RESEARCH

Paternal diabetes programs sex-dependent placental alterations and fetal overgrowth

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Abstract

The aim of this study was to evaluate the paternal programming of sex-dependent alterations in fetoplacental growth and placental lipid metabolism regulated by peroxisome proliferator-activated receptor (PPAR) target genes in F1 diabetic males born from F0 pregestational diabetic rats. F1 control and diabetic male rats were mated with control female rats. On day 21 of gestation, F2 male and female fetoplacental growth, placental lipid levels, and protein and mRNA levels of genes involved in lipid metabolism and transport were evaluated. Fetal but not placental weight was increased in the diabetic group. Triglyceride, cholesterol and free fatty acid levels were increased in placentas of male fetuses from the diabetic group. The mRNA levels of *Pparα* and *Pparγ coactivator* 1α (*Pgc-1α*) were increased only in placentas of male fetuses from the diabetic group. Protein levels of PPARα and PGC-1α were decreased only in placentas of male fetuses from the diabetic group. No differences were found in *Pparγ* mRNA and protein levels in placentas from the diabetic group. The mRNA levels of genes involved in lipid synthesis showed no differences between groups, whereas the mRNA levels of genes involved in lipid oxidation and transport were increased only in placentas of male fetuses from the diabetic group. In conclusion, paternal diabetes programs fetal overgrowth and sex-dependent effects on the regulation of lipid metabolism in the placenta, where only placentas of male fetuses show an increase in lipid accumulation and mRNA expression of enzymes involved in lipid oxidation and transport pathways.

Key Words

- paternal diabetes
- placenta
- fetal programming
- peroxisome proliferator-activated receptor
- lipids

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Introduction

The growing prevalence of obesity and type 2 diabetes has been related not only to sedentary lifestyle, the ingestion of hypercaloric diets and the genetic background but also to the fetal programming of glucose intolerance, insulin resistance, dyslipidemia and type 2 diabetes (Fernandez-Twinn *et al.* 2019). As a result of the exposure of the fetus to an intrauterine environment affected by some of these maternal insults, such as malnutrition, obesity and type 1, type 2 and gestational diabetes, these metabolic disorders will also be evident in postnatal life (Barker *et al.* 2002, Mitanchez 2015). Another consequence related to maternal disorders is increased birth weight, which has also been described as one of the risks of developing obesity, diabetes, hypertension and cardiovascular diseases (Barker 2006). It is thus clear that the maternal environment plays a dominant role in programming adult-onset diseases in

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the offspring. However, although the paternal metabolic status during sperm maturation before conception may also result in alterations in fetoplacental development that impact the offspring's long-term health (Li *et al.* 2016), much less is known about its contribution to such a phenomenon.

The main determinant of intrauterine growth is the placental supply of maternal nutrients and oxygen to the fetus. Placental lipid content depends on the maternal supply and placental *de novo* lipid synthesis (Capobianco *et al.* 2005, Lager & Powell 2012). The multiple factors that influence proper placental transport of nutrients include placental morphology and blood flow and abundance of transporters in the trophoblast (Larqué *et al.* 2014, Segura *et al.* 2017). Fatty acids (FAs) are lipid nutrients that can be internalized from the maternal circulation through different transport proteins. Both placental lipoprotein lipase (LPL) and endothelial lipase (EL), also named lipase G (LIPG), hydrolyze the lipoproteins, triglycerides and phospholipids transported in maternal blood, followed by the release of FAs and further internalization by the trophoblast (Barrett *et al.* 2014). Non-sterified FAs are internalized in the trophoblast through facilitated diffusion by transmembrane proteins such as fatty acid translocase (FAT/CD36), placental plasma membrane FA-binding proteins (FABPs) and FA transport proteins (FATPs). Once within the trophoblast, FAs can also bind intracellular FABPs, which drive their destiny to utilization, storage or transport to the fetal side (Lewis *et al.* 2018*a*).

Lipid metabolic pathways are regulated by different proteins, whose expression is in turn regulated by peroxisome proliferator-activated receptors (PPARs), which are ligand-activated transcription factors able to transactivate or transrepress several genes (Mirza *et al.* 2019). PPARs are also involved in the regulation of diverse anti-inflammatory and developmental pathways (Wahli & Michalik 2012). We have previously found that both the levels and activity of the three PPAR isotypes (PPARα, PPARδ and PPARγ) are altered in different periods of embryonic, fetal and placental development, as well as in placental and fetal tissues at term and in the offspring of female diabetic rats (Capobianco *et al.* 2005, Fornes *et al.* 2018, Roberti *et al.* 2020). The three PPAR isotypes regulate lipid metabolism by differentially regulating multiple enzymes such as fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (Scd-1), acetyl CoA carboxylase 1 (ACC1) and acetyl CoA carboxylase 2 (ACC2), involved in lipid anabolism; acyl-CoA oxidase (ACO) and carnitine palmitoyl-transferase 1 (CPT-1), involved in lipid oxidation; and LPL, EL, FATPs and CD36, involved in lipid transport (Munday & Hemingway 1999, Feng *et al.* 2000, Tontonoz & Spiegelman 2008, Monsalve *et al.* 2013, Barchuk *et al.* 2018).

During pregnancy, the placenta is exposed to a variety of environmental signals that can alter placental formation and thus fetal and placental weight (Sferruzzi-Perri & Camm 2016). Both neonatal and placental weight are involved in the fetal programming of metabolic diseases (Godfrey & Barker 1995). Programmed fetal changes arise through epigenetic mechanisms that affect the structure and function of individual organs in the offspring. If the developing gonads of the offspring are altered by an adverse intrauterine environment, changes programmed *in utero* may also be transmitted to subsequent generations (Li *et al.* 2016). We have previously shown that mild pre-gestational maternal diabetes (F0 generation) is associated with increased fetal and placental growth (Martínez *et al.* 2011*a*,*b*). Moreover, both the male and female offspring (F1 generation) of these mild diabetic rats have altered lipid metabolism, poor insulin and glucose handling and develop type 2 diabetes by 5 months of age (Capobianco *et al.* 2015). This is a model of type 2 diabetes induced by intrauterine programming with sex-dependent alterations in lipid levels and increased lipid peroxidation and nitric oxide levels in the F1 male and female offspring's hearts (Capobianco *et al.* 2015). The increasing evidence regarding sex-dimorphic pathophysiological mechanisms of several diseases such as type 2 diabetes mellitus and its complications highlights the importance of studying the biological factors responsible for sex differences in diabetes risk and outcome (Kautzky-Willer *et al.* 2016).

The evidence that environmental factors before conception and during sperm development determine the health of the offspring led us to hypothesize that paternal diabetes programs disorders in fetoplacental metabolism and fetal overgrowth (Watkins *et al.* 2018, Velazquez *et al.* 2019). Thus, the aim of this study was to evaluate the F2 fetoplacental growth, placental lipid levels and placental expression of PPARs and PPAR target genes involved in pathways related to lipid synthesis, oxidation and transport to find sex-dependent alterations transmitted by F1 diabetic males born from F0 pre-gestational diabetic rats.

Materials and methods

Animals

Outbred albino Wistar rats were bred in our animal facility with free access to commercial rat chow (Gepsa, Distribuidora Izaguirre, Buenos Aires, Argentina) and tap water. The rats were kept in a room at a controlled

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temperature of 20°C, on a 12 h light:12 h darkness cycle. The animal protocol was approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD no. 2587/2018), which follows the recommendations of the 'Guide for the Care and Use of Laboratory Animals' of the US National Institutes of Health (NIH Publication, 8th Edition, 2011; http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader (Accessed March 15, 2011)).

Experimental design

Mild diabetic rats (F0 generation) were obtained by injecting 2-day-old female neonates with streptozotocin (90 mg/kg, s.c., Sigma-Aldrich) diluted in citrate buffer (0.05 M pH 4.5, Sigma-Aldrich, St.Louis, MO, USA), as previously described (Portha *et al.* 1979, Capobianco *et al.* 2008) (Fig. 1). Control rats (F0 generation) were obtained by injecting female neonates with citrate buffer alone. Diabetes, defined as fasting blood glucose higher than 130 mg/dL, was confirmed in 2-month-old rats prior to mating. Blood glucose levels were 150–250 mg/dL in diabetic rats and 88–101 mg/dL in controls. The characteristics of this mild pre-gestational diabetic model have been reported previously (Jawerbaum & White 2010, White *et al.* 2015).

Then, F0 control and mild diabetic female rats were mated with control males as previously described ($n=7$) (Capobianco *et al.* 2016). The number of neonates per dam was adjusted to eight after birth, and only one male from each dam was used for this experimental design. The male offspring from mild diabetic rats ($n=7$) developed diabetes (glycemia values: 140–190 mg/dL) at 5 months of age, by intrauterine programming (F1 generation) (Capobianco *et al.* 2015). Glycemia was measured by using reagent strips and a glucometer (Accu-Chek, Bayer Diagnostics, Buenos Aires, Argentina) in blood samples collected from the tail vein. Seven healthy and seven diabetic F1 male rats (control and diabetic groups, respectively) were weighed and mated with seven control female rats, respectively ($n=7$). The first day of pregnancy was confirmed by the presence of sperm cells in vaginal smears. On day 21 of gestation, pregnant female rats were euthanized by decapitation after brief exposure to CO_2. All fetuses (F2 generation) and placentas were weighed. The sex of fetuses was determined under a stereomicroscope as previously described (Fornes *et al.* 2018). Fetal plasma was collected by decapitation and pooled separately into plasma from male fetuses and plasma from female fetuses from each pregnant rat ($n=7$ pregnant rats in each experimental group mated with a different F1 male (father)). Placentas from F2 male and female fetuses were randomly selected and dissected in fragments of 100 ± 10 mg. Placental explants were stored at −80°C for lipid content analysis by thin-layer chromatography and protein evaluation by Western blot or preserved in RNAlater® (Ambion, Inc., Austin, TX, USA) for gene expression studies by real-time reverse transcription (RT-qPCR).

Male rats were also euthanized by decapitation. Paternal and fetal plasma was obtained and stored at −80°C for metabolic determinations.

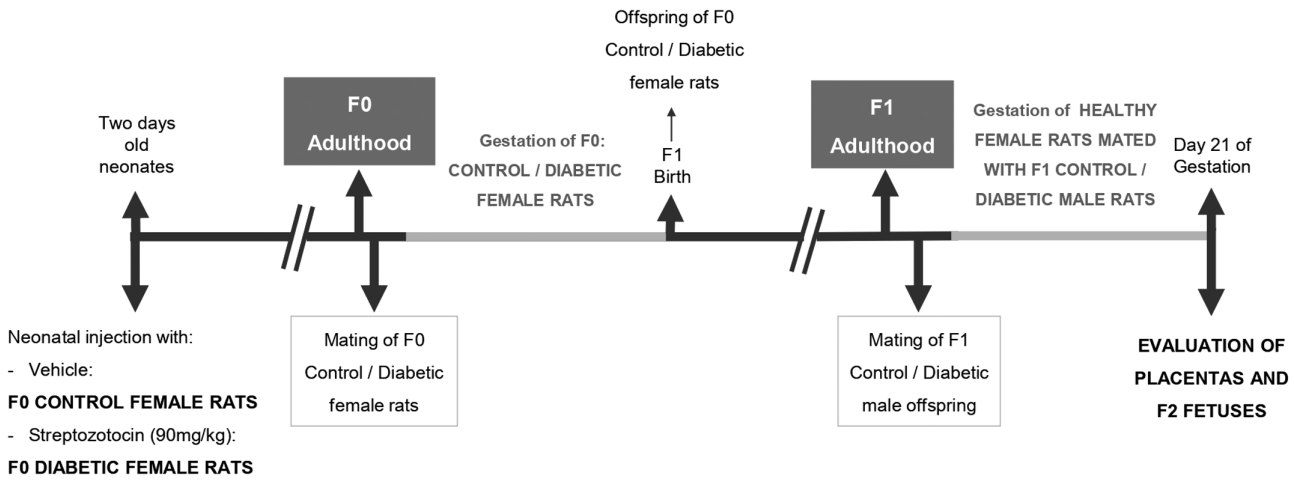

Figure 1
Experimental design. A rat model of mild diabetes was induced in Wistar rats by injecting streptozotocin (90 mg/kg) to 2-days-old female neonates (F0 generation). Control female neonates were injected with vehicle. Control and diabetic female rats were mated with control male rats. The male offspring of mild diabetic rats (F1 generation) developed type 2 diabetes by intrauterine programming. Control and diabetic male rats were mated with healthy female rats ($n=7$). On day 21 of gestation, pregnant female rats and F1 male rats were euthanized. Plasma from F1 males and F2 fetuses was collected and placentas from the F2 generation were used for biochemical and molecular analyses.

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Plasma metabolic parameters

Glycemia, triglyceridemia and cholesterolemia were evaluated in paternal plasma (at 5 months of age) and fetal plasma (on day 21 of gestation) from control and diabetic rats by spectrophotometric enzymatic assays (Wiener lab. Rosario, Argentina), as previously described (Capobianco *et al.* 2015).

Insulin levels were evaluated in paternal plasma from control and diabetic male rats at 5 months of age by a commercial assay kit (Mercodia Ultrasensitive Rat Insulin ELISA kit, Uppsala, Sweden) (Capobianco *et al.* 2015).

Determination of lipid concentrations

Placental explants of male and female fetuses from control and diabetic male rats were homogenized in 1 mL PBS. Protein content was determined in 10 µL of homogenate by the Bradford assay (Bradford 1976). Placental lipids were extracted from 500 µL of homogenates by three rounds of organic extraction with methanol:chloroform (2:1, v:v), following the method of Bligh and Dyer (1959). Volumes of lipid extraction containing 400 mg of proteins were developed by chromatography in thin silica gel plates (Merck, Darmstadt, Germany) by using hexane:ether:acetic acid (80:20:2, v:v:v) as the developing solvent mixture. Lipid species were stained with iodine vapors, identified and quantified by comparison with known amounts of standards on the same plate and analyzed densitometrically with Image J software. Results are expressed as micrograms per milligram of protein.

Measurement of mRNA expression by Real-time RT-qPCR analyses

Placental RNA from male and female fetuses from control and diabetic male rats was isolated from placental explants using RNAzol® (MCR Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Samples were subjected to a two-phase separation step and then the RNA was precipitated and resuspended in RNase-free sterile water. The concentration of total RNA was determined by using the NanoDrop 100 spectrophotometer (Thermo Scientific, IL, USA). cDNA was synthesized by incubating 2 µg of extracted RNA in a first-strand buffer containing 200 U MML-V enzyme (Promega, WI, USA), 7.5 mM random primer hexamers (Promega) and 0.5 mM of each of all four dNTPs (Invitrogen, Carlsbad, CA, USA). The reaction mixture was incubated for 60 min at 37°C, followed by 15 min at 70°C. cDNA (2.5 µL) was used to perform the amplification in a 10 µL reaction buffer containing a 20 mM dNTPs mix (Solis BioDyne, Tartu, Estonia), GoTaq Polymerase (Promega), Eva Green 20x in water (Biotium, CA, USA) and gene-specific primers (Table 1).

Real-time RT-qPCR was performed on a Corbett Rotor-Gene 6000 system (Qiagen, MD, USA) and the conditions in all cases started with a denaturation step at 95°C and were followed by up to 40 cycles of denaturation (95°C), annealing (melting temperature) and primer extension (72°C). Negative controls were performed without cDNA template. The mRNA levels were corrected with the geometric mean of the 60S ribosomal protein L32 (L32) and the light chain of the major histocompatibility complex β2-microglobulin (*β2µG*) by using the $2^{-\Delta\Delta Ct}$ method. Relative mRNA levels are reported as fold value of the control.

Western blot analysis

Placental explants from male and female fetuses from control and diabetic male rats were homogenized in 500 µL ice-cold buffer D (250 mM sucrose, 10 mM Hepes-Tris, pH 7.4 with protease inhibitors). Proteins were separated

Table 1 Primers used in real-time RT-qPCR.

Gene	Primer sequences
Pgc-1α	5′-AATGCAGCGGTCTTAGCACT-3′ Forward
	5′-GTGTGAGGAGGGTCATCGTT-3′ Reverse
Pparα	5′-TCACACGATGCAATCCGTTT-3′ Forward
	5′- GGCCTTGACCTTGTTCATGT-3′ Reverse
Pparγ	5′-CCCTGGCAAAGCATTTGTAT-3′ Forward
	5′-ACTGGCACCCTTGAAAAATG-3′ Reverse
Fas	5′-TCGAGACACATCGTTTGAGC-3′ Forward
	5′-CCCAGAGGGTGGTTGTTAGA-3′ Reverse
Acc1	5′-CCAGACCCTTTCTTCAGCAG-3′ Forward
	5′-AGGACCGATGTGATGTTGCT-3′ Reverse
Acc2	5′-CAAAGCCTCTGAAGGTGGAG-3′ Forward
	5′-CTCGTCCAAACAGGGACACT-3′ Reverse
Scd-1	5′-GCTTCCAGATCCTCCCTACC-3′ Forward
	5′-CAACAACCAACCCTCTCGTT-3′ Reverse
Aco	5′-CCAATCACGCAATAGTTCTGG-3′ Forward
	5′-CGCTGTATCGTATGGCGAT-3′ Reverse
Cpt1-L	5′-AGGGGCCTTTCTGTGTACCT-3′ Forward
	5′-TGTGCCTGCTCTCATACTGG-3′ Reverse
Fatp1	5′-CGCTTTCTGCGTATCGTCTG-3′ Forward
	5′-AAGATGCGTGGGATCGTGTC-3′ Reverse
Lipg	5′-TCTATTGTTGCTTCCCGGCG-3′ Forward
	5′-CGGTGGGTTTATGGTGCTCA-3′ Reverse
Lpl	5′-CCCTAAGGACCCCTGAAGAC -3′ Forward
	5′-GGCCCGATACAACCAGTCTA-3′ Reverse
Cd36	5′-CGAAGGCTTGAATCCTAACGAA-3′ Forward
	5′-CCGATGGTCCCAGTCTCATT-3′ Reverse
L32	5′-TGGTCCACAATGTCAAGG-3′ Forward
	5′-CAAAACAGGCACACAAGC-3' Reverse
β2µG	5′-CCGTGATCTTTCTGGTGCTT-3′ Forward
	5′-ATTTGAGGTGGGTGGAACTG-3′ Reverse

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by SDS-PAGE and transferred to nitrocellulose membranes (35-V constant, overnight at 4°C) as described previously (Capobianco *et al.* 2016). The membranes were then stained with Ponceau S solution to evaluate the quality of the transfer. The membranes were blocked for 1 h at room temperature in 5% (w/v) nonfat milk in TBS-Tween, and membranes were incubated overnight at 4°C with the following primary antibodies (diluted in 1% bovine serum albumin in TBS-Tween): rabbit polyclonal IgG antibody against PPARα (1:200), PPARγ (1:100) or PGC-1α (1:200) (Cayman Chemical Co., Ann Arbor, MI, USA); mouse monoclonal IgG antibody anti-CD36 (1:150) (Santa Cruz Biotechnology, Inc., CA, USA); and rabbit polyclonal IgG antibody anti-actin, used for the normalization for unequal loading (1:500) (Sigma-Aldrich). Then, the membranes were washed and incubated with the appropriate peroxidase-conjugated secondary antibody (1:5000) (Jackson Immuno Research Laboratories, Inc, Baltimore, MD, USA) and visualized using ECL detection solution (Thermo Scientific). The images were captured in a G: BOX gel imaging system (Syngene), and the relative intensity of protein signals was quantified by densitometric analysis using the ImageJ Software, as previously (Capobianco *et al.* 2016). Results are expressed as the relative intensity of each target protein normalized against actin.

Statistical analysis

Data are presented as the mean ± S.D. GraphPad Prism 5 was used for the statistical analysis (GraphPad Software Inc., La Jolla, CA, USA). Normality of the variable distribution was assessed with the Shapiro–Wilk test using the SPSS 19 software. Homogeneity of variance was verified with the Levene's test and in the cases where homogeneity of variance was not verified, the variance function Varldent was applied to the model (Infostat 2017 software). Statistical differences between the two groups were evaluated by unpaired, two-tailed Student's *t*-test. For multiple comparisons, two-way ANOVA followed by Bonferroni's *post hoc* test was used with paternal condition (control vs diabetic) and sex as factors. A P value less than 0.05 was considered statistically significant.

Results

Paternal and fetoplacental data

In this model of diabetes induced by intrauterine programming, 5-month-old F1 males born from F0 diabetic mothers showed increased glycemia values ($P < 0.0001$), insulinemia ($P = 0.0028$), triglyceridemia ($P = 0.042$) and cholesterolemia ($P = 0.013$) when compared to the control group (Table 2). The body weight was similar in control and diabetic groups.

In the plasma of F2 male and female fetuses, glycemia values of the control and diabetic groups were similar. Triglyceride concentrations were higher in the diabetic group than in the control group (plasma of male fetuses: $P = 0.0018$; plasma of female fetuses: $P = 0.0086$). Besides, triglyceridemia was increased in male fetuses when compared to females in the diabetic group ($P = 0.038$). Cholesterol in plasma of F2 males, but not of female fetuses from the diabetic group, was increased when compared to control ($P = 0.009$). No significant differences in cholesterol concentrations were found in male and female fetuses within the control and diabetic groups (Table 3).

The number of male and female fetuses within a litter was similar when the control and diabetic groups were compared (Table 3), with no significant sex-dependent differences and litter male–female distribution was also similar (1:0.8 in the control group and 1:0.9 in the diabetic group).

Fetal weight was increased in both male and female fetuses ($P = 0.044$ for both male and female fetuses) of the diabetic group, with no sex-dependent differences within the control and diabetic groups. Placental weight and efficiency were similar in control and diabetic groups, without sex-dependent differences (Table 3).

Lipid content in the placenta

Considering the alterations in lipid metabolism observed in fetal plasma, we next evaluated lipid levels in the placentas from fetuses fathered by control and diabetic male rats. The placentas of male fetuses from the diabetic

Table 2 Paternal data. Paternal glycemia, insulinemia, triglyceridemia, cholesterolemia and body weight of F1 offspring of control and diabetic female rats. Values represent mean ± S.D. obtained from seven rats in each experimental group (n = 7). Statistical analysis: Student's t-test for paternal data. Different letters denote significant differences between groups, P < 0.05.

Paternal data	F1 offspring of control female rats	F1 offspring of diabetic female rats
Glycemia (mg/dL)	94 ± 5[a]	144 ± 10[b]
Insulinemia (μg/L)	0.95 ± 0.12[a]	1.22 ± 0.15[b]
Triglyceridemia (mg/dL)	114 ± 11[a]	139 ± 25[b]
Cholesterolemia (mg/dL)	132 ± 6[a]	158 ± 20[b]
Weight (g)	533 ± 47[a]	545 ± 12[a]

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Table 3 Fetal data. Glycemia, triglyceridemia, cholesterolemia, number of fetuses per liter, placental efficiency and placental and body weight of F2 fetal offspring of control and diabetic male rats. Values represent mean ± S.D. obtained from seven rats in each experimental group ($n = 7$). Statistical analysis: two-way ANOVA (factors: paternal condition and sex) in conjunction with Bonferroni's *post hoc* test for fetal data. Different letters denote significant differences between groups, $P < 0.05$.

	F2 offspring of control male rats		F2 offspring of diabetic male rats		*P-value*		
Fetal data	Male fetuses	Female fetuses	Male fetuses	Female fetuses	Paternal condition	Sex	Interaction Paternal condition* Sex
Glycemia (mg/dL)	45 ± 7[a]	47 ± 6[a]	45 ± 10[a]	45 ± 12[a]	0.773	0.773	0.773
Triglyceridemia (mg/dL)	94 ± 8[a]	101 ± 5[a,b]	103 ± 4[b]	111 ± 5[c]	0.0027	0.0003	0.8256
Cholesterolemia (mg/dL)	97 ± 26[a]	116 ± 6[a,b]	124 ± 12[b]	132 ± 13[b]	0.0353	0.0016	0.372
Number of fetuses per litter	6 ± 1[a]	8 ± 2[a]	6 ± 2[a]	7 ± 3[a]	0.074	0.539	0.539
Fetal weight (g)	3.14 ± 0.46[a]	2.99 ± 0.40[a]	3.58 ± 0.29[b]	3.43 ± 0.27[b]	0.0038	0.286	>0.99
Placental weight (mg)	620 ± 74[a]	595 ± 74[a]	640 ± 99[b]	620 ± 56[a]	0.4486	0.4486	0.9325
Placental efficiency	5 ± 0.81[a]	5 ± 0.94[a]	6 ± 0.44[a]	6 ± 0.33[a]	0.0007	>0.99	>0.99

group showed an increase in the levels of triglycerides (Fig. 2A, $P = 0.004$), cholesterol (Fig. 2B, $P = 0.0005$) and free FAs (Fig. 2C, $P = 0.005$) when compared to the control group. The levels of cholesteryl esters were similar in both groups (Fig. 2D).

Regarding lipid levels in the placentas of female fetuses from the diabetic group, there were no differences between groups in the levels of any of the four lipid species evaluated (Fig. 2A, B, C and D).

No significant differences were found for the evaluated placental lipid levels between placentas from male and female fetuses within the control and diabetic groups (Fig. 2).

Placental mRNA and protein expression of PPARα, PPARγ and PGC-1α

To understand the origin of the lipid alterations found in the placentas of fetuses fathered by diabetic males, we decided to study the mRNA and protein expression of the nuclear receptors *PPARα*, *PPARγ* and their coactivator *PGC-1α*, involved in the transcription of genes that regulate lipid metabolism.

In the placentas of male fetuses from the diabetic group, the mRNA expression of *Pparα* and *Pgc-1α* was increased (Fig. 3A, $P < 0.0001$), whereas their protein expression was decreased (Fig. 3C, $P < 0.0001$ and Fig. 3D, $P = 0.036$, respectively), when compared to controls. The mRNA and protein expression of PPARγ did not vary significantly between groups (Fig. 3A and B, respectively).

In the placentas of female fetuses, the mRNA and protein expression of PPARα, PGC-1α and PPARγ in the diabetic group were similar to that in the control group (Fig. 3A, B, C and D).

Placental mRNA expression of genes involved in the regulation of lipid synthesis and oxidation

To investigate whether the different regulation of the lipid levels found in the placentas of male and female fetuses fathered by diabetic males was related to the activation of PPARs, we assessed the mRNA expression of target genes of PPARα, involved in lipid oxidation, and PPARγ, involved in lipid synthesis. The genes involved in lipid synthesis here evaluated were *Fas*, *Acc1*, *Acc2* and *Scd-1*, whereas those involved in lipid oxidation here evaluated were *Aco* and *Cpt1-L*.

The mRNA expression of *Fas*, *Acc1*, *Acc2* and *Scd-1* in the placentas of male fetuses from the diabetic group showed no significant differences when compared to the control group (Fig. 4A). In the placentas of female fetuses, the mRNA expression of these enzymes involved in lipid synthesis showed no differences between groups (Fig. 4A).

The mRNA expression of *Aco* was increased in the placentas of male fetuses from the diabetic group when compared to the control group ($P = 0.0003$), whereas that of *Cpt1-L* was similar in both groups (Fig. 4B). In the placentas of female fetuses, the mRNA expression of these enzymes involved in lipid oxidation showed no differences between groups (Fig. 4A).

Placental mRNA expression of genes involved in the regulation of lipid transport and placental protein expression of CD36

The placental transfer of lipids drives the growth and development of the fetus. To study the availability of lipid transporters in the placenta, we next assessed the mRNA expression of proteins involved in lipid transport. We found an increase in the mRNA expression of *Fatp1* ($P < 0.0001$),

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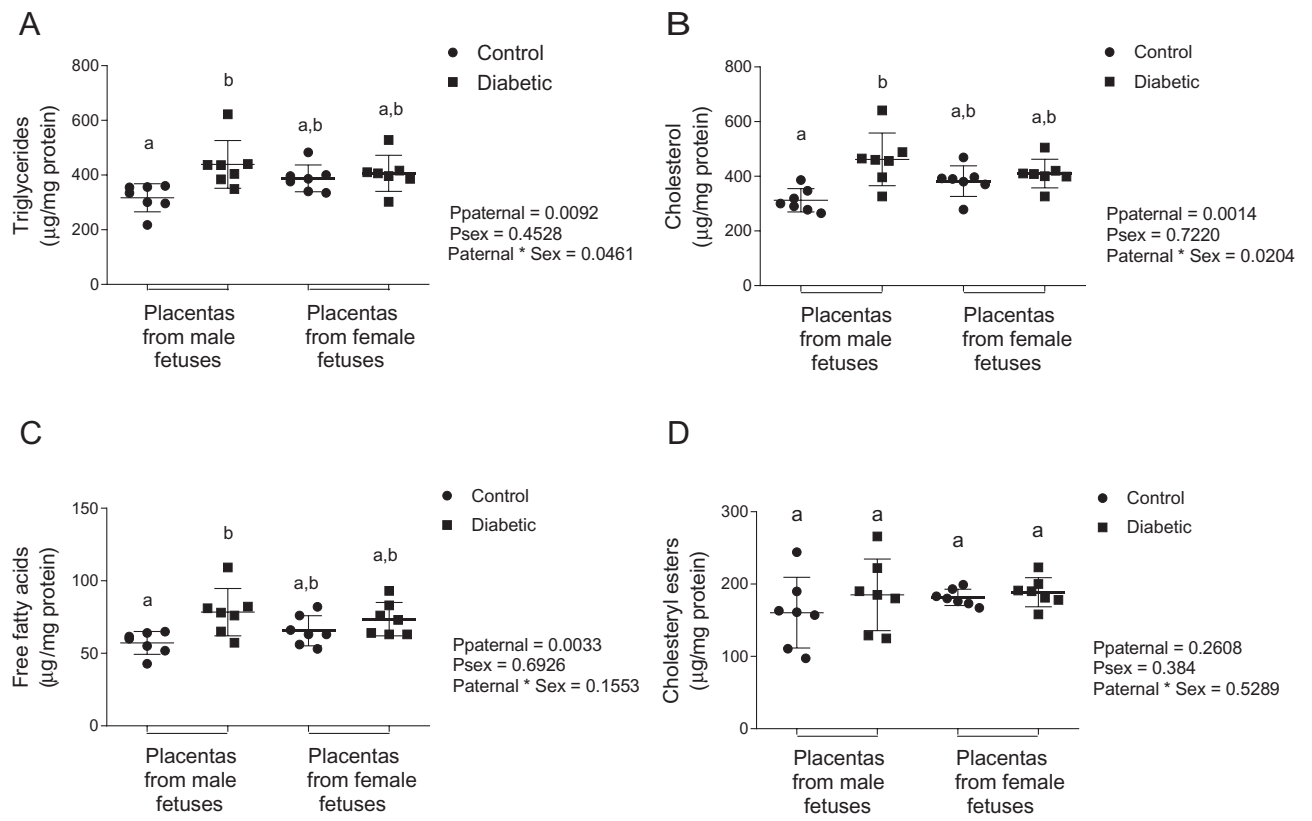

Figure 2

Placental lipid concentrations. The levels of triglycerides (A), cholesterol (B), free fatty acids (C) and cholesteryl esters (D) were evaluated in the placentas from male and female fetuses fathered by control and diabetic male rats. Values represent mean ± S.D. obtained from seven placentas in each experimental group ($n = 7$). Statistical analysis: two-way ANOVA in conjunction with Bonferroni's test. Different letters denote significant differences between groups. $P < 0.05$.

Lipg ($P < 0.0001$), *Lpl* ($P < 0.0001$) and *Cd36* ($P = 0.0159$) (Fig. 5A), as well as in the protein expression of CD36 ($P = 0.004$) (Fig. 5B) in the placentas of male fetuses from the diabetic group when compared to the control group.

In the placentas of female fetuses from the diabetic group, the mRNA expression of *Fatp1*, *Lipg*, *Lpl* and *Cd36* as well as the protein expression of CD36 showed no significant differences when compared to the control group (Fig. 5A and B, respectively).

Discussion

Metabolic disorders leading to diabetes, obesity and cardiovascular diseases can be transmitted through the paternal germ line, but the mechanisms underlying the transmission from the father's environment to his offspring are unclear. In the present study, we found not only an increase in paternal glycemia and insulinemia but also an increase in triglyceridemia and cholesterolemia, indicating paternal altered carbohydrate and lipid profiles (known markers of alterations in the paternal germline) such as those found in animal models of paternal high-fat diet (Ng *et al.* 2010). Similarly, using a non-genetic mouse model of diabetes, Wei *et al.* (2014) showed that paternal diabetes induces fasting hyperglycemia, glucose intolerance and insulin insensitivity in their male offspring. The experimental model of diabetes here developed in F1 males is unique in rats, where the F0 mother is not genetically diabetic and her offspring develop diabetes in adulthood with no intervention other than having been gestated in the uterus of a diabetic mother (Capobianco *et al.* 2015). This model of type 2 diabetes induced by intrauterine programming in the F1 male offspring is useful to study the transmission of metabolic alterations to the F2 fetal offspring. Although glycemia values in the F2 fetuses from F1 diabetic fathers were normal, the increase found in fetal cholesterolemia and triglyceridemia demonstrates lipid metabolic alterations that can be leading to the offspring's fetal overgrowth observed. In particular, increased fetal growth is a strong evidence of the programming of alterations later in life (Harder *et al.* 2007, Hanson & Gluckman 2008).

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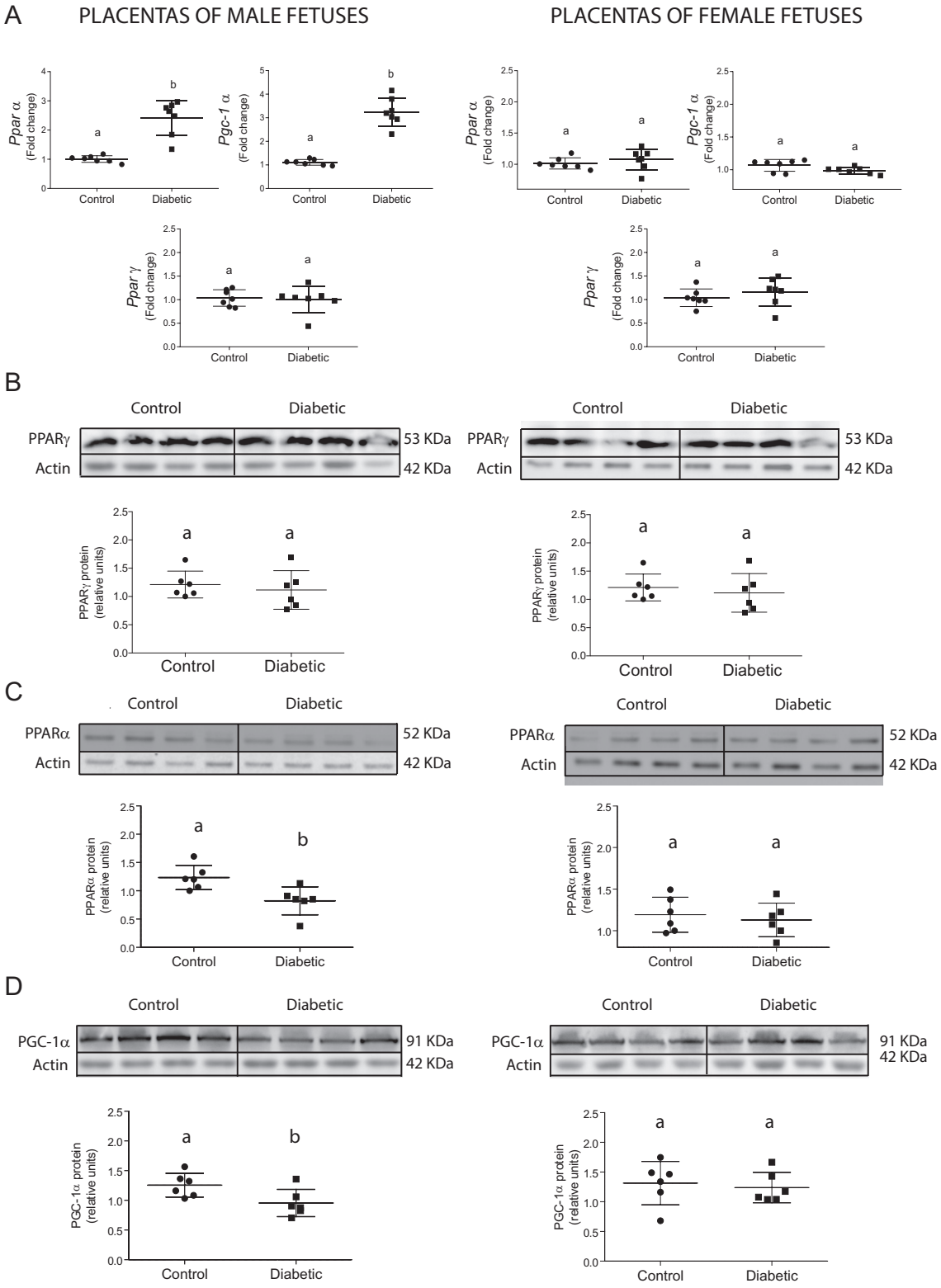

Figure 3

Placental expression of PPARα, PGC-1α and PPARγ. mRNA expression of *Pparα*, *Pgc-1α* and *Pparγ* (A) and protein expression of PPARγ (B), PPARα (C) and PGC-1α (D) in placentas from male and female fetuses fathered by control and diabetic male rats. Values represent mean ± S.D. obtained from six to seven placentas in each experimental group ($n = 6–7$). Statistical analysis: Student's *t*-test. Different letters denote significant differences between groups. $P < 0.05$.

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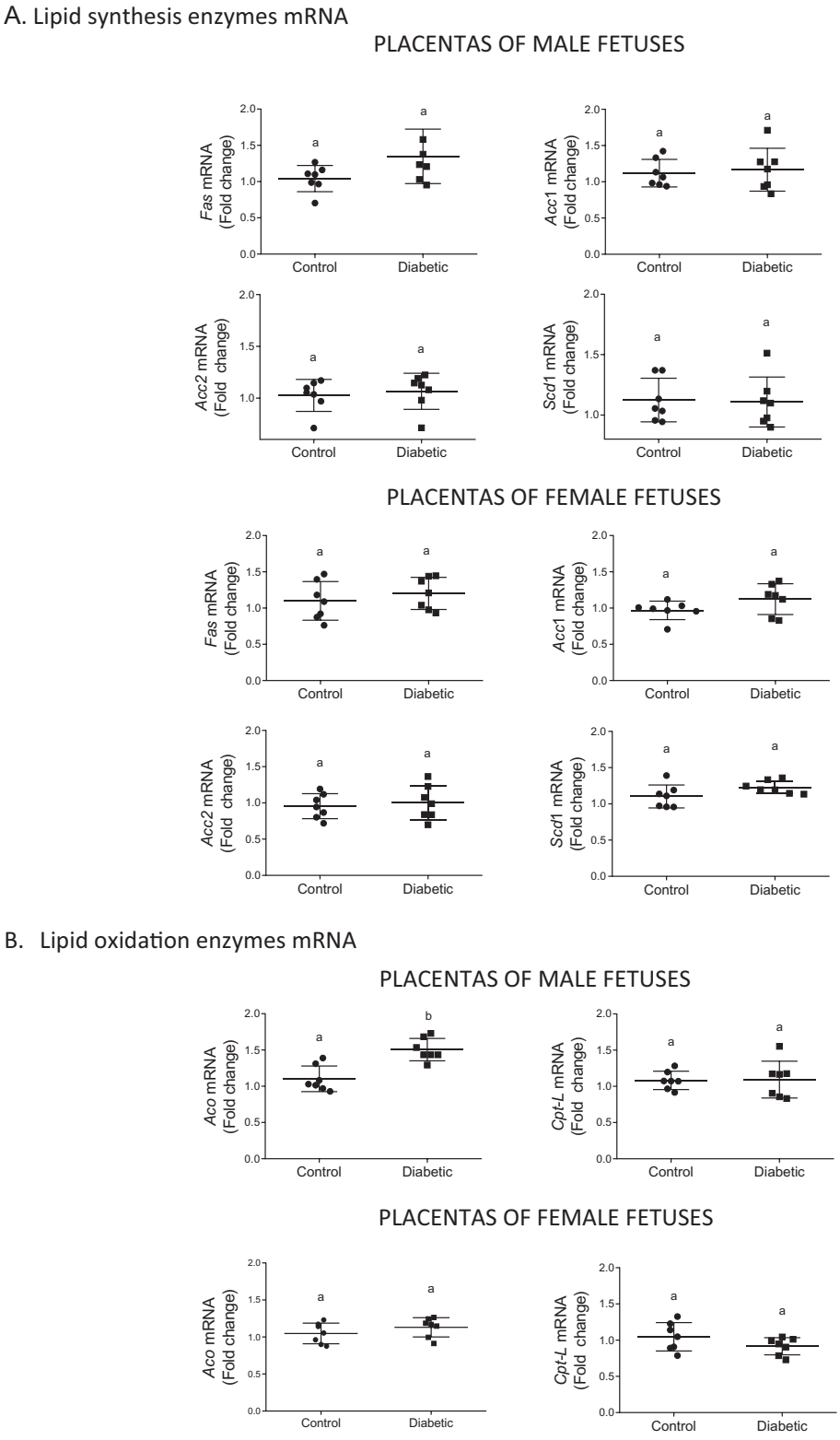

Figure 4

Placental mRNA expression of genes involved in lipid synthesis and oxidation. mRNA expression of *Fas*, *Acc1*, *Acc2* and *Scd-1* genes involved in lipid synthesis (A) and mRNA expression of *Aco* and *Cpt1-L* genes involved in lipid oxidation (B) in the placentas from male and female fetuses fathered by control and diabetic male rats. Values represent the mean ± S.D. obtained from 6 to 7 placentas in each experimental group (n = 6–7). Statistical analysis: Student's *t*-test. Different letters denote significant differences between groups. $P < 0.05$.

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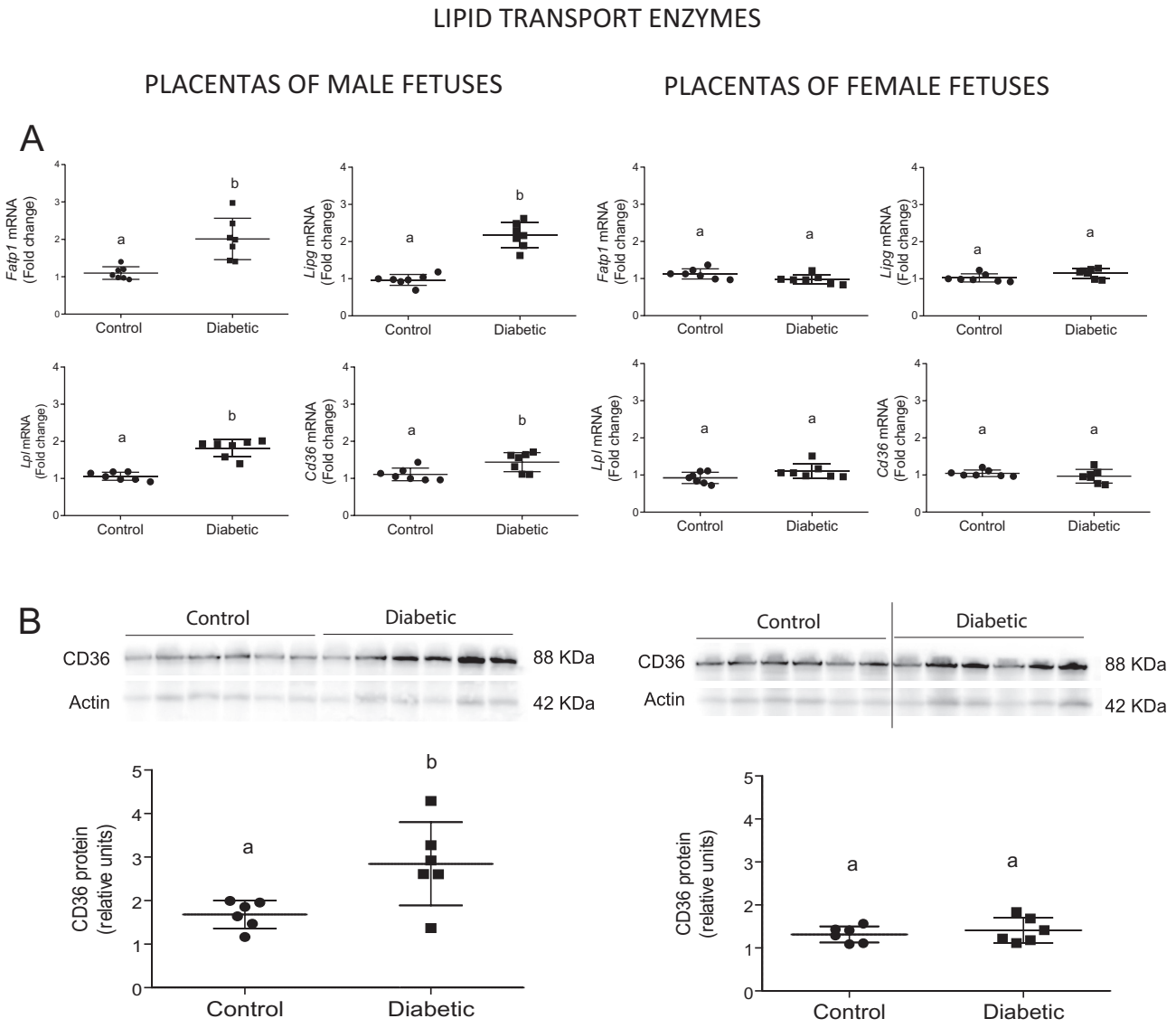

Figure 5
Placental mRNA expression of genes involved in lipid transport and CD36 protein expression. mRNA expression of *Fatp1*, *Lipg*, *Lpl*, and *Cd36* (A) and protein expression of CD36 (B) in placentas from male and female fetuses fathered by control and diabetic male rats. Values represent the mean ± S.D. obtained from 6 to 7 placentas in each experimental group (n = 6–7). Statistical analysis: Student's t-test. Different letters denote significant differences between groups. $P < 0.05$.

The placenta is genetically half the father's and is the main determinant of fetal nutrient supply and normal growth (Sferruzzi-Perri & Camm 2016). Among the nutrients that govern fetal growth, FAs have several functions regarding growth and development (Lewis *et al.* 2018*b*). In this work, placental weight and efficiency were normal in all the litters of F2 fetuses fathered by diabetic males, without variation within litters. However, the increase observed in the accumulation of lipids in the placentas from F2 male fetuses from the diabetic group is clear evidence that the alterations in placental lipid metabolism are related to factors that come from the paternal germline.

Our novel findings showing an increase in lipid accretion in placentas of male fetuses from diabetic rats compared to controls but not in placentas of female fetuses suggest that both sexes turn on/off different mechanisms to regulate lipid metabolism and fetal overweight, as reviewed by Gabory *et al.* (2013).

In the same way, maternal obesity and diabetes impact differently on lipid metabolism in female and male

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offspring (Fornes *et al.* 2018, Savva *et al.* 2021). The increase in male placental lipid levels seems not to be related to an increase in the mRNA expression of lipid synthesis enzymes. On the other hand, the increase in the mRNA expression of *Aco*, an oxidation enzyme, found in the placentas of male fetuses but not in the placentas of female fetuses, could be a pathway activated to stop, or at least ameliorate, the increase in placental lipid levels.

Since the increase in the placental lipid levels could not be associated with an increase in the expression of lipid synthesis genes, we evaluated the mRNA expression of proteins involved in lipid transport and found an increase in *Fatp1, Lipg*, *Lpl* and *Cd36* expression in the placentas of male fetuses fathered by male diabetic rats. Because of the relevance of CD36 as a placental transporter (Segura *et al.* 2017, Chassen *et al.* 2018), we decided to further evaluate the CD36 protein expression and found that it was also increased in the placentas of male fetuses fathered by diabetic male rats.

Both membrane transport and placental metabolism (esterification, β-oxidation or biosynthesis of bioactive lipids) determine placental lipid content, which may also be related to the uptake of FAs from the maternal side and the supply of FAs to the fetus (Perazzolo *et al.* 2017). Also, studies in the placenta suggest that ATP-binding cassette transporters play a role in cholesterol efflux to the mother (Aye *et al.* 2010), a mechanism that could be interesting to study in this model. In a rat model of gestational diabetes mellitus, we have previously found that lipids are accumulated in the liver of male fetuses but not in that of female fetuses (Fornes *et al.* 2020). This fact, together with the increase in the expression of mRNA of lipid transport proteins here found in the placentas of male fetuses fathered by diabetic male rats, suggests a putative relationship between fetal lipid profile and overgrowth with the placental lipid accretion. However, the mechanisms involved in the linking pathways will require further research.

As a limitation of this study, maternal plasma was not collected, and so we were not able to address whether mating with a diabetic male could influence the metabolic state or health of pregnant rats. However, previous studies by Patterson *et al.* (2021) in a mouse model of paternal obesity have shown that maternal metabolism is not altered by paternal hyperglycemic condition. Besides, since not only sperm but also seminal plasma can act on maternal uterine inflammatory responses, this should be studied in future works in our model of paternal diabetes (Bromfield 2014).

The participation of PPARs in placental lipid metabolism and regulation of antioxidant and anti-inflammatory pathways is well known (Jawerbaum & Capobianco 2011). The increase in the mRNA levels of *Pparα* and its coactivator *Pgc-1α* in placentas of male fetuses suggests that this pathway could be activated to increase lipid oxidation and to downregulate oxidative stress, another evidence of the altered programming that could arise via the paternal germline (Soubry 2018). On the other hand, we found a decrease in the protein levels of PPARα and PGC-1α. The difference in the regulation of mRNA and protein levels of PPARα and PGC-1α in the diabetic group could be attributed to the epigenetic transmission of information from the paternal lineage, where miRNAs (small non-coding RNAs) are the possible candidates that regulate the rate of PPAR biosynthesis by different pathways in male and female fetuses (Fornes *et al.* 2020).

The placentas of female fetuses showed no differences in the expression of these molecules. The placenta could be sensing the accelerated growth of the fetus and trying to stop this growth by regulating the translation rate of the master genes that command lipid metabolism, thus buffering the supply of FAs to the fetal circulation (Jansson & Powell 2013).

Since paternal diabetes affects the sperm epigenome, the epigenetic heredity via paternal sperm was the motivation to hypothesize that the father's environmental information controls the offspring's phenotype and should be regarded as important as genetic factors in determining the risk for diabetes or obesity. The best sncRNAs candidates involved in the epigenetic transmission of information from the paternal lineage are the tRFs and miRNA subtypes (Portha *et al.* 2019). Additional analyses are needed to determine the mechanism by which male offspring born from diabetic mothers may transmit growth and metabolic defects to their offspring. Such a mechanism may be mediated by programmed changes in the morphology of testes and quality, genome and epigenome of the sperm, and seminal fluid of diabetic fathers, born from diabetic mothers (Bromfield 2014).

In conclusion, paternal diabetes programs fetal overgrowth and sex-dependent alterations in fetoplacental lipid metabolism. The intergenerational transfer of these metabolic alterations to the fetus could have adverse consequences in the offspring's later life.

A better understanding of the link between the father's well-being and the development and health of his offspring will ultimately guide public health policies in the future.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
D F contributed to conceptualization, performed the animal experiments, methodology and data analysis and helped draft the manuscript. F H assisted with animal experiments and methodology. C G and S R assisted with animal experiments. V W assisted with animal experiments and contributed to reviewing the manuscript. A J contributed to discussing and reviewing the manuscript and to the acquisition of funding. E C contributed to conceptualization, funding acquisition, project administration, animal experiments and writing of the original draft of the manuscript. All the authors have read and approved the final manuscript.

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