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Highlights

- Chronic maternal CAF diet impairs placental growth and pup´s weight at birth in Wistar rats.
- VEGF and IGF mRNAs are deregulated in placental CAF-fed dams.
- Alterations in VEGF and IGF placental systems are associated with modifications in DNA methylation levels

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Epigenetic disruption of placental genes by chronic maternal cafeteria diet in rats

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Running title: Placental genes epigenetic disruption after CAF diet

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Abstract

Maternal diet has impact on reproduction, fetal development and offspring behavior, although molecular mechanisms remained unknown. Our aims were to assess 1) the effects of a cafeteria (CAF) diet (western diet habits) on female reproductive performance, fetal and placental parameters on gestational day 21 (GD21) and litter size and pup weight at birth; and 2) placental mRNA expression and epigenetic regulation of insulin-like growth factor (*Igf*) and vascular endothelial growth factor (*Vegf*) and their

receptors. Female Wistar rats were fed with control (CON) or CAF diet from weaning until parturition. At week 14 after diets started, females were mated and half of the animals were euthanized on GD21 to evaluate reproductive parameters including the pregnancy rate, number of corpora lutea, implantation sites and resorption sites. Moreover, fetal weight and length, placental weight and placental index were recorded. Placentas were collected for mRNA quantification and DNA methylation analysis.The remaining animals were allowed to give birth and the number and weight of the pups were evaluated.

CAF diet did not affect reproductive performance or fetal weight and length. However, CAF-fed animals showed a decrease in placental weight and index and the pups exhibited a low birth weight. Additionally, we found an upregulation of *Igf2* and a down regulation of *Vegf* placental mRNA expression in CAF dams, associated with methylation status changes of their promoters. We conclude that female chronic CAF diet consumption impairs feto-placental development and could be explained by an epigenetic disruption of *Igf* and *Vegf* systems.

Keywords: Cafeteria diet – Feto-placental development – Vascular Endothelial Growth Factor – Insulin-like Growth Factor 2 - DNA Methylation

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Abbreviations

AHR: aryl hydrocarbon receptor

ARNT: aryl hydrocarbon nuclear translocator

CAF: cafeteria

CLs: corpora lutea

CON: control

CpG: cytosine-phosphate-guanine-dinucleotide

GD: gestational day

Elk-1: ETS like-1 protein

HFD: high-fat diet

HSHF: High Sugar/ High Fat

ICR: imprinting control region

Igf: Insulin-like Growth Factor

Igf1: Insulin-like Growth Factor 1

Igf1R: Insulin-like Growth Factor 1 Receptor

Igf2: Insulin-like Growth Factor 2

Igf2R: Insulin-like Growth Factor 2 Receptor

IS: number of implantation sites

LPS: lipopolysaccharide

RS: resorption sites

TFs: transcription factors

Vegf: Vascular Endothelial Growth Factor

VegfR: Vascular Endothelial Growth Factor Receptor

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1. Introduction

Multiple lifestyle factors, such as physical activity, social interaction, smoking and dietary habits, affects positively and negatively our health [1 - 3]. The daily consumed food has influence on determining our overall health condition, affecting the predisposition to develop metabolic syndromes, brain dysfunctions and reproductive disorders, among others [4, 5]. In this sense, the type of diet and its nutrient composition can improve or disturb fertility and pregnancy outcomes [4, 6]. Taking into account that food consumption can be modified, it is necessary to review dietary habits to enhance our health and avoid critical consequences in the short- and long-term.

Cafeteria (CAF) diet is a murine model on which nutritional program simulates Western diets habits [7]. The CAF diet reflects variety, palatability, and energy density food of the majority of Western population diets. In addition, the composition of this diet implies an unbalanced diet with predominantly fat energy content (49%) at the expense of low protein content (7%) [8]. Several studies that evaluated CAF diet or high-fat diet (HFD) effects on reproduction detected different consequences on fertility [9 - 12], placental function [13 - 15], fetal weight [9, 16] and weight of pups at birth [16]. However, the possible mechanism involved in impairment of feto-placental development has not been determined.

Placental structure and functions have been optimized by an evolutionary process to maintain the pregnancy as well as fetal health, growth and nutrition. The placenta is the place of nutrients and waste vascular interexchange between mother and fetus, taking critical job on fetal growth and development related process [17]. Vascular Endothelial Growth Factor (*Vegf*) and Insulin-like Growth Factor (*Igf*) play a key role during placental and fetal development [18 - 20]. The *Vegf* system, composed of *Vegf* and its

receptor (*VegfR*), is crucial for angiogenesis which implies an important piece on vascular placental function [20, 21]. Particularly, *Vegf* is essential for blood vessel formation during early embryogenesis [21]. Interestingly, some authors found an altered vessels density in placenta and a correlation between *Vegf* down-regulation and low placental and fetal weight [22 - 24]. On the other hand, *Igf* system, integrated by *Igf1* and *Igf2* and their receptors (*Igf1R* and *Igf2R*), has pivotal role on promotion of feto-placental growth and development. In this sense, *Igf* system knockout mouse models provoked a reduction of 40-55% in birth weight, reflecting the essential role of this system on fetal development [19 - 25].

Several studies show that intrauterine environment influenced by maternal nutrition experience or environmental pollutants, produces changes in the epigenetic profile of placental genes, affecting fetal and placental development [26 - 29]. Epigenetic state of cells is critical for the cell function. One of the most studied epigenetic mechanisms is DNA methylation which occurs primarily as addition of a methyl group to a cytosine base in a cytosine-phosphate-guanine-dinucleotide (CpG). Regions with a high frequency of CpG sites are known as CpG island. These regions are mostly located in promoter regions of genes and can be associated with regulatory elements such as transcription factors (TFs) and repressors [30, 31]. Cytosine methylation is generally related with transcriptional silencing due to a decrease of the TF binding capacity resulting in a minor expression of the studied gene [32]. Importantly, these epigenetic marks can be heritable and produce manifestations in health and diseases after the exposure ended.

The present study was conducted to investigate the effects of chronic maternal consumption of CAF diet on rat reproductive performance, feto-placental parameters on gestational day 21 (GD21) and pup birth weights. In addition, we analyze the

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implications of key placental systems (*Igf* and *Vegf* systems) along with the underlying epigenetic mechanisms.

2. Materials and Methods

2.1 Animals and experimental design

Wistar female rats were obtained from the Department of Human Physiology of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina). All animal housing and handling were in accordance with the principles set out in the Declaration of Helsinki. The experiments were designed and performed to match closely to the 3R (replacement, reduction, refinement) principles of animal welfare. Additionally, we comply with the ARRIVE guidelines. Beside this all animal´s procedures were approved by Ethical Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina) (number of approved project *PICT 2014-1522*). In addition, all the procedures were designed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences (Commission on Life Science, National Research Council, Institute of Laboratory Animal Resources, 1996).

Female rats were weaned at 21 days of age and randomly housed two per cage, at 22 ± 2 °C and with a 12-h light-dark cycle, and fed with either standard chow control (CON) diet or CAF diet (N=20 per group) from weaning. All animals had free access to pellet laboratory chow (Cooperación, Buenos Aires, Argentina) and tap water supplied *ad libitum* in glass bottles with rubber stoppers surrounded by a steel ring. The average body weight of each group was equivalent (36 ± 2g). The standard chow (Cooperación,

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ACA Nutrición Animal, Buenos Aires, Argentina) provided 12.55 KJ/g, 5% energy as fat, 23% as protein and 72% as carbohydrate. As we mentioned, the CAF diet reflects variety, palatability, and energy density food of Western diet habits. The CAF diet was composed of standard chow and parmesan cheese, cheese flavoured snacks, crackers, sweet biscuits, pudding, and chocolate. All these items are low in essential micronutrient density (Supplementary Table 1). This diet provided an average of 20.29 KJ/g, 49% of energy as fat, 7% as protein, and 44% as carbohydrate, in addition to that provided by the standard chow. Together with the standard chow, three of the CAF items were provided in excess quantities and were changed every other day, by replacing all the food with new items, in order to maintain the variety. Therefore, the animals did not receive the same food items for more than two consecutive days. [8]. Body weights were recorded weekly, and food intake daily, during the whole treatment period (18 weeks), defined as pre-gestational period (week 1 to 14) and gestational period (week 15 to 18). Food intake was determined by the weight difference between the offered and the remaining food, adjusted to the waste by collecting food spillage. Energy intake was calculated using the energy contents of each food (kcal/g). Daily macronutrient intake was expressed as a percentage of total daily energy consumption at 14 week (pre-gestational period) and at 18 week (gestational period).

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2.2 Evaluation of reproductive performance

The oestrous cycle was monitored by vaginal smears during two weeks before mating to determine if the CAF diet alters the duration of any particular phase of the oestrous cycle. Vaginal smears were obtained daily from lavage fluid collected by flushing the vagina with phosphate-buffered saline and were examined under a light microscope.

The stage of the oestrous cycle was determined based upon vaginal cytology as described in Brack et al. (2006) [33].

On 14th week of treatment (after CAF animals showed a weight difference respect CON animals), each female at the proestrus stage were caged with a male with fertility proved. Next morning, vaginal smears were performed to check the presence of spermatozoa [33]. The day after mating was defined as gestational day 1 (GD1) evaluating the presence of sperm on the smear. We determine the pregnancy rate as the number of females that get pregnant in relation to the total number of females that were individually housed with a male x 100. After sperm detection, each dam was singled caged and kept with their respective diet. The male rats used for mating were fed with standard chow and were maintained in identical environmental conditions to ensure minimal variation from paternal factors.

Ten dams per diet group were briefly exposed to CO2 and sacrificed by decapitation at 12.00 pm on GD21 to evaluate the reproductive performance. Trunk blood was collected, samples were centrifuged, and serum was immediately used or frozen and stored at −80 °C until further use. The ovaries were dissected, and the number of profusely irrigated corpora lutea (CLs) was counted under direct visualization by using a stereomicroscope (Leica Corp., Buffalo, NY, USA). The two-horned uteri were removed and visually inspected to quantify resorption sites (RS) and the number of implantation sites (IS). The RS were defined as endometrial sites with an appended amorphous mass without a fetus. The number of IS was defined as the result of the total number of placentas with fetuses plus the total number of RS. With these data, we calculated the rate of pre-implantation loss as follows: [number of CLs – number of IS/ number of CLs] x 100 [34]. A total of 10 animals per group were maintained until

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parturition with their respective diet. The litter size was monitored and the weight of each pup at birth was registered.

2.3 Feto-placental parameters

To determine the effects of the diet on fetal and placental development, on GD21 each fetus and placenta pair was removed from the uterus and weighed. The placental index was calculated as follows: placental weight/ fetal body weight. In addition, the fetal body length from the top of the head to the bottom of the buttocks (crown-rump length) was measured using a caliper. A total of three placental tissues per each dam selected randomly were collected and stored at -80°C until RNA and DNA extraction.

2.4 Reverse transcription and quantitative real-time polymerase chain reaction

Individual whole placental tissue were homogenized in TRIzol® reagent, and total RNA was extracted following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The concentration of total RNA was estimated by measuring the absorbance at 260 nm and 280 nm in a NanoDrop Lite Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the sample was stored at -80°C until needed.

Equal quantities (1 µg) of total RNA were reverse-transcribed into cDNA with Moloney Murine Leukemia Virus reverse transcriptase (300 units; Promega, Madison, WI, USA) using 200 pmol of random primers (Promega, Madison, WI). Twenty units of ribonuclease inhibitor (RNA out) (Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture were added to each reaction tube at final volume of 30 µl of 1× reverse transcriptase buffer. Reverse transcription was performed at 37 °C for 90 min and at 42 °C for 15 min. Reactions were stopped by heating at 80 °C for 5 min and cooling on ice.

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The mRNA expression of *Igf1*, *Igf1R*, *Igf2*, *Igf2R*, *Vegf* and *VegfR* was quantified by real-time RT-PCR. Each reverse-transcribed product was diluted with RNAse free water to a final volume of 60 µl and further amplified in duplicate using the Real-Time DNA Step One Cycler (Applied Biosystems Inc., Foster City, CA, USA). L19 was used as a housekeeping gene. The primer sequences are described in Table 1. For cDNA amplification, 5 µl of cDNA was combined with HOT FIREPol Eva Green qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) and 10 pmol of each primer (Invitrogen, Carlsbad, CA) to a final volume of 20 µl. After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 52–60 °C for 15 s, and extension at 72 °C for 15 s. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, and these reactions did not yield any consistent amplification. The relative expression levels of each target were calculated based on the cycle threshold (C_T) method [35]. The C_T for each sample was calculated using the Step One Software (Applied Biosystems Inc.) with an automatic fluorescence threshold (*Rn*) setting. The efficiency of the PCR reactions for each target was assessed by the amplification of serial dilutions (over five orders of magnitude) of cDNA fragments of the transcripts under analysis. Accordingly, the fold expression over control values was calculated for each target using the relative standard curve, which was designed to analyze real-time PCR data [36]. For all experimental samples, the relative target quantity was determined using the standard curve, normalized to the relative quantity of the reference gene, and finally divided by the normalized target value of the control sample. No significant differences in C_T values were observed for L19 among the various experimental groups.

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2.5 Bioinformatics

Taking into account the results, we analyzed the promoter regions of *Igf2* and *Vegf* genes identifying CpG Islands, using MethPrimer program (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi; ***RRID***: SCR_010269), and detecting restriction sites for *BstU* I (New England BioLab, Beverly, MA, USA), *Tai* I (Thermo Scientific, Wilmington, DE, USA), and *Sac II* (Promega, Madison, WI). In addition, *Igf2*/*H19* imprinting control region (ICR) region was analyzed. Potential binding sites for transcription factors were predicted using the bioinformatic tool PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3; RRID: SCR_016926) (Messeguer et al., 2002). To analyze methylation sensitive regions, PCR primers were designed with the online software NCBI Primer-BLAST (National Center for Biotechnology; https://www.ncbi.nlm.nih.gov/tools/primer-blast/; RRID: SCR_003095).

2.6 Methylation-sensitive analysis

The methylation status of the gene promoters in experimental groups was evaluated by using a combination of digestion with methylation-sensitive restriction enzymes and subsequent qRT-PCR analysis [37, 38]. Genomic DNA from placentas was isolated using the phenol/chloroform/isoamyl alcohol extraction. The concentration of total DNA was assessed by A_{260}, and DNA was stored at 2-8 °C until needed. Equal quantities of DNA (1 μg) were digested with 5 units of *Tai* I (10X enzyme buffer, 3 h at 65 °C), 10 units of *Sac* II (10X enzyme buffer, 3 h at 37° C) or 10 units of *BstU* I (1X enzyme buffer, 1 h at 50 °C). The digestion products were purified using the phenol/chloroform extraction.

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An optimized qRT-PCR protocol was used to analyze the relative methylation levels of various regions of the gene promoters (Table 2). The procedure for DNA amplification, the analysis of the product purity and the C_T for each sample were above-described in the section 2.4. A region devoid of methylation-sensitive restriction sites was amplified as an internal control. When a CpG-rich site is methylated, enzymatic digestion is not possible, allowing amplification of the fragment. In contrast, if the CpG-rich site is not methylated, enzyme cleaves the DNA and prevents amplification of the fragment. The relative degree of promoter methylation was calculated by plotting C_T values against the log input (internal control: IC), which yielded standard curves for the quantification of unknown samples [36]. The efficiency of the qRT-PCR reactions and the fold expression over control values for each target gene were calculated as previously described [39].

2.7 Serum assessment

Fasting serum metabolites (glucose, triglycerides, and cholesterol) were assessed by a commercially available assay (Wiener Laboratorios, Argentina).

2.8 Statistical analysis

G Power software (http://www.gpower.hhu.de/; RRID:SCR_013726) was used to determine the sample size [40]. To confirm the normal distribution of the data and variance homogeneity, Shapiro–Wilk test and Levene's test were performed. Weekly body weights, nutrient intake and energy intake were analyzed using repeated-measured ANOVA followed by Bonferroni post-test while feto-placental parameters and serum parameters were analyzed using Student´s T test. The analysis of pregnancy rate was assessed using Fisher´s exact test. The number of CLs, IS and RS as well as relative mRNA expression and DNA methylation levels in placentas was analyzed using Mann-

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Whitney U test. All the data are expressed as the means ± SEM and was statistically analyzed using the IBM SPSS Statistics 19 software (IBM Inc.; RRID: SCR_002865), considering significant differences at $p<0.05$.

3. Results

3.1 CAF diet influences dam´s body weight, nutrient and energy intake

Dams fed with CAF diet increased their body weights during pre-gestational period, from week 10 of dietary treatment and maintained these differences during the following 4 weeks ($p <0.05$) (Fig. 1A). Thus, on the 14th week of treatment, when CAF animals were heavier than CON animals, females were mated with male rats. During pregnancy (gestational period), CAF rat's body weight remained heavier than CON animals ($p <0.05$) (Fig. 1A). Also CAF group showed a higher energy intake during pre-gestational and gestational period ($p <0.05$) (Fig. 1B). In addition, CAF dams reported a greater percentage of their intake as fat and lesser as protein during pre-gestational and gestational period ($p <0.05$) (Fig. 1C). Throughout the entire dietary intervention, animals fed with CAF consumed excessive amounts of the different highly palatable items presented (85% of the total daily energy intake), related to the balanced feed (15% of total daily energy intake) (Figure not shown).

Regarding serum assessments (glucose, cholesterol and triglycerides), we did not find differences between CAF and control fed dams (Table 3).

3.2 Reproductive performance of CAF-fed dams on GD21

CAF-fed rats did not have alterations in oestrous cycle (data not shown) or pregnancy rates (Fig. 2A). The fertility test showed a normal response in CAF animals, without

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differences neither in the pregnancy rate nor number of CLs (Fig. 2B). Besides, neither changes in the number of IS nor in the number of RS on GD21 were detected between control and CAF-fed rats (Fig. 2C and D).

3.3 Effects of CAF diet on feto-placental development

Maternal CAF diet affected the feto-placental development. We detected a lower placental weight of CAF-fed dams compared with control ($p < 0.05$) (Fig. 3A). The fetal weight and length did not change between groups (Fig. 3B and C). The placental index calculated as placental/fetal weight ratio, showed a decrease in CAF group ($p < 0.01$) (Fig. 3D). Finally, the pups were weighted one day after parturition, and pups of CAF-fed dams showed a lower weight compared to CON ($p < 0.001$) (Fig. 3E). Last, litter size was optimal for all dams (CON: 12 ± 1; CAF: 11 ± 1) without difference between groups.

3.4 CAF diet alters the expression of *Igf* and *Vegf* placental system

As CAF diet altered the placental weight and placental index, we decided to evaluate the expression of molecules involved on placental function, determining the changes of expression on GD21-placentas. Regarding *Igf* placental system, maternal CAF diet revealed neither changes in *Igf1* nor its receptor (*Igf1R*) (Fig. 4A and B); however, CAF diet produced an increase in *Igf2* expression ($p < 0.01$), while its receptor (*Igf2R*) remained unchanged (Fig. 4C and D). Concerning *Vegf* placental system, *Vegf* expression was lower in CAF than in CON group ($p < 0.01$) and its receptor (*VegfR*) did not change (Fig. 4E and F).

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3.5 *Igf2* and *Vegf* placental genes showed modifications in DNA methylation levels in response to maternal CAF diet

To evaluate if the alteration in the transcript levels of *Igf2* and *Vegf* genes are related to DNA methylation modifications, we first analyzed *in silico* the promoter regions of those genes (Fig. 5 and 6). To search for potential sites of DNA methylation, we analized the promoter region for CpG islands and checked for restriction sites for *BstU* I and *Tai* I, *Sac* II enzymes. Moreover, we searched for TFs that regulate these putative sites of DNA methylation (Fig. 5 and 6). Then, to determine whether altered transcript levels of placental genes are associated with differential DNA methylation due to CAF administration, we determined the methylation status of the regulatory regions of *Igf2* and *Vegf* genes. Regarding *Igf* system, we evaluated the methylation status in the *Igf2* promoter and the *Igf2-H19* ICR. We found a reduction in methylation levels of two sites, *BstU* I/*Sac* II (*Igf2*) and *BstU* I (*Igf2-H19* ICR), in the placentas of rat fed with CAF diet ($p < 0.05$) (Fig. 6). In addition, the promoter region of *Vegf* showed an increase in the methylation state at the *BstU* I site in CAF group ($p < 0.05$), while a decrease in the *Tai* I site was found ($p < 0.01$) (Fig. 5). The predictive analysis of TFs revealed that some of these changes were observed at potential binding sites for regulatory proteins such as aryl hydrocarbon receptor (AHR), aryl hydrocarbon nuclear translocator (ARNT) and ETS like-1 protein (Elk-1).

4. Discussion

The aim of the present study was to determine the effects of a chronic maternal CAF diet on reproductive performance, feto-placental parameters on GD21 and pup weight at birth. In addition, we analyze the implications of key placental systems: *Igf* and *Vegf*, along with the underlying epigenetic mechanisms. To our knowledge, this is the first

work to show that, the impairment in feto-placental development and the lower pup birth weights due to a maternal CAF diet could be associated, at least in part, with an epigenetic disruption of *Igf* and *Vegf* systems.

In our work, CAF diet administered from weaning until maturity produced an increase in body weight and energy intake, as it has been shown in other studies [8, 9, 16, 41 - 43]. Additionally, those differences were remained before and during whole pregnancy in agreement with other authors [9, 43]. These changes were accompanied by a significant increase in fat and a decrease in protein content, as was reported previously by Akyol et al. (2009) [9]; Bayol et al. (2007) [54] and Rossetti et al. (2019) [43].

In non-pregnant rats, our CAF diet model generated overweight without impairments in glucose, triglycerides, cholesterol and insulin, although an increase in leptin concentration was found [8]. In the present study we showed no disturbance of some metabolic parameters such as, glucose, triglycerides and cholesterol in pregnant rats. Akyol et al. (2009) [9] found that plasma glucose and cholesterol concentrations at day 5 or 20 of gestation were unaffected significantly by cafeteria feeding, although the CAF-fed dams showed a tendency of increase in plasma glucose concentrations at GD20. In addition, Ong and Muhlhausler et al. (2011) [72] also found no differences in glucose, leptin and insulin levels of dams fed with junk-food diet and control diet at the end of lactation, while they detect an increase in plasma NEFA in junk-food dams. Altogether these data indicate controversial results about CAF maternal diet effect on metabolic alterations indicating that further studies are needed.

We found no differences in breeding success between the control and CAF dams, as was previously suggested by Akyol et al. (2009) [9].However, other studies showed association between CAF feeding and reproductive impairments [10, 11, 44 -

46] as a consequence of different metabolic and endocrine alterations, such as insulin resistance. Differences between these studies and our results on reproduction could be associated to dietary items, timing and length of diet exposition and rat strain tendencies. Importantly, some diets include high content of sugar and/or fat (in most cases it is composed of a single solid food), do not representing in an appropriate way the human diet habits. Even when the diets consist in human foods, these could differ on types and quantities of items. In this sense, it is hard to identify whether the effects of a CAF diet could be attributed to specific item composition or maternal obesity [16]. Nevertheless, a greater range of novel food, including several highly palatable-energy dense items, may have a combined effect and produced a larger impact on food intake and adiposity and consequently could influence on physiology and metabolism [16]. Regardless of the differences between several animal models impacts on reproduction, it is well known that the obesity impairs reproductive performance, produce ovulatory disruption and alter the hypothalamic-pituitary-ovarian axis in humans [12, 70] and rodents [11, 71]. Additionally, several human and experimental animal studies describe that maternal obesity could alter the placental and fetal developmental trajectory predisposing to unfavourable outcomes [73, 74]. In this sense, it is crucial to know the implications of nutrition and obesity on reproductive health despite our model did not alter the fertility.

At the date, scientific reports evidence that maternal nutrition has implications on feto-placental development [47 - 50]. Particularly, some authors reported that the placenta reacts to a HFD and CAF diet and the fetal growth is affected [9, 51]. Our findings show that CAF feeding before and during pregnancy produced a decrease in placental weight and index, without changes on fetal length and weight on GD21. Similarly, Akyol et al. (2009) [9] observed a decrease in placental weight, although they

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also reported low fetal weight. Neither the length of dietary exposition nor strain rat tendencies seems to be the critical points on this discrepancy. Although, it is not possible to conclude definitively what might have caused these differences, we could speculate that the dietary items and quality would have any role. In fact, maternal diets poor in essential nutrient and protein intake (such as CAF diet) might have detrimental effects on placental development and growth. Thus, understanding placental adaptations to nutritional adversities may reveal mechanisms underlying the effects on the offspring and the developmental origins of later diseases [75].

In optimal conditions, the placenta matures while the fetal weight increases, reflecting a decrease of the placental index [52]. There are associations between atypical placental weight and/or placental index with maternal health impairments [52]. Often, the placenta becomes more efficient and supports more mass of fetus per gram placenta. These placental adaptations optimize fetal growth [53]. In this context, we propose that the placenta of CAF-fed dams has not enough functional capacity to resolve the impairments produced by CAF diet. As a consequence, a low placental weight on GD21 was found with a probably impact on weight of pups, similar to reported by George and colleagues (2019) [16]. In this sense, it is possible that the placenta is not able to transfer enough nutrients and oxygen after GD21 to support normal fetal growth prior to birth. In addition, Bayol et al. (2007) [54] suggested that the reduced maternal protein intake that characterized the CAF diet would affect the offspring's body mass at birth and at weaning. In this sense, Rolls et al. (1986) [76] reported that changes in the protein intake of the CAF diet modify the production and composition of breast milk; although other authors reported no differences in the protein content of the lactation milk between CON and CAF-fed dams [77-79]. While we did not find apparent differences during lactation period in maternal behavior between CON and CAF-fed

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dams [43]; two studies described alterations in maternal care of CAF fed dams, including increased licking/grooming [80] and arched nursing and nesting [81]. Thus, it is an ongoing challenged to clarify if maternal care has more influence on pups than the diet itself. These results are great of interest considering that there are strong associations between reduced weight at birth and during early-life and disease in adult life, including obesity [54], insulin resistance [55], altered brain function [56] and increased cardiovascular risk [57].

Igf system is important on fetal and placental growth. *Igf1* is involved in fetus development; its administration produces a high fetal weight, while its ablation decreases it [58]. Meanwhile, *Igf2* overexpression generates placental and fetal overgrowth and its deletion produce the opposite effects [59, 60]. Our findings showed no differences of *Igf1* and *Igf1R* on placental tissues between groups, which could be related with the normal fetal weight on GD21 found on CAF-fed dams. Instead, *Igf2* gene expression showed an increase in GD21, without changes on its receptor. Similarly, High Sugar/ High Fat (HSHF) diet produced a low fetal and placental weight on GD16 with an upregulation of IGF2 placental gene expression; on GD19, fetal weight and *Igf2* expression were normalized, but not the placental weight [61]. Meanwhile, Gao et al. (2012) [47] found that a low protein diet reduced fetal and placental weight and the *Igf2* placental gene expression on GD14 and GD18; on GD21 fetal weight remained lower and placental weight and *Igf2* expression were normalized. *Igf2* contributions to adaptation to maternal protein restriction are apparently ineffective in promoting fetal growth [47]. It could be suggested that the upregulation of *Igf2* placental gene expression is playing a role on placental adaptations to optimize the fetal growth. We wonder whether dietary items or other intrinsic mechanisms are the cause of our finding, and whether upregulation of *Igf2* is playing a role on fetal normal weight

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but it is inefficient to keep an optimal development until birth. This cannot be concluded with this study and would require further investigations.

Vegf and *VegfR* are angiogenic factors and their well working is crucial for the nutrient transport. This system has a key role on endothelial cell proliferation, migration and tube-like structure formation [62]. In our study, we found a decrease in placental *Vegf* gene expression of CAF-fed dams, without changes in *VegfR* on GD21. Hu et al. (2019) [63] reported a down regulation of *Vegf* placental expression associated with changes in the density of placental vessels in dams feeding with high energy diet. Similarly, Wang et al. (2019) [64] reported that HFD produced a decrease in placental weight together with a down regulation of placental *Vegf* expression. Salvolini et al. (2019) [24] reported a link between obesity and an increase of *Vegf*, suggesting that this up regulation could be a compensatory mechanism for placental blood flow changes. Last, Bao et al. (2019) [23] found low fetal and placental weight with a decrease in *Vegf* placental levels, after lipopolysaccharide (LPS) treatment. Taking all together, it is clear that maternal diet affects the *Vegf* regulation however, it is still unknown the specifically role of this deregulation on fetal and placental development.

To explain the changes of transcription levels of placental genes, we evaluate the methylation status of *Vegf* and *Igf2* promoter regions and analyze these regions looking for potential TFs binding sites. TFs are proteins with the ability to bind to a specific upstream regulatory sequence on genes and regulate their transcription [65]; methylation can block TFs binding and interfere with mRNA expression [66]. First, we detected an increase in a potential binding site for AHR, ARNT and Elk-1 factors in the CpG Island of the *Vegf* promoter.. This epigenetic alteration could be associated with the down regulation of *Vegf*. At the same time, the *Vegf* promoter region showed a decrease in the methylation state at other site, which is a potential binding site for Elk-1.

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This could be a compensatory mechanism of *Vegf* down-regulation, considering the essential role of this factor on placental and fetal optimal development [63]. In addition, we found a reduction in methylation in *Igf2-H19* ICR and *BstU* I/*Sac* II site in DMR2 region of the *Igf2* promoter, which could explain the up regulation of *Igf2* mRNA levels. The first one is a CTCF6 site which is a possible site to binding a highly conserved transcription factor that can act as either transcriptional activator or repressor, and its function is regulated through an epigenetic mechanism [67]. The second one is included in a CpG Island and it is a potential binding site for Elk-1. Taking into account that all these TFs (AHR, ARNT and Elk-1) are involved on angiogenic responses as vascular remodeling [68, 69], mainly process related with placental development, it is possible that the changes of methylation levels of these regions could affect the normal physiology of placentas. However, further studies are needed to clarify the cause-effect relationship between placental gene dysregulation in response to CAF diet. Due to the limitations of the technique, some methylation-targeted CG sites were not included in our analysis. In addition, it would be interesting to analyze the implication of histone modifications or miRNA or to perform experiments using DNA methyltransferase (DNMT) inhibitors that block the epigenetic effects in this experimental model.

5. Conclusion

Our work is reflecting the influence of one of the most important factors of lifestyle, the maternal diet, on feto-placental development. We detected that CAF diet affects the placental weight and reduced the pup´s birth weight. Regarding the feto-placental impaired growth, we consider that the placenta has a key role. Taking into consideration the changes observed on *Igf2* and *Vegf* is clear that CAF-fed dams suffer disturbances of *Igf* and *Vegf* placental systems. These findings could be a cause or an effect of placental disturbances, reflected by low placental weight on GD21 and the low weight of pups at

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birth; although this is still unknown. To the best of our knowledge, this is the first study that evaluates the impact of a CAF diet on key placental systems methylation profile and the possible consequences of these epigenetic changes on feto-placental development. It is also important to considerer that these epigenetic changes could have a long-lasting effect later in life and in the offspring. However, it is still unclear if the dysfunction in placenta parameters reported are due to the components of the diet or other intrinsic mechanisms of the mother, although the varied of foods included on this work could has essential implications. However further studies are needed to clarify the regulatory mechanisms involved. In this sense, identifying how CAF diet dysregulate target genes will allow us the development of prevention strategies to improve especially human but also animal reproductive health.

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Figure Legends

Figure 1: (A) Body weight, (B) Energy intake and (C) Nutrient intake of cafeteria diet (CAF diet) and control diet (CON) fed animals, during pre-gestation period (weeks 1-14), and gestation period (weeks 15-18). Week 1 corresponds to postnatal day 21. All results are expressed as the mean ± SEM (N=20/Group). Asterisks indicate significant differences compared to the CON group after repeated-measured ANOVA followed by Bonferroni post-test (*: $p < 0.05$).

Figure 2: Reproductive performance of cafeteria diet (CAF) and control diet (CON) fed animals on gestational day 21 (GD21). (A) The pregnancy rates were calculated by the average of females that were pregnant with a fertile male. (B) Number of corpora lutea (CLs) and (C) Number of implantation sites (IS) are expressed as the mean ± SEM for each experimental group. (D) Number of resorption sites (RS) were plotted to each individual pregnant rat and the horizontal lines are the mean of each group with the corresponding SEM.

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Figure 3: Effects of cafeteria diet (CAF diet) and control diet (CON) on feto-placental development parameters on gestational day 21 (GD21). (A) Placental weight (mg), (B) fetal weight (mg), (C) fetal length (cm), (D) placental index calculated as placental weight/ fetal weight ratio, on GD21 and (E) weight of pups at birth (mg). All results are expressed as the mean ± SEM for each experimental group. Asterisks indicate significant differences compared to the CON group (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Figure 4: Analysis of relative mRNA levels of Insulin Like Growth Factor (IGF) and Vascular Endothelial Growth Factor (VEGF) systems in placental tissue of cafeteria (CAF) and control (CON) diet fed dams on gestational day 21 (GD21). (A) IGF1, (B) IGF1R, (C) IGF2, (D) IGF2R, (E) VEGF and (F) VEGFR. All results are expressed as the mean ± SEM for each experimental group. Asterisks indicate significant differences compared to the CON group (*: $p<0.05$; **: $p<0.01$).

Figure 5: Methylation analysis of Vascular Endothelial Growth Factor (*Vegf*) in placental tissue of cafeteria (CAF) and control (CON) diet fed dams on gestational day 21 (GD21). Predicted binding sites for transcription factors, CpG islands and CG target sites for digestion by the methylation-sensitive restriction enzymes *Tai* I and *BstU* I are indicated. Methylation levels of promoters in CAF rats are showed as fold changes from those of CON rats. All results are expressed as the mean ± SEM for each experimental group. Asterisks indicate significant differences (*: $p<0.05$; **: $p<0.01$). Elk-1: ETS like-1 protein, AHR: aryl hydrocarbon receptor, ARNT: aryl hydrocarbon nuclear translocator.

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Figure 6: Methylation analysis of Insulin-like Growth Factor 2 (*Igf2*) and its imprinting control region (*Igf2-H19*) in placental tissue of cafeteria (CAF) and control (CON) diet fed dams on gestational day 21 (GD21). Predicted binding sites for transcription factors, CpG islands and CG target sites for digestion by the methylation-sensitive restriction enzymes *Tai* I, *BstU* I and *Sac* II are indicated. Methylation levels of promoters in CAF rats are showed as fold changes from those of CON rats. All results are expressed as the mean ± SEM for each experimental group. Asterisks indicate significant differences (*: $p<0.05$). Elk-1: ETS like-1 protein, CTCF: CCCTC-binding factor.

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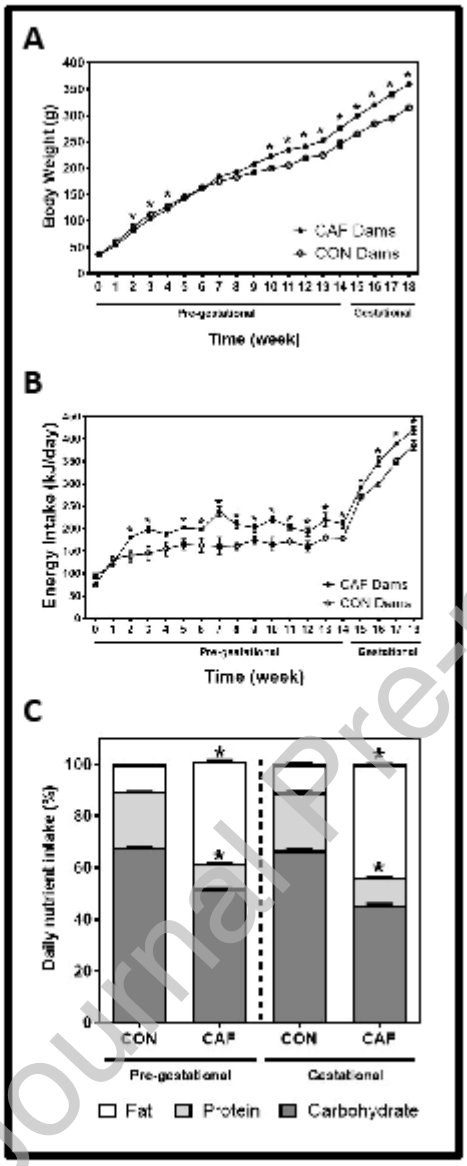

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Fig. 1

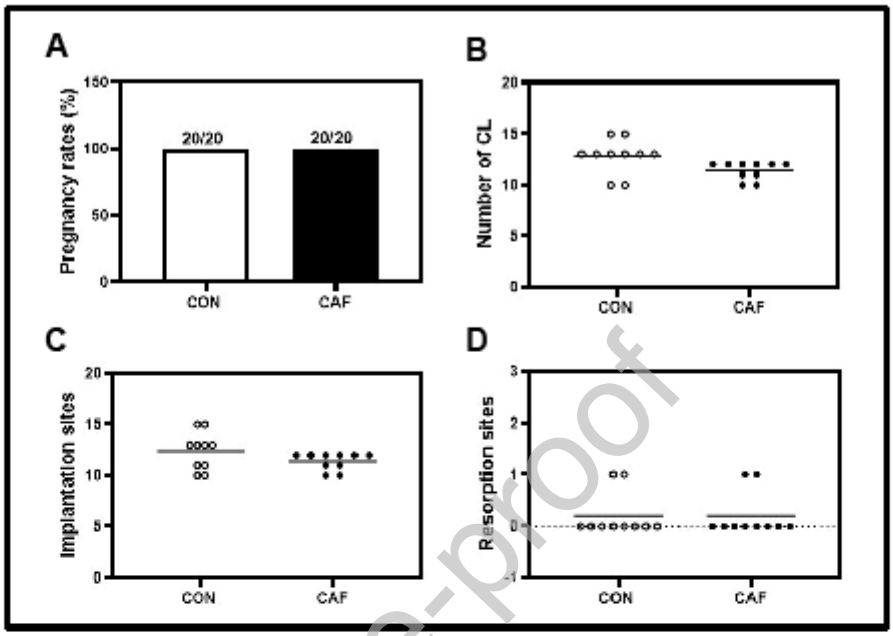

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Fig. 2

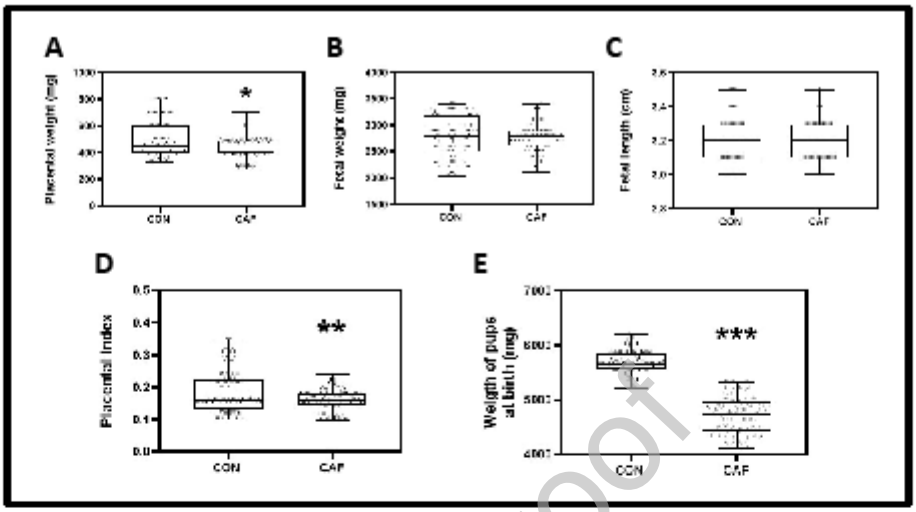

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Fig. 3

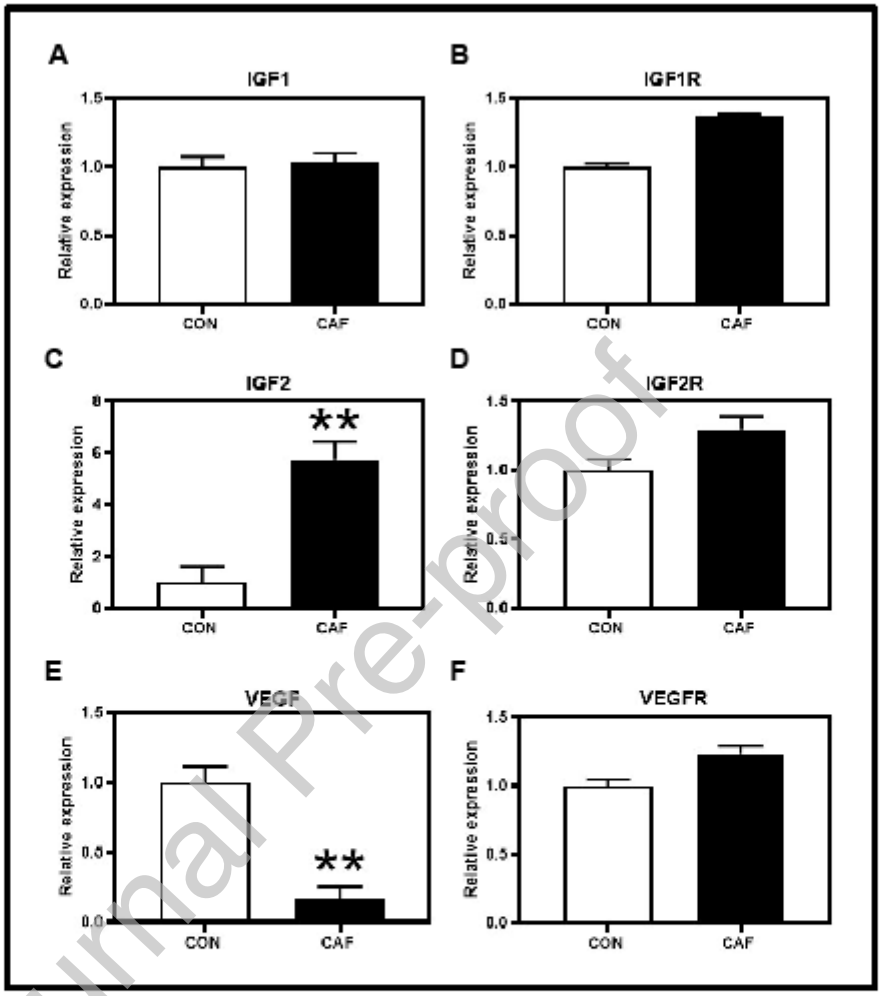

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Fig. 4

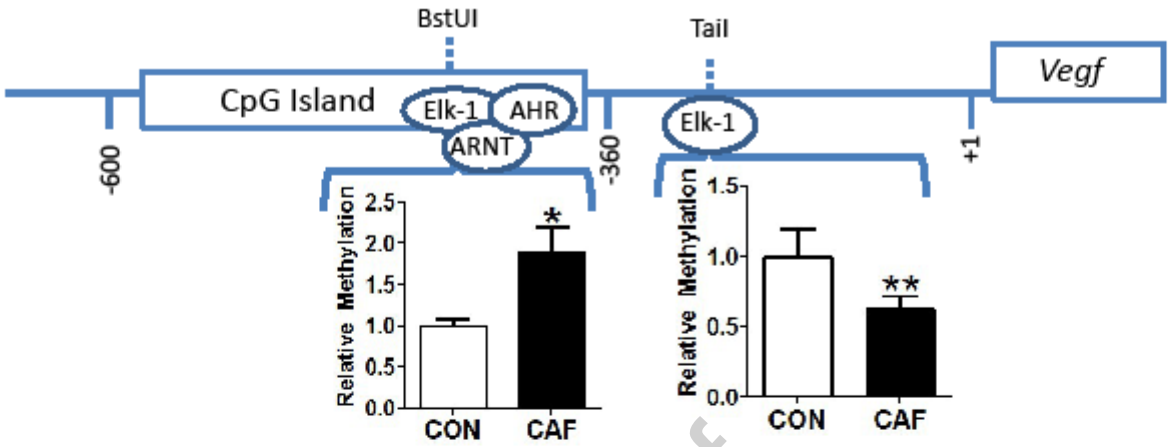

Fig. 5

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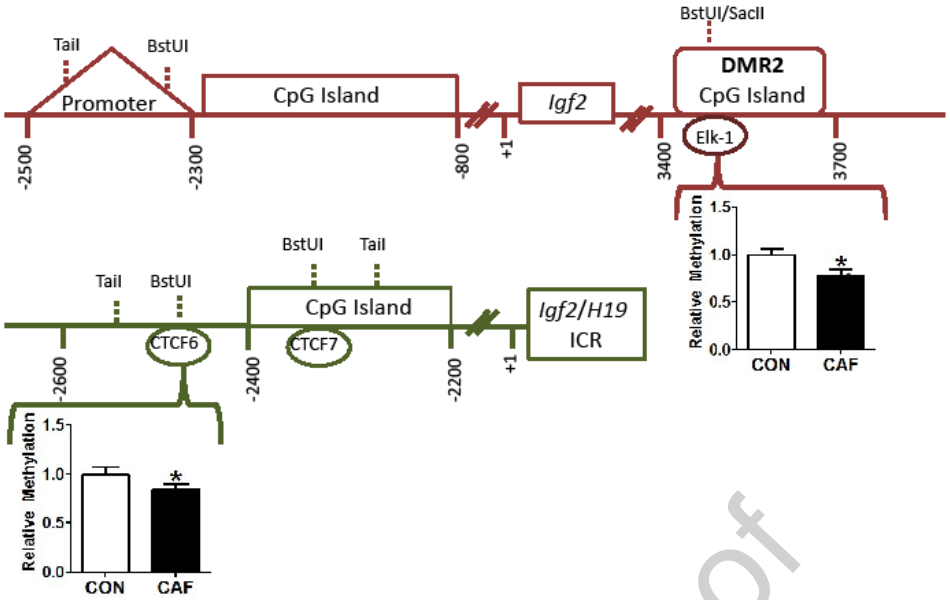

Fig. 6

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Table 1 - Primers and PCR products for real-time quantitative RT-PCR

Target	Primer Sequences	Size (bp)
L19	F: 5´- GAAATCGCCAATGCCAACTC -3´ R: 5´- ACCTTCAGGTACAGGCTGTC -3´	290
Igf1	F: 5´- CTCAAGGATGGCGTCTTCAC -3´ R: 5'-GAACTTGCTCGTTGGACAGG-3'	137
Igf1R	F: 5´- CTCAAGGATGGCGTCTTCAC -3´ R: 5'-GAACTTGCTCGTTGGACAGG-3'	115
Igf2	F: 5´- TGTTAGGAAGGTGCTCGGAG -3´ R: 5'- TGTAGAGCTCCAGACCTCCT -3'	219
Igf2R	F: 5´- AAGCTCTCACTTCCCTGCAT -3´ R: 5'- GAACTTCCCTCTTCTGGCCT -3'	203
Vegf	F: 5´- TATCTTCAAGCCGTCCTGTG -3´ R: 5'- TCTCCTATGTGCTGGCTTTG -3'	156
VegfR	F: 5´- TGCAGGAAACCATAGCAGGA -3´ R: 5'- GTATAGTCCCCTGCGTCCTC -3'	184

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Table 2 - Sequences of primer oligonucleotides for PCR amplification to evaluate methylation sensitive sites in promoters.

Target	Primer Sequences	Size (bp)
Vegf - IC	F: 5´- CCTCATAAGATCCTCATAAC -3´ R: 5' - AAAAGGTTACTCCACCATCT - 3'	116
Vegf - *Tai* I/ *BstU* I	F: 5´- CGGGGAGATCGTGAACTTGG -3´ R: 5' - AGCTGGCAAGGACGTATGGG - 3'	153
Igf2 - IC	F: 5´- TGGGGTGAGACAAAGAAATC -3´ R: 5' - TCCCATCCAGGTGTCAATAT - 3'	143
Igf2 - Prom *Tai* I/ *BstU* I	F: 5´- TAATCCTCTAACTGGGCACA -3´ R: 5' - ACTAAATCCTGGGTGTCCAT - 3'	159
Igf2 - DMR2 *BstU* I/ *Sac* II	F: 5´- ATTCGACACCTGGAGACAGT -3´ R: 5'- CTTTGGGTGGTAACACGATC -3'	149
Igf2 - CTCF6 *Tai* I/ *BstU* I	F: 5´- GACACTTGTCTTTCTGGAGG -3´ R: 5'- TATAGGAGTATGCTGCCACC -3'	138
Igf2 - CTCF7 *Tai* I/ *BstU* I	F: 5´- CATTTCTCGGGTAACTCCTTCG -3´ R: 5'- AACCCCAAATCTATGCCACG -3'	154

IC: Internal control

Table 3 - Concentrations of glucose, cholesterol and triglycerides in maternal serum samples

Group	Glucose (mmol/L)	Cholesterol (mmol/L)	Triglycerides (mmol/L)
CON	1.85 ± 0.11	2. 49 ± 0.18	1.86 ± 0.32
CAF	2.56 ± 0.17	2.12 ± 0.11	2.17 ± 0.23

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