RESEARCH ARTICLE

Intrauterine programming of lipid metabolic alterations in the heart of the offspring of diabetic rats is prevented by maternal diets enriched in olive oil

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Scope: Maternal diabetes can program metabolic and cardiovascular diseases in the offspring. The aim of this work was to address whether an olive oil supplemented diet during pregnancy can prevent lipid metabolic alterations in the heart of the offspring of mild diabetic rats.
Methods and results: Control and diabetic Wistar rats were fed during pregnancy with either a standard diet or a 6% olive oil supplemented diet. The heart of adult offspring from diabetic rats showed increases in lipid concentrations (triglycerides in males and phospholipids, cholesterol, and free fatty acids in females), which were prevented with the maternal diets enriched in olive oil. Maternal olive oil supplementation increased the content of unsaturated fatty acids in the hearts of both female and male offspring from diabetic rats (possibly due to a reduction in lipoperoxidation), increased the expression of Δ6 desaturase in the heart of male offspring from diabetic rats, and increased the expression of peroxisome proliferator activated receptor α in the hearts of both female and male offspring from diabetic rats.
Conclusion: Relevant alterations in cardiac lipid metabolism were evident in the adult offspring of a mild diabetic rat model, and regulated by maternal diets enriched in olive oil.

Keywords:
Diabetes in pregnancy / Intrauterine programming / Lipid metabolism / Offspring's heart / Olive oil supplementation

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1 Introduction

Although increasing clinical and basic evidence has shown the involvement of adverse intrauterine programming in the etiology of diabetes and cardiovascular diseases [1–3], the mechanisms involved are still largely unclear. Cardiac dysfunction in diabetes has been related to maladaptation in cardiac energy metabolism and intramyocardial lipid accumulation [4, 5]. Impaired lipid metabolism and the ectopic deposition of fat have been found involved in the long-term programming of diseases [6] and related to the generation of a proinflammatory environment [7]. Peroxisome proliferator activated receptor α (PPARα) is a ligand-activated transcription factor with key functions in the regulation of lipid metabolism, antioxidant and antiinflammatory pathways, highly relevant in cardiac energy metabolism, cardiovascular remodeling, and vascular homeostasis [8–11]. PPARα is one of the three identified PPARs, nuclear receptors that are activated by endogenous ligands of lipid nature, including diverse unsaturated fatty acids [12].

Both alterations in the expression of PPARα and overaccumulation of lipids have been found in the heart in the experimental models of adverse intrauterine programming due to protein restriction [13]. Besides, studies have shown that diabetes experimentally induced in rats during late

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Abbreviations: **ANOVA**, analysis of variance; **LP**, lipoperoxidation; **NOP**, nitric oxide production; **PPARα**, peroxisome proliferator activated receptor α

gestation leads to the programming of cardiovascular alterations in the offspring [14–16]. Damage in the offspring's heart has also been found in severe models of pregestational diabetes [17, 18]. In a pregestational but mild experimental model of diabetes, we have previously found that the fetus accumulates lipids in different fetal organs, including the liver, lung, and heart; organs in which the activation of PPARα regulates the expression of genes involved in lipid metabolism [19–21]. This mild diabetic model is neonatally induced by streptozotocin, has been thoroughly characterized during pregnancy, and is particularly relevant as its glycemia ranging 150–230 mg/dL resembles the levels of glycemia often seen in human diabetic pregnancies [22]. In addition, this model is characterized by maternal and fetal glucose and lipid metabolic impairments, and increased placental and neonatal weight [23], characteristics of human diabetic pregnancies and markers of an adverse intrauterine programming [24]. Whether this model leads to an intrauterine programming of alterations in lipid metabolism in the heart of adult offspring is unknown.

On the other hand, due to epidemiological data, it is well known that diets enriched in olive oil, such as the Mediterranean diet, are able to prevent cardiovascular diseases [25, 26]. Sex differences in the intrauterine programming of metabolic and cardiovascular diseases have been observed in different experimental models of intrauterine growth restriction, obesity, and diabetes [14, 27, 28]. Experimentally, we have previously demonstrated that diets enriched in 6% olive oil (highly enriched in oleic acid, an unsaturated fatty acid that activates PPARα) are able to regulate lipid-metabolic pathways as well as oxidative and nitrative pathways in different fetal organs of diabetic rats, including the fetal heart [19, 20]. In this work, we hypothesize that maternal diabetes leads to gender-dependent alterations in lipid metabolism in the heart of offspring from diabetic rats, and that these alterations are prevented by maternal diets enriched in 6% olive oil. Therefore, the aim of this work was to evaluate metabolic parameters as well as lipid content, fatty acid composition, expression of Δ6 desaturase and PPARα, lipid peroxidation, and nitric oxide production (NOP) in the hearts of adult female and male offspring from control and diabetic rats, as well as to identify putative beneficial effects of maternal diets enriched in olive oil.

2 Materials and methods

2.1 Animals

Forty-five outbred female Albino Wistar rats bred in our animal facility, fed ad libitum with commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina), were mated with males and randomly assigned to generate the control or the diabetic group as they became pregnant. In the diabetic group, a mild diabetic model was induced, as previously described [21, 22], by injecting the two-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; Fig. 1). In the control group, the litters were injected with citrate buffer alone. The litters were not cross-fostered. The diabetic state was confirmed in two-month-old rats prior to mating. Rats were considered diabetic when they presented fasting glycemia values higher than 130 mg/dL. Average glycemia of the animals prior to mating was 93 ± 6 mg/dL in control and 183 ± 2 mg/dL in diabetic groups. No siblings were used in either the control or the diabetic group. The animal protocol was approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD N° 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) available at http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader.

2.2 Experimental design

As schematized in Fig. 1, control and diabetic female rats were mated with control males. The presence of sperm cells in vaginal smears confirmed the first day of pregnancy. On this day, both control and diabetic animals were randomized into two different groups: (Group 1) Animals fed with a standard diet ($n = 8$ control mothers and $n = 8$ diabetic mothers), and (Group 2) animals fed until parturition with a standard diet supplemented with 6% olive oil (a supplemented diet that is 354% enriched in oleic acid, a PPAR activator, when compared to the standard diet; $n = 8$ control mothers and $n = 8$ diabetic mothers). The composition of the diets was as follows: (i) standard diet (g/100g): proteins (25); carbohydrates (50); fat (5); major fatty acids 16:0 (0.58), 18:0 (0.16), 18:1 (1.27), 18:2 (1.99), 18:3 (0.73) and (ii) olive oil supplemented diet (g/100g): proteins (24); carbohydrates (48); fat (11); major fatty acids 16:0 (1.55), 18:0 (0.26), 18:1 (5.77), 18:2 (2.41), 18:3 (0.57) [19]. Food intake did not change in the control and diabetic olive oil supplemented groups when respectively compared to the control and diabetic nonsupplemented groups (control: 66 ± 3 g/kg/day, control supplemented with olive oil: 67.5 ± 2.5 g/kg/day, diabetic: 76.6 ± 3.5 g/kg/day ($p < 0.05$ versus control), and diabetic supplemented with olive oil: 72.5 ± 2.7 g/kg/day). No treatments were performed on the offspring. The offspring was not cross-fostered and no siblings were used in the same experiment. Four male and four female offspring from each mother were euthanized at the fifth month of age through decapitation. Blood was collected in heparinized tubes and plasma was preserved at −80°C. The hearts were explanted, weighed, and from each male or female offspring of each mother the whole heart was either preserved at −80°C for further analysis of lipid content, fatty acid composition, or nitric oxide production/lipoperoxidation (NOP/LP), or preserved at −20°C in RNA stabilization

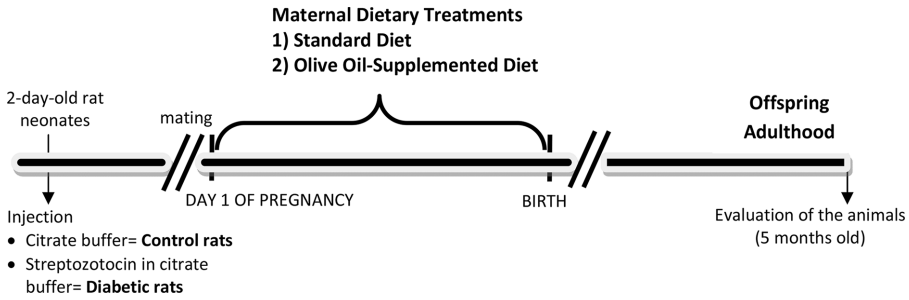

Figure 1. Experimental design: Diabetes was induced in Wistar rats by neonatal administration of streptozotocin. Control and diabetic female rats were mated with control males. During pregnancy, rats were fed with a standard diet enriched or not with 6% olive oil. No further animal treatments were performed. The offspring from control and diabetic rats were studied at five months of age.

solution (RNAlater, Invitrogen, CA, USA) for further evaluation of the expression of Δ6 desaturase and PPARα.

2.3 Plasma metabolic measures

Glycemia values were measured by the Accu-chek reagent strips and by a glucometer Accu-chek (Bayer Diagnostics, Buenos Aires, Argentina) in blood obtained from the tail vein of the mothers. Adult offspring glycemia and triglyceridemia were measured in plasma by spectrophotometric enzymatic assays (Wiener lab, Rosario, Argentina). Insulinemia was measured by using a commercial assay kit (Mercodia Ultrasensitive Rat Insulin ELISA kit, Uppsala, Sweden), according to the manufacturer´s instructions.

2.4 Lipid content

Hearts from one female and one male offspring from each rat were each homogenized in 1 mL PBS and protein content in the homogenates measured by the Bradford assay [29]. Lipids in the homogenates were extracted by three rounds of organic extraction in methanol:chloroform (2:1), as previously stated [21]. The lipids extracted (equivalent to 400 μg of protein) were developed by TLC 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. Lipid species stained with iodine vapors were identified and quantified by comparison with known amounts of standards run on the same plate. Image J software was used for the densitometric analysis [21].

2.5 Fatty acid composition of tissue lipids

FAME of heart lipids extracted from one female or one male adult offspring from each rat were prepared by reaction with 5% HCl in methanol at 70°C for 2 h. After cooling, water was added and FAME were extracted with chloroform. FAME were analyzed by GC on a Thermo Focus Gc (Thermo Scientific, Waltham, MA, USA) and by GC-MS on a Shimadzu GCMS-QP5050A (Shimadzu Corporation, Kyoto, Japan) as previously described [20].

2.6 mRNA expression by RT-PCR

Heart RNA was extracted from one female and one male adult offspring from each rat for the determination of Δ6 desaturase (fatty acid desaturase 2) and PPARα mRNA expression by RT-PCR, as previously determined [19]. The following primers were used: Δ6 desaturase (forward: 5′-ATCTGCCCTACAACCACCAG-3′ and reverse: 5′-GTGTGACCCACACAAACCAG-3′), PPARα (forward: 5′-TCACACAATGCAATCCGTTT-3′ and reverse: 5′-GGCCTTGACCTTGTTCATGT-3′), and ribosomal protein L30, used as an internal control, (forward: 5′-CCATCTTGGCGTCTGATCTT-3′ and reverse: 5′-GGCGAGGATAACCAATTTC-3′), which were designed using the Primer 3 software and used previously [19].

2.7 Lipidperoxidation measurement

LP was assessed by evaluating the concentrations of thiobarbituric acid reactive substances, a method widely used to assess peroxidation of fatty acids. Briefly, the heart from one female or one male adult offspring from each rat was homogenized in 100 mM Tris-HCl buffer (0.1 mM, pH: 7.4) and thiobarbituric acid reactive substances, evaluated as previously described [21].

2.8 Nitric oxide production

NOP was evaluated by measuring the concentration of NO stable metabolites, nitrates/nitrites, as previously determined [19]. For this, the heart of one female or one male adult offspring from each rat was homogenized in 1 mL Tris-HCl buffer pH 7.6, and an aliquot was separated for protein analysis. After reducing nitrates to nitrites using nitrate reductase enzyme, nitrites were measured by using a commercial assay kit (Cayman Chemical Co. Ann Arbor, MI, USA), according to the manufacturer's instructions.

2.9 Statistical analysis

Data are presented as the mean ± SEM. Groups were compared by two-way analysis of variance (ANOVA) in conjunction with Bonferroni's test where appropriate. A p-value less than 0.05 was considered statistically significant.

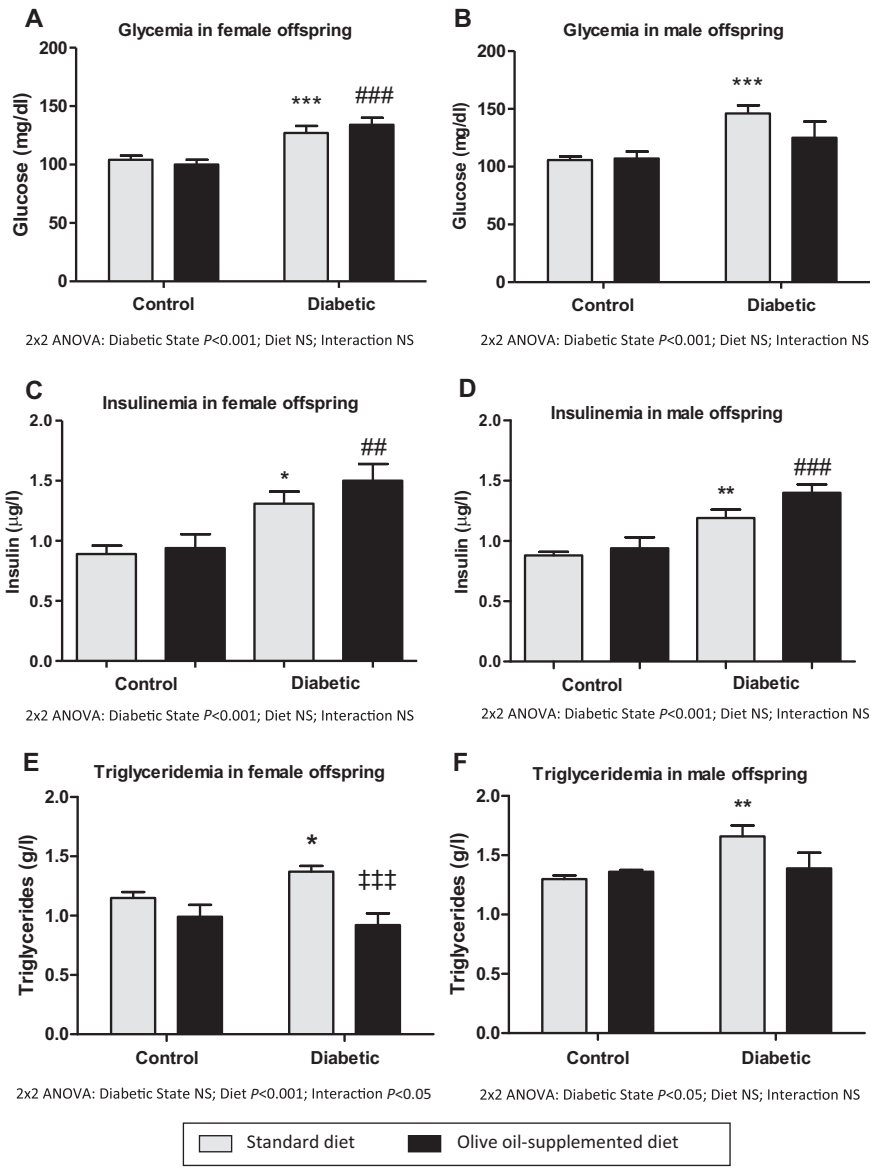

Figure 2. Effect of maternal diet supplemented or not with 6% olive oil on glycemia, insulinemia, and triglyceridemia in five-month-old offspring from control and diabetic rats. (A) Glycemia in female offspring, (B) glycemia in male offspring, (C) insulinemia in female offspring, (D) insulinemia in male offspring, (E) triglyceridemia in female offspring, and (F) triglyceridemia in male offspring. Values represent mean ± SEM obtained from eight rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. Posttest significant results: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control with standard diet; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus control with olive oil supplemented diet; $^{\ddagger\ddagger\ddagger}p < 0.001$ versus diabetic with standard diet. NS, nonsignificant for the two-way ANOVA.

3 Results

3.1 Serum parameters, body weight, and heart weight in the offspring of diabetic rats: Regulation by maternal diets enriched in olive oil

Glycemia values were increased in both female and male adult offspring from nondietary-supplemented diabetic rats when compared to controls (Fig. 2A and B, $p < 0.001$). Glycemia values were similar in the female offspring from diabetic rats that received or not the olive oil supplementation (Fig. 2A). Glycemia values showed no significant changes in the male offspring from the olive oil supplemented diabetic group when compared both to the nondietary-supplemented diabetic group and the olive oil supplemented control group (Fig. 2B).

Insulin concentrations were increased in both female and male adult offspring from nondietary-supplemented diabetic rats when compared to controls (Fig. 2C and D, $p < 0.05$), and were also increased in the male and female offspring from olive oil supplemented control and diabetic groups compared to the respective olive oil supplemented control groups (Fig. 2C and D, $p < 0.01$).

Triglyceridemia was found increased in female ($p < 0.05$) and male ($p < 0.01$) adult offspring from nondietary-supplemented diabetic rats when compared to controls (Fig. 2E and F). Triglyceridemia was significantly reduced in the female offspring from olive oil supplemented diabetic rats when compared to the nondietary-supplemented diabetic group ($p < 0.001$, Fig. 2E). Triglyceridemia values showed no significant changes in the male offspring from the olive oil supplemented diabetic group when compared both to the

nondietary-supplemented diabetic group and to the olive oil supplemented control group (Fig. 2F).

The body weight in the female offspring from the nondietary-supplemented and the olive oil supplemented diabetic rats was similar to those of controls (Supporting Information Table 1). The male offspring from the nondietary-supplemented diabetic rats showed increased body weight when compared to controls ($p < 0.05$). No changes in body weight were observed when the male offspring from olive oil supplemented diabetic rats were compared to both the nondietary-supplemented diabetic group and to the olive oil supplemented control group (Supporting Information Table 1). The heart showed no differences in weight in the groups evaluated (Supporting Information Table 1).

3.2 Lipid overaccumulation in the heart of the offspring from diabetic rats: Regulation by maternal diets enriched in olive oil

In the heart, the adult female offspring from nondietary-supplemented diabetic rats showed increased concentrations of phospholipids when compared to controls ($p < 0.05$), while the maternal supplementation with olive oil prevented this increase (Fig. 3A). Indeed, maternal olive oil supplementation reduced heart phospholipid content in both the offspring of control ($p < 0.001$) and diabetic rats ($p < 0.05$) when respectively compared to the nondietary-supplemented control and diabetic groups (Fig. 3A). Differently, the phospholipid content was similar and there was no effect of maternal treatments with olive oil when the male offspring of diabetic and control rats were evaluated (Fig. 3B).

The content of cholesterol in the hearts of female offspring from nondietary-supplemented diabetic rats was also increased compared to controls ($p < 0.05$, Fig. 3C). Moreover, the heart of female offspring from control and diabetic rats, fed the olive oil supplemented diet, showed reduced cholesterol concentrations compared to the respective nondietary-supplemented control and diabetic groups ($p < 0.05$, Fig. 3C). In the heart of male offspring, no differences in cholesterol concentrations were evident in the experimental groups evaluated (Fig. 3D).

Triglyceride concentrations remained unchanged in the hearts of female offspring from nondietary-supplemented diabetic rats when compared to controls or to the olive oil supplemented diabetic group (Fig. 3E). On the other hand, triglyceride concentrations increased in the hearts of male offspring from nondietary-supplemented diabetic rats ($p < 0.05$), an alteration prevented by the maternal treatment with olive oil ($p < 0.01$, Fig. 3F).

Free fatty acid concentrations, increased in the heart of female offspring from nondietary-supplemented diabetic rats ($p < 0.01$), were decreased by maternal diets enriched in olive oil in both the offspring of control ($p < 0.05$) and diabetic ($p < 0.001$) animals (Fig. 3G). In male offspring from diabetic rats, no changes in free fatty acid concentrations were evident in the diabetic group compared to controls or compared with the olive oil supplemented diabetic group (Fig. 3H).

3.3 Fatty acid composition in the heart of the offspring from diabetic rats: Regulation by maternal diets enriched in olive oil

Despite the important changes in lipid content in the heart of the female offspring from diabetic rats compared to controls, only a reduction in palmitoleic acid was observed in the nondietary-supplemented diabetic group when compared to controls ($p < 0.05$, Table 1). Interestingly, the maternal diet enriched in olive oil led to a decrease in stearic acid ($p < 0.05$, a saturated fatty acid) and arachidonic acid ($p < 0.05$, an *n*-6 PUFA), and to an increase in palmitoleic acid and oleic acid ($p < 0.05$, MUFA) and α-linolenic acid ($p < 0.01$, an *n*-3 PUFA) in the female offspring of diabetic rats compared to the nondietary-supplemented diabetic group (Table 1).

In the heart of male offspring from the nondietary-supplemented diabetic group, there were no changes in fatty acid composition when compared to controls (Table 1). Interestingly, in the male offspring of control rats fed the olive oil supplemented diet, an increase in oleic acid (*n*-9), linoleic acid (*n*-6) and α-linolenic acid (*n*-3), and a decrease in arachidonic acid (*n*-6) and docosahexanoic acid (*n*-3) were found when compared to the nondietary-supplemented control group ($p < 0.05$, Table 1). Besides, in the heart of male offspring of diabetic rats fed the olive oil supplemented diet, oleic acid was increased ($p < 0.05$), and both arachidonic acid (*n*-6) and docosahexanoic acid (*n*-3) were decreased ($p < 0.01$) when compared to the nondietary-supplemented diabetic group (Table 1).

3.4 Expression of Δ6 desaturase and PPARα in the heart of the offspring from diabetic rats: Regulation by maternal diets enriched in olive oil

The changes observed in fatty acid composition led us to analyze the expression of Δ6 desaturase, a target of PPARα in different tissues [30]. In the heart of female offspring from nondietary-supplemented diabetic rats, Δ6-desaturase expression was reduced when compared to controls ($p < 0.05$, Fig. 4A). No changes in Δ6-desaturase expression were evident in the hearts of female offspring from control and diabetic rats when the olive oil supplemented groups were compared to the respective nondietary-supplemented ones (Fig. 4A). Differently, in the hearts of male offspring, there were no changes in Δ6-desaturase expression when the nontreated control and diabetic groups were compared, but an increase in Δ6-desaturase expression was evident in both the olive oil supplemented control ($p < 0.05$) and diabetic ($p < 0.001$) groups when compared to the respective nondietary-supplemented groups (Fig. 4B).

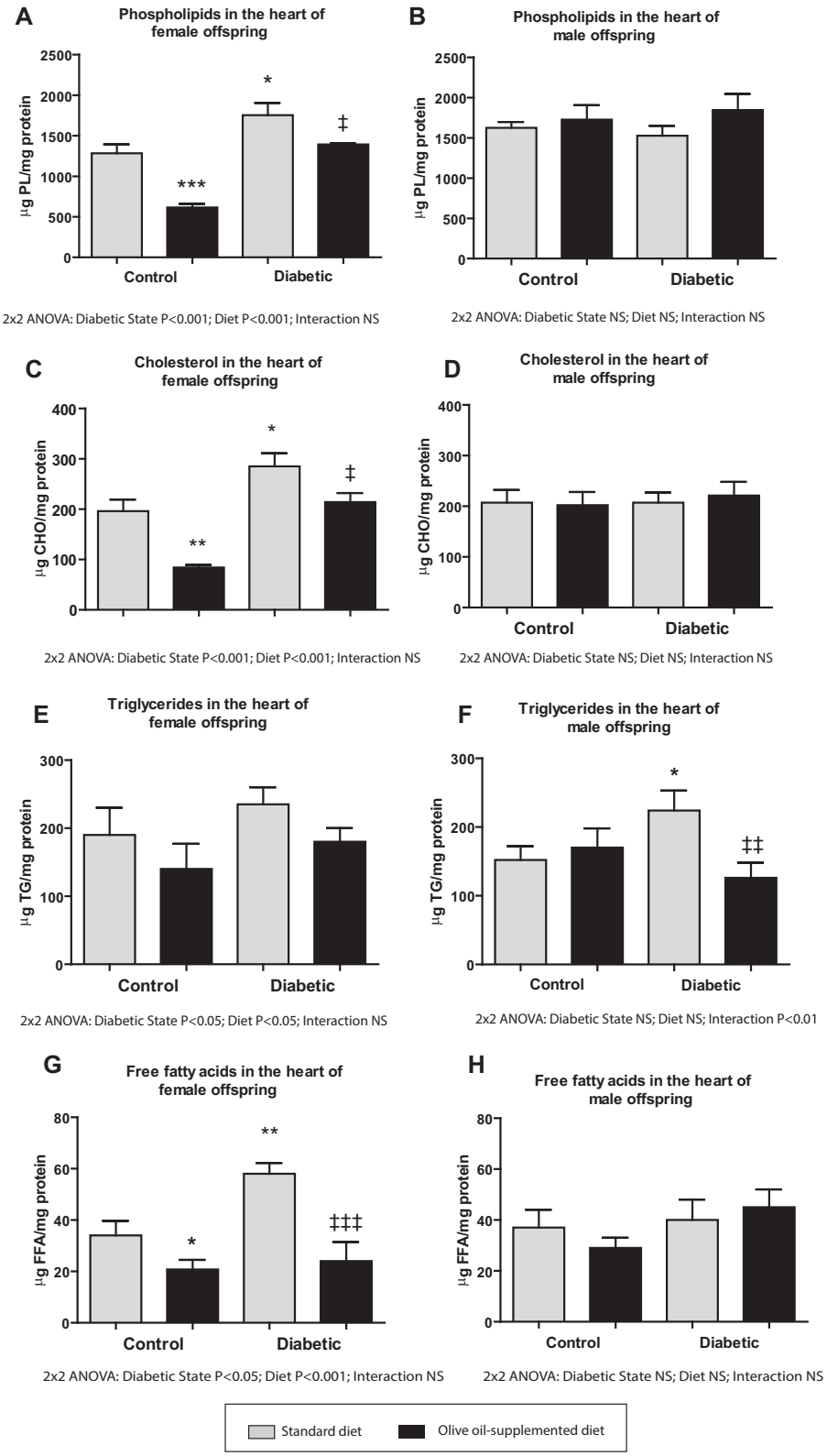

Figure 3. Effect of maternal diet supplemented or not with 6% olive oil on lipid content in the heart of five-month-old offspring from control and diabetic rats. (A) Phospholipids in the heart of female offspring, (B) phospholipids in the heart of male offspring, (C) cholesterol in the heart of female offspring, (D) cholesterol in the heart of male offspring, (E) triglycerides in the heart of female offspring, (F) triglycerides in the heart of male offspring, (G) free fatty acids in the heart of female offspring, and (H) free fatty acids in the heart of male offspring. Values represent mean ± SEM obtained from eight rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. Posttest significant results: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control with standard diet; $^{‡}p < 0.05$, $^{‡‡}p < 0.01$, $^{‡‡‡}p < 0.001$ versus diabetic with standard diet. NS, nonsignificant for the two-way ANOVA.

Table 1. Effect of maternal diet supplemented with 6% olive oil on the composition of major fatty acids in the heart of adult offspring from control and diabetic rats

	Control offspring		Diabetic offspring		2 × 2 ANOVA results diabetic state-diet-interaction
	Standard diet	Standard diet supplemented with 6% olive oil	Standard diet	Standard diet supplemented with 6% olive oil	
(A) Females					
C16:0 palmitic acid	21.6 ± 0.4	21.1 ± 0.9	21.1 ± 1.2	21.9 ± 1.1	NS-NS-NS
C16:1 palmitoleic acid (*n*-7)	1.3 ± 0.2	1.1 ± 0.1	0.7 ± 0.1*	1.2 ± 0.1‡	NS-NS-$p < 0.05$
C18:0 stearic acid	20.2 ± 1.7	18.5 ± 1.8	22.0 ± 1.0	16.0 ± 2.0‡	NS-$p < 0.05$-NS
C18:1 oleic acid (*n*-9)	11.9 ± 2.1	14.7 ± 1.2	10.1 ± 1.0	16.4 ± 2‡	NS-$p < 0.05$-NS
C18:2 linoleic acid (*n*-6)	24.1 ± 0.6	25.1 ± 0.3	25.0 ± 0.2	27.8 ± 1.6	NS-NS-NS
C18:3 α-linolenic acid (*n*-3)	0.9 ± 0.1	1.2 ± 0.1	0.7 ± 0.09	1.3 ± 0.1‡‡	NS-$p < 0.01$-NS
C20:3 dihomogamma linolenic acid (*n*-6)	0.24 ± 0.04	0.16 ± 0.02	0.16 ± 0.02	0.16 ± 0.01	NS-NS-NS
C20:4 arachidonic acid (*n*-6)	12.2 ± 0.9	10.9 ± 0.9	12.2 ± 0.9	8.9 ± 2‡	NS-$p < 0.01$-NS
C22:5 docosapentaenoic acid (*n*-3/*n*-6)	1.14 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	NS-NS-NS
C22:6 docosahexaenoic acid (*n*-3)	3.2 ± 0.2	2.7 ± 0.3	3.5 ± 0.5	2.3 ± 0.9	NS-NS-NS
(B) Males					
C16:0 palmitic acid	20.4 ± 0.8	20.3 ± 1.0	19.5 ± 0.3	21.2 ± 0.7	NS-NS-NS
C16:1 palmitoleic acid (*n*-7)	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.08	NS-NS-NS
C18:0 stearic acid	19.3 ± 0.5	16.8 ± 1.4	21.0 ± 1.0	17.9 ± 0.7	NS-$p < 0.05$-NS
C18:1 oleic acid (*n*-9)	12.4 ± 0.7	15.6 ± 1.0*	11.6 ± 0.9	15.3 ± 0.8‡	NS-$p < 0.01$-NS
C18:2 linoleic acid (*n*-6)	26.7 ± 0.2	29.5 ± 0.7*	26.7 ± 0.2	27.8 ± 0.5	NS-$p < 0.01$-NS
C18:3 α-linolenic acid (*n*-3)	1.0 ± 0.05	1.5 ± 0.1**	0.9 ± 0.1	1.2 ± 0.1	$p < 0.05$-$p < 0.001$-NS
C20:3 dihomogamma linolenic acid (*n*-6)	0.16 ± 0.02	0.15 ± 0.02	0.16 ± 0.01	0.14 ± 0.01	NS-NS-NS
C20:4 arachidonic acid (*n*-6)	11.9 ± 0.6	9.7 ± 0.9*	12.9 ± 0.5	9.8 ± 0.5‡‡	NS-$p < 0.01$-NS
C22:5 docosapentaenoic acid (*n*-3/*n*-6)	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.04	0.9 ± 0.1	NS-NS-NS
C22:6 docosahexaenoic acid (*n*-3)	2.7 ± 0.2	1.7 ± 0.1***	3.0 ± 0.2	2.0 ± 0.1‡‡‡	NS-$p < 0.001$-NS

Values represent mean ± SEM of the percent composition of each fatty acid in the heart of (A) female and (B) male offspring. $n = 8$ offspring from eight different mothers. Two-way ANOVA in conjunction with Bonferroni's test was performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control with standard diet; ‡$p < 0.05$, ‡‡$p < 0.01$, ‡‡‡$p < 0.001$ versus diabetic with standard diet. NS, nonsignificant.

When PPARα expression was analyzed, no changes were found in the hearts of female and male offspring from nondietary-supplemented diabetic rats when compared to the respective control groups. Maternal dietary treatments with olive oil led to an increase in PPARα expression in the hearts of female and male offspring from both control and diabetic rats when compared, respectively, to the nondietary-supplemented control and diabetic groups ($p < 0.05$, Fig. 4C and D).

3.5 Increased lipoperoxidation and nitric oxide production in the heart in the heart of the offspring from diabetic rats: Regulation by maternal diets enriched in olive oil

Lipoperoxidation was increased in the heart of the female offspring of nondietary-supplemented diabetic rats when compared to controls ($p < 0.01$, Fig. 5A). Besides, the maternal dietary supplementation with olive oil was able to prevent this increase ($p < 0.001$, Fig. 5A). LP was also increased in the heart of male offspring from nondietary-supplemented diabetic rats compared to controls ($p < 0.01$, Fig. 5B). Besides, in the heart of the male offspring of both control and diabetic rats, the maternal dietary treatment with olive oil led to a reduction in LP when compared, respectively, to the nondietary-supplemented control and diabetic groups ($p < 0.05$, Fig. 5B). Nitric oxide production, which was increased in the hearts of adult female and male offspring of nondietary-supplemented diabetic rats when compared to controls ($p < 0.01$), was only reduced by the maternal dietary treatments with olive oil in the heart of male offspring from diabetic rats ($p < 0.001$, Fig. 5D).

4 Discussion

The findings of this work demonstrate that mild maternal diabetes leads to an increased lipid content and lipoperoxidation in the heart of adult offspring, and that these alterations are prevented by maternal treatments with diets supplemented with olive oil. Moreover, the maternal olive oil supplemented diets, enriched in the PPARα activator oleic acid, increased the percentage of oleic acid, changed PUFAs composition, and increased PPARα expression in the heart of the adult

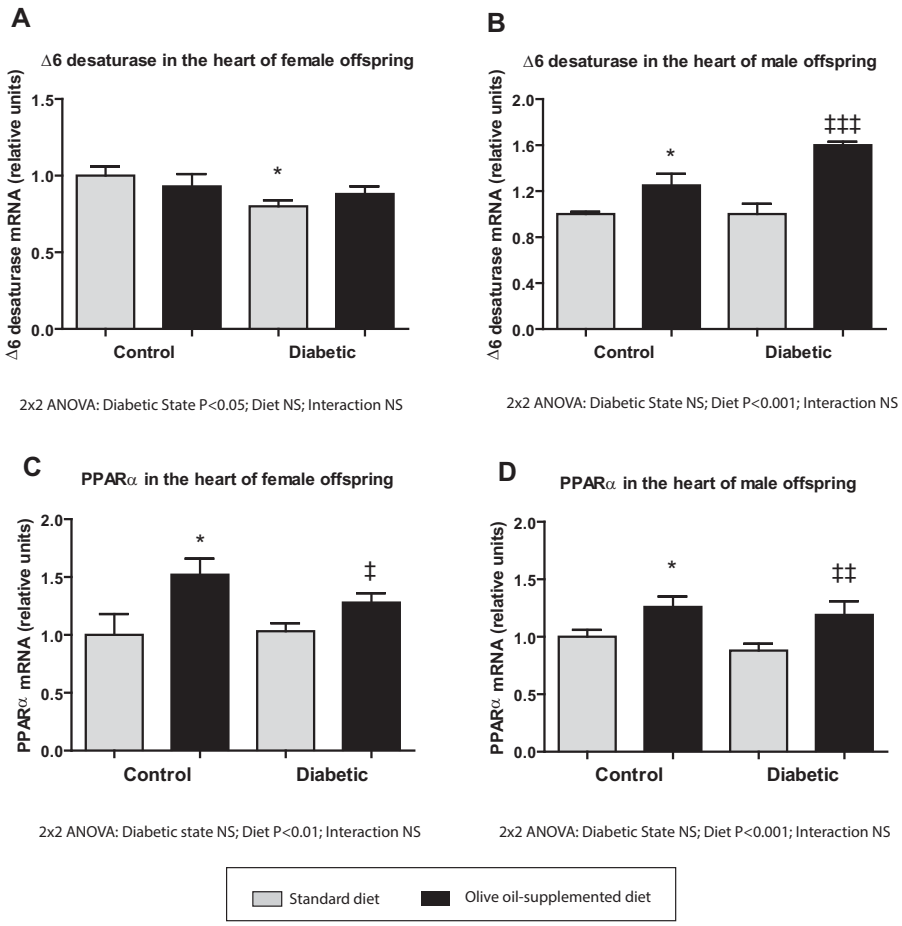

Figure 4. Effect of a maternal diet supplemented with 6% olive oil on the mRNA expression of Δ6 desaturase and PPARα in the heart of five-month-old offspring from control and diabetic rats. (A) Δ6-desaturase mRNA in the heart of female offspring, (B) Δ6-desaturase mRNA in the heart of male offspring, (C) PPARα mRNA in the heart of female offspring, and (D) PPARα mRNA in the heart of male offspring. Values represent mean ± SEM obtained from eight rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. Posttest significant results: $^{*}p < 0.05$ versus control with standard diet; $^{‡}p < 0.05$, $^{‡‡}p < 0.01$, $^{‡‡‡}p < 0.001$ versus diabetic with standard diet. NS, nonsignificant for the two-way ANOVA.

offspring of diabetic rats, showing the impact of this dietary treatment on the next generation.

Overaccumulation of lipids in the heart has been related to increases in cardiomyocyte apoptosis, myocardial fibrosis, contractile dysfunction, impaired mitochondrial energetics, and increased oxidative stress [7, 31, 32]. Although the mechanisms responsible for the overaccumulation of different lipid species in the heart of females and males are unknown, they may be due to changes in lipid metabolism that are highly dependent on multiple factors, including hormonal influences, mitochondrial function, and epigenetic changes [33–35].

Intrauterine programming of cardiovascular impairments and regulation of PPAR-dependent pathways have also been found to be sex-dependent [6, 35, 36]. PPARα regulates multiple genes of lipid catabolism [9]. Oleic acid, present in high concentrations in olive oil, is a natural agonist that interacts with the ligand-binding domain of PPARα and leads to its activation [37, 38]. In this work, the diets enriched in olive oil in the diabetic mothers prevented lipid overaccumulation in the offspring´s heart, possibly due to the observed upregulation of PPARα, which may contribute to increasing PPARα signaling and stimulating lipid-catabolic pathways. Previously, we have shown changes in PPARα expression and signaling in the heart of fetuses from diabetic rats fed an olive oil supplemented diet during pregnancy [19]. Future studies, addressing putative epigenetic changes that regulate PPARα expression in the offspring of rats fed an olive oil supplemented diet during pregnancy, are needed to understand how PPARα agonist effects pass to the next generation. Studies performed in experimental models of intrauterine growth restriction have shown epigenetic regulation of PPARα expression in the offspring´s heart related to an adverse intrauterine programming [13].

Different works performed in severe models of diabetes characterized by hyperglycemias over 300 mg/dL and low insulinemia have shown relevant changes in fatty acid composition of heart, most of which are related to a percent reduction of PUFAs [39, 40]. Differently, in this work, only minor changes in fatty acid composition were found when comparing the offspring of nondietary-supplemented control and diabetic groups, probably due to the mild diabetic model obtained by intrauterine programming and characterized by hyperglycemia values around 140 mg/dL and hyperinsulinemia. On the other hand, it was interesting to find many changes in fatty acid composition when the groups fed the olive oil supplemented diet were compared to the nonsupplemented ones. In the heart of female offspring from diabetic

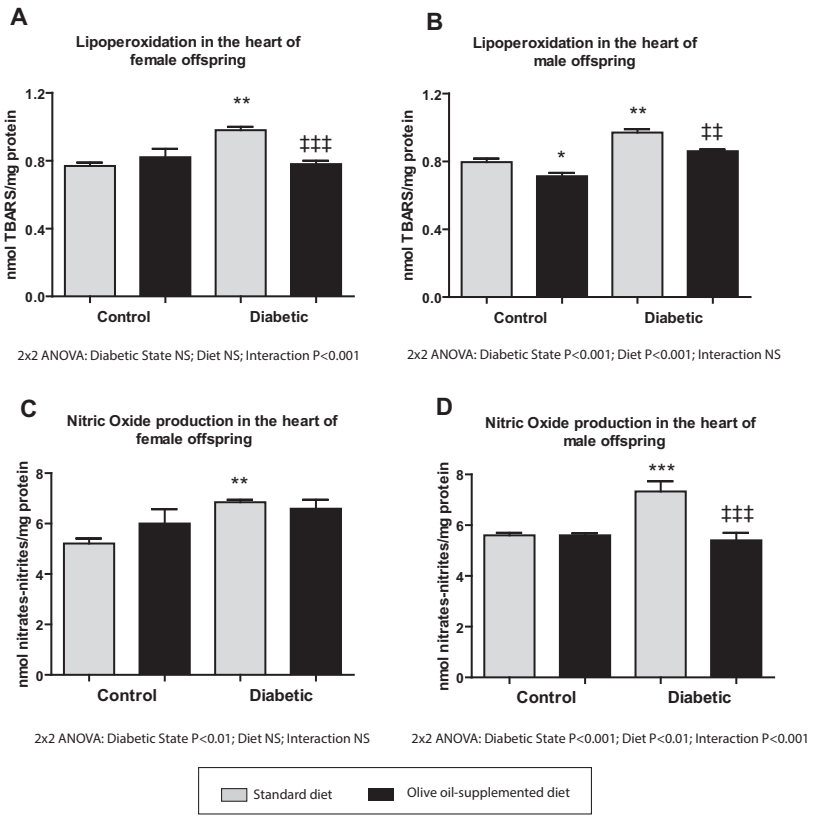

Figure 5. Effect of a maternal diet supplemented with 6% olive oil on LP and NOP in the heart of five-month-old offspring from control and diabetic rats. (A) LP in the heart of female offspring, (B) LP in the heart of male offspring, (C) NOP in heart of female offspring, and (D) NOP in the heart of male offspring. Values represent mean ± SEM obtained from eight adult offspring from eight different mothers, eight rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. Posttest significant results: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control with standard diet; $^{‡‡}p < 0.01$, $^{‡‡‡}p < 0.001$ versus diabetic with standard diet. NS, nonsignificant for the two-way ANOVA.

rats fed the supplemented diet, the observed reduction of saturated fatty acids and increase in MUFA and *n*-3 PUFAs are likely to be beneficial to cardiac health. Saturated fatty acids play a role in cardiac and vascular dysfunction [41]. It is well known that unsaturated fatty acids play important roles in membrane fluidity, and as mediators of signal transduction, cellular differentiation, and metabolic homeostasis [42, 43]. Increased MUFA, *n*-3 PUFAs, and an *n*-3/*n*-6 ratio have been found related to the prevention of cardiovascular events [26, 44, 45]. This increase in the content of unsaturated fatty acids was even found in the heart of male offspring from control rats fed the olive oil enriched diet, although not in the female group fed the same diet, and the putative transgenerational, dietary- and sex-dependent mechanisms involved will require further research.

On the other hand, in the heart of male offspring from both control and diabetic rats, maternal diets enriched in olive oil, together with the increase in MUFA, led to a decrease in both arachidonic acid (*n*-6) and docosahexaenoic acid (*n*-3). This occurred even when the expression of Δ6 desaturase, a rate-limiting enzyme in the synthesis of arachidonic and docosahexanoic acids from their respective precursors of the *n*-6 and the *n*-3 series, was increased in the heart of male offspring from diabetic rats fed the olive oil supplemented diet. This suggests that the synthesis of PUFAs from their precursors is probably not a main limitation, and that their utilization may be increased in the heart of male offspring from both control and diabetic rats fed the olive oil supplemented diet. On the other hand, it is known that PPARα agonists can increase the expression of Δ6 desaturase [30], and if this occurs during fetal development, this might lead to epigenetic changes that in turn lead to its overexpression in the offspring. Besides, changes in insulin sensitivity that can regulate Δ6-desaturase expression [46] may also occur in the offspring of the rats fed the olive oil supplemented diet, an issue that deserves to be evaluated in future studies.

Several works have addressed the capacity of diets enriched in olive oil to prevent not only lipid accumulation but also a proinflammatory and prooxidant state that protects the heart from adverse effects [25, 26]. In this work, we found that the increased lipoperoxidation evident in the hearts of both female and male offspring from diabetic rats was prevented by the maternal diets enriched in olive oil. On the other hand, the overproduction of nitric oxide, an indicator of a proinflammatory environment [47], was also prevented but only in the heart of male offspring from diabetic rats fed the olive oil supplemented diet. These results confirm a close relationship between lipid overaccumulation, oxidative and nitrative stress in the damaged heart [48], and, interestingly, evidence the capacity to be regulated in the next generation with a dietary treatment during pregnancy.

Finally, of note, the amelioration of alterations in the heart lipid metabolism in the offspring from diabetic rats fed the

olive oil supplemented diet occurs in parallel with some beneficial effects evident in the sera. As serum metabolic parameters can predict cardiometabolic risk [34], the observed ameliorations in the offspring´s heart may be related to the improvement of serum parameters, an issue that requires further evaluation.

In conclusion, development in the intrauterine environment of a mild diabetic experimental model programs lipid deposition and lipoperoxidation in the heart of the adult offspring in a sex-dependent manner. A maternal olive oil supplemented diet given only during pregnancy prevents the abnormal intrauterine programming of heart lipid metabolism in the adult offspring. Due to this beneficial effect, and being aware of the limitations of an experimental study, further evaluation of the clinical relevance of these studies is warranted.

E.C. and A.J. designed the study. E.C., M.P., V.C., D.F., I.C., and R.C. conducted the research. E.C., V.C., M.M., and A.J. analyzed the data. A.J. wrote the manuscript. E.C., V.C., R.H., and M.M. reviewed and edited the manuscript. All the authors have read and approved the final manuscript.

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5 References

[1] Higa, R., Jawerbaum, A., Intrauterine effects of impaired lipid homeostasis in pregnancy diseases. *Curr. Med. Chem.* 2013, *20*, 2338–2350.

[2] Zohdi, V., Lim, K., Pearson, J. T., Black, M. J., Developmental programming of cardiovascular disease following intrauterine growth restriction: findings utilising a rat model of maternal protein restriction. *Nutrients* 2014, *7*, 119–152.

[3] Visentin, S., Grumolato, F., Nardelli, G. B., Di Camillo, B. et al., Early origins of adult disease: low birth weight and vascular remodeling. *Atherosclerosis* 2014, *237*, 391–399.

[4] Miki, T., Yuda, S., Kouzu, H., Miura, T., Diabetic cardiomyopathy: pathophysiology and clinical features. *Heart Fail. Rev.* 2013, *18*, 149–166.

[5] Nakanishi, T., Kato, S., Impact of diabetes mellitus on myocardial lipid deposition: an autopsy study. *Pathol. Res. Pract.* 2014, *210*, 1018–1025.

[6] Pruis, M. G., van Ewijk, P. A., Schrauwen-Hinderling, V. B., Plosch, T., Lipotoxicity and the role of maternal nutrition. *Acta Physiol.* 2014, *210*, 296–306.

[7] Wende, A. R., Abel, E. D., Lipotoxicity in the heart. *Biochim. Biophys. Acta* 2010, *1801*, 311–319.

[8] Wahli, W., Michalik, L., PPARs at the crossroads of lipid signaling and inflammation. *Trends Endocrinol. Metab.* 2012, *23*, 351–363.

[9] Lefebvre, P., Chinetti, G., Fruchart, J. C., Staels, B., Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. *J. Clin. Invest.* 2006, *116*, 571–580.

[10] Smeets, P. J., de Vogel-van den Bosch, H. M., Willemsen, P. H., Stassen, A. P. et al., Transcriptomic analysis of PPARalpha-dependent alterations during cardiac hypertrophy. *Physiol. Genomics* 2008, *36*, 15–23.

[11] Yongming, P., Zhaowei, C., Yichao, M., Keyan, Z. et al., Involvement of peroxisome proliferator-activated receptors in cardiac and vascular remodeling in a novel minipig model of insulin resistance and atherosclerosis induced by consumption of a high-fat/cholesterol diet. *Cardiovasc. Diabetol.* 2015, *14*, 6.

[12] Hihi, A. K., Michalik, L., Wahli, W., PPARs: transcriptional effectors of fatty acids and their derivatives. *Cell Mol. Life Sci.* 2002, *59*, 790–798.

[13] Slater-Jefferies, J. L., Lillycrop, K. A., Townsend, P. A., Torrens, C. et al., Feeding a protein-restricted diet during pregnancy induces altered epigenetic regulation of peroxisomal proliferator-activated receptor-alpha in the heart of the offspring. *J. Dev. Orig. Health Dis.* 2011, *2*, 250–255.

[14] Katkhuda, R., Peterson, E. S., Roghair, R. D., Norris, A. W. et al., Sex-specific programming of hypertension in offspring of late-gestation diabetic rats. *Pediatr. Res.* 2012, *72*, 352–361.

[15] Segar, E. M., Norris, A. W., Yao, J. R., Hu, S. et al., Programming of growth, insulin resistance and vascular dysfunction in offspring of late gestation diabetic rats. *Clin. Sci.* 2009, *117*, 129–138.

[16] Agoudemos, M., Reinking, B. E., Koppenhafer, S. L., Segar, J. L. et al., Programming of adult cardiovascular disease following exposure to late-gestation hyperglycemia. *Neonatology* 2011, *100*, 198–205.

[17] Corrigan, N., Treacy, A., Brazil, D. P., McAuliffe, F. M., Cardiomyopathy and diastolic dysfunction in the embryo and neonate of a type 1 diabetic mouse model. *Reprod. Sci.* 2013, *20*, 781–790.

[18] Holemans, K., Gerber, R. T., Meurrens, K., De Clerck, F. et al., Streptozotocin diabetes in the pregnant rat induces cardiovascular dysfunction in adult offspring. *Diabetologia* 1999, *42*, 81–89.

[19] Kurtz, M., Capobianco, E., Martinez, N., Roberti, S. et al., PPAR ligands improve impaired metabolic pathways in fetal hearts of diabetic rats. *J. Mol. Endocrinol.* 2014, *53*, 237–246.

[20] Kurtz, M., Capobianco, E., Careaga, V., Martinez, N. et al., Peroxisome proliferator-activated receptor ligands regulate lipid content, metabolism, and composition in fetal lungs of diabetic rats. *J. Endocrinol.* 2014, *220*, 345–359.

[21] Martinez, N., White, V., Kurtz, M., Higa, R. et al., Activation of the nuclear receptor PPARalpha regulates lipid metabolism in foetal liver from diabetic rats: implications in diabetes-induced foetal overgrowth. *Diabetes Metab. Res. Rev.* 2011, *27*, 35–46.

[22] Jawerbaum, A., White, V., Animal models in diabetes and pregnancy. *Endocr. Rev.* 2010, *31*, 680–701.

[23] White, V., Jawerbaum, A., Mazzucco, M. B., Gauster, M. et al., Diabetes-associated changes in the fetal insulin/insulin-like growth factor system are organ specific in rats. *Pediatr. Res.* 2015, *77*, 48–55.

[24] Plagemann, A., Harder, T., Dudenhausen, J. W., The diabetic pregnancy, macrosomia, and perinatal nutritional programming. *Nestle Nutr. Workshop Ser. Pediatr. Program* 2008, *61*, 91–102.

[25] Stradling, C., Hamid, M., Taheri, S., Thomas, G. N., A review of dietary influences on cardiovascular health: part 2: dietary patterns. *Cardiovasc. Hematol. Disord. Drug Targets* 2014, *14*, 50–63.

[26] Schwingshackl, L., Hoffmann, G., Monounsaturated fatty acids, olive oil and health status: a systematic review and meta-analysis of cohort studies. *Lipids Health Dis.* 2014, *13*, 154.

[27] Sardinha, F. L., Fernandes, F. S., Tavares do Carmo, M. G., Herrera, E., Sex-dependent nutritional programming: fish oil intake during early pregnancy in rats reduces age-dependent insulin resistance in male, but not female, offspring. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2013, *304*, R313–R320.

[28] Grigore, D., Ojeda, N. B., Alexander, B. T., Sex differences in the fetal programming of hypertension. *Gend. Med.* 2008, *5* (Suppl A), S121–S132.

[29] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, *72*, 248–254.

[30] Tang, C., Cho, H. P., Nakamura, M. T., Clarke, S. D., Regulation of human delta-6 desaturase gene transcription: identification of a functional direct repeat-1 element. *J. Lipid Res.* 2003, *44*, 686–695.

[31] McGavock, J. M., Victor, R. G., Unger, R. H., Szczepaniak, L. S. et al., Adiposity of the heart, revisited. *Ann. Intern. Med.* 2006, *144*, 517–524.

[32] Elezaby, A., Sverdlov, A. L., Tu, V. H., Soni, K. et al., Mitochondrial remodeling in mice with cardiomyocyte-specific lipid overload. *J. Mol. Cell. Cardiol.* 2015, *79*, 275–283.

[33] Vijay, V., Han, T., Moland, C. L., Kwekel, J. C. et al., Sexual dimorphism in the expression of mitochondria-related genes in rat heart at different ages. *PLoS One* 2015, *10*, e0117047.

[34] Manson, J. E., Bassuk, S. S., Biomarkers of cardiovascular disease risk in women. *Metabolism* 2015, *64*, S33–S39.

[35] Gabory, A., Attig, L., Junien, C., Sexual dimorphism in environmental epigenetic programming. *Mol. Cell. Endocrinol.* 2009, *304*, 8–18.

[36] Benz, V., Kintscher, U., Foryst-Ludwig, A., Sex-specific differences in type 2 diabetes mellitus and dyslipidemia therapy: PPAR agonists. *Handb. Exp. Pharmacol.* 2012, *214*, 387–410.

[37] Keller, H., Dreyer, C., Medin, J., Mahfoudi, A. et al., Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. USA* 1993, *90*, 2160–2164.

[38] Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J. et al., Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell* 1999, *3*, 397–403.

[39] Black, S. C., Katz, S., McNeill, J. H., Influence of omega-3 fatty acid treatment on cardiac phospholipid composition and coronary flow of streptozocin-diabetic rats. *Metabolism* 1993, *42*, 320–326.

[40] Gudbjarnason, S., el-Hage, A. N., Whitehurst, V. E., Simental, F. et al., Reduced arachidonic acid levels in major phospholipids of heart muscle in the diabetic rat. *J. Mol. Cell. Cardiol.* 1987, *19*, 1141–1146.

[41] Mann, J. I., Diet and risk of coronary heart disease and type 2 diabetes. *Lancet* 2002, *360*, 783–789.

[42] Sampath, H., Ntambi, J. M., Polyunsaturated fatty acid regulation of gene expression. *Nutr. Rev.* 2004, *62*, 333–339.

[43] Spector, A. A., Yorek, M. A., Membrane lipid composition and cellular function. *J. Lipid Res.* 1985, *26*, 1015–1035.

[44] Sanders, T. A., Protective effects of dietary PUFA against chronic disease: evidence from epidemiological studies and intervention trials. *Proc. Nutr. Soc.* 2014, *73*, 73–79.

[45] dos Santos, A. L., Weiss, T., Duarte, C. K., Gross, J. L. et al., Dietary fat composition and cardiac events in patients with type 2 diabetes. *Atherosclerosis* 2014, *236*, 31–38.

[46] Brenner, R. R., Hormonal modulation of delta6 and delta5 desaturases: case of diabetes. *Prostaglandins Leukot. Essent. Fatty Acids* 2003, *68*, 151–162.

[47] Lappas, M., Hiden, U., Desoye, G., Froehlich, J. et al., The role of oxidative stress in the pathophysiology of gestational diabetes mellitus. *Antioxid. Redox Signal* 2011, *15*, 3061–3100.

[48] Guzzardi, M. A., Iozzo, P., Fatty heart, cardiac damage, and inflammation. *Rev. Diabet. Stud.* 2011, *8*, 403–417.

