An extra virgin olive oil-enriched diet improves maternal, placental and cord blood parameters in GDM pregnancies.

Dalmiro Gomez Ribot[1,2,3], Esteban Diaz[3], María Victoria Fazio[3], Hebe Lorena Gómez[3], Daiana Fornes[1,2], Silvia Beatriz Macchi[3], Carlos Alberto Gresta[3], Evangelina Capobianco[1,2], Alicia Jawerbaum[1,2].

[1]Universidad de Buenos Aires (UBA). Facultad de Medicina, Argentina

[2]CONICET – UBA. Laboratory of Reproduction and Metabolism, Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Buenos Aires, Argentina.

[3]Hospital General de Agudos Dr. Ignacio Pirovano, Buenos Aires, Argentina.

Correspondence author: Alicia Jawerbaum. Laboratory of Reproduction and Metabolism, CEFYBO. CONICET. School of Medicine, University of Buenos Aires, Paraguay 2155 (1121), Phone: (+5411) 52853605. Argentina. Email: a.jawerbaum@gmail.com.

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Abstract

Aims: To address the effect of a diet enriched in extra virgin olive oil (EVOO) on maternal metabolic parameters and placental proinflammatory markers in Gestational diabetes mellitus (GDM) patients. **Methods:** Pregnant women at 24-28 weeks of gestation were enrolled: 33 GDM patients which were randomly assigned or not to the EVOO-enriched group and 17 healthy controls. Metabolic parameters were determined. Peroxisome proliferator activated receptor (PPAR) γ and PPARα protein expression, expression of microRNA (miR)-130a and miR-518d (which respectively target these PPAR isoforms) and levels of proinflammatory markers were evaluated in term placentas. Matrix metalloproteinases (MMPs) activity was evaluated in term placentas and umbilical cord blood. **Results:** GDM patients that received the EVOO-enriched diet showed reduced pregnancy weight gain (GDM-EVOO:10.3±0.9, GDM:14.2±1.4, $P=0.03$) and reduced triglyceridemia (GDM-EVOO:231±14, GDM:292±21, $P=0.02$) compared to the non-EVOO-enriched GDM group. In GDM placentas, the EVOO-enriched diet did not regulate PPARγ protein expression or miR-130a expression, but prevented the reduced

PPARα protein expression (*P*=0.02 vs GDM) and the increased miR-518d expression (*P*=0.009 vs GDM). Increased proinflammatory markers (interleukin-1β, tumor necrosis factor-α and nitric oxide overproduction) in GDM placentas were prevented by the EVOO-enriched diet (respectively *P*=0.001, *P*=0.001 and *P*=0.01 vs GDM). MMPs overactivity was prevented in placenta and umbilical cord blood in the EVOO-enriched GDM group (MMP-9: respectively *P*=0.01 and *P*=0.001 vs GDM). **Conclusions:** A diet enriched in EVOO in GDM patients reduced maternal triglyceridemia and weight gain and has anti-inflammatory properties in placenta and umbilical cord blood, possibly mediated by the regulation of PPAR pathways.

1. INTRODUCTION

Gestational diabetes mellitus (GDM) is a prevalent disease that increases the risks of maternal, placental, and perinatal adverse outcomes and induces long-term adverse effects on the offspring´s later life[1,2]. Adverse outcomes in GDM have been related to an intrauterine proinflammatory environment[3]. Indeed, proinflammation is a common alteration in gestational diseases and can influence the placental development and function, the fetal development and the offspring´s later life[4,5].

Our previous studies performed in experimental models of diabetes and pregnancy allowed us to identify changes in pathways regulated by peroxisome proliferator activated receptors (PPARs) in embryos, fetuses and placentas, related to a proinflammatory environment[6]. PPARs are ligand activated transcription factors capable of regulating

metabolic and anti-inflammatory pathways, as well as intrauterine development[7,8]. PPARs are nutrigenomic agents, being monounsaturated fatty acids (MUFAs, the main components of olive oil), PPARs endogenous ligands[9,10]. In experimental models of diabetes and pregnancy, our previous studies have shown that diets enriched in extra virgin olive oil (EVOO) lead to anti-inflammatory effects, as shown by the ability of these diets to prevent increased levels of proinflammatory cytokines, nitric oxide overproduction, and matrix metalloproteinases (MMPs) overexpression in placentas, embryos and different fetal organs[11-16]. Studies have also shown that although maternal EVOO-dietary treatments do not prevent programming of metabolic diseases in the offspring of diabetic animals, this treatment reduces the levels of proinflammatory markers in the offspring´s heart and prevents hypertriglyceridemia in the adult offspring of diabetic rats[11,16].

In women with pregestational and gestational diabetes, placental levels and expression of PPARγ and PPARα are reduced[17,18]. Besides, expression of microRNA-518d, a microRNA that targets PPARα, is increased in the placentas of GDM patients[19]. Whether microRNAs that regulate PPARs are related to PPARγ changes and whether alterations in placental microRNAs that target PPARs can be prevented by diets enriched in PPAR ligands are unknown.

EVOO is the main vegetable oil that composes the Mediterranean diet, which is increasingly being considered as a medical treatment[20,21]. The Mediterranean diet has been found associated with a lower incidence of GDM[22]. Out of pregnancy, an EVOO-enriched diet has beneficial effects on metabolic and cardiovascular diseases, as

demonstrated in the PREDIMED study and other clinical studies[23-25]. In pregnant women, studies addressing a diet enriched in EVOO and pistachios reduced the incidence of GDM[26]. No previous studies addressing a putative beneficial effect of an EVOO-enriched diet in GDM pregnancies have been conducted so far. In this work, we conducted a randomized clinical trial and tested the hypothesis that a maternal diet enriched in EVOO ameliorates triglyceridemia in the mothers, regulates placental PPAR protein levels, modulates the expression of microRNAs that regulate PPARs expression, and reduces pro-inflammatory markers in the placenta and the umbilical cord blood from GDM patients.

2. METHODS

2.1 Study design

Pregnant women with a singleton fetus at the time of GDM diagnosis (between 24 to 28 weeks) were randomly assigned in a 1:1 manner to receive a diet with additional three tablespoons of crude EVOO daily (36 g/day). GDM was diagnosed according to Latin American Diabetes Association (ALAD)/ Argentine Society of Diabetes (SAD) diagnostic criteria, based on glycemia values either at fasting (>99 mg/dl in two measurements) or after the universal p75 g oral glucose tolerance test (>140 mg/dl at 2 h)[27]. Control women were recruited at the same gestational age. Exclusion criteria included BMI over 30 kg/m^2 before pregnancy, multiple pregnancies and concurrent pathologies including: thrombophilia, preeclampsia, pregestational diabetes, vascular or

renal complications associated with chronic hypertension, anemia with total hemoglobin below 8 g/dL and positive serology for HIV, VDRL, Hepatitis B or Chagas disease. Potential participants received written and oral information about the trial and had at least 24 h to decide their participation. This clinical trial was approved on 14th January 2016 by the Ethics and Research Committee of the Pirovano Hospital (Review Board Project: DI-2016-29-HGAIP) and registered at the Ministry of Health of the City of Buenos Aires on October 2016, IF-2016-22533767. Participants were recruited at Hospital de Agudos Dr. Ignacio Pirovano, Buenos Aires, Argentina, from December 2016 to December 2018. The study was undertaken in accordance with the Declaration of Helsinki and followed the 2010 Consort guidelines. All women that agreed to participate provided their written informed consent.

All eligible women received standardized personal advice on healthy eating and appropriate medical care. The study was not blinded, as those women in the EVOO-enriched group received commercial bottles of EVOO at the nutrition visits to ensure adherence. In the three experimental groups (Control, GDM, GDM-EVOO), the women received dietary indications to follow a nutritional plan with the following composition: 2100-2400 Kcal/day; carbohydrates 48-50%, proteins 18-20% and lipids 30-32%. In the intervention group, women were indicated to include three tablespoons of EVOO daily (36 g/day). The EVOO was indicated to be consumed uncooked and within the main meals. The group that did not receive the EVOO-enriched diet was indicated to include none to one tablespoon of EVOO daily (0 to 12 g per day). Follow-up appointments to the obstetrics and nutrition professionals in charge of the study were frequent (every 1 to

4 weeks according to the gestational age and requirements). To assess adherence to the EVOO dietary intervention, questionnaires were performed and diet composition was evaluated at each nutritional visit. Adherence was considered good if reports indicated a daily EVOO consumption over 26 g/day 5 to 7 times a week, regular if daily EVOO consumption was over 26 g/day 3 or 4 times a week and bad if daily EVOO consumption was lower than 26 g/day or lower than 3 times a week. Clinical data of the participants were recorded at each visit. At GDM diagnosis, women received the dietary treatment and the indications for glucose monitoring. In subsequent visits, insulin was added if target blood-glucose values were not achieved[27].

2.2 Metabolic parameters and pregnancy outcomes

Eight-hour fasting blood was collected at enrolment (gestational weeks 24 to 28) and at gestational week 37. Plasma glucose, fructosamine and glycated hemoglobin were evaluated using an Abbott autoanalyzer (Architect C8000). Triglycerides were evaluated by colorimetric methods (Wiener lab, Rosario, Argentina). Gestational weight gain (defined as the difference in maternal weight between the reported weight at term and prior to pregnancy) was determined. Maternal and fetal complications were reported. Neonatal and placental weight were obtained at birth.

2.3 Placenta and umbilical cord blood sampling

Umbilical cord blood was collected before placental delivery and citrate plasma immediately obtained and conserved at -80°C. Placental tissues were collected from

centrally located cotyledons, avoiding decidua and membrane layers. Placental tissues were stored in 10% formalin for immunohistochemical studies, immersed in RNA-later® solution and stored at -80°C for PCR studies, or immediately stored at -80°C for Western Blot, zymography and nitric oxide production evaluation.

2.4 Western blot analysis

Placental explants were homogenized, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (35V constant, overnight at 4°C), as previously described[28]. The membranes were stained with Ponceau Red staining solution for total proteins (Sigma-Aldrich, St Louis, MO, USA) to confirm proper transfer. After blocking for 1 h the membranes were incubated overnight at 4°C with PPARγ antibody (1:100, Cayman Chemical Co., MI, USA), PPARα antibody (1:100, Cayman Chemical Co.) or actin antibody (1:500, Sigma-Aldrich), which was used as a loading control. After washing, the membranes were incubated with the appropriate peroxidase conjugated secondary antibody, visualized using ECL detection solution (Thermo Scientific) and captured in an ImageQuant LAS 4000 (GE Healthcare Life Sciences, NJ, USA). The relative intensity of protein signals was quantified by densitometric analysis using the ImageJ Software (NIH, MD, USA). Results are expressed as protein of interest /actin protein ratio.

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

Total RNA and microRNA were isolated from placental explants (100 mg) using RNAzol® (MCR Inc., OH, USA) and their concentrations determined using the NanoDrop spectrophotometer.

For microRNA evaluation, cDNA was obtained using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, CA, USA). The relative expression of miR-130a and of miR-518d was determined using the TaqMan detection system (Applied Biosystems), the appropriate primers (assay ID 000454 and 002389 respectively), and the U6 spliceosomal RNA (assay ID 001973) as endogenous control (Applied Biosystems).

From total RNA, cDNA was synthesized incubating 1 µg of extracted RNA in a buffer containing 200 U MML-V enzyme (Promega, WI), 7.5 mM random primer hexamers and 0.5 mM of each dNTPs, as previously[28]. The reaction mixture was incubated for 60 min at 37°C followed by 15 min at 70°C. Then, 2.5 µl of cDNA 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 primer (Cu/Zn SOD, forward: 5′-ACAAAGATGGTGTGGCCGAT-3′, reverse: 5′-AACGACTTCCAGCGTTTCCT-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 (62°C) and primer extension (72°C). mRNA levels were normalized to the 60s ribosomal protein L30 levels (L30 primer: forward: 5′- TGATCAGACAAGGCAAAGCG-3′, reverse: 5′-GCCACTGTAGTGATGGACACC-3′).

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, MD).

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 Immunohistochemistry

Placentas were paraffinized and serially cut in 5-µm-thick sections for further evaluation of interleukin 1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) immunostaining. Sections were deparaffinized, rehydrated through a graded series of ethanol and the endogenous peroxidase activity was blocked. The sections were processed overnight using anti-TNF-α primary antibody (mouse monoclonal antibody, 1:100 dilution, Santa Cruz Biotechnology, CA, USA) and anti-IL-1β primary antibody (mouse monoclonal antibody, 1:100 dilution, Santa Cruz Biotechnology) in a humidified chamber at room temperature and then incubated with the biotinylated anti-mouse secondary antibody (anti-mouse IgG, dilution 1:200, Vector Laboratories, CA) for 1 h. Sections were incubated with the Avidin-Biotin-Complex (Vectastain, Vector Laboratories, USA) for 1 h and then the stain was developed with 3,3'-diaminobenzidine, as previously described[15]. Control sections were generated by omitting the primary antibody. Two skilled blinded observers evaluated three sections per placenta. Immunoreactivity intensity was quantified with the ImageProPlus software. Data are shown as relative to a value of 1 assigned to the mean values in the control group.

2.7 Nitric oxide production

Nitric oxide production was determined by measuring the concentration of its stable metabolites nitrates/nitrites, as reported previously[28]. Briefly, placental explants were homogenized in 1 ml Tris-HCl buffer pH 7.6, and an aliquot was separated for protein analysis. After reducing nitrates to nitrites by using nitrate reductase enzyme, nitrites were measured by the Griess reaction using a commercial assay kit (Cayman Chemical Co.).

2.8 Gelatinase activity of matrix metalloproteinases

Zymography was performed to evaluate the gelatinase activity of matrix metalloproteinases (MMP) 2 and 9, as previously described[15]. Briefly, placental explants were homogenized in 50 mM Tris, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 1% Triton X-100. Then, 30 µg of protein of the homogenates was mixed with loading buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 50 mM Tris-HCl, pH 6.8) and subjected to a 7.5% SDS-PAGE containing 1 mg/ml gelatin (type A from porcine skin). Gels were rinsed, stained with Coomassie blue and destained with 10% acetic acid and 30% methanol in water. The areas of proteolytic activity appeared as negatively stained bands in a dark background. MMPs were identified by their molecular weights and a positive internal control (conditioned medium of human fibrosarcoma HT-1080 cells). The enzymatic activity was quantified using ImageJ software and expressed as arbitrary densitometric units. Data are shown as relative to a value of 1 assigned to the mean value for MMP activity in the control group.

2.9 Study endpoints

The primary endpoint was a change in PPARγ and PPARα levels and placental markers of a prooxidant/proinflammatory state (TNFα and IL-1β levels, nitric oxide production, SOD expression, and MMPs gelatinase activity). Second endpoints were changes in maternal metabolic control, triglyceridemia, maternal weight gain, neonatal and placental weight as well as microRNAs that target PPARγ and PPARα (miR-130a and miR-518d respectively).

2.10 Statistical analysis

Data are presented as the mean ± SEM. Groups were compared by one-way ANOVA followed by Bonferroni´s post-hoc test to compare all groups to each other, Student´s *t*-test or chi-square test as appropriate (Graphpad Prism 8 software). Normality of the variable distribution was corroborated with the Shapiro-Wilk test. Homogeneity of variance was evaluated with the Levene´s test, and in the cases where homogeneity of variance was not verified, the variance function Varldent was applied to the model (Infostat 2017 software). A *P* value lower than 0.05 was considered statistically significant.

3 RESULTS

3.1 Metabolic parameters and pregnancy outcomes

As shown in the study Flow chart (Supplementary Figure 1), we were able to obtain the complete samples at term of 45 women (15 of the Control group, 15 of the GDM group

and 15 of the GDM-EVOO-enriched group (GDM-EVOO). Adherence to the EVOO dietary intervention was good in 80% of the GDM patients (Table 1). Age and pre-pregnancy weight were similar between the three groups (Table 1). Total weight gain was increased in the GDM group (P=0.02 GDM vs. Control), an alteration prevented by the maternal diet enriched in EVOO (P=0.03 GDM-EVOO vs. GDM) (Table 1). A very good metabolic control was achieved in both the GDM and the GDM-EVOO group, as shown by the fasting blood glucose, fructosamine and HbA1c levels at term (Table 1). GDM patients were treated with diet or diet and insulin, with no significant changes between the two groups (P=0.14, Table 1). At enrolment, triglyceridemia values in the groups evaluated showed no changes (Table 1). Differently, at term, triglyceridemia was increased in the GDM group compared to Controls (P=0.04), an alteration prevented in the GDM group that received the EVOO-enriched diet (P=0.02 GDM-EVOO vs. GDM) (Table 1). Regarding the pregnancy outcomes, gestational age at delivery, Cesarean delivery rate, maternal complications, neonatal complications, neonatal weight and placental weight were similar in the three groups evaluated (Table 2).

3.2 PPARs and microRNAs that regulate PPARs expression

We next addressed PPAR pathways, and found reduced protein expression of PPARγ in the placentas from GDM patients that received or not the diet enriched in EVOO compared to controls (P=0.007 GDM vs. Control, P=0.03 GDM-EVOO vs. Control, Figure 1). Differently, the reduced protein expression of PPARα in the placentas from GDM patients was prevented by the diet enriched in EVOO (P=0.02 GDM vs. Control,

P=0.02 GDM- EVOO vs. GDM, Figure 1). To determine whether microRNAs that target PPARs are involved in the changes observed, we evaluated the expression of miR-130a, which targets PPARγ in different tissues[29], and of miR-518d, which targets PPARα in the placenta[19]. The expression of miR-130a showed no changes in the groups evaluated (Figure 1), but miR-518d expression, which was increased in the placenta of GDM patients (*P*=0.003 vs. Control), was reduced in the placentas of the GDM patients that received the EVOO-enriched diet (*P*=0.009 vs. GDM, Figure 1).

3.3 Regulation of the proinflammatory environment

As PPARs are largely involved in the regulation of the proinflammatory environment and the EVOO diet is enriched in MUFAs, which are PPAR ligands, we next evaluated proinflammatory markers in term placentas from the GDM patients that received or not the EVOO-enriched diet. We found that TNF-α levels were increased in the placentas from GDM patients compared to controls (*P*=0.0002), an alteration prevented by the maternal diet enriched in EVOO (*P*=0.0002 vs. GDM) (Figure 2). Similarly, IL-1β levels were increased in the GDM group compared to controls (*P*=0.0001), an alteration prevented by the maternal diet enriched in EVOO (*P*=0.0001 vs. GDM) (Figure 2). The production of nitric oxide was increased in the GDM group compared to controls (*P*=0.03), an alteration prevented by the maternal diet enriched in EVOO (*P*=0.01 vs. GDM) (Figure 2). Moreover, the gene expression of the antioxidant enzyme Cu-Zn superoxide dismutase was reduced in the placentas from GDM patients compared to controls (*P*=0.004), an alteration prevented by the maternal diet enriched in EVOO

(*P*=0.004 vs. GDM) (Figure 2). Finally, the activity of MMPs was measured both in the placenta and the umbilical cord blood. MMP-2 activity showed no changes, whereas MMP-9 activity was increased in the placentas from GDM patients compared to controls (*P*=0.003), an alteration prevented by the maternal diet enriched in EVOO (*P*=0.01 vs. GDM) (Figure 3). Moreover, MMP-2 and MMP-9 activities were increased in the umbilical cord blood from GDM patients compared to controls (*P*=0.03 and *P*=0.009 respectively), alterations prevented by the maternal diet enriched in EVOO (*P*=0.03 and *P*=0.001 vs. GDM, respectively) (Figure 3).

4. DISCUSSION

The study presented here is the first to address the effects of an EVOO-enriched diet in GDM pregnancies. In GDM mothers, the EVOO-enriched diet reduced triglyceridemia and weight gain. In the placenta of GDM patients, the maternal EVOO-enriched diet did not regulate miR-130a expression or PPARγ levels, but did regulate miR-518d expression and PPARα levels. Anti-inflammatory effects were observed in the placenta and the umbilical cord blood in the GDM patients that received the EVOO-enriched diet. If replicated in a larger number of patients, these results would have important clinical implications as a feasible dietary treatment that provides benefits to GDM mothers and their placentas, with possible beneficial effects on the offspring´s later life.

Maternal nutrition is crucial in GDM metabolic control. Modified diet interventions favorable influence outcomes related to maternal glycemia and birth weight[30] and the

follow up of nutritional advices, such as consumption of low-glycemic index carbohydrates, has largely improved the metabolic control in this prevalent gestational disease[31,32]. In the last years, the nutrigenomic concepts brought to a new dimension the interaction of nutrients and the genome, with implications in multiple diseases, including GDM[33]. PPARs are ligand activated transcription factors that respond to nutrients to bring transcriptional regulation of metabolic, developmental and proinflammatory pathways[7]. Endogenous PPAR ligands have lipid nature and can be incorporated through the diet[9]. Dietary unsaturated fatty acids are endogenous PPAR ligands that can efficiently be transferred through the placenta and the fetus to act as PPAR activators[6,34]. Considering this, the low levels of PPARα and PPARγ previously observed in the placentas from GDM patients[17] provide a rationale to provide PPAR agonists from the maternal diet to the placenta in GDM pregnancies.

Our previous studies performed in experimental models of diabetes and pregnancy have shown that a maternal EVOO dietary treatment regulates PPAR pathways and reduces proinflammatory markers in rat embryos, fetuses, placentas and in the offspring´s heart[6,11-13,15]. The dose of olive oil that shows these beneficial effects in animal models of diabetes and pregnancy provides half of the lipid-derived calories (5% in an 11% lipid content diet). Based on this, in this translational clinical study, we provided a diet that brings about half of the lipid-derived calories by the olive oil (14% in a 30% lipid content diet). This amount of EVOO added to the diet was similar to that reported to be beneficial in different clinical studies, including those evaluating the ability of an EVOO-enriched diet to prevent GDM induction in a general population[21,26].

The PREDIMED and other clinical studies have addressed the ability of EVOO- enriched diets to ameliorate cardiovascular diseases and improve lipid metabolic profiles [21,23,25]. Here, the fact that triglycerides and weight gain are reduced to control values in the GDM patients that received the EVOO-enriched diet is clinically relevant and deserves to be studied in larger populations. The mechanisms involved are likely to be related to effects on maternal target organs, including the liver, through mechanisms which are possibly related to the activation of PPARα[35].

Other pregnancy outcomes, including gestational age, Cesarean delivery, maternal and neonatal complications, and neonatal and placental weight did not change between the groups evaluated, and a larger number of patients would be needed to address putative changes in these and other maternal/perinatal outcomes. Indeed, a limitation of this work was the limited number of patients included in this study. Another limitation is that we did not quantify overall caloric intake and macronutrient consumption, and thus we cannot rule out that differences in caloric intake could explain some of the benefits observed. A third limitation of this work is the lack of use of operator-independent biomarkers of fatty acid consumption such as circulating fatty acids[36].

In this study, we focused in the placenta and the putative changes in PPARs expression. Similar to that previously found in a rat model of GDM[11], we here found that the maternal EVOO-enriched diet did not prevent the reduced PPARγ levels, but induced anti-inflammatory effects in the placenta. Indeed, PPAR ligands are provided by the EVOO-enriched diet, and thus, even without changing PPARγ levels, the activity of this nuclear receptor may be increased, in turn leading to the observed anti-inflammatory

effects[37]. Besides, the expression of miR-130, a microRNA that targets PPARγ and is related to PPARγ changes in the livers of GDM rats[29], was similar in the three groups evaluated, suggesting that other epigenetic regulators are those related to the observed PPARγ changes induced by GDM. Zhao et al. showed that the reduced PPARα levels in GDM placentas are related to increased levels of miR-518d[19]. In this work, together with confirming this result, we found that the EVOO-enriched diet was able to prevent both increased miR-518d expression and reduced PPARα levels. This points to the miR-518d/PPARα pathway as a relevant component in the placental anti-inflammatory effects observed.

In non-pregnant subjects, EVOO-enriched diets have been found to exert potent anti-inflammatory effects in different tissues[21,24]. In this work, the anti-inflammatory effects observed in the placentas from the GDM patients that received the EVOO-enriched diet included reductions in cytokine levels, regulation of antioxidant enzymes gene expression and reduction of nitric oxide overproduction. These proinflammatory markers have been previously found altered in GDM placentas and are difficult to modulate even with a good metabolic control[3,5]. Also, the ability of the EVOO-enriched diet to prevent MMPs activity in the placenta and the umbilical cord blood observed in GDM patients is supported by previous studies addressing the effect of diets added with EVOO as negative regulators of MMPs in placentas from diabetic rats and in different human diseases[15,38], and provides evidence of the capacity of the benefits of the EVOO-enriched diet to reach the fetal compartment.

5. CONCLUSIONS

This randomized clinical study allowed identifying, for the first time, the ability of an EVOO-enriched diet in GDM pregnancies to reduce maternal triglyceridemia and weight gain, which are clinically relevant parameters, thus suggesting a putative benefit that should be addressed in larger populations. Also, this clinical study provides evidence of the capacity of an EVOO-enriched diet to induce placental anti-inflammatory effects, at least partly mediated by the activation/regulation of PPAR pathways. Anti-inflammatory effects were also evidenced in the umbilical cord blood, suggesting benefits in the perinatal period and the offspring´s later life. Further studies addressing long term effects of this dietary treatment will be needed to establish its clinical significance in the offspring´s later life.

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Conflict of interest

The authors report no conflicts of interest.

Author contributions

DGR: Protocol and project development, Data acquisition, Data analysis, Interpretation of the data. ED: Protocol and project development, Interpretation of the data, Manuscript editing. MVF and HLG: Protocol and project development, Manuscript editing. DF: Data acquisition and Data analysis. SBM and CAG: Project development, Manuscript editing. EC: Data acquisition, Data analysis, Manuscript editing. AJ: Conceptualization, Project development, Manuscript writing. Funding acquisition. All authors have read and approved the final manuscript.

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FIGURE LEGENDS

Supplementary Figure 1. Flow chart and trial overview

FIGURE 1. Protein expression of PPARγ and PPARα and expression of microRNAs that target these PPAR isotypes in term placentas of GDM patients that received or not a maternal diet enriched in three tablespoons of EVOO per day from weeks 24-28 of pregnancy until term and in controls. **a.** PPARγ. **b.** PPARα. **c.** miR-130a (targeting PPARγ). **d.** miR-518d (targeting PPARα). Values represent mean ± SEM. Statistical analysis: one-way ANOVA in conjunction with Bonferroni´s test. $^{‡}P<0.05$ and $^{‡‡}P<0.01$ vs. Control, $^{§}P<0.05$, $^{§§}P<0.01$ vs. GDM.

FIGURE 2. Evaluation of proinflammatory and prooxidant markers in term placentas of GDM patients that received or not a maternal diet enriched in three tablespoons of EVOO per day from weeks 24-28 of pregnancy until term and in controls. **a.** TNF-α. Representative images and densitometric analysis. **b.** IL-1β. Representative images and densitometric analysis. **c.** Nitric oxide production (evaluated through the determination

of its stable metabolites nitrates/nitrites). **d.** Gene expression of Cu-Zn SOD. Values represent mean ± SEM. Statistical analysis: one-way ANOVA in conjunction with Bonferroni´s test. ‡P<0.05 and ‡‡P<0.01, ‡‡‡P<0.001 vs. Control. §P<0.05 and §§P<0.01, §§§P<0.001 vs. GDM.

FIGURE 3. Gelatinase activity of MMP-2 and MMP-9 in term placentas and umbilical cord blood in GDM patients that received or not a maternal diet enriched in three tablespoons of EVOO per day from weeks 24-28 of pregnancy until term and in controls. **a.** MMP-2 in term placentas. **b.** MMP-9 in term placentas. **c.** MMP-2 in umbilical cord blood. **d.** MMP-9 in umbilical cord blood. Values represent mean ± SEM. Statistical analysis: one-way ANOVA in conjunction with Bonferroni´s test. ‡P<0.05 and ‡‡P<0.01 vs. Control. §P<0.05 and §§P<0.01 vs. GDM.

TABLE 1

	Control (n=15)	GDM (n=15)	GDM-EVOO (n=15)	*P-value*
Adherence to dietary treatment	-	-	Good: 80% (12/15) Regular: 20% (3/15) Bad: None	-
Age (years)	27.4±1.9	29.6±1.6	31.1±1.6	>0.99 GDM vs. Control >0.99 GDM-EVOO vs. GDM 0.39 GDM-EVOO vs. Control
Pre-pregnancy weight (kg)	57.5±1.9	62.3±2.0	61.3±2.0	0.27 GDM vs. Control >0.99 GDM-EVOO vs. GDM 0.53 GDM-EVOO vs. Control
Total weight gain (kg)	9.9±0.7	14.2±1.4	10.3±0.9	0.02 GDM vs. Control 0.03 GDM-EVOO vs. GDM >0.99 GDM-EVOO vs. Control
Fasting blood glucose at enrolment (mg/dL)	81.4±1.9	88.0±2.7	86.0±1.8	0.11 GDM vs. Control >0.99 GDM-EVOO vs. GDM 0.43 GDM-EVOO vs. Control
Fasting blood glucose at term (mg/dL)	81.3±1.6	89.0±3.6	87.6±2.7	0.16 GDM vs. Control >0.99 GDM-EVOO vs. GDM 0.34 GDM-EVOO vs. Control
Blood fructosamine at enrolment (mg/dL)	-	183.0±6.1	181.1±4.2	0.80 GDM-EVOO vs. GDM
Blood fructosamine at term (mg/dL)	-	185.8±5.8	189.6±2.6	0.55 GDM-EVOO vs. GDM

HbA1c at enrolment (%)	-	5.0±0.08	4.9±0.10	0.44 GDM-EVOO vs. GDM
HbA1c at term (%)	-	5.4±0.10	5.5±0.06	0.39 GDM-EVOO vs. GDM
Blood triglycerides at enrolment (mg/dL)	185±8	209±10	219±15	0.43 GDM vs. Control >0.99 GDM-EVOO vs. GDM 0.12 GDM-EVOO vs. Control
Blood triglycerides at term (mg/dL)	238±6	292±21	231±14	0.04 GDM vs. Control 0.02 GDM-EVOO vs. GDM >0.99 GDM-EVOO vs. Control
Treatment	-	Diet only: 73% (11/15) Diet+Insulin: 27% (4/15)	Diet only: 93% (14/15) Diet+Insulin: 17% (1/15)	0.14 GDM-EVOO vs. GDM

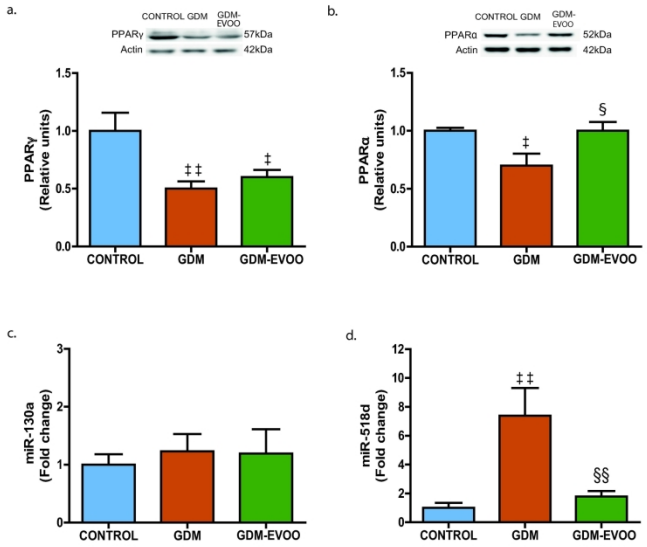
Table 1. Pregnancy and metabolic control data. Data are presented as mean ± SEM. Statistical analysis: One-way ANOVA in conjunction with Bonferroni´s test or Student´s *t* test on continuous variables and chi-square test for categorical variables.

TABLE 2

Parameter	Control (n=15)	GDM (n=15)	GDM-EVOO (n=15)	P-value
Gestational age at delivery (weeks)	38.1±0.3	37.6±0.3	37.8±0.3	0.73 GDM vs. Control >0.99 GDM-EVOO vs. GDM >0.99 GDM-EVOO vs. Control
Cesarean delivery	40% (6/15)	47% (7/15)	40% (6/15)	0.91
Maternal complications	None	None	None	-
Neonatal complications	0% (0/15)	26.7% (4/15) Respiratory Distress Syndrome (1) Hyperbilirubinemia (3)	13.3% (2/15) Hypoglycemia and Macrosomia (1) Hyperbilirubinemia (1)	0.10
Neonatal weight (g)	3312±168	3205±113	3332±135	>0.99 GDM vs. Control >0.99 GDM-EVOO vs. GDM >0.99 GDM-EVOO vs. Control
Placental weight (g)	599±27	613±40	672±32	>0.99 GDM vs. Control >0.66 GDM-EVOO vs. GDM >0.39 GDM-EVOO vs. Control

Table 2. Pregnancy outcomes. Data are presented as mean ± SEM. Statistical analysis: One-way ANOVA in conjunction with Bonferroni´s test on continuous variables and chi-square test for categorical variables. Maternal complications considered: pyelonephritis, cholestasis, preeclampsia, pregnancy-induced hypertension and threatened preterm labor. Neonatal complications considered: shoulder dystocia, brachial plexus injury, clavicle fracture, respiratory distress syndrome, intrauterine growth restriction, hypoglycemia, hyperbilirubinemia, birth defects and perinatal mortality.

Figure 1.



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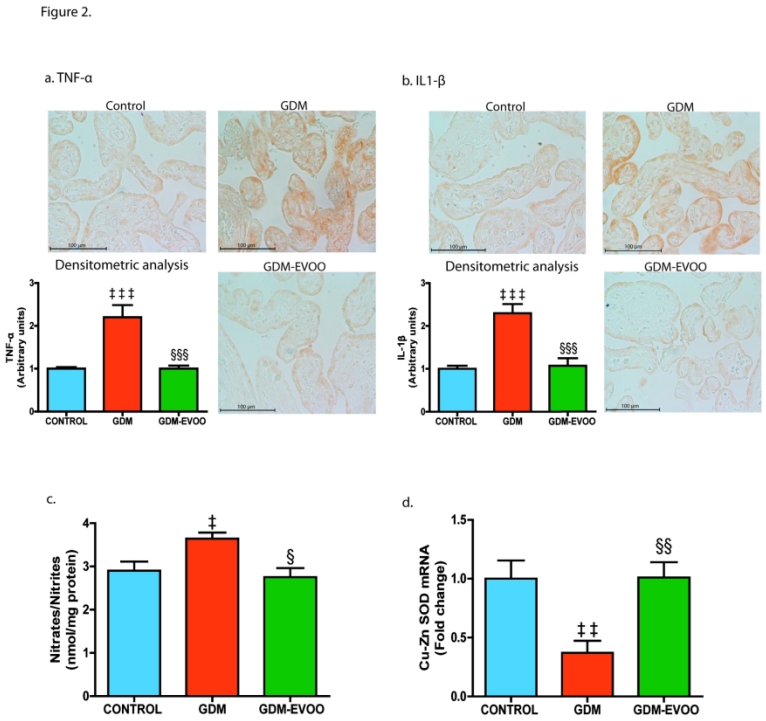
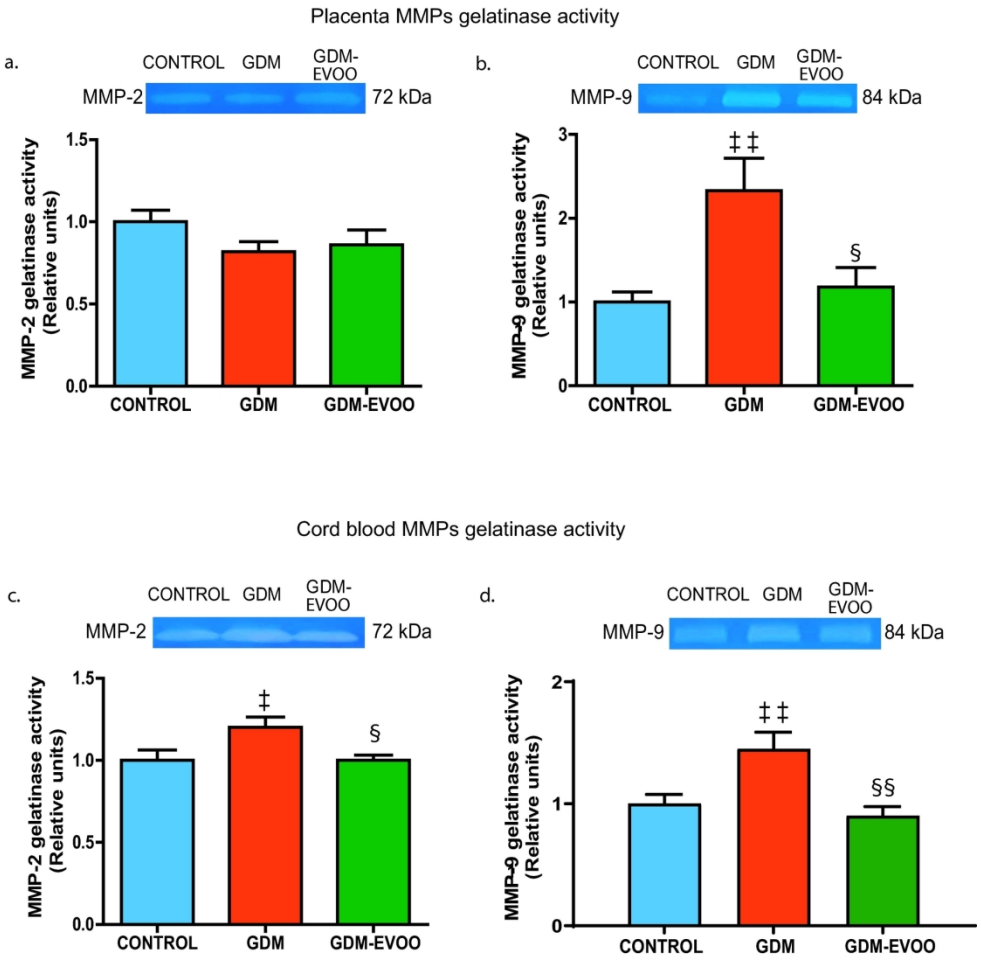


FIGURE 2. Evaluation of proinflammatory and prooxidant markers in term placentas of GDM patients that received or not a maternal diet enriched in three tablespoons of EVOO per day from weeks 24-28 of pregnancy until term and in controls. a. TNF-α. Representative images and densitometric analysis. b. IL-1β. Representative images and densitometric analysis. c. Nitric oxide production (evaluated through the determination of its stable metabolites nitrates/nitrites). d. Gene expression of Cu-Zn SOD. Values represent mean ± SEM. Statistical analysis: one-way ANOVA in conjunction with Bonferroni´s test. ‡P<0.05 and ‡‡P<0.01, ‡‡‡P<0.001 vs. Control. §P<0.05 and §§P<0.01, §§§P<0.001 vs. GDM.

Figure 3.



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