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Sex-dependent changes in lipid metabolism, PPAR pathways and microRNAs that target PPARs in the fetal liver of rats with gestational diabetes

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ABSTRACT

Gestational diabetes mellitus (GDM) is a prevalent disease that impairs fetal metabolism and development. We have previously characterized a rat model of GDM induced by developmental programming. Here, we analyzed lipid content, the levels of the three PPAR isotypes and the expression of microRNAs that regulate PPARs expression in the liver of male and female fetuses of control and GDM rats on day 21 of pregnancy. We found increased levels of triglycerides and cholesterol in the livers of male fetuses of GDM rats compared to controls, and, oppositely, reduced levels of triglycerides, cholesterol, phospholipids and free fatty acids in the livers of female fetuses of GDM rats compared to controls. Although GDM did not change PPARα levels in male and female fetal livers, PPARγ was increased in the liver of male fetuses of GDM rats, a change that occurred in parallel to a reduction in the expression of miR-130, a microRNA that targets PPARγ. In livers of female fetuses of GDM rats, no changes in PPARγ and miR-130 were evidenced, but PPARδ was increased, a change that occurred in parallel to a reduction in the expression of miR-9, a microRNA that targets PPARδ, and was unchanged in the liver of male fetuses of GDM and control rats. These results show clear sex-dependent changes in microRNAs that target different PPAR isotypes in relation to changes in the levels of their targets and the differential regulation of lipid metabolism evidenced in fetal livers of GDM pregnancies.

1. Introduction

Gestational diabetes (GDM) is a prevalent metabolic disease, characterized by impaired glucose tolerance with onset or first recognition during pregnancy and associated with maternal, fetal and neonatal adverse outcomes and long-term consequences for both the mother and the child (Damm et al., 2016; Hod et al., 2015; Lappas et al., 2011). It is widely recognized that GDM is associated with alterations in both glucose and lipid metabolism. Moreover, maternal lipid impairments in GDM reach the fetal compartment and are related to the adverse fetal outcomes (Herrera and Desoye, 2016; Higa and Jawerbaum, 2013). Experimental models of diabetes and pregnancy are valuable to study the mechanisms involved in fetal impairments (Jawerbaum and White, 2010). Alterations in lipid concentrations have been found in fetal tissues in diabetic experimental models in which maternal insulin levels are reduced (Higa and Jawerbaum, 2013), but poorly addressed in diabetic models characterized by insulin resistance.

Recently, we have characterized a GDM model induced by developmental programming in the adult offspring of streptozotocin-induced diabetic rats (maternal glycemia in F0 generation: 150–250 mg/dl). In this model, the pregnant F1 offspring of these mild diabetic rats develop GDM and show both maternal and fetal hyperglycemia and hyperinsulinemia (Capobianco et al., 2016). In addition, the placentas of these GDM rats show altered levels of peroxisome proliferator activated receptors (PPARs) (Capobianco et al., 2016), nuclear receptors with key functions in developmental processes and in the regulation of inflammatory and metabolic pathways (Jawerbaum and

Abbreviations: Gestational diabetes mellitus, GDM; peroxisome proliferator activated receptors, PPARs; microRNAs, miRs.

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Capobianco, 2011; Wahli and Michalik, 2012). PPARs are ligand activated transcription factors that heterodimerize with retinoid-X-receptors. Their ligand binding leads to the release of corepressors and recruitment of coactivators and allows the transcription of multiple target genes containing PPAR response elements (PPRE) in their promoters (Wahli and Michalik, 2012). The three PPAR isotypes (PPARγ, PPARα and PPARδ) are highly involved in the regulation of lipid metabolism and have been found to differentially regulate multiple enzymes involved in both lipid oxidative processes, such as acyl-CoA oxidase (ACO) and carnitine palmitoyltransferase 1 (CPT-1), and lipid anabolic processes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC1) (Lefebvre et al., 2006; Pawlak et al., 2015; Reilly and Lee, 2008; Rogue et al., 2010).

In this work, aiming to address the fetal impact of lipid metabolic alterations in GDM rats, we focused on the fetal liver. It is known that, in the adult, the liver is a main site of lipid metabolic regulation by the three PPAR isotypes (Liss and Finck, 2017). PPARγ is an important determinant of liver fat accumulation, as demonstrated by the increased PPARγ expression in animal models of fatty liver diseases (Inoue et al., 2005) and studies showing that genetic disruption of PPARγ reduces hepatic steatosis (Matsusue et al., 2003). PPARα is highly expressed in the liver, where it plays a major role in fasting regulations (Lefebvre et al., 2006). This PPAR isotype is an important target in metabolic diseases due to its involvement in energy balance, lipid metabolism and inflammation, and its pharmacological ligands include hypolipidemic fibrates (Wahli and Michalik, 2012). The third isotype, PPARδ, has been less studied in liver lipid metabolism, but has been found involved in both oxidative and lipogenic processes and plays a role in adipogenesis (Lee et al., 2006; Luquet et al., 2005; Reilly and Lee, 2008).

Recently, multiple studies have identified the relevant role of microRNAs as epigenetic regulators of PPARs (Portius et al., 2017). MicroRNAs are endogenous small noncoding RNAs of approximately 22 nucleotides which bind and target mRNAs to mediate their decay, destabilization or translation inhibition, thus regulating the abundance of target proteins (Dumortier et al., 2013; Holley and Topkara, 2011). PPARγ was identified as a target of miR-130a/b in preadipocytes, human hepatocellular carcinoma tissue and hepatic cell lines (Huang et al., 2015; Lee et al., 2011; Tu et al., 2014). Both PPARα and PPARδ are targets of miR-9, a microRNA with relevant roles in carcinogenesis, inflammation, fibrosis and metabolism (Drakaki et al., 2015; Tang et al., 2008; Thulin et al., 2013; Wang et al., 2016). In addition, miR-122, the most highly expressed miRNA in the liver, implicated in several important aspects of liver physiology and pathology, has been shown to target PPARδ in the liver (Gatfield et al., 2009; Tsai et al., 2012). In this way, the translation of the three PPAR isotypes has been found to be regulated by microRNA in different systems and cell types, although it is still unknown if this regulation occurs in the fetus (Portius et al., 2017).

Sex-dependent differences in lipid metabolism, PPAR pathways and microRNAs have been described (Benz et al., 2012; Dai and Ahmed, 2014; Kautzky-Willer et al., 2016). These differences are possibly related to the levels of sex hormones and their receptors, which interact with PPAR pathways (Rando and Wahli, 2011). The aim of this work was to analyze putative changes in the levels of lipids, the three PPAR isotypes, and microRNAs that target these PPARs in the liver of female and male fetuses of GDM rats. Our findings point to sex-dependent changes in fetal liver accumulation, which occur in relationship with the levels of different PPAR isoforms and the expression of microRNAs that target these PPARs.

2. Materials and methods

2.1. Animals

Albino Wistar rats bred in our animal facility were fed *ad libitum* with commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina). Mild diabetic rats (F0 generation) were obtained by injecting 2-day-old neonates with streptozotocin (90 mg/kg, s.c, Sigma-Aldrich, St. Louis, MO, USA) diluted in citrate buffer (0.05 M pH 4.5, Sigma-Aldrich), as previously (Jawerbaum and White, 2010; Martinez et al., 2012). Control F0 rats were injected with citrate buffer alone. The diabetic state was confirmed in the adult animals prior to mating. Rats were considered diabetic when they presented fasting glycemia values higher than 130 mg/dl. Blood glucose values in mild diabetic rats were 150–250 mg/dl and below 100 mg/dl in controls. Control and mild diabetic female adult rats (F0) were mated with control adult males. The presence of sperm cells in vaginal smears confirmed the first day of pregnancy. No treatments were performed in the offspring of control and mild diabetic animals (F1 generation) until the third month of age. At this time, glycemia values were determined and normoglycemia found in the offspring from both control and mild diabetic rats. Previous studies have shown induction of GDM in three-month-old offspring from mild diabetic rats mated with control males (Capobianco et al., 2016). In this work, the three-month-old female offspring of mild diabetic rats that would develop GDM and of control rats were mated with control males. The presence of sperm cells in vaginal smears confirmed the first day of pregnancy. F1 animals were euthanized on day 21 of pregnancy to obtain maternal and fetal blood and fetal livers that were preserved at −80 °C. Studies were performed in tissues from eight F1 offspring in each group, each rat obtained from a different F0 mother. The animal protocol was approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD Nº 1497/2013), School of Medicine, University of Buenos Aires, and conducted according to the Guide for the Care and Use of Laboratory Animals, US National Institutes of Health (NIH Publication, 8th Edition, 2011) http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader.

2.2. Plasma metabolic measures

Blood glucose and triglyceride levels were evaluated by spectrophotometric enzymatic assays (Wiener lab. Rosario, Argentina) and insulin levels by a commercial assay kit (Mercodia Ultrasensitive Rat Insulin ELISA kit, Uppsala, Sweden) in maternal and fetal plasma from pregnant rats on day 21 of pregnancy.

2.3. Determination of lipid concentrations

Liver from female or male fetuses were homogenized in 1 ml phosphate buffered saline (PBS) and protein content in the homogenates measured by the Bradford assay. Tissue lipids were extracted from 500 μl of each homogenate by three rounds of organic extraction in methanol:chloroform (2:1), following the method of Bligh & Dyer. The lipids extracted (equivalent to 400 μg of protein) were developed by thin layer chromatography in 0.2 mm silica gel plates (Merck, Darmstadt, Germany), using hexane:ether:acetic acid (80:20:2, v:v:v) as the developing solvent mixture, as previously performed (Kurtz et al., 2010). Samples were developed with known amounts of lipid standards in the same plate. After the development, lipid species were stained with iodine vapors and the plate was scanned for further identification and quantification of the lipid species. Densitometric analysis of the area intensity of each spot was performed with the ImageJ

software. Quantification was performed by extrapolation from the standard curves built with the densitometric values of standards run in the same plate. The lipid content was expressed as μg lipids/mg protein.

2.4. Western blot analysis

Livers of female or male fetuses (100 mg) were homogenized in 500 μl in ice-cold buffer D (250 mM sucrose, 10 mM Hepes-Tris, pH 7.4 with protease and phosphatase inhibitors). Proteins from liver homogenates were separated by SDS-PAGE and transferred to nitrocelullose membranes (35 V constant, overnight at 4 °C), as previously performed (Capobianco et al., 2016). Blocking was carried out for 1 h at room temperature in 5% non-fat milk in TBS-Tween and membranes were incubated in primary antibody (diluted in 1% BSA in TBS-Tween) overnight at 4 °C. The primary antibodies used were: rabbit polyclonal IgG antibody against either PPARα (1:200) (Cayman Chemical Co., Ann Arbor, MI, USA), PPARγ (1:100) (Cayman Chemical Co.) and PPARδ (1:100) (Cayman Chemical Co.), or a rabbit polyclonal IgG antibody against actin (1:500) (Sigma-Aldrich) used as an internal control. The identity of PPARs was established by the use of molecular weight standards, which allows the identification of the band revealed at the expected size of ~50–58 kDa, which was absent in the negative control experiments performed in the absence of primary antibody. Two bands corresponding to PPARγ1 and PPARγ2 were identified in the presence of PPARγ antibody at the expected sizes of ~53 and ~57 kDa, respectively. After washing with TBS-Tween 0.05%, the membranes were treated with HRP conjugated secondary antibody (1:5000) (Jackson Immuno Research Laboratories, Inc, Baltimore, USA) for 1 h and washed several times. The specific signals were visualized using ECL detection solution (Thermo Scientific, Illinois, USA) and acquired in an ImageQuant LAS 4000 (GE Healthcare Life Sciences, New Jersey, USA). Densitometry analysis was performed with ImageJ software. Results are expressed as the relative intensity of each PPAR isotype normalized against actin.

2.5. Total RNA and microRNA isolation, and qRT-PCR analysis

Total RNA and microRNA were isolated from livers of female or male fetuses (100 mg) using RNAzol® (MCR Inc., Cincinnati, USA) according to the manufacturer's recommendations. The concentrations of total RNA and microRNA were determined using the NanoDrop spectrophotometer.

From total RNA, cDNA was synthesized incubating 1 μg of extracted RNA in a buffer containing 200 U MML-V enzyme (Promega, Wisconsin, USA), 7.5 mM random primer hexamers and 0.5 mM of each of all four dNTPs, as previously (Capobianco et al., 2016). 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 10 μl reaction buffer containing dNTPs mix 20 mM, GoTaq Polymerase (Promega), Eva Green 20x and gene specific primers (FAS, forward: 5′-TCGAGACACATCGTTTGAGC-3′, reverse: 5′-CCCAGAGGGTGGTTGTTAGA-3′; ACO, forward: 5′-CCAATCACG-CAATAGTTCTGG-3′, reverse: 5′-CGCTGTATCGTATGGCGAT-3′; and CPT-1a, forward: 5′-AGGGGCCTTTCTGTGTACCT-3′, reverse: 5′-TGTGCCTGCTCTCATACTGG-3′). ACC1, forward: 5′-CCA-GACCCTTTCTTCAGCAG-3′, reverse: 5′-AGGACCGATGTGATGTTGCT-3′; AR, forward: 5′-ACTGAGGACCCATCCCAGAA-3′, reverse: 5′-GTACAAGCTGTCTCTCGCCA-3′; ERα, forward: 5′-GCA-CATTCCTTCCTTCCGTC-3′, reverse: 5′-CTCGTTCCCTTGGATCTGGT-3′. The qPCR conditions started with a denaturation step at 95 °C for 5 min and followed by up to 40 cycles of denaturation (95 °C), annealing (60 °C) and primer extension (72 °C). mRNA levels were normalized to the 60s ribosomal protein L32 levels (L32 primer: forward: 5′-TGGTCCACAATGTCAAGG-3′, reverse: 5′-CAAAA-CAGGCACACAAGC-3′).

For microRNAs evaluation, cDNA was obtained using the Taq-Man MicroRNA reverse transcription kit (Applied Biosystem, California, USA). The relative expression of miR-122, miR-130a and miR-9 was determined using the TaqMan detection system (Applied Biosystem), the appropriate primers were (assay ID) 002245, 000454 and 000583 respectively, and U6 spliceosomal RNA (assay ID 001973), used as endogenous control (Applied Biosystem).

From total RNA and microRNA the course of PCR amplification was followed in each cycle by the fluorescence measurement on Corbett Rotor-Gene 6000 (QIAGEN, Maryland, USA). Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. Relative mRNA and microRNA levels are reported as fold value of the control.

2.6. Statistical analysis

Data are presented as the mean ± SEM. GraphPad Prism 5 was used for the statistical analysis (GraphPad Software). Statistical differences between the two groups were evaluated by unpaired, two-tailed *t*-test. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Metabolic determinations and growth parameters

In a previous study, we showed that the offspring of mild diabetic rats have normal blood glucose and insulin values at three months of age, but develop GDM when mated with control males (Capobianco et al., 2016). Similarly, in this work, we found that the offspring of mild diabetic animals, which showed normal blood glucose values at three months of age before mating (glycemia: control offspring 102 ± 5 mg/dl, diabetic offspring 110 ± 4 mg/dl), showed increased glycemia and insulinemia values on day 21 of pregnancy ($p < 0.01$, Table 1), indicating that these animals develop GDM. In the fetuses of GDM rats, increased body weights were observed in both males and females compared to those of control rats ($p < 0.05$), although no changes were evidenced in the fetal livers of males and female fetuses when the respective control and GDM groups were compared (Table 1). Glycemia ($p < 0.01$) and

Table 1
Maternal and fetal glycemia, insulinemia and triglyceridemia, as well as fetal body weight and fetal liver weight in 21-day pregnant offspring of diabetic rats that develop GDM and of control rats. Values represent mean ± SEM obtained from 8 rats in each experimental group. Statistical analysis: Student *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control.

	Offpring of Control rats	Offpring of Diabetic rats
Maternal Data		
Glycemia (mg/dl)	107 ± 8	136 ± 4 **
Insulinemia (μg/l)	1.19 ± 0.08	1.58 ± 0.09 **
Triglyceridemia (g/l)	1.99 ± 0.11	2.50 ± 0.14 *
Male Fetal Data		
Glycemia (mg/dl)	47 ± 3	62 ± 3 **
Insulinemia (μg/l)	2.88 ± 0.18	3.62 ± 0.16 **
Triglyceridemia (g/l)	0.51 ± 0.04	0.48 ± 0.03
Fetal weight (g)	3.21 ± 0.09	3.54 ± 0.10 *
Liver weight (mg)	298 ± 12	317 ± 15
Female Fetal Data		
Glycemia (mg/dl)	49 ± 3	80 ± 5 ***
Insulinemia (μg/l)	2.80 ± 0.06	3.08 ± 0.08 *
Triglyceridemia (g/l)	0.47 ± 0.03	0.49 ± 0.04
Fetal weight (g)	3.07 ± 0.09	3.34 ± 0.08 *
Liver weight (mg)	301 ± 5	311 ± 9

insulinemia ($p < 0.05$) were increased in the male and female fetuses from GDM rats when compared to controls (Table 1). In addition, triglyceride levels were increased in GDM mothers compared to controls ($p < 0.05$), although unchanged in male and female fetuses from the control and GDM groups (Table 1). This prompted us to analyze whether lipids, which can be transferred through the placenta and accumulated in fetal tissues (Herrera and Desoye, 2016), are accumulated in the fetal livers of GDM animals.

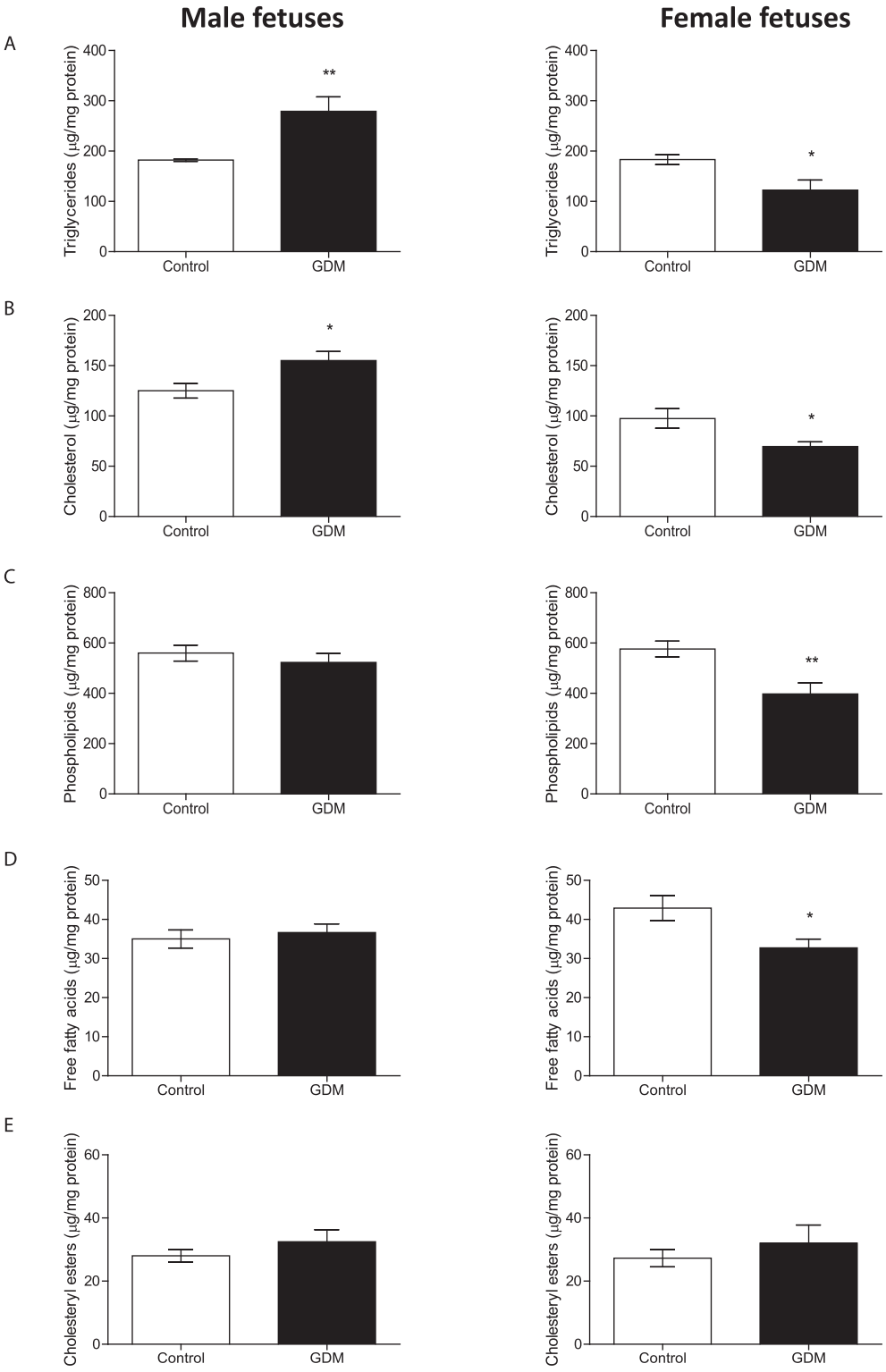

Fig. 1. Lipid concentrations in the livers of male and female fetuses of 21-day pregnant offspring of diabetic rats that develop GDM and of control rats. A. Triglycerides. **B.** Cholesterol. **C.** Phospholipids. **D.** Free fatty acids. **E.** Cholesteryl esters. Values represent mean ± SEM obtained from 8 rats in each experimental group. Statistical analysis: Student *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$.

3.2. Lipid content in the fetal liver

Important sex-dependent changes were evidenced when the fetal liver content was analyzed in a sex-dependent manner. Indeed, triglycerides ($p < 0.01$) and cholesterol ($p < 0.05$) were found increased in the liver of male fetuses of GDM rats compared to controls (Fig. 1), whereas no changes in phospholipids, free fatty acids and cholesteryl esters were found when the control and GDM male groups were compared. On the other hand, opposite changes were evidenced in the livers of female fetuses from GDM rats, which showed reduced triglyceride ($p < 0.05$), cholesterol ($p < 0.05$), phospholipids ($p < 0.01$) and free fatty acids ($p < 0.05$) levels compared to controls, without differences in cholesteryl esters concentrations when the control and GDM female groups were compared (Fig. 1).

3.3. Protein levels of PPARγ, mRNA levels of enzymes involved in lipid synthesis and mRNA levels of sex hormone receptors in the fetal liver

The accumulation of lipids in the livers of male fetuses of GDM rats prompted us to analyze the levels of PPARγ, a nuclear receptor with crucial functions in adipogenesis and found increased in hepatic steatosis (Matsusue et al., 2003; Poulos et al., 2016; Wahli and Michalik, 2012). Levels of both PPARγ1 and PPARγ2 were increased in the liver of male fetuses of GDM rats compared to controls ($p < 0.05$, Fig. 2). Differently, PPARγ1 and PPARγ2 levels were unchanged in the fetal liver of female fetuses of GDM rats compared to controls (Fig. 2).

The expression of FAS, a PPARγ target gene, was increased in the liver of male fetuses of GDM rats compared to controls ($p < 0.05$, Fig. 3A). Also, the expression of the lipogenic gene ACC1 was highly increased in the liver of male fetuses of GDM rats compared to controls ($p < 0.001$, Fig. 3B). The expression of estrogen receptor α (ERα), involved in the inhibition of lipogenic pathways, was reduced in the liver of male fetuses of GDM rats compared to controls ($p < 0.05$, Fig. 3C), whereas the expression of androgen receptor (AR), mostly involved in liver lipolytic pathways in males and liver lipogenic pathways in females, was increased in the liver of male fetuses of GDM rats compared to controls ($p < 0.01$, Fig. 3D). A different expression of genes involved in lipid metabolism was evidenced in the liver of female fetuses from GDM rats, which showed unaltered mRNA levels of FAS, ACC1, ERα and AR when compared to controls (Fig. 3).

3.4. PPARα and PPARδ protein levels and ACO and CPT-1 mRNA levels in the fetal liver from GDM rats

The observed reduction of lipid concentrations in the liver of female fetuses led us to analyze the levels of PPARα, a PPAR isotype highly involved in fatty acid oxidation in the liver (Lefebvre et al., 2006; Liss and Finck, 2017). No changes in PPARα protein expression were found in the livers of both male and female fetuses of GDM rats when compared to controls (Fig. 4A).

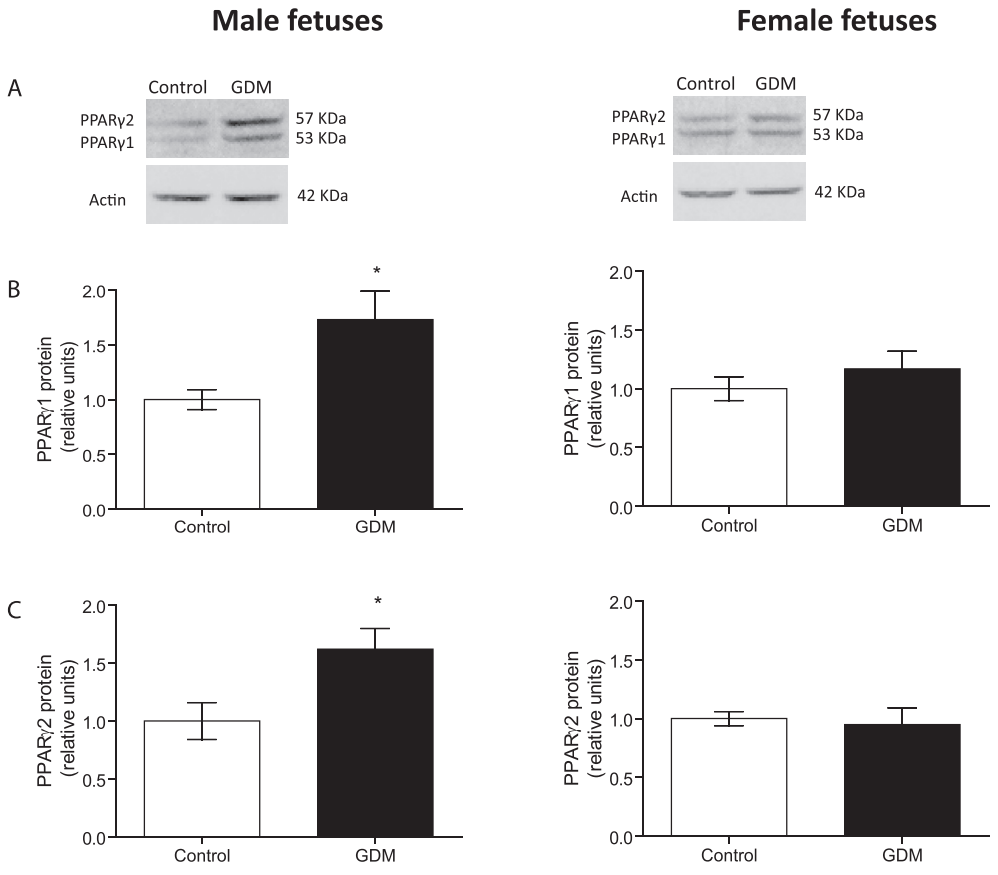

Fig. 2. PPARγ levels in the livers of male and female fetuses of 21-day pregnant offspring of diabetic rats that develop GDM and of control rats. A. Representative immunoblot. **B.** PPARγ1. **C.** PPARγ2. Values represent mean ± SEM obtained from 8 rats in each experimental group. Statistical analysis: Student *t*-test: $^{*}p < 0.05$.

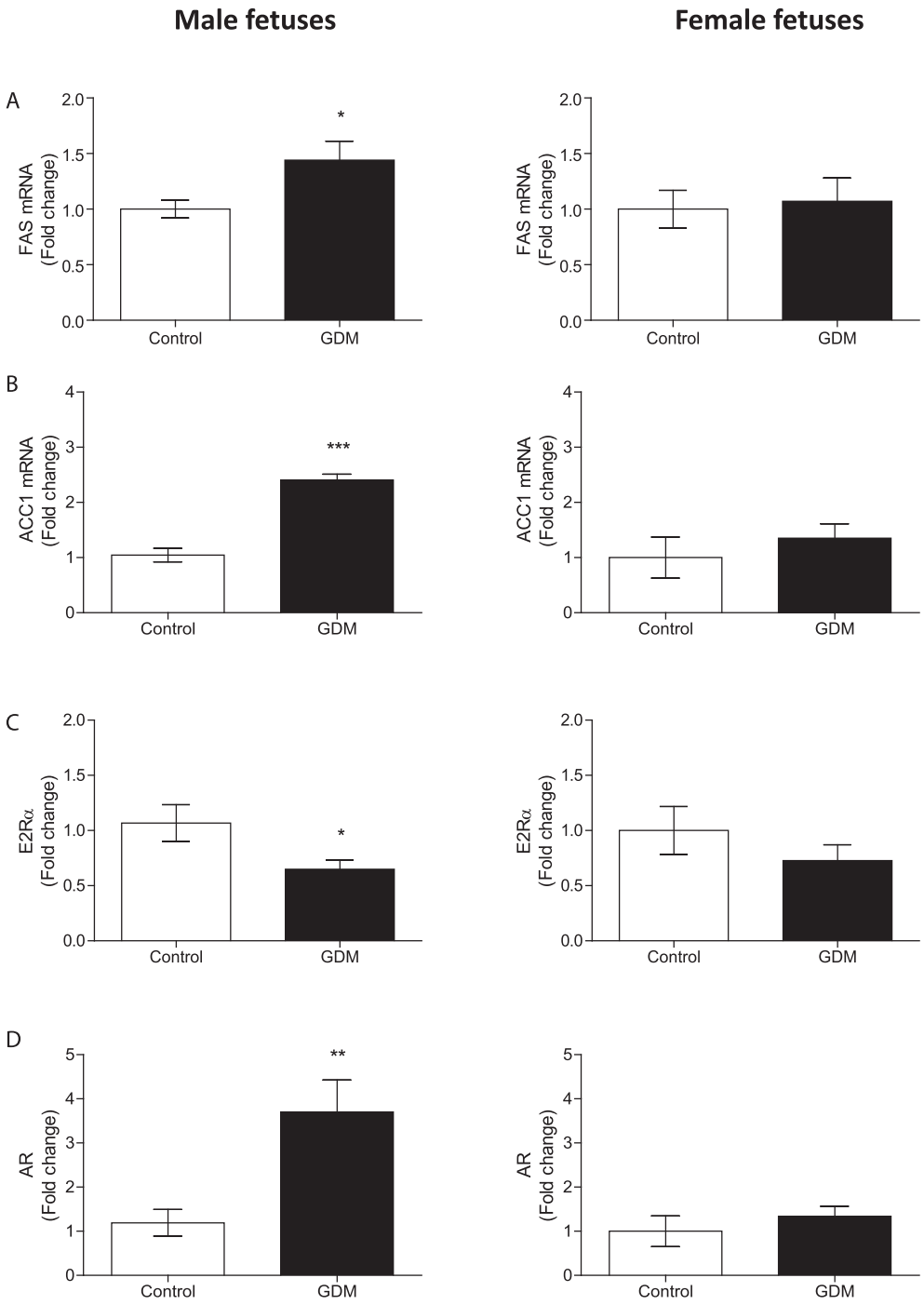

Fig. 3. Expression of FAS, ACC1, ERα and AR in the livers of male and female fetuses of 21-day pregnant offspring of diabetic rats that develop GDM and of control rats. A. FAS mRNA levels, **B.** ACC1 mRNA levels, **C.** ERα mRNA levels, **D.** AR mRNA levels. Values represent mean ± SEM obtained from 8 rats in each experimental group. Statistical analysis: Student *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Differently, the levels of PPARδ, a PPAR isotype associated with both lipogenic and lipid oxidation processes in different tissues (Lee et al., 2006; Reilly and Lee, 2008), were increased in the fetal livers of both male and female fetuses of GDM rats when compared to controls ($p < 0.01$, Fig. 4B). Besides, the mRNA levels of ACO and CPT-1, target genes of both PPARα and PPARδ involved in fatty acid oxidation (Lefebvre et al., 2006; Reilly and Lee, 2008), were increased in the livers of female fetuses ($p < 0.01$) although not in those of male fetuses in the GDM group compared to controls (Fig. 4C and D).

3.5. Expression of miR-130 and miR-9 in the fetal liver from GDM rats

The observed increases in PPARγ and PPARδ in the fetal livers of GDM rats prompted us to analyze the levels of miR-130, miR-9 and miR-122. Indeed, miR-130 targets and negatively regulates PPARγ expression, while miR-9 and miR-122 target and negatively regulate PPARδ expression in different tissues (Gatfield et al., 2009; Lee et al., 2011; Thulin et al., 2013). We found that miR-130 was reduced in the livers of male fetuses of GDM rats compared to controls

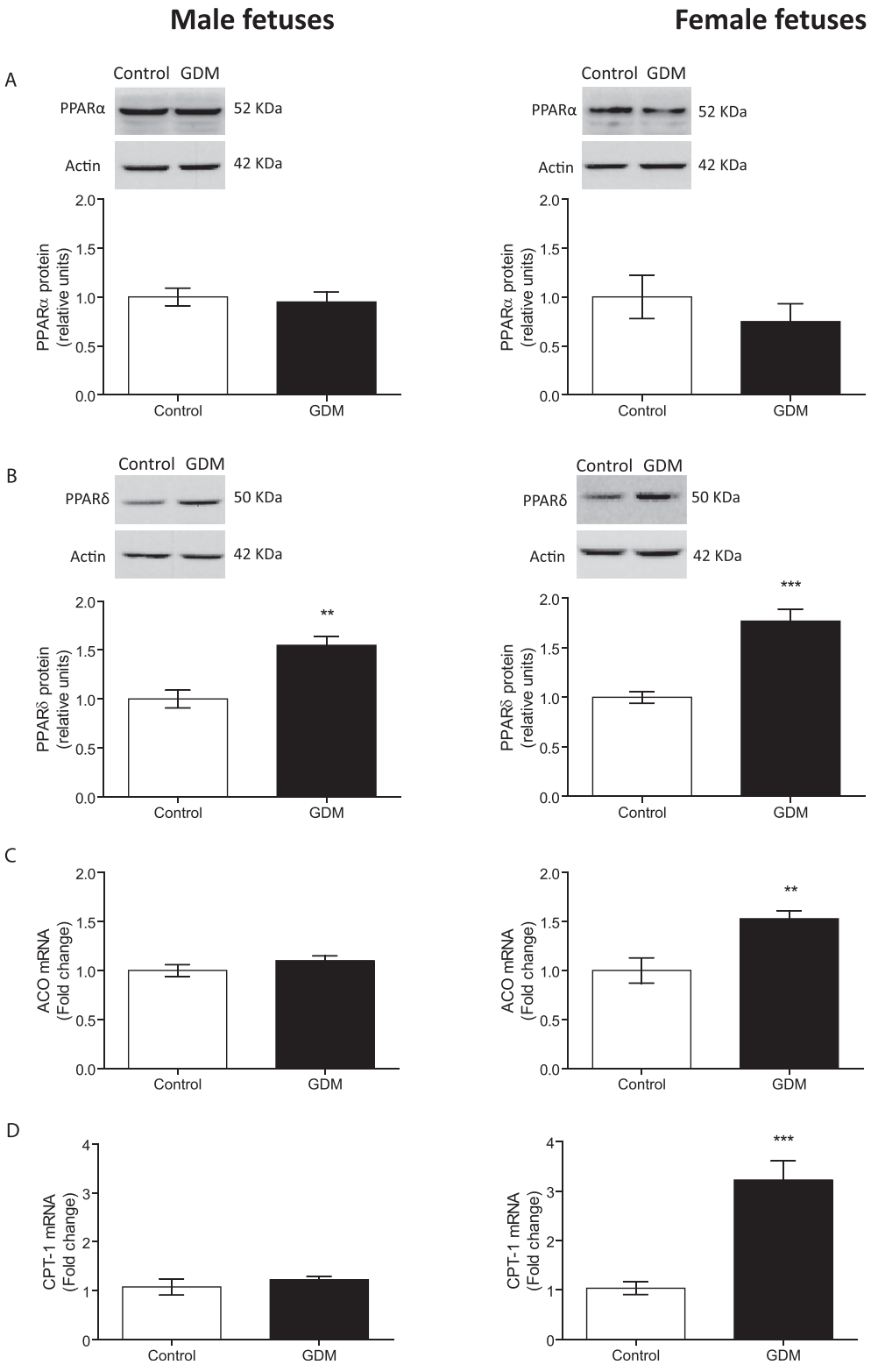

Fig. 4. PPARα and PPARδ levels and expression of fatty acid oxidation enzymes in the livers of male and female fetuses of 21-day pregnant offspring of diabetic rats that develop GDM and of control rats. A. PPARα protein levels. **B.** PPARδ protein levels. **C.** Acyl-CoA (ACO) mRNA levels. **B.** Carnitine palmitoyl transferase 1 (CPT-1) mRNA levels. Values represent mean ± SEM obtained from 8 rats in each experimental group. Statistical analysis: Student *t*-test: $^{**}p < 0.01$, $^{***}p < 0.001$.

($p < 0.05$), although no changes were evidenced in the fetal livers of female fetuses of the control and GDM groups (Fig. 5A). Besides, miR-9 was reduced in the livers of female fetuses of GDM rats compared to controls ($p < 0.001$), although unchanged in the livers of male fetuses of the control and GDM groups (Fig. 5B). On the other hand, the expression of miR-122 was reduced in the livers of male fetuses of GDM rats compared to controls ($p < 0.01$, Fig. 5C). Differently, values of miR-122 expression were unaltered in the

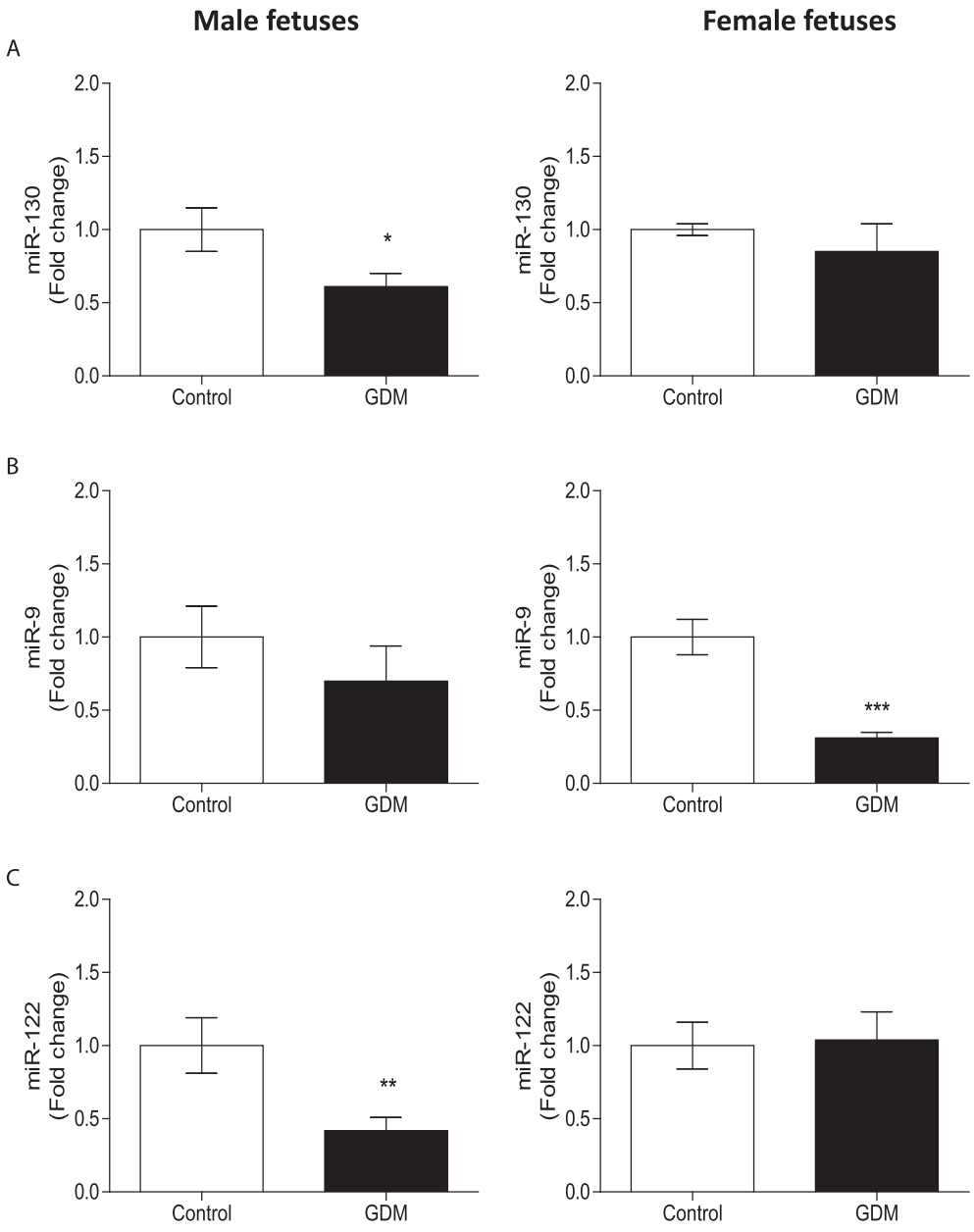

Fig. 5. Expression of microRNAs that target PPARs in the livers of male and female fetuses of 21-day pregnant offspring of diabetic rats that develop GDM and of control rats. A. miR-130. **B.** miR-9. **C.** miR-122. Values represent mean ± SEM obtained from 8 rats in each experimental group. Statistical analysis: Student *t*-test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

livers of female fetuses of GDM rats when compared to controls (Fig. 5C).

4. Discussion

The availability of an experimental model of GDM allowed us to study the impact of this gestational disease on the fetal liver, a target organ in the regulation of lipid metabolism. As main findings in this work, we identified opposite alterations in lipid accretion in the fetal liver according to the fetal sex, changes that occurred in parallel to alterations in the expression of different PPAR isotypes and microRNAs that regulate PPARs expression.

GDM is a metabolic disease resulting both from insulin resistance during pregnancy and a lack of an appropriate adaptation to pregnancy in the maternal pancreas (Catalano et al., 2003). Increased glucose levels in the maternal compartment will reach the fetal compartment and lead to both fetal hyperglycemia and fetal hyperinsulinemia (Desoye and Nolan, 2016), which are related to increased fetal lipids in circulation and/or lipid accretion in different fetal tissues (Herrera and Desoye, 2016; Higa and Jawerbaum, 2013).

In this work, GDM rats, generated by developmental programming in the offspring of mild diabetic rats (Capobianco et al., 2016), showed maternal and fetal hyperglycemia and hyperinsulinemia. Besides, although circulating triglycerides were increased in the maternal circulation, no changes in fetal triglyceridemia were observed. Aiming to address whether lipid accretion is evidenced in GDM fetuses, we focused our studies in the fetal liver, key organ in lipid metabolism (Liss and Finck, 2017). Striking sex-dependent differences were found in fetal liver lipid accretion, as males

showed increased levels of triglycerides and cholesterol, while females showed reduced levels of most lipid species analyzed. As male and female fetuses showed similar basal lipid levels in liver, female and male fetuses seem to respond oppositely to the adverse intrauterine environment in GDM pregnancies. In both humans and experimental models of metabolic diseases, clear sex-dependent changes in lipid metabolism have been evidenced as a result of both differential developmental processes and the effect of sex steroid hormones (Benz et al., 2012; Kautzky-Willer et al., 2016). Although further work is needed to clarify how lipid metabolism is affected by sex at the fetal stage, we here propose that sex-dependent changes in microRNAs that differentially regulate PPAR receptors, which exert differential actions in accretion and oxidation in the fetal livers, play a role in this regulation.

As PPARs have crucial functions in the regulatory adaptations of lipid metabolism to adverse metabolic conditions (Jawerbaum and Capobianco, 2011; Wahli and Michalik, 2012), we focused in their putative changes in the fetal liver from GDM rats. Previous studies have clearly shown that PPARγ1 and PPARγ2 are expressed in the liver and that their excess in hepatocytes contributes to fatty liver (Inoue et al., 2005; Yamazaki et al., 2011; Yu et al., 2003). In this work, we found increased PPARγ1 and PPARγ2 levels in the liver of male fetuses from GDM rats, an alteration that occurred in parallel to an increase in the PPARγ target gene FAS and the gene expression of ACC1 and would lead to increased lipid content. Possibly contributing to the increased lipid accretion, ERα was found reduced in the liver of male fetuses of the GDM group. Indeed, ERα is the estrogen receptor mostly expressed in the fetal liver (Kuiper et al., 1997) and is involved in the inhibition of lipogenic pathways and FAS and ACC1 expression in the adult liver (Qiu et al., 2017; Shen and Shi, 2015). Differently, AR, whose expression was found increased in the liver of male fetuses of GDM rats, has been reported to have lipolytic actions in the liver of adult males and lipogenic actions in the liver of adult females and in some non-alcoholic fatty acid liver disease conditions such as anabolic-androgenic steroid use (Schwingel et al., 2015; Shen and Shi, 2015). Further research addressing sex hormone levels in the fetuses, their role in the fetal liver and their interaction with PPARs are needed to understand the role of androgens in the sex-dependent changes in lipid accretion observed in the GDM fetuses.

Although PPARα is highly involved in lipid oxidative pathways in the liver (Lefebvre et al., 2006; Liss and Finck, 2017), no changes in PPARα protein levels were evidenced in the livers of male and female fetuses from GDM rats. Differently, PPARα was found increased in fetal livers in experimental models of pregestational diabetes (Martinez et al., 2011), suggesting the presence of differential PPARα responses to intrauterine metabolic alterations in pregestational and gestational diabetic experimental models.

On the other hand, PPARδ, which is involved both in oxidative processes and in adipogenesis (Hansen et al., 2001; Lee et al., 2006; Luquet et al., 2005), was found increased in the livers of both male and female fetuses from GDM rats. Interestingly, the PPARδ target genes ACO and CPT-1 were found increased only in the livers of female fetuses of GDM rats, in which lipid content was reduced. This suggests a role of PPARδ in liver lipid oxidation in female fetuses exposed to the adverse metabolic environment. Similarly, activation of PPARδ has been shown to upregulate oxidative pathways in different cell types and tissues in different adverse metabolic conditions (Kurtz et al., 2010; Liss and Finck, 2017; Reilly and Lee, 2008). Besides, activation of PPARδ can also lead to upregulation of target genes involved in fatty acid synthesis, as shown in livers from db/db mice (Lee et al., 2006), indicating the ability of PPARδ activation to lead to lipid oxidation or accumulation in different metabolic contexts (Lee et al., 2006; Reilly and Lee, 2008). Morever, both PPARδ and PPARγ signaling are precisely coordinated during adipogenesis (Hansen et al., 2001). Whether a simultaneous increase in PPARγ and PPARδ levels is that leading to a lipogenic pathway in the fetal livers from male fetuses deserves to be further studied.

Of interest, in this study, changes in the levels of PPARs occurred in parallel to changes in microRNAs that target these nuclear receptors. Different microRNAs have been described as PPAR targets, probably due to the relevant regulatory role of microRNAs in metabolic regulation (Dumortier et al., 2013; Portius et al., 2017). Indeed, miR-130 has been described to target PPARγ in different cell types and tissues (Huang et al., 2015; Lee et al., 2011; Tu et al., 2014), and found in this work reduced only in the liver of male fetuses from GDM rats, in which PPARγ levels were increased. On the other hand, miR-9 has been found to target PPARα and PPARδ in different tissues (Portius et al., 2017; Thulin et al., 2013). As PPARα levels were found unchanged in the fetal liver from GDM rats, probably this microRNA did not target PPARα in the fetal liver or other compensatory change occurred. Indeed, microRNA targets are usually subject to the action of multiple microRNAs in both direct and indirect forms (Dumortier et al., 2013). Besides, the translation inhibition/destabilization roles of the mRNA targets are dependent on the cell type and the specific biological context (Dumortier et al., 2013). Interestingly, in the liver of female fetuses of GDM rats, PPARδ increases occurred in parallel to a reduction in miR-9 expression, whereas in the liver of male fetuses of GDM rats, PPARδ increases occurred in parallel to a reduction in miR-122. Although both miR-9 and miR-122 have been described to target PPARδ in different tissues and cell types (Gatfield et al., 2009; Portius et al., 2017), the findings of sex-dependent changes in these microRNAs evidenced in relation to the levels and function of PPARδ in fetal livers opens new questions regarding the possible involvement of different microRNAs in leading to an increase in PPARδ that exert opposite roles in the regulation of lipid metabolism.

Sex-dependent changes in microRNA expression have been evidenced in different studies (Dai and Ahmed, 2014; Dumortier et al., 2013). In this work, the sex-dependent changes observed in the microRNAs evaluated in the fetal livers of GDM rats deserve further studies. Indeed, as these microRNAs are regulators of PPAR pathways and of other multiple targets in metabolic/inflammatory pathways (Dumortier et al., 2013; Wang et al., 2016; Zampetaki and Mayr, 2012), they may constitute relevant markers of fetal metabolic alterations in GDM.

In conclusion, the results of this study show that sex-dependent differences in GDM offspring start *in utero,* in the fetal liver, which shows alterations that may lead to different responses and adaptations to challenges in the life of the offspring. To our knowledge, this is the first time that sex-dependent changes in lipid content, expression of microRNAs that target PPARs, and PPAR levels are evidenced in the livers of fetuses of GDM rats. Our results suggest that the PPAR changes may result from alterations in microRNAs that target PPAR levels and lead to different lipid metabolic pathways. This would lead to opposite changes in liver lipid concentrations in male and female fetuses, which may in turn lead to different adaptive responses in the offspring's later life.

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