Molecular and Cellular Endocrinology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Induction of uterine hyperplasia after cafeteria diet exposure

María Paula Gastiazoro[a], Marlise Guerrero-Schimpf[a], Milena Durando[a], Gisela Paola Lazzarino[a], María Florencia Andreoli[a], Oliver Zierau[b], Enrique Hugo Luque[a], Jorge Guillermo Ramos[a], Jorgelina Varayoud[a,*]

[a] Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral (UNL) – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina

[b] Molecular Cell Physiology and Endocrinology, Institute for Zoology, Technische Universität Dresden, 01062, Dresden, Germany

ARTICLE INFO

Keywords:
Uterus
Cafeteria diet
Hyperplasia
Estrogen receptor
Endometrial proliferation

ABSTRACT

Our aim was to evaluate whether chronic administration of CAF affects the uterus and induces the morphological and molecular changes associated with endometrial hyperplasia. Female Wistar rats exposed to CAF from weaning for 20 weeks displayed increased energy intake, body weight and fat depots, but did not develop metabolic syndrome. The adult uteri showed an increase in glandular volume fraction and stromal area. The epithelial proliferation rate and protein expression of oestrogen receptor alpha (ERα) were also increased. The CAF diet enhanced leptin serum levels and the long form of leptin receptor (Ob-Rb) mRNA expression in the uterus. No changes were detected in either insulin serum levels or those of insulin growth factor I (IGF-I) mRNA expression. However the levels of IGF-I receptor (IGF-IR) mRNA were lower in CAF-fed animals. Overall, the results indicate that our rat model of the CAF diet produces morphological and molecular changes associated with uterine hyperplasia and could predispose to endometrial carcinogenesis.

1. Introduction

Many risk factors of endometrial cancer have been described such as, early age at menarche, nulliparity, late-onset menopause, exposure to exogenous oestrogens (without a progesterone component), diabetes and obesity (Parazzini et al., 1991; Amant et al., 2005). Currently, many groups have demonstrated that obesity increases the risk of endometrial cancer (Thompson, 2010) but the role of each diet component is less clear. It has been speculated that Asian diets, typically characterized by a lower intake of fat and higher intake of fish, soy products, and cruciferous vegetables, compared with Western diets, may contribute to lower endometrial cancer risk (Messina et al., 2006).

Endometrial cancer is often preceded by the occurrence of precursor lesions and is accompanied by incessant oestrogen stimulation usually referred to as oestrogen dominance (Lacey and Chia, 2009; Kirschner et al., 1982). In normal conditions, the estrogenic effects initiate a succession of biochemical reactions in uterine cells in anticipation of the possible pregnancy, involving cell hypertrophy and hyperplasia (Nephew et al., 2000).

Oestrogens act mainly through oestrogen receptor alpha (ERα) to promote cell proliferation, differentiation and growth. ERα is expressed in all uterine cells, including glandular and luminal epithelium and mesenchyma (stromal and myometrial cells) (Wang et al., 2000). The oestrogen receptor beta (ERβ) plays a less dominant role in the mature uterus and only modifies the effects of ERα (Koehler et al., 2005). Together with oestradiol (E2), insulin-like growth factors I (IGF-I) and II (IGF-II) and their signalling pathways also play significant roles in the regulation of uterine growth and differentiation during the oestrous cycle. Estrogen boosts uterine IGF-I gene expression and provokes endometrial proliferation. The results of the actions of IGF-I and IGF-II are facilitated mainly by activation of the IGF-I receptor (IGF-IR) (McCampbell et al., 2008).

In addition, other circulating hormones regulate uterine functional differentiation. Fat tissue has been proved to be an endocrine organ that synthesizes and secretes polypeptide hormones and adipokines having the capacity to produce effects on the function of many tissues, among them the uterus. Leptin exerts direct effects on proliferation and invasion, as well as the production of angiogenic proteins in tumourous endometrial cells through the long form of leptin receptor (Ob-Rb) activation (Tartaglia et al., 1995; Carino et al., 2008; Gao et al., 2009).

* Corresponding author. Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina. Casilla de Correo 242, Santa Fe, Argentina.

E-mail addresses: paulagastiazoro@gmail.com (M.P. Gastiazoro), marliseluciana@gmail.com (M. Guerrero-Schimpf), mdurando@fbcb.unl.edu.ar (M. Durando), gplazzarino@fbcb.unl.edu.ar (G.P. Lazzarino), mfandreoli@fbcb.unl.edu.ar (M.F. Andreoli), oliver.zierau@tu-dresden.de (O. Zierau), eluque@fbcb.unl.edu.ar (E.H. Luque), gramos@fbcb.unl.edu.ar (J.G. Ramos), varayoud@fbcb.unl.edu.ar (J. Varayoud).

https://doi.org/10.1016/j.mce.2018.06.007
Received 7 March 2018; Received in revised form 12 June 2018; Accepted 13 June 2018

Abbreviations

CAF diet	cafeteria diet
E2	estradiol
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERK	extracellular signal-regulated kinase
GE	glandular epithelium
IGF-I	insulin growth factor I
IGF-II	insulin growth factor II
IGF-IR	insulin growth factor I receptor
IL-6	interleukin 6
IOD	integrated optical density
IR	insulin receptor
JAK	januskinases 2
LE	luminal epithelium
Ob-Rb	long form of leptin receptor
PS	periglandular stroma
RIA	radioimmunoassay
SS	subepithelial stroma
STAT3	ignal transducers and activators of transcription 3
Vim	vimentin

Besides, hyperleptinaemia is a frequent characteristic of obese women, who are more likely to develop endometrial cancer than women with normal weight, suggesting that the adipose tissue plays a direct role through the hormone leptin (Petridou et al., 2002).

It is well known that obesity could predispose to endometrial cancer, but the effect of chronic administration of a westernized diet was not determined. In our experiment, western diet habits are reflected by the cafeteria diet (CAF), an experimental rodent diet model composed by a variety of highly savoury and energy-dense foods with a prevalence in Western society (a life style linked to the current obesity pandemic) (Sampey et al., 2011). Recently, in our laboratory, Lazzarino et al. found that the CAF diet differentially modifies the expression of feeding-related genes by affecting the DNA methylation mechanisms in individual hypothalamic nuclei (Lazzarino et al., 2017). In another work, the CAF diet was linked with increased weight gain, abdominal fat, and serum interleukin 6 (IL-6) levels, in addition to more damage in the kidney (chronic interstitial inflammation and glomerular sclerosis), heart (coronary perivascular fibrosis and steatosis), and liver (liver weight, portal fibrosis, apoptosis, and steatosis) in comparison with a high fat diet (Zeeni et al., 2015).

In the present work, we speculate that chronic administration of the CAF diet might induce uterine changes associated with endometrial hyperplasia. According to this, we assessed possible changes caused by CAF diet on the uterine morphology, the expression of oestrogen-sensitive genes and epithelial cell proliferation. In addition, we determined the expression of IGF-I, IGF-IR and Ob-Rb as molecular regulators of endometrial proliferation.

2. Materials and methods

2.1. Animals

All procedures in this study were approved by the Institutional Ethic Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina) and were performed in accordance with the principles and procedures outlined in the Guide of the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences (Commission on Life Sciences, National Research Council, Institute of Laboratory Animal Resources, 1996).

2.2. Experimental design

Female Wistar rats were obtained at the Department of Human Physiology of the School of Biochemistry and Biological Sciences (UNL) where they were bred, weaned at 21 days of age, and randomly divided into two groups: control diet group (CON) (n = 6) and cafeteria diet group (CAF) (n = 6). The animals of the CON diet group were fed with a standard chow diet and the animals of the CAF diet group were fed with the diet described below. The diets were administered from weaning and for 20 weeks and water was administered *ad libitum*. Rats were housed two per cage and maintained in controlled conditions (22 ± 2 °C and 12-h light-dark cycle). The standard chow (Cooperación, ACA Nutrición Animal, Buenos Aires, Argentina) provided 3 kcal/g, 5% energy as fat, 23% protein and 72% carbohydrate. The CAF diet was composed of food items selected to reproduce the diversity, palatability, and energy density of the modern Western diet. The CAF diet incorporated standard chow, aside from french fries, parmesan cheese, cheese-flavored snacks, crackers, sweet biscuits, cookies, pudding, peanut butter, and chocolate. This diet supplied an average of 4.85 kcal/g, 49% of energy as fat, 7% as protein, and 44% as carbohydrate, in addition to that provide by standard chow. Three of the CAF foods were offered in excess quantities and were changed every day, by supplanting all the food with new items for more than two consecutive days. During the experimental time, body weights were registered once a week and food intake every day. Food intake was measured by the weight difference between the accessible 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) and the average intake.

The oestrous cycle was monitored by vaginal smears during two weeks to determine if the CAF diet alters the duration of particular phases of the oestrous cycle. Vaginal smears were obtained daily from lavage fluid collected by flushing the female's 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 by Montes & Luque (Montes and Luque, 1988).

All animals were weighed and sacrificed on the dioestrus stage of the oestrous cycle after 20 weeks of treatment. Trunk blood was collected, samples were centrifuged, and serum was immediately used or frozen and stored at −80 °C until further use. Perigonadal and retroperitoneal fat pads were isolated and weighed. The uteri were sampled, and one uterine horn from each rat was placed immediately in liquid nitrogen and stored at −80 °C for RNA extraction. The other uterine horn was fixed by immersion in 4% paraformaldehyde buffer for 6 h at 4 °C and processed for histological studies (morphometric and immunohistochemical analysis).

2.3. Serum assessments

Fasting serum metabolites (glucose, triglycerides, and cholesterol) were assessed by a commercially available assay (Wiener Laboratorios, Argentina). Serum insulin levels were estimated by radioimmunoassay (RIA) using an anti-rat insulin antibody (Sigma, St. Louis, Missouri, USA) and standard rat insulin provided by Laboratorios Beta (Buenos Aires, Argentina). The circulating levels of leptin were determined by specific RIA (Giovambattista et al., 2006). Total E2 levels were measured using competitive RIA kits (Immunotech, Marseille, France) (Matthews et al., 1985).

2.4. Immunohistochemistry

A standard immunohistochemical technique was performed, following protocols previously described by our laboratory (Muñoz-de-Toro et al., 1998). Briefly, uterine longitudinal sections (5 μm thick)

were deparaffinized and rehydrated in graded ethanol. After microwave pretreatment for antigen retrieval, the endogenous peroxidase activity and nonspecific binding sites were blocked. Samples were incubated in a humid chamber with the specific primary antibody (overnight at 4 °C) and then with the corresponding biotin-conjugated secondary antibody (30 min at room temperature). Reactions were developed using the avidin-biotin-peroxidase method and diaminobenzidine (DAB) (Sigma-Aldrich) as a chromogen substrate. Samples were dehydrated and mounted with permanent mounting medium (Eukitt, Sigma-Aldrich). A monoclonal antibody against Ki67 was used to evaluate epithelial cell proliferation and the expression of steroid hormone receptors ERα and progesterone receptor (PR) was performed using specific antibodies described in Table 1. For Ki67 immunodetection, the samples were counterstained with Mayer haematoxylin (Biopur, Rosario, Argentina). Each immunohistochemical run included negative controls in which the primary antibody was replaced by non-immune horse serum (Sigma-Aldrich).

2.5. Histological analysis

2.5.1. Determination of the glandular volume fraction

Uterine samples embedded in paraffin were cut into 5-µm sections, mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma-Aldrich) and stained with haematoxylin and eosin for light microscopy (Olympus BH2, Tokyo, Japan). The volume fraction of uterine glands was calculated by applying the formula given by Weibel (1969): $Vv = Pi/P$, where *Vv* is the estimated volume fraction of the object under study, *Pi* is the number of incidents points over glands, and *P* is the number of incidents points over all cells in the studied population (stroma). To obtain the data for the point-counting procedure, a glass disk with a squared grid of 0.8 mm × 0.8 mm was inserted into a focusing eyepiece. The results were expressed as *Vv x 100*.

2.5.2. Determination of the stromal area

Vimentin is a cytoskeletal protein expressed in mesenchymal-derived cells. To estimate the uterine stromal area, vimentin protein expression was evaluated. A standard immunohistochemical technique was developed using an antibody against vimentin. The expression of vimentin was quantified in the periglandular stroma (PS) zone defined as the 20-µm-wide area around the glands, and in the subepithelial stroma (SS) zone defined as the 300-µm-wide area adjacent to the luminal epithelium, from the basement membrane toward the outer layers. The image analysis system and process are described below (item 2.7 of the M&M).

2.6. Quantification of cell proliferation

Tissue sections were evaluated using an Olympus BH2 microscope with a Dplan 40X objective (numerical aperture = 0.65; Olympus). The proliferation indices of the luminal epithelium and the glandular epithelium were quantified by considering the percentage of epithelial Ki67-positive cells in a total of 2000 cells/section.

2.7. Quantification of protein expression by image analysis

The expression of ERα, PR and vimentin proteins in the uterine cells was evaluated by image analysis, using Fiji of Image J as previously described (Ramos et al., 2002). Briefly, the images were recorded with a Spot Insight V3.5 colour video camera, attached to a microscope (Olympus) and converted to a grey scale. The integrated optical density (IOD) was measured as a linear combination of the average grey intensity and the relative area occupied by the positive cells (Ramos et al., 2001, 2002). Because the IOD is a dimensionless parameter, the results were expressed as arbitrary units. The IODs of ERα and PR were evaluated in the luminal and glandular epithelium (LE and GE, respectively) of each tissue section, and in the SS (subepithelial stroma; 300-µm-wide area adjacent to the epithelium, from the basement membrane toward the outer layers). The IOD of vimentin was evaluated in the SS and PS, as mentioned above. At least 10 fields of each histological compartment were recorded in each section, and two sections per animal were evaluated. Correction of unequal illumination (shading correction) and measurement system calibration were performed with a reference slide.

2.8. RNA extraction and reverse transcription

Individual uterine horns 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.

2.9. Quantitative real-time polymerase chain reaction

The mRNA expression of IGF-I, IGF-IR and Ob-Rb 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 2. 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 (Higuchi et al., 1993). The C_T for each sample was calculated using the Step One Software (Applied Biosystems Inc. Foster City, CA, USA) 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 (Cikos et al., 2007).

Table 1
Antibodies used for immunohistochemistry.

Antibodies	Dilution	Supplier
Primary		
Anti-Ki67 (clone MIB-5)	1/50	Dako Corp. (Carpinteria, CA, USA)
Anti-Vimentin (clone V9)	1/50	Novocastra (Newcastle upon Tyne, UK)
Anti-ERα (clone 6F-11)	1/100	Novocastra (Newcastle upon Tyne, UK)
Anti-PR (A0098)	1/200	Dako Corp. (Carpinteria, CA, USA)
Secondary		
Anti-mouse (B8774)	1/100	Sigma (St. Louis, MO)
Anti-rabbit (B8895)	1/200	Sigma (St. Louis, MO)

Table 2
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
Ob-Rb	F: 5′- AGGATGAGTGTCAGAGTCAA- 3′ R: 5′- CTCTTCATCAGTTTCCACTG- 3′	80
IGF-I	F: 5′- CTCAAGGATGGCGTCTTCAC- 3′ R: 5′- GAACTTGCTCGTTGGACAGG- 3′	137
IGF-IR	F: 5′- CTCAAGGATGGCGTCTTCAC- 3′ R: 5′- GAACTTGCTCGTTGGACAGG- 3′	115

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.

2.10. Statistics

Sample size was determined using G Power software (Faul et al., 2007). In all cases the actual power value was higher than 0.90. The results are expressed as the mean ± SD. All data were analysed using a Mann-Whitney *U* test using the IBM SPSS Statistics 19 software (IBM Inc.). $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*) were accepted as significant.

3. Results

3.1. CAF diet influences on body weight, adipose tissue and food intake

The group of animals that received the CAF diet showed normal growth, without differences with the control animals, from the beginning of treatment until week 13 (Fig. 1). The weight of the CAF-fed animals then started to show an increase at 14 weeks of treatment (weights in week 14: CON: 215.8 g ± 4.35 vs CAF: 229.8 g ± 13.00, $p < 0.05$). In addition, the CAF diet also increased the perigonadal and retroperitoneal fat depots expressed as a percentage of body weight ($p < 0.01$, Fig. 1). These results are in accordance with the 21% increase in energy intake found in CAF-fed rats, which presented a higher daily caloric intake throughout the experiment. Nevertheless, the rats on the CAF diet were not hyperphagic as they showed a decrease in their daily food intake, defined by the weight of food, in comparison with that of the control animals. Thus, the elevated energy uptake resulted from the high-energy content of the palatable food.

3.2. CAF diet effects on metabolic and endocrine endpoints

In relation to metabolic parameters, we detected that serum leptin levels were increased four-fold in CAF-fed rats (CON: 1.80 ± 1.26 ng/ml, CAF: 6.99 ± 4.11 ng/ml, $p < 0.001$) (Table 3). No changes were detected in the serum levels of insulin or in those of any other metabolic parameters. The CAF diet did not alter the day of the first vaginal opening, which was from postnatal day 40–45 in both groups. In addition, CAF-fed animals exhibited neither alterations in serum E2 levels nor in the oestrous cycle. The oestrous cycle lasted 5 days in all animals in both groups, without differences in the length of each phase.

3.3. CAF diet induced uterine morphological changes

The uteris of control animals revealed typical morphological features of the dioestrous stage: a simple columnar luminal epithelium, simple tubular glands lined with simple cuboidal epithelium, being surrounded with a stratified endometrial stroma and two typical layers of myometrium (Fig. 2 A). CAF-fed rats showed morphological changes that resembled uterine hyperplasia. An increased glandular volume fraction (CON: 4.77 ± 0.76vs CAF: 8.27 ± 1.87, $p < 0.001$) (Fig. 2A and B) was observed. Moreover, a higher expression of vimentin was detected in both the SS and the PS (Fig. 3A and B).

3.4. CAF diet deregulates the expression of ERα

We determined the expression of two classical nuclear steroid hormone receptors, ERα and PR. CAF-fed animals showed deregulation of ERα. The CAF diet increased ERα expression in all uterine compartments: SS, GE and LE (Fig. 4A and B). PR expression was not affected by CAF treatment.

3.5. CAF diet induces an increase of uterine cell proliferation

To determine if the CAF diet affects uterine cell proliferation, we quantified Ki67 expression in glandular and luminal epithelial cells. The results indicated that CAF-fed animals showed a higher level of Ki67 expression in both compartments: GE: CON: 63.52 ± 11.46% vs CAF: 76.06 ± 3.62% ($p < 0.05$) and LE: CON: 40.64 ± 11.53% vs CAF: 68.11 ± 5.38% ($p < 0.01$) (Fig. 5A and B).

3.6. CAF diet alters the expression of molecules related to uterine proliferation control

The higher proliferation of CAF uterine cells led us to evaluate which molecular pathways could be affected. Therefore, we decided to evaluate IGF-I and IGF-IR expression at the transcriptional level. CAF-fed animals showed a decreased uterine IGF-IR expression, without changes in IGF-I expression (Fig. 6A and B). In addition, we evaluated

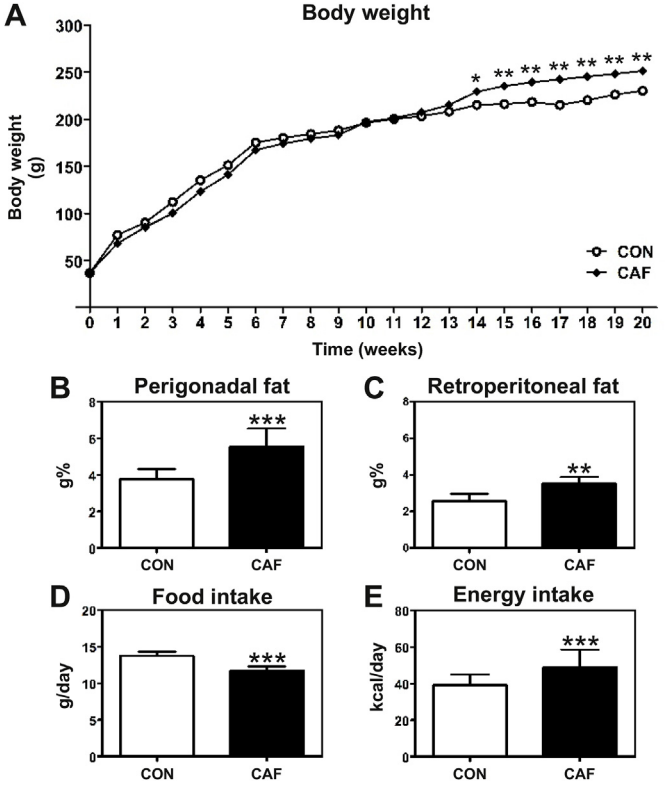

Fig. 1. (A) Body weight of rats fed with standard chow (CON) or cafeteria diet (CAF) for 20 weeks. Perigonadal fat (B), retroperitoneal fat (C), expressed as a percentage of body weight. Food intake (D) and energy intake (E), expressed as g/day and kcal/day, respectively. Values in the bar graph are the mean ± SD (6 rats per group). Data were analysed with the Mann Whitney Test. **, $p < 0.01$; ***, $p < 0.001$ vs. the control group.

Table 3
Serum parameters and hormones of rats fed with control diet (CON) or a Cafeteria diet (CAF).

Parameter	CON	CAF	p value
Triglycerides (mmol/l)	103.29 ± 35.23	83.55 ± 30.93	0.26
Cholesterol (mmol/l)	186.19 ± 24.67	195.71 ± 13.49	0.49
Glucose (mmol/l)	6.22 ± 0.57	6.03 ± 0.38	0.36
Insulin (mU/l)	29.12 ± 10.61	33.46 ± 20.19	0.60
Leptin (ng/ml)	1.80 ± 1.26	6.99 ± 4.11***	< 0.001
Estradiol (ng/ml)	0.026 ± 0.009	0.023 ± 0.009	0.45

CON: Control Diet; CAF: Cafeteria Diet.
Data shown are presented as the mean ± SD (n = 10/group). *** indicates significant differences at $p < 0.001$; Mann-Whitney test.

uterine Ob-Rb because it is well known that leptin exerts direct effects on proliferation in cancerous endometrial cells across Ob-Rb stimuli. We found that CAF-fed animals showed an increased expression of uterine Ob-Rb (Fig. 6C).

4. Discussion

Several risk factors for endometrial cancer are associated with different life style factors. Since obesity is a conclusive risk factor for endometrial cancer, increased dietary fat uptake was hypothesized to be associated with endometrial cancer risk. Currently, epidemiological studies concerning the subject are unconvincing (Jochems et al., 2018; Welti et al., 2017; Ollberding et al., 2012). Some results indicate a positive association between total and saturated fat intake and endometrial cancer. Cohort studies indicate that higher monounsaturated fatty acid intake is associated with a reduced risk of developing endometrial cancer (Zhao et al., 2016).

All data published until today, suggest that additional studies on dietary patterns are necessary (Grosso et al., 2017). It is imperative to investigate whether life-long dietary habits may contribute to disease risk. The present study was created to estimate the possible impact of the westernized diet on the uterus through chronic administration of the CAF diet. We chose the CAF diet because it reflects the daily food consumption of the Western population, as it, among other reasons, is a more palatable diet.

In previous studies the CAF diet produced metabolic changes, including an increase in triglycerides, glucose, insulin, and total cholesterol levels (Goularte et al., 2012; Sagae et al., 2012; Lalanza et al., 2014). In our experiment, the CAF diet generated slight obesity, which was due in part to an increased energy intake, fat pads and body weight, but no development of metabolic syndrome. These apparently discrepancies could be explained because the different composition of CAF diets used.

Our CAF diet is composed of lower protein content and higher fat content, compared to those of other CAF diets. Different reports indicate that a lower dietary protein content could affect normal growth (de Oliveira et al., 2018; Furuta and Murakami, 2018; Arsenault and Brown, 2017). In fact, using a model of male rats fed with a low-protein diet, protein reduction at puberty contributed to energy and metabolic disorders with long-term consequences (de Oliveira et al., 2018). In our study, the growth of the CAF rats from the beginning of the experiment until week 13 was slightly lesser than that of the control rats, but not statistically different. Perhaps, the lower protein content was compensated for the high fat content, producing a significant increase in body weight, detected after week 14. Another difference between our study and other similar studies is the timing of diet exposure and the susceptibility of rat strains. In relation to reproductive parameters, some authors indicated that the CAF diet induces reproduction impairment (Bazzano et al., 2015, 2017). They determined that the CAF diet produces a lower E2 secretion as a central failure related to the impairment ovulation. They propose that the lower E2 level may induce a prolonged dioestrus in the rat, leading to reproductive dysfunction (Bazzano et al., 2015). Another published work found no differences in E2 serum levels but detected follicle-derived preovulatory progesterone surge inhibition during the pro-oestrus phase and, consequently, inhibition of the preovulatory gonadotropin surge (Sagae et al., 2012). In the present work, E2 serum levels, the time of vaginal opening and the oestrous cycle were not affected. However, differences related to diet composition, time of exposure and/or strain of rats should not be minimized when interpreting the results presented herein.

As we mentioned before, the primary goal of our work was to investigate if the chronic administration of the CAF diet might induce the uterine effects associated with endometrial hyperplasia. We detected different morphological and molecular changes that indicated that the

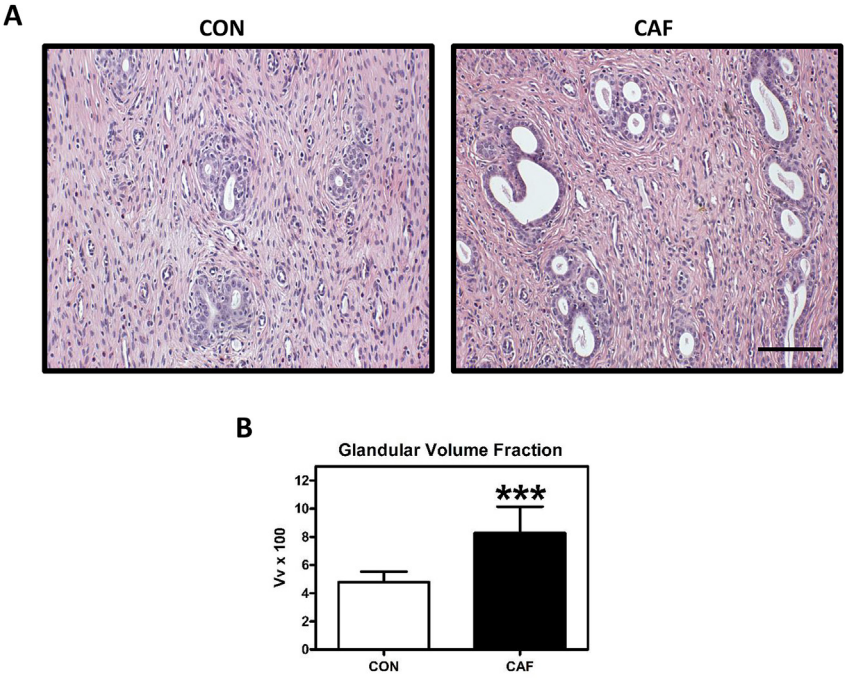

Fig. 2. (A) Representative photomicrographs of uterus staining with Haematoxylin-Eosin of control and CAF rats. Scale bar: 50 μm. (B) Quantification of the glandular volume fraction. The results are expressed as Vvx100. Values in the bar graph are the mean ± SD (6 rats per group). Data were analysed with the Mann Whitney Test. ***, $p < 0.001$ vs. the control group.

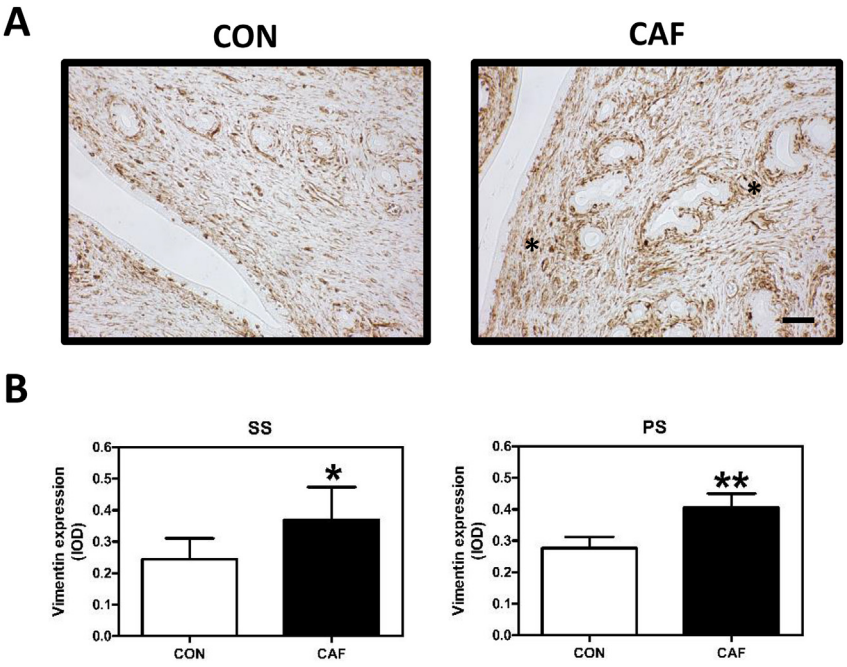

Fig. 3. (A) Representative photomicrographs of vimentin expression in control and CAF rats. Scale bar: 50 μm. (B) Quantification of vimentin expression in subepithelial (SS) and periglandular (PS) stroma. The results are expressed as IOD. Values in the bar graph are the mean ± SD (6 rats per group). Data were analysed with the Mann Whitney Test. *, $p < 0.05$; **, $p < 0.01$ vs. the control group.

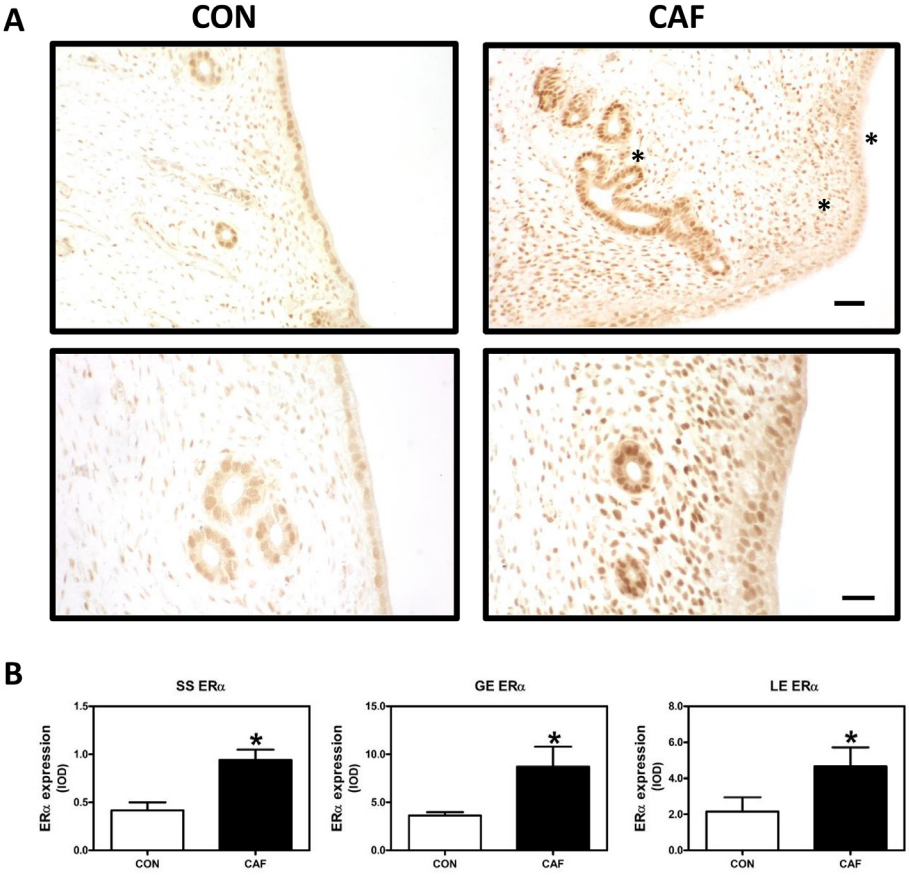

Fig. 4. (A) Representative photomicrographs of immunohistochemical detection of ERα on SS, GE and LE. Scale bar: 50 μm. (B) Quantification of ERα expression. The results are expressed as IOD. Values in the bar graph are the mean ± SD (6 rats per group). Data were analysed with the Mann Whitney Test. *, $p < 0.05$ vs. the control group.

CAF diet affects the normal uterine functional differentiation during the oestrous cycle. Few studies have shown a close relationship between the higher ERα uterine expression and the endometrial hyperplasia (Chakraborty et al., 2005; Pieczynska et al., 2011). ERα is almost exclusively situated in the nucleus and is involved in the most important mechanism of oestrogen action–the genomic mechanism responsible for cell growth and proliferation. The gradual increase in ERα density in normal proliferative endometrium, which is greater in simple hyperplasia, and is maximal in complex endometrial hyperplasia, proves the growth and proliferative effect of activated ERα. The fact that ERα density decreases in atypical endometrial hyperplasia, which is much smaller in low-grade and even greater in high-grade adenocarcinomas, demonstrates that ERα is involved only in growth and proliferation of cells with conserved architecture (normal or benign hyperplasic cells).

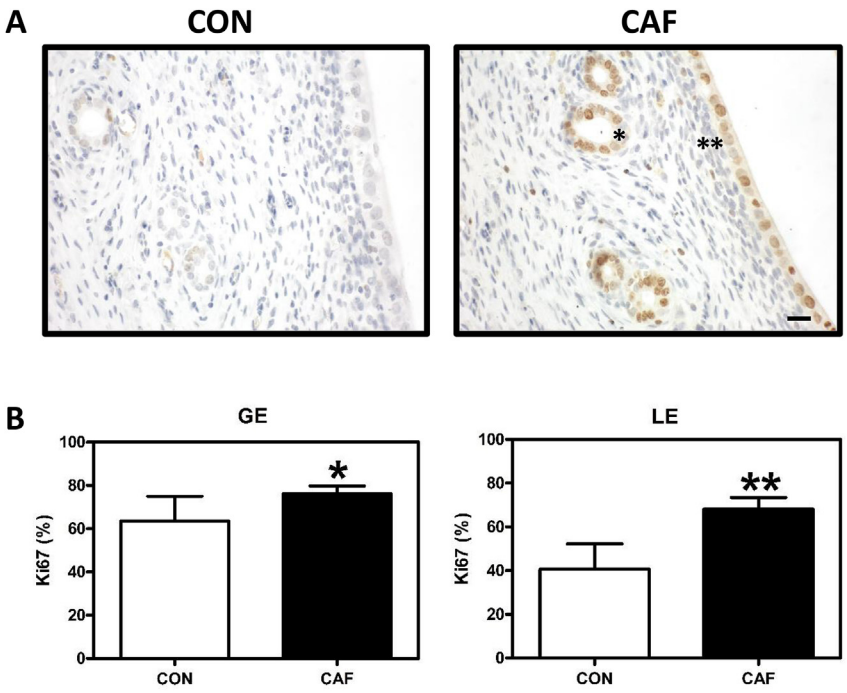

Fig. 5. (A) Representative photomicrographs of immunohistochemical detection of Ki67 on LE and GE. Scale bar: 50 μm. (B) Quantification of Ki67 expression. Results are expressed as percentage. Values in bar graph are mean ± SD (6 rats per group). Data were analysed with the Mann Whitney Test. *, $p < 0.05$; **, $p < 0.01$ vs the control group.

Atypical, especially malignant cells, expressed significantly less ERα, where lower expression is a sign of poor prognosis and/or a high-grade lesion (Chakraborty et al., 2005; Tica et al., 2016). In CAF animals, we detected an increase of glandular area and stromal compartments accompanied by an elevated proliferation rate and ERα expression.

Leptin and insulin are defined as regulators of endometrial proliferation (Villavicencio et al., 2010). Previous studies indicate that circulating insulin/IGF as well as the sex hormone axes are significantly dysregulated by obesity and diabetes and during endometrial cancer development (Merritt et al., 2016). Where as insulin predominantly acts through the insulin receptor (IR), IGFs bind to the IGF-IR, the IR, and the hybrid IR/IGF-IR (Pollak, 2012; Brouwer-Visser and Huang, 2015). Oestrogen and insulin/IGF signalling result in downstream mitogenic and anti-apoptotic effects and converge on the AKT signalling pathway. Different expression levels of these pathway components were detected between pre and post-menopausal women. In the postmenopausal women IGF-II showed a lower expression compared with that in the premenopausal proliferative phase (Lacey and Chia, 2009). The same result was detected in our CAF-fed rats, with a lower expression of IGF-IR compared with that of the control rats. However, the insulin serum level and the IGF-I mRNA expression did not change in CAF animals. Given that CAF animals were not obese or diabetic, we propose that changes in leptin levels could explain the induction of endometrial hyperplasia.

Previous results indicate that significantly high serum leptin levels are a usual characteristic of individuals with endometrial hyperplasia and cancer in comparison to women having normal endometrium. This characteristic correlates to their body mass indices. Leptin has been demonstrated to exert significant effects on endometrial proliferation (Cymbaluk et al., 2008). Leptin has been shown to have, through Ob-Rb activation, immediate effects on proliferation, production of angiogenic proteins and invasion of malignant endometrial cells (Tartaglia et al., 1995; Gao et al., 2009; Amjadi et al., 2016). It is well know that endometrium has leptin receptors. In our experiment, CAF animals showed an increase in serum leptin levels together with a high expression of Ob-Rb mRNA. Previous results indicate that the Ob-Rb overexpression in uterine hyperplasia may increase its susceptibility to malignancy in obese patients as a consequence of the hyperleptinaemia (Mendez-Lopez et al., 2017).

In the uterus, epithelial-mesenchymal crosstalk also plays an important role in the development of epithelial lesions. Confirmation of the control exerted by the stroma on differentiation through different tissue recombination studies (mixing uterine or vaginal stroma and epithelia) has revealed that the fate of epithelial cells depends on stromal/mesenchymal signalling (Kurita et al., 2001). In mice, the stromal compartment is sufficient to induce endometrial cancer, and

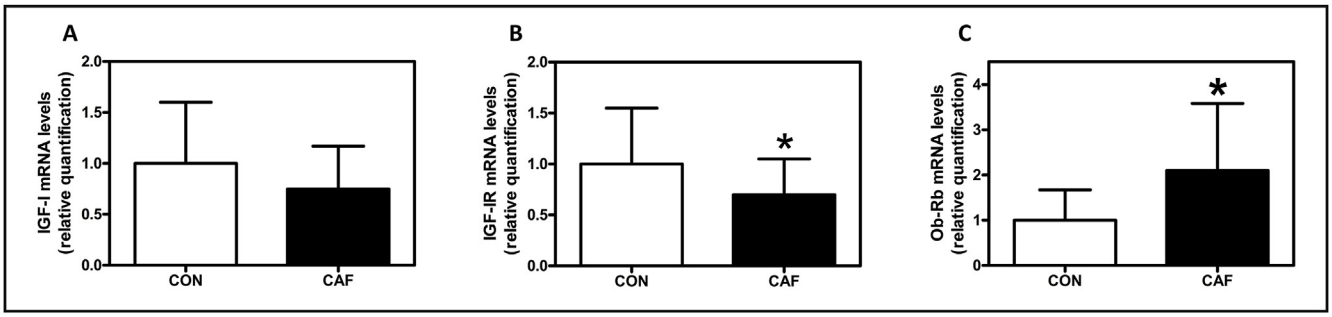

Fig. 6. Expression of molecules related to proliferation control. Relative expression of IGF-I, IGF-IR and Ob-RbmRNA levels (A, B and C, respectively). Samples were normalized to L19 mRNA expression, and the relative amounts of mRNA were calculated using the standard curve method. Each column represents the mean ± SD (6 rats per group). *, $p < 0.05$ vs. the control group.

comparable changes in human endometrial cancer patients are observable, suggesting that mesenchymal cells play an important role in the aetiology of endometrial cancer (Tanwar et al., 2011). According to the World Health Organization 1994, an abundant cellular stroma is a histological change present in the endometrial hyperplasia (Sanderson et al., 2017). In our study, we detected that peri-glandular stroma and subepithelial stroma areas increased in CAF-fed rats. Taking into account the role of stromal cells in epithelial lesions, our results indicate that the changes detected in the stromal cells could contribute to epithelial proliferation detected in CAF-fed rats. Some mediators of stromal control have been proposed (Senol et al., 2016; Tanwar et al., 2012). The dysregulation of the signalling of the mammalian target of rapamycin complex 1 (mTORC1) in stromal cells plays an important role in the pathogenesis of uterine diseases (Tanwar et al., 2012).

Endometrial hyperplasia is a central clinical topic, as it has the potential to transform into cancer. In this sense, is crucial to take life style into consideration because it could generate endometrial hyperplasia. Thus, we believe that in our animals, the CAF diet (and probably the increased level of leptin), but not obesity (which is absent in our animals), could be producing the endometrial hyperplasia. In future studies we would like to evaluate the gene pathways affected by the leptin hormone to induce the endometrial proliferation. In vitro studies indicate that the knockdown of Ob-Rb disrupts the capacity of leptin to promote cell growth. Leptin stimulates endometriotic epithelial cells Janus Kinases 2 (JAK2) activation, signal transducers, as well as the activation of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK) (Oh et al., 2013). In future studies, we propose to determine if these signal transducers could be affected in CAF fed animals as one possible mechanism to explain the endometrial hyperplasia.

Declaration of interest

The authors declare they have no actual or potential competing financial interests.

Acknowledgments

We thank Juan Grant and Juan C. Villarreal for technical assistance and animal care.

This work was supported by grants from the Universidad Nacional del Litoral (CAI+D 50420150100085LI) and the Argentine National Agency of Scientific and Technological Promotion (ANPCyT) (PICT 1522). These funding sources had no involvement in study design; collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication. MPG is a fellow of ANPCyT and MGS and GL are fellows of CONICET. EHL, MD, JGR and JV are Career Investigators of the CONICET. OZ is fellow at the Molecular Cell Physiology & Endocrinology, Faculty of Biology, Technische Universität Dresden.

References

Amant, F., Moerman, P., Neven, P., Timmerman, D., Van Limbergen, E., Vergote, I., 2005. Endometrial cancer. Lancet 366, 491–505.

Amjadi, F., Mehdipoor, R., Zarkesh-Esfahani, R., Javanmard, H., 2016. S.H.Leptin serves as angiogenic/mitogenic factor in melanoma tumor growth. Adv. Biomed. Res. 5 2277–9175.

Arsenault, J.E., Brown, K.H., 2017. Effects of proteins or amino-acid supplementation on the physical growth of young children in low-income countries. Nutr. Rev. 75, 699–717.

Bazzano, M.V., Torelli, C., Pustovrh, M.C., Paz, D.A., Elia, E.M., 2015. Obesity induced by cafeteria diet disrupts fertility in the rat by affecting multiple ovarian targets. Reprod. Biomed. Online 31, 655–667.

Bazzano, M.V., Paz, D.A., Elia, E.M., 2017. Obesity alters the ovarian glucidic homeostasis disrupting the reproductive outcome of female rats. J. Nutr. Biochem. 42, 194–202.

Brouwer-Visser, J., Huang, G., 2015. S.IGF2 signaling and regulation in cancer. Cytokine Growth Factor Rev. 26, 371–377.

Carino, C., Olawaiye, A.B., Cherflis, S., Serikawa, T., Lynch, M.P., Rueda, B.R., Gonzalez, R.R., 2008. Leptin regulation of proangiogenic molecules in benign and cancerous endometrial cells. Int. J. Canc. 123, 2782–2790.

Chakraborty, S., Khurana, N., Sharma, J.B., Chaturvedi, K.U., 2005. Endometrial hormone receptors in women with dysfunctional uterine bleeding. Arch. Gynecol. Obstet. 272, 17–22.

Cikos, S., Bukovská, A., Koppel, J., 2007. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC. Mol. Biol. 8, 113.

Cymbaluk, A., Chudecka-Glaz, A., Rzepka-Górska, I., 2008. 2008. Leptin levels in serum depending on Body Mass Index in patients with endometrial hyperplasia and cancer. Eur. J. Obstet. Gynecol. Reprod. Biol. 136, 74–77.

de Oliveira, J.C., de Moura, E.G., Miranda, R.A., de Moraes, A.M.P., Barella, L.F., da Conceicao, E.P.S., et al., 2018. Low-protein diet in puberty impairs testosterone output and energy metabolism in male rats. J. Endocrinol. 237, 243–254.

Faul, F., Erdfelder, E., Lang, A.G., Buchner, A., 2007. G*Power: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav. Res. Meth. 39, 175–191.

Furuta, C., Murakami, H., 2018. A novel concept of amino acid supplementation to improve the growth of young malnourished male rats. Ann. Nutr. Metab. 72, 231–240.

Gao, J., Tian, J., Lv, Y., Shi, F., Kong, F., Shi, H., Zhao, L., 2009. Leptin induces functional activation of cyclooxygenase-2 through JAK2/STAT3, MAPK/ERK, and PI3K/AKT pathways in human endometrial cancer cells. Canc. Sci. 100, 389–395.

Giovambattista, A., Piermaría, J., Suescun, M.O., Calandrea, R.S., Gaillard, R.C., Spinedi, E., 2006. Direct effect of ghrelin on leptin production by cultured rat white adipocytes. Obesity 14, 19–27.

Goularte, J.F., Ferreira, M.B., Sanvitto, G.L., 2012. Effects of food pattern change and physical exercise on cafeteria diet-induced obesity in female rats. Br. J. Nutr. 108, 1511–1518.

Grosso, G., Bella, F., Godos, J., Sciacca, S., Del Rio, D., Ray, S., Galvano, F., Giovannucci, E.L., 2017. Possible role of diet in cancer: systematic review and multiple meta-analyses of dietary patterns, lifestyle factors, and cancer risk. Nutr. Rev. 75, 405–419.

Higuchi, R., Fockler, C., Dollinger, G., Waston, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11, 1026–1030.

Jochems, S.H.J., Van Osch, F.H.M., Bryan, R.T., Wesselius, A., van Schooten, F.J., Cheng, K.K., Zeegers, M.P., 2018. Impact of dietary patterns and the main food groups on mortality and recurrence in cancer survivors: a systematic review of current epidemiological literature. BMJ. Open. 8, e014530.

Kirschner, M.A., Schneider, G., Ertel, N.H., Worton, E., 1982. Obesity, androgens, estrogens, and cancer risk. Canc. Res. 42, 3281–3285.

Koehler, K.F., Helquero, L.A., Haldosén, L.A., Warner, M., Gustafsson, J.A., 2005. Reflections on the discovery and significance of estrogen receptor beta. Endocr. Rev. 26, 465–478.

Kurita, T., Cooke, P.S., Cunha, G.R., 2001. Epithelial-stromal tissue interaction in paramesonephric (Mullerian) epithelial differentiation. Dev. Biol. 240, 194–211.

Lacey, J.V., Chia, V.M., 2009. 2009. Endometrial hyperplasia and the risk of progression to carcinoma. Maturitas 63, 39–44.

Lalanza, J.F., Caimari, A., del Blas, J.M., Torregrosa, D., Cigarroa, I., Pallàs, M., Capdevila, L., Arola, L., Escorihuela, R.M., 2014. Effects of a post-weaning cafeteria diet in young rats: metabolic syndrome, reduced activity and low anxiety-like behavior. PLoS One 9, e85049.

Lazzarino, G.P., Andreoli, M.F., Rossetti, M.F., Stoker, C., Tschopp, M.V., Luque, E.H., Ramos, J.G., 2017. 2017. Cafeteria diet differentially alters the expression of feeding-related genes through DNA methylation mechanisms in individual hypothalamic nuclei. Mol. Cell. Endocrinol. 450, 113–125.

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., Turner, R.C., 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28, 412–419.

McCampbell, A.S., Walker, C.L., Broaddus, R.R., Cook, J.D., Davies, P.J., 2008. Developmental reprogramming of IGF signaling and susceptibility to endometrial hyperplasia in the rat. Lab. Invest. 88, 615–626.

Mendez-Lopez, L.F., Zavala-Pompa, A., Cortés-Gutiérrez, E.I., Cerda-Flores, R.M., Davila-Rodriguez, M.I., 2017. Leptin receptor expression during the progression of endometrial carcinoma is correlated with estrogen and progesterone receptors. Arch. Med. Sci. 13, 228–235.

Merritt, M.A., Strickler, H.D., Einstein, M.H., Yang, H.P., Sherman, M.E., Wentzensen, N., Brouwer-Visser, J., Cossio, M.J., Whitney, K.D., Yu, H., Gunter, M.J., Huang, G., 2016. S.Insulin/IGF and sex hormone axes in human endometrium and associations with endometrial cancer risk factors. Cancer Causes Control 27, 737–748.

Messina, M., Nagata, C., Wu, A.H., 2006. Estimated Asian adult soy protein and isoflavone intakes. Nutr. Canc. 55, 1–12.

Montes, G.S., Luque, E.H., 1988. Effects of ovarian steroids on vaginal smears in the rat. Acta Anat. 133, 192–199.

Muñoz-de-Toro, M.M., Maffini, M.V., Kass, L., Luque, E.H., 1998. Proliferative activity and steroid hormone receptor status in male breast carcinoma. J. Steroid Biochem. Mol. Biol. 67, 333–339.

Nephew, K.P., Long, X., Osborne, E., Burke, K.A., Ahluwalia, A., Bigsby, R.M., 2000. Effect of estradiol on estrogen receptor expression in rat uterine cell types. Biol. Reprod. 62, 168–177.

Oh, H.K., Choi, Y.S., Yang, Y.I., Kim, J.H., Leung, P.C., Choi, J.H., 2013. Leptin receptor is induced in endometriosis and leptin stimulates the growth of endometriotic epithelial cells through the JAK2/STAT3 and ERK pathways. Mol. Hum. Reprod. 19, 160–168.

Ollberding, N.J., Lim, U., Wilkens, L.R., Setiawan, V.W., Shvetsov, Y.B., Henderson, B.E., Kolonel, L.N., Goodman, M.T., 2012. Legume, soy, tofu, and isoflavone intake and endometrial cancer risk in postmenopausal women in the multiethnic cohort study. J. Natl. Cancer Inst. 104, 67–76.

Parazzini, F., La Vecchia, C., Bocciolone, L., Franceschi, S., 1991. The epidemiology of endometrial cancer. Gynecol. Oncol. 41, 1–16.

Petridou, E., Belechri, M., Dessypris, N., Koukoulomatis, P., Diakomanolis, E., Spanos, E., Trichopoulos, D., 2002. 2002. Leptin and body mass index in relation to endometrial

cancer risk. Ann. Nutr. Metab. 46, 147–151.
Pieczynska, B., Wojtylak, S., Zawrocki, A., Biernat, W., 2011. Analysis of PTEN, estrogen receptor alpha and progesterone receptor expression in endometrial hyperplasia using tissue microarray. Pol. J. Pathol. 62, 133–138.
Pollak, M., 2012. The insulin and insulin-like growth factor receptor family in neoplasia: an update. Nat. Rev.Cancer 12, 159–169.
Ramos, J.G., Varayoud, J., Sonnenschein, C., Soto, A.M., Muños-de-Toro, M., Luque, E.H., 2001. Prenatal exposure to low doses of bisphenol A alters the periductal stroma and glandular cell function in the rat ventral prostate. Biol. Reprod. 65, 1271–1277.
Ramos, J.G., Varayoud, J., Bosquiazzo, V.L., Luque, E.H., Muñoz-de-Toro, M., 2002. Cellular turnover in the rat uterine cervix and its relationship to estrogen and progesterone receptor dynamics. Biol. Reprod. 67, 735–742.
Sagae, S.C., Menezes, E.F., Bonfleur, M.L., Vanzela, E.C., Zacharias, P., Lubaczeuski, C., Franci, C.R., Sanvitto, G.L., 2012. Early onset of obesity induces reproductive deficits in female rats. Physiol. Behav. 105, 1104–1111.
Sampey, B.P., Vanhoose, A.M., Winfield, H.M., Freemeran, A.J., Muehlbauer, M.J., Fueger, P.T., Newgard, C.B., Makowski, L., 2011. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. Obesity 19, 1109–1117.
Sanderson, P.A., Critchley, H.O.D., Williams, A.R.W., Arends, M.J., Saunders, P.T.K., 2017. New concepts for an old problem: the diagnosis of endometrial hyperplasia. Hum. Reprod. Update 23, 232–254.
Senol, S., Sayar, I., Ceyran, A.B., Ibiloglu, I., Akalin, I., Firat, U., Kosemetin, D., Engin Zerk, P., Aydin, A., 2016. Stromal clues in endometrial carcinoma: loss of expression of β-catenin, epithelial-mesenchymal transition regulators, and estrogen-progesterone receptor. Int. J. Gynecol. Pathol. 35, 238–248.
Tanwar, P.S., Zhang, L., Roberts, D.J., Teixeira, J.M., 2011. Stromal deletion of the APC tumor suppressor in mice triggers development of endometrial cancer. Canc. Res. 71, 1584–1596.
Tanwar, P.S., Kaneko-Tarui, T., Zhang, L., Tanaka, Y., Crum, C.P., Teixeira, J.M., 2012. Stromal liver kinase B1 [STK11] signaling loss indices oviductal adenomas and endometrial cancer by activating mammalian target of rapamycin complex 1. PLoS Genet. 8, e1002906.
Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Wool, E.A., Monroe, C.A., Tepper, R.I., 1995. Identification and expression cloning of a leptin receptor, OB-R. Cell 83, 1263–1271.
Thompson, R., 2010. Preventing cancer: the role of food, nutrition and physical activity. J. Fam. Health Care 20, 100–102.
Tica, A.A., Tica, O.S., Georgescu, C.V., Pirici, D., Bogdan, M., Ciurea, T., Mogoanta, S.Ş., Georgescu, C.C., Comanescu, A.C., Balşeanu, T.A., Ciurea, R.N., Osiac, E., Buga, A.M., Ciurea, M.E., 2016. GPER and ERalpha expression in abnormal endometrial proliferations. Rom. J. Morphol. Embryol. 57, 413–418.
Villavicencio, A., Aguilar, G., Argüello, G., Dünner, C., Gabler, F., Soto, E., Gaete, F., Peñaloza, P., Celis, M., Rojas, C., 2010. The effect of overweight and obesity on proliferation and activation of AKT and ERK in human endometria. Gynecol. Oncol. 117, 96–102.
Wang, H., Eriksson, H., Sahlin, L., 2000. Estrogen receptors alpha and beta in the female reproductive tract of the rat during the estrous cycle. Biol. Reprod. 63, 1331–1340.
Weibel, E.R., 1969. Stereological principles for morphometry in electron microscopic cytology. Int. Rev. Cytol. 26, 235–302.
Welti, L.M., Beavers, D.P., Caan, B.J., Sangi-Haghpeykar, H., Vitolins, M.Z., Beavers, K.M., 2017. Weight fluctuation and cancer risk in postmenopausal women: the Women's Health initiative. Cancer Epidemiol. Biomark. Prev. 26, 779–786.
Zeeni, N., Dagher-Hamalian, C., Dimassi, H., Faour, W.H., 2015. Cafeteria diet-fed mice is a pertinent model of obesity-induced organ damage: a potential role of inflammation. Inflamm. Res. 64, 501–512.
Zhao, J., Lyu, C., Gao, J., Du, L., Shan, B., Zhang, H., Wang, H.Y., Gao, Y., 2016. Dietary fat intake and endometrial cancer risk: a dose response meta-analysis. Medicine 95, 4121.