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High-glucose levels reduce fatty acid oxidation and increase triglyceride accumulation in human placenta

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23 **Word count:** 242 in abstract; 4062 in main text.

24

25 ## Abstract

26 **Aim/hypothesis:** Placenta of women with gestational diabetes mellitus (GDM) exhibits an
27 altered lipid metabolism. The mechanism by which GDM is linked to alterations in placental lipid
28 metabolism remains obscure. We hypothesized that high-glucose levels reduce mitochondrial fatty
29 acid oxidation (FAO) and increase triglyceride accumulation in human placenta.

30 **Methods:** To test this hypothesis, we measured FAO, fatty acid esterification, *de novo* fatty
31 acid synthesis, triglyceride levels and carnitine palmitoyltransferase activities (CPT) in placental
32 explants of women with GDM or with no pregnancy complication.

33 **Results:** In women with GDM, FAO was reduced by ~30% without change in mitochondrial
34 content, and triglyceride content was 3-fold higher than control group. Likewise, in placental
35 explants of women with no complication high-glucose levels reduced by ~20% FAO and
36 esterification increased linearly with increasing fatty acids concentrations. However, *de novo* fatty
37 acid synthesis remained unchanged between high-and–low glucose levels. In addition, high-glucose
38 levels increased triglycerides content ~2-fold compared to low-glucose levels. Furthermore,
39 etomoxir-mediated inhibition of FAO enhanced by ~40% esterification capacity, and elevated by
40 1.5-fold triglycerides content in placental explants of women with no complications. Finally, high-
41 glucose levels reduced ~70% CPT-I activity, and ~25% phosphorylation levels of acetyl-CoA
42 carboxylase in placental explants of women with no complications.

43 **Conclusion:** We reveal an unrecognized regulatory mechanism on placental fatty acid
44 metabolism by which high-glucose levels reduce mitochondrial FAO through inhibition of CPT-I,
45 shifting flux of fatty acids away from oxidation towards the esterification pathway, leading to
46 accumulation of placental triglycerides.

47 **Keywords:** Carnitine palmitoyltransferase I, de novo fatty acid synthesis, esterification of fatty
48 acids, fatty acid oxidation, gestational diabetes mellitus, hyperglycemia, placenta, triglycerides.

49 **Abbreviations:** Acetyl-CoA carboxylase, ACC; Carnitine palmitoyltransferase I, CPT-I;
50 Carnitine palmitoyltransferase II, CPT-II; Fatty acid oxidation, FAO; Free fatty acids, NEFA;
51 GDM, Gestational diabetes mellitus.

52 # Introduction

53 Pregnancies affected by gestational diabetes mellitus (GDM) are characterized by various
54 degrees of maternal glucose intolerance, hyperglycemia and hyperinsulinemia (6). Several
55 epidemiological studies have shown that GDM is independently associated with adverse perinatal
56 outcomes (9, 36, 42). The main adverse outcome of maternal diabetes is fetal macrosomia, which is
57 characterized by fetal fat accretion and overgrowth (27, 42). The HAPO (Hyperglycemia and
58 Adverse Pregnancy Outcome) Study Cooperative Research Group has demonstrated an association
59 between maternal hyperglycemia and fetal macrosomia (1, 26), suggesting that maternal
60 hyperglycemia is a contributing factor to fetal macrosomia by enhancing substrate availability to the
61 fetus, stimulating excessive growth and formation of adipose tissue (13, 34).
62 The underlying mechanisms by which maternal hyperglycemia translate into fetal adiposity are
63 incompletely understood. In 1954, Pedersen proposed that maternal hyperglycemia results in
64 augmented transplacental glucose transfer leading to hyperglycemia in the fetus, which stimulates
65 the production and secretion of insulin by the fetal pancreatic beta-cells. Hence, glucose surplus and
66 hyperinsulinemia would play a direct role in the accumulation of fat in fetal adipose tissue (30, 31).
67 However, Szabo et al. proposed a different hypothesis to explain fetal macrosomia in diabetic
68 women. The hypothesis postulates that high maternal plasma free fatty acids levels (NEFA),
69 secondary to maternal insulin resistance, lead to increased transplacental transfer of NEFA to the
70 fetus, which are subsequently transported to fetal adipocytes and esterified into triglycerides. In this
71 scenario, maternal hyperglycemia does not contribute directly to fetal fat accretion in the form of
72 energy oversupply, but rather maternal glucose is used as a source of the glycerol, necessary for

73 NEFA esterification (41). Several clinical studies have reinforced the idea that elevated maternal
74 plasma triglyceride levels may account for fetal fat accretion (15, 22, 23, 28, 38).

75 Recently, it has been shown that placental lipid metabolism is altered in placentas from diabetic
76 women (16, 25, 34, 35). These findings have prompted the notion that placental lipid metabolism
77 may represent a regulatory step towards fetal macrosomia (14, 34, 39, 41). In this study, we aimed
78 to further understand the role of maternal hyperglycemia on the regulation of placental lipid
79 metabolism. To this end, we tested the hypothesis that high-glucose levels inhibit placental fatty
80 acid oxidation leading to enhanced NEFA esterification and accumulation of placental triglycerides.

81 Methods

82 Study subjects

83 The study was performed on placentas from pregnancies monitored at the Department of
84 Obstetrics and Gynecology, University Hospital "Puerta del Mar" (HUPM). Patient samples were
85 obtained after written informed consent in accordance with the HUPM Ethics Committee
86 requirements and the Declaration of Helsinki. Patients were eligible among consecutive pregnant
87 women attending our antenatal clinic who were planned to deliver by an elective Caesarean section
88 due to clinical reasons other than diabetes, and potentially not affecting placental metabolism
89 (breech presentation or prior Caesarean section). This was so to rule out potential effects of labor on
90 placental energy metabolism. Specific exclusion criteria included women under the age of 18,
91 smokers or those with a history of long-chain 3-hydroxyacyl-CoA deficiency, hemolysis elevated
92 liver function syndrome or acute fatty liver of pregnancy, preeclampsia, chronic hypertension, or
93 other co-morbid disease. The diabetic group was composed of 8 gestational diabetic women. Only
94 cases needing insulin therapy for metabolic control were eligible and offered to participate in the
95 study in order to include only cases with clear metabolic impairment. Maternal diabetes mellitus

96 was defined as an abnormal glucose tolerance according the criteria defined by the National
97 Diabetes Data Group (18), which have been accepted by the Spanish Group of Diabetes in
98 Pregnancy (11). Screening was performed using a two-steps approach in pregnant women between
99 24-28 weeks of gestation. The initial screening procedure consisted of a 50-g glucose challenge
100 test, with a 1-h blood glucose cut-off set at ≥7.76 mmol/l. Women with a positive screening test
101 underwent a confirmatory 3-hour 100-g oral glucose tolerance test (fasting glucose ≥5.82 mmol/l;
102 1-hour, ≥10.54 mmol/l; 2-hour, ≥9.15 mmol/l; and 3-hour, ≥8.04 mmol/l). Gestational diabetes
103 mellitus was defined when two or more plasma glucose measurements were equal or higher than the
104 cut-off points. Insulin therapy was indicated if more than one-third of capillary peripheral glucose
105 measurements were higher than the targets (>5.27 mmol/l fasting, >5.82 mmol/l preprandrial and
106 >7.76 mmol/l 1-hour postprandrial). In total, 14 women with no pregnancy complication
107 participated in the control group. Randomly chosen subsets of either 6 or 8 controls were used for
108 the experiments as indicated in the legend of Figures. Demographics and baseline data, as well as
109 perinatal variables, are shown in Table 1. All Caesarean sections were performed at term. Placental
110 samples and fasting maternal blood samples from control and GDM group were obtained at the time
111 of the elective Caesarean section. At this time no significant differences were found in lipids,
112 glycemia nor insulinemia levels. Neonatal anthropometric measurements were performed
113 immediately at delivery as usual. Fetuses of women with GDM showed a slight tendency to have
114 higher birthweight and placental weight was significantly higher in this group.

115 **Biochemical parameters**

116 All biochemical parameters were analyzed at the Clinical laboratory, HUPM, using reagents and
117 modular systems from Roche Diagnostics. Plasma insulin was measured by
118 electrochemiluminiscence immunoassay (ECLIA) by E-170 using 20 μl of sample. Plasma
119 glucose, triglycerides, total cholesterol and high-density lipoprotein cholesterol (HDL-c) were

120 measured by standard enzymatic methods by C-711 using between 2-3 µl of sample. Low-
121 density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald-Fredrickson
122 formula.

123 **Placental explants culture**

124 Term placenta obtained from elective Caesarean section was placed on ice and arrived to the
125 laboratory within 10-15 minutes of delivery. Then, decidual tissue and large vessels were removed
126 from villous placenta by blunt dissection on aseptic culture conditions. Afterwards, small fragments
127 of villous tissues (~100 mg wet weight) were rinsed twice in cold-PBS and 6 explants were
128 transferred to each well of a 6-well plate containing 2 ml of culture medium (RPMI-1640
129 supplemented with 5 mmol/l glucose, 10% FBS (vol/vol), 100 units/ml penicillin G, and 100 µg/ml
130 streptomycin) and maintained at 37ºC in a humidified atmosphere of 5% CO_2/ 95% O_2 for 1h prior
131 to experiments. Villous explant viability and morphological integrity was assessed by XTT (XTT
132 kit, Roche) and haematoxylin-eosin staining respectively.

133 **Materials**

134 Cell culture reagents (RPMI-1640 medium without glucose and fetal bovine serum) were from
135 Invitrogen/Gibco, California, USA. The [9,10-^{3}H]-palmitic acid, [^{3}H]-H_2O, D-[^{14}C(U)]-glucose and
136 L-[N-methyl-^{14}C]carnitineHCl were from PerkinElmer, Massachusetts, USA. Etomoxir and
137 essentially fatty acid-free bovine serum albumin were from Sigma, St. Louis, USA.

138 **Fatty acid solution preparation**

139 Stock of fatty acid solution was prepared by conjugating palmitate with essentially fatty acid-
140 free bovine serum albumin (BSA) to generate a stock solution of 25% (wt/vol) BSA, 4 mmol/l
141 palmitate in glucose-free culture medium. Stock solution was filtered-sterilized and diluted into the
142 final culture medium to give concentrations of 1.25% BSA, 0.1 or 0.2 mmol/l palmitate.

143 **Fatty acid oxidation assay in placental explants**

144 Mitochondrial FAO assays were performed ex vivo in placental explants as described
145 previously (2, 32) with the following modifications. Freshly isolated villous explants were
146 incubated in culture media supplemented with low (5 mmol/l) or high (11 mmol/l) glucose
147 concentrations, and in the presence of 1.25% BSA, 0.1 mmol/l cold palmitate, and 18500 Bq/ml
148 [^{3}H]-Palmitate at 37ºC for 18h. The glucose concentration in culture medium for the experiments in
149 which glucose was not an experimental factor was 5 mmol/l. Glucose was added to media from a
150 sterile stock solution of 1 mol/l glucose. At the end of the incubation period, the medium was
151 collected, and tritiated water determined by the vapor-phase equilibration method of Hughes et al
152 (21). FAO was defined as nmol of palmitate per mg of tissue per hour.

153 **Esterification into total lipids in placental explants**

154 The esterification rate in placental explants was determined as previously described with some
155 modifications (5). Briefly, after similar incubation conditions to those used for measurements of β-
156 oxidation, with low or high glucose levels in the presence of 1.25% BSA, 0.1 mmol/l cold
157 palmitate, and 18500 Bq/ml [^{3}H]-palmitate for 18h, explants were washed 3 times with 2 ml of ice-
158 cold PBS and homogenized in 500 µl of PBS. An aliquot of 100 µl was used to extract the lipid
159 content from samples according to Bligh and Dyer (3). Afterwards, the radioactive content was
160 determined by liquid scintillation counting. Esterification was defined as nmol of palmitate per mg
161 of tissue per hour.

162 **De novo lipid synthesis in placental explants**

163 *De novo* lipid synthesis was determined using [^{14}C]-glucose according to the procedure
164 described by Brown et al. with some modifications (5). Villous Placental explants from control
165 group were incubated in RPMI-1640 culture media with low- or high-glucose levels (5 mmol/l and

166 11 mmol/l respectively) and 37000 Bq/ml [^{14}C]-glucose at 37ºC for 18h. At the end of the
167 incubation period, culture media were discarded and explants collected, rinsed 3 times with 2 ml of
168 ice-cold PBS, followed by homogenization in 500 µl of PBS. After a total lipid extraction (as
169 described for measurements of placental esterification rate), the radioactive content was determined.
170 De novo lipid synthesis is expressed as pmol per mg of tissue per hour.

171 **Placental triglyceride determination**

172 Placental triglyceride determination was determined as previously described (33). Frozen
173 placental explants from control and GDM group (~20 mg) were used for experiments showed in
174 Figure 1. For the rest of the experiments, placental explants were preincubated in low or high-
175 glucose in the presence of 0.1 mmol/l palmitate for 18h as described above. Tissues were
176 homogenized in 400 µl HPLC-grade acetone. After incubation with agitation at room temperature
177 overnight, aliquots of 5 µl of acetone-extracted lipid suspension were used to determined
178 triglyceride concentrations using a triglyceride reagent kit (Biosystems, Barcelona, Spain). Proteins
179 were quantified using the bicinchoninic acid method (Thermo Scientific, Madrid, Spain). Placental
180 lipid content was defined as mg of triglyceride per mg of total placental proteins.

181 **Western blot analysis**

182 Placental explants from control group were preincubated in RPMI-1640 culture media
183 containing low- or high-glucose levels for 18h. At the end of the incubation period culture media
184 were discarded, explants were collected and washed with ice-cold PBS, followed by
185 homogenization in lysis buffer (20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1
186 mmol/l EGTA, 1% (v/v) Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-
187 glycerophosphate, 1 mmol/l Na_3VO_4, 1 µg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride))
188 plus protease inhibitors (Protease Inhibitor Cocktail, Sigma, St. Louis, MO). After 10 min. on ice,

189 extracts were sonicated and centrifugated at 18,000 X *g* for 10 minutes at 4ºC. Pellets were
190 discarded and solubilized proteins (40-60 μg/sample) were resolved by 5% SDS-PAGE for
191 phospho-acetyl-CoA Carboxylase (p-ACC) and 10% SDS-PAGE for actin, and electrotransferred
192 onto polyvinylidene difluoride filters for immunoblotting by conventional means. After probing
193 with specific p-ACC antibody (1:1000, Cell Signaling, Barcelona, Spain), the membranes were
194 stripped and reprobed with antibody against actin (1:3000, Sigma). Signals were detected by
195 chemiluminiescence (Immun-Start western chemiluminiescence kit, Bio-Rad, Madrid, Spain), and
196 band densitometry was quantified with the ImageJ software (NIH, USA.

197 **Mitochondrial citrate synthase assay**

198 As an index of mitochondrial content, citrate synthase activity was measured using the Citrate
199 Synthase Assay kit (Sigma, St. Louis, USA) according to manufacturer's instructions, in placenta
200 from control and GDM group. Protein content was determined as above. Citrate synthase activity
201 was defined as nmol/ml/min.

202 **CPT assay**

203 Activities of carnitine palmitoyltransferase I (CPT-I) and carnitine palmitoyltransferase II
204 (CPT-II) were determined in the direction of acyl-carnitine formation, using [^{14}C]-carnitine as
205 substrate (4). Briefly, placental explants were preincubated in RPMI-1640 culture media containing
206 low- or high-glucose levels at 37ºC for 18h. At the end of the incubation period culture media were
207 discarded, explants were collected and washed with ice-cold PBS priory homogenization in lysis
208 buffer (5 mmol/l Tris-HCl, pH 7.2, 150 mmol/l KCl) with a glass homogenizer. For assay of CPT-I,
209 100 μl of cell homogenate, in which the mitochondria remain largely intact, was incubated in the
210 presence of 50 μmol/l palmitoyl-CoA, 500 μmol/l carnitine and 9250 Bq/ml [^{14}C]-carnitine, in a
211 30ºC shaking water bath for 10 min. For assay of CPT-II, a portion of the homogenate was adjusted

212 to 1 % (w/v) of the detergent octylglucoside, which solubilizes the mitochondrial membranes,
213 inactivating CPT-I and releasing CPT-II from the mitochondrial matrix in active form. Afterwards,
214 reactions were stopped by adding 500 µl 1.2 *N* HCl and palmitoyl-[^{14}C]-carnitine was extracted by
215 adding 500 µl of 1-butanol. Radioactive content was determined by liquid scintillation counting.

216 **Statistical analysis**

217 Statistical analysis of data was performed using the SPSS software (SPSS, Inc., Chicago, IL).
218 Distributions were checked with a histogram and the Kolmogorov-Smirnov test. When a variable
219 was distributed normally, data were presented as mean ± S.D. In cases of non-normal distribution,
220 data were shown as median and interquartile range. Comparisons were done by using the Mann
221 Whitney's U test or ANOVA. Differences were considered significant at $p<0.05$.

222 Results

223 **Reduced fatty acid oxidation and elevated triglyceride levels in placentas from women with**
224 **gestational diabetes**

225 To reveal the metabolic characteristics of placentas from women with GDM, we determined the
226 FAO capacity in placental explants from control and diabetic women. As shown in Figure 1A, FAO
227 was reduced by ~30% in placentas of women with gestational diabetes compared with the control
228 group. A reduction in FAO capacity could be explained by a lower mitochondrial number in the
229 GDM group. However, as assessed by citrate synthase activity, mitochondrial content was similar
230 between placental explants from control and diabetic women, suggesting that the molecular
231 mechanism underlying reduced FAO capacity in diabetic group may be related to other factors
232 rather than to mitochondrial number (Fig1B). Coinciding with reduced FAO, triglyceride levels in
233 the GDM group were 3-fold higher compared to control group (Fig1C). Taken together, these

234 results indicate an association between reduced FAO capacity and accumulation of triglycerides in
235 placentas from diabetic women.

236 **Effect of high-glucose levels on fatty acid oxidation and triglyceride levels in explants of**
237 **human placenta.**

238 Maternal hyperglycemia is a hallmark of women with gestational diabetes. Therefore, it is
239 reasonable to hypothesize that the impaired ability of placentas from women with GDM to oxidize
240 fatty acids is a direct consequence of placental glucose surplus environment, leading to
241 accumulation of placental triglycerides. To test this hypothesis, we measured the effect of low- or
242 high-glucose levels on FAO in placental explants from control group. As shown in Figure 2A, high-
243 glucose levels significantly reduced the FAO rate in placental explants. In parallel, high-glucose
244 levels enhanced fatty acid esterification in the presence of 0.1 and 0.2 mmol/l palmitate (Fig2B).
245 Likewise, esterification augmented at increasing concentrations of palmitate from 0.1 to 0.2 mmol/l,
246 (Fig2B). However, *de novo* fatty acid synthesis using [^{14}C]-glucose as carbon source remained
247 unchanged (Fig2C). Similar findings were found for *de novo* fatty acid synthesis using [^{14}C]-acetate
248 as carbon source (data not shown). High-glucose levels significantly increased by ~2-fold the
249 placental triglyceride content (Fig2D), consistent with the expectation that fatty acids are
250 preferentially directed towards esterification under that condition. Taken together, these data
251 indicate that high glucose levels alter the placental triglycerides content through inhibition of FAO.

252 **Etomoxir-mediated inhibition of fatty acid oxidation increases triglyceride accumulation in**
253 **placental explants**

254 To gain further insight into the molecular mechanism by which high glucose levels alter
255 placental fatty acid partitioning, we used etomoxir, a specific and irreversible inhibitor of the
256 carnitine palmitoyltransferase I (CPT-I), to evaluate the impact of inhibition of mitochondrial fatty

257 acid entry on FAO, fatty acid esterification and the storage pool of triglycerides in placenta from
258 healthy women. Etomoxir treatment significantly inhibited FAO capacity in placental explants
259 (Fig3A), resulting in augmented esterification (Fig3B), and higher placental triglyceride content
260 (Fig3C).

261 **High-glucose levels decreases carnitine palmitoyltransferase I activity in placental explants**

262 We further investigated the mechanisms by which high-glucose reduced FAO capacity in
263 human placental explants. To this end, we measured the activity of CPT-I and CPT-II in placental
264 explants from control group preincubated in low- or high-glucose levels for 18h. As shown in
265 Figure 4A-B, high-glucose levels reduced by ~70% the activity of CPT-I, whereas CPT-II activity
266 remained unchanged as expected. Because malonyl-CoA is a physiological regulator of CPT-I
267 activity, we quantified the phosphorylation levels of ACC, the enzyme that catalyzes the ATP-
268 dependent carboxylation of acetyl-CoA to form malonyl-CoA. Interestingly, phosphorylation levels
269 of ACC were reduced by ~25% in the presence of high-glucose levels (Figure 4C), suggesting an
270 increased production of malonyl-CoA in placental explants.

271 Discussion

272 The availability of maternal nutrients to the fetus is regulated by the placenta involving three
273 main mechanisms: direct transfer of nutrients, placental consumption of nutrients and placental
274 conversion of nutrients into alternative fuel sources (19). Direct transfer has been considered the
275 main mechanism by which placenta regulates the nutrient-exchange between the mother and the
276 fetus (19). However, the placenta exhibits a high metabolic activity, which is severely affected by
277 the intrauterine milieu of diabetic and/or obese women. Specifically, studies performed on placentas
278 from diabetic women have shown major changes in expression levels of genes involved up-
279 regulation of pathways of lipid synthesis and transplacental lipid fluxes (16, 25, 34, 35). These

280 findings have spurred the notion that alterations in placental lipid pathways perhaps contribute to
281 fetal fat accumulation and adiposity in diabetic women (8, 34).
282 The FAO pathway has not been evaluated in placenta from GDM women. In this study, we
283 demonstrated that these women exhibited lower FAO oxidation capacity without change in
284 mitochondrial content. To explain these observations, we hypothesized that lower FAO capacity
285 may be related to maternal hyperglycemia, a hallmark of GDM women. However, the metabolic
286 environment of women with GDM is characterized also by the presence of excessive NEFA levels
287 and pro-inflammatory cytokines (6, 35), which makes difficult to tease apart the causing factor
288 involved in reduced placental FAO observed in these women. Thus, we attempted to mimic
289 maternal milieu of women with GDM in our *ex vivo* studies, using low-and-high glucose levels, and
290 low-and-high NEFA levels. Therefore, a limitation of this study is that although our *ex vivo* culture
291 conditions for placental explants clearly allowed mechanistic studies; they may not accurately
292 reflect a GDM milieu and replicate *in vivo* pathology. Thus, our findings in placenta from women
293 with GDM may be explained by other factors related to obesity, such as elevated NEFA and/or pro-
294 inflammatory cytokines, rather than maternal hyperglycemia. However, obesity is not a
295 confounding factor in the phenotype of the GDM women group in our study population (BMI was
296 similar between both groups), which supports the notion that only GDM related factors, such as
297 higher glucose levels, may trigger the observed modifications. Although glycemia and insulinemia
298 levels were only determined in the fasting state, it may be highlighted that the absence of
299 differences between the two groups may be also attributed to the prescription of a strict metabolic
300 control in patients with GDM. Along this line, there were no differences in the levels of
301 glycosylated haemoglobin between the two groups. Nevertheless, further studies are warranted to
302 investigate regulation of FAO pathways using placental explants from women with type I diabetes,
303 type II diabetes, and obese non-diabetic women.

304

305

306

307 Using placental explants from women with no pregnancy complication, we demonstrated that
308 high-glucose levels inhibited FAO and increased triglyceride accumulation. These results are in
309 agreement with our findings in placentas from GDM women. Because *de novo* fatty acid synthesis
310 remained unchanged, and because etomoxir-mediated inhibition of CPT-I recapitulated the effects
311 of high-glucose on FAO and esterification pathways, we thought that the mechanistic link between
312 high-glucose levels and lower FAO was inhibition of CPT-I activity by its physiological inhibitor
313 malonyl-CoA, which is synthesized from glucose-derived acetyl-CoA by ACC. Following this
314 rationale, we demonstrated that CPT-I activity and phosphorylation of ACC was significantly
315 decreased by high-glucose levels. Because phosphorylation of ACC inhibits its enzymatic activity,
316 our results support the notion that FAO is diminished by high-glucose levels through decreased
317 ACC phosphorylation and enhanced production of malonyl-CoA levels in placental explants, which
318 resulted in lower CPT-I activity. Interestingly, this mechanism results in a shift of fatty acid
319 partitioning away from the β-oxidation pathway towards esterification, allowing the accumulation
320 of triglycerides in human placenta.

321 These alterations in lipid metabolism mediated by high-glucose levels beg for two important
322 questions: 1) What are the consequences of triglycerides accumulation in placenta? 2) Is placental
323 storage of triglycerides a contributing factor to fetal macrosomia? Several studies have
324 demonstrated that maternal serum triglyceride levels are associated with abnormal fetal growth in
325 women with GDM, type 1 and type 2 diabetes (17, 38), spurring the notion that increased maternal
326 lipid availability results in fetal fat accretion. In a hypothetical scenario of maternal triglycerides
327 oversupply and elevated lipolysis rate at the maternal-placental side, esterification of NEFA into

328 triglycerides in placental cells may indicate a regulatory system to limit maternal fatty acids transfer
329 to the fetus, and serve as a protective mechanism against fetal macrosomia. However, there is no
330 data about the lipolysis rate of very low-density lipoproteins and chylomicron remnants in placentas
331 from women with GDM. Thus, although placental lipid metabolism has been proposed as a
332 regulatory step towards fetal macrosomia (14, 34, 39, 41), it is still missing a direct evidence
333 demonstrating that unbalanced triglycerides storage in placental cells results in augmented
334 transplacental delivery of adipogenic substrates to the fetus. On the other hand, accumulation of
335 triglycerides or its harmful intermediaries, such as ceramide and diacylglycerol, in trophoblast cells
336 may exacerbate the basal pro-inflammatory state of pregnancy. In this hypothetical scenario,
337 accumulation of triglycerides in placental cells would trigger inflammatory pathways in trophoblast
338 cells and deleterious effects on placental and fetal metabolism. Several studies support the idea that
339 GDM and/or obesity induces inflammatory pathways in placenta (7, 12, 24, 35).
340 Our results on fatty acid partitioning contrast with early studies performed by Pathmapeura et
341 al. in trophoblast isolated from normal term human placentas. They showed that low- or high-
342 glucose (0,5-18 mmol/L) levels had not significant effects on FAO and esterification processes in
343 cultured trophoblast exposed to short (2h) or longer (24h) periods of time (29). The differences
344 between both studies may be explained by the experimental models employed. Firstly, Pathmapeura
345 et al. used cultured trophoblast isolated from human placentas, whereas we used placental explants.
346 The latter technique allows the possibility to investigate trophoblast function in a context that
347 contains other cell types (fibroblasts, macrophages, endothelial cells, etc.) and retains the cellular
348 architecture of the tissue *in vivo*. Secondly, trophoblast cells were maintained in culture media for
349 16h prior initiation of experimental procedures, whereas placental explants were only maintained in
350 culture media for 1h. Finally, they investigated the effects of glucose levels on fatty acid

351 partitioning for 24h in the presence of 0.25 mmol/l non-esterified fatty acids (palmitate:oleate ratio
352 1:1), whereas we used 0.2 mmol/l palmitate as a source of non-esterified fatty acids.
353 We showed that placenta from healthy women can incorporate [^{14}C]-glucose into lipids,
354 corroborating previous studies concerning the *de novo* fatty acid synthesis capacity of human
355 placenta (10, 20, 40). Whereas high-glucose did not result in a significant increase in *de novo* lipid
356 synthesis in placental explants, FAO was decreased, suggesting an increase in glucose-derived
357 malonyl-CoA. Under these experimental conditions ACC activity appeared to function primarily as
358 a regulator of the FAO pathway, rather than a regulator of the *de novo* fatty acid synthesis pathway.
359 A similar role for ACC has been described in tissues with low *de novo* fatty acid synthesis capacity,
360 such as skeletal and cardiac muscle (37). Early studies suggested that *de novo* fatty acid synthesis
361 pathway plays a minor role in triglyceride accumulation in diabetic placenta, consistent with our
362 observation on *ex vivo* metabolism (10, 20, 39, 40). Finally, we acknowledge that a limitation of our
363 study is that placental explants were preincubated in the absence of insulin, which is present in the
364 *in vivo* milieu and it is required for *de novo* lipid synthesis. Therefore, taken into consideration our
365 experimental conditions without insulin and given the non-significant trend towards increased
366 [^{14}C]-glucose incorporation into lipid in the presence of high glucose, we cannot conclude that
367 elevations in glucose do not increase placental *de novo* lipid synthesis *in vivo*.
368 In conclusion, we demonstrate that high-glucose levels alter the metabolic partitioning of fatty
369 acids in human placenta, shifting flux of fatty acids away from oxidation towards the esterification
370 pathway, leading to accumulation of placental triglycerides. The mechanistic link between high-
371 glucose levels and lower FAO capacity is through reduced activity of the enzyme CTP-I, which
372 regulates the first step of the entry of long-chain acyl-CoA into the mitochondrial matrix for β-
373 oxidation. These findings shed light on the biochemical mechanisms by which maternal
374 hyperglycemia may regulate placental lipid pathways in diabetic mothers.

375 **Acknowledgments:** We thank Dr. Nicholas F. Brown (CardioMetabolic Disease Research
376 Boehringer Ingelheim Pharmaceuticals Inc., USA) for thoughtful discussions of the ideas in this
377 report.
378 **Funding:** This study was supported by a grant from the Carlos III Health Institute
379 (CP08/00106), the Spanish Ministry of Science and Innovation (SAF2009-11282) and the FP7-
380 PEOPLE-2009-RG (PIRG06-GA-2009-25369) to GP; grant from the Consejería de Salud, Junta de
381 Andalucía (Nº0269/05.2005) to JLB; and grant from the Carlos III Health Institute (PI11/00676) to
382 FB.
383 **Duality of interest:** The authors declare no conflict of interest in the research.Contribution
384 statement: FV, VS and IC performed experiments, analyzed data, and revised the manuscript for
385 important intellectual content. FB, JB and GP design experiments, conceived the work, participated
386 in clinical data collection, analyzed data, and revised the manuscript for important intellectual
387 content. GP wrote the manuscript. All authors approved the final version to be published.

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505 # Figure legends

506 **Figure 1. Fatty acid oxidation is reduced in placenta from women with gestational**
507 **diabetes.** (a) Mitochondrial fatty acid oxidation. A subset of 8 placentas from women with no
508 pregnancy complication (control group, n=8) and gestational diabetic women (GDM, n=8) were
509 used to obtain villous explants as described in "Methods" section. The explants were preincubated
510 with 0.1 mmol/l (18500 Bq/ml) palmitate for 18 hours, and the production of [^{3}H]-water was
511 determined as described in the "Methods" section. Values are Mean ± S.D. for 8 independent
512 experiments in triplicate. Significance is indicated (*$p<0.05$) relative to control group. (b)
513 Mitochondrial content. Citrate synthase activity, an indicator of mitochondrial content, was assayed
514 in placental explants from control and GDM group. Values are Mean ± S.D. for 8 independent
515 experiments in duplicate. $p=0.845$ relative to control group. (c) Placental triglyceride content.
516 Frozen placental tissues (~100 mg) from control (n=8) and GDM group (n=8) were used to quantify
517 placental triglyceride content as described in the "Methods" section. Values are Mean ± S.D. for 8
518 independent experiments in triplicate. *$p<0.05$ relative to control group.

519 **Figure 2. High-glucose levels inhibit fatty acid oxidation in placentas from healthy women.**
520 (a) Effect of high-glucose levels on fatty acid oxidation. A subset of 6 placentas from women with
521 no pregnancy complication described in table 1 was used to obtain villous explants. Placental
522 explants from control group were incubated at 5 (5 Gl) or 11 (11 Gl) mmol/l glucose in the presence
523 of 0.1 (0.1 Pa) mmol/l palmitate for 18 hours. Afterwards, [^{3}H]-water was determined as described
524 in the "Methods" section. Values are Mean ± S.D. for 6 independent experiments in triplicate.
525 Significance is indicated (*$p<0.05$) relative to 5 mmol/l glucose. (b) Effect of high-glucose levels
526 on fatty acid esterification. A subset of 6 placentas from women with no pregnancy complication
527 described in table 1 was used to measure the esterification capacity. Placental explants from control
528 group were incubated at 5 (5 Gl) or 11 (11 Gl) mmol/l glucose in the presence of 0.1 (0.1 Pa) or 0.2

529 (0.2 Pa) mmol/l palmitate for 18 hours. Afterwards, [^{3}H]-palmitate incorporation into total lipids
530 was determined as described in the "Methods" section. Mean ± S.D. for 6 independent experiments
531 in triplicate. *p<0.05 relative to 5 mmol/l glucose; †p<0.05 relative to 0.1 mmol/l palmitate. (c)
532 Effect of high-glucose levels on de novo lipid synthesis. A subset of 4 placentas from women with
533 no pregnancy complication described in table 1 was used to obtain villous explants. Placental
534 explants were incubated at low (5 mmol/l) or high (11mmol/l) glucose levels in the presence of
535 [^{14}C]-glucose for 18 hours. Afterwards, [^{14}C]- glucose incorporation into total lipids was determined
536 as described in the "Methods" section. Mean ± S.D. for 4 independent experiments in triplicate.
537 *p<0.05 relative to low glucose. (d) Effect of high-glucose levels on placental triglyceride content.
538 The same subset of placentas used for fatty acid oxidation and esterification experiments described
539 above was used to measure triglyceride content. Placental explants were incubated as described
540 above and the triglyceride content was determined as described in the "Methods" section. Mean ±
541 S.D. for 5 independent experiments in triplicate. *p<0.05 relative to 5 mmol/l glucose.

542 **Figure 3. Etomoxir-dependent inhibition of fatty acid oxidation increases triglyceride**
543 **accumulation in placentas from healthy women.** (a) Fatty acid oxidation in placental explants
544 treated with various concentrations of etomoxir. A subset of 6 placentas from women with no
545 pregnancy complication described in table 1 was used to asses FAO capacity. Placental explants
546 were incubated in the absence or presence of 50 μmol/l, 100 μmol/l or 200 μmol/l etomoxir with
547 0.1 mmol/l (18500 Bq/ml) palmitate for 18 hours, and the production of [^{3}H]-water was determined
548 as described in the "Methods" section. Mean ± S.D. for 6 independent experiments in triplicate is
549 shown. *p<0.05 relative to untreated placental explants; †p<0.05 relative to 200 μmol/l etomoxir-
550 treated placental explants. (b) Fatty acid esterification in placental explants treated with various
551 concentrations of etomoxir. The same subset of placental explants described in panel A were used
552 to assess esterification into total lipids as described in the "Methods" section. Mean ± S.D. for 6

553 independent experiments in triplicate. *p<0.05 relative to untreated placental explants. (c) The same
554 subset of placental explants described in panel A were used to assess triglycerides content as
555 described in the "Methods" section. Mean ± S.D. for 6 independent experiments in triplicate.
556 *p<0.05 relative to untreated placental explants.

557 **Figure 4. High-glucose levels inhibit carnitine palmitoyltransferase I activity and reduce**
558 **phosphorylation levels of acetyl-CoA carboxylase.** A subset of 6 placentas from women with no
559 pregnancy complication described in table 1 was used to obtain villous explants and perform the
560 following experiments. Carnitine palmitoyltransferase I activity (a) and carnitine
561 palmitoyltransferase II activity (b) were determined as described in the "Methods" section in
562 placental explants incubated at low (5 mmol/l) or high (11 mmol/l) glucose concentrations for 18
563 hours. Mean ± S.D. for 6 independent experiments in duplicate is shown. *p<0.05 relative to 5
564 mmol/l glucose. (c) Western blot analysis of phospho-acetyl-CoA carboxylase (p-ACC) in protein
565 extracts from placental explants incubated at low (5 mmol/l) or high (11 mmol/l) glucose
566 concentrations for 18 hours. In the upper panel is shown a representative picture of the western blot.
567 In the lower panel the *y*-axes represents the ratio of phosphorylated acetyl-CoA carboxylase *versus*
568 β-actin in arbitrary units. Mean ± S.D. for 4 independent experiments in triplicate. *p<0.05 relative
569 to 5 mmol/l glucose.

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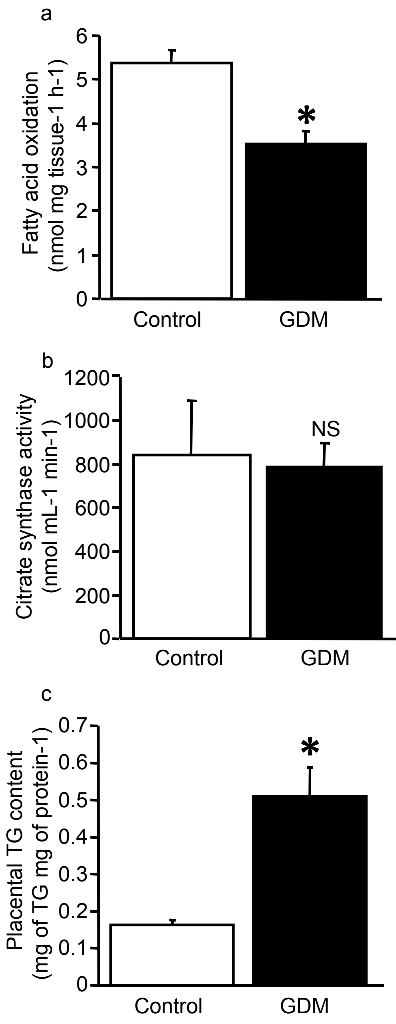

Figure 1

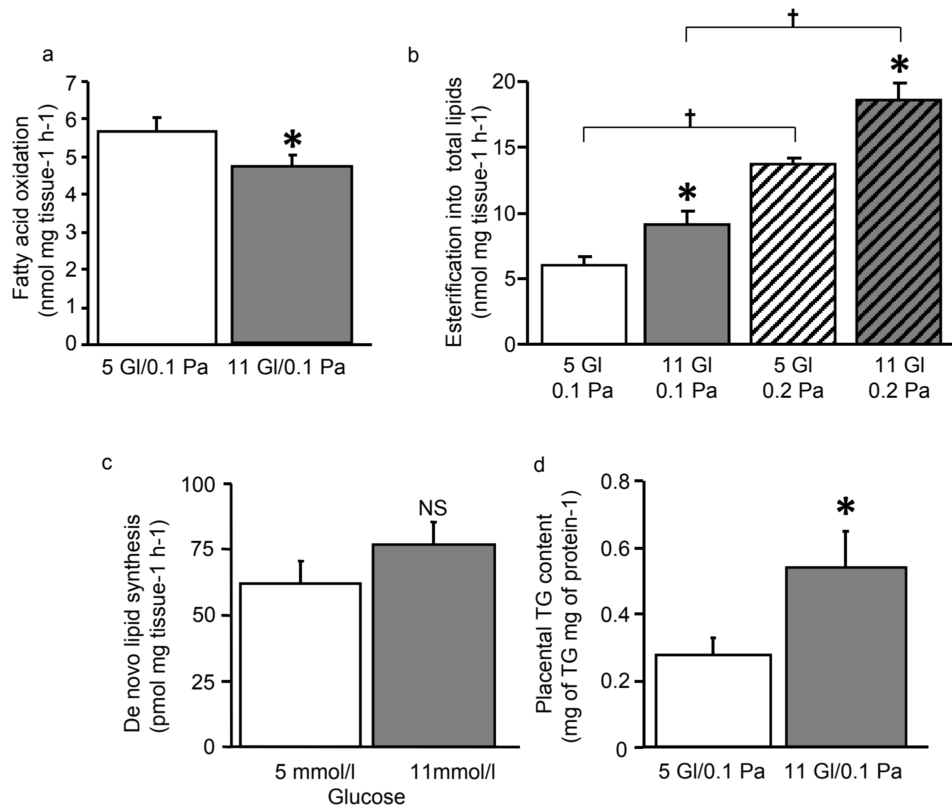

Figure 2

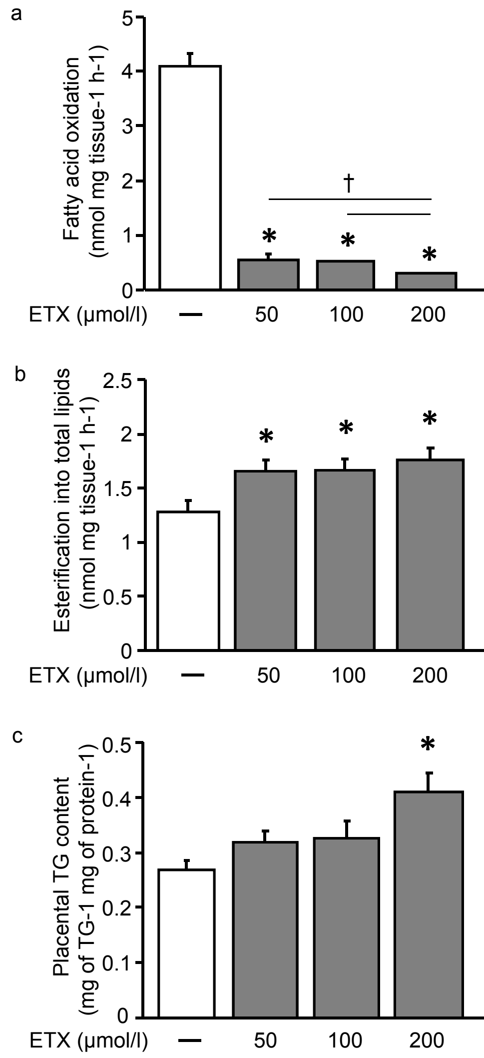

Figure 3

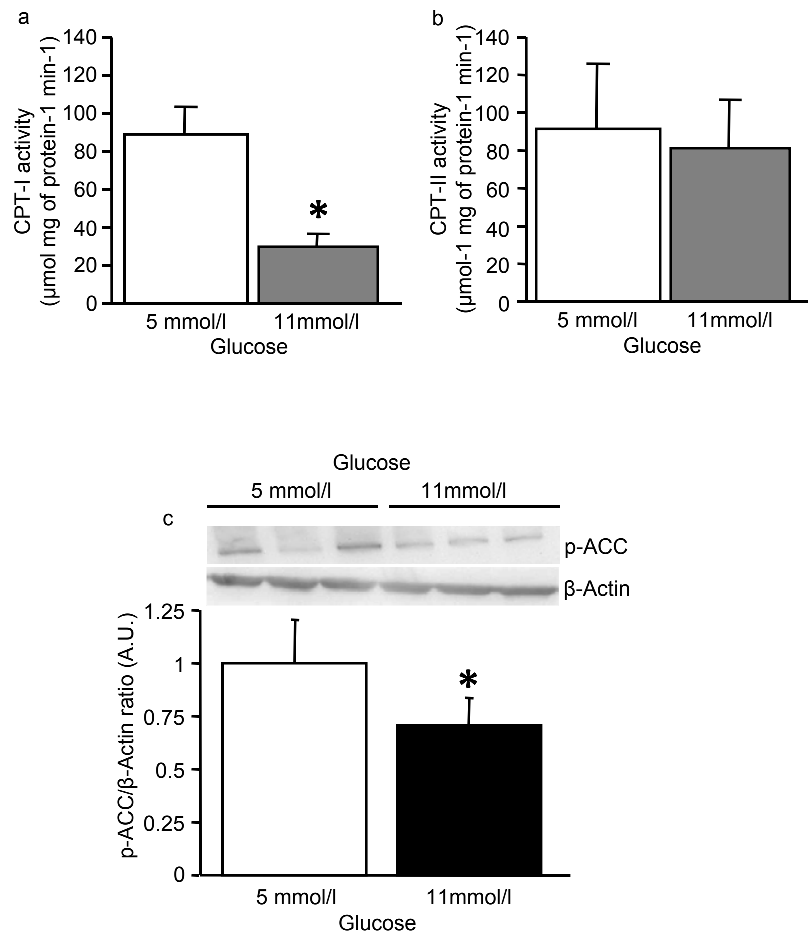

Figure 4

Table 1. Anthropometrics and metabolic data of the study population.

	Control group (n=14)	GDM Group (n=8)
Delivery mode	Caesarean section No labour	Caesarean section No labour
Maternal age (yr)	33.4 ± 4.6	36.3 ± 2.0
Gestational age (wk)	37.4 ± 1.81	39 ± 1.0
Maternal pregravid BMI	23.7 ± 4.8	25.8 ± 5.3
Maternal glucose (mg/dL)	77.8 ± 14.6	78,6 ± 10,2
Maternal insulin (pmol/L)	8.8 ± 4.6	8.6 ± 1.4
Maternal triglycerides (mg/dL)	185.8 ± 66.5	195 ± 12.2
Maternal Total Cholesterol (mg/dL)	252.8 ± 64.2	233 ± 47.6
Maternal HDL Cholesterol (mg/dL)	101,2 ± 54.4	62,33 ± 10.2
Maternal LDL Cholesterol (mg/dL)	121.8 ± 48,9	135.6 ± 37.8
Maternal HbA1c (%)	5.3 ± 0.3	5.3 ± 0.1
Placental weight (g)	510 ± 75	612 ± 74*
Birthweight (g)	3048 ± 591	3186 ± 362

When a variable is normally distributed, data are given as mean ± SD. *GDM*, Gestational diabetes mellitus; *BMI*, Body mass index; *HbA1C*, hemoglobin A_{1c}. *HDL*, High-density lipoprotein; *LDL*, Low-density lipoprotein. *$p<0.05$ vs control group.