alters the metabolic partitioning of fatty acids towards triglyceride accumulation through inhibition of FAO and stimulation of esterification and de novo fatty acid synthesis.

**HGF decreases carnitine palmitoyltransferase I activity in human placental explants**

Because HGF increased glucose transport and utilization, in parallel with diminished FAO, we hypothesized that the mechanistic link between accelerated glucose metabolism and lower FAO was inhibition of CPT-I activity by its physiological inhibitor malonyl-CoA, which is synthesized from glucose-derived acetyl-CoA by acetyl-CoA carboxylase (ACC) activity. Therefore, to gain further insight into the molecular mechanism by which HGF inhibits FAO in placental explants, we measured the activity of CPT-I and CPT-II in placental explants preincubated without (control) or with HGF (30 ng/mL) for 18h. As shown in Figure 7, HGF reduced by ~70% the activity of CPT-I (Fig. 5A), whereas CPT-II activity remained unchanged as expected (Fig. 5B).

**Discussion**

In obese women, the risk of macrosomia is 2-fold higher than in control women [2], leading to heavier neonates because of an increase in fat but not lean body mass [32]. Excessive fat accumulation in adipose tissue of newborns predispose them to suffer from metabolic diseases later in life [4], which perpetuates a vicious cycle of obesity and diabetes. At first glance, hypertriglyceridemia in plasma from obese women, secondary to maternal insulin resistance, would facilitate non-esterified free fatty acids delivery to the adipocytes of the fetus through potential
mechanisms such as alteration of concentration gradients across the placental barrier, and changes in
the levels and/or activity of lipoprotein lipase, fatty acid-binding proteins and fatty acid transporters
in syncytiotrophoblast cells [33]. Thus, it has been shown that maternal obesity modulates placental
fatty acid transporters and placental fatty acid transport and metabolism [34, 35].

Maternal obesity during pregnancy is also associated with placental accumulation of immune
cells, such as macrophages and neutrophils, lipid accumulation in the placental villi stroma and
syncytiot, and elevated pro-inflammatory cytokines leading to lipotoxicity and inflammation [5-7,
33, 36]. In this work, we showed that HGF is elevated in placental tissue and amniotic fluid of obese
women during early- mid pregnancy, and identified mechanisms by which HGF regulated placental
lipid accumulation. However, the molecular and biochemical mechanisms by which obesity is related
to enhance pro-inflammatory cytokines expression in placenta have not been entirely clarified. A
conceivable mechanism by which placental HGF production is augmented could be related to the
lipotoxic and pro-inflammatory placental environment. Excessive circulating maternal free fatty
acids and/or elevated glucose levels, which reduce fatty acid oxidation and enhance esterification
through inhibition of carnitine palmitoyltransferase I [23], would lead to the accumulation of
triglycerides in placenta. This accumulation of lipids may trigger activation of toll-like receptors
(TLR) in trophoblast and/or macrophages, which would signal through phosphorylation of the c-Jun
N-terminal kinase (JNK) and the inhibitor of κB (IxB), leading to activation of the nuclear factor-κB
(NF-κB) and activator protein 1 (AP-1), the primary mediators of inflammatory responses. This
signaling pathway could be further stimulated by interleukin 1-beta (IL-1β), tumor necrosis alpha
(TNF-α) and interleukin 6 (IL-6), constituting a vicious cycle. Nonetheless, signaling pathways
involved in the production/ secretion of HGF in placentas from obese women remains unknown.
Using placental explants from women with no pregnancy complications, we demonstrate that at similar concentration to that present in amniotic fluid of obese women during early-mid pregnancy, HGF inhibited FAO and increased triglyceride accumulation. A limitation of this study is that we performed a cross-sectional study regarding HGF levels in amniotic fluid. Thus, in the absence of a longitudinal study, we cannot assume that HGF levels during the third trimester are similar to those measured during the second trimester. Nonetheless, these HGF levels augmented Glut-1 protein levels, glucose transport and metabolism. We evaluated whether the mechanistic link between HGF and lower FAO was inhibition of CPT-I activity by its physiological inhibitor malonyl-CoA, which is synthetized from glucose-derived acetyl-CoA by ACC. This mechanism results in a shift of fatty acid partitioning away from the β-oxidation pathway toward esterification, allowing the accumulation of triglycerides in placenta. This notion is in agreement with our findings in placentas from pregnancies complicated by GDM, where we showed that accelerated glucose metabolism in placental explants lowered FAO [23]. The impact of other pro-inflammatory cytokines on lipid metabolism has not been extensively studied. In human trophoblast cell cultures IL-6, but not TNF-α, stimulated fatty acid accumulation [37]. This effect was not explained by an increased expression of lipoprotein lipase or fatty acid binding proteins. Although the mechanism by which IL-6 stimulates trophoblast fatty acid accumulation remains to be established, it is plausible to hypothesize that would be mediated by inhibition of FAO and/or stimulation of de novo fatty acid synthesis. Finally, this knowledge about HGF-mediated accelerated glucose metabolism and its impact on placental lipid metabolism, may offer new therapeutic targets for clinical management of pregnancies complicated by obesity.
Earlier studies suggested that *de novo* fatty acid synthesis pathway plays a minor role on accumulation of triglycerides in placentas from pregnancies complicated with diabetes [23, 38]. However, we demonstrated that HGF stimulated *de novo* fatty acid synthesis in placental explants from women with no pregnancy complication. If this HGF-mediated regulation on fatty acid synthesis would translate directly into the *in vivo* situation of placentas from obese women, the glucose-derived malonyl-CoA, synthesized by the activity of ACC, would not merely serve as a regulator of FAO pathway; but in addition, it would serve as a regulator of the *de novo* fatty acid synthesis. Consequently, *de novo* fatty acid synthesis pathway in placenta from pregnancies complicated with obesity would significantly contribute to placental triglycerides accumulation and augmented transplacental fatty acids delivery to fetal adipocytes.

Although it is known that HGF participates in the regulation of glucose transport in different cells types [14], no study has addressed weather HGF might regulate glucose metabolism in human placenta. The current study demonstrates for the first time that HGF increases glucose uptake and utilization in human placental explants *ex vivo*. Our studies establish that, in addition to behaving as a growth factor, HGF has additional biological features as a modulator of glucose metabolic flux in placental cells engaged in glucose homeostasis control. Interestingly, HGF significantly increased total Glut-1 expression in human placental explants, which is associated with augmented glucose uptake. Furthermore, inactivation of PI3K signaling pathway completely abolished HGF-mediated effect on glucose uptake. We have previously showed that HGF also activates PI3K and ERK1/2 signaling pathways in parallel with up-regulation of Glut-1 levels in skeletal muscle cells [14]. Likewise, pharmacological inhibition of these pathways also abolished glucose uptake in these cells [14]. Also, Di Simone et al. showed that resistin, a cytokine elevated in serum of third trimester
normal pregnancies, stimulated glucose uptake and Glut-1 levels in trophoblast cells through activation of ERK1/2 pathway [39]. Collectively, these results highlight an underappreciated role for cytokines in the control of glucose transport and metabolism in human placenta.

In conclusion, HGF is a mechanistic link between the intrauterine pro-inflammatory milieu of pregnancies complicated with obesity and its associated placental lipotoxicity. Because most of maternal cytokines do not cross the human placental barrier, we propose that synthesized HGF within the placenta itself exacerbates lipotoxicity through biochemical mechanisms involving augmented glucose transport, fatty acid esterification and de novo fatty acid synthesis, which lead to placental triglyceride accumulation. Finally, our results suggest that placental inflammation may play an indirect role on fetal macrosomia modifying the availability of nutrient supply to the fetus.

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References


**Figure legends**

**Figure 1. HGF levels in amniotic fluid and placentas from obese pregnant women.** HGF levels in amniotic fluid. t-HGF (A) and a-HGF (B) were measured in amniotic fluid from control (n=29) and obese (n=12) women by ELISA (see details in Table 2). Values are means ± SEM. *p<0.05 relative to control group by unpaired t-test. (C) Western blot analysis of HGF in placental...
tissue from control and obese women. Frozen placental tissues (~100 mg) from control (n=8) and obese (n=10) groups (see details in Table 1) were used to quantify placental HGF content. β-actin expression was determined to ensure similar protein loading. Top: the y-axis represents the ratio of HGF vs. β-actin in arbitrary units (A.U). Bottom: a representative picture of the western blot is shown. Data are means ± SEM. *p<0.05 relative to control group by unpaired t-test.

**Figure 2.** HGF stimulates glucose uptake and metabolism in human placental explants. (A) Stimulation of glucose uptake in placental explants. Placental explants from control women (n=3; Table 1) were incubated with different concentrations of HGF for 18h, and 2-DOG uptake was measured. Values are means ± SEM for 3 independent experiments in triplicate. *p<0.05 relative to control without HGF by ANOVA. (B) Effect of cytochalasin B (CB) on 2-DOG uptake mediated by HGF. Placental explants from control women (n=4; Table 1) were treated with HGF (30 ng/ml) for 18h in the presence or the absence of CB at 10 µM or 50 µM. Afterward, 2-DOG uptake was determined. Values are means ± SEM for 4 independent experiments in triplicate. *p<0.05 relative to control without CB and without HGF; †p<0.05 relative to HGF without CB by ANOVA. (C) Effect of HGF on glycolysis in placental explants. Placental explants from control women (n=7; Table 1) were treated with HGF (30 ng/ml) for 18h. Afterward, glycolysis was determined. Values are represented as percentage of control for 7 independent experiments in triplicate. *p<0.05 relative to control by unpaired t-test.

**Figure 3.** Augmented Glut-1 protein levels and activation of PI3K signaling pathway are required for HGF-mediated stimulation of glucose transport in human placental explants. (A) Western blot analysis of Glut-1 in placental explants. Explants from control women (n=4; Table 1) were preincubated with or without HGF (30 ng/mL) for 18h, and protein extracts were subjected to
immunoblot analysis using anti-Glut-1 antibody. Top: the y-axis represents the ratio of Glut-1 vs. β-actin in arbitrary units (A.U.). Bottom: a representative picture of the western blot is shown. Data are means ± SEM for 4 independent experiments. *p<0.05 relative to control by unpaired t-test. (B) Western blot analysis of PI3K-signaling pathway activation mediated by HGF in placental explants. Explants from control women (n=4; Table 1) were preincubated without (time 0) or with HGF (30ng/mL) for the indicated times. Afterward, protein extracts were subjected to immunoblot analysis using anti-PKB or anti-p-PKB antibody. Top: the y-axis represents the ratio of p-PKB vs. PKB in arbitrary units (A.U.). Bottom: a representative picture of the western blot is shown. Data are means ± SEM for 4 independent experiments. *p<0.05 relative to time 0 by ANOVA. (C) Effect of wortmannin-mediated inhibition of PI3K signaling pathway on 2-DOG uptake. Placental explants from control women (n=4; Table 1) were preincubated with 100 nmol/L wortmannin (WM) for 30 min before and during chronic (18h) incubation with HGF (30 ng/mL). At the end of the incubation period, the 2-DOG uptake was measured. Values are means ± SEM for 4 independent experiments in triplicate. *p<0.05 relative to control without HGF; †p<0.05 relative to HGF without WM by ANOVA.

Figure 4. HGF inhibits fatty acid oxidation and increases triglycerides content in human placental explants. (A) Effect of HGF on fatty acid oxidation. Placental explants from control women (n=7; Table 1) were incubated in the absence (control) or presence of HGF (30 ng/mL) for 18h. Afterward, [³H]-water was determined. Values are means ± SEM for 7 independent experiments in triplicate. Significance is indicated *p<0.05 relative to control by unpaired t-test. (B) Effect of HGF on fatty acid esterification. The same subset of placental explants described in panel A was used to assess esterification into total lipids. Values are represented as percentage of control for 7
independent experiments in triplicate. Significance is indicated *p<0.05 relative to control by unpaired t-test. (C) Effect of HGF on de novo lipid synthesis. Placental explants from control women (n=7; Table 1) were used to assess de novo fatty acid synthesis in the absence (control) or presence of HGF (30 ng/mL) plus [14C]-acetate for 18h. Afterward, [14C]-acetate incorporation into total lipids was determined. Values are represented as percentage of control for 7 independent experiments in triplicate. Significance is indicated *p<0.05 relative to control by unpaired t-test. (D) Placental explants from control women (n=4; Table 1) were used to assess the effect of HGF on placental TG accumulation. Values are means ± SEM for 4 independent experiments in triplicate. Significance is indicated *p<0.05 relative to control by unpaired t-test.

Figure 5. HGF inhibits carnitine palmitoyltransferase I activity. (A) Effect of HGF on CPT-I activity. Placental explants from control women (n=4; Table 1) were incubated in the absence (control) or presence of HGF (30 ng/mL) for 18h. Afterward, CPT-I activity was determined. Values are means ± SEM for 4 independent experiments in duplicated. Significance is indicated *p<0.05 relative to control by unpaired t-test. (B) Effect of HGF on CPT-II activity. The same placental explants used in panel A were used to assess CPT-II activity. Values are means ± SEM for 4 independent experiments in duplicated. Significance is indicated *p<0.05 relative to control by unpaired t-test.

Contributors

Francisco Visiedo, I declare that I participated in sample collection, experimental work, data analysis and figures/tables and manuscript elaboration, and that I have seen and approved the final version. I have no conflicts of interest to declare.
Fernando Bugatto, I declare that I participated in sample collection, experimental work, data analysis and figures/tables and manuscript elaboration, and that I have seen and approved the final version. I have no conflicts of interest to declare.

Cristina Carrasco-Fernández, I declare that I participated in sample collection, experimental work, data analysis and figures/tables and manuscript elaboration, and that I have seen and approved the final version. I have no conflicts of interest to declare.

Ana Sáez-Benito, I declare that I participated in sample collection, experimental work, data analysis and figures/tables and manuscript elaboration, and that I have seen and approved the final version. I have no conflicts of interest to declare.

Rosa María Mateos, I declare that I participated in sample collection, experimental work, data analysis and figures/tables and manuscript elaboration, and that I have seen and approved the final version. I have no conflicts of interest to declare.

Irene Cózar-Castellano, I declare that I participated in experimental work, data analysis and figures/tables and manuscript elaboration, and that I have seen and approved the final version. I have no conflicts of interest to declare.

José L. Bartha, I declare that I participated in sample collection, data analysis and figures/tables and manuscript elaboration, and that I have seen and approved the final version. I have no conflicts of interest to declare.

Germán Perdomo, I declare that I participated in work conceptualization, experimental design, experimental work, data analysis and figures/tables, and I wrote the manuscript, and that I have seen and approved the final version. I have no conflicts of interest to declare.
Table 1. Placental explants samples. Maternal and fetal characteristics from the studied population used for placental explants experiments.

<table>
<thead>
<tr>
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<th>Control group (n=26)</th>
<th>Obese group (n=10)</th>
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<tbody>
<tr>
<td>Delivery mode</td>
<td>Caesarean section</td>
<td>Caesarean section</td>
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<tr>
<td>Maternal age (yr)</td>
<td>30.0 ± 5.7</td>
<td>31.8 ± 4.0</td>
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<tr>
<td>Gestational age at delivery (wk)</td>
<td>38.3 ± 1.81</td>
<td>38.6 ± 0.9</td>
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<td>Maternal pregravid BMI (kg/m²)</td>
<td>21.4 ± 1.5</td>
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<td>Maternal plasma glucose (mg/dL)</td>
<td>72.8 ± 6.6</td>
<td>80.6 ± 15.3</td>
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<td>Maternal plasma insulin (pmol/L)</td>
<td>6.5 ± 3.7</td>
<td>16.1 ± 9.4*</td>
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<tr>
<td>Maternal plasma triglycerides (mg/dL)</td>
<td>182.0 ± 35.8</td>
<td>226.5 ± 48.6</td>
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<tr>
<td>Maternal plasma total cholesterol (mg/dL)</td>
<td>249.8 ± 32.0</td>
<td>288.4 ± 35.1*</td>
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<tr>
<td>Maternal plasma HDL-cholesterol (mg/dL)</td>
<td>73.3 ± 21.2</td>
<td>68.0 ± 18.0</td>
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<tr>
<td>Maternal plasma LDL-cholesterol (mg/dL)</td>
<td>144.9 ± 24.3</td>
<td>158.7 ± 40.5</td>
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<tr>
<td>Placental weight (g)</td>
<td>528 ± 106</td>
<td>617 ± 42*</td>
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<tr>
<td>Birthweight (g)</td>
<td>3225 ± 309</td>
<td>3235 ± 279</td>
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</table>

Data are given as mean ± SEM. BMI, Body mass index; HDL, High-density lipoprotein; LDL, Low-density lipoprotein. *p<0.05 vs. control group by unpaired t-test.
**Table 2. Amniotic fluid samples.** Maternal and fetal characteristics from the studied population used for amniotic fluid collection.

<table>
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<th>Control group (n=29)</th>
<th>Obese group (n=12)</th>
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</thead>
<tbody>
<tr>
<td>Maternal age (yr)</td>
<td>35.61 ± 5.02</td>
<td>35 ± 1.1</td>
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<td>Gestational age at amniocentesis (wk)</td>
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<td>16 (16-17)</td>
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<td>Gestational age at delivery (wk)</td>
<td>40.0 ± 1</td>
<td>39 ± 1.7</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>22.69 ± 1.3</td>
<td>34.6 ± 2.56*</td>
</tr>
<tr>
<td>Newborn weight (g)</td>
<td>3258.52 ± 475</td>
<td>3357 ± 579</td>
</tr>
</tbody>
</table>

Data are given as mean ± SE. Gestational age at amniocentesis is listed as median (interquartile range). *p<0.05 vs. control group by unpaired t-test.
Figure 1

A

**Control**

**Obese**

**Total-HGF (ng/mL)**

B

**Control**

**Obese**

**Activated-HGF (ng/mL)**

C

**Control**

**Obese**

**HGF levels (A.U.)**

MW (kDa)

69 —

42 —

HGF

β-Actin

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Figure 3

(A) Glut-1 levels (A.U.)

Control | HGF (30 ng/mL)

MW (kDa):
- 50
- 42

(B) p-PKB/PKB levels (A.U.)

Time (min):
- 0
- 15
- 30
- 60

MW (kDa):
- 60

Figure 3
Figure 3
Figure 4

A. Fatty acid oxidation (nmol/h/g of tissue)

B. Esterification (% of control)

C. De novo fatty acid synthesis (% of control)

D. Triglycerides (mg/mg of protein)

Control vs. HGF (30ng/mL)
Figure 5

A

CPT-I activity
(nmol/min/mg of protein)

Control
HGF (30ng/mL)

*  

B

CPT-II activity
(nmol/min/mg of protein)

Control
HGF (30ng/mL)

n.s.