RESEARCH

Addition of olive oil to diet of rats with mild pre-gestational diabetes impacts offspring β-cell development

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Abstract

Maternal diabetes impairs fetal development and increases the risk of metabolic diseases in the offspring. Previously, we demonstrated that maternal dietary supplementation with 6% of olive oil prevents diabetes-induced embryo and fetal defects, in part, through the activation of peroxisome proliferator-activated receptors (PPARs). In this study, we examined the effects of this diet on neonatal and adult pancreatic development in male and female offspring of mothers affected with pre-gestational diabetes. A mild diabetic model was developed by injecting neonatal rats with streptozotocin (90 mg/kg). During pregnancy, these dams were fed a chow diet supplemented or not with 6% olive oil. Offspring pancreata was examined at day 2 and 5 months of age by immunohistochemistry followed by morphometric analysis to determine number of islets, α and β cell clusters and β-cell mass. At 5 months, male offspring of diabetic mothers had reduced β-cell mass that was prevented by maternal supplementation with olive oil. PPARα and PPARγ were localized mainly in α cells and PPARβ/δ in both α and β cells. Although Pparβ/δ and Pparγ RNA expression showed reduction in 5-month-old male offspring of diabetic rats, Pparβ/δ expression returned to control levels after olive-oil supplementation. Interestingly, *in vitro* exposure to oleic acid (major component of olive oil) and natural PPAR agonists such as LTB4, CPC and 15dPGJ2 also significantly increased expression of all Ppars in αTC1–6 cells. However, only oleic acid and 15dPGJ2 increased insulin and Pdx-1 expression in INS-1E cells suggesting a protective role in β-cells. Olive oil may be considered a dietary supplement to improve islet function in offspring of affected mothers with pre-gestational diabetes.

Key Words
- diabetes
- pregnancy
- olive oil
- PPARs

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Introduction

The *in utero* environment plays an important role in the development of the fetus and the neonate (Harris *et al.* 2017, Marciniak *et al.* 2017). Pre-gestational diabetes affects organogenesis and increases the risk of congenital malformations (Correa *et al.* 2008). Moreover, both pre-gestational and gestational diabetes raise the

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predisposition to insulin resistance early in life (Catalano *et al.* 2003, Lacroix *et al.* 2013) and the development of type 2 diabetes (Sobngwi *et al.* 2003, Chon *et al.* 2014). In humans, exposure to hyperglycemia *in utero* leads to differential effects on male and female offspring upon the risk of developing diabetes later in life (Sobngwi *et al.* 2003, Mauvais-Jarvis 2018).

It has been shown that not only the quantity (Ojha *et al.* 2013), but the quality and ratio of components of the maternal diet also affects both fetal and postnatal development (Herring *et al.* 2018). Importantly, lipids play a role in fetal development and variations to dietary fat composition has major implications, both short- and long-term, on offspring health (Herrera 2002, Berti *et al.* 2016). For example, oleic acid, a monounsaturated fatty acid (MUFA) given in pregnancy to diabetic rats lowers phospholipids, cholesterol and free fatty acid content in foetuses (Capobianco *et al.* 2008*a*). Furthermore, in humans, the addition of olive oil to the diet in individuals with type 2 diabetes has beneficial effects on blood glucose and reducing insulin resistance (Ryan 2000).

Previous research from our group has indicated that an enriched olive-oil diet given during gestation to mothers with mild diabetes improves fetal and placental development (Capobianco *et al.* 2008*a*; Martinez *et al.* 2012, Kurtz *et al.* 2014*a*), mainly by reducing the intrauterine inflammatory environment and regulating lipid metabolic pathways such as fatty acid uptake, lipid synthesis and catabolism (Jawerbaum & Capobianco 2011). Furthermore, these beneficial effects were identified to be mediated, in part, by the activation of the entire nuclear peroxisome proliferator-receptor (PPAR) family, namely PPARα, PPAR β/δ and/or PPARγ. The three members of this family of nuclear receptors are involved in feto-placental development, cell proliferation, differentiation, and metabolism (carbohydrate, lipid, protein) (Rees *et al.* 2008). Nevertheless, PPARs represent critical sensors of environmental dietary stimuli and are crucial in the regulation of metabolism. As primary regulators of lipid metabolism at the cellular level, they help maintain metabolic homeostasis when energy or lipid dietary composition is altered (Bordoni *et al.* 2006).

In maternal diabetes, PPARs levels and transcriptional activity are impaired in the placenta and different fetal organs, with further influence on the postnatal stage (i.e. the fetal origins of metabolic diseases) (Rees *et al.* 2008, Jawerbaum & Capobianco 2011, Jawerbaum & White 2017). PPARs function as critical transcription factors when activated by unsaturated fatty acids, which are efficiently transported through the placenta to the uterus (Herrera 2002, Bordoni *et al.* 2006). Furthermore, dietary supplements enriched in 6% olive oil or safflower oil during diabetic pregnancies have been shown to activate PPARs, leading to the prevention of the metabolic and pro-inflammatory impairments in the fetus with evident results in the offspring, including the increase in insulin secretion (Capobianco *et al.* 2008*a*, 2015, Higa *et al.* 2010).

While PPARs are ubiquitous in the pancreas during normal embryonic development (Braissant & Wahli 1998) and play an important role in pancreatic glucose and lipid metabolism, the role of *in utero* pancreatic PPAR activation remains elusive.

A previous report has indicated that oleic acid (the major component of olive oil), a natural activating ligand of PPARs, exhibits anti-diabetic and anti-inflammatory properties in the INS-1E pancreatic cell line (Ravnskjaer *et al.* 2010). A rat model of maternal mild diabetes is useful to study the programming of the pancreatic development and the putative beneficial effects of the intervention with a normolipidemic diet enriched in oleic acid. Therefore, we hypothesize that olive-oil supplementation during pregnancy to a mild pre-gestational diabetic rat will activate PPARs *in utero* and might benefit the pancreas of the offspring postnatally.

Materials and methods

Animals

The *in vivo* experiments followed the Principles of Laboratory Animals Care (NIH publication number 85-23, https://olaw.nih.gov/policies-laws/phs-policy.htm) and were approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAL, Resolution CD Nº 3170/2015; School of Medicine, UBA, Argentina). Eight male and sixteen female adult Albino Wistar rats were purchased from the certified animal facilities of the School of Exact and Natural Sciences, University of Buenos Aires (UBA, Argentina). The rats were housed in the animal facilities of the Center for Pharmacological and Botanical Studies (CEFYBO-UBA-CONICET, Argentina) on a 12 h light:12 h darkness cycle with humidity maintained at a 45–60% and temperature between 21 ± 2°C. The rats had *ad libitum* access to food and water throughout the study. For mating the female rats, two females were placed in one cage with a male and mating was confirmed by the presence of spermatozoa in the vaginal smear the following morning. Two days after birth, female neonates from each rat were randomly selected to rendered diabetic by a s.c. injection of streptozotocin (90 mg/kg,

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Sigma-Aldrich) diluted in citrate buffer (0.05 M, pH 4.5, Sigma-Aldrich), as described before (Kurtz *et al.* 2010), or received citrate buffer alone (controls). The health of the rats and the environmental parameters were checked and recorded daily. At 2 months of age, prior to mating to control males, female offspring were confirmed diabetic by a fasting glucose reading (higher than 130 mg/dL) with a hand-held glucometer (Accu-Check, Roche Diagnostics) without anesthesia from lancing the tail vein. Twenty-four female rats (8 control and 16 diabetic rats) were housed in separate cages (two female: one male rat ratio) and pregnancy was confirmed by the presence of spermatozoa in vaginal smears the next morning. No adverse effects were observed by the dietary interventions or procedures detailed subsequently. At day 1 of pregnancy, rats were allocated into three groups ($n=8$ each). The number of rats used was determined by statistical power analysis. Eight control (C) and eight diabetic (D, randomly selected) rats received a normal commercial chow diet composed of (g/100 g): carbohydrates (50); proteins (25); 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); calories: 325 kcal/100 g (Asociacion Cooperativa Argentina, Buenos Aires, Argentina). A third group of eight diabetic rats randomly selected were supplemented with 6% olive oil (a supplement diet that is 354% enriched in oleic acid, PPAR activator) (D+OO) in the pellet. The composition of the diet was described previously (Capobianco *et al.* 2015) and contains: (1) normal standard diet (composition listed previously) and (2) olive-oil-supplemented diet (g/100 g): carbohydrates (48); proteins (24); 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); calories: 340 kcal/100g. Food and water were provided *ad libitum*. Food intake was similarly increased in the diabetic group that received or not the olive-oil dietary treatment: control: 67 ± 3 g/kg/day, maternal diabetes: 75 ± 3 g/kg/day, maternal diabetes+olive oil: 73 ± 3 g/kg/day. Weight gain was similar in the evaluated groups (control: 132 ± 8 g, maternal diabetes: 135 ± 9 g, maternal diabetes+olive oil: 119 ± 10 g). Fasting glycemia values, evaluated on day 20 of pregnancy, were similar in the diabetic group that received or not the olive-oil dietary treatment (control: 101 ± 10 mg/dL, maternal diabetes: 229 ± 19 mg/dL, maternal diabetes+olive oil: 208 ± 10 mg/dL). After birth, all the rats were fed with a normal chow diet. Body weight and food intake were measured bi-weekly. Maternal body weight was similar at weaning in the evaluated groups (control: 315 ± 14 g, maternal diabetes: 318 ± 12 g, maternal diabetes+olive oil: 324 ± 15 g). Offspring weight was evaluated on a per litter basis. Each litter was weighted on day 2 of pregnancy and litter was adjusted to three males and three females. Housing conditions were maintained as described previously. At postnatal day 2 and at 5 months old, two female and two male rats per litter were kiled by decapitation at 12:00 h. Pancreata were dissected immediately and fixed in 4% formalin or immersed in RNAlater (RNA later, Invitrogen). Glycemia was also measured before killing by a hand-held glucometer (Accu-Check, Roche Diagnostics) on postnatal day 2 and after 6 h fasting at 5 months old rats from blood obtained by lancing the tail vein.

Immunofluorescence

After a 24-h fixation in 4% buffered formalin (west-Chester, PA, USA) pancreata were dehydrated and embedded in paraffin (University Hospital, Pathology Lab, London, ON, Canada) and sectioned in 5-µm sections and mounted in Superfrost-Plus slides (Fischer Scientific). In order to localize α-cells and β-cells within the islets of Langerhans, dual immunofluorescence was performed. Three 5-µm sections, separated by at least 50 µm, were deparaffinized in xylene, rehydrated in descending ethanol series (100%, 90%, 70%) and washed in tap water. Tissues were then blocked with 1–2 drops of Sniper (Biocare Medical, Concord, CA, USA) for 5 min. All antisera were diluted in antibody diluent solution (DakoCytomation). Tissues were then incubated overnight at 4°C in a humidified chamber with 1:50 guinea pig anti-insulin (Abcam) and 1:750 rabbit anti-glucagon (Novus Biologicals, Centennial, CO, USA) primary antibodies. Slides were then rinsed and incubated for 60 min in darkness in a humidified chamber with its correspondent secondary antibodies (Invitrogen) 1:500 Donkey anti-guinea pig (Alexa Flour 555), and 1:500 Donkey anti-rabbit (Alexa 488) fluorescent secondary antibodies and DAPI (Sigma-Aldrich) was used to counterstain nuclei. Coverslip was applied with the addition of an anti-fade mounting solution (Life Technologies). To establish the specificity of all antibodies, controls included substitution of the primary antibody with non-immune serum or omission of the secondary antibody.

To further identify the co-localization of β-cells with the different PPAR isotypes (α, β/δ and γ) within the islets of Langerhans, dual immunofluorescence was performed. After deparaffinization as described previously, tissues were treated with citrate buffer pH 6 in a decloaking chamber for 20 min for antigen retrieval. Slides were left to cool at room temperature, washed in PBS and blocked with 1–2 drops of Sniper (Biocare Medical) for 10 min at room temperature.

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All antisera were diluted in antibody diluent solution (DakoCytomation). Primary antibodies anti-mouse insulin (1:200) (Sigma-Aldrich) with anti-rabbit PPARα (1:100) (Abcam) or anti-rabbit PPARβ/δ (1:50) (Santa Cruz Biotechnology) and anti-rabbit PPARγ (1:50) (Santa Cruz Biotechnology) were incubated in a humidified chamber for 48 h at 4°C. Slides were then rinsed and incubated for 60 min in darkness in a humidified chamber with its correspondent secondary antibodies (Invitrogen) at a concentration of (1:500) Donkey anti-mouse (AlexaFluor 555) and (1:500) Donkey anti-rabbit (Alexafluor 488) at room temperature. This was followed by two 5-min PBS washes. Nuclear counterstain DAPI (Sigma-Aldrich, 1:500) was applied before the addition of coverslip with anti-fade mounting solution (Life Technologies). To establish the specificity of all antibodies, controls included substitution of the primary antibody with non-immune serum or omission of the secondary antibody.

Morphometric analysis

Analysis of pancreatic sections was performed using a Carl Zeiss Axioskop transmitted light and fluorescent microscope (Carl Zeiss) with QImaging Micro Publisher 3.3 Real Time viewing camera (QImaging, Burnaby, BC, Canada). Digital images were captured with 40× or 2.5× objectives lens. Image analysis of sections and quantification of areas of interest was performed using ImageJ v1.51s software (NIH) (Chamson-Reig *et al*. 2009). Data processing and statistical analysis were performed using Excel v16.12 (Microsoft) and GraphPad Prism v7.00 (GraphPad Software).

For each analysis, five male and five female animals per group (one male and one female per litter, a total of 30 rats) were randomly selected. Subsequently, three random sections (separated for at least 50 µm) from each pancreas were analyzed. Multiple fields of view were assessed upon the entire pancreas, to ensure all islets were analyzed. For each section, the following measurements were determined: total pancreatic area, islet area and total area occupied by α and β-cells and β-cell mass in mg (total β cell area × pancreatic weight/total pancreatic area). Islets were separated by size, clusters (<500 µm^2), small (500–5000 µm^2), medium (5000–10,000 µm^2) and large (>10,000 µm^2).

RNA extraction and qPCR

At 5 months of age, eight female and eight male pancreata (one male and one female per litter) per group of treatment (a total of 48 rats) were dissected and immersed immediately in RNAlater (Ambion) and stored at −80°C until RNA extraction. Total RNA was extracted using Qiagen RNeasy Plus kit (Qiagen) according to the manufacturer's specifications and stored until further analysis. Sample yield and purity was quantified by absorbance at 260 and 280 nm (value 1.7–2) using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). Transcript abundance for Pparα, β/δ, γ, insulin and Pdx-1 was analyzed using quantitative RT PCR (RT-qPCR). RNA was amplified using designed primers (Primer-BLAST A, NCBI, NIH) with a Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, Thermo Fisher Scientific) following manufacturer's protocol. Primer sequences are in Table 1. Relative quantification was performed using $2^{-\Delta\Delta\ Ct}$ method with β-actin as the housekeeping gene. Data was determined as the relative expression ratio to control samples.

Cell culture

To further understand the direct effects of oleic acid on the expression profile of Ppars in the endocrine pancreas, two different cell lines were utilized as they represent specific endocrine cells. INS-1E cells is a β-cell line (insulin) and αTC1–6 is an α-cell line (glucagon). INS-1E cells were cultured in RPMI1640 supplemented with 11 mM glucose, 1 mM pyruvate, 10 mM HEPES, 100 µM β-mercaptoethanol, 10% FBS and penicillin/streptomycin. αTC1–6 cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 5.5 mM glucose, 2.5% FBS and 15% horse serum. For each cell line, 10^5 cells per well were cultured in 6-well plates for 24 h. Media were replaced with serum free media and cells were treated in the presence or absence of 5 µM oleic acid (OA) (major component of olive oil) (Sigma-Aldrich) (Vassiliou *et al*. 2009), 1 µM 15 deoxy$\Delta^{12,14}$prostaglandin J2 (15dPGJ2)

Table 1 Primer sequences for q-PCR.

PPARα	Forward	TCCTCTGGTTGTCCCCTTGA
PPARα	Reverse	TGTCAGTTCACAGGGAAGGC
PPARβ/δ	Forward	GCTCCTGCTCACTGACAGAT
PPARβ/δ	Reverse	CGTGGCCACTTCCTCTTTCT
PPARγ	Forward	CCTGTTGACCCAGAGCATGG
PPARγ	Reverse	GGTCCACAGAGCTGATTCCG
Pdx-1	Forward	CCGCGTTCATCTCCCTTTC
Pdx-1	Reverse	TGCCCACTGGCTTTTCCA
Insulin	Forward	CCCGGCAGAAGCGTGGCATT
Insulin	Reverse	CATTGCAGAGGGGTGGGCGG
β-Actin	Forward	CGCGAGTACAACCTTCTTGC
β-Actin	Reverse	ATACCCACCATCACACCCTG

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(Cayman) (Capobianco *et al.* 2008*a*), 0.1 μM Leukotriene B4 (LTB4) (Cayman) (Martínez *et al.* 2011) and 1 μM Carbaprostacyclin (CPC) (Cayman) (Higa *et al.* 2007) for 24 h. At the end of incubation, total RNA was isolated using Qiagen total RNA isolation kit (RNeasy mini kit) according to the supplier's protocol. cDNA synthesis and real-time PCR were performed using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Thermo Fisher Scientific). Primers specific for Pparα, Pparβ/δ and Pparγ, Pdx-1 and insulin were used (Table 1). Relative expression levels were determined using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were presented as mean±S.E.M. One-way ANOVA followed by Bonferroni post-hoc test was used to compare all groups. In case of comparison of only two groups, we used *t*-test. The differences were considered statistically significant at $P<0.05$. Statistical analysis was performed using Graph Pad Prism Version 7 (GraphPad).

Results

Body weight, pancreas weight and fasting glycemia

To understand the general effects of postnatal diabetes and the dietary supplementation with 6% olive oil on maternal outcomes, we measured body weight, pancreas weight and glycemia at day 2 and 5 months in male and female offspring. At day 2, there were no statistical differences between body and pancreas weight and/or glycemia within all treatment groups in both sexes (Table 2). By 5 months of age, male and female offspring exhibited no changes in body weight and pancreas weight within all the groups. However, glycemia, was significantly increased in the male and female offspring from the diabetic dam groups, compared to the controls ($P<0.05$) (Table 3). Interestingly, the gestational dietary treatment with olive oil in diabetic mothers prevented the increased glycemia in the 5-month male offspring but not the female offspring ($P<0.05$ vs control group) (Table 3).

Pancreatic morphometry

To examine if alterations in pancreatic development underlie the changes in postnatal glycemia observed, the percentage of islet area was measured to determine the effects of the olive-oil supplementation on the postnatal endocrine pancreas in male and female offspring born to diabetic mothers.

At postnatal day 2, the percentage of islet area was statistically different in males between control and diabetic rats receiving olive oil and diabetic rats given vehicle ($P<0.05$) (Fig. 1A), This was not seen in females (Fig. 1C). However, at 5 months of age, the percentage of

Table 2 Weight and glycemia in 2-day-old female and male offspring.

	Control	Maternal diabetes	Maternal diabetes + olive oil
Females			
Body weight (g)	6.32 ± 0.14	6.32 ± 0.15	6.60 ± 0.12
Pancreas weight (g)	0.025 ± 0.003	0.027 ± 0.001	0.024 ± 0.001
Glycemia (mg/dL)	64 ± 2	69 ± 5	70 ± 3
Males			
Body weight (g)	6.42 ± 0.20	6.60 ± 0.16	6.20 ± 0.11
Pancreas weight (g)	0.022 ± 0.002	0.028 ± 0.001	0.031 ± 0.001
Glycemia (mg/dL)	61 ± 3	65 ± 4	69 ± 5

All values were expressed as mean ± S.E.M. ($n = 6$–8 per litter/group). Body weight was evaluated on a per litter basis. Pancreas weight and glycemia were evaluated on two females and two males per litter. Significant differences between treatment groups determined by one-way ANOVA ($P < 0.05$).

Table 3 Weight and glycemia in 5-month old female and male offspring.

	Control	Maternal diabetes	Maternal diabetes + olive oil
Females			
Body weight (g)	383 ± 15	421 ± 11	380 ± 17
Pancreas weight (g)	1.03 ± 0.10	0.88 ± 0.062	0.83 ± 0.07
Glycemia (mg/dL)	104 ± 6[a]	133 ± 6[b]	140 ± 9[b]
Males			
Body weight (g)	460 ± 18	499 ± 5	512 ± 17
Pancreas weight (g)	0.99 ± 0.09	0.97 ± 0.06	0.92 ± 0.02
Glycemia (mg/dL)	102 ± 12[a]	147 ± 11[b]	120 ± 12[ab]

All values were expressed as mean ± S.E.M. ($n = 6$–8 litters/group). Body weight was determined on a per adjusted-litter basis. Pancreas weight and glycemia were evaluated in two females and two males per litter. Significant differences between treatment groups determined by one-way ANOVA. Different letters represent means that are significantly different from one another according to Bonferroni post-hoc test ($P < 0.05$).

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islet area was significantly reduced ($P<0.05$) in the male offspring from diabetic rats compared to the control group and significantly increased to control values ($P<0.05$) in the male offspring of diabetic mothers that received the olive-oil-supplemented diet (Fig. 1B). No differences between groups were seen in the females at the same age (Fig. 1C and D).

In addition, there was a significant decrease of the β-cell mass in the male offspring from diabetic rats ($P<0.01$) that was prevented by the dietary maternal treatment with olive oil (Fig. 2B). Females at either age did not exhibit any differences in β-cell mass between experimental treatments (Fig. 2C and D). In view of these results and given females did not demonstrate any differences in the islet architecture in postnatal life, we decided to focus further analysis only in males.

Islet number and size distribution in male offspring at 5 months of age was examined and the number of small (5000–10,000 μm²) and large (>10,000 μm²) islets of diabetic rats supplemented with olive oil was significantly increased ($P<0.05$) compared to either control or diabetic rats fed the standard chow diet (Fig. 3). Total number of islets was also increased in the adult offspring of diabetic rats fed with the diet supplemented with olive oil compared to the diabetic group that did not receive the olive oil ($P<0.001$) (Fig. 3). At this age, there was a

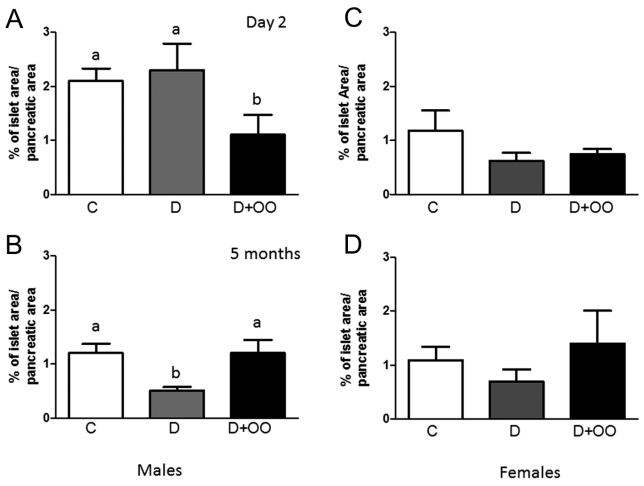

Figure 1
Representation of the percentage of islets in total pancreas area in offspring at day 2 and 5 months of age: graphs A (males) and C (females) represents day 2 and graphs B (males) and D (females) 5 months of age. Bars represent control animals (white), diabetic animals (grey) and diabetic treated with olive oil (black). All values were expressed as mean ± S.E.M. ($n = 6–8$ animals/group). Significant differences between treatment groups were determined by one-way ANOVA. Different letters represent means that are significantly different from one another according to Bonferroni post-hoc test ($P < 0.05$).

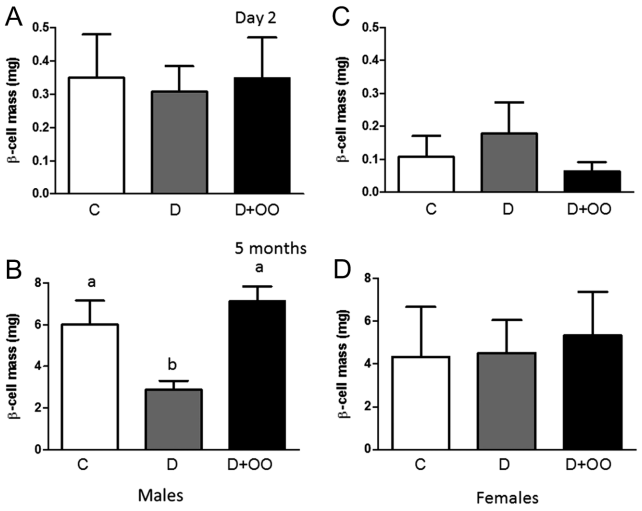

Figure 2
Representation of the total β-cell mass in offspring pancreata at day 2 and 5 months of age: graphs A (males) and C (females) day 2 and graphs B (males) and D (females) 5 months of age. Bars represent control animals (white), diabetic animals (grey) and diabetic treated with olive oil (black). All values were expressed as mean ± S.E.M. ($n = 6–8$ animals/group). Significant differences between treatment groups were determined by one-way ANOVA. Different letters represent means that are significantly different from one another according to Bonferroni post-hoc test ($P < 0.05$).

significant increase in islets clusters (2–3 cells) budding near pancreatic ducts (most of them β-cell origin) (Fig. 4A) of diabetic rats fed the olive-oil-supplemented diet compared to the diabetic group ($P<0.01$). However, no changes in α-cell clusters were observed between the groups (Fig. 4B). Collectively, this resulted in an increase in the total number of clusters in the pancreas of olive-oil-supplemented diabetic rat offspring compared to diabetic rats alone (Fig. 4C).

Effects of maternal olive-oil supplementation on pancreatic PPAR expression and their distribution within the endocrine pancreas in the offspring

Real-time quantitative PCR was employed to assess the effects of olive-oil supplementation during pregnancy on postnatal pancreatic PPAR gene expression. No differential expression on the steady-state levels of PPARα was seen at 5 months of age (Fig. 6A). By immunofluorescence, PPARα was localized to the mantle of the islet (α cell area) suggesting co-localization with glucagon secreting cells (Fig. 5A). However, pancreatic PPARβ/δ expression was significantly reduced ($P<0.01$) in the adult offspring of diabetic rats compared to controls but was restored in the offspring of diabetic rats that received olive oil as a supplement during pregnancy (Fig. 6B). By immunofluorescence,

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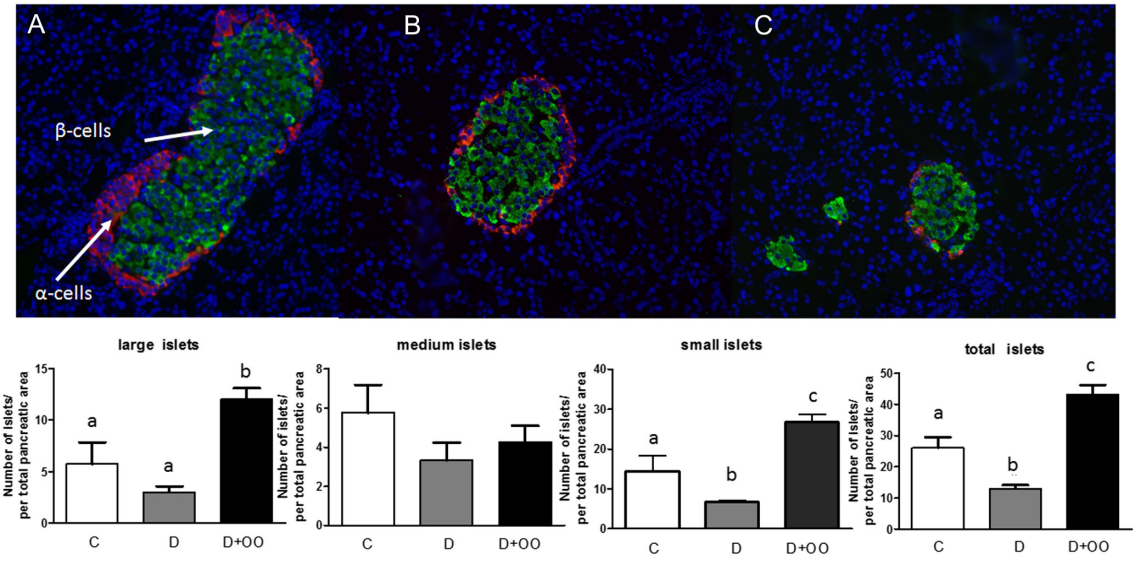

Figure 3
Representative microphotographs of large (A), medium (B) and small islets (C). Arrows indicate β cells (red) and α cells (brown). Column graphs represent the distribution of islets by size within the different treatments from male 5-month-old rats. Bars represent control animals (white), diabetic animals (grey) and diabetic treated with olive oil (black). All values were expressed as mean ± S.E.M. ($n = 6–8$ animals/group). Significant differences between treatment groups were determined by one-way ANOVA. Different letters represent means that are significantly different from one another according to Bonferroni post-hoc test ($P < 0.05$).

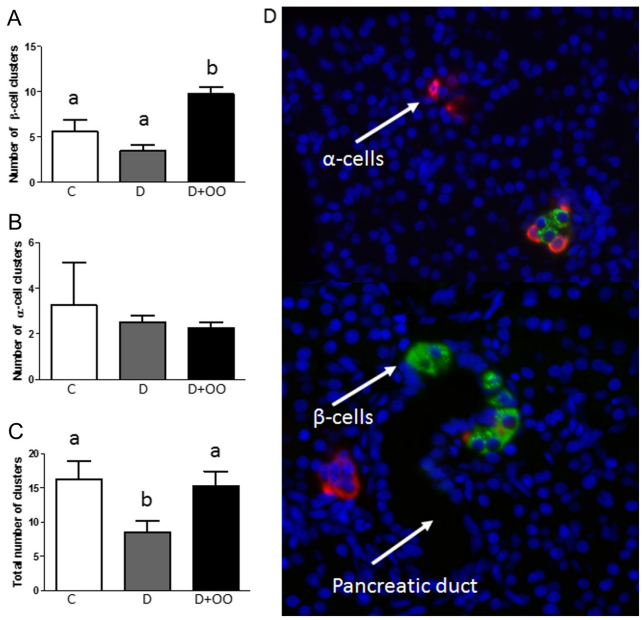

Figure 4
Representative microphotograph of beta cell clusters near ducts. The arrows identify insulin secreting β cells. On the left-hand side, graphs represent the number of (A) β, (B) α and (C) total clusters per total pancreas area in male offspring at 5 months of age. Bars represent control animals (white), diabetic animals (grey) and diabetic treated with olive oil (black). All values were expressed as mean ± S.E.M. ($n = 6–8$ animals/group). Significant differences between treatment groups were determined by one-way ANOVA. Different letters represent means that are significantly different from one another according to Bonferroni post-hoc test ($P < 0.05$).

PPARβ/δ was also localized at the mantle of the islet (to the cytoplasm of α cells) and the nuclei of β-cells (Fig. 5B) suggesting some role in β-cell differentiation or function. Finally, PPARγ was significantly reduced ($P<0.05$) in the pancreas of the offspring of diabetic rats regardless of olive-oil supplementation (Fig. 6C). Moreover, PPARγ was also localized in the area where α cells are present in the rat islets by dual immunofluorescence (Fig. 5C).

Examining the direct effects of oleic acid on pancreatic PPAR expression

When both cell lines were treated with oleic acid for 24 h, INS-1E cells showed a differential profile of PPAR gene expression compared to αTC1–6 cells. Treatment with OA reduced gene expression of PPARα (~20%) and PPARγ (~96%) and increased gene expression of PPARβ/δ (~20%) compared to corresponding controls in INS-1E cells. Meanwhile in αTC1–6, OA increased the gene expression of PPARα, PPARβ/δ and PPARγ mRNA around ~310%, ~260%, and ~410 %, respectively, compared to controls. These results show for the first time that PPAR expression can be induced also in α-cells. Mainly, in this case, oleic acid significantly increased the gene expression of PPARα ($P<0.01$), PPARβ/δ ($P<0.05$) and PPARγ ($P<0.01$) in αTC1–6 cells compared to INS-1E cells (Fig. 7A).

In order to confirm if these differences seen in INS-1E cells and αTC1–6 cells occur in the presence of other PPAR

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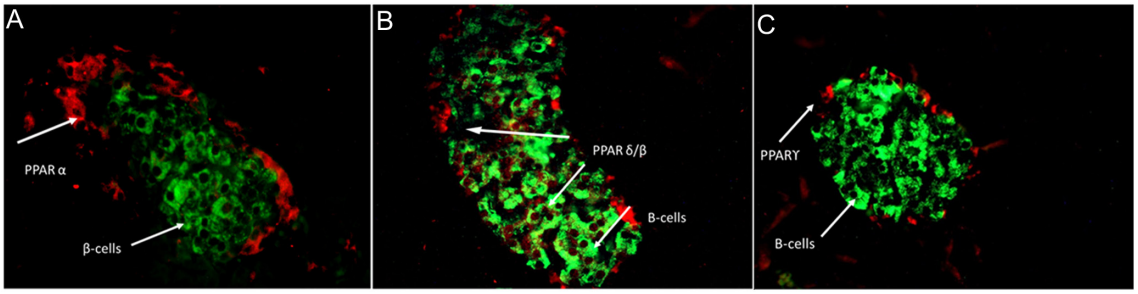

Figure 5
Representative microphotographs of PPARα, PPARβ/δ and γ. (A) Arrows identify PPARα (red) and insulin (green). (B) Arrows identify PPARβ/δ (red) in the nucleus of β-cells and insulin (green). (C) Arrows identify PPARγ (red) and insulin (green).

natural agonists, we tested and compared LTB4, CPC and 15dPGJ2. LTB4, a PPARα agonist, significantly increased mRNA expression of all PPARs in α cells compared to β cells with different levels of significance (Fig. 7B) ($P<0.01$), ($P<0.05$), ($P<0.05$), respectively. Also, CPC, a PPARβ/δ agonist, did not have any effect on any of the PPARs in INS-1E cells. Although, in αTC1–6 cells, CPC significantly increased the expression of PPARα (~297%) and PPARγ (279%) (Fig. 7C). Moreover, 15dPGJ2 (PPARγ agonist) increased the expression of PPARγ mRNA in INS-1E cells (~191%) with no effect on αTC1–6. However, 15dPGJ2 significantly increased PPARβ/δ gene expression (~127%) in αTC1–6 cells (Fig. 7D). These results imply that PPARs can be induced in either α- or β-cells and that their expression depends on the stimulus.

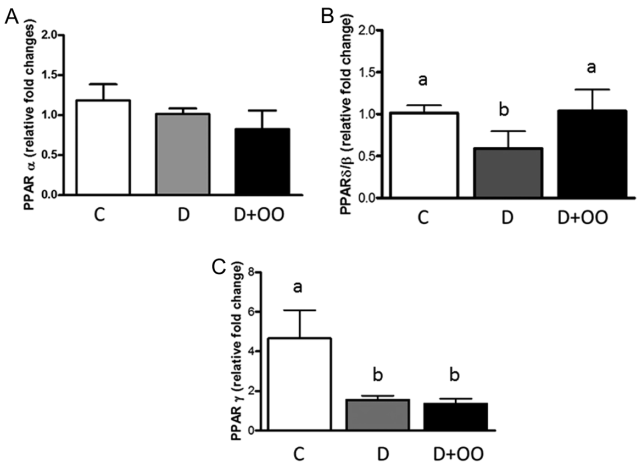

Figure 6
Expression of PPARα (A), PPARβ/δ (B) and PPARγ (C) by q-PCR. Bars represent control animals (white), diabetic animals (grey) and diabetic treated with olive oil (black). *P < (0.05) and ***P < 0.001. All values were expressed as mean ± S.E.M. (n = 6–8 animals/group). Significant differences between treatment groups were determined by one-way ANOVA. Different letters represent means that are significantly different from one another according to Bonferroni post-hoc test (P < 0.05).

Interestingly, when the downstream PPAR target genes (e.g. Pdx-1 and insulin) were measured after OA, 15dPGJ2 and LTB4 treatments in INS-1E cells, both target genes were significantly increased, while treatment of the cells with CPC had no effect (Fig. 8).

Discussion

Population studies had shown that dietary habits resulting high in monounsaturated fatty acids (MUFAs) attributed

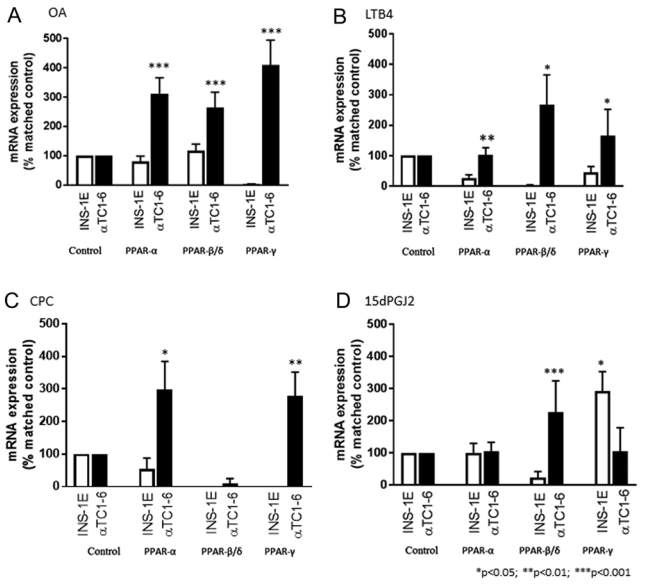

Figure 7
INS-1E cells and αTC1-6 cells treated with oleic acid (OA; 5 μM), leukotriene B4 (LTB4; 0.1 μM), 15 deoxy prostaglandin J2 (15dPGJ2; 1 μM), and carbaprostacyclin (CPC; 1 μM) for 24 h. Gene expression levels of PPARα, PPARβ/δ and PPARγ were determined following treatment with (A) OA, (B) LTB4 (C) 15dPGJ2, and (D) CPC using qRT-PCR. Levels of PPARα, PPARβ/δ and PPARγ were normalized to an internal control, β-actin. The normalized levels of transcripts were shown as relative percent to that of non-treated control. *t*-test analysis (α = 0.05) was performed to compare the levels of difference between αTC1-6 cells and INS-1E cells for each gene. *P < (0.05); **P < 0.01; ***P < 0.001.

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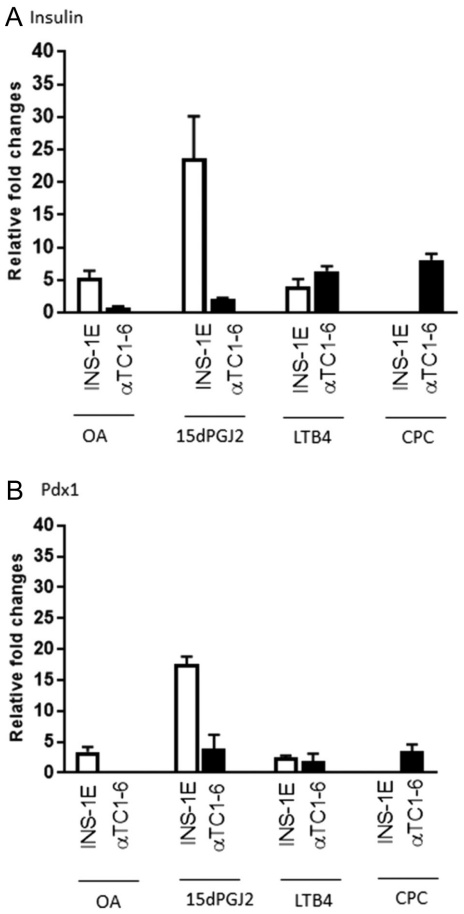

Figure 8
mRNA expression levels of (A) insulin and (B) Pdx-1 after treatment of INS-1E cells and αTC1-6 cells with OA, PGJ2, LTB4 and CPC determined by qRT-PCR. Log2 fold changes were calculated compared to the respective controls.

to olive-oil consumption leads to overall reduced inflammatory markers and better health outcomes (Jiménez-Gómez *et al.* 2009, Schwingshackl & Hoffmann 2014). Moreover, oleic acid, the major component of olive oil, reduces LDL and total cholesterol levels with beneficial consequences on blood sugar control and reducing insulin resistance, culminating in a better management of type 2 diabetes (T2D). Oleic acid also reverses inflammation in obesity (Ryan 2000). Aside from regulating glycemia in adulthood, olive oil is promising during development as well (Jawerbaum & Capobianco 2011).

Previously, we have shown that daily dietary olive-oil supplementation administered to mildly diabetic rats during pregnancy improved the development of the placenta and fetus with beneficial effects in different organs, including the heart and the lung (Kurtz *et al.* 2014*a*,*b*, Capobianco *et al.* 2008*a*). The benefit to these organs has been attributed to PPARs activation and restoration of PPAR levels (Capobianco *et al.* 2008*b*, 2015, Kurtz *et al.* 2014*a*). Specifically, these PPARs have anti-inflammatory functions and their expression is reduced in the placenta from diabetic rats and from pre-gestational and gestational diabetic patients (Capobianco *et al.* 2005, 2013, Martínez *et al.* 2008, 2011, Wieser *et al.* 2008, Arck *et al.* 2010, Holdsworth-Carson *et al.* 2010). Moreover, impaired PPAR pathways and levels of PPARs endogenous ligands in the placenta are rescued with olive-oil supplementation (Capobianco *et al.* 2008*a*). Besides, we previously found that maternal olive-oil supplementation increases insulin levels and decreases triglycerides in the 5-month-old offspring from diabetic rats (Capobianco *et al.* 2015). The proposed dietary supplementation provides half of the calories from lipids derived from olive oil, and thus, it would be feasible to be recommended in humans, as it corresponds to three daily spoons of olive oil. Therefore, we proposed that maternal dietary manipulations (such as the addition of olive oil) may add to the benefits of the tight insulin monitoring to mothers with pre-gestational or gestational diabetes on the development of the pancreata in postnatal life.

In rodents, the pancreas and other metabolic organs are not fully developed at birth. During e-18.5 to e-20.5, β-cells duplicate in number (Kaung 1994). Any alterations in this period are of importance as it determines the health or predisposition to develop disease of the individual long-term. By weaning, the quantity and quality of β cells are finally defined (Kaung 1994, Petrik *et al.* 1999, Zhang *et al.* 2005). Tight regulation of β-cell mass is required for preserving insulin secretion capacity over a life time.

In this study, by postnatal day 2, no significant differences were observed between treatments and sexes with respect to β-cell mass (mg), suggesting that at this time point the effects of maternal status of disease or diet had not manifested. However, by 5 months of age, males of diabetic mothers were overtly glucose intolerant (Capobianco *et al.* 2015) and exhibited reduced β-cell mass. Interestingly, maternal olive-oil supplementation prevented fasting hyperglycemia in the male offspring due, in part, to prevention of β-cell mass and total pancreatic islet loss. The benefits of olive-oil supplementation in these offspring could also be attributed to higher number of larger islets and clusters near the ducts (mainly β-cells). Nevertheless, the increased β-cell number observed does not always account for hyperinsulinemia and hypoglycaemia (Zhang *et al.* 2005) or changes in plasma insulin levels (Nguyen *et al.* 2006). Given females did not show any glycemic differences within treatments at either

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developmental age, further studies were only conducted in males.

Previous studies have demonstrated that, during development, PPARα, β/δ and γ are ubiquitously distributed in the pancreas and play essential roles in the regulation of its cellular differentiation, proliferation and metabolism (Braissant *et al.* 1996). During late gestation (i.e. gestational day 18.5), PPARα is expressed in the pancreas during a period when cells adapt from high-fat oxidation to high-glucose oxidation (Gremlich *et al.* 2005). Deletion of PPARα in *ob/ob* mice developed pancreatic β-cell dysfunction characterized by reduced mean islet area and decreased insulin secretion in response to glucose *in vitro* and *in vivo* (Lalloyer *et al.* 2006). Although PPAR isoforms are expressed in islets (Braissant *et al.* 1996), their function is still unclear.

In the adult rat pancreas, they are expressed in both the exocrine and the endocrine pancreas and by *in situ* hybridization and gene expression analysis PPARα and have lower expression when compared with PPARβ/δ (Braissant *et al.* 1996). PPARβ/δ is highly expressed in β-cells (Iglesias *et al.* 2012). Furthermore, in the diabetic male offspring at 5 months of age, the expression of PPARβ/δ was reduced significantly but restored to control levels in the offspring of diabetic mothers treated with olive oil. In this group, PPARβ/δ was found in the nucleus of β cells suggesting that may be involved in β-cell differentiation and proliferation as observed histologically. Under normal cell culture conditions, we further determined that the steady-state levels of PPARβ/δ mRNA were increased in both INS1-E and αTC1–6 cells. Activation of PPARβ/δ by its ligands increases fatty acid oxidation capacity in INS-1E cells, enhances glucose stimulated insulin secretion (GSIS) in islets and protects GSIS against the effects of prolonged fatty acid exposure (Cohen *et al.* 2011). In db/db mice, prolonged treatment with PPARβ/δ agonists (GW501516) reduced blood glucose by improving insulin sensitivity and islet function (Yang *et al.* 2016). A recent report indicates that a pharmacological ligand of PPARβ/δ amplifies the adaptive insulin secretory response of β-cells upon exposure to increasing concentrations of glucose in both INS-1E and rat isolated islets (Winzell *et al.* 2010). Others suggested that PPARβ/δ is a master regulator of functions associated with each step of insulin secretion (granule biosynthesis, vesicle trafficking and exocytosis) and may have a repressive role controlling β-cell mass and insulin exocytosis (Hellemans *et al.* 2007) with a protective effect against metabolic stress in β-cells (Ravnskjaer *et al.* 2010).

Taking into consideration all the observations listed previously, we suggest that PPARβ/δ may have had a role in restoring the β-cell mass in the male offspring of diabetic rats that received the olive-oil supplementation during pregnancy. In contrast, PPARα gene expression did not differ between treatment groups at 5 months of age, and it was only localized in the cytoplasm of α cells, suggesting that it has minimal effects on β-cell gene expression in adulthood. Furthermore, PPARα mRNA was also increased in αTC1–6 cells after OA exposure. Finally, we examined the expression of PPARγ and our studies showed a reduced expression of PPARγ in the 5-month pancreas of the offspring of diabetic rats, which was not prevented by the maternal treatment with olive oil, and that this PPAR isotype was only localized in α cells. While it has been shown that PPARγ is localized in both β cells (Braissant *et al.* 1996) and in α cells (Laybutt *et al.* 2002, Gupta *et al.* 2008), we were only able to detect its localization in α cells. This PPAR isotype has been previously demonstrated to represses glucagon transcription in the islets (Rosen *et al.* 2003) and its signaling is implicated in the regulation of β-cell proliferation in adults. Mice with deleted expression of PPARγ in β-cell had significant islet hyperplasia but, despite this alteration in β-cell mass, no effect on glucose homeostasis was noted (Rosen *et al.* 2003, Moibi *et al.* 2007). Another study showed that PPARγ may regulate Pdx-1 transcription (Kim *et al.* 2002, Gupta *et al.* 2008), glucokinase (Kim *et al.* 2000), glucose transporter (Glut2) (Gupta *et al.* 2008) and indirectly β-cell function and mass (Laybutt *et al.* 2002). In our hands, the *in vitro* experiments showed that exposure with OA increased PPARγ mRNA only in αTC1–6 cells where it could be acting as a repressor.

In order to elucidate if the *in vitro* effects of OA was specific, we tested both cell lines with three different natural PPAR agonists and found that they were differentially expressed in both cell lines. To this end, the expression of PPARs did not show a common characteristic among all three isotypes of PPARs in β cells. However, aside of OA, all administered agonists dramatically reduced PPARβ/δ expression in β-cells. Although, PPARs downstream target genes such as PDX-1 and insulin were significantly increased in (INS-1E cells) after OA, 15dPGJ2 and LTB4 treatments.

Another important point to address from these results is the novelty that both OA and LTB4 increased the expression levels for all three isotypes of PPARs in αTC1–6 cells. However, such pattern was not shown in INS-1E cells, suggesting that OA has a differential effect in α cell compared to β cell *in vitro*.

In summary, this study has identified the distribution and the expression of PPARs in different cell populations

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within the endocrine pancreas when maternally exposed to a diet enriched in oleic acid. We also demonstrated histologically that PPARβ/δ is present in the β cells at 5 months postnatal, while PPARα and γ are only in α cells suggesting a different role of these PPARs in the adult endocrine pancreas. To further elucidate the underlying molecular mechanisms involved, we also examined the direct effects of oleic acid in α- and β-cell lines. We first found that all PPARs were differentially expressed in both cell lines. Interestingly, all PPAR isoforms were present in αTC1–6 cells when stimulated by oleic acid. The up-regulated gene expression of all three PPARs in αTC1–6 cells suggests a role for these nuclear receptors in a counter regulatory mechanism between α and β cells which are likely important for the maintenance of β-cell survival and function. Furthermore, we showed for the first time to our knowledge *in vivo* and *in vitro* the presence of PPARs in α cells. Collectively, based on these observations, we suggest that, in male offspring of mildly diabetic mothers, the early exposure of 6% olive oil may have permitted a normal pancreas development, likely due to the indirect effect of the maternal diet (e.g. oleic acid) on the intrauterine micro-environment (Capobianco *et al.* 2008*a*) or by a direct effect by the activation of PPARs during fetal pancreatic development. Both interactions may have programmed β cells *in utero* and rescued male offspring from an early onset of T2D in adulthood.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

AJ and EA designed the study, while BT, EV and FA performed all experiments and data analysis. DBH assisted with real-time PCR experiments and data analysis. EA, AJ, and DBH assisted in preparation and revisions of the manuscript.

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