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Lifestyle and reproductive health: effects of diet and glyphosate exposure on fertility, feto-placental development and uterine carcinogenesis

María Paula Gastiazoro

Argentine Director: Prof. Dr. Jorgelina Varayoud

German Director: Prof. Dr. Oliver Zierau

Places of execution: Instituto de Salud y Ambiente del Litoral-UNL-CONICET, Santa Fe, Argentina
and Institut für Zoology-TUD, Dresde, Germany.

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Publications related with the thesis:

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- **Gastiazoro MP**, Durando M, Milesi MM, Lorenz V, Vollmer G, Varayoud J, Zierau O. Glyphosate induces epithelial mesenchymal transition-related changes in human endometrial Ishikawa cells via estrogen receptor pathway. Mol Cell Endocrinol. 2020 Jun 15;510:110841. Doi: 10.1016/j.mce.2020.110841. Epub 2020 Apr 28. PMID: 32360565.

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List of Abbreviations

AHR	aryl hydrocarbon receptor
AMPA	aminomethylphosphonic acid
ARNT	aryl hydrocarbon nuclear translocator
BPA	Bisphenol A
CAF	cafeteria
CLs	corpora lutea
CON	control
CpG	cytosine-phosphate-guanine-dinucleotide
DDT	dichloro diphenyl trichloroethane
DMEM/F12	Dulbecco's modified Eagle's medium F12
DMSO	Dimethylsulfoxide
E_2	estradiol
EDC	Endocrine Disruptors Chemicals
EDSP	Endocrine Disruptor Screening Program
EFSA	European Food Safety Authority
Elk-1	ETS like-1 protein
EMT	Epithelial Mesenchymal Transition
EPA	Environmental Protection Agency
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GD	gestational day
GE	glandular epithelium
GBH	Glyphosate Based Herbicide

Gly	pure Glyphosate
HFD	High Fat Diet
HFHS	High Sugar/High Fat diet
H&E	Haematoxylin and Eosin
IARC	International Agency for Research on Cancer
IGF	Insuline like growth factor
IGFR	Insuline like Growth Factor Receptor
IHC	Immunohistochemistry
IOD	integrated optical density
IR	insulin receptor
IS	implantation site
ITS	Insulin-Transferrin-Selenium A
IUGR	Intrauterine Growth Restriction
JAK 2	Janus Kinases 2
LE	luminal epitheium
LGA	Large Gestational Age
mTORC1	mammalian target of rapamycin complex 1
Ob-Rb	long form of leptin receptor
PBS	phosphate buffer saline
PND	postnatal day
PR	progesterone receptor
PS	periglandular stroma
RfD	reference dose
RS	resorption site
SGA	Small Gestational Age
SS	subepithelial stroma
STAT3	signal transducers and activators of transcription 3

TF	transcription factor
TCDD	2,3,7,8-tetraclorodibenzo-p-dioxina
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

Abstract

Lifestyle and reproductive health: effects of diet and glyphosate exposure on fertility, feto-placental development and uterine carcinogenesis

CHAPTER I – *Cafeteria Diet*

Part I: Evaluation of body weight and metabolic approaches
Part II: Fertility and feto-placental development
Part III: Endometrial Hyperplasia

CHAPTER II – *Exposure to Glyphosate*

Part I: Evaluation of body weight and food intake
Part II: Fertility and feto-placental development
Part III – *In vitro*: Gly effects on endometrial carcinogenesis-related process

Lifestyle factors, such as physical activity, social interaction, smoking, dietary habits, and environmental pollution, could influence positively or negatively our health. The foods that compose our diet take part on determining our overall health condition, including reproductive health and risk of cancer. In like manner, environmental pollution could affect our health, by different routes of exposure. We select two lifestyle factors in the present work:

1- **Cafeteria (CAF) diet** is a murine model on which nutritional program simulates Western diets habits. The CAF diet reflects variety, palatability, and energy density food of Western population diets. In addition, the composition implies an unbalanced diet with predominantly fat energy content (49%) at the expense of lower protein content (7%).

2- **Glyphosate** (Gly) (N-phosphonomethyl glycine) is the most frequently used herbicide in the world, for agriculture, forestry, urban and home applications. Gly is the active ingredient of a number of broad-spectrum herbicide formulations, named Glyphosate Based Herbicides (GBHs). In recent years, there has been criticism on the use of GBHs and the usage has gained considerable concern in the world since increasing levels of Gly have been detected in different sources.

Our aim was to evaluate the effects of these two lifestyle factors, the administration of CAF diet and the exposure to Gly, on female fertility and endometrial carcinogenesis-related process.

In the FIRST CHAPTER, we propose to study the effects of chronic administration of CAF diet. In the PART I we determine if CAF diet affects body weight and metabolic parameters; in the PART II we investigate the effects on rat reproductive performance, feto-placental parameters, and the weight of pups at birth. Additionally, we evaluate the placental health determining the expression of Insuline Like Growth Factor -IGF- and Vascular Endothelial Growth Factor -VEFG- gene systems on gestational day 21 (GD21), and possible epigenetic modifications of these genes. Finally, in the PART III we evaluate if chronic exposure to a CAF diet might induce uterine changes associated with endometrial hyperplasia evaluating uterine morphology, the expression of estrogen-sensitive genes, epithelial cell proliferation and the expression of molecular regulators of endometrial proliferation (such as IGF1, IGF1 receptor (IGF1R) and the long form of leptin receptor (Ob-Rb). Female Wistar rats were fed a control (CON) or CAF diet from the moment they were weaned (postnatal day 21, PND21) until parturition. For the PART I the CAF diet was administered during 20 weeks and the animal weight and energy intake was recorded every week. At the moment of sacrifice (160 days-old) trunk blood was collected for metabolic determinations and perigonadal and retroperitoneal fat pads were isolated and weighted. For the PART II, after 14th week of feeding, females were mated and half of the animals of each group were euthanized during pregnancy (at GD21). The rest of the animals were maintained until parturition with the diets to assess the weight of pups at birth. The reproductive outcome was assessed by evaluating the pregnancy rate, and at GD21 we determine the number of corpora lutea, implantation sites and resorption sites. Moreover, fetal weight and length, placental weight and placental index (placental weight/ fetal weight ratio) were recorded to evaluate feto-placental parameters. Additionally, placentas were collected for quantification of mRNA expression and for determination of epigenetic changes by the evaluation of DNA methylation levels. We study the expression of mRNA expression of IGF1, IGF1R, IGF2, IGF2R, VEGF and VEGFR and according to the changes we defined the epigenetic studies. For the PART III, after 20 weeks of treatment, the uterine samples were collected to evaluate endometrial carcinogenesis-related process by determination of uterine morphology, rate of proliferation and the expression of regulators of uterine growth.

In the SECOND CHAPTER, we evaluate the second selected lifestyle factor, using an oral chronic exposure to a safe dose of Gly (commercial formulation of a GBH). In the PART I we determine the effects of GBH on body weight and food intake after 10

weeks of treatment. In the PART II we investigate the effects of GBH on rat reproductive performance and feto-placental parameters on GD19. And last, in the PART III we evaluate the influence of Gly on endometrial carcinogenesis-related process using an *in vitro* model. In the last experiment, we analize the effects of Gly on Epithelial Mesenchymal Transition (EMT)-related process using a human endometrial carcinoma cell line (Ishikawa cells). We performed the evaluation of cell migration and invasion after Gly exposure, and expression of EMT-related markers. Additionally we determine if Gly effects are mediated via Estrogen Receptor (ER)-dependent pathway. To perform the experimental procedures of PART I and II, female Wistar rats were fed with a diet provided with a laboratory pellet chow-based paste (CON group), or a similar diet but using a paste supplemented with GBH in a dose of 2 mg of Gly/kg bw/day, starting at weaning (PND21). For the PART I the exposure was performed during 10 weeks. The body weight and energy intake was recorded every week. For the PART II, after 10 weeks of treatment, females were mated and euthanized during pregnancy at GD19. We determine the reproductive outcome evaluating the pregnancy rate, and at GD19 we quantify the number of corpora lutea, implantation sites and resorption sites. In addition, fetal weight and length, placental weight and placental index were recorded to evaluate feto-placental parameters at GD19. Last, for the PART III (*in vitro* experiment), we aimed to determine whether Gly induces EMT-related changes in a human endometrial carcinoma cell line (Ishikawa cells). Besides, we evaluated whether the ER pathway is involved in these changes. Ishikawa cells were exposed to Gly (0.2 μM and 2 μM) or 17β-estradiol (E_2: 10^{-9} M) and an ER antagonist (Fulvestrant: 10^{-7} M) was co-administrated with Gly or E_2.

Concerning the FIRST CHAPTER, the chronic administration of CAF diet produced an increased energy intake, body weight and fat depots, but the rats did not develop metabolic syndrome. The effects of CAF diet on female fertility and fetal parameters revealed neither changes on reproductive performance nor fetal weight and length on GD21. Nevertheless, CAF diet produced a lower placental weight and lower placental index on GD21. Surprisingly the pups of CAF-fed dams exhibited a low birth weight. When we evaluate the placental health, we found an upregulation of IGF2 and a down regulation of VEGF placental mRNA expression in CAF dams. Importantly, these changes were associated with modifications in DNA methylation levels of their respective promoter regions. The other study, focused on CAF diet effects on

endometrial carcinogenesis-related process, showed that CAF diet induces uterine hyperplasia during adulthood. The uterus of CAF animals showed an increase of glandular volume fraction and stromal area. The epithelial proliferation rate and protein expression of ERα were also increased. Besides, we determined that CAF diet enhanced leptin serum levels and uterine Ob-Rb mRNA expression. No changes were detected in either insulin serum levels or uterine IGF1 mRNA expression. However, we detected a low expression of uterine IGF1R mRNA in CAF-fed animals.

Regarding the SECOND CHAPTER, the animals displayed an increased body weight after chronic exposure to oral safe dose of GBH. The study of GBH effects on reproductive performance did not show impairments at the pregnancy rates. The fertility test performed on GD19 revealed neither changes in the number of CLs nor in the number of resorption sites, nevertheless we observed a significant increase in the percentage of pre-implantation loss of GBH-exposed animals. Regarding feto-placental parameters non alterations were detected neither placental weight nor placental index on GD19. However GBH induces a lower fetal weight and length in GD19, indicating an impairment on fetal development. Finally, *in vitro* studies showed that Gly increased cell migration and invasion ability of endometrial cancer cell line compared to vehicle, as did E_2. Moreover, a down regulation of E-cadherin mRNA expression was observed in response to Gly, similar to E_2-effects. These results show that Gly promotes EMT-related changes in Ishikawa cells. When an ER antagonist (Fulvestrant: 10^{-7} M) was co-administrated with Gly, all changes were reversed, suggesting that Gly might promote EMT-related changes via ER-dependent pathway.

Overall, the results indicate that chronic consumption of CAF diet starting at an early age produces: 1) morphological and molecular changes associated with uterine hyperplasia and 2) effects on placental growth and lower weight of pups at birth, associated with epigenetic disruption of IGF and VEGF systems critical to fetal and placental growth and development.

Concerning GBH studies, our results showed that GBH exposure induced pre-implantation loss and alteration of fetal development on GD19. Furthemore, our results are interesting evidence of Gly effects on endometrial cancer progression via the ER-dependent pathway.

In conclusion, the results increase the evidence about the lifestyle as a key partaker of health. Taking into account that food consumption can be modified and the exposure to herbicides can be regulated, it is necessary to review diet composition and lifestyle habits to improve our health and avoid possible consequences in the short- and long-term.

Resumen

Los factores del estilo de vida, como la actividad física, la interacción social, el tabaquismo, los hábitos alimentarios y la contaminación ambiental, pueden influir positiva o negativamente en nuestra salud. Los alimentos que componen nuestra dieta intervienen en la determinación de nuestro estado de salud general, incluida la salud reproductiva y el riesgo de cáncer. De igual manera, la contaminación ambiental podría afectar nuestra salud, por diferentes vías de exposición. Seleccionamos dos factores de estilo de vida para el presente trabajo:

1- La **dieta de cafetería (CAF)** es un modelo murino en el que el programa nutricional imita los hábitos alimentarios de las poblaciones occidentales. La dieta CAF refleja la variedad, la palatabilidad y la densidad energética de los alimentos de la población occidental. Además, su composición implica una dieta desequilibrada con un contenido energético predominantemente graso (49%) a expensas de un menor contenido de proteínas (7%).

2- **El glifosato (Gli)** (N-fosfonometilglicina) es el herbicida más utilizado en el mundo, para aplicaciones agrícolas, forestales, urbanas y domésticas. Gli es el ingrediente activo de una serie de formulaciones de herbicidas de amplio espectro, denominadas herbicidas a base de glifosato (HBG). En los últimos años, ha habido críticas sobre el uso de HBG y su uso ha generado una considerable preocupación en el mundo ya que se han detectado niveles crecientes de Gli en diferentes fuentes.

Nuestro objetivo fue evaluar los efectos de estos dos factores de estilo de vida, la administración de dieta CAF y la exposición a Gli, sobre la fertilidad femenina y sobre procesos relacionados con la carcinogénesis endometrial.

En el PRIMER CAPÍTULO nos proponemos estudiar los efectos de la administración crónica de la dieta CAF. En la PARTE I determinamos los efectos de la dieta CAF sobre el peso corporal y los parámetros metabólicos. En la PARTE II investigamos los efectos de la dieta CAF crónica sobre el rendimiento reproductivo de las ratas, los parámetros feto-placentarios y el peso de las crías al nacer. Adicionalmente, evaluamos la salud placentaria determinando la expresión de los sistemas de genes del factor de crecimiento similar a la insulina -IGF- y del factor de crecimiento endotelial vascular -VEFG- en el día 21 de gestación (DG21), y posibles modificaciones epigenéticas de estos genes. Finalmente, en la PARTE III evaluamos si la exposición crónica a una dieta

CAF podría inducir cambios uterinos asociados a hiperplasia endometrial evaluando la morfología uterina, la expresión de genes sensibles a estrógenos, la proliferación de células epiteliales y la expresión de reguladores moleculares de la proliferación endometrial (como IGF1, receptor de IGF1 (IGF1R) y la variante larga del receptor de leptina (Ob-Rb). Las ratas Wistar hembras fueron alimentadas con una dieta de control (CON) o CAF desde el momento en que fueron destetadas (día 21 postnatal, DPN21) hasta el parto. En la PARTE I se administró la dieta CAF durante 20 semanas y se registró semanalmente el peso y la ingesta energética del animal. En el momento del sacrificio (160 días de edad) se extrajo sangre del tronco para las determinaciones metabólicas y se aislaron tejidos adiposos perigonadales y retroperitoneales y para la PARTE II, después de la semana 14 de alimentación con la dieta, las hembras fueron apareadas y la mitad de los animales de cada grupo fueron sacrificados durante la gestación (en DG21). El resto de los animales se mantuvieron hasta el parto con las dietas correspondientes para evaluar el peso de las crías al nacer. El resultado reproductivo se evaluó mediante la determinación de la tasa de preñez, y en DG21 determinamos el número de cuerpos lúteos, sitios de implantación y sitios de reabsorción. Además, se registraron el peso y la longitud fetal, el peso de la placenta y el índice placentario (relación peso placentario / peso fetal) para evaluar los parámetros feto-placentarios. Además, se recolectaron placentas para la cuantificación de la expresión génica a nivel de ARNm y para la determinación de cambios epigenéticos mediante la evaluación de los niveles de metilación del ADN. Estudiamos la expresión de ARNm de IGF1, IGF1R, IGF2, IGF2R, VEGF y VEGFR y según los cambios definimos los estudios epigenéticos. Para la PARTE III, después de 20 semanas de tratamiento, se recolectaron las muestras uterinas para evaluar el proceso relacionado con la carcinogénesis endometrial mediante la evaluación de la morfología uterina, la tasa de proliferación y los reguladores del crecimiento uterino.

En el SEGUNDO CAPÍTULO, evaluamos los efectos de una exposición crónica oral a una dosis segura de un Gli (un HBG comercial) sobre el desempeño reproductivo y el desarrollo feto-placentario. Además, nuestro objetivo es determinar si la exposición a Gli promueve el proceso relacionado con la carcinogénesis endometrial utilizando un modelo *in vitro*. En la PARTE I determinamos los efectos de HBG sobre el peso corporal y la ingesta de alimentos después de 10 semanas de tratamiento. En la PARTE II investigamos los efectos de HBG sobre el rendimiento reproductivo de ratas y los parámetros fetoplacentarios en DG19. Y por último, en la PARTE III evaluamos la

influencia de Gli en el proceso relacionado con la carcinogénesis endometrial utilizando un modelo *in vitro*. Investigamos los efectos de Gli en el proceso relacionado con la transición mesenquimal epitelial (TEM) utilizando una línea celular de carcinoma endometrial humano (células de Ishikawa). Realizamos la evaluación de la migración e invasión celular después de la exposición a Gli y la expresión de marcadores relacionados con la TEM. Además, determinamos si los efectos de Gli están mediados por la vía dependiente del receptor de estrógeno (RE). Para ello, las ratas Wistar hembras fueron alimentadas con una dieta provista de una pasta a base de pellet de laboratorio (grupo CON), o una dieta similar pero utilizando una pasta suplementada con HBG en una dosis de 2 mg de Gli / kg bw / día, a partir del destete (DPN21). Para la PARTE I la exposición se realizó durante 10 semanas. Se registró semanalmente el peso corporal y la ingesta energética. Para la PARTE II, después de 10 semanas de tratamiento, las hembras fueron apareadas y sacrificadas durante el embarazo en DG19. Determinamos el resultado reproductivo evaluando la tasa de embarazo, y en DG19 cuantificamos el número de cuerpos lúteos, sitios de implantación y sitios de reabsorción. Además, se registraron el peso y la longitud fetal, el peso de la placenta y el índice placentario para evaluar los parámetros fetoplacentarios en DG19. Por último, para la PARTE III (experimento *in vitro*), nuestro objetivo fue determinar si Gli induce cambios relacionados con la TEM en una línea celular de carcinoma endometrial humano (células de Ishikawa). Además, evaluamos si la vía RE está involucrada en estos cambios. Las células de Ishikawa se expusieron a Gli (0,2 µM y 2 µM) o 17β-estradiol (E_2: 10-9 M) y se co-administró un antagonista de RE (Fulvestrant: 10^{-7} M) con Gli o E_2.

En el PRIMER CAPÍTULO, la administración crónica de la dieta CAF produjo un aumento del aporte energético, del peso corporal y de los depósitos de grasa, pero las ratas no desarrollaron síndrome metabólico. Los efectos de la dieta CAF sobre la fertilidad femenina y los parámetros fetales no revelaron cambios en el rendimiento reproductivo ni en el peso y la longitud fetal en DG21. Sin embargo, la dieta CAF produjo un peso placentario más bajo y un índice placentario más bajo en DG21. Sorprendentemente, las crías de madres alimentadas con CAF exhibieron un bajo peso al nacer. Cuando evaluamos la salud de la placenta, encontramos una up-regulación de IGF2 y una down-regulación de la expresión de ARNm placentario de VEGF en madres alimentadas con CAF. Es importante destacar que estos cambios se asociaron con modificaciones en los niveles de metilación del ADN de sus respectivas regiones

promotoras. El otro estudio, centrado en los efectos de la dieta CAF en el proceso relacionado con la carcinogénesis endometrial, mostró que la dieta CAF induce hiperplasia uterina durante la edad adulta. El útero de los animales CAF mostró un aumento de la fracción de volumen glandular y del área de estroma. También aumentaron la tasa de proliferación epitelial y la expresión de proteínas de REα. Además, determinamos que la dieta CAF mejoró los niveles séricos de leptina y la expresión de ARNm de Ob-Rb uterino. No se detectaron cambios ni en los niveles séricos de insulina ni en la expresión de ARNm de IGF1 uterino. Sin embargo, detectamos una baja expresión de ARNm de IGF1R uterino en animales alimentados con CAF.

Con respecto al SEGUNDO CAPÍTULO, los animales mostraron un aumento de peso corporal después de la exposición crónica a una dosis segura de HBG. El estudio de los efectos de GBH sobre el rendimiento reproductivo no mostró alteraciones en las tasas de embarazo. La prueba de fertilidad realizada en DG19 no reveló cambios en el número de CL ni en el número de sitios de reabsorción, sin embargo, observamos un aumento significativo en el porcentaje de pérdida pre-implantatoria en animales expuestos al HBG. En cuanto a los parámetros feto-placentarios no se detectaron alteraciones el peso placentario como así tampoco en el índice placentario en DG19. Sin embargo, HBG induce un menor peso y longitud fetal en DG19, lo que indica un deterioro en el desarrollo fetal. Finalmente, los estudios *in vitro* mostraron que Gli promovió la migración celular y la capacidad de invasión de la línea celular de cáncer de endometrio en comparación con el vehículo, al igual que E_2. Además, se observó una down-regulación de la expresión de ARNm de E-cadherina en respuesta a Gli, similar a los efectos de E_2. Estos resultados muestran que Gli promueve los cambios relacionados con el proceso de TEM en las células de Ishikawa. Cuando un antagonista de RE (Fulvestrant: 10^{-7} M) fue co-administrado con Gli, todos los cambios se revirtieron, lo que sugiere que Gli podría promover cambios relacionados con el proceso TEM a través de la vía dependiente de RE.

En general, los resultados indican que el consumo crónico de la dieta CAF a partir de una edad temprana produce: 1) cambios morfológicos y moleculares asociados con la hiperplasia uterina y que podrían predisponer a la carcinogénesis endometrial; 2) efectos sobre el crecimiento placentario y menor peso de las crías al nacer. Estos efectos

podrían explicarse, al menos en parte, por una alteración epigenética de los sistemas IGF y VEGF críticos para el crecimiento y desarrollo fetal y placentario.

Con respecto a los estudios de HBG, nuestros resultados mostraron que la exposición a HBG indujo la pérdida previa a la implantación y la alteración del desarrollo fetal en DG19. Además, nuestros resultados son una evidencia interesante de los efectos de Gli en la progresión del cáncer de endometrio a través de la vía dependiente de RE.

En conclusión, los resultados aumentan la evidencia sobre el estilo de vida como un autor clave del estado de salud. Teniendo en cuenta que se puede modificar el consumo de alimentos y se puede regular la exposición a herbicidas, es necesario revisar la composición de la dieta y los hábitos de vida para mejorar nuestra salud y evitar posibles consecuencias a corto y largo plazo.

Zuzammenfassung

Lifestyle-Faktoren wie körperliche Aktivität, soziale Interaktion, Rauchen, Ernährungsgewohnheiten als auch Umweltverschmutzung können unsere Gesundheit positiv oder negativ beeinflussen. Die Lebensmittel, aus denen sich unsere Ernährung zusammensetzt, beinflussen unseren allgemeinen Gesundheitszustand, einschließlich der reproduktiven Gesundheit und des Krebsrisikos. Ebenso könnte Umweltverschmutzung unsere Gesundheit auf verschiedenen Expositionswegen beeinträchtigen. In der vorliegenden Arbeit wählen wir zwei Lifestyle-Faktoren aus:

1- **Die Cafeteria (CAF) -Diät** ist ein Nagetier-Futtermodell, bei dem das Ernährungspezifika westliche Ernährungsgewohnheiten simuliert wird. Die CAF-Diät spiegelt die Vielfalt, die Schmackhaftigkeit und die Energiedichte von Nahrungsmitteln der modernen westlichen Diäten wider. Darüber hinaus impliziert die Zusammensetzung eine unausgewogene Ernährung mit überwiegend Fettenergiegehalt (49%) auf Kosten eines niedrigeren Proteingehalts (7%).

2- **Glyphosat (Gly)** (N-Phosphonomethylglycin) ist das weltweit am häufigsten verwendete Herbizid für Land-, Forst-, Stadt- und Haushaltsanwendungen. Gly ist ein Wirkstoff einer Reihe von Breitbandherbizidformulierungen, die auch als Herbizide auf Glyphosatbasis (GBHs) bezeichnet werden. In den letzten Jahren wurde Kritik an der Verwendung von GBHs geäußert und dieses hat weltweit zu erheblicher Besorgnis geführt, da in verschiedenen Quellen steigende Gly-Spiegel festgestellt wurden.

Mein Ziel war es, die Auswirkungen dieser beiden Lifestyle-Faktoren, CAF-Diät und Gly-Exposition, auf die Fruchtbarkeit und den krebsbedingten Prozess zu bewerten.

Im ERSTEN KAPITEL schlagen wir vor, die Auswirkungen der chronischen Verabreichung einer CAF-Diät auf die Fruchtbarkeit, die Entwicklung fetalen Plazentaanteils und den Prozess der Endometriumkarzinogenese zu untersuchen. In TEIL I bestimmen wir die Auswirkungen einer chronischen CAF-Diät Exposition auf das Körpergewicht und die Stoffwechselparameter. In TEIL II untersuchen wir die Auswirkungen einer chronischen Exposition gegenüber einer CAF-Diät auf die Reproduktionserfolg von Ratten, die fetoplazentaren Parameter und das Gewicht von Neugeborenen bei der Geburt. Zusätzlich bewerten wir die plazentare regulatorische Genexpression (Insuline Like Growth Factor -IGF- und Vascular Endothelial Growth Factor -VEFG- Systeme) am embryonalen Tag 21 (GD21) als auch die mögliche

epigenetische Modifikationen dieser Gene. Schließlich bewerten wir in TEIL III, ob eine chronische Exposition gegenüber einer CAF-Diät Uterusveränderungen hervorrufen kann, die in eine Endometriumhyperplasie münden können, und bewerten die Uterusmorphologie, die Expression östrogenabhängiger Gene, die Epithelzellproliferation und die Expression molekularer Regulatoren der Endometriumproliferation (wie z.B..IGF1, IGF1-Rezeptor (R) und die lange Form des Leptinrezeptors (Ob-Rb). Zu diesem Zweck wurden weibliche Wistar-Ratten nachdem dem Absetzen (posnataler Tag 21, PND21) mit Kontrolldiät (CON) oder CAF-Diät gefüttert. Für TEIL I wurde die CAF-Diät über 20 Wochen verabreicht und das Tiergewicht und die Energieaufnahme wurden jede Woche aufgezeichnet. Nach dem Töten wurde Blut für Stoffwechselbestimmungen gesammelt und perigonadale und retroperitoneale Fettpolster wurden isoliert und gewogen. Für TEIL II wurden nach der 14. Fütterungswoche die Weibchen gepaart und je die Hälfte der Tiere jeder Gruppe während der Trächtigkeit eingeschläfert (GD21). Der Rest der Tiere wurde bis zur Geburt mit der jeweiligen Nahrung gehalten, um das Gewicht der Welpen bei der Geburt zu bestimmen. Das reproduktive Ergebnis wurde durch Auswertung der Trächtigkeitsrate bewertet, und am GD21 bestimmten wir die Anzahl der Corpora lutea, Implantationsstellen und Resorptionsstellen. Darüber hinaus wurden das Gewicht und die Länge des Fötus, das Plazentagewicht und der Plazentaindex aufgezeichnet, um die Parameter der Fetoplazenta zu bewerten. Zusätzlich wurden Plazenten zur mRNA-Quantifizierung und DNA-Methylierungsanalyse gesammelt. Wir untersuchen die Expression der Gene IGF1, IGF1R, IGF2, IGF2R, VEGF und VEGFR und ihrer Promotorregionen. Für TEIL III wurden nach 20-wöchiger Behandlung die Uterusproben gesammelt, um die Uterusmorphologie und molekulare Probleme zu bewerten.

Im ZWEITEN KAPITEL schlagen wir vor, die Auswirkungen einer oralen chronischen Exposition gegenüber einer als sicher betrachteten Dosis eines kommerziellen GBH auf die Reproduktionsleistung und die Entwicklung der Feto-Plazenta zu untersuchen. Darüber hinaus wollen wir mithilfe eines In-vitro-Modells feststellen, ob die Gly-Exposition den mit der Endometriumkarzinogenese verbundenen Prozess fördert. In TEIL I bestimmen wir die Auswirkungen einer oralen chronischen Exposition gegenüber einer sicheren Dosis kommerziellen GBH auf das Körpergewicht und die Nahrungsaufnahme nach 10-wöchiger Behandlung. In TEIL II untersuchen wir die Auswirkungen einer oralen chronischen Exposition gegenüber einer sicheren Dosis

kommerziellen GBH auf die Reproduktionsleistung von Ratten und die fetoplazentaren Parameter auf GD21. Und schließlich bewerten wir in TEIL III den Einfluss von Gly auf den Prozess im Zusammenhang mit der Endometriumkarzinogenese anhand eines In-vitro-Modells: Wir untersuchen die Auswirkungen von Gly auf den Prozess im Zusammenhang mit dem epithelialmesenchymalen Übergang (EMT) in einer menschlichen Endometriumkarzinom-Zelllinie und bewerten ihn Zellmigration und -invasion nach Gly-Exposition und Expression von EMT-verwandten Markern; Zusätzlich bestimmen wir, ob Gly-Effekte auf den vom Östrogenrezeptor (ER) abhängigen Weg vermittelten. Zu diesem Zweck wurden weibliche Wistar-Ratten mit CON-Diät gefüttert, die mit einer Paste auf Basis der normalen Futterpellets-Chow- oder mit GBH-Diät, die wiederum aus dieser Paste bestand, welcher GBH in einer Dosis von 2 mg Glyphosat / kg Körpergewicht / Tag zugesetzt war war, beginnend mit dem Absetzen (PND21). . Für TEIL I wurde die Verabreichung von Diäten während 10 Wochen durchgeführt. Das Körpergewicht und die Energieaufnahme wurden jede Woche aufgezeichnet. Für TEIL II wurden die Weibchen nach 10-wöchiger Behandlung während der Trächtigkeit am GD19 gepaart und eingeschläfert. Wir bestimmen das Reproduktionsergebnis anhand der Schwangerschaftsrate und quantifizieren am Tag E19 die Anzahl der Corpora lutea, Implantationsstellen und Resorptionsstellen. Zusätzlich wurden Gewicht und Länge des Fötus, Plazentagewicht und Plazentaindex aufgezeichnet, um die Parameter der Fetoplazenta bei GD19 zu bewerten. Zuletzt wollten wir für PART III (In-vitro-Experiment) bestimmen, ob Gly EMT-bedingte Veränderungen in einer menschlichen Endometriumkarzinom-Zelllinie (Ishikawa-Zellen) induziert. Außerdem haben wir untersucht, ob der ER -vermittelte-Pfad an diesen Änderungen beteiligt ist. Ishikawa-Zellen wurden mit Gly (0,2 µM und 2 µM) oder 17β-Östradiol (E2: 10^{-9} M) behandelt, und ein ER-Antagonist (Fulvestrant: 10^{-7} M) wurde zusammen mit Gly oder E_2 verabreicht.

In Bezug auf das ERSTE KAPITEL zeigten Ratten, die 20 Wochen lang CAF ausgesetzt waren, eine erhöhte Energieaufnahme, ein erhöhtes Körpergewicht und erhöhte Fettdepots, entwickelten jedoch kein metabolisches Syndrom. Die Auswirkungen der CAF-Diät auf die Reproduktionsbewertung zeigten weder Veränderungen der Reproduktionsleistung noch des Gewichts und der Länge des Fötus am GD21. Trotzdem zeigten Plazentagewicht und -index nach CAF-Verzehr einen Rückgang am GD21, und die Neugeborenen der CAF-gefütterten Muttertieren zeigten ein geringes Geburtsgewicht. Darüber hinaus fanden wir eine Hochregulation von IGF2

und eine Herunterregulation der VEGF-Plazenta-mRNA-Expression in CAF-Muttertieren. Wichtig ist, dass diese Änderungen mit Modifikationen der DNA-Methylierungsniveaus ihrer jeweiligen Promotorregionen verbunden waren. Schließlich induziert die CAF-Diät im Erwachsenenalter eine Uterushyperplasie. Die CAF-Tiere zeigten eine Zunahme des Drüsenvolumenanteils und der Stromalfläche. Die epitheliale Proliferationsrate und die Proteinexpression von ERα waren ebenfalls erhöht. Die CAF-Diät erhöhte die Leptinserumspiegel und die Ob-Rb-mRNA-Expression in der Gebärmutter. Weder in den Insulinserumspiegeln noch in denen der IGF1-mRNA-Expression wurden Veränderungen festgestellt. Die mRNA Spiegel an IGF1R- waren jedoch bei CAF-gefütterten Tieren niedriger.

In Bezug auf das ZWEITE KAPITEL zeigten die Tiere nach chronischer Exposition gegenüber einer oralen sicheren Dosis von GBH eine Zunahme des Körpergewichts. Die Untersuchung der Auswirkungen von GBH auf die Reproduktionsbewertung ergab keine Beeinträchtigungen der Schwangerschaftsraten. Andererseits ergab der an GD19 durchgeführte Fertilitätstest weder eine Änderung der Anzahl der CLs noch der Anzahl der Resorptionsstellen. Dennoch beobachteten wir einen signifikanten Anstieg des Prozentsatzes des Verlusts vor der Implantation bei GBH-exponierten Tieren. In Bezug auf fetoplazentare Bestimmungen wurden bei GD19 weder Plazentagewicht noch Plazentaindex Veränderungen festgestellt. GBH induziert jedoch in GD19 ein geringeres Gewicht und eine geringere Länge des Fötus. Schließlich zeigten In-vitro-Studien, dass Gly ebenso wie E_2. die Zellmigration und die Invasionsfähigkeit der Endometriumkrebs-Zelllinie im Vergleich zum Vehikel erhöhteDarüber hinaus wurde eine Herunterregulierung der E-Cadherin-mRNA-Expression als Reaktion auf Gly beobachtet, ähnlich wie bei E_2-Effekten. Diese Ergebnisse zeigen, dass Gly EMT-bedingte Veränderungen in Ishikawa-Zellen fördert. Wenn ein ER-Antagonist (Fulvestrant: 10^{-7} M) zusammen mit Gly verabreicht wurde, konnten alle Änderungen inhibiert werden, was darauf hindeutet, dass Gly EMT-bedingte Änderungen über den ER-abhängigen Weg fördern könnte.

Insgesamt deuten die Ergebnisse darauf hin, dass unser Rattenmodell der CAF-Diät morphologische und molekulare Veränderungen im Zusammenhang mit Uterushyperplasie hervorruft, die widerumfür die Endometriumkarzinogenese prädisponieren könnte. Außerdem zeigen unsere Ergebnisse, dass die chronische CAF-Ernährung bei Müttern sowohl das Plazentawachstum als auch das Gewicht des

Nachwuchses bei der Geburt reduzieren. Darüber hinaus könnten diese Effekte zumindest teilweise durch eine epigenetische Störung der IGF- und VEGF-Systeme erklärt werden.

In Bezug auf GBH-Expositionsstudien sind unsere Ergebnisse interessante Hinweise auf GBH-Effekte auf reproduktive Bewertungen, die einen Verlust vor der Implantation und Veränderungen der fetalen Länge an Tag GD19 belegen. Darüber hinaus belegen unsere Studien Gly-Effekte auf den progressionsbedingten Prozess des Endometriumkarzinoms über den ER-abhängigen Weg.

Im Allgemeinen ist diese Arbeit ein weiterer Beweis dafür, dass unser Lebensstil ein wesentlicher Bestandteil unserer Gesundheit ist. Unter Berücksichtigung der Tatsache, dass der Lebensmittelkonsum geändert und die Exposition gegenüber Umweltchemikalien z.B. Herbiziden reguliert werden kann, ist es notwendig, die Ernährungs- und Lebensgewohnheiten zu überprüfen, um unsere Gesundheit zu verbessern und negative Konsequenzen kurz- und langfristig zu vermeiden.

Chapter I

Cafeteria diet

I. Introduction

1. Lifestyle factors

Multiple lifestyle factors, such as physical activity, social interaction, smoking and dietary habits, could affect positively and negatively our health (Figure 1) (Abbate et al. 2020; Chiodi et al. 2020; Carlos et al. 2018). The foods that compose our diet habits have influence on determining our overall health condition, including reproductive health (Sharma et al. 2013; Winship et al. 2019) and risk of cancer (Grosso et al. 2017; Rezende et al. 2019). In this sense, the type of diet and its nutrient composition could improve or disturb fertility performance and pregnancy outcomes (Sharma et al. 2013; Grieger et al. 2020). Several studies have shown evidence of an association between individual nutrients of foods and risk of cancer (Bail et al. 2016; WCRF/AICR 2018; Bodén et al. 2019; Grosso et al. 2017). Taking into account that diet is a modifiable lifestyle factor, the examination of dietary habits and its association with reproductive impairments and cancer related process risk, is crucial to prevent critical consequences in the short- and long-term.

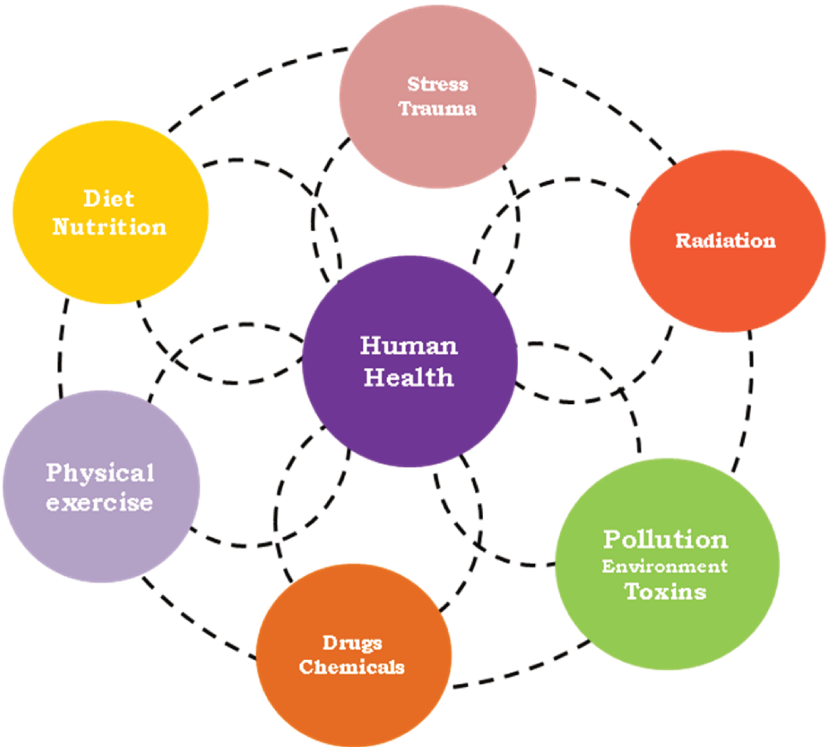

Figure 1: Lifestyle factors such as Drugs - Chemicals, Pollution – Environment – Toxins, Radiation, Stress – Trauma, Diet – Nutrition and Physical exercise, have influence on health and could produce positive or negative outcomes.

1.2 Cafeteria diet

Diverse animal models have been developed to induce obesity in rodents. These are usually based on different diets containing high fat, high sugar or palatable foods often consumed by humans (Lalanza et al. 2014). However, there is substantial variability in the composition of the diets in each animal model of study. The diets which consist of feed with a high content of sugar and / or additional fat (in most cases it is composed of a single solid food), do not represent in an appropriate way the human diet habits but are optimal to represent metabolic pathologies such as metabolic syndrome or obesity.

Thus, in order to study the effects of "junk food", a variety of foods currently consumed by humans are used in animal studies, allowing free choice of animals (Buettner et al. 2007). These diets are rich in sugars and/or fats content, which is why they are considered very palatable, and have a high caloric load. Particularly, the "cafeteria" diet (CAF) is an experimental dietary model rich in fat and sugar (not as single solid food, rather as a main nutrient on human foods), reflecting the variety of highly palatable and energy-dense foods prevalent in Western society (Figure 2) and associated with the current obesity pandemic (Sampey et al. 2011). In addition, the composition of this diet implies an unbalanced diet with predominantly fat energy content (49%) at the expense of lower protein content (7%) (Lazzarino et al. 2017). CAF diet is designed and composed of balanced food (Standard Chow Laboratory food) and a selection of "supermarket items foods" frequently consumed for humans. The items food with high energy content and palatability are varied, allowing the animal free choose the food according to its needs. Therefore, this diet has the advantage of allowing novelty, choice and variety, key characteristics of the modern human food environment. In several studies, CAF diet is used as an obesogenic diet. In adult animals it has been reported that the CAF diet has the ability to increase body weight and adiposity and induces hyperphagia, and disturbs the metabolic parameters generating metabolic syndrome (Lalanza et al. 2014). However, most studies in this field have focused on the effects of CAF diet as a promoter of metabolic disorders, while the effects on reproduction and cancer-related events have been less studied.

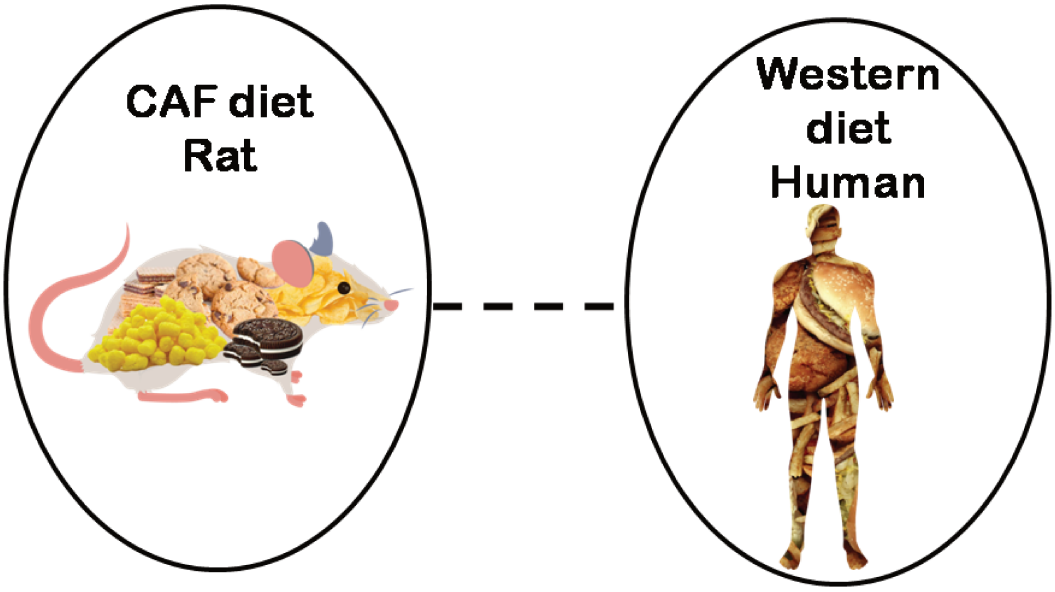

Figure 2: "Cafeteria" (CAF) diet is an animal experimental dietary model that mimics the variety of highly palatable and energy-dense foods prevalent in Western society.

2. CAF diet and Fertility

2.1. CAF diet and Reproductive Performance

It has been estimated that 10% of women worldwide are infertile, remaining the infertility as a highly prevalent global condition (WHO 2017). Decreasing the number of people affected by infertility has become a top priority for many health organizations, including Healthy People 2020. Lifestyle factors can be modified to enhance overall well-being and they are ultimately under one's own control. They play a key role in determining reproductive health and can positively or negatively influence fertility (Sharma et al. 2013). In this sense, the type of diet and its nutrient composition could improve or disturb fertility performance and pregnancy outcomes (Sharma et al. 2013; Grieger et al. 2020).

Several studies have evaluated the effects of CAF maternal diet composed by palatable food on fertility (Akyol et al. 2009; Bazzano et al. 2015, 2017; Sagae et al. 2012; Kannan et al. 2019a, b). Two studies indicated that CAF diet induces subfertility and sub-fecundity and delays conception, without affecting mating index (Bazano et al. 2015; 2017). For his part, Sagae et al. reported that CAF diet alters female rat reproduction by reducing the number of oocytes and preantral follicles, as well as the thickness of the follicular layers; nonetheless, they observed an optimal sexual receptiveness after CAF diet administration (Sagae et al. 2012).

Regarding Kannan et al. studies, CAF diet induced sub-fecundity, sub-fertility and delayed conception in rats (Kannan et al. 2019a; b). On the opposite, Akyol et al. did not detect rat fertility impairments after CAF diet administration (Akyol et al. 2009).

It is important to highlight, that these studies have several differences on the experimental design as time of dietary administration, rats strain, and metabolic implications, which could explain some discrepancies. At this point is important for us, to make clear that CAF diet has implications on reproduction performance. On the discussion section of this chapter we will analyze in detail possible basis and mechanism.

2.2. CAF diet and feto-placental development

Several human and experimental animal studies describe that perinatal non optimal environment such as maternal nutrition (deficits or excess), could alter fetal and offspring developmental trajectory predisposing to unfavourable outcomes (Gao et al. 2012; Dhobale et al. 2016; Gabory et al. 2016; Howell et al. 2017).

The link between maternal environment and offspring health has lead to the concept of "developmental programming", which takes place when the fetus grows under influences of maternal environment (Figure 3) (Marciniak et al. 2017; Castro-Rodriguez et al. 2020). If the maternal environment is not optimal, the fetal development will not be either resulting on possible permanent health outcomes throughout the whole life (Perrone et al. 2016). Particularly, maternal nutrition is one of the main factors which act in early life to program the risk for adverse long-term outcomes later in life (Barker 1998; Howell et al. 2017; Perrone et al. 2016). Although the fetal programming mechanism had not elucidate, the effects of intrauterine adverse background have been confirmed for diseases in adult life such as metabolic dysfunction, cardiovascular diseases, cancer, increased vulnerability to infections (among others) (Perrone et al. 2016).

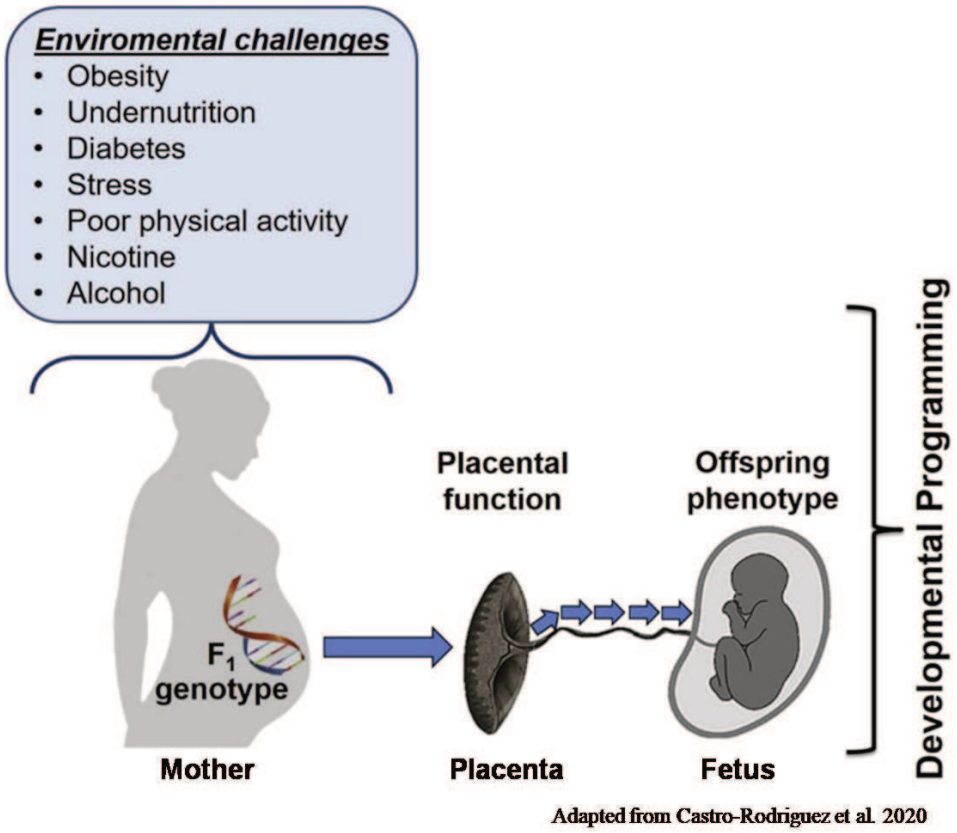

Figure 3: Maternal environment influence on developmental programming during fetal growth. The placenta is a key intermediary. Figure adapted from Castro-Rodriguez et al. 2020.

In addition to adverse long-term outcomes, maternal and pregnancy non optimal environment could have immediately effects on the placental and fetal growth. The evidences indicate that fetal and placental weight and the weight of pups at birth could be affected in rat studies and birth weight impairments were observed in humans in a non optimal environment (Howell et al. 2017). Sharma defines Intrauterine Growth Restriction (IUGR) as a rate of fetal growth minor than normal for the growth potential of a specific infants as per the race and gender of the fetus (Sharma et al. 2016). Small Gestational Age (SGA) is often used indistinctly of IUGR, although there are differences. To SGA diagnosis, the birth weight is minor than 10^{th} percentile for that particular age of the population norms on the growth register charts. SGA does not consider in-utero growth, whereas IUGR considers it. Moreover, IUGR infant could birth with an appropriate weight as per gestation, but may have suffered from any in-utero growth deceleration as a result of perinatal non optimal conditions. Thus IUGR is

a clinical definition and applied to neonates with clinical evidences of malnutrition (Battaglia et al. 1967; Sharma et al. 2016). On the other hand, Large Gestational Age (LGA) refers to neonatal birth weight larger than the 90th percentile for a given gestational age. In contrast to LGA, fetal macrosomia is defined as an absolute birth weight above a specified threshold regardless of gestational age (Silasi et al. 2018).

Placental structure and functions have a key role on the pregnancy process and fetal health. 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 (Murphy et al. 2006). Pregnancy complications such as diabetes, IUGR, pre-eclampsia, and hypoxia are associated with placental dysfunction and fetal programming (Perrone et al. 2016; Capobianco et al. 2016).

Some authors reported that the placenta and feto react to a CAF diet showing a reduction in placental weight as well as the fetal weight (Akyol et al. 2009; Crew et al. 2016). Otherwise, CAF maternal feeding was associated with lower offspring birth weight and body weight in early postnatal life (George et al. 2019). However, the possible mechanism to explain these effects has not been determined.

2.3. Placental systems

Vascular Endothelial Growth Factor (VEGF) and Insulin like Growth Factor (IGF) play a key role during placental and fetal development (Soto et al. 2017; Mangwiro et al. 2018; Chen et al. 2013).

2.3.1. IGF placental system

IGF system has pivotal role on fetal growth and placental function and development (Bauer et al. 1998; van Kleffens et al. 1998). The IGFs stimulate cellular mitosis and differentiation in several of cell types and are particularly implicated in fetal and placental growth (van Kleffens et al. 1998). IGF system knockout mouse models provoked a reduction of 40-55% in birth weight, reflecting the essential role of this system on fetal development (Elzein et al. 2016; Mangwiro et al. 2018). Particularly, IGF1 is involved in fetus development; its administration produces a high fetal weight, while its ablation decreases the fetal weight (Sferruzzi-Perri et al. 2006). Meanwhile, IGF2 overexpression generates placental and fetal overgrowth and its deletion produce the opposite effects (De Chiara et al. 1990; Baker et al. 1993), showing its role on placental growth (Matthews et al. 1999) besides fetal development (White et al. 2017).

At the date, there is no evidence linking CAF maternal diet with deregulation of IGF placental system and feto-placental growth. However, some authors associated unbalanced diet with feto-placental growth impairments and deregulation of IGF system related molecules (Gadd et al. 2000; Jansson et al. 2006; Mangwiro et al. 2018).

2.3.2 VEGF placental system

The VEGF system is crucial for angiogenesis which implies an important piece on vascular placental function (Chen et al. 2013; Shibuya et al. 2013). This system has a key role on endothelial cell proliferation, migration and tube-like structure formation (Dumont et al. 1994). Particularly, VEGF is essential for blood vessel formation during early embryogenesis. VEGF and VEGFR are angiogenic factors and they optimal work is crucial for the nutrient transport (Shibuya et al. 2013). Interestingly, some authors found an altered vessels density in placenta and a correlation between VEGF down-regulation and low placental and fetal weight (Wang et al. 2013; Bao et al. 2019; Salvolini et al. 2019).

To our knowledge, there no evidence of CAF diet effects on placental angiogenesis. Nevertheless, several authors reported correlation between maternal high-energy diet and/or obesity with placental angiogenesis, showing changes on VEGF placental expression (Hu et al. 2019) and deregulation of angiogenesis (Dubova et al. 2011; Salvolini et al. 2019).

2.3.3 Epigenetic mechanism

Epigenetic alterations of certain genes have been proposed as another possible adaptive reaction to an intrauterine environment (Salam et al. 2014). Several studies suggest that intrauterine environment influenced by maternal nutrition experience could produces changes in the epigenetic profile of placental genes, affecting fetal and placental development (Desgagne et al. 2014; Panchenko et al. 2015; Hillman et al. 2014; Kappil et al. 2016). 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 could be associated with regulatory elements such as transcription factors (TF) and repressors (Jacobs et al. 2017;

Jones et al. 2001). Cytosine methylation is generally related with transcriptional silencing due to a decreased of the TF binding capacity resulting in a diminution expression of the studied gene (Schübeler et al. 2015). The epigenetic mechanisms might modify gene expression without changing the DNA sequence itself (Panchenko et al. 2015). Importantly, these epigenetic marks could be heritable and could have manifestations in health and diseases after the exposure ending.

3. CAF diet and uterine carcinogenesis

Many risk factors of endometrial cancer have been described such as, early age at menarche, nulliparity, late-onset menopause, exposure to exogenous estrogens (without a progesterone component), diabetes and obesity (Parazzini et al. 1991; Amant et al. 2005). Currently, many studies have showed that obesity increases the risk of endometrial cancer (Thompson et al. 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 estrogen stimulation usually referred to as estrogen dominance (Lacey and Chia 2009; Kirschner et al. 1982). Endometrial cells can proliferate leading to the gradual development of endometrial hyperplasia (Grady et al. 1995; Persson et al. 2000), precursor lesion of endometrial cancer (Bokhman et al. 1983; Armstrong et al. 2012).

In normal conditions, the estrogenic effects initiate biochemical reactions in uterine cells previous a possible pregnancy, involving cell hypertrophy and hyperplasia (Nephew et al. 2000). Estrogens act mainly through estrogen 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 estrogen 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 estradiol (E_2), IGF1 and IGF2 and their signalling pathways also play significant roles in the regulation of uterine growth and differentiation during the oestrous cycle. Estrogen boosts uterine IGF1 gene expression and stimulates endometrial proliferation. The results of the actions of IGF1 and IGF2 are facilitated mainly by activation of the IGF1 receptor (IGF1R) (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 endometrial neoplastic cells through the long form of leptin receptor (Ob-Rb) activation (Tartaglia et al. 1995; Carino et al. 2008; Gao et al. 2009). 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 and diet could predispose to endometrial cancer (Dunneran et al. 2019; Owuor et al. 2018), but the effect of chronic administration of a westernized diet was not determined.

II. Goals

1. Main goal

We propose to study the effects of chronic administration of Cafeteria diet on fertility, feto-placental development and endometrial carcinogenesis-related process.

2. Specific goals

PART I:

To determine the effects of chronic administration of CAF diet on body weight and metabolic parameters.

PART II:

To investigate the effects of chronic administration of CAF diet on rat reproductive performance, feto-placental parameters, and the weight of pups at birth.

To evaluate placental regulatory gene expression (IGF and VEFG systems) on gestational day 21 (GD21), and possible epigenetic modifications of these genes.

PART III:

To evaluate if chronic administration of CAF diet might induce uterine changes associated with endometrial hyperplasia by evaluating uterine morphology, the expression of estrogen-sensitive genes and epithelial cell proliferation.

To determine the expression of molecular regulators of endometrial proliferation (IGF1, IGF1R and Ob-Rb).

III. Materials & Methods

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. 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 (postnatal day 21, PND21), and randomly divided into two groups: control diet group (CON) and cafeteria diet group (CAF). 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 respective diets and water were 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. The administration of the

diets was maintained from PND21 to the moment when we detected a difference of body weight between CAF and CON animals (week 14th of treatment).

3. PART I

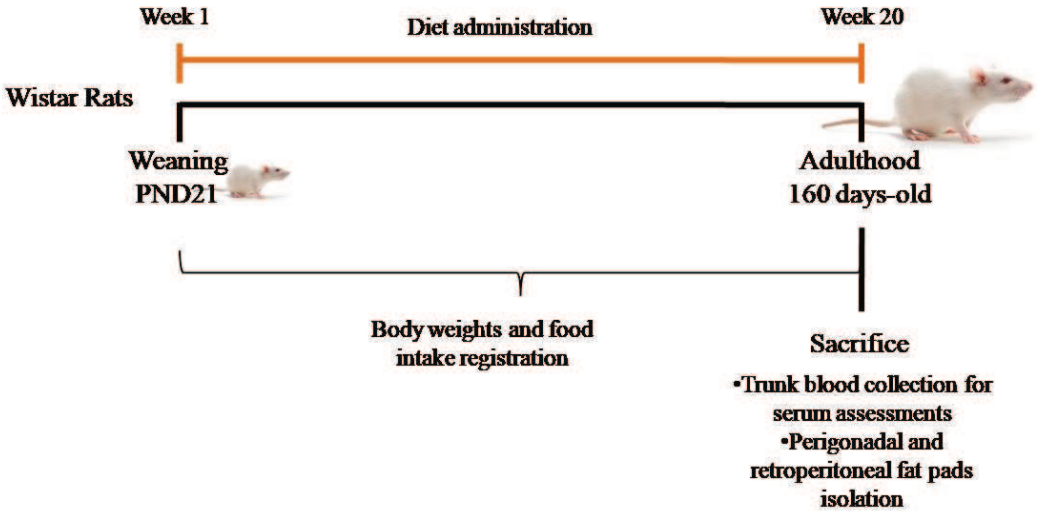

Figure 4: Experimental design to evaluate the effects of chronic administration of cafeteria (CAF) diet on body weight and metabolic parameters. PND: postnatal day.

3.1 Determination of body weight and metabolic parameters

During the experimental time, body weights were registered once a week and food intake every day, from the beginning of administration of each diet (PND21) and during the whole treatment. 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.

Ten animals per group were weighed and euthanized on the dioestrous stage of the oestrous cycle after 20 weeks of treatment (160 days-old). Vaginal smears were obtained daily from lavage fluid collected by flushing the female's vagina with physiological solution 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). We assessed the oestrous cycle during two weeks and determine the dioestrous stage to be sure that all animals were sacrificed at the same stage of the cycle.

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.

3.2 Serum assessments

Fasting serum metabolites (glucose, triglycerides, and total cholesterol) were assessed by commercially available assays (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 E_2 levels were measured using competitive RIA kits (Immunotech, Marseille, France) (Matthews et al. 1985).

4. PART II

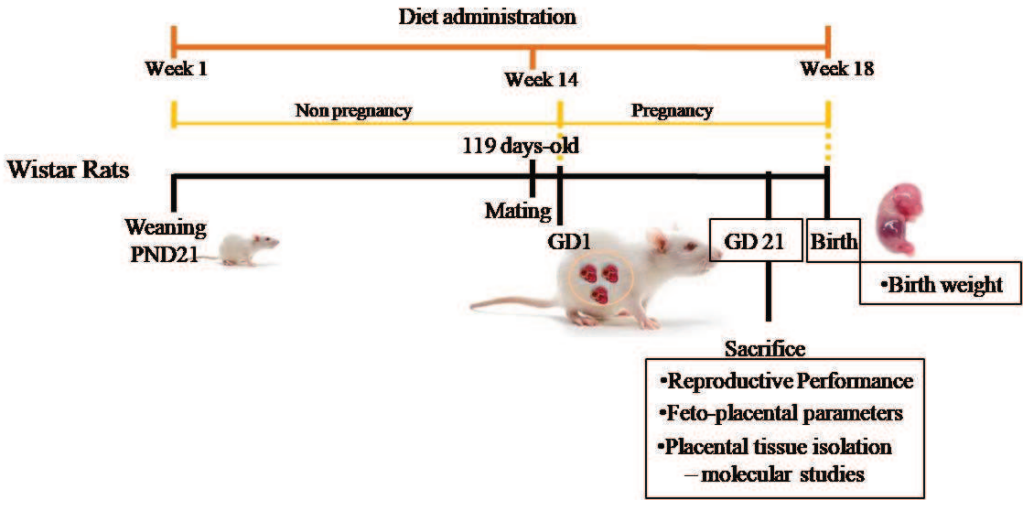

Figure 5: Experimental design to investigate the effects of chronic administration of cafeteria (CAF) diet on rat reproductive performance, feto-placental parameters, and the weight of pups at birth. PND: postnatal day; GD gestational day.

4.1 Evaluation of reproductive performance

Other animals of both experimental groups (N=10 per group) were used to evaluate reproductive performance. On the 14th week of diet administration (119 days-old, after

CAF animals showed a weight difference respect CON animals), females at the proestrus stage were caged with males with proved fertility. The male rats were fed with standard chow and were maintained in identical environmental conditions to ensure minimal variation from paternal factors. Every morning, vaginal smears were performed to check the presence of spermatozoa (Montes and Luque, 1988). The first day after mating on which a sperm-positive smear was detected, was considered gestational day 1 (GD1). We determine the pregnancy rate as the number of pregnant females/ number of females housed with a male x 100. After sperm-positive smear detection, each dam was singled caged and continued with the respective diet.

Ten dams per diet group were euthanized on GD21 to evaluate the reproductive performance. The ovaries were dissected, and the number of profusely irrigated corpora lutea (CLs) was counted by direct visualization using a stereomicroscope (Leica Corp., Buffalo, NY, USA). The two-horned uteri were removed and visually inspected to identify the number of 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 calculate the rate of pre-implantation loss as follows: [number of CLs – number of IS/ number of CLs] x 100 (Perobelli et al. 2012). A total of 10 animals per group were maintained until parturition with the respective diet. The litter size was monitored and the weight of pups at birth was registered.

4.2 Feto-placental parameters

To determine the effects of the diet on fetal and placental development, the fetuses and the placentas pairs were removed from uteri and weighed on GD21. 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. A total of three placental tissues per each dam selected randomly were collected and stored at -80°C until RNA isolation.

4.3. Placental regulatory gene expression

4.3.1 RNA isolation

Individual placentas 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.

4.3.2 Reverse transcription (RT)

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.

4.3.3 Quantitative real-time polymerase chain reaction (RT-qPCR)

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 (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). 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 experimental groups.

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

Table 1: Primers and PCR products for real-time quantitative RT-PCR. Table adapted from Gastiazoro et al. (*in evaluation*).

4.3.4. Bioinformatics to Epigenetic studies

We analyzed the promoter regions of placental 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) 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). 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).

4.3.5. 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 (Bruce et al. 2008; von Kanel et al. 2010). 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.

An optimized qRT-PCR protocol was used to analyze the relative methylation levels of different 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 section 4.3.2 and 4.3.3. 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 (Cikos et al. 2007). The efficiency of the qRT-PCR reactions and the fold expression over control values for each target gene were calculated as previously described (Rossetti et al. 2015).

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 2: Sequences of primer oligonucleotides for PCR amplification to evaluate methylation sensitive sites in promoters. Table adapted from Gastiazoro et al. (*in evaluation*).

5. PART III

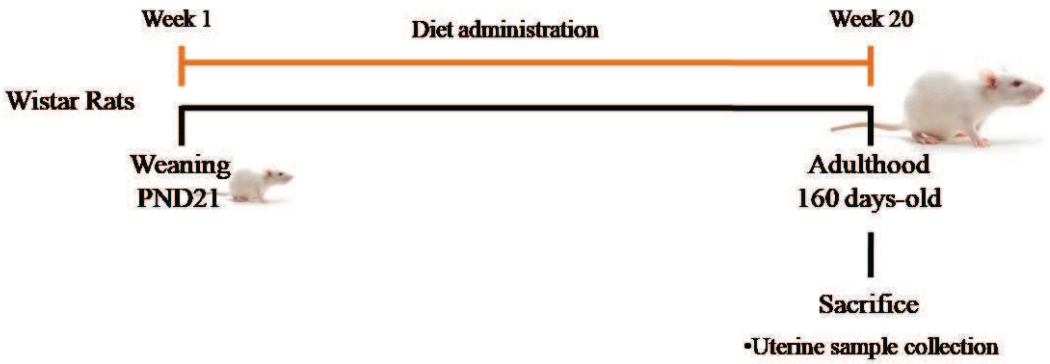

Figure 6: Experimental design to evaluate the effects of chronic administration of cafeteria (CAF) diet on uterine tissue of adult rats. PND: postnatal day.

5.1. Evaluation of endometrial hyperplasia

Ten animals per group were weighed and euthanized on the dioestrous stage of the oestrous cycle after 20 weeks of treatment (160 days-old).

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).

5.2 Study of protein expression by Immunohistochemistry (IHC)

5.2.1 Tissue processing

The uterine biopsies were fixed for 6 h in buffer formalin 10% v / v in PBS (phosphate buffer saline) pH 7.5 to preserve the histological structures of the tissue. Then two washes with PBS (pH 7.5) of 10 min each were carried out, and finally the samples were preserved in a 70% alcohol solution until they were processed by routine histological techniques for their inclusion in paraffin. During the histological processing, the samples were dehydrated in alcohols of increasing graduation, and placed in Bioclear (Biopack, Argentina) (non-aqueous, non-polar solvent, miscible in molten paraffin). In this way, the alcohol present in the tissues was replaced by Bioclear, which allowed the samples to be cleared. For inclusion in paraffin, the samples were placed in an oven at 60 ºC inside a bottle with molten paraffin. The heat caused the evaporation of the solvent remains and in this way, the spaces occupied by Bioclear were impregnated by paraffin. Finally, the samples were placed in a container with molten paraffin that was allowed to solidify at room temperature. This procedure allowed the obtaining of a paraffin block called a "block" with the tissue inside. Once the plug was obtained, longitudinal uterine sections of 5 µm thickness were obtained using a microtome (Leica, Jung RM2025, Leica Instruments GMT, Nussioch, Germany) and mounted on slides pre-treated with tissue adhesive (3-aminopropyl triethoxysilane, Sigma-Aldrich) and dried in an oven at 37°C for 24 hours.

5.2.2 Immunohistochemistry (IHC)

The IHC was carried out following a protocol previously optimized in our laboratory (Muñoz de Toro et al. 1998).

Uterine sections were deparaffinized and rehydrated in graded ethanol solutions. Then the antigen retrieval was performed by immersion of the uterine slides in 0.01 M citrate buffer pH 6.0 and subjected to heating in a microwave oven (the details of the procedure

are described in Table 3). Next, the glasses were placed in a 3% solution of hydrogen peroxide (H2O2) 30 volumes (vol) diluted with methanol for 15 min to block the endogenous peroxidase activity. To block non-specific binding-sites, the sections were incubated 30 min at room temperature (RT) with a solution of normal horse serum (NHS, English: Normal Horse Serum) diluted 1/20 in PBS and added with 1.5 % skim milk powder (Sigma Aldrich). The sections were incubated in a humid chamber with the specific primary antibody (14-16 hrs at 4°C), and then with their corresponding biotinylated secondary antibody (30 min at RT). Finally, incubation was carried out with the streptavidin-peroxidase complex (Sigma-Aldrich) (30 min at RT). For the development of the reaction, a solution containing 2.3 mg of the chromogen diaminobenzidine (DAB, Sigma-Aldrich) dissolved in 3.3 ml of 0.05 M Tris-HCl buffer (pH 7.5) and added with 5 µl of H2O2 30 vol. The reaction time was 10 min at RT. Finally, the histological sections were dehydrated and mounted with a permanent mounting liquid (Eukitt). In all cases, negative specificity controls were performed, replacing the primary antibody with a non-immune serum, and positive controls, including a tissue section whose positive reaction for the protein of interest was previously verified. The general protocol used is shown in Table 3 and the antibodies used in the IHC assays of this stage are detailed in Table 4. For Ki67 immunodetection, the samples were counterstained with Mayer haematoxylin (Biopur, Rosario, Argentina).

Table 3 - IHC general protocol

Deparaffinization and rehydration	
Bioclear 1, 2 and 3	3 min. ea.
Alcohol 100°, 96° and 70°	3 min. ea.
PBS	5 min.
Antigen retrieval	
Microwave hot treatment using citrate buffer 0.01M pH 6.0	Warm the buffer alone for 3 min. at 100% potency, add the samples and warm for 11 min. at 100% potency. Leave for 20 min. with microwave turned off.
PBS	5 min.
Blocking endogenous peroxidase activity	
3% solution of hydrogen peroxide, 30 volumes, diluted with methanol	15 min.
PBS	15 min.

Blocking non-specific binding-sites	
Normal Horse Serum diluted 1/20 in PBS, with 1.5 % skim milk powder	30 min. RT (humid chamber)
Primary antibody	
Primary antibody incubation	14 - 16 hs. 4°C (humid chamber)
PBS	15 min.
Biotinylated Secondary antibody	
Secondary antibody incubation	30 min. RT (humid chamber)
PBS	15 min.
Streptavidin-peroxidase complex	
Streptavidin-peroxidase complex incubation	30 min. RT (humid chamber)
PBS	5 min.
Developing	
2.3 mg of the chromogen diaminobenzidine (DAB) dissolved in 3.3 ml of 0.05 M Tris-HCl buffer (pH 7.5) and 5 µl of H_2O_2 30 vol.	10 min. RT
H_2O (distilled)	5 min.
Counterstain with Mayer haematoxylin (optional)	
Dehydratation and mounting	
Alcohol 70°, 96° and 100°	1 min. ea.
Bioclear 1 and 2	2 and 5 min. ea.
Mount with permanent mounting liquid (Eukitt)	

Table 4 - Antibodies used for IHC

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)

5.2.3. Histological analysis

5.2.3.1. Determination of the glandular volume fraction

Uterine samples were stained with haematoxylin and eosin for histological analysis using an Olympus BH2 microscope with a Dplan 40X objective (numerical aperture=0.65; Olympus). 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 (uterine stroma). To obtain the data for the point-counting procedure, a glass disk with a squared grid of 0.8mm×0.8mm was inserted into a focusing eye piece. The results were expressed as Vv x 100.

5.2.3.2. 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 used as stromal marker. 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 was performed using Fiji of Image J as previously described (Ramos et al. 2002). Briefly, the images were recorded with a Spot Insight V3.5 color 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 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 (40X objective), and two sections per animal were evaluated. Correction of unequal illumination (shading correction) and measurement system calibration were performed with a reference slide.

5.2.3.3. 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.

5.2.3.4. Quantification of ERα and PR expression by image analysis

The expression of ERα and PR proteins in the uterine cells was evaluated by image analysis, using Fiji of Image J as above described (section 5.2.3.2).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). At least 10 fields of each histological compartment (40X objective) 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.

5.3 Expression of molecular regulators of endometrial proliferation

The expression of IGF1, IGF1R and Ob-Rb were evaluated in uterine horns. The primer sequences are described in Table 5. The RNA isolation, reverse transcription and Quantitative real-time polymerase chain reaction (RT-qPCR) were performed as described above (sections 4.3.1; 4.3.2; 4.3.3).

Table 5 - 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
IGF1	F: 5´- CTCAAGGATGGCGTCTTCAC -3´ R: 5′-GAACTTGCTCGTTGGACAGG-3′	137
IGF1R	F: 5´- CTCAAGGATGGCGTCTTCAC -3´ R: 5′-GAACTTGCTCGTTGGACAGG-3′	115

Table 5: Primers and PCR products for real-time quantitative RT-PCR. Table adapted from Gastiazoro et al. 2018.

6. Statistical analysis

G Power software (http://www.gpower.hhu.de/; RRID:SCR_013726) was used to determine the sample size (Faul et al. 2007). 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, energy intake, serum parameters as well as feto-placental 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 the whole parameters regarding endometrial carcinogenesis-related process were analyzed using Mann-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 $p<0.05$.

IV. Results

Part I: Evaluation of body weight and metabolic approaches

1. CAF diet increases dam´s 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 (from 21 days-old to 116 days-old) (Figure 7). The weight of CAF-fed animals then started to show a significant increase at 14 weeks of treatment (weights in week 14: CON: 215.8 g ± 4.35 vs CAF: 229.8 g ± 13.00, $p < 0.05$). The age of the animals when we detected the weight´s differences with controls was 119 days-old (during adulthood). In addition, the CAF diet also increased the perigonadal and retroperitoneal fat depots expressed as a percentage of body weight ($p < 0.01$, Figure 7). 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 that received 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.

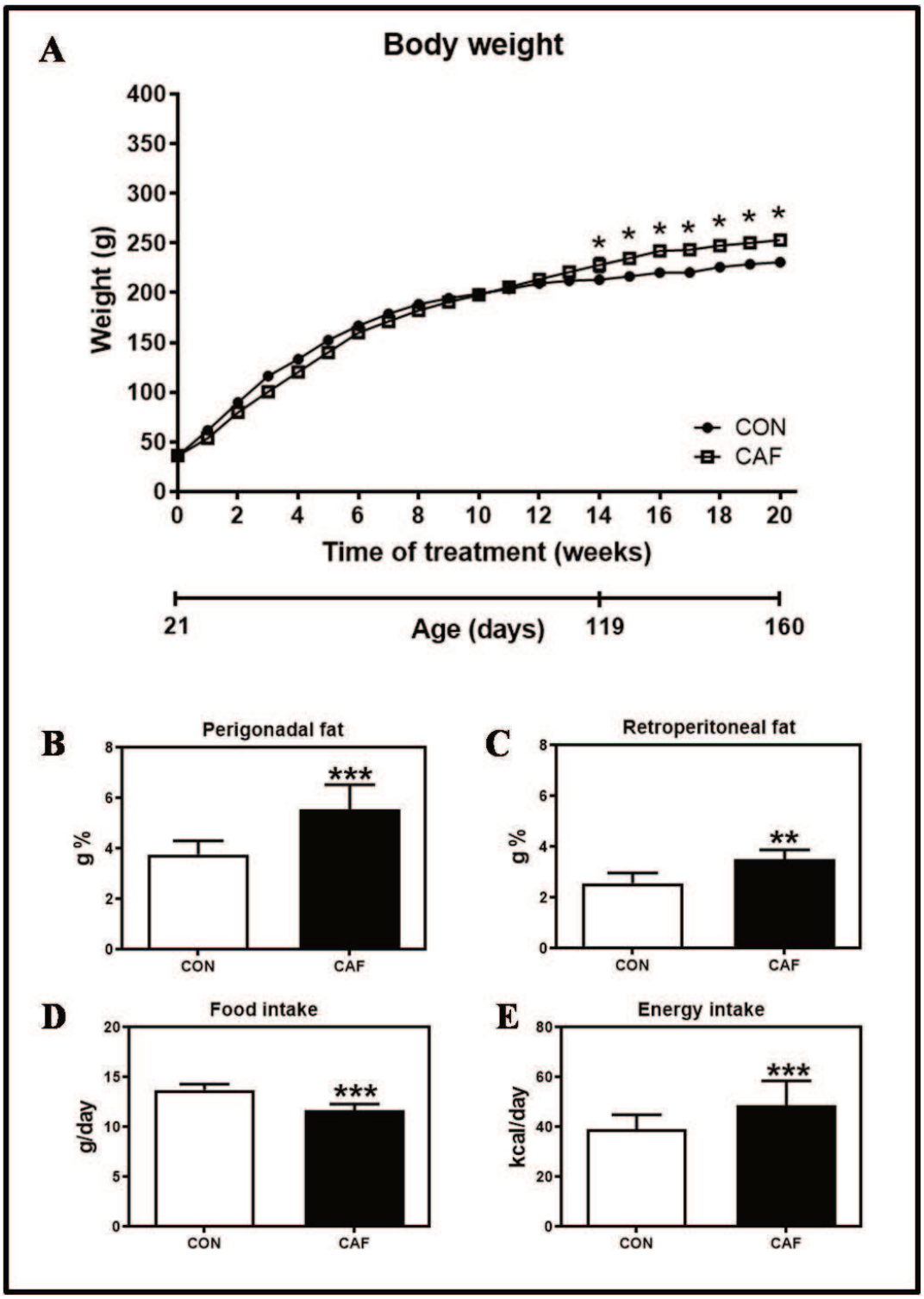

Figure 7: (A) Body weight of rats fed with standard chow (CON) or cafeteria (CAF) diet for 20 weeks. The time of treatment indicate the duration of CAF or CON diet administration expressed in weeks. The other line indicates the age of animals when the experiment began (postnatal day 21: PND21). At the time when the animals showed a

different weight the animals are 119 days-old. 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 ± SEM (N=10/Group). Asterisks indicate significant differences (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). Figure adapted from Gastiazoro et al. (*in evaluation*).

2. CAF diet alters leptin levels: an endocrine and metabolic partaker

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 6). No changes were detected in the serum levels of insulin or in those of any other metabolic parameters. In addition, CAF-fed animals exhibited neither alteration in serum E_2 levels (Table 6) nor in the duration of oestrous cycle phases (data not shown).

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)	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

Table 6: Serum metabolic parameters and hormone serum levels. Data shown are presented as the mean ± SEM (N=10/Group). Asterisks indicate significant differences (*: $p<0.05$). Table adapted from Gastiazoro et al. 2018.

Part II: Fertility and feto-placental development

1. CAF diet did not alter the reproductive performance

CAF fed rats did show not alteration of pregnancy rates (Figure 8A). The fertility test showed a normal response in CAF animals, without differences in the number of CLs (Figure 8B). Besides, neither changes in the number of IS nor in the number of RS on GD21 were detected between control and CAF fed rats (Figure 8C and D).

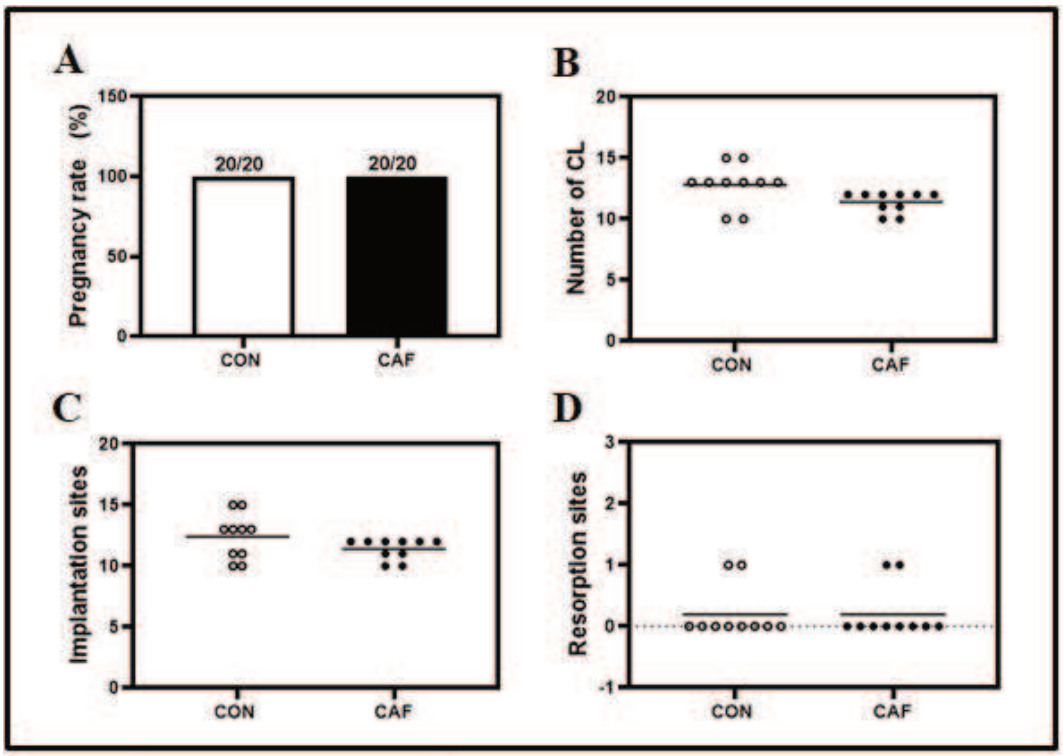

Figure 8: Reproductive performance of cafeteria diet (CAF) and control diet (CON) fed animals. (A) The pregnancy rates were calculated by the average of females that were pregnant with a fertile male. (B) Number of corpora lutea (CLs) and (C) Number of implantation sites (IS) are expressed as the mean ± 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 (N=10/Group). B, C and D parameters were evaluated on gestational day 21 (GD21). Figure adapted from Gastiazoro et al. (*in evaluation*).

2. CAF diet disturbs the feto-placental parameters and birth weight

Maternal CAF diet affected the feto-placental development. We detected a lower placental weight of CAF fed dams compared with control (CON: 502.40 ± 12.30 mg; CAF: 431.30 ± 8.40 mg; $p< 0.05$) (Figure 9A). The fetal weight and length did not change between groups (Figure 9B and C). The placental index calculated as placental/fetal weight ratio, showed a decrease on CAF group (CON: 0.189 ± 0.006; CAF: 0.160 ± 0.003; $p<0.001$) (Figure 9D), as a consequence of lower placental weight. However the litter size was normal in both groups (CON: 12.4 ± 1.5; CAF: 11.4 ± 1), the pups weight at birth was affected. We weighted the pups one day after parturition,

and CAF fed dam´s pups showed a lower weight (CON: 5695.00 ± 19.03 mg; CAF: 4721.00 ± 30.15 mg; $p<0.001$) (Figure 10).

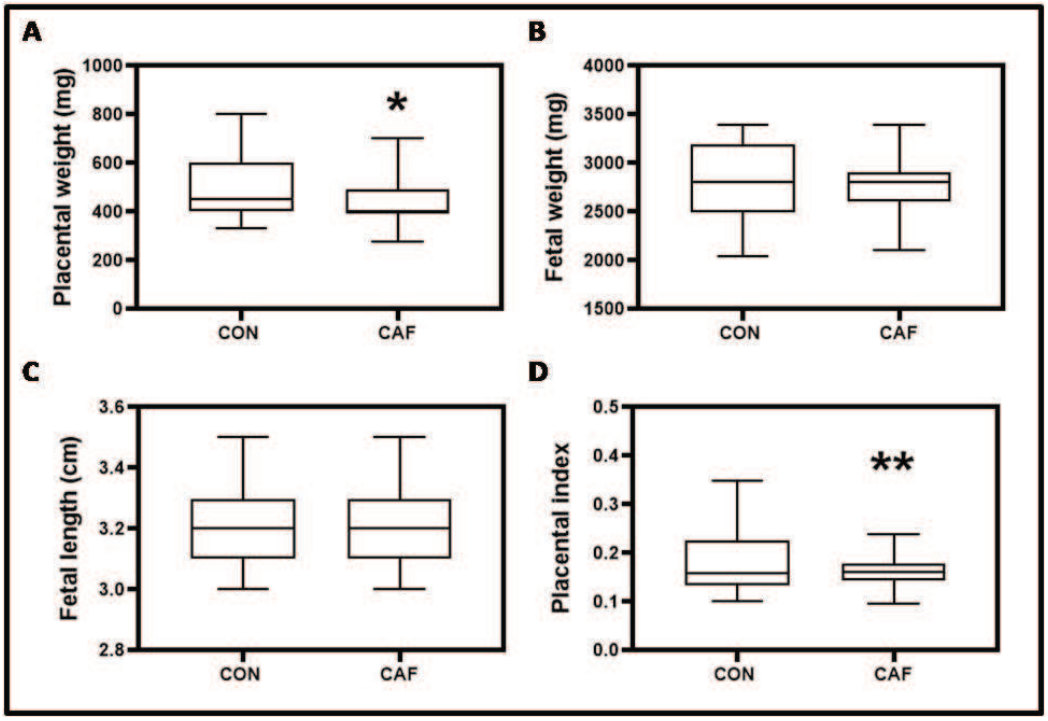

Figure 9: Effects of cafeteria (CAF) diet and control (CON) diet on feto-placental development parameters on gestational day 21 (GD21). (A) Placental weight (mg), (B) fetal weight (mg), (C) fetal length (cm), (D) placental index calculated as placental weight/ fetal weight ratio, on GD21. All results are expressed as the mean ± SEM for each experimental group (N total fetuses and placentas=127 CON/ 118 CAF; N mothers 10/Group). Asterisks indicate significant differences (*: $p<0.05$; **: $p<0.01$). Figure adapted from Gastiazoro et al. (*in evaluation*).

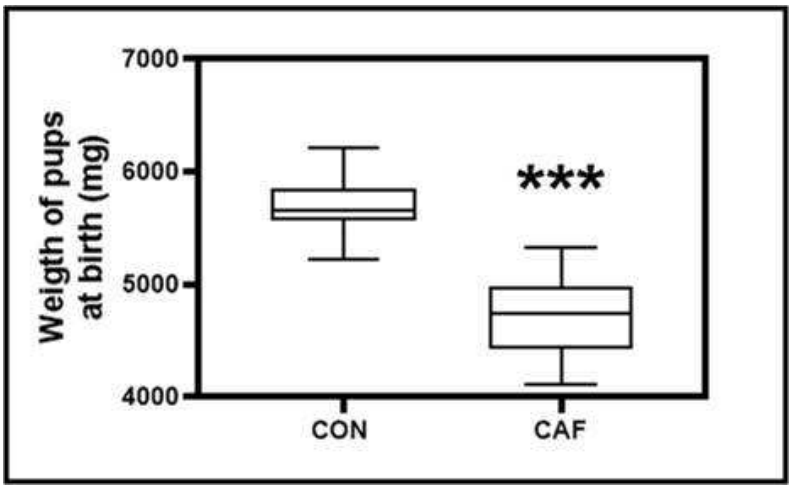

Figure 10: Effects of cafeteria (CAF) diet and control (CON) diet on weight of pups at birth (mg). The results are expressed as the mean ± SEM for each experimental group (N pups = 121 CON/ 124 CAF; N mothers 10/Group). Asterisks indicate significant differences compared to the control (***: $p < 0.001$). Figure adapted from Gastiazoro et al. (*in evaluation*).

3. CAF diet alters the expression of IGF and VEGF placental system molecules

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) (Figure 11 A and B); however, the diet produced an increase of IGF2 expression, while its receptor (IGF2R) remained unchanged (Figure 11C and D). Concerning VEGF placental system evaluation, VEGF expression was lower on CAF group-placentas and its receptor (VEGFR) did not change (Figure 11E and F).

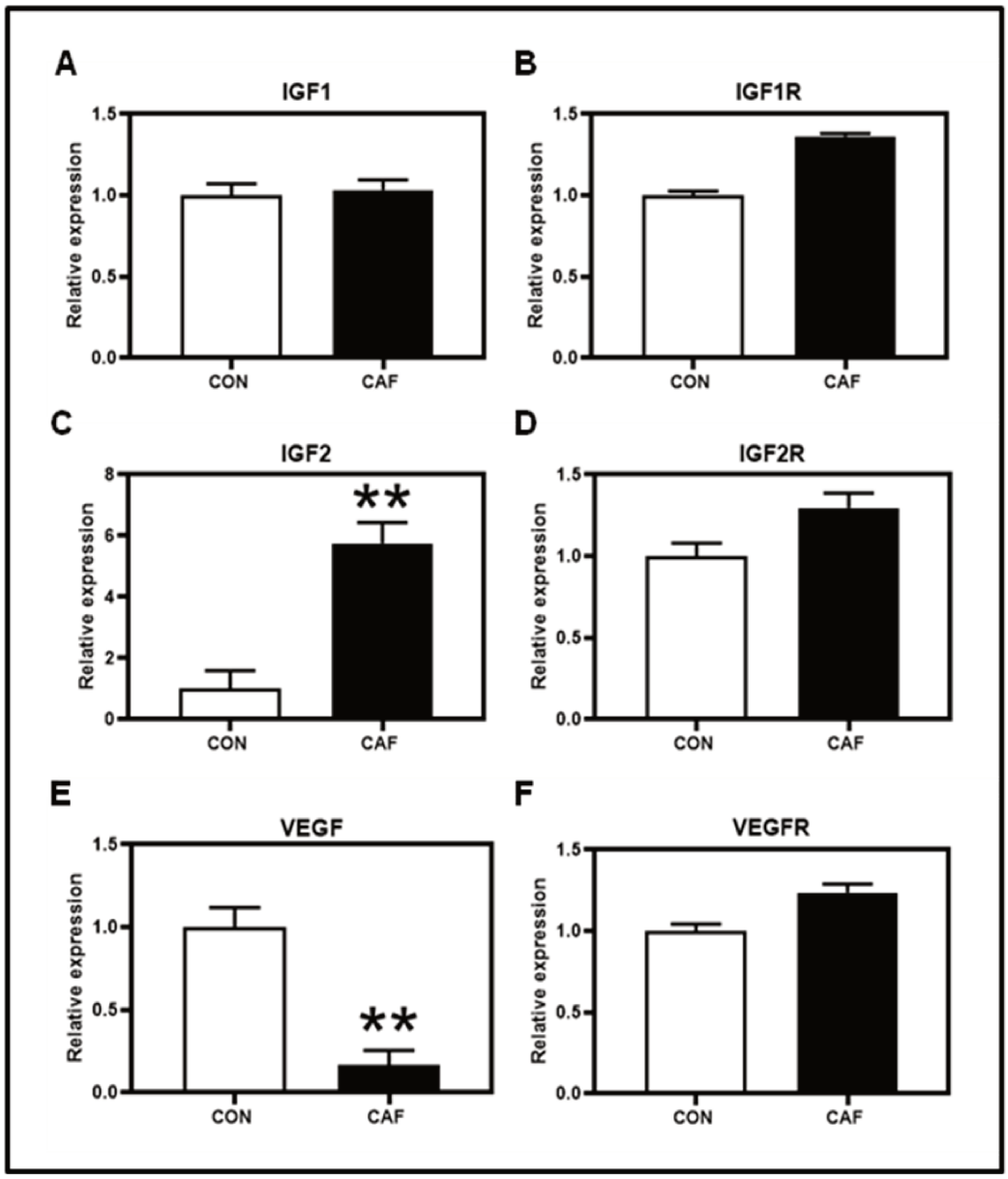

Figure 11: Analysis of relative mRNA levels of Insulin like Growth Factor (IGF) and Vascular Endothelial Growth Factor (VEGF) system in placental tissue of cafeteria (CAF) and control (CON) diet fed dams on gestational day 21 (GD21). (A) IGF1, (B) IGF1R, (C) IGF2, (D) IGF2R, (E) VEGF and (F) VEGFR. All results are expressed as the mean ± SEM for each experimental group (N placentas=10/Group; N mothers 10/Group). Asterisks indicate significant differences compared to the control (**: $p<0.01$). Figure adapted from Gastiazoro et al. (*in evaluation*).

3.1. Epigenetic disruption of IGF2 and VEGF placental genes produced by maternal CAF diet

To evaluate if the alteration in the mRNA levels of IGF2 and VEGF genes are related to DNA methylation modifications, we analyzed *in silico* the promoter regions of those genes (Figure 12 and 13) and we determined the methylation state in the CON and CAF groups (Figure 12 and 13). Regarding IGF system, we analyzed 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 (Figure 12). In addition, the promoter region of VEGF showed an increase in the methylation state at the *BstU* I site in CAF group, while a decrease at the *Tai* I site was found (Figure 13). The predictive analysis of TF 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 Elk-1 (ETS like-1 protein).

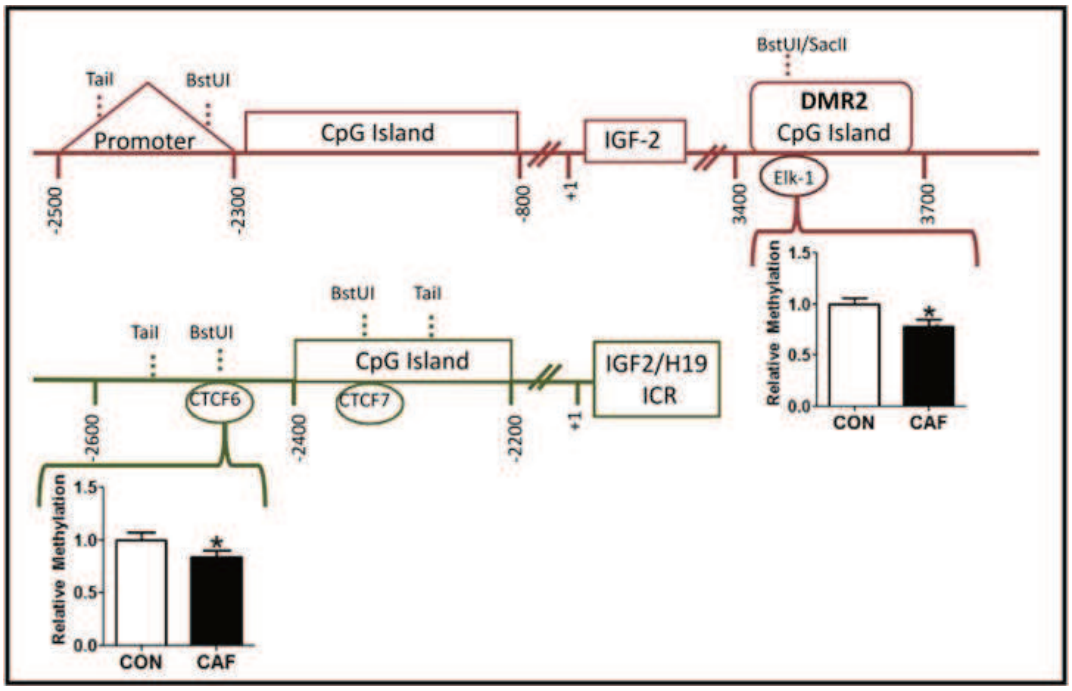

Figure 12: Methylation analysis of Insulin-like Growth Factor 2 (IGF2) and its imprinting control region (IGF2-H19) in placental tissue of cafeteria (CAF) and control (CON) diet fed dams on gestational day 21 (GD21). Predicted binding sites for transcription factors, CpG islands and CG target sites for 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 (N placentas=10/Group). Asterisks indicate significant differences (*: p<0.05). Elk-1: ETS like-1 protein, CTCF: CCCTC-binding factor. Figure adapted from Gastiazoro et al. (*in evaluation*).

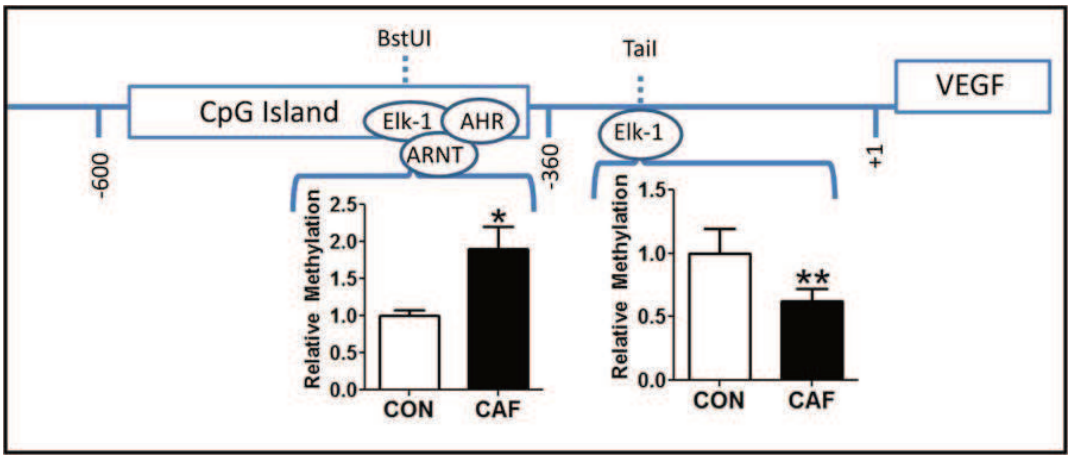

Figure 13: Methylation analysis of Vascular Endothelial Growth Factor (VEGF) in placental tissue of cafeteria (CAF) and control (CON) diet fed dams on gestational day 21 (GD21). Predicted binding sites for transcription factors, CpG islands and CG target sites for digestion by the methylation-sensitive 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 (N placentas=10/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. Figure adapted from Gastiazoro et al. (*in evaluation*).

Part III: Endometrial Hyperplasia

1. CAF diet induced uterine morphological changes

The uterus 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 (Figure 14A). CAF-fed rats showed morphological

changes that resembled uterine hyperplasia. An increased glandular volume fraction (CON: 4.77 ± 0.76 vs. CAF: 8.27 ± 1.87, $p < 0.001$) (Figure 14A and B) was observed. Moreover, a higher expression of vimentin was detected in both stromal studied regions (the SS and the PS) (Figure 15 A and B).

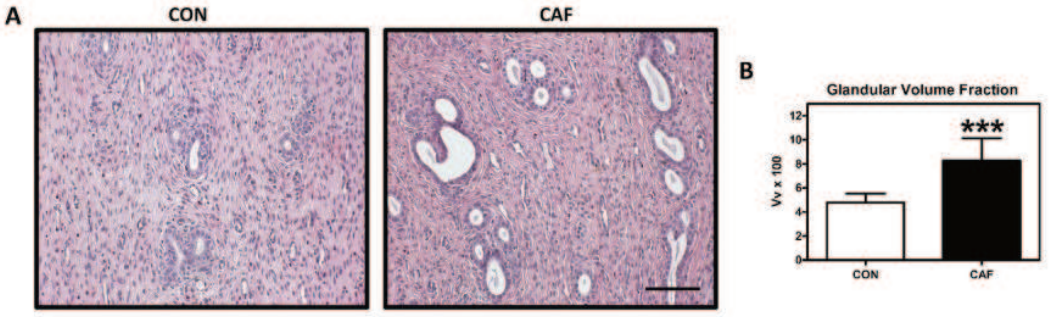

Figure 14: (A) Representative photomicrographs of uterus staining with Haematoxylin-Eosin of cafeteria diet (CAF) group and control group (CON). Scale bar: 50 µm. (B) Quantification of the glandular volume fraction. The results are expressed as Vvx100. Values in the bar graph are the mean ± SEM (N=10/Group). Data were analysed with the Mann Whitney Test. Asterisks indicate significant differences (***: $p < 0.001$). Figure adapted from Gastiazoro et al. 2018.

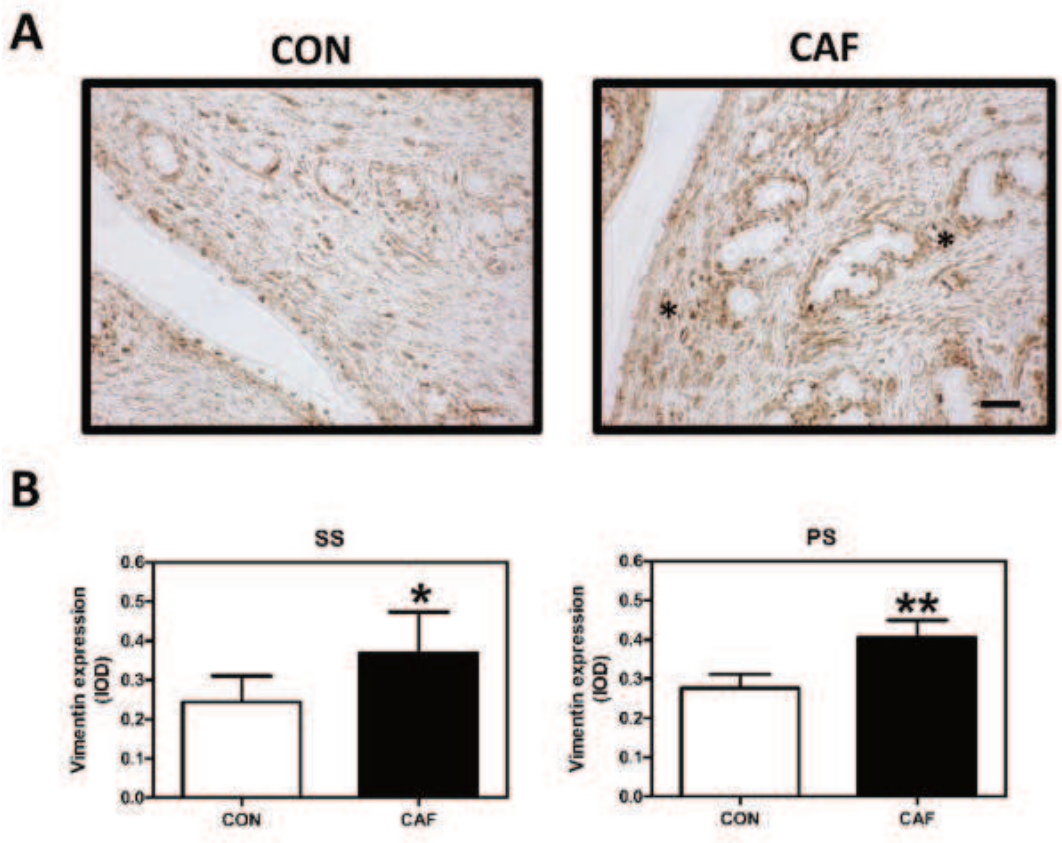

Figure 15: (A) Representative photomicrographs of vimentin expression in cafeteria (CAF) diet group and control (CON) group. Scale bar: 50 µm. (B) Quantification of vimentin expression in subepithelial (SS) and periglandular (PS) stroma. The results are expressed as integrated optical density (IOD). Values in the bar graph are the mean ± SEM (N=10/Group). Data were analysed with the Mann Whitney Test. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$). Figure adapted from Gastiazoro et al. 2018.

2. 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 (Figure 16A and B). PR expression was not affected by CAF treatment.

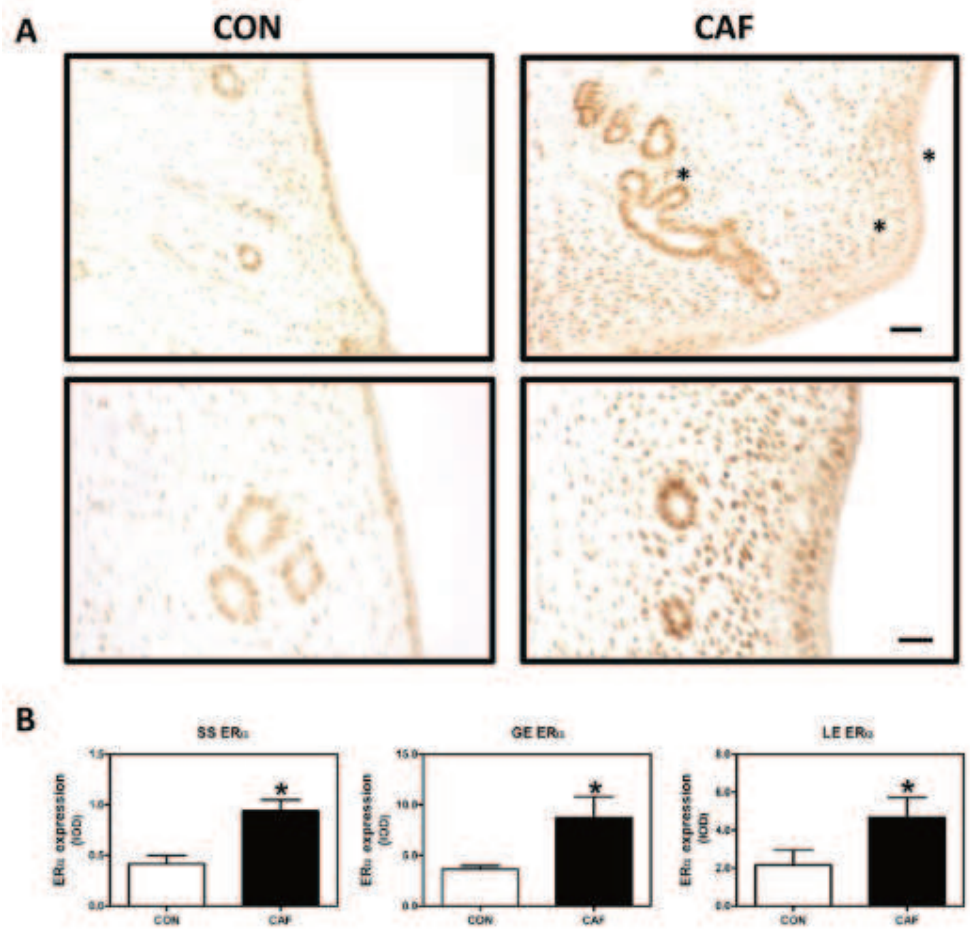

Figure 16: (A) Representative photomicrographs of immunohistochemical detection of ERα on Subepithelial stroma (SS), glandular epithelium (GE) and luminal epithelium (LE). Scale bar: 50 µm. (B) Quantification of ERα expression. The results are expressed as integrated optical density (IOD). Values in the bar graph are the mean ± SEM (N=10/Group). Data were analysed with the Mann Whitney Test. Asterisks indicate significant differences (*, $p < 0.05$). Figure adapted from Gastiazoro et al. 2018.

3. CAF diet induces an increase of uterine epithelial proliferation

To determine if the CAF diet affects uterine cellular 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% ($p < 0.01$) (Figure 17A and B).

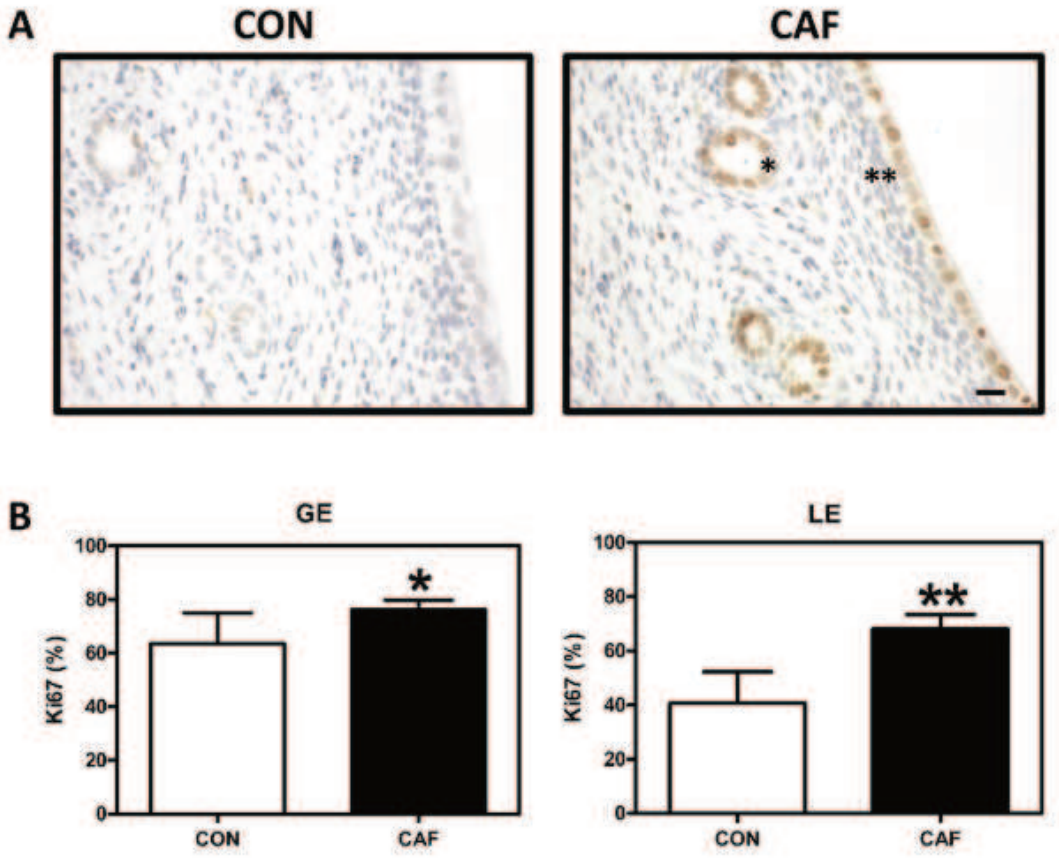

Figure 17: (A) Representative photomicrographs of immunohistochemical detection of Ki67 on LE and GE. Scale bar: 50 µm. (B) Quantification of Ki67 expression. Results are expressed as percentage. Values in bar graph are mean ± SEM (N=10/Group). Data were analysed with the Mann Whitney Test. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$). Figure adapted from Gastiazoro et al. 2018.

4. 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 IGF1 and IGF1R expression at the transcriptional level. CAF-fed animals showed a decreased uterine IGF1R expression, without changes in IGF1 expression (Figure 18A and B). In addition, we evaluated 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 (Figure 18C).

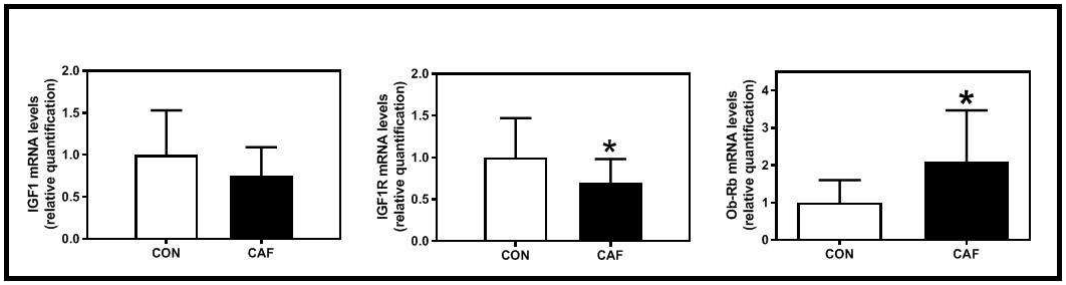

Figure 18: Expression of molecules related to proliferation control. Relative expression of Insulin like Growth Factor 1 (IGF1), its receptor (IGF1R) and long form of leptin receptor (Ob-Rb) mRNA levels. Samples were normalized to L19 mRNA expression, and the relative amounts of mRNA were calculated using the standard curve method. Each column represents the mean ± SEM (N=10/Group). Data were analysed with the Mann Whitney Test. Asterisks indicate significant differences (*, $p < 0.05$). Figure adapted from Gastiazoro et al. 2018.

V. Discussion

The aim of the present chapter was to study the effects of chronic administration of Caf diet on metabolic parameters, fertility, feto-placental development and endometrial carcinogenesis-related process.

In our work, CAF diet administered from weaning until adulthood produced an increase on body weight and energy intake, as it has been shown in others studies (Akyol et al. 2009; Goularte et al. 2012; Lalanza et al. 2014; Lazzarino et al. 2017; George et al. 2019; Rossetti et al. 2020). It is important to comment that those differences were remained before and during whole pregnancy and these results were published in other work of our group (Rossetti et al. 2020). Our CAF diet model generated overweight without metabolic impairments which could be explained by the different diet items in comparison with other diet models. Taking into account the response of animals to CAF diet, we could propose that any alteration observed in our CAF diet model would be produced by the dietary administration and not due to metabolic consequences produced by the diet.

Several studies showed that 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 work, the CAF diet generated

particular metabolic changes, characterized by increased energy intake, fat pads and body weight, but no metabolic syndrome. These apparent discrepancies with other authors 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 other CAF diets. Different reports indicate that 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 energetic 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.

A part of the aim of the present chapter was to determine the effects of a chronic maternal CAF diet on reproductive performance, feto-placental parameters on GD21 and the weight of pups at birth. In addition, we analyze the health of placenta by evaluating key placental systems: IGF and VEGF, along with the underlying epigenetic mechanisms.

In terms of reproductive performance, we observed that some parameters that we evaluated remained optimal, independently of the diet. Consistent with our results, Akyol et al. (2009) reported no alteration on reproductive outcome on CAF-fed animals and normal litter size. Contrary, other studies showed association between CAF feeding and reproductive impairments (Bazzano et al. 2015; 2017; Sagae et al. 2012; Kannan et al. 2019a, b). In general, these reports showed different metabolic and endocrine alterations that could explain the reproductive impairments, such as insulin resistance. We could suggest that the differences between these studies and our results on reproduction could be associated to dietary items, timing and length of diet administration and rat strain tendencies. It is important to highlight that there is a significant variability in the composition of diets of each study, some of which consist in a high content of sugar and/or additional 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 only in human foods, could differ on types and quantities of food items. In this sense, is hard to identify whether the observed damage on the different parameter

evaluated must be attributed to specific nutrient diet composition or maternal overweight (George et al. 2019). Nevertheless, a greater range of novel food including several items produced a larger impact on food intake and adiposity (George et al. 2019) and consequently could affect the rest of studied parameters.

At the date, scientific reports evidence that maternal nutrition has implications on feto-placental development (Gao et al. 2012; Dhobale et al. 2016; Gabory et al. 2016; Howell et al. 2017). Particularly, some authors reported that the placenta reacts to a high-fat diet (HFD) and CAF-fed dams and affected the fetal growth (Akyol et al. 2009; Lin et al. 2019). Our findings show that CAF feeding before and during pregnancy produced a lower placental weight, without changes on fetal length and weight on GD21. Similarly, Akyol et al. (2009) observed a decreased in placental weight, although they also reported lower fetal weight. Neither the length of dietary administration nor strain rat tendencies seems to be the critical point on this discrepancy. Although, it is not possible to conclude definitively what might have caused these differences, one could speculate that the dietary items and quality was not exactly the same. Besides, we found that the placental index of CAF-fed dams showed a decreased in comparison with CON fed dams on GD21. In optimal conditions, the placenta matures while the fetal weight increases, reflecting a decrease of the placental index (Macdonald et al. 2014). There are associations between atypical placental weight and/or placental index with maternal health impairments (Macdonald et al. 2014). Often, the placenta becomes more efficient and supports more mass of fetus per gram placenta. These placental adaptations optimize fetal growth (Sferruzzi-Perri et al. 2011). In this context, we propose that the placenta of CAF-fed dams has not enough functional capacity to resolves the impairments produced by this kind of diet, provoking a lower placental weight on GD21 and probably with consequences on weight of pups at birth. Other study, in coincidence with our results showed low weight of pups at birth after CAF maternal diet (George et al. 2019), but without analyze the health of the palcenta. These authors agree at the point that variations between species, timing and type of maternal exposure could be important factors on the different effects of maternal nutrition. Nevertheless, we propose that a possible mechanism to explain the observed effect of CAF maternal diet on reduction of placental weight and low weight of pups at birth is related with key placental systems. Additionally, possible epigenetic implications as mechanism of effects of the diet have not been studied.

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 (Sferruzzi-Perri et al. 2006). Meanwhile, IGF2 overexpression generates placental and fetal overgrowth and its deletion produce the opposite effects (De Chiara et al. 1990; Baker et al. 1993). Our findings indicate that the gene expression of IGF1 and its receptor IGF1R did not show differences in placental tissues between groups, which could be related with the normal fetal weight on GD21 found in CAF-fed dams. Instead, IGF2 gene expression showed an increase in GD21 of dams feeding with this diet, without changes in its receptor. Similarly, a study reported that the administration of 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 (Sferruzzi-Perri et al. 2013). Meanwhile, Gao et al. (2012) 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 low and placental weight and IGF2 expression were normalized. IGF2 contributions to adaptation to maternal protein restriction are apparently ineffective in promoting fetal growth (Gao et al. 2012). 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 but is inefficient to keep an optimal development until birth. Unfortunately, with this study we could not conclude that question and we consider it require further investigations.

VEGF and VEGFR are angiogenic factors and their well functioning is crucial for the nutrient transport. This system has a key role on endothelial cell proliferation, migration and tube-like structure formation (Dumont et al. 1994). In our study, we found a decrease of VEGF placental gene expression of CAF-fed dams, without changes in its receptor on GD21. Hu et al. (2019) reported a down regulation of VEGF protein expression in placenta and the density of placental vessels in dams feeding with high energy diet. Similarly, Wang et al. (2013) reported that HFD produced a decreased in placental weight together with a down regulation of VEGF expression in placenta. Salvolini et al. (2019) reported a link between obesity and an increase in VEGF, suggesting that this up regulation could be a compensatory mechanism for placental blood flow changes. Last, Bao et al. (2019) 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 specific role of this deregulation on fetal and placental development.

Taking into account the changes observed on mRNA expression of VEGF and IGF2, we decide to evaluate the methylation status of their respective promoter regions and to analyze these regions looking for potential TFs. TFs are proteins with the ability to bind to a specific upstream regulatory sequence on genes and regulate their transcription (Tapia et al. 2011); the methylation can block TFs binding and interfers with mRNA expression (Tate and Bird, 1993). First, we detected an increase in the methylation state at *BstU* I site in the CpG Island of the VEGF promoter, which is a potential binding site for AHR, ARNT and Elk-1. 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 the *Tai* I site, which is potential binding site for Elk-1. Although this effect is not reflected in mRNA, it could be explained as a compensatory mechanism of VEGF down-regulation, consideration the essential role of this factor on placental and fetal optimal development (Hu et al. 2019). In addition, we found a reduction in methylation levels at *BstU* I site 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 site is a CTCF6 which is a possible site to binding a highly conserved TF that can act as either transcriptional activator or repressor, and it function is regulated through an epigenetic mechanism (Gonzalez-Rodriguez et al. 2016). The second site (*BstU* I/*Sac* II*)* 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 (Li et al. 2017; Wu et al. 2014), a mainly process related with placental development, it is possible that these regions could be potential regulatory methylation-sensitive sites. 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 the analysis. In addition, it would be interesting to analyze the implication of histone modifications or miRNA or to perform experiments by using DNMT inhibitors that block the epigenetic effects in this experimental model.

Several risk factors for endometrial cancer are associated with different lifestyle 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. Part of the present chapter was developed to estimate the possible impact of the westernized diet on the uterus through chronic administration of the CAF diet.

As we mentioned before 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 E_2 secretion as a central failure related to the impairment ovulation. They proposed that the lower E_2 level may induce a prolonged dioestrus in the rat, leading to reproductive dysfunction (Bazzano et al. 2015). Another published work found no differences in E_2 serum levels but detected follicle-derived preovulatory progesterone surge inhibition during the proestrus phase and, consequently, inhibition of the preovulatory gonadotropin surge (Sagae et al. 2012). In the present thesis work, E_2 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 neglected when interpreting the results presented herein. The primary goal of this part 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 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 estrogen action–the genomic mechanism responsible for cell growth and proliferation. The gradual increase in ERα density in normal proliferative endometrium, which is higher in a 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 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). 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 a high ERα expression. Leptin and insulin are defined as regulators of endometrial proliferation (Villavicencio et al. 2010). Previous studies indicated that circulating insulin/IGF as well as the sex hormone axes is significantly dysregulated by obesity and diabetes and during endometrial cancer development (Merritt et al. 2016). Whereas insulin predominantly acts through the insulin receptor (IR), IGFs bind to the IGF1R, the IR, and the hybrid IR/IGF1R (Pollak et al. 2012; Brouwer-Visser and Huang 2015). Estrogen 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 post-menopausal women IGF2 showed a lower expression compared with that in the pre-menopausal proliferative phase (Lacey and Chia 2009). The same result was detected in our CAF-fed rats, with a lower expression of IGF1R compared with that of the control rats. However, the insulin serum level and the IGF1 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. The control exerted by the stroma on differentiation through different tissue recombination studies (by 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 comparable changes in human endometrial cancer patients are observable, suggesting that mesenchymal cells play an important role in the etiology of endometrial cancer (Tanwar et al. 2011). According to the World Health Organization (WHO, 1994), an abundant cellular stroma is a histological change presents 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).

VI. Conclusion

Our work is reflecting the influence of one of the most important factors of lifestyle, the maternal diet, on feto-placental development. At first, our CAF diet did not produce metabolic impairments withoutconsequences on reproduction. However CAF diet affected feto-placental development with consequences on birth weight. Regarding the feto-placental impaired growth, we consider that the placenta has a key role on the observed results. Taking into consideration the changes observed on IGF2 and VEGF is clear that CAF-fed dams suffered disturbances of IGF and VEGF placental systems. These findings could be cause or effect of placental disturbances, reflected on low placental weight on GD21 and low weight of pups at birth; although this is still unknown. In the same way, it is still unclear if the dysfunctional placenta and fetal growth outcome reported are due to the components of the diet or other intrinsic mechanisms of the mother. To the best of our knowledge, this is the first study of the impact of a CAF diet model on key placental systems methylation profile and the possible consequences of these epigenetic changes on fetoplacental development. It

seems that the type of foods included on this thesis work has essential implication in epigenetic modification. It is also important to considerer that these epigenetic changes could have a long-lasting effect later in life and in the offspring. 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 human and animal reproductive health.

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). For future studies, we are propoing to determine if these signal transducers could be affected in CAF fed animals as one possible mechanism to explain the endometrial hyperplasia.

Chapter II

Exposure to Glyphosate

I. Introduction

1. Glyphosate and Glyphosate Based Herbicides

Glyphosate (Gly) (N-phosphonomethyl glycine) is the active ingredient of a number of broad-spectrum herbicide formulations, named Glyphosate Based Herbicides (GBHs). Currently, Gly is the most frequently used herbicide in the world, for agriculture, forestry, urban and home applications (Guyton et al. 2015). In recent years, the use of GBHs is being voiced and has gained considerable concern in the world since increasing levels of Gly have been detected in different sources (Ronco et al. 2016; Gillezeau et al. 2019). These alarming levels of pollution might negatively impact on biodiversity in a number of ecosystems and also might affect human health (Niemann et al. 2015; Myers et al. 2016) (Figure 19).

Among the herbicide formulations available in our country, those that contain Gly as an active principle represent approximately 80% (Berman et al. 2018). These series of formulations have a wide spectrum of action (non-selective) and are used to eradicate weeds in agricultural areas and for post-harvest chemical desiccation. In addition to traditional uses in agriculture, GBHs are used in homes, in the maintenance of public spaces and in the control of aquatic flora (Székács et al. 2018). It is estimated that the use of this type of herbicide has increased ~100 times globally between 1974 and 2014, with an abrupt increase since 1996 with the introduction of genetically modified herbicide-tolerant crops (such as soybeans, corn and cotton), and the consequent expansion of the areas planted, both in our country, and in the rest of the world (Benbrock et al. 2016; CASAFE 2012). The massive use of the herbicide led to the appearance of weeds resistant to GBHs, which led to an increase in the doses and number of applications of the product (Fischer et al. 2014).

1.2. Environmental levels, occupational and environmental exposure

In Argentina, the environmental concentrations of Gly and its main metabolite, aminomethylphosphonic acid (AMPA), have been evaluated in different matrices that surround the main cereal and oilseed production areas. The data revealed the presence of Gly and AMPA in the water and sediments of rivers, streams and lagoons of rural and suburban areas in the provinces of Buenos Aires (Loughlin et al. 2017; Aparicio et al. 2017; Peruzzo et al. 2013), Santa Fe (Ronco et al. 2016), Córdoba (Bonansea et al. 2017) and Entre Ríos (Ronco et al. 2016; Primost et al. 2017). The most contaminated

areas revealed concentrations of Gly in water of 700 µg / l in the province of Buenos Aires (Loughlin et al. 2017), and of 2.15 and 3.29 mg / kg in sediments of rivers in the provinces of Córdoba and Entre Ríos, respectively (Ronco et al. 2016; Primost et al. 2017). In addition, elevated levels of Gly and AMPA were detected in soil samples from rural towns and agricultural fields, virtually ubiquitous, and at Gly concentrations up to 3.89 and 5.0 mg / kg (Loughlin et al. 2017; Primost et al. 2017; Avila-Vazquez et al. 2017). Besides, levels of these compounds were detected in the dust derived from the erosion of a field in the province of La Pampa, one year after the last application of the herbicide (Mendez et al. 2017). This shows that dust from fields where the herbicide is applied could be a major source of air pollution. In turn, Gly and AMPA residues have been detected in a series of foods for human and animal consumption, in soy-based food formulas for children and even in bottled water (Figure 19) (Rodrigues et al. 2018; Zoller et al. 2018; Rendon-von Osten et al. 2017; Bai et al. 2016). In Argentina, very high levels of Gly, above the magnitude of mg / kg, were detected in transgenic soybean seeds and plants in fields of the Santa Fe and Salta provinces. Some of these samples exceeded the maximum residue limit for food of 20 mg / kg established by the European Union (Arregui et al. 2004; Test-Biotech 2013).

Biomonitoring studies conducted on human samples detected the presence of Gly in the urine of individuals living in both urban and rural areas of Europe and the United States (Niemann et al. 2015; Mills et al. 2017). In the latter country, a study conducted in pregnant women found a positive correlation between urine Gly levels and its residence in rural areas (Parvez et al. 2018). Another study performed in Thailand detected Gly in the serum of pregnant women and umbilical cord blood at concentrations that were in the range of 0.21-89.1 ng/ml and 0.2-94.9 ng/ml, respectively. The presence of Gly in pregnant women at the time of delivery was strongly associated with factors such as their own occupations as farmers, and its residence close to growing areas or with family members who performed agricultural tasks (Kongtip et al. 2017). Together, the evidence suggests that there is a risk of environmental and occupational exposure to Gly both locally and globally, which has aroused interest and concern to know its effects on human health.

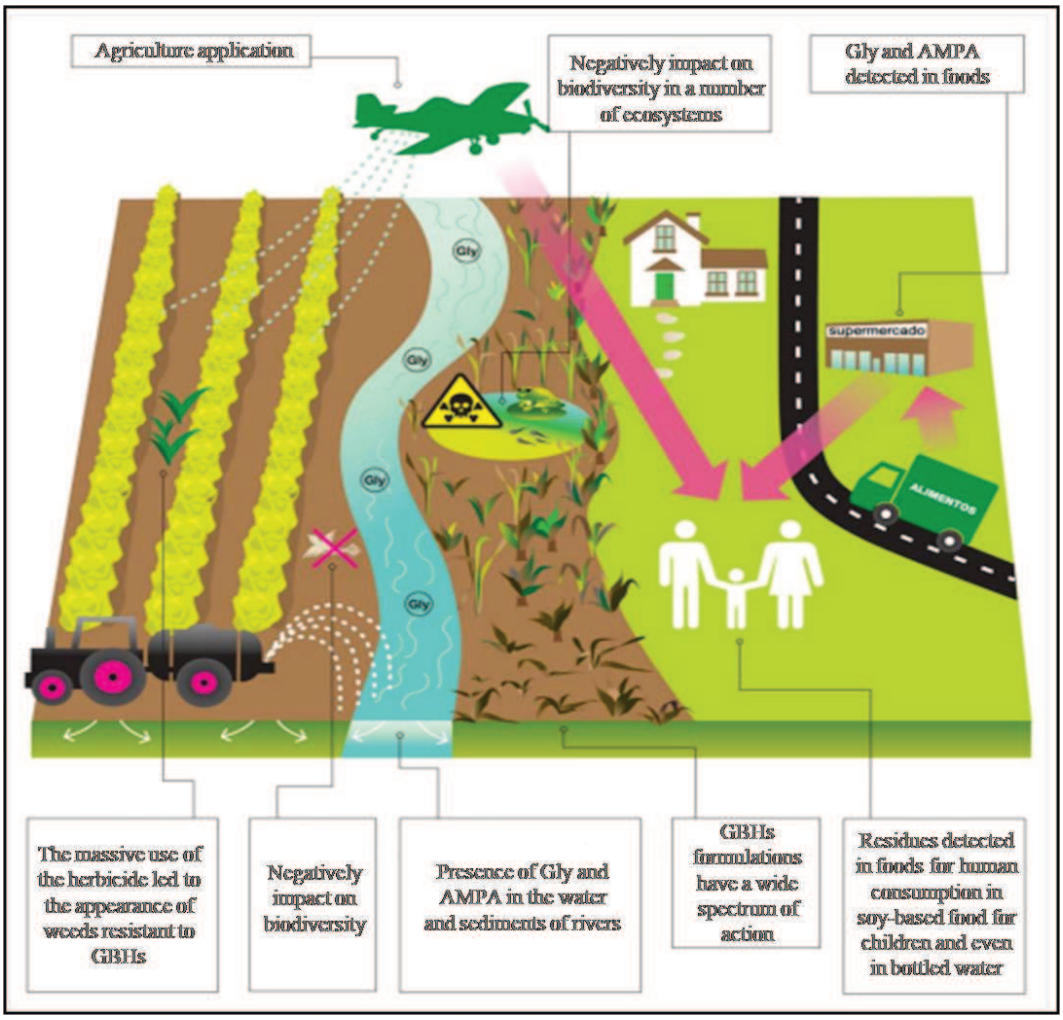

Figure 19: Effects of glyphosate (Gly), Glyphosate Based herbicides (GBHs) and its main metabolite aminomethylphosphonic acid (AMPA) on environment and human health. Figure taked and adapted from Riley et al. 2011.

2. Glyphosate effects on reproduction and development

In recent years, an issue of concern has focused on the toxicological properties of the chemicals used in the formulations of GBHs. These formulations are marketed as mixtures containing Gly and different co-formulants (solvents, penetrants, preservatives, and surfactants) whose identity is often not disclosed because it is considered confidential business information. Several studies comparing the effects of GBHs, their co-formulants, and technical grade Gly revealed that GBHs are more toxic than the active ingredient, suggesting that co-formulants may also be considered a source of toxicity (Mesnage et al. 2019; Defarge et al. 2016).

Studies in animal models have shown that exposure to GBHs during developmental periods such as gestation, neonatal and pre-pubertal stages, induces adverse effects on the reproductive system of animals. Female rats exposed to low doses of GBH by subcutaneous injection during neonatal period, exhibited impaired uterine development both at the neonatal and the prepuberal periods (Guerrero Schimpf et al. 2017), with long-term consequences on fertility. Other results indicated that GBH exposure induced post-implantation embryo loss (Ingaramo et al. 2016) and provoked impairments in endometrial decidualization process (Ingaramo et al. 2017). On the other hand, lambs exposed to a low doses of GBH via subcutaneous injection, manifested histofunctional impairments and an altered differentiation of the ovaries and uterus (Alarcón et al. 2019). Another study done in female rats perinatally exposed to an oral low dose of GBH, showed higher pre-implantation loss rate and adverse outcome on fetal development and structural congenital anomalies in F2 offspring of GBH-exposed rats (Milesi et al. 2018). *In vitro* studies have shown toxic cellular effects at low doses by using embryonic, placental and Sertoli cells (Benachour et al. 2007; Richard et al. 2005; Liz Oliveira Cavalli et al. 2013).

Taking into consideration all these results, in the present study we propose a model of exposure including two interesting points: 1) oral administration of a low dose of GBH, and 2) chronic exposure from the weaning to adulthood.

In vitro and *in vivo* studies have documented different effects induced by Gly and its formulations in hormone-dependent tissues, such as testis, ovary, uterus and mammary gland, which would indicate a possible activity as endocrine disrupting chemical (EDC) (Cassault-Meyer et al. 2014; Perego et al. 2017; Varayoud et al. 2017; Altamirano et al. 2018). Some of the most relevant effects included the inhibition of the aromatase enzyme, responsible for the irreversible conversion of androgens to estrogens, the induction of proliferation of breast cancer cells through the activation of ERα (Thongprakaisang et al. 2013; Mesnage et al. 2017), and alterations in the steroidogenesis process. Previously, we determined the estrogenic activity of a GBH using an *in vivo* model, and we detected that GBH produced modifications in the regulation of estrogen-sensitive genes, similar to those induced by E_2 (Varayoud et al. 2017). Likewise, other studies of our laboratory indicated that exposure to GBH affected the development of the mammary gland of male (Altamirano et al. 2018) and female (Zanardi et al. 2020) rats, which was accompanied by an increase in highly

proliferative mammary gland structures, and an increase in cell proliferation and in the expression of ERα (Altamirano et al. 2018; Zanardi et al. 2020).

Despite the antecedents reported in the scientific literature, the EPA (Environmental Protection Agency) through the EPA Endocrine Disruptor Screening Program (EDSP), stated that Gly does not present the properties of an EDC, using a weight-of-evidence approach, that the existing data do not indicate that Gly has the potential to interact with the estrogen, androgen or thyroid signaling pathways (EPA 2015). Therefore, the potential of Gly as EDC remains uncertain.

3. Glyphosate as a potential carcinogen

In relation to the properties as a carcinogen, the background on the effects of Gly and its formulations is controversial. In 2015, the International Agency for Research on Cancer (IARC, WHO) reclassified glyphosate as "probable carcinogenic to humans" (group 2A), including in its analysis more than 500 scientific works carried out around the world that used so much Gly like their commercial formulations (IARC 2017). This classification is based on the fact that although the evidence of carcinogenesis in humans is limited, tests in laboratory animals are sufficient. In addition, genotoxic effects and oxidative stress were detected in cells of various species that were proposed as mechanisms of carcinogenicity. In contrast, the European Food Safety Authority (EFSA) has concluded that the herbicide not show carcinogenic or mutagenic properties, based on all available evidence for Gly (the active ingredient in formulations), and including unpublished data from the industry (Portier et al. 2016; Tarazona et al. 2017). The EFSA stated that "glyphosate is unlikely to pose a cancer risk to people and that the evidence does not support its classification with respect to carcinogenic potential" (EFSA 2015). Due to the above, the carcinogenic potential of Gly and its formulations remains uncertain.

A study carried out on inhabitants of Monte Maíz (in Córdoba province, Argentina), whose main economic activity is agriculture, revealed that cancer incidence, prevalence, and mortality are between two and three times higher than the reference values for the entire nation (Avila-Vasquez et al. 2017) (cancer incidence: 706/100,000 persons vs. 217/100,000; cancer prevalence: 2123/100,000 persons vs. 883.82/100,000 and mortality: 383/100,000 persons vs. 115.13/100,000, respectively; Globocan 2012, WHO). The authors highlighted that levels of Gly and its metabolite AMPA were

higher in comparison with other agrochemicals were detected in different zones of the same town. It is important to mention that sampling belonging to a children´s playground contained 68 times more Gly that sampling belonging to a farm field of corn resistant to Gly. Similarly, the highest concentration of Gly, AMPA and other pesticides (more than 3000 ppb) were found in a soil sample from the sidewalk next to pesticides deposits (Avila-Vasquez et al. 2017). Evidence for the carcinogenic effects of Gly in humans is supported by studies of occupational exposure to the herbicide in populations of the United States, Canada and Sweden that showed increased risks for non-Hodgkin lymphoma (Eriksson et al. 2008; De Roos et al. 2003; McDuffie et al. 2001). Added to this, *in vivo* and *in vitro* experimental trials regarding the effects of Gly or its commercial formulations reinforce this evidence. George et al. (2010) observed that a commercial Gly formulation acted as a "promoter" in tumor development in a mouse melanoma model (George et al. 2010). Other authors attributed to Gly the increased incidence of renal tubular carcinoma, hemangiosarcoma, and pancreatic islet cell adenoma in murine models (IARC 2017).

Evidence for the possible activity of Gly as EDC has sparked interest and concern about its effects on development/differentiation, reproductive capacity, and tumor development in animals and humans. As we mentioned before, there are a few studies that evaluate the effects of GBH in rats (Guerrero Schimpf et al. 2017, Milesi et al. 2018) and lambs (Alarcón et al. 2019) after acute exposure by oral or subcutaneous routes. So far, there are not available studies on the consequences of chronic low doses exposure to GBH administrated via oral on female reproduction.We consider that an oral chronic exposure to low doses of the herbicide through the food seems to reflect the real human exposure through the life.

4. Endometrial carcinogenesis-related process

One process associated with carcinogenesis (endometrial and others) is the epithelial-mesenchymal transition (EMT) (Kalluri et al. 2009). EMT is a cell-biological program that allows the polarized epithelial cell phenotype to convert to mesenchymal cell phenotype. The epithelial cell phenotype implies a normal interaction with basement membrane via cell basal surface, named the immotile cells. However, the mesenchymal cell phenotype includes enhanced migratory capacity and invasiveness, and decrease of the epithelial gene expression; consequently these cells are called the motile cells (Kalluri et al. 2009; Yang and Weinberg 2008). Moreover, EMT has been suggested as

the first and key step from migration and invasion of cancer cells (Zhai et al. 2016). During cancer progression process, cells loose the cell-cell adhesion and gain migratory and invasive features, which are evidenced by the down-regulation of epithelial markers, like E-cadherin (Figure 20) (Yang and Weinberg 2008).

In the present study we propose to determine if Gly affected EMT as a key process related to endometrial carcinogenesis. Different EDCs were shown to promote EMT process via ER dependent pathway (Zhai et al. 2016; Chen et al. 2010). Given that Gly has been reported to trigger the activation of estrogenic signals, we hypothesized that it could be a pathway through which the herbicide could promote cancer-related outcomes.

To our knowledge, until now there is no evidence connecting Gly with EMT-related changes.

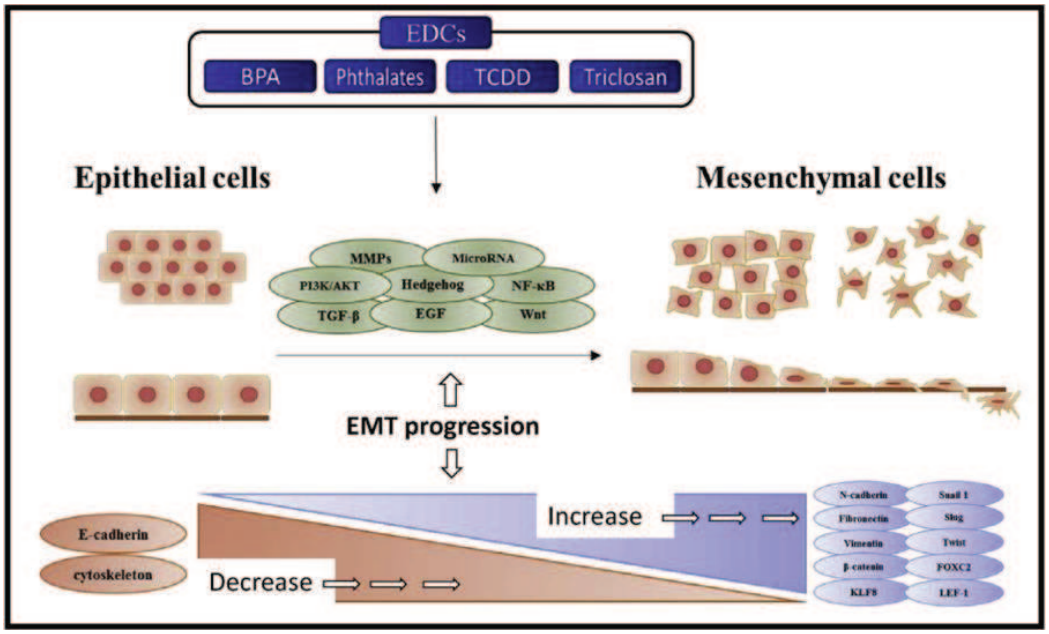

Figure 20: Potential effects of Endocrine Disruptors (EDCs) such as Bisphenol A (BPA), phthalate, 2,3,7,8-tetraclorodibenzo-p-dioxina (TCDD) and triclosan on the Epithelial Mesenchymal Transition (EMT) process in cancer metastasis.. Through the EMT process, primary cancer cells preferentially lose their cell-cell adhesion to gain mesenchymal properties and are transformed to migratory and invasive cancer cells, which break through the basement membrane and enter the bloodstream through intravasation to move to the metastatic sites. Epithelial cells express high levels of E-cadherin, and mesenchymal cells express such as vimentin. EDCs such as BPA, phthalates, TCDD, and triclosan have the potential to regulate EMT markers and

migration via several signaling pathways associated with the EMT program. Figure adapted from Lee et al. 2017.

II. Goals

1. Main goal

We propose to study the effects of a chronic exposure to a safe dose of a commercial GBH on reproductive performance and feto-placental development. In addition we aim to determine if Gly exposure promote endometrial carcinogenesis related-process.

2. Specific goals

PART I:
To determine the effects of an oral chronic exposure to a safe dose of commercial GBH on body weight and food intake.

PART II:
To investigate the effects of an oral chronic exposure to a GBH on rat reproductive performance and feto-placental parameters.

PART III:
To evaluate the influence of exposure to Gly on endometrial carcinogenesis-related process, using an *IN VITRO* model*:*

-To investigate the effects of Gly on EMT-related process in a human endometrial carcinoma cell line, by evaluating cell migration and invasion after Gly exposure, and expression of EMT-related markers.

-To determine if Gly effects on human endometrial carcinoma cell line are mediated via ER-dependent pathway.

III. Materials & Methods

1. Substances

The Gly formulation used in this study was Roundup FULL II, a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt (equivalent to 54% w/v of glyphosate acid), as its active ingredient, coadjuvants and inert ingredients. This GBH was chosen based on the fact that it is one of the herbicides most commonly used in

Argentina, and that it is representative of formulations with high content of Gly indicated against weeds difficult to eradicate.

2. 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).

We used inbred Wistar-derived strain rats that were bred in the Department of Human Physiology (Universidad Nacional del Litoral), and housed in a controlled environment (22 ± 2 °C; lights on from 06:00 to 20:00 h) in stainless steel cages with wood bedding.

3. Experimental design

Female Wistar rats were bred, weaned at 21 days of age (PND21), and randomly divided into two groups: control diet group (CON) (n= 15) and glyphosate based herbicide group (GBH) (n= 15). The animals of the CON diet group were fed with a laboratory pellet chow-based paste and the animals of the GBH group were fed with a laboratory pellet chow-based paste provided with paste supplemented with GBH in a dose of 2 mg of Gly/kg bw/day. This dose of Gly is in the order of magnitude of the reference dose (RfD) of 1 mg/kg bw/day, which is based on maternal and developmental toxicity studies (EPA 2017). Moreover, the dose evaluated is in the order of magnitude of the Gly residue levels found in Argentina from different environmental matrixes such as soybean grains, open-reservoir tank water, soil and sediment (Demonte et al. 2018; Primost et al. 2017; TestBiotech 2013). The laboratory chow-based paste was prepared by blending optimized quantities of pellet chow (Nutrición Animal, Santa Fe, Argentina) and water; for GBH treatment groups a Gly commercial formulation was added to the water according to the above described dose. The mixture was covered and stood overnight, after that it was homogenized to form a paste and chow balls were prepared to each treatment. The pellet-based paste for control and GBH groups was prepared freshly, i.e., the same day the food was replaced. We checked for Gly integrity in the dietary matrix by measuring its concentration during three consecutive days by Ultra performance liquid chromatography–tandem mass spectrometer (UHPLC–MS/

MS) according to the protocols described in Oulkar et al. (2017). A preliminary study was carried out to ensure that the addition of GBH does not alter food consumption, and to estimate the average amount of chow-based paste daily consumed during a period of time (for details please see Milesi et al. 2018). These data allowed us to calculate the amount of the active ingredient (glyphosate) to add to the pellet chow. Tap water was supplied *ad libitum* in glass bottles with rubber stoppers surrounded by a steel ring. The respective diets and water were administered *ad libitum*.

4. PART I

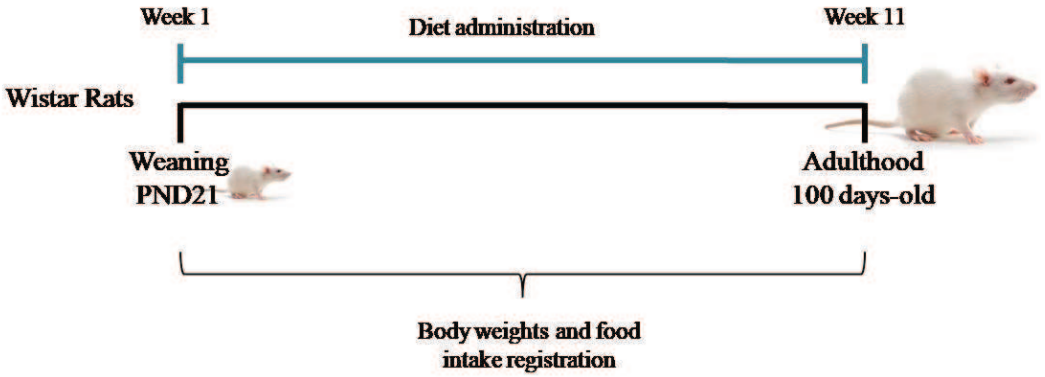

Figure 21: Experimental design to evaluate the effects of a chronic exposure to a safe dose of commercial Glyphosate Based Herbicide (GBH) on body weight and food intake. PND: postnatal day.

4.1 Determination of body weight and food intake

During the experimental time, body weights and food intake were registered three times per week, from the beginning of administration of each diet (PND21) and during the whole treatment (11 weeks; n=15 per group). Food intake was measured by the weight difference between the accessible and the remaining food, adjusted to the waste by collecting food spillage.

5. PART II

5.1 Evaluation of reproductive performance

After the 11th week of diet administration (100 days-old), we evaluated the reproductive performance according to the method described in Chapter I, PART II, section 4.1. For this part of the thesis the evaluation of reproductive parameters during pregnancy was performed on GD19 (n=15 per group).

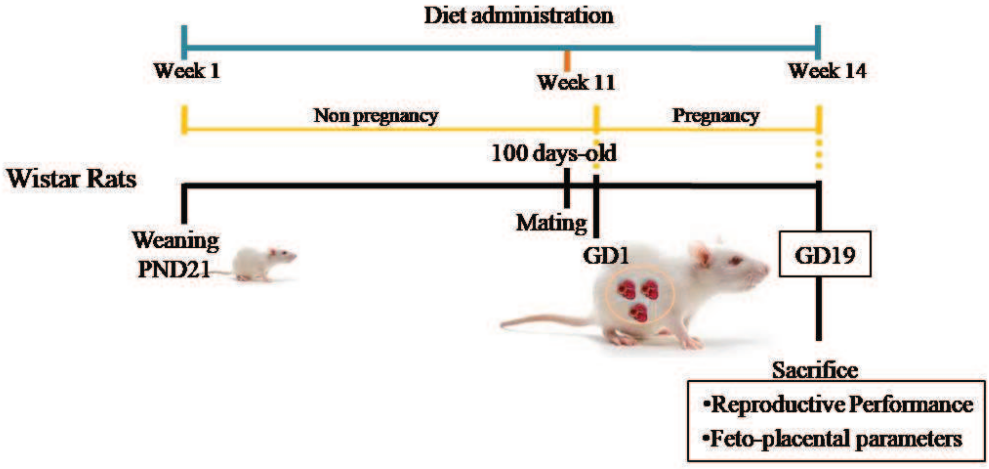

Figure 22: Experimental design to evaluate the effects of chronic exposure to a Glyphosate Based Herbicide (GBH) on rat reproductive performance and feto-placental parameters. PND: postnatal day.

5.2 Feto-placental parameters

The feto-placental parameters were determined at GD19 according to the method described in Chapter I, PART II, section 4.2.

6. PART III

IN VITRO

6.1 Substances

All reagents and chemicals were of analytical grade. Glyphosate (CAS N° 1071-83-6) used as the PESTANAL® analytical standard (purity grade ≤100%) and E_2 (purity ≥98%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Fulvestrant (ICI 182,780; purity ≥99%) was purchased from Tocris Bioscience (Minneapolis, Minnesota, USA).

6.2 Cell culture

The human endometrial adenocarcinoma cell line Ishikawa was provided by Masato Nishida, Department of Obstetrics and Gynecology, University of Tsukuba. These cells were obtained from an endometrial adenocarcinoma of a 39-year-old woman in 1985 by Nishida, and established as ER- and PR-positive cell line (Nishida et al. 1985). The cells were cultured in Dulbecco's modified Eagle's medium F12 (DMEM/F12) (Biowest, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% Insulin-Transferrin-Selenium A (ITS) (Gibco-BRL, Grand Island, NY, USA) and maintained in culture 75 cm^2 flask at 5% CO_2 and 37 °C. The medium was replaced every 48 h. Ishikawa cells were grown to 80% of confluence and enzymatically detached by trypsin and (0.05%) EDTA at 37 °C.

6.3 Treatment conditions

The concentrations in our experiment were chosen based on the RfD of 1 mg of glyphosate/kg b.w. per day established by U.S. EPA (US EPA, 2017). We worked in a range between 0.2 µM and 200 µM (0.2 µM, 2 µM, 20 µM and 200 µM), an interval of concentrations that include the RfD. A 1 mM Gly stock solution was prepared with ethanol and different volumes were added during the treatment to achieve the corresponding Gly concentration. In detail, to final Gly concentration 0.2 µM in a 6-well plate with 2 ml media per well, we added 0.4 µl of Gly stock solution; to final Gly concentration 2 µMwe added 4 µl; to final Gly concentration 20 µM we added 40 µl of Gly stock solution and to final Gly concentration 200 µM we added 400 µlof Gly stock solution. As a positive control we used E_2 at 10^{-9} M (Keiler et al. 2015). ER antagonist Fulvestrant was tested at concentration of 10^{-7} M (Tsutsumi et al. 2011). Dimethylsulfoxide (DMSO) was used as vehicle for E_2 and Fulvestrant and added in a way that the DMSO concentration in the test did not exceed 0.1%. Ethanol was used as vehicle for Gly and added in a way that Ethanol concentration in the test did not exceed 2%. No difference between DMSO and Ethanol was detected among assays. For that reason, vehicle is shown as a unique condition in the results. To carry out the treatments, the cells were seeded in a 6-well plate for 24 h to establish adherent monolayer after that the corresponding drug-treatment was added depending on the assay. The medium with the corresponding amount of Gly, E_2, or Fulvestrant was renewed every 24 h.

6.4 Trypan blue dye exclusion assay

We determined the viability of Ishikawa cells after treatment with selected concentrations of Gly by trypan blue dye exclusion assay. The aim was to determine whether the different concentrations affected the cell viability in a significant way. Four concentrations of Gly were tested, Gly 0.2 (0.2 μM), Gly 2 (2 μM), Gly 20 (20 μM) and Gly 200 (200 μM). The exclusion criterium was to reject the concentrations that cause a viability ≤ 80% and show statistical significant difference respect to vehicle.

Cells were seeded in a total volume of 2 ml at a density of 200,000 cells/well in a 6-well plate until monolayer formation (24 h). The next day, the cells were incubated with Gly concentrations previously described and incubated at 5% CO_2 and 37 °C for 24 h. The cells were washed twice with PBS and all supernatant was collected in Falcon tubes (one per each well). Then the cells were detached using trypsin/EDTA for 7 min at 37 °C. Trypsin reaction was stopped by cell culture medium addition. The cell suspension was collected in a Falcon tube and centrifuged at 900 g for 5 min. The supernatant was discarded, and we prepared a cell suspension with 10 μl of cells and 10 μl of 0.5% Trypan Blue (Merck, U.S) to be counted on the haemocytometer (Neubauer counting chamber). We determined Viability (%) as follows: viable cell count (unstained cells count)/total cells count (stained cells count + unstained cell count) x 100. We expressed the results as Viability (%) relative to vehicle.

6.5 Scratch-wound healing assay

The Ishikawa cells were seeded in 6-well plates at density of 200,000 cells/well with DMEM/F12 - FBS 10% - ITS 1% medium and incubated at 5% CO_2 and 37 °C with vehicle, E_2 or different Gly concentrations with/without addition of Fulvestrant for 24 h. Then the monolayers were scratched using a sterile 200 μl micropipette tip, and washed with PBS twice to remove the detached cells. The different treatments were renewed and the DMEM/F12 - FBS 10% - ITS 1% medium was replaced with DMEM/F12 - FBS 1% - ITS 1% medium. The cells were incubated during a 72-h period, renewed the media and treatments every 24 h. The 72-h period was selected based on the frame time of incubation on which only under optimal condition (positive control) be close to closure the scratch (Liang et al. 2007). In order to measure the percentage of wound closure area by migrating cells, 8–10 images (from each treatment group) of the wounded cell monolayers were taken at 0 and 72 h after scratching at 40X using an Olympus CK40 Inverted Microscope (Olympus, Japan) coupled with a camera Canon Power Shot G9 (Canon, Japan). The average area of wound covered was calculated as

percentage of wound closure after 72 h = (uncovered area at 0 h – uncovered area at 72 h)/uncovered area at 0 h x 100. The uncovered areas were determined using Image J software. The results were expressed as % wound closure after 72 h relative to vehicle.

6.6 Transwell Invasion assay

The invasion assay was conducted using Falcon cell culture insert 8 µm pore size placed into a 24-well plate. The inserts were uniformly coated with Matrigel® basement membrane matrix (Corning Life Science, U.S.) for 1 h at 37 °C before cells were added. Cells were treated with vehicle, E_2 or different Gly concentrations with/without addition of Fulvestrant for 24 h and then, harvested by trypsinization. Cells (1 × 105 cells/well) were seeded into the upper chamber in 200 µl of DMEM/F12-FBS 1% - ITS 1% medium, while the bottom of the chamber was incubated with 750 µl DMEM/F12-ITS 1% medium containing FBS 20% as a chemoattractant. After 48 h of incubation, cells migrating from the top chamber to the lower surface of the insert, as a result of cell invasion through Matrigel®, were fixed 3 min with formaldehyde 4% and 20 min with methanol. Finally, the cells were stained 20 min with crystal violet 0.1%. To quantify the number of invasive cells, all the slides with stained cells were photographed at 40X using an Olympus CK40 Inverted Microscope (Olympus, Japan) coupled with a camera Canon Power Shot G9 (Canon, Japan). Images were analysed using the cell counting tool in Image J software. We determined the number of invasive cells after 48 h. The results were expressed as number of invasive cells after 48 h relative to vehicle.

6.7 Determination of gene expression using Ishikawa cells

The Ishikawa cells were seeded in 6-well plates with DMEM/F12 FBS 10% - ITS 1% medium and incubated at 5% CO_2 and 37 °C with vehicle, E_2 or different Gly concentrations with/without addition of Fulvestrant for 24 h. Total RNA from cultured cells was isolated after treatment using Tri-Fast™ (Peqlab VWR, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed by enzymatic digestion (RQ1 DNase, Promega, U.S.) and checked by PCR. First-strand cDNA synthesis was performed by mixing 2 µgof digested RNA with MMLV reverse transcriptase (Promega, U.S.) and Oligo (dT) 12–18 primers (Eurofins MWG Operon, Germany). Quantitative real-time PCR was applied for cDNA amplification with SybrGreen I as the detection dye using the iCycler iQ™ Real-Time PCR Detection System (BioRad, U.S.). 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 56–60 °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, yielding no consistent amplification. To quantify the expression relative to the vehicle-treated cells, the ΔΔCT method was used (Pfaffl 2001).

7. Statistical analysis

PART I and II: G Power software (http://www.gpower.hhu.de/; RRID:SCR_013726) was used to determine the sample size (Faul et al. 2007). To confirm the normal distribution of the data and variance homogeneity, Shapiro–Wilk test and Levene's test were performed. Body weights, food intake, as well as feto-placental 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 were analyzed using Mann-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 $p<0.05$.

PART III: Data are presented as the mean ± SEM of three independent experiments. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls comparison test using the Graph-Pad Prism software Version 5.03 (San Diego, CA, USA). p values < 0.05 were regarded as statistically significant.

IV. Results

Part I: Evaluation of body weight and food intake

1. GBH exposure increases body weight

The exposure to GBH began on PND21. Then, 8 days after the beginning of treatment (PND30) we detected an increase on body weight of GBH- exposed animals (CON: 57.33 g ± 2.25 vs. GBH: 88.96 g ± 1.72, $p < 0.05$). As we showed in Figure 23, the high body weight was maintained during the whole treatment. When we analyzed if animals modified the food intake as a consequence of GBH exposure, the results indicate that not differences were detected between food intake of CON and GBH-treated rats (CON: 22.01 g/day ± 0.34 vs. GBH: 23.52 g/day ± 0.65).

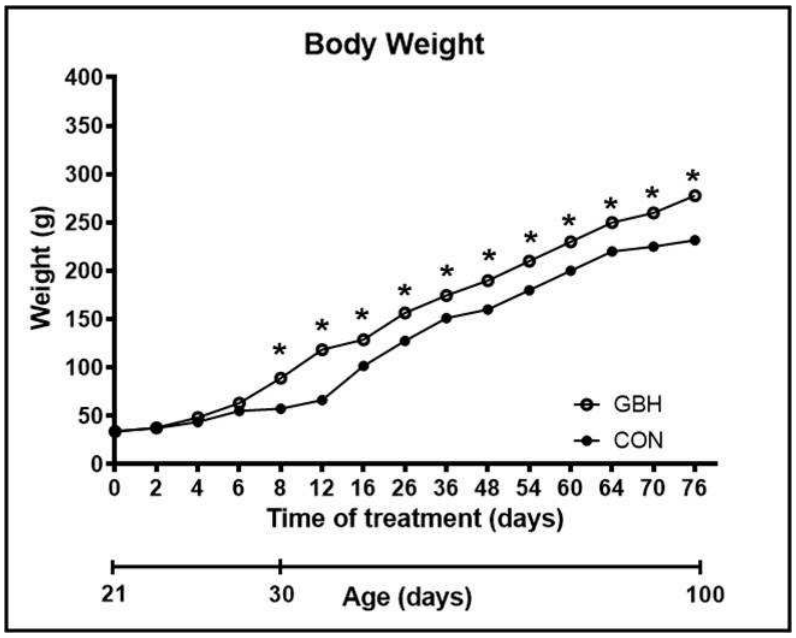

Figure 23: Body weight of rats fed with standard chow (CON) or Glyphosate Based Herbicide (GBH). The time of treatment indicate the duration of GBH or CON diet administration expressed in days. The other line indicates the age of animals when the experiment began (postnatal day 21: PND21). GBH group showed an increase in the body weight 8 days after the beginning of treatment (postnatal day 30: PND30). Values in the bar graph are the mean ± SEM (N=15/Group). Asterisks indicate significant differences (*: $p<0.05$).

Part II: Fertility and feto-placental development

1. GBH affects the reproductive performance producing an increase in the pre-implantation loss

We determine the effects of chronic GBH exposure on reproductive performance. First we evaluate if the animals have impairments to get pregnant, determining the the pregnancy rates. Figure 24A showed that the all animals of both groups (CON and GBH) get pregnant, indicating that this parameter was not affected after GBH exposure. Then, during pregnancy, we performed a fertility test on GD19. The results revealed neither change in the number of CLs (Figure 24 B) nor in the number of RS (Figure 24 C). However, when we analyzed the percentage of pre-implantation loss (calculated as

follows [number of CLs – number of IS/number of CLs] x 100), a significant increase was detected in GBH-exposed group (Figure 24D).

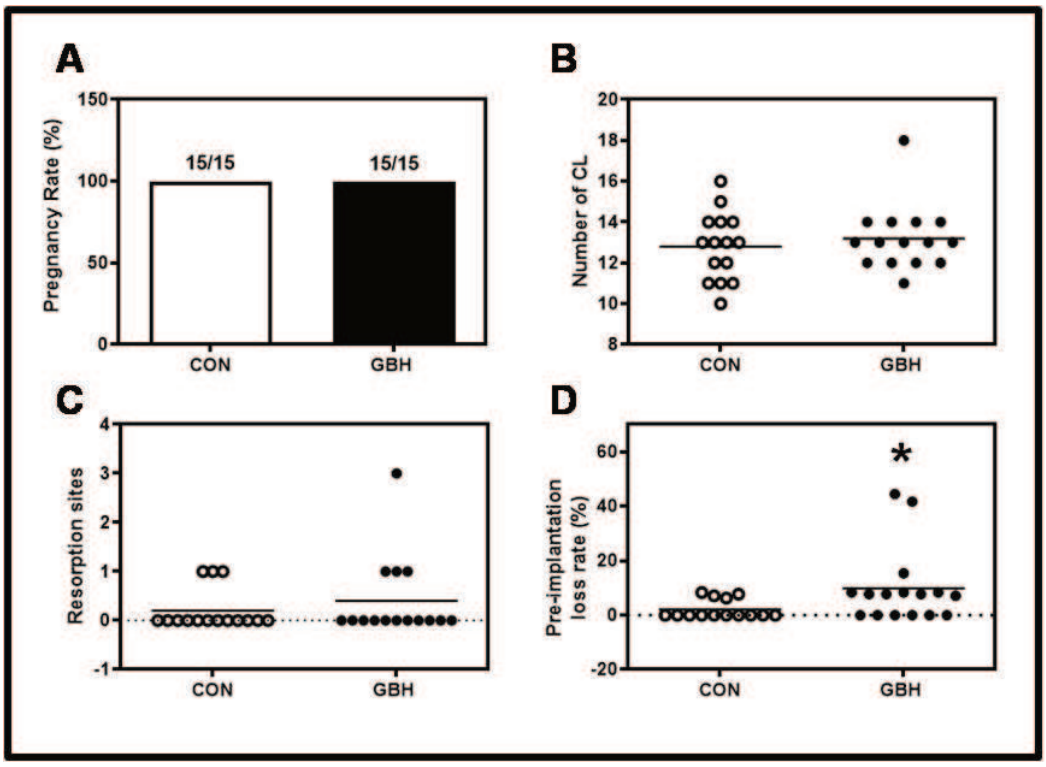

Figure 24: Reproductive performance of Glyphosate Based Herbicide (GBH) and control diet (CON) fed animals. (A) The pregnancy rates were calculated by the average of females that were pregnant with a fertile male, (B) Number of corpora lutea (CLs), (C) Number of resorption sites (RS) and (D) Pre-implantation loss rate (%) are expressed as the mean ± SEM for each experimental group and were plotted to each individual pregnant rat and the horizontal lines are the mean of each group with the corresponding SEM (N=15/Group). B, C and D parameters were evaluated on gestational day 19 (GD19). Asterisks indicate significant differences (*: $p<0.05$).

2. GBH disturbs the fetal parameters on GD19

Oral chronic exposure to a GBH affected the fetal development. We detected a low fetal weight and length on GBH females (Figure 25 B and C). The placental weight and placental index did not change between groups (Figure 25 A and D). The litter size was not different between groups (CON: 12.2 ± 1.4; GBH: 11.6 ± 1.2).

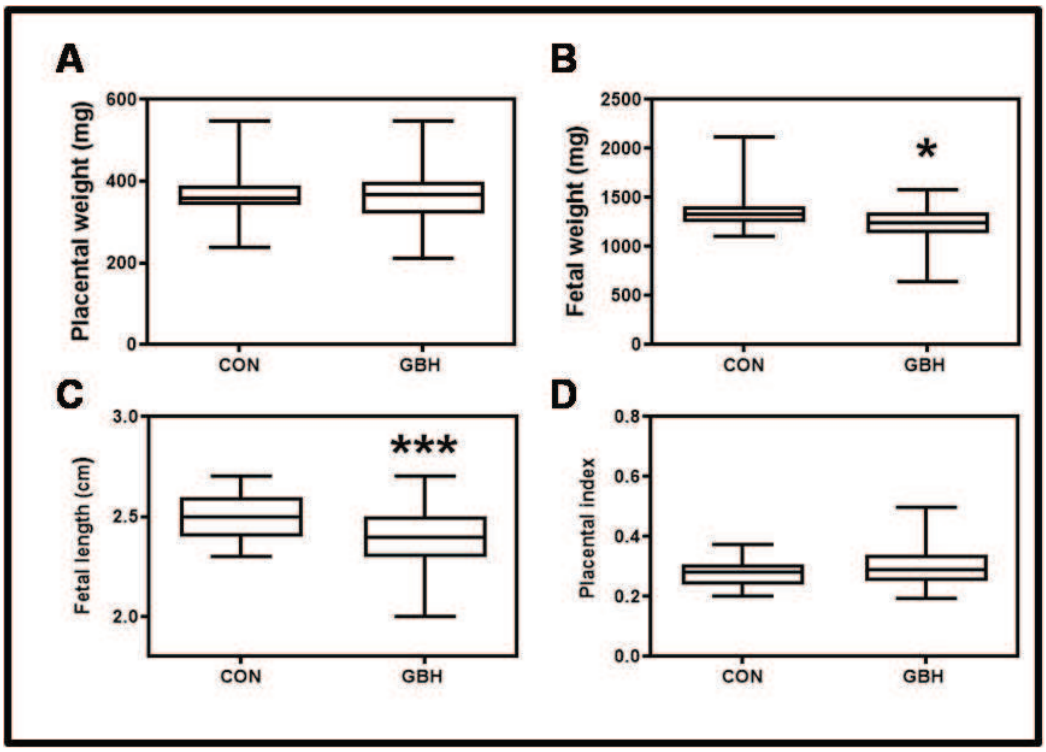

Figure 25: Effects of glyphosate based herbicide (GBH) and control diet (CON) on feto-placental development parameters on gestational day 19 (GD19). (A) Placental weight (mg), (B) fetal weight (mg), (C) fetal length (cm), (D) placental index calculated as placental weight/fetal weight ratio. All results are expressed as the mean ± SEM for each experimental group (N total fetuses and placentas=196 CON/ 182 CAF; N mothers 15/Group). Asterisks indicate significant differences (*: $p < 0.05$; ***: $p < 0.001$).

Part III – In vitro: Gly effects on endometrial carcinogenesis-related process

1. Gly alters the cell viability of Ishikawa cells

Trypan blue exclusion assay was conducted to identify the effects of Gly on cell viability of Ishikawa cells and determine the optimal concentration of Gly for subsequent experiments. Initially, we decided to test four concentrations, including the RfD established by EPA on the interval of concentrations studied. Our exclusion criterium was to reject the concentrations that cause a viability ≤80% and show statistical significant difference respect to vehicle, for considering them toxic. As shown in Figure 26, the two highest tested concentrations (Gly 20 and Gly 200) showed statistical significant difference respect to vehicle and the cell viability was lower than 80%. For that reason these two concentrations were excluded. Contrary, Gly 0.2 and Gly 2 did not show differences respect to vehicle and the cell viability was kept above

80%. Based on these results, we ensured that the cell survival is not altered by the two lower concentrations and we decided to perform all the experiments with Gly 0.2 and Gly 2.

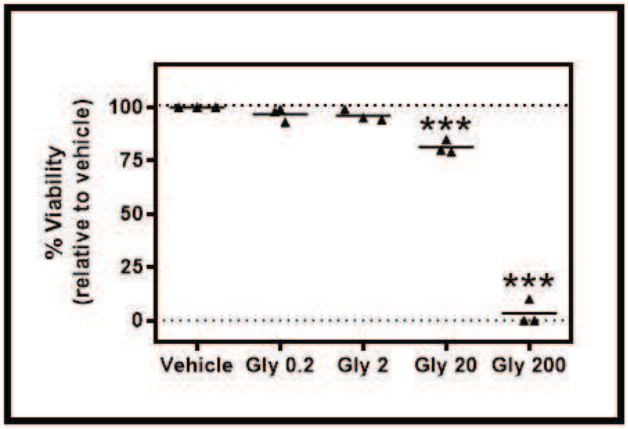

Figure 26: Effects of Glyphosate (Gly) treatment on cell viability of Ishikawa endometrial cancer cell. Cell viability was evaluated by Trypan Blue Exclusion Assay. The results are presented as percentage of viability (% Viability) and expressed as a percentage of the vehicle. Data were expressed as mean ± SEM. Asterisks indicate significant differences (***$p < 0.001$ vs. vehicle).

2. Gly affects EMT process by induction of cell migration via ER pathway

Migratory activity of Ishikawa cells was measured by scratch-wound healing assay. Figure 27 (A and C) shows the effects of Gly exposure on the percentage of wound closure after 72 h (relative to vehicle) evidencing that Gly 0.2 and Gly 2 promoted the migration of Ishikawa cells (Gly 0.2: 172.81 ± 20.79% of the vehicle, $p < 0.01$; Gly 2: 160.56 ± 3.97% of the vehicle, $p < 0.001$) as did E_2 (336.92 ± 20.04% of the vehicle, $p < 0.001$). After Ishikawa cells were treated with E_2 plus Fulvestrant or Gly plus Fulvestrant, the percentage of wound closure after 72 h did not change compared with vehicle (Figure 27 B and C). In other words, the E_2 or Gly effects were reversed by Fulvestrant.

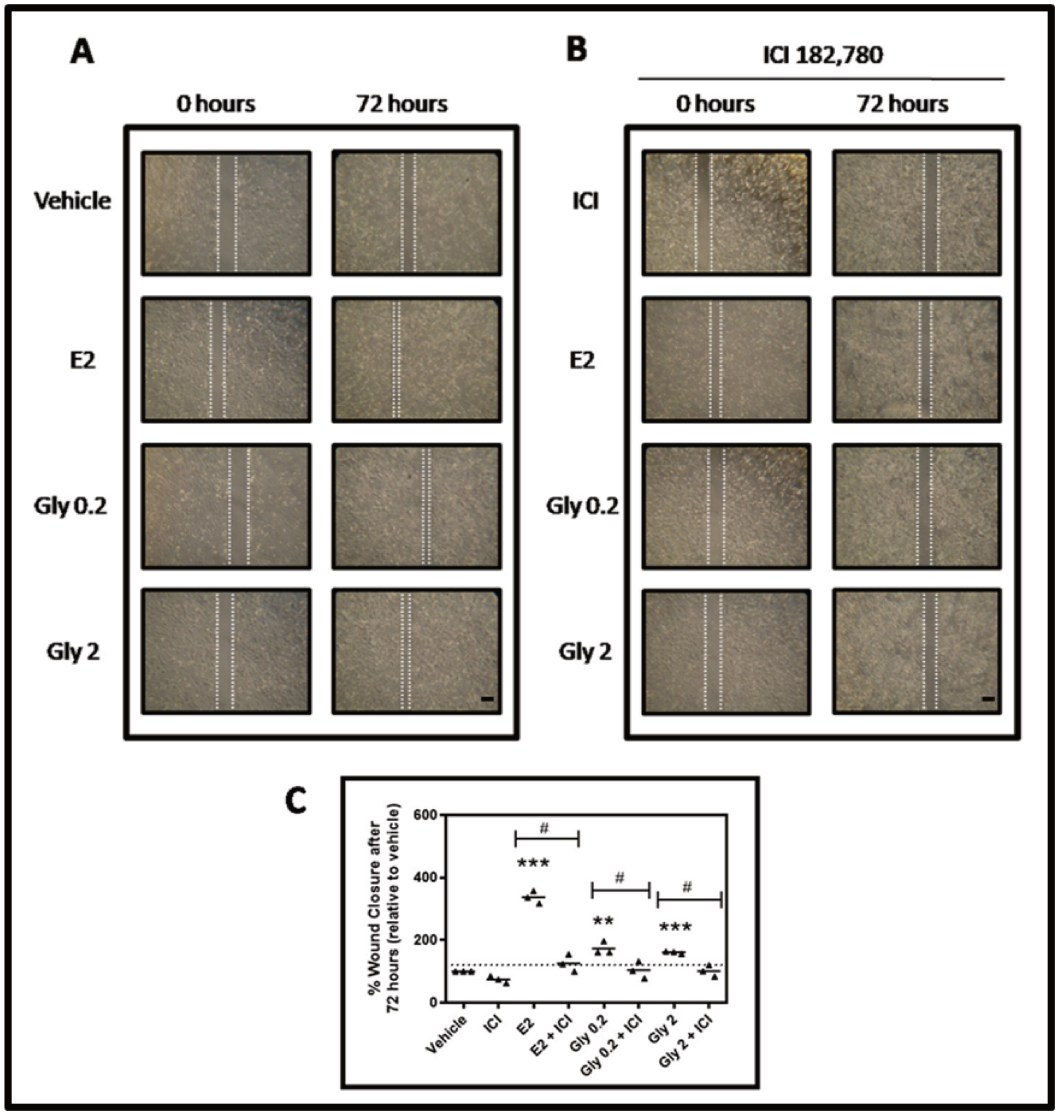

Figure 27: Effects of Glyphosate (Gly) and Fulvestrant (ICI 182,780) treatment on migration ability of Ishikawa endometrial cancer cell. Migration ability was evaluated by the Scratch-Wound Healing Assay. The results are presented as percentage of wound closure after 72 hours relative to the vehicle control. The images presented were captured using a magnification of 40X. Scale bar: 50 μm. Data were expressed as mean ± SEM. Asterisks indicate significant differences: * $p<0.05$ vs. vehicle, ** $p<0.01$ vs. vehicle, *** $p<0.001$ vs. vehicle, #: indicates means values of the mixture of ICI 182,780 with E_2, Gly 0.2 or Gly 2 were significantly reduced from E_2, Gly 0.2 or Gly 2 alone.

3. Gly induced cell invasion via ER pathway

To investigate the invasiveness of Ishikawa cells, transwell invasion assay was conducted. Figure 28 (A and B) shows the effects of Gly exposure on the number of invaded cells compared to vehicle, evidencing that Gly 0.2 promoted the invasion of Ishikawa cells (Gly 0.2: 135.96 ± 22.47% of the vehicle; $p < 0.05$) as did E_2 (E_2: 170.92 ± 9.06% of the vehicle; $p < 0.001$). Gly 2 did not promote the invasion ($p>0.05$). After Ishikawa cells were treated with E_2 plus Fulvestrant or Gly plus Fulvestrant, the number of invasive cells after 48 h did not change, indicating that Fulvestrant attenuated the Gly 0.2 and E_2 effects.

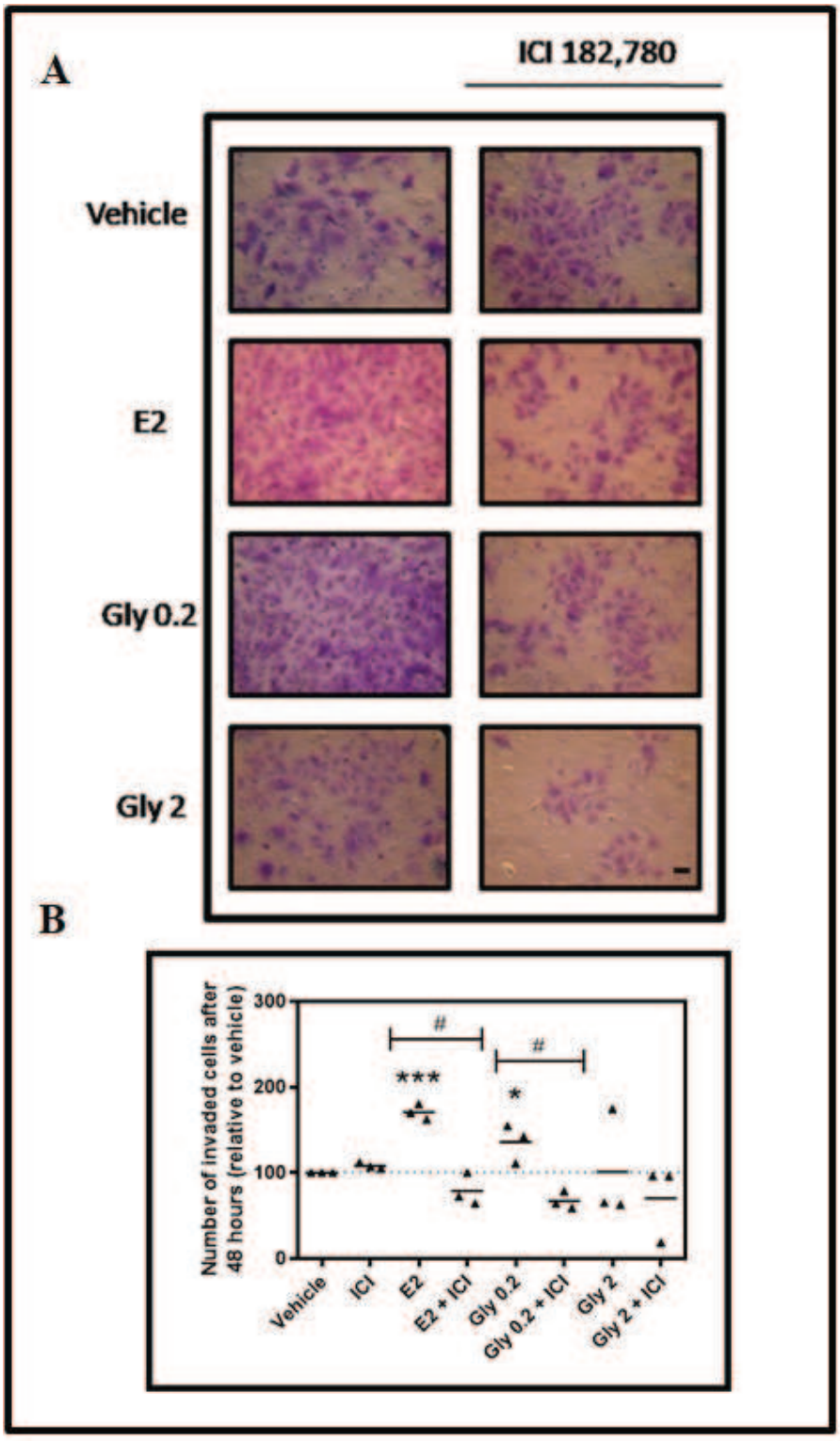

Figure 28: Effects of Glyphosate (Gly) and Fulvestrant (ICI 182,780) treatment on invasion ability of Ishikawa endometrial cancer cell. Invasion ability was evaluated by the Transwell Invasion Assay with Matrigel®. The results are expressed as number of invasive cells after 48 hours and presented as a percentage of the vehicle. The images

presented were captured using a magnification of 40X. Scale bar: 50 µm. Data were expressed as mean ± SEM. Asterisks indicate significant differences: * $p<0.05$ vs. vehicle, ** $p<0.01$ vs. vehicle, *** $p<0.001$ vs. vehicle, #: indicates means values of the mixture of ICI 182,780 with E_2, Gly 0.2 or Gly 2 compared with E_2, Gly 0.2 or Gly 2 alone.

4. Gly down-regulates E-Cadherin mRNA expression via ER pathway

E-cadherin and vimentin mRNA expression was determined 24 h after the application of different concentrations of Gly or E_2. Figure 29A shows that Gly down regulated E-cadherin mRNA expression of Ishikawa cells ($p < 0.01$), as did E_2 ($p < 0.001$). Gly 0.2 produced a more pronounced effect on down regulation of E-cadherin mRNA expression in comparison with Gly 2 (Figure 29 A). Vimentin mRNA expression showed no changes with any of the treatments (Figure 29 B). After combinatorial treatment with E_2 plus Fulvestrant or Gly plus Fulvestrant, E-cadherin mRNA expression was restored and even up regulated respect to the vehicle (Figure 29 A). This up regulation has higher potency to Gly 2 than E_2 and Gly 0.2.

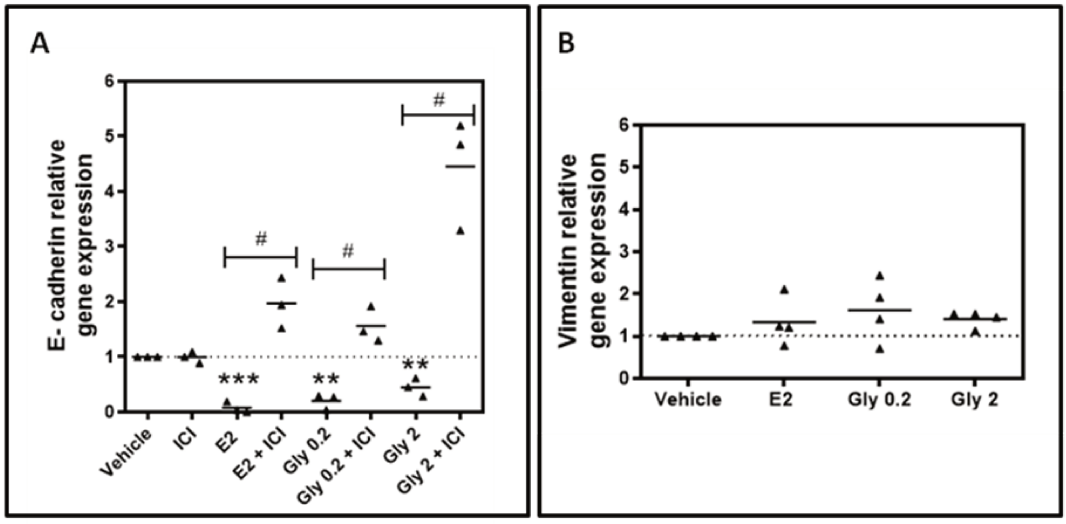

Figure 29: Effects of Glyphosate (Gly) and Fulvestrant (ICI 182,780) treatment on E-Cadherin gene expression **(A)** and on Vimentin gene expression **(B)** on Ishikawa endometrial cancer cell. Relative gene expression was quantified by RT-qPCR. The samples were normalized to the housekeeping gene RPS18. Data were expressed as mean ± SEM. Asterisks indicate significant differences: ** $p<0.01$ vs. vehicle, *** $p<0.001$ vs. vehicle, #: indicates means values of the mixture of ICI 182,780 with E_2, Gly 0.2 or Gly 2 were significantly reduced from E_2, Gly 0.2 or Gly 2 alone.

V. Discussion

The aim of the present chapter was to study the effects of oral chronic GBH exposure on body weight and food intake, fertility, feto-placental development and endometrial carcinogenesis-related process.

We worked with an oral exposure model in which GBH was offered to the animals through food by means of the incorporation of GBH into a laboratory pellet chow-based paste. Like it was reported by Milesi et al. (2018) this model proved to be successful since rats consumed a similar amount of food regardless of the treatment received. In a study performed by Beuret et al. (2005), GBH was administered during the gestational period through drinking water, and a reduction in water and food consumption was reported. Additionally, it is important to highlight that in our model the real Gly doses achieved are in the same order of magnitude as the theoretical doses (Milesi et al. 2018).

Regarding the effects of oral chronic GBH exposure on body weight and food intake, surprisingly we observed a sustained difference on body weight between groups while the food intake was similar in all animals. Several researchers have started to define if EDCs could act as obesogens, get consideration to their possible etiological incidence of obesity. There is epidemiological and experimental evidence supporting the idea that environmental contaminants could have endocrine and metabolic effects. A great number of these contaminants are present in the food chain and accumulate in the fat mass after absorption (Fénichel et al. 2017; Tordjman et al. 2016). Diverse research works propose that some of these EDCs could have a role in the global epidemic of obesity, diabetes (diabetogens), as well as in hormone-dependent cancer (Baillie-Hamilton et al. 2002; Newbold et al. 2008; Arvelo et al. 2016). Although it is not the aim of this thesis, would be interesting to perform more studies to evaluate the possible role of GBH as environmental obesogen as was documented for Bisphenol A (BPA) and dichloro diphenyl trichloroethane (DDT) (González-Casanova et al. 2020).

Several studies have been determined that GBH exposure impairs female fertility. Milesi et al. (2018) reported that perinatal oral exposure to a GBH impairs female rat fertility, increasing the rate of pre-implantation loss. These authors concluded that perinatal oral exposure to a safe dose of GBH disrupts critical hormonal and uterine targets during the endometrial receptive period, possibly implicated on the implantation failures (Lorenz et al. 2019; 2020). Besides, postnatal subcutaneous exposure to a safe dose of GBH (2 mg/kg/day) alters the reproductive performance on Wistar rats, but in

this case the results showed a higher rate of post-implantation loss as a consequence of alterations in the decidualization process (Ingaramo et al 2016; 2017). In our experiment, oral chronic GBH exposure to a safe dose provoked pre-implantation loss in Wistar rats. Taking into account all the results, we could conclude that GBH exposure affects rat reproductive performance, regardless the model of exposure. Particularly, GBH and other herbicides or environmental pollutants may migrate from dust and air from application zones, foods or drink water to the human media resulting in a chronic exposure during the whole life. In this sense, animal model studies which applies oral and chronic exposure to low doses of GBH or other compounds, reflects in a better way the humans routes and times of exposure making more representative models of study. Taking into account that there are no evidence of oral chronic exposure to GBH on reproductive outcomes and that the results of this thesis showed that GBH produced an increase of pre-implantation losses, it would be interesting to evaluate the possible mechanism that provoked these effects in our model of study. For the future we are proposing to determine if the physiological uterine processes that support embryo implantation could be altered in GBH-exposed animals.

Concerning our findings on fetal development, we observed a lower fetal weight and length in GD19, after oral chronic exposure to GBH. In our laboratory, Milesi et al. (2018) detected similar results in F2 generation of rats exposed perinatally to different doses of GBH. Several epidemiological researches have been linked low birth weigth and length of pups with environmental exposure to pesticides (Burdorf et al. 2011; Quintana et al. 2017). Some studies which evaluated effects of GBH exposure during pregnancy founding alterations as developmental retardation (Dallegrave et al. 2003) and central nervous system impairments (Gallegos et al. 2016), between others. However, there is no evidence of an oral chronic exposure to a safe dose of GBH on fetal development, and it would be interesting to deepen in the possible mechanism related whith our findings. Here, even we did not observe impairments in placenta parameters, it could be a cause. As we mention in the Chapter I, placenta has a key role on fetal growth and molecular mechanism of IGF and VEGF genes systems could be implicated. In this sense, in the future it would be interesting to study if IGF and VEGF genes systems are altered in the placentas, and similar to CAF experiment, the weight of pups at birth is affected as a consequence of GBH exposure.

The controversy about the carcinogenic properties of Gly has been intensified after IARC re-classification of Gly as "probably carcinogenic to humans" (Guyton et al. 2015). This classification was based on a small number of epidemiological studies following occupational exposures, as well as, rodent experimental studies. These studies determined associations between Gly and renal tubule carcinoma, haemangiosarcoma, pancreatic islet cell adenoma, skin tumors, and diverse mechanistic data (Myers et al. 2016). In addition, a few evidence from human cell lines *in vitro* studies and animal models indicate that Gly has a genotoxic potential, produces chromosomal damage, and induces oxidative stress (Stur et al. 2019; IARC 2017). On the other hand, concerning to properties of Gly as a possible EDC, it was speculated that GBH disrupts estrogen synthesis through aromatase deregulation (Benachour et al. 2007). Therefore, Gly may be involved in diseases related to hormonal physiology, such as breast and uterine cancer (Gasnier et al. 2009). Regarding breast cancer, some researchers reported that, Gly activates ERα in MCF7 (Mesnage et al. 2017) and in T47D cell lines (Thongprakaisang et al. 2013). In the study of Mesnage et al. (2017) a ligand-independent mechanism of action was proposed for Gly. Further, two additional studies indicate that a commercial formulation of Gly and pure Gly deregulates intracellular pathways in MCF7 and MDA-MB468 (ER (−)) cell lines (Stur et al. 2019; De Almeida et al. 2018) by inducing cell damage independently of estrogenic pathways. In relation to uterine cancer, little information on Gly and GBHs is available. Our previous *in vivo* studies indicate that early life exposure to a commercial formulation of Gly affects uterine organogenesis (Guerrero Schimpf et al. 2017) with long-term consequences on uterine physiology and rat fertility (Ingaramo et al. 2016; 2017). In addition, we detected an increased uterine sensitivity to exogenous estradiol treatment suggesting a possible estrogenic effect of the herbicide (Guerrero Schimpf et al. 2018). These effects have been linked to the development and progression of uterine cancer (Folkerd and Dowsett, 2010). Also, Gly proved to affect the ER expression in different models of exposure (Thongprakaisang et al. 2013; Mesnage et al. 2017; Lorenz et al. 2019; Gomez et al. 2019, Zanardi et al. 2020). However, there was no report about Gly risks associated with cancer progression in estrogen responsive uterine cancer cell lines such as Ishikawa cells.

In estrogen-dependent cancers, such as endometrial cancer, E_2 was found to increase the metastatic potential by inducing EMT, migration, and invasion of cancer cells via the ER-dependent pathway (Lee et al. 2017). Taking this into account, EDCs with

estrogenic activity have been emerging as another risk factor for estrogen-responsive cancers (Scsukova et al. 2016). Moreover, EDCs can affect not only initiation but also promotion and metastasis of hormone dependent cancers (Lee et al. 2017). As typical EDCs, BPA and nonylphenol stimulate the migration of ovarian cancer cells by the EMT process (Kim et al. 2015). In addition, BPA promoted the migration and invasion ability of RL95-2 human endometrial carcinoma cells via the induction of EMT and COX-2 gene expression through MAPK pathway-dependent up-regulation (Wang et al. 2015). In the present study we studied whether the herbicide Gly produces similar effects as E_2 on EMT-related process using the Ishikawa cell line. The EMT process has been considered to be closely associated with malignant transformation and the initiation of cancer metastasis (Thiery, 2009; Chaffer and Weinberg, 2011). To initiate EMT process, it is crucial for the cells to acquire migratory and invasive capabilities gaining motile abilities (Yang and Weinberg, 2008). In our study we determined the migratory activity of Ishikawa cells by scratch-wound healing assay. We detected that Gly increases the percentage of wound closure after 72 h, implying that Gly promotes migration of Ishikawa cells as well as E_2, although with a lower potency. Additionally, we found that Gly stimulates the invasion of endometrial cancer cells using the transwell invasion assay. This stimulation of invasiveness was produced by the low concentration of Gly (Gly 0.2) and the high concentration of Gly (Gly 2) did not induce cellular invasion. Similar to other EDCs, this dose effect could be related with a non-monotonic dose-response curve (Hill et al. 2018). Nevertheless, more studies are needed to better characterize this phenomenon. Beside the EMT, cancer cells can acquire diverse cell invasion modes for efficient metastatic spread according to the context of epithelial dedifferentiation, which are characterized by the further loss of epithelial features. Particularly, E-cadherin is a key gatekeeper of epithelial state or phenotype (Peinado et al. 2007). Consequently, the partial loss of E-cadherin has been associated with carcinoma progression and poor prognosis in various human and mouse tumors (Vincent-Salomon and Thiery, 2003). *In vivo* and *in vitro* mouse tumor models have been showed that induced expression of E-cadherin in certain invasive carcinoma cells can inhibit their ability to invade and metastasize. Conversely, blocking E-cadherin function in non-invasive tumor cells activates their invasiveness and metastatic potential (Frixen et al. 1991; Perl et al. 1998). In our study, the treatment of Ishikawa cells with Gly produced a down regulation of E-cadherin mRNA expression. This down regulation present high potency after E_2 treatment compared to Gly (both concentrations). At the

same time, the E-cadherin down regulation has high potency after Gly 0.2 treatment compared with Gly 2. As mentioned above, E-cadherin repression is often considered to be the critical event in the EMT program (Gonzalez and Medici, 2014) as well as an appropriated indicator of cancer progression and poor prognosis in multiple types of tumor (Bruner and Derksen, 2018). Another interesting finding of our work is that all changes induced by Gly were reversed when a co-treatment with an ER antagonist (Fulvestrant) was applied. These results suggest that the herbicide could act via ER-dependent pathway. Our results are in accordance with others works that showed that Gly activates estrogen response element (ERE) transcription activity on T47D-KBluc cells which was inhibited by Fulvestrant (Thongprakaisang et al. 2013; Mesnage et al. 2017). Interestingly, the co-treatment combining Gly or E_2 with an ER antagonist, not only reverses the effects but also produces an up regulation of E-cadherin mRNA expression. At the same time, it is important to note that the two concentrations of Gly are associated with different potency of effects. Nonetheless, more studies are necessary to further elucidate this topic. A study that estimated Gly binding energy to ERα predicted a weak and unstable interaction compared to E_2, suggesting that activation of this receptor by Gly is via a ligand-independent mechanism (Mesnage et al. 2017). Taking together, our present and previous results indicate that Gly promotes estrogenic effects in different models, and thereby increasing evidence of Gly having EDCs properties.

In conclusion, the results show that Gly promotes EMT process through the down regulation of E-Cadherin and increase cell migration and invasion abilities. Moreover, all changes could be prevented by the application of Fulvestrant (ER antagonist). According to our knowledge, these are the first pieces of evidence showing Gly effects on endometrial cancer cell progression via the ER-dependent pathway. Our findings, in accordance with others, suggest that Gly might increase the risk of aggravating the disease for cancer patients. Further studies are needed to shed light on the *in vivo* effects of Gly on the EMT process and cancer metastasis to contribute to the knowledge about carcinogenic potential of the herbicide.

VI. Conclusion

Here, we detected effects of an oral chronic exposure to a safe dose of GBH on female rat body weigth, reproduction performance, feto-placental development and promotion of cancer-related process.

In this animal model, GBH exposure is associated with body weigth gain sugesting that this herbicide would be act as an obesogenic environmental pollutant. This finding was surprisingly and worrying, and challenge us to study in deep this aspect.

Concerning the reproduction performance and feto-placental parameters, there is some evidence of GBH effects on these topics, as we mention during this thesis manuscript. However, the oral chronic exposure to a safe dose of GBH, give us a more real evidence of human exposure and possible consequences on health. In this sense, we enhance the evidence about the effects of environmental agents applications and the necessary evaluation of the effects of the most used herbicide in Argentina.

Regarding GBH implication on cancer-related process, we used an *in vitro* model of exposure using endometrial human cells. Together with all the background, our findings improve the evidence of Gly as a possible carcinogen for humans, according with the classification proposed by IARC in 2015.

Overall conclusions and Future perspectives

Thus, it can be concluded that our lifestyle have a key role in our health by altering weigth gain and metabolic approaches, reproductive performance and feto-placental development, and influence on cancer-related process.

However, new prospective and deep studies are needed in order to enhance our conclusions and improve our lifestyle.

We propose to:

1-Promote healthy lifestyle and diet habits to avoid consequences on the human health.

2-Promote awareness of the ecosystem and human health taking into account the principle of precautionary: "*When there is danger of serious or irreversible damage, the absence of information or scientific certainty should not be used as a reason to postpone the adoption of effective measures to prevent the degradation of the environment*" (Ley Argentina General de Ambiente N° 25675, Artículo 4, 6 de Noviembre de 2002).

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