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Gynecological tumors: influence of lifestyle factors on its development

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Publications related to the thesis:

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List of Abbreviations

3-MC	3-methylcholanthrene
6-PN	6-prenylnaringenin
8-PN	8-prenylnaringenin
AHR	aryl hydrocarbon receptor
AHRR	aryl hydrocarbon receptor repressor
AlkP	Alkaline phosphatase
AMPA	aminomethylphosphonic acid
ARNT	aryl hydrocarbon receptor nuclear translocator
BCA	bicinchoninic acid
BDS	botanical dietary supplements
C3	complement C3
CAF	cafeteria
CLU	clusterin
CYP1A1	cytochrome P450 1A1
CYP1B1	cytochrome P450 1B1
DCC	dextran charcoal-treated FCS
DESIGNER	Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources
DMEM/F12	Dulbecco's modified Eagle's medium F12
DMSO	dimethyl sulfoxide
DMX	desmethylxanthohumol
DRE	dioxin response element
E2	17β-estradiol
EDC	endocrine-disrupting chemical
EDSP	Endocrine Disruptor Screening Program
EFSA	European Food Safety Authority

EPA	Environmental Protection Agency
ER	estrogen receptor
ERE	estrogen response element
ESR1	estrogen receptor α
ESR2	estrogen receptor β
FBS	fetal bovine serum
FRT	female reproductive tract
GBH	glyphosate-based herbicides
GE	glandular epithelium
GnRH	gonadotropin releasing hormone
HRT	hormone replacement therapy
IARC	International Agency for Research on Cancer
IOD	integrated optical density
ITS	Insulin-Transferrin-Selenium A
IX	isoxanthohumol
KO-Hops	knock-out Hops
LE	luminal epithelium
OECD	Organization for Economic Cooperation and Development
P4	progesterone
PCNA	proliferating cell nuclear antigen
PI3K	phosphatidylinositol 3-kinase
PND	postnatal day
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
RfD	reference dose
RUWW	Relative uterine wet weight
SS	subepithelial stroma

TCDD	2,3,7,8-tetraclorodibenzo-p-dioxina
WHI	Women's Health Initiative
XH	xanthohumol

Abstract

Multiple modifiable lifestyle factors can positively or negatively affect the Women's health. Some of the negative effects are associated with the development of neoplastic diseases. Here, we have selected three factors from three different lifestyle areas: a pesticide, a diet choice, and an herbal remedy. The exposure to certain pesticides is associated with the development of endometrial cancer. Among pesticides, we are focused on glyphosate-based herbicides (GBHs), due to their high application in the world. In addition, the risk of endometrial cancer is associated with unhealthy diets. Considering that unhealthy diets are popular in a big part of the world, we are interested in the study of the cafeteria (CAF) diet, an unbalanced diet with predominantly fat energy content at the expense of lower protein content, which provides a highly relevant model in terms of mimicking human eating patterns. The hops (Humulus lupulus L.) is an herbal remedy used as a "safer" alternative to hormone replacement therapy (HRT). Thus, its characterization regarding potential beneficial health effects and risks has been on demand.

In the FIRST CHAPTER, we proposed to study whether GBH exposure at a low dose alone or added to the CAF diet, predisposes to develop preneoplastic and/or neoplastic lesions in the uterus of Wistar rats. In the first part, we evaluated the long-term effects of GBH alone by using a neonatal exposure model. Female pups were injected with vehicle or GBH on postnatal day (PND) 1, 3, 5 and 7. On PND 600, serum samples were collected to assess 17β-estradiol (E2) and progesterone (P4) levels, and uterine samples were obtained to determine proliferation index and the expression of steroid hormone receptors, by immunohistochemistry. Animals exposed to GBH increased the estrogen receptor α (ESR1) expression in the preneoplastic glandular lesions. In the second part, we evaluated the effects of subchronic GBH exposure in rats fed with CAF diet. Thus, rats were fed from PND 21 until PND 240 with lab chow (Control) or CAF diet. On PND 140, one group of rats fed with CAF diet also received GBH through food, yielding three experimental groups: Control, CAF, and CAF+GBH. On PND 240, serum samples were collected to assess E2 and P4 levels, fat depots were collected to determine adiposity and uterine samples were obtained for histological studies (morphometric and immunohistochemical analysis). CAF and CAF+GBH animals showed an increase in the adiposity index. The serum levels of P4 were increased in CAF+GBH group, respect to CAF one. In the uterus, CAF and CAF+GBH induced morphological and molecular changes associated with endometrial hyperplasia. The addition of GBH increased the thickness of subepithelial stroma and the density of abnormal gland in comparison with Control animals. Moreover, CAF+GBH rats showed reduced phosphatase and tensin homolog (PTEN) and p27 expression, respect to Control rats.

These results indicate that the addition of GBH exacerbates the CAF-effects on uterine preneoplastic lesions and that the PTEN/p27 signaling pathway seems to be involved.

In the SECOND CHAPTER, we proposed to determine the estrogenic effects of hops extract by using *in vivo* and *in vitro* studies and to evaluate the molecular mechanisms of anti-carcinogenic process. In the first part, we evaluated the effects of hops extract and its bioactive compounds 6-prenylnaringenin (6-PN) and 8-prenylnaringenin (8-PN) on estrogen receptor α (ERα) and aryl hydrocarbon receptor (AHR) signaling pathways in a human endometrial cancer cell line. Hops extract, 8-PN and 6-PN showed estrogenic activity. Hops extract and 6-PN activated both ERα and AHR pathways. 6-PN increased the expression of the tumor suppressor gene (*AHRR*), and that of genes involved in the estrogen metabolism (*CYP1A1, CYP1B1*). Although 6-PN might activate the detoxification and genotoxic pathways of estrogen metabolism, hops extract as a whole only modulated the genotoxic pathway by an up-regulation of *CYP1B1* mRNA expression. These data demonstrate the relevant role of 6-PN contained in the hops extract as a potential modulator of estrogen metabolism due to its ERα and AHR agonist activity. In the second part, we evaluated the estrogenic effect of hops extract and an extract reduced in xanthohumol (named knock-out Hops: KO-Hops) by using a 3-day uterotrophic assay in Wistar rats. At PND 49, rats were subjected or not to ovariectomy or sham surgery. After 14 days, the animals were exposed for 72 h with vehicle (Control, SHAM and Ovx groups), E2 (4 µg/kg bw/day), Hops or KO-Hops at 8, 40 and 200 mg/kg bw/day, respectively. Animals were sacrificed 24 h after the last treatment day. The ovariectomy reduced the relative uterine weight and this effect was reversed by E2 treatment, validating the uterotrophic assay. In general, no estrogenic uterine effects at morphological and molecular levels (protein and mRNA) were observed in response to Hops extracts, independently of their composition (Hops and KO-Hops). However, we observed an increased glandular density by the exposure to Hops extract at 40 mg/kg bw/day accompanied by a reduced protein ESR1 expression, and an increased glandular density by the exposure to KO-Hops at 8 mg/kg bw/day. Our results showing a weak estrogenic effect on the uterus suggest a minimal probability to induce endometrial hyperplasia and cancer associated. Thus, Hops compounds could be a safer alternative to the conventional HRT but this topic deserves more studies.

In conclusion, since our lifestyle has a key role in our reproductive health and can influence the development of uterine pathologies, we encourage promoting a healthy lifestyle to minimize negative consequences on female human health.

Resumen

Muchos factores que componen el estilo de vida pueden influir positiva o negativamente en la salud de las mujeres. Algunos de ellos son modificables y estan asociados al desarrollo de distintos tipos de cáncer. En la presente tesis, seleccionamos tres factores: un pesticida, un tipo de dieta considerada poco saludable y una hierba medicinal. La exposición a ciertos pesticidas está relacionada con el desarrollo de cáncer de endometrio. Entre los pesticidas, nos interesan particularmente los herbicidas a base de glifosato (HBG) por su gran uso a nivel mundial. Por otra parte, el riesgo de desarrollar cáncer de endometrio también está asociado con alimentación poco saludable, la cual es cada vez más frecuente a nivel mundial. A nivel experimental, la dieta de cafetería (CAF) es un tipo de dieta desequilibrada con un alto contenido graso a expensas de un menor contenido proteico que imita los hábitos alimentarios de las poblaciones occidentales. El hops (Humulus lupulus L.) es una hierba medicinal utilizada como una alternativa más segura a la terapia de reemplazo hormonal (TRH). Por lo tanto, es necesario realizar una exhaustiva caracterización para conocer sus posibles efectos sobre la salud.

En el PRIMER CAPÍTULO, nos propusimos evaluar si la exposición a un HBG a bajas dosis suministrado solo o con una dieta CAF, predispone al desarrollo de lesiones preneoplásicas y/o neoplásicas en el útero de ratas Wistar. En la primera parte, evaluamos los efectos a largo plazo de la exposición a un HBG mediante un modelo de exposición aguda durante el periodo postnatal. Para ello, las crías hembras recibieron inyecciones subcutáneas en los días postnatales (DPN) 1, 3, 5 y 7 con vehículo o HBG. Los animales fueron sacrificados el DPN 600. En muestras de suero, se determinaron los niveles de 17β-estradiol (E2) y progesterona (P4). En el tejido uterino, se determinó la proliferación celular y expresión de receptores de hormonas esteroides, mediante inmunohistoquímica. Los animales expuestos a HBG exhibieron una mayor expresión del receptor de estrógenos α (ESR1) en lesiones glandulares preneoplásicas. En la segunda parte, nos propusimos evaluar si la exposición subcrónica a bajas dosis de un HBG exacerba los efectos inducidos por CAF. En DPN 21, las ratas hembras fueron asignadas al azar a uno de los siguientes grupos: Control (alimentadas con dieta estándar), CAF (alimentadas con CAF) y CAF+HBG (alimentadas con CAF y a partir del DPN 140 co-expuestas a un HBG a través del alimento). Los animales fueron sacrificados el DPN 240. Se obtuvieron muestras de suero para la determinación de niveles de E2 y P4, tejido adiposo para evaluar el índice de adiposidad y muestras uterinas para análisis morfológico y expresión de proteínas. Los grupos CAF y CAF+GBH presentaron un mayor índice de adiposidad respecto al grupo Control. El grupo CAF+HBG presentó mayores niveles de P4 respecto al grupo CAF. En el útero, CAF y CAF+GBH produjeron alteraciones morfológicas y moleculares asociadas con hiperplasia

endometrial. La adición de HBG incrementó el espesor del estroma subepitelial y la densidad de glándulas anormales respecto al grupo Control. A nivel de proteínas, la expresión de PTEN (del inglés *phosphatase and tensin homolog*) y p27, moléculas supresoras de tumor que inhiben la proliferación celular, fue menor en el grupo CAF+HBG que en el grupo Control. Por lo tanto, la adición del HBG exacerbó los efectos producidos por la dieta CAF sobre las lesiones preneoplásicas, posiblemente a través de la vía de señalización PTEN/p27.

En el SEGUNDO CAPÍTULO, nos propusimos evaluar el efecto estrogénico del extracto hops mediante estudios *in vivo* e *in vitro* y evaluar mecanismos moleculares anti-cancerígenos. En la primera parte, evaluamos los efectos del hops y sus compuestos bioactivos, 6-prenilnaringenina (6-PN) y 8-prenilnaringenina (8-PN) sobre las vías de señalización del receptor de estrógeno α (REα) y del receptor de hidrocarburos de arilos (AHR), utilizando una línea celular de carcinoma endometrial humano. Hops, 8-PN y 6-PN mostraron actividad estrogénica. Hops y 6-PN activaron tanto la vía del REα como la vía del AHR. A su vez, 6-PN incrementó la expresión del ARNm del gen supresor tumoral (*AHRR*) y de los genes involucrados en el metabolismo de estrógenos (*CYP1A1, CYP1B1*). Si bien el 6-PN podría incrementar la activación de las vías de detoxificación y genotoxicidad del metabolismo de estrógenos, el hops tuvo efecto sólo sobre la vía genotóxica mediante la regulación hacia arriba de la expresión del ARNm del *CYP1B1*. Esto demuestra el potencial efecto del 6-PN en el hops como modulador de las vías de metabolización de estrógenos a través de la activación del AHR y del REα. En la segunda parte, evaluamos el efecto estrogénico del hops y un extracto reducido en xantohumol (llamado *knock-out* Hops: KO-Hops) en ratas, mediante el ensayo uterotrófico. Para ello, ratas Wistar de 49 días de edad fueron o no ovariectomizadas, o sometidas a cirugía SHAM. Luego de 14 días, las ratas fueron alimentadas durante 72 horas con vehículo (grupos Control, SHAM y Ovx), Hops o KO-Hops a distintas dosis (8 mg/kg/día, 40 mg/kg/día o 200 mg/kg/día) o inyectadas con E2 (4 µg/kg/día). Los animales fueron sacrificados 24 horas después de finalizado el tratamiento y se obtuvieron muestras uterinas. La ovariectomía redujo el peso relativo del útero, efecto revertido por la exposición a E2. De este modo, validamos el ensayo uterotrófico. En general, ambos extractos de Hops, independientemente de su composición, no produjeron efectos estrogénicos en el útero tanto a nivel morfológico como molecular (proteína y ARNm). Sin embargo, en los animales expuestos a Hops (40 mg/kg/día) se observó un incremento en la densidad glandular acompañada por una reducción en la expresión proteica del ESR1. A su vez, los animales expuestos a KO-Hops (8 mg/kg/día) también presentaron un aumento en la densidad glandular. Estos resultados muestran un efecto estrogénico débil en el útero por lo que la probabilidad de inducir hiperplasia y cáncer

endometrial sería muy baja. Por lo tanto, los suplementos a base de Hops podrían ser una alternativa segura a la TRH. Sin embargo, son necesarios más estudios para ser concluyentes respecto a su seguridad uterina.

En conclusión, dado que nuestro estilo de vida puede influir en el desarrollo de patologías uterinas, fomentamos la promoción de un estilo de vida saludable para minimizar posibles consecuencias negativas sobre la salud humana femenina.

Zusammenfassung

Eine Reihe von Lifestyle-Faktoren können die Gesundheit von Frauen positiv oder negativ beeinflussen. Einige dieser negativen Wirkungen sind mit der Entwicklung neoplastischer Erkrankungen verbunden. Hierfür werden drei Faktoren aus drei unterschiedlichen Lifestyle-Gebieten untersucht: ein Pestizid, eine Ernährungsform und eine Heilpflanze. Die Exposition gegenüber bestimmten Pestiziden wird mit der Entwicklung von Gebärmutterkrebs in Verbindung gebracht. Aufgrund der hohen weltweiten Anwendung von Herbiziden auf Glyphosatbasis (kurz „GBH“: *Glyphosate-Based Herbicides*) konzentriert sich die vorliegende Arbeit auf diese. Außerdem wird das Risiko für Gebärmutterkrebs mit ungesunder Ernährung, welche auf der ganzen Wel immer häufiger anzutreffen ist, in Verbindung gebracht. In Anbetracht der Tatsache, dass ungesunde Ernährung weit verbreitet ist, wird die Cafeteria-Diät (CAF), eine unausgewogenen Ernährung mit überwiegendem Energieanteil auf Fettbasis, und niedrigerem Proteingehalt untersucht. Dieses Modell spiegelt die typischen Essgewohnheiten von Bevölkerungen in den westlichen Ländern wider. Beim Hopfen (Humulus lupulus L.) handelt es sich um ein pflanzliches Arzneimittel, welches als sinnvolle Alternative zur Hormonersatztherapie (HET) verwendet wird. Aus diesem Grund ist eine Charakterisierung hinsichtlich möglicher gesundheitsfördernder Wirkungen und Risiken nötig.

Im ERSTEN KAPITEL wird untersucht, ob eine GBH-Exposition mit niedrigen Dosen auch in Kombination mit einer CAF-Diät, Wistar-Ratten für präneoplastische und/oder neoplastische Läsionen der Gebärmutter anfälliger macht. Im ersten Teil werden die langfristigen Auswirkungen der GBH-Exposition anhand eines Modells der akuten Exposition in der postnatalen Periode bewertet. Weiblichen neugeborenen Ratten wurden dafür am postnatalen Tag (PND) 1, 3, 5 und 7 entweder Vehikel oder GBH injiziert. Am PND 600 wurden die Tiere getötet. Anhand von Serumproben wurden die 17β-Östradiol (E2)- und Progesteron (P4)-Spiegel nachgewiesen. Durch die Durchführung imnmunhistochemischer Verfahren wurden im Gebärmuttergewebeder der Proliferationsindex und die Expression von Steroidhormonrezeptoren bestimmt. Bei Tieren, die GBH ausgesetzt waren, erhöhte sich die Expression des Östrogenrezeptors α (ESR1) in den präneoplastischen Drüsenläsionen. Im zweiten Teil wurde untersucht, ob eine niedrig dosierte, subchronische GBH-Exposition die durch eine CAF-Diät hervorgerufenen Folgen bei Ratten verschlimmert. Bei dieser Untersuchung wurden Ratten von PND 21 bis PND 240 mit Standard-Laborfutter (Kontrolle) oder CAF-Diät gefüttert. Am PND 140 erhielt eine Gruppe von Ratten, die mit CAF-Diät gefüttert wurden, zusätzlich auch GBH über das Futter, wodurch sich drei experimentelle Gruppen ergaben: Kontrolle, CAF und CAF + GBH. Am PND 240 wurden die Tiere getötet. Darauf wurden Serumproben entnommen, um die E2- und P4-Spiegel zu bestimmen,

außerdem wurden Fettdepots entnommen, um Adipositas zu quantifizieren und Uterusproben wurden für histologische Untersuchungen entnommen (für morphometrische und immunhistochemische Analyse). Die CAF- und CAF+GBH- Gruppen zeigten einen höheren Adipositas-Index als die Kontrollgruppe. Die Serumspiegel von P4 waren in der CAF+GBH-Gruppe im Vergleich zu der CAF-Gruppe erhöht. Im Uterus induzierten CAF und CAF+GBH morphologische und molekulare Veränderungen im Zusammenhang mit Endometriumhyperplasie. Die Zugabe von GBH erhöhte die Dicke des subepithelialen Stromas und die Dichte des anormalen Drüsenbereichs im Vergleich zu den Kontrolltieren. Darüber hinaus zeigten CAF+GBH-Ratten im Vergleich zu Kontrolltieren eine reduzierte Phosphatase- und Tensin-Homolog- (PTEN) sowie p27-Expression, daher Tumorsuppressoren, welche die Zellproliferation hemmen. Diese Ergebnisse verdeutlichen, dass die Zugabe von GBH die Auswirkungen der CAF-Diät bezogen auf die Entstehung von präneoplastischen Läsionen verstärkt und dass der PTEN/p27-Signalweg beteiligt zu sein scheint.

Im ZWEITEN KAPITEL wurden die östrogenen Auswirkungen von Hopfenextrakt durch In-vivo- und In-vitro-Studien untersucht und die molekularen Mechanismen des potentiellen antikarzinogenen Prozesses bewerten. Im ersten Teil wurden die Auswirkungen von Hopfenextrakt und seinen bioaktiven Verbindungen, 6-Prenylnaringenin (6-PN) und 8-Prenylnaringenin (8-PN), auf die Signalwege des Östrogenrezeptors α (ERα) und des Aryl-Hydrocarbon-Rezeptor (AHR) in einer humanen Endometriumkarzinom-Zelllinie untersucht. Der Hopfenextrakt, 8-PN und 6-PN, zeigten östrogene Aktivität. Der Hopfenextrakt und 6-PN aktivierten sowohl den ERα- als auch den AHR-Weg. 6-PN hat die Expression des mRNA des Tumorsuppressorgens (*AHRR*) und der Gene, die am Östrogenstoffwechsel beteiligt sind (*CYP1A1, CYP1B1*), erhöht. Obwohl 6-PN die Entgiftungs- und genotoxischen Wege des Östrogenstoffwechsels aktivieren könnte,wirkte sich der Hopfenextrakt nur auf den genotoxischen Weg durch eine Hochregulierung der *CYP1B1*-mRNA-Expression aus. Diese Daten zeigen diepotenziellen Auswirkungen des im Hopfenextrakt enthaltenen 6-PN als Modulator des Östrogenstoffwechsels, durch seine ERα- und AHR-agonistischen Aktivität. Im zweiten Teil wurde die östrogene Wirkung von Hopfenextrakt und einem Xanthohumol-reduzierten Extrakt (genannt Knock-out-Hopfenextrakt: KO-Hopfen) mittels eines 3-tägigen uterotrophen Assay an Ratten untersucht. Bei PND 49 wurden die Wistar-Ratten einer Ovariektomie oder einer Sham-Operation unterzogen. Nach 14 Tagen wurden die Tiere 72 Stunden lang mit dem Vehikel (Kontroll-, SHAM- und Ovx-Gruppen), Hopfen- oder KO-Hopfenextrakt in verschiedenen Dosen (8, 40 bzw. 200 mg/kg /Tag) behandelt oder mit E2 (4 µg/Kg/Tag) injiziert. Die Tiere wurden 24 Stunden nach dem letzten Behandlungstag getötet und es wurden Gebärmutterproben entnommen. Die Ovarektomie verringerte das

relative Uterusgewicht; dieser Effekt wurde durch die E2-Behandlung umgekehrt, wodurch der uterotrophe Assay validiert wurde. Es wurden größtenteils keine östrogenen uterinen Wirkungen auf morphologischer und molekularer Ebene (Protein und mRNA) als Reaktion auf Hopfenextrakte beobachtet, unabhängig von ihrer Zusammensetzung. Wir beobachteten jedoch eine erhöhte Drüsendichte durch die Exposition gegenüber Hopfenextrakt bei 40 mg/kg Körpergewicht/Tag, begleitet von einer reduzierten ESR1-Expression, so wie eine erhöhte Drüsendichte durch die Exposition gegenüber KO-Hopfen bei 8 mg/kg Körpergewicht/Tag. Anhand dieser Ergebnisse lässt sich eine schwache östrogene Wirkung auf den Uterus belegen, sodass die Wahrscheinlichkeit, dass Endometriumhyperplasie und ein damit verbundener Endometriumkrebs hervorgerufen wird, sehr gering ist. Daraus lässt sich die Schlussfolgerung ziehen, dass Hopfenverbindungen möglicherweise eine sicherere Alternative zur herkömmlichen Hormonersatztherapie (HET) sein könnten, aber dieses Thema bedarf noch weiterer Untersuchungen bezüglich der Gesundheit der Gebärmutter.

Zusammenfassend lässt sich sagen, da unser Lebensstil eine Schlüsselrolle bei der Entwicklung von Gebärmuttererkrankungen spielt empfehlen wir abschließend, einen gesunden Lebensstil zu fördern, um negative Auswirkungen auf die Gesundheit von Frauen zu minimieren.

General introduction

1. Gynecological tumors

Cancer is among the leading causes of mortality worldwide, representing a major public health issue (Siegel et al., 2022). Ongoing research on cancer estimated that a large percentage of cancer is preventable by behavior modification (Anand et al., 2008; Friedenreich et al., 2021; Stewart, 2012). Despite this, the incidence of cancer in the world is expected to rise over the next years (Soerjomataram & Bray, 2021).

Gynecological tumors are a group of tumors originated in the female reproductive tract (FRT) that includes uterine, ovarian, vulvar, vaginal, and fallopian tube cancer (Ledford & Lockwood, 2019). Uterine cancer of humans includes the cancer of the cervix uteri (cervical cancer) and the cancer of the corpus uteri (which includes mostly endometrial cancer) (Weiderpass & Labrèche, 2012). Cervical cancer is caused by persistent infection with human papilloma virus, whereas endometrial cancer is mainly due to hormonal imbalance (Memon & El-Turki, 2018). The incidence of ovarian, cervical and vaginal cancer has decreased over the years, whereas the incidence of endometrial and vulvar cancer has increased (Ledford & Lockwood, 2019). During the last decades, the cancer survival for the most common types of cancer has improved with an exception of uterine cancers. This reflects a lack of major treatment advances in this field (Siegel et al., 2022).

Endometrial cancer is the most common gynecological tumor (Braun et al., 2016; Giordano & Macaluso, 2016) and affects mainly women between 55 and 64 years of age (Baiden-Amissah et al., 2021). In 2020, 417,336 new cases and 97,000 deaths were reported worldwide (Baiden-Amissah et al., 2021; Sung et al., 2021). The incidence of endometrial cancer is predicted to continue to rise in the coming decades, owing to the increasing aging population and the effect of lifestyle factors on its development (Baiden-Amissah et al., 2021; Raglan et al., 2019). Given the high prevalence of endometrial cancer among women, it is necessary to improve prevention (as early detection and therapy), including education on risk factors that can be modified. Thus, the present thesis focuses on the mechanistic study of potential positive and negative lifestyle factors on the uterine lesions that can induce endometrial cancer. To understand the mechanism involved in this pathology we used *in vitro and in vivo* studies. For *in vivo* studies, we used a rodent model. Thus, in the following sections, readers will be introduced into the functional and morphological characterization of rat uterus.

2. The development of the rat uterus

The FRT typical for rodents consists of a pair of ovaries, each attached to an oviduct connected to two bicornuate uterine horns, which are connected to a cervix to separate it from the vaginal canal (Herington et al., 2018). The FRT is derived from a pair of paramesonephric or Müllerian ducts (MDs) of mesodermal origin, which gives rise to the oviduct, uterus, cervix, and vagina (Spencer et al., 2012).

The development of the rat uterus begins prenatally but is not fully developed or differentiated until the birth (Spencer et al., 2012). The process of postnatal uterine morphogenesis includes: (1) organization and stratification of endometrial stroma, (2) differentiation and growth of myometrium (Brody & Cunha, 1989), and (3) the development of endometrial glands from the luminal epithelium (LE) (Spencer et al., 2012). The origin of endometrial glands is not observed until postnatal day (PND 9) and concludes at PND 15 (Guerrero Schimpf et al., 2021). Normal uterine growth and morphogenesis depend on cell proliferation (Guerrero Schimpf et al., 2021; Nanjappa et al., 2015). During this period, the uterus is highly sensitive to exposure to certain compounds that can alter the normal course of uterine development with lasting consequences (Guerrero Schimpf et al., 2021).

2.1. Overview of the anatomy and histology of the rat uterus

The uterus is an organ essential for the transport, storage, and maturation of spermatozoa. It provides an embryotrophic environment for conceptus (embryo/fetus and associated extraembryonic membranes), survival and development, and is necessary for the delivery of the offspring (Gray et al., 2001; Spencer et al., 2012). As shown in Figure 1, the histological organization of the adult rat uterus consists of the endometrium, the myometrium and perimetrium. The endometrium consists of a simple columnar LE surrounded by mesenchymal (i.e., stromal) cells that contain endometrial glands lined by simple cuboidal epithelial cells, blood vessels, and immune cells. The myometrium is the smooth muscle component and includes an inner circular layer and an outer longitudinal layer with an intervening vascular layer. The myometrium is surrounded by serosa, the perimetrium (Brody & Cunha, 1989; Gray et al., 2001; Spencer et al., 2012).

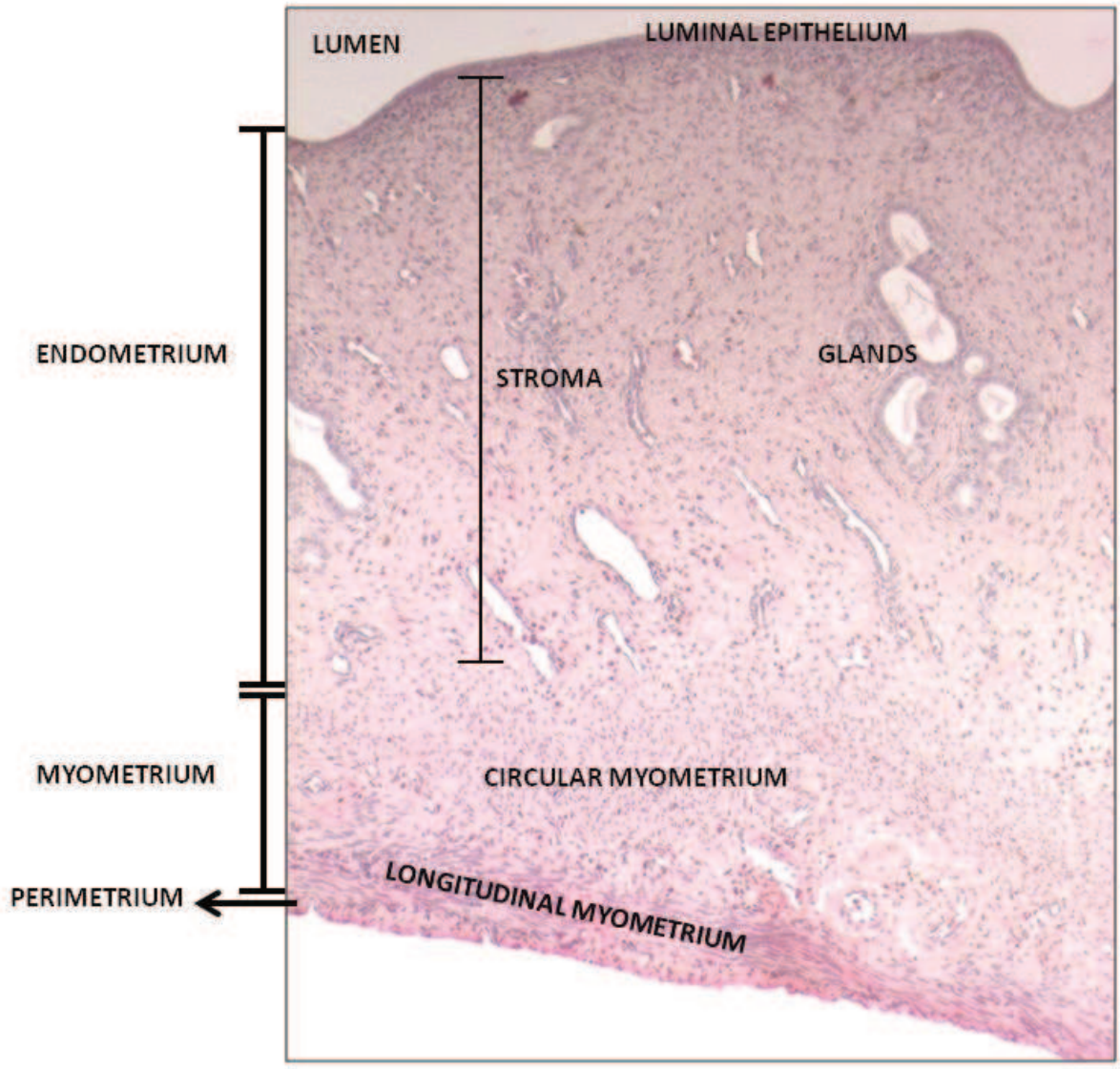

Figure 1. Representative photomicrograph of the longitudinal section of uterus stained with hematoxylin and eosin. The histological organization of adult rat uterus consists of the endometrium (luminal epithelium and stroma), the myometrium (circular and longitudinal) and perimetrium.

3. Steroid hormone receptors in the uterus

The ovarian steroids, 17β-estradiol (E2) and progesterone (P4), are key regulators of growth, differentiation, and physiological functions of many tissues, including the reproductive tract (Björnström & Sjöberg, 2005). The biological effect of E2 is mediated by estrogen receptor (ER) α and β, and the biological effect of P4 is mediated by progesterone receptor (PR).

Estrogen receptors, α and β, are expressed throughout the FRT and play essential roles in female reproduction, demonstrated by infertile in various knock-out and knock-in mice lines

(Peluso, 2018). In rat uterus, both ERs are expressed in epithelial, stromal, and myometrial cells.

Progesterone receptor plays an important role in the differentiation of endometrium, control of implantation, maturation of mammary epithelium, and modulation of gonadotropin releasing hormone (GnRH) pulsatility (Ellmann et al., 2009). Its role in female fertility was confirmed in infertile mice that lack PR (Peluso, 2018). In the rodent uterus, the expression of PR is modulated by receptor-mediated E2 actions, depending on the uterine compartments (Binder et al., 2015). In ovariectomized rats, the exposure to exogenous E2 reduces PR levels in the LE and the glandular epithelium (GE), whereas increases PR levels in the stroma (Binder et al., 2015; Varayoud & Durando et al., 2017).

4. Uterine lesions: pre and neoplastic lesions

Endometrial hyperplasia is one of the most common gynecological diseases in the world, with an incidence of 200,000 new cases per year in Western countries (Chandra et al., 2015; Singh, G., & Puckett, 2022). It is characterized by an increase in gland-to-stroma ratio compared to normal endometrium (Mills & Longacre, 2010; Sanderson et al., 2016). It is a proliferative process of endometrial glands with architectural alterations that result in variably shaped and irregularly distributed glands (Dixon et al., 2014; Refaie & El-Hussieny, 2017; Sanderson et al., 2016; Siegel et al., 2021; Sobczuk & Sobczuk, 2017). These alterations can be grouped into: I) glands with squamous metaplasia (stratified epithelium composed by two or three layers of cells), II) glands with cellular anomalies (cylindrical epithelium, low nuclei/cytoplasm ratio, cytoplasmic borders undefined, or cells with dispersed chromatin and atypical arrangement), III) cystic glands (enlarged lumen and flat epithelium), IV) glands with daughter glands (these glands have various sizes and shapes (round, elongated, tortuous) and formed daughter glands or glands inside the epithelium or inside the mother gland lumen, or on the outer surface of the mother gland) and V) conglomerate of glands (individual glands are closed to each other almost without intervening stroma) (Bosquiazzo et al., 2013; Gunin et al., 2001). The presence of these glandular alterations is considered preneoplastic lesions (Gunin et al., 2001).

Endometrial hyperplasia can progress to the most common gynecological cancer, endometrial cancer (Dixon et al., 2014; Refaie & El-Hussieny, 2017; Sanderson et al., 2016; Siegel et al., 2022). Endometrial cancer occurs mainly in postmenopausal women (Weiderpass & Labrèche, 2012). Traditionally it has been divided, based on histological, clinical, and molecular behavior, into estrogen-dependent type I and the less common, but clinically more aggressive, estrogen-independent type II (Gibson & Saunders, 2014; Sanderson et al., 2016). The type I, which comprises approximately 80% of endometrial

cancer cases, is related to excess unopposed estrogenic stimulation of the endometrium (Sanderson et al., 2016). This type of tumor is frequently preceded by endometrial hyperplasia (Ryan et al., 2005). The prognosis of patients with endometrial cancer type I is favorable and is often amenable to surgical treatment (Sanderson et al., 2016). The type II or estrogen-independent is associated with endometrial atrophy and with a worse clinical prognosis (Huvila et al., 2021; Sanderson et al., 2016). Unlike type I, type II is usually not preceded by endometrial hyperplasia (Ryan et al., 2005).

4.1. Proliferative signaling pathway involved in endometrial carcinogenesis

Both endometrial cancer and endometrial hyperplasia are associated with high E2 and low P4 levels in circulation; which lead to an unopposed estrogen stimulation (Sanderson et al., 2016; Sobczuk & Sobczuk, 2017). The estrogen stimulation induces cell proliferation, differentiation and growth through the ER (Wang et al., 2000). Estrogens induce DNA instability, cellular hyperplasia and neoplastic transformation of epithelial cells into carcinomas (Gibson & Saunders, 2014; Martin et al., 1973). The proliferative effects of E2 are counteracted by P4, which asserts its actions via PR (Martin et al., 1973; Sanderson et al., 2016). The expression of PR in endometrial hyperplasia is variable with high, low or similar expression with respect to normal endometrium (Sanderson et al., 2016).

The transition from normal endometrium to carcinoma involves a stepwise accumulation of alterations favoring cell proliferation (Enomoto et al., 1991). As the severity of endometrial lesions increases, the cell proliferation increases, too (Masjeed et al., 2017; Yoshida et al., 2012). This proliferative process observed from benign to atypical and then to malignant uterine lesions is accompanied by estrogen receptor α (ESR1)-expressing cells (Yoshida et al., 2012). While, the expression of ESR1 is increased in endometrial hyperplasia (Kreizman-Shefer et al., 2014; Masjeed et al., 2017), the ESR1 expression may increase or decrease as the hyperplasia progresses to malignancy (Kreizman-Shefer et al., 2014; Prat et al., 2007; Yoshida et al., 2012). Therefore, the diagnosis of these lesions is based on morphological criteria hand in hand with immunohistochemical markers (Dore et al., 2021; Sobczuk & Sobczuk, 2017).

Since endometrial hyperplasia can progress to malignant endometrial lesions (Sanderson et al., 2016), it is useful to have a prognostic marker of cancer progression. One of them is the phosphatase and tensin homolog (PTEN), a tumor suppressor gene located on chromosome 10q23.3 (Kimura et al., 2004). Its loss of immunohistochemical expression in endometrial hyperplasia is associated with an increased risk of endometrial cancer (Raffone et al., 2019). The role of PTEN in tumor suppression was confirmed by frequent endometrial abnormalities that develop in PTEN-deficient mice (Mutter et al., 2000a). In fact, PTEN is mutated in up to

80% of endometrial cancer and in up to 50% of premalignant endometrial lesions, such as endometrial hyperplasia (Mutter et al., 2000a).

Several studies have suggested that the tumor suppression action of PTEN is through the activation of p27 (Erkanli et al., 2006; Hlobilková et al., 2003; Restuccia & Hemmings, 2010; Weng, 2001). p27 is a cyclin-dependent kinase inhibitor that acts on the cell cycle and controls cell proliferation, differentiation, and death (Abbastabar et al., 2018; An et al., 2002; Weng, 2001; Zhu et al., 2001). Zhu et al. (2001) showed that PTEN induces G1 cell cycle arrest and indirectly induces p27 activity in endometrial cancer cells line by modulating the levels of cyclin D3. This shows that p27 may be a target of the PTEN cell cycle arrest pathway (Zhu et al., 2001). p27 acts as a tumor suppressor too and its loss expression occurs in around 50% of endometrial hyperplasia and carcinoma (Erkanli et al., 2006). Therefore, the inactivation of PTEN and p27, which are known regulators of cell proliferation, are important early events in uterine carcinogenesis (Horree et al., 2007; Kimura et al., 2004; McCampbell et al., 2016).

5. Lifestyle factors and endometrial carcinogenesis

Multiple lifestyle factors, such as dietary habits, physical activity, weight management, medications, and environmental exposures can affect positively or negatively human health (Abbate et al., 2020; Griban et al., 2020; Sharma et al., 2013). The lifestyle factors can be grouped into non-modifiable and modifiable risk factors (Midha et al., 2016; Nindrea et al., 2017; Tafrishi et al., 2020). Non-modifiable factors are those factors that cannot be voluntarily altered through lifestyle practices (Ng & Chew, 2020). They include age, gender, personal and family medical history, among others (Midha et al., 2016; Ng & Chew, 2020; Nindrea et al., 2017; Serre & Sasongko, 2012). In contrast, modifiable factors allow intervention and include dietary habits, physical activity, environmental chemicals, and medical treatments (Eid et al., 2019; Midha et al., 2016; Ng & Chew, 2020). However, in the case of environmental chemicals, in some cases are not possible to voluntarily choose whether or not to be exposed.

Besides hormonal actions, many lifestyle factors are strongly associated with the incidence of different types of cancer (Anand et al., 2008; Friedenreich et al., 2021; Moore & Brewer, 2017; Stewart, 2012). For female reproductive cancer, exposure to environmental pollutants, such as pesticides, increases the risk of developing cancer (Shah et al., 2018; Stewart, 2012; Taketa et al., 2016). Among pesticides, we are interested in the herbicide glyphosate (N-(phosphonomethyl)-glycine), due to the high application in the world (Benbrook, 2016). If the glyphosate is a carcinogen is still under debate, because of conflicting data and interpretation (Guyton et al., 2015; Leon et al., 2019; IARC, 2015; Myers et al., 2016).

However, there is plenty of scientific evidence pointing to glyphosate as an agent that can modify the features of different hormone-dependent cells (Mesnage et al., 2017; Richard et al., 2005; Thongprakaisang et al., 2013).

The risk of endometrial cancer is strongly associated with different dietary patterns, particularly those diets with a high content of sugar and/or additional fat (Dunneram et al., 2019; Moore & Brewer, 2017; Si et al., 2017). Taking into account that the type of diet can be modified voluntarily, it seems more likely to reduce the incidence of cancer (Dunneram et al., 2019; Midha et al., 2016). Thus, and considering that unhealthy diets are popular in a big part of the world, we are interested in the study of the cafeteria (CAF) diet, which provides a highly relevant model in terms of mimicking human eating patterns (Lalanza & Snoeren, 2021).

On the other hand, and as we mentioned, medical treatment is one of the lifestyle factors that can be modified. It is known that during the menopausal period, women suffer uncomfortable symptoms attributed to the reduced secretion of E2 and P4 (Bruce & Rymer, 2009; Nelson et al., 2005). The typical treatment to alleviate those symptoms is the hormone replacement therapy (HRT) (Angioli et al., 2018). However, the use of HRT is highly debated after the Women's Health Initiative (WHI) study. That study found associations between the HRT and an increased risk of developing hormone-dependent cancer, including endometrial cancer (D'Alonzo et al., 2019). Thus, many women have turned to herbal remedies as an alternative to HRT (Moore et al., 2017). Based on the increased use of herbal remedies, their characterization regarding potential beneficial health effects and risks has been on demand. In such context, we are interested in hops (*Humulus lupulus* L.), which is a widely used agent to replace the HRT (Bolton et al., 2019; Dietz et al., 2016).

In contrast to glyphosate and the CAF diet, which can be classified as clear negative lifestyle factors, we propose hops as a factor with a positive influence on women's health.

CHAPTER I: Evaluation of the carcinogenic effect of glyphosate-based herbicide on the rat uterus

1. Introduction

1.1. Glyphosate-based herbicide

Glyphosate (N-phosphonomethyl glycine) is the active ingredient of broad-spectrum herbicide formulations commercially available as glyphosate-based herbicides (GBHs). These formulations consist of glyphosate in its salt form and polar surfactants, which enhance the herbicidal action of glyphosate by increasing its solubility in water as well as inducing its penetration and absorption in the plant (Soares et al., 2021). Currently, GBHs are the most frequently used herbicide in the world with more than hundreds of formulations commercialized under different brands (Soares et al., 2021). The uses of glyphosate vary greatly from one country to another. While the use of glyphosate has increased in the world, in some countries like Germany its use has been reduced (Antier et al., 2020).

The GBHs have a wide spectrum of action (non-selective) and are used to control weeds in a wide array of crop lands and for post-harvest chemical desiccation. In addition to the traditional uses in agriculture, GBHs are used in homes, in the maintenance of public spaces and in the control of aquatic flora (Székács & Darvas, 2018). The use of GBHs 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 in the world (Benbrook, 2016). The expanded use of this herbicide has led to the appearance of weeds resistant to GBHs, leading to an increase in the doses and the number of applications of the herbicide (Fischer et al., 2014).

1.1.1. Occupational and environmental exposure

The presence of glyphosate and its primary metabolite, aminomethylphosphonic acid (AMPA), was found in several environmental matrices, such as water, soil, and air (Pérez et al., 2021; Van Bruggen et al., 2018). Despite the low persistence of glyphosate in the environment, the frequency of its application impacts on the magnitude of the environmental effect (Mamy et al., 2010). Climatic conditions and soil composition can influence on the persistence of glyphosate and it was suggested a typical half-life in the field of 47 days. In water, the half-life varies from a few days to 91 days (Henderson et al., 2010). In addition to the environmental contamination, glyphosate has also been detected in drinking water and several groups of food for human consumption including honey, cereals and cereal products, fruits and vegetables, animal-derived products and even in baby food (Bai & Ogbourne, 2016; Rendon-von Osten & Dzul-Caamal, 2017; Rodrigues & de Souza, 2018; Soares et al.,

2021; Zoller et al., 2018). Among them, the greatest concern is water contamination because glyphosate was found in levels that were higher than the maximum residue limits admitted (Soares et al., 2021). In Argentina, high levels of glyphosate, 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 products exceeded the maximum residue limit for food of 20 mg/kg established by the European Union (Arregui et al., 2004; Test-Biotech, 2013). Glyphosate was also detected in the muscle tissue of fish living in the Salado river of Santa Fe (Lajmanovich et al., 2023). Therefore, this herbicide constitutes a real threat to humans, as confirmed in different biomonitoring studies by the presence of glyphosate residues in urine and serum samples of individuals living in urban (Gillezeau et al., 2019; Grau et al., 2022) and rural areas (Gillezeau et al., 2019; Kongtip et al., 2017; Parvez et al., 2018). In fact, in Germany, a study conducted on children and adolescents found that half of the first-void urine samples had glyphosate levels above the limit of quantification of 0.1 µg/L (Lemke et al., 2021). All these antecedents alert us to the high risk of environmental and occupational exposure to glyphosate and highlight the need to evaluate the effects of GBH on human and animal health.

1.1.2. Metabolism

The high use of glyphosate and consequent accumulation in the environment has increased concerns about the possible side effects of this compound on animal and human health (Torretta et al., 2018). However, very few studies intended to explain the pharmacokinetics of glyphosate. Previous studies in rats showed that the absorption of orally administered glyphosate is low; with only about 20 to 30% of the administered dose being absorbed (WHO & FAO, 2017) and even at high dose, the absorption is lower (Williams et al., 2000). The elimination half-life of glyphosate from the plasma is between 10 and 15 h, depending on the route of administration (Anadón et al., 2009; WHO & FAO 2017). As an example, glyphosate is absorbed more slowly through the gastrointestinal tract after oral administration than intravenous dosing (Anadón et al., 2009). The elimination half-life of glyphosate found in the plasma of rats was comparable to that of human studies (Connolly et al., 2019; Zoller et al., 2020). The half-life of glyphosate in human urine samples was estimated between 3 and 14 h (Connolly et al., 2019).

Glyphosate is excreted mostly unchanged in the feces and secondarily in the urine, and only 1% undergoes metabolism, originating AMPA (WHO & FAO, 2017; Williams et al. 2000). Both glyphosate and its metabolite are excreted after 48 h, and after 7 days practically all of them have been eliminated from the body (EFSA, 2015; WHO & FAO, 2017). However, our knowledge of pharmacokinetic glyphosate is still incomplete. And it seems that more studies

are necessary to have a deeper understanding of the metabolic pathways of this compound; taking into account the extent of the usage of GBHs around the world.

1.1.3. Glyphosate-based herbicides and carcinogenesis

The potential carcinogenic effect of glyphosate has been in the center of debates, because of conflicting data and interpretation. In 2015, the International Agency for Research on Cancer (IARC) reclassified glyphosate as "probable carcinogenic to humans" (group 2A), including in its analysis more than 500 scientific works that used glyphosate and GBH (IARC, 2017). This classification was based on limited epidemiologic evidence in humans, mainly for non-Hodgkin lymphoma, and significant evidence of carcinogenicity in animal models (Novotny, 2022). In addition, genotoxic effects and oxidative stress were detected in cells of various species that were proposed as mechanisms of carcinogenicity (Novotny, 2022). In contrast to the IARC, the European Food Safety Authority (EFSA) concluded that the herbicide does not exhibit mutagenic or carcinogenic properties, based on all available evidence for glyphosate, and also 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 according to US Environmental Protection Agency (EPA) it is "not probable to be carcinogenic" (EFSA, 2015; 2021). However, the European Commission became more restrictive and the permission for glyphosate was renewed for a period of only 5 years instead of the usual 10 years (Arcuri & Hendlin, 2019). Furthermore, the EFSA recommended new preventive measures regarding its use like, for example the application of glyphosate in public gardens and playgrounds is strongly discouraged (Arcuri & Hendlin, 2019).

But why are the evaluations done by the EFSA and the IARC so contrasting? It may be because of the different methodologies followed for each one. While the IARC examined only published studies, the EFSA relied mostly on unpublished studies funded by herbicide manufacturers (Portier et al., 2016). In addition, the IARC examined both glyphosate and GBH, whereas the EFSA examined only glyphosate as a pure substance (Portier et al., 2016; Tarazona et al., 2017). Given that glyphosate formulations are regarded as more toxic than the compound in its technical grade (Benachour & Séralini, 2009; Defarge et al., 2016; Mesnage et al., 2014; Tsui & Chu, 2003), safety evaluations focused on glyphosate alone can consequently underestimate its toxicity (Vandenberg et al., 2017). Even though the EFSA and the US EPA have declared non-conclusive evidence of carcinogenic effects (EFSA, 2017; U.S. EPA, 2015), its use has been restricted and banned in some countries (Meftaul et al., 2020). This decision was based on some studies, including those of the IARC, suggesting that both glyphosate and GBHs have probable carcinogenic effects (Guyton et al., 2015; Leon et al., 2019; IARC, 2015; Myers et al., 2016).

Several studies in animal models have evaluated the potential carcinogenic effect of glyphosate; however, the results are highly variable and depend on time, route, and doses of herbicide exposure. Just as examples of the latest, rodents orally and chronically exposed to glyphosate have developed tumors in kidney, liver, skin and adrenal cortex (reviewed in Portier, 2020); but in mice exposed topically to GBH in a skin carcinogenicity test, George et al. (2010) showed a tumor-promoting effect, not an initiating one. In our own experience and by using an early postnatal exposure to GBH (during the first week of life), we showed an increased proportion of hyperplastic ducts associated with a fibroblastic-like stroma in the mammary gland of aging female rats (Zanardi et al., 2020). Furthermore, with the same experimental protocol, Guerrero Schimpf et al. (2017) detected uterine luminal epithelial hyperplasia in rats at early postnatal life. Such lesions found in the mammary glands and in the uterus are considered preneoplastic lesions (Sanderson et al., 2016; Singh et al., 2000).

Another controversial point is the impact of glyphosate exposure on human populations. In a study on the health condition of the inhabitants of a city in Argentina, the cancer incidence, prevalence, and mortality was two or three times higher (Vazquez et al., 2017) than the reference values for the entire country (Globocan 2012, WHO). Breast, colon and prostate cancer were the most prevalent types of cancer found in that city, whose conspicuously the main economic activity is intensive agriculture (Vazquez et al., 2017). Many human epidemiological studies have evaluated the relationship between glyphosate and the risk of cancer. Some of those studies found no association between glyphosate exposure and melanoma, neither colon, rectum, bladder, kidney and blood cancer (Andreotti et al., 2018; De et al., 2005; Ward et al., 2023). Others found that glyphosate is associated with the development of multiple myeloma (De et al., 2005), and large B-cell lymphoma (Leon et al., 2019). Regarding the link between glyphosate exposure and non-Hodgkin's lymphoma, the studies are not conclusive. Some studies (Eriksson et al., 2008; Hardell et al., 2002; Zhang et al., 2019) demonstrated that non-Hodgkin's lymphoma is more frequent in populations highly exposed to glyphosate, whereas others found no association (Andreotti et al., 2018; De et al., 2005; Leon et al., 2019).

The effect of glyphosate as an endocrine-disrupting chemical (EDC) has also been highly debated alongside the carcinogenic effect, but its classification is still unclear. First, the GBH was suggested to have endocrine disrupting properties by inhibiting aromatase activity in human placental and embryonic cell lines (Richard et al., 2005). In breast cancer cells lines, glyphosate mimics estrogen activity through interaction with its two receptors, ERα and ERβ, based on the inhibited effect of ER antagonists on glyphosate action (Thongprakaisang et al., 2013). Similar activated ER pathway was observed in breast cancer cells too by Mesnage et al. (2017), but through a ligand-independent mechanism. In a human

endometrial cancer cells line, glyphosate promotes cell invasion and migration via ER pathway (Gastiazoro et al., 2020). The deregulation of the estrogen pathway by GBH has also been studied *in vivo*. In ovariectomized rat uterus, GBH is able to modulate the expression of estrogen sensitive genes (Varayoud & Durando et al., 2017) and enhances the uterine sensitivity to E2 (Guerrero Schimpf et al., 2018). Moreover, neonatal exposure to a GBH alters uterine morphology and the expression pattern of key proteins for uterine differentiation in pre-pubertal rats (Guerrero Schimpf et al., 2017), and impairs endometrial decidualization at adulthood (Ingaramo et al., 2016, 2017). However, no evidence of potential interaction of glyphosate with endocrine pathways has been detected in the Endocrine Disruptor Screening Program (EDSP) conducted by the US EPA (US EPA 2015). Thus, both the carcinogenic and endocrine disrupting potential of glyphosate and GBHs remains uncertain.

1.2. Cafeteria diet

Cafeteria (CAF) diet, also called “Junk Food Diet” or “Western Diet” is a rodent dietary model that reflects the variety of highly palatable and energy dense foods prevalent in Western society (Lalanza & Snoeren, 2021; Sampey et al., 2011). The composition of this diet implies an unbalanced diet with predominantly fat energy content (49%) at the expense of low protein content (7%) (Lazzarino et al., 2019). The CAF diet model is based on offering the animals balanced food (Standard Chow laboratory food) plus varied and highly palatable foods frequently consumed by humans. These items foods are changed periodically, to encourage the interest of the animals in new foods. Therefore, this diet has the advantage of allowing novelty, choice and variety (Lalanza et al., 2014; Lalanza & Snoeren, 2021). This diet provides a highly relevant model in terms of mimicking human eating patterns because laboratory rodents eat the same ultraprocessed, unhealthy but tasty, products that humans consume every day (Lalanza & Snoeren, 2021).

1.2.1. Cafeteria diet and carcinogenesis

Several studies have explored and produced evidence of the relationship between diet and endometrial cancer risk (Dunneram et al., 2019; Moore & Brewer, 2017; Si et al., 2017). The type of diet chosen can modify the susceptibility to develop uterine tumors. A prospective cohort study in women showed that one of the major risk factors of endometrial cancer is the high energy intake, associated or not with overweight (Furberg & Thune, 2003). Also, those diets with high fat content and glycemic load increase the risk of this type of cancer (Littman et al., 2001; Mulholland et al., 2008). In contrast, the Mediterranean diet, which is characterized by a combination of highly complex carbohydrates in fiber and polyunsaturated fatty acids, is associated with a decreased risk of endometrial cancer (Filomeno et al., 2015). Another controversial point was added to this topic, after the study of

the WHI. In that study it was demonstrated that the endometrial cancer risk is not associated with the quality of diet, at least in postmenopausal women (George et al., 2015; Prentice et al., 2007). Other studies of dietary influences on endometrial cancer risk have focused on specific foods or nutrients, separately. However, those studies failed to mimic the complexity of the human diet, and the interactions between all components were lost (Si et al., 2017). Thus, the evaluation of dietary patterns and their association with cancer risk could provide a better estimation of the relation between food habits and health (Si et al., 2017).

In a previous work of our laboratories (Gastiazoro et al., 2018), CAF diet was administered to female rats from weaning until adulthood. Those females exhibited overweight respect to animals fed with standard diet accompanied by an increase in serum leptin levels (Gastiazoro et al., 2018). These high leptin levels were associated with high levels of leptin receptor in the uterus of CAF animals (Gastiazoro et al., 2018). Moreover, the animals fed with CAF diet exhibited endometrial hyperplasia (Gastiazoro et al., 2018), perhaps as a consequence of high leptin levels because of its role as regulator of endometrial proliferation (Villavicencio et al., 2010). It is important to highlight that endometrial hyperplasia is a central clinical topic, as it has the potential to transform into cancer (Sanderson et al., 2016). Also, some studies performed in animals and humans have demonstrated that this diet is associated with the development of some types of cancer, such as breast (Dianatinasab et al., 2020; Foroozani et al., 2022), urinary bladder (Dianatinasab et al., 2020) and colorectal (Benninghoff et al., 2020) cancer. Taking account that unhealthy diets like the CAF diet are widespread in Western society and might predispose to cancer, it is necessary to increase research efforts and investments in this field.

Based on these antecedents, we hypothesize that:

a) Proliferative process could be involved in the alterations observed in the morphology of glands in the uterus of aging female rats exposed to a low dose of GBH during the first week of life.

b) The addition of subchronic GBH during adulthood could worsen the effect induced by the CAF diet alone.

2. Goals

2.1. Main goal

To evaluate whether the GBH exposure alone or added to the CAF diet, predispose to develop preneoplastic and/or neoplastic uterine lesions.

2.2. Specific goals

EXPERIMENT I. To study the carcinogenic effect of a GBH by using a neonatal exposure model in aging Wistar rats, we proposed:

a) to determine the effect of GBH on the body weight (bw) of the animals along the experiment;
b) to measure the ovarian steroid hormone levels at sacrifice;
c) to determine the expression of some proteins involved in the proliferation process by immunohistochemistry, in the preneoplastic lesions.

EXPERIMENT II. To study whether the addition of a subchronic low-dose of a GBH impacts on the endometrial hyperplasia induced by CAF diet in Wistar rats, we proposed:

a) to determine the bw during and at the end of treatments;
b) to calculate the adiposity index at sacrifice;
c) to measure the ovarian steroid hormone levels at sacrifice;
d) to analyze the incidence of uterine preneoplastic lesions;
e) to quantify the uterine expression of steroid hormone receptors and their signaling pathways.

3. Materials and Methods

3.1. Diet composition

Laboratory rodent chow (Cooperación, ACA Nutrición Animal, Buenos Aires, Argentina) was used for the standard diet. This diet provided 3.00 kcal/g, containing 5% energy as fat, 23% as protein and 72% as carbohydrate. This diet is composed of 6% of raw fiber and 10% of minerals, with a relative humidity of 12% (Andreoli et al., 2015; Kass et al., 2012).

The CAF diet was composed of standard chow plus parmesan cheese, cheese-flavored snacks, crackers, salty and sweet biscuits, cookies, pudding, peanut butter, and chocolate (Lazzarino et al., 2019). All these CAF items are low in essential micronutrient density (Gastiazoro et al., 2022). This diet provided an average of 4.85 kcal/g, containing 49% energy as fat, 7% as protein, and 44% as carbohydrate, in addition to that provided by the standard chow (Gastiazoro et al., 2022; Lazzarino et al., 2019).

3.2. Glyphosate formulation

The GBH used in this study was Roundup FULL II® (Monsanto, Argentina). This formulation is soluble in water and contains 66.2% glyphosate potassium salt (active ingredient), with an equivalent to 54% glyphosate acid. This formulation, as those of other GBHs, also contains coadjuvants and inert ingredients not specified in the herbicide label.

Both in *EXPERIMENT I* and *II*, GBH was administered in a dose of 2 mg of glyphosate/kg bw/day, although in a different route of administration (as it is described in 3.4 item). The chosen dose 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 (U.S. EPA, 2017). Also, the evaluated dose is in the order of magnitude of the environmental levels detected in Argentina, including in soil, sediment and particulate matter (Bonansea et al., 2018; Mac Loughlin et al., 2022a, 2022b; Primost et al., 2017; Ramirez Haberkon et al., 2021), but also in soybean leaves and stems (Arregui et al., 2004).

3.3. Animals

Rats were housed and handled in accordance with the principles set out in the Declaration of Helsinki. The experiments were designed and performed to match closely to the 3R (replacement, reduction, refinement) principles of animal welfare. In addition, we complied with the ARRIVE guidelines. Beside this, all the experimental protocols used in this Chapter of the PhD thesis were approved by the Institutional Ethics Committee of the Facultad de Bioquímica y Ciencias Biológicas (FBCB) of the Universidad Nacional del Litoral (UNL), Santa Fe, Argentina, and performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the U.S.

National Academy of Sciences. Animals were treated humanely and with regard for the alleviation of suffering.

Rats of a Wistar-derived strain bred at the Instituto de Salud y Ambiente del Litoral (ISAL; UNL-CONICET) were used. Animals were maintained in a controlled environment (22 ± 2°C; 14 h of light from 06:00 am to 08:00 pm) in stainless steel cages with sterile pine wood shavings as bedding. Rats had free access to pellet laboratory chow and tap water in glass bottles with rubber stoppers. We have previously tested the rat diet (pellet chow and water) for glyphosate residues by using ultra-high-performance liquid chromatography-tandem mass spectrometry and found no detectable levels (Milesi et al., 2018).

3.4. Experimental procedures

3.4.1. EXPERIMENT I

Pups were obtained from eight timed-pregnant rats housed singly. After delivery (PND 0), pups were sexed according to the anogenital distance and litters of eight pups (preferably four males and four females) were left with each mother (Guerrero Schimpf et al., 2017). Female pups were cross-fostered among the mothers to minimize the use of siblings. This schedule allows including no more than two siblings in each group. Female pups from each foster mother were randomly assigned to one of the following postnatal treatment groups: 1) Control group, receiving saline solution (n = 11), and 2) GBH group, receiving a commercial formulation of glyphosate diluted with saline solution (2 mg glyphosate/kg bw/day, n = 10). The number of animals per group was determined according to previous works of the ISAL (Alarcon et al., 2020; Guerrero Schimpf et al., 2017, 2018; Zanardi et al., 2020).

As shown in Figure 2, the treatments were administered by subcutaneous injections in the nape of the neck every 48 h from PND 1 to PND 7. On each treatment day, a glyphosate solution was prepared according to the average bw of the pups, so as to administer 2 mg glyphosate/kg bw in a fixed volume of 40 µL. As previously reported (Guerrero Schimpf et al., 2017), the postnatal treatment with GBH did not alter the maternal care or nursing of the experimental groups. In addition, the treatment led to no signs of local reaction or acute or chronic toxicity.

At weaning (PND 21), the offspring were kept under standard laboratory animal husbandry conditions. Along the experiment, animals were weighed on each treatment day (PND 1, 3, 5 and 7) as well as 24 h after the end of the experiment (PND 8), and then weekly (from PND 9 up to PND 30) or monthly (from PND 31 up to PND 600).

To sacrifice all animals at the same stage of the estrous cycle, vaginal smears were performed every morning (Montes & Luque, 1988) starting on PND 570. Briefly, vaginal

secretion was collected with a plastic pipette filled with 500 µL of saline solution (NaCl 0.9%) by inserting the tip of the pipette into the vaginal canal. The pipette bulb was firmly but gently depressed to expel the saline into the vagina and the saline was drawn back into the dropping pipette which was removed from the vaginal canal. Vaginal fluid was placed on glass slides and observed under an optical microscope to determine the stage of the estrous cycle, according to the predominant cells present (Montes & Luque, 1988; Manservisi et al., 2018). All animals were sacrificed by decapitation in the morning at the estrus closest to 600 days of age and the uterus was obtained. According to ethical rules, in those cases of possible suffering from the animal, the sacrifice was performed before, and during the course of the study. Trunk blood was collected, samples were centrifuged and serum was stored at −20 °C until hormone assays were performed.

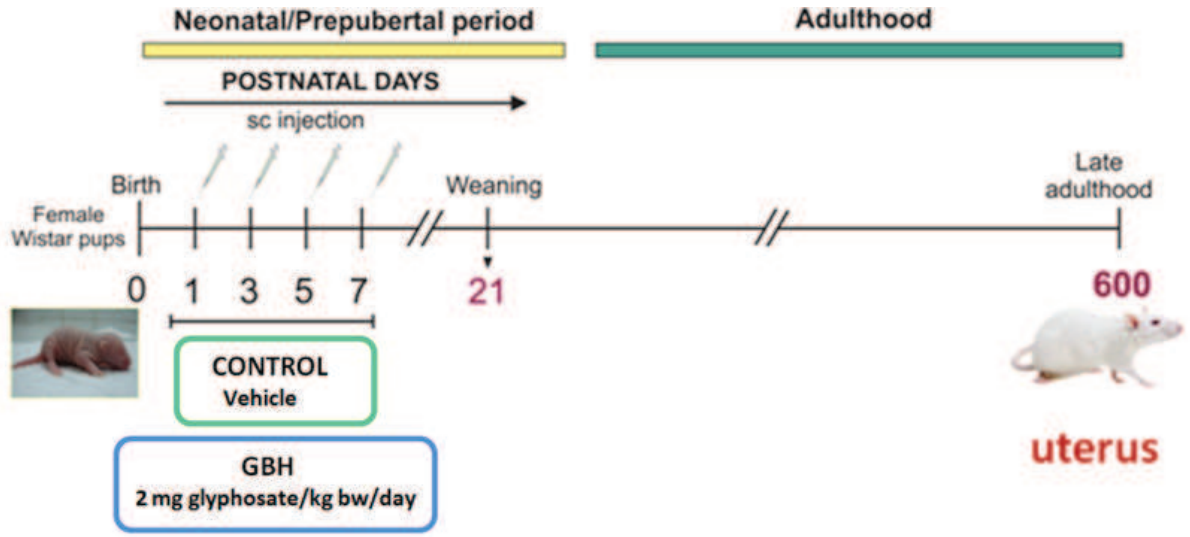

Figure 2. Schematic representation of the experimental protocol used in *EXPERIMENT I*, to study the effects of neonatal exposure to glyphosate-based herbicide (GBH) in aging female Wistar rats. Sc, subcutaneous.

3.4.2. EXPERIMENT II

The timeline of experimental handling is shown in Figure 3. The animals were fed either with the standard chow diet or with the CAF diet from weaning (PND 21) until PND 240. For the CAF diet treatment, together with the standard chow, three of the CAF food items were offered in excess quantities and changed every other day, by supplanting them with new items, in order to maintain the variety. Therefore, the animals did not receive the same food items for more than two consecutive days (Lazzarino et al., 2017).

From PND 140, animals fed with standard chow received a pellet chow-based paste with added water (Control group, n=8). Animals fed with CAF diet were randomly divided into two groups: one group received a pellet chow-based paste with added water (CAF group, n=8)

and the other one received a pellet chow-based paste supplemented with GBH in a dose of 2 mg of glyphosate/kg bw/day (CAF+GBH group, n=10). The number of animals per group was determined according to previous works of the ISAL (Ingaramo et al., 2019; Varayoud & Durando et al., 2017).

The dose of glyphosate was calculated based on the average bw and food intake during the treatment period. The laboratory chow-based paste was prepared for each experimental group by blending optimized quantities of pellet chow and water, according to Milesi et al. (2018). For the GBH treatment, the glyphosate commercial formulation was diluted with water to obtain 2 mg of glyphosate/kg bw/day. The mixture was covered and left to stand overnight, and then homogenized to form a paste and prepare chow balls with the paste. This pellet-based paste was prepared the same day the food was replaced.

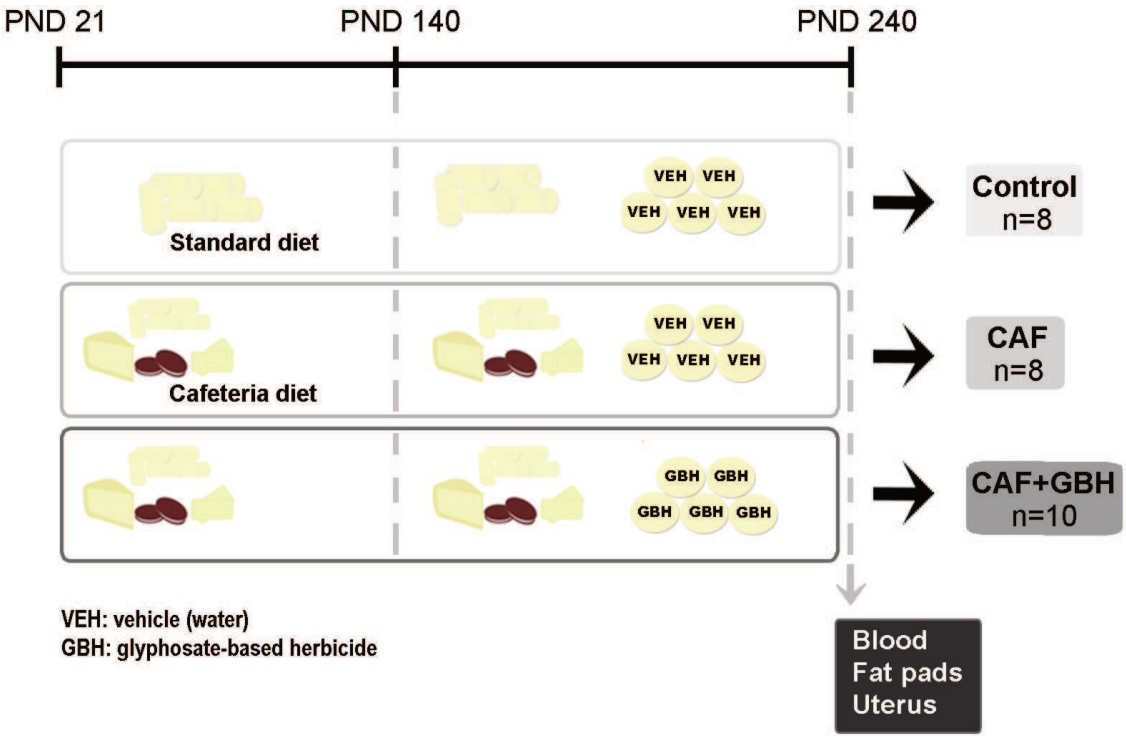

Figure 3. Schematic representation of the experimental protocol used in *EXPERIMENT II,* to study the effects of the addition of a subchronic low dose of a glyphosate-based herbicide (GBH) in rats fed with cafeteria (CAF) diet. PND, postnatal day.

The bw of each animal was recorded on PNDs 21, 140 and 240. The food intake was recorded every day and determined by the weight difference between the food offered and the remaining food, adjusted to the waste by collecting food spillage. Energy intake was calculated using the energy contents (kcal/g) and average intake of each food item. To sacrifice all animals at the same stage of the estrous cycle, vaginal smears were performed during the two weeks before sacrifice. All animals were sacrificed by decapitation in the

morning of the second day of diestrus closest to PND 240. Food was withdrawn 8 h before sacrifice. Trunk blood was collected and glucose concentration was measured using an Accu-Chek Performa Nano meter (Roche Diagnostics, Mannheim, Germany). Blood samples were centrifuged and serum was stored at −20°C until hormone assays. The uterus was sampled; and retroperitoneal, perigonadal, and subcutaneous fat depots were isolated and weighed to calculate the adiposity index (Rossi et al., 2013).

We also performed a pilot experiment in order to determine the uterine effects of GBH alone. Animals were fed with standard diet from PND 21 until PND 140 and from PND 140 were also exposed to GBH in a dose of 2 mg of glyphosate/kg bw/day. At sacrifice, the morphology of the uterus of these animals was similar to that of Control animals. Thus, we decided not to include this experimental group in the thesis.

3.5. Hormone assays

The serum levels of E2 and P4 were determined by electrochemiluminescence immunoassay, using Elecsys Estradiol III and Elecsys Progesterone III (Cobas®, Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's specifications.

3.6. Tissue processing

The uterine biopsies were fixed for 6 h in buffer formalin 10% v/v in phosphate buffer saline (PBS) 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 60ºC inside a bottle with molten paraffin. The heat caused the evaporation of the remaining solvent and 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 (RT). This procedure allowed the obtaining of a paraffin block with the tissue inside.

Uterine samples embedded in paraffin were longitudinally cut into 5 µm sections using a microtome (Leica, Jung RM2025, Leica Instruments GMT, Nussioch, Germany) and mounted on slides coated with 3-aminopropyl tri-ethoxysilane (Sigma–Aldrich, Argentina S.A.) for histological studies (morphometric and immunohistochemical analysis).

3.7. Hematoxylin and eosin stain

Following the protocol detailed in Table 1, uterine sections were deparaffinized and rehydrated in graded ethanol solutions to be then stained with Mayer hematoxylin and eosin. Then, the histological sections were dehydrated and mounted with a permanent mounting liquid (Eukitt®, Sigma–Aldrich, Argentina S.A.).

Table 1. Hematoxylin and eosin protocol staining.

Deparaffinization and rehydration	
Bioclear 1, 2 and 3	5 min ea.
Alcohol 100°, 96° and 70°	1 min ea.
Water wash	2 min
Staining	
Mayer hematoxylin	4 min
Water wash	2 min
Ammonia water	30 sec
Water wash	2 min
Eosin	2 min
Dehydratation and mounting	
Alcohol 70° and 96°	2 sec ea.
Alcohol 100°	30 sec
Bioclear 1 and 2	5 min ea.
Mount with permanent mounting liquid	

3.8. Immunohistochemistry

A standard immunohistochemical technique was performed, following protocols previously described by us (Varayoud & Durando et al., 2017; Zanardi et al., 2020). Uterine longitudinal sections were deparaffinized and rehydrated in graded ethanol solutions. Then, the antigen retrieval was performed by immersion of the 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 2). Next, the slides were placed in a 3% solution of hydrogen peroxide (H_2O_2) 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 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, Argentina S.A.). The sections were incubated in a humid chamber with the specific primary antibody (14 to 16 h at 4°C), and then with their

corresponding biotinylated secondary antibody for 30 min at RT. Finally, incubation was carried out with the streptavidin-peroxidase complex (Sigma–Aldrich, Argentina S.A.) for 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 H_2O_2 30 vol were used. The reaction time was 10 min at RT. For Ki67 and p27 immunodetection, samples were counterstained with Mayer´s hematoxylin (Biopur, Rosario, Argentina). Finally, the histological sections were dehydrated and mounted with a permanent mounting liquid (Eukitt®, Sigma–Aldrich, Argentina S.A.). 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 primary and secondary antibodies used in the immunohistochemical assays are detailed in Table 3.

Table 2. Immunohistochemical 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 the 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 (see Table 3)	14 - 16 h 4°C (humid chamber)
PBS	15 min
Biotinylated Secondary antibody	
Secondary antibody incubation (see Table 3)	30 min RT (humid chamber)
PBS	10 min
Streptavidin-peroxidase complex	
Streptavidin-peroxidase complex incubation	30 min RT (humid chamber)
PBS	10 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
Distilled water	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	

Table 3. Antibodies used for immunohistochemistry.

Antibodies	Dilution	Supplier
Primary		
Anti-ESR1 (clone 6F-11)	1/200	Novocastra (Newcastle upon Tyne, UK)
Anti-PR	1/400	Dako Corporation (Carpinteria, CA, USA)
Anti-Ki67 (clone MIB-5)	1/50	Dako Corporation (Carpinteria, CA, USA)
Anti-PTEN	1/1500	Generated and validated in the ISAL (Bracho et al., 2020)
Anti-p27	1/1200	Santa Cruz Biotechnology, Inc
Secondary		
Anti-mouse (B8774)	1/100	Sigma-Aldrich (St. Louis, MO, USA)
Anti-Rabbit (B8895)	1/200	Sigma-Aldrich (St. Louis, MO, USA)

ESR1, estrogen receptor α; PR, progesterone receptor; PTEN, phosphatase and tensin homolog.

3.9. Histological analysis

EXPERIMENT II

3.9.1. Determination of luminal epithelial hyperplasia

The number of luminal epithelial layers was quantified using a Dplan 40× focusing eyepiece (numerical aperture=0.65; Olympus Optical Co., Ltd., Tokyo, Japan). Luminal epithelial hyperplasia was established as a LE with more than four cellular layers. A total of 15 fields were evaluated and the results were expressed as the percentage of incidence of luminal epithelial hyperplasia, as previously described (Guerrero Schimpf et al., 2017).

3.9.2. Determination of stroma and myometrium thickness

The thickness of the subepithelial stroma (SS), circular myometrium, and longitudinal myometrium layers was analyzed by image analysis, using the Fiji software (Figure 4). Briefly, the images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope with a Dplan 20× focusing eyepiece (numerical aperture = 0.40). To spatially calibrate the Image Pro-Plus analyzer, square grids from Neubauer's chamber images were captured. The length of each uterine compartment (SS, circular myometrium, and longitudinal myometrium) was measured (Figure 4), on at least 10 fields per animal.

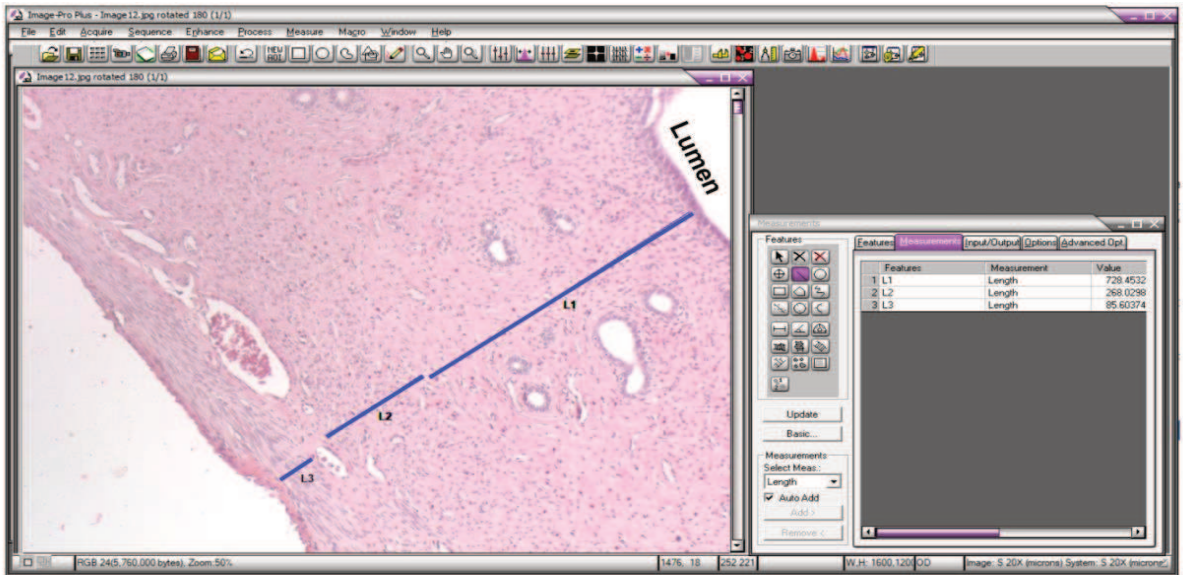

Figure 4. Determination of the thickness of stroma and myometrium in a longitudinal section of the uterus stained with hematoxylin and eosin. Following spatial calibration of the Image Pro-Plus software, point-to-point length measurements were made in the uterine compartment of interest. Blue lines L1, L2 and L3 represent the thickness of the subepithelial stroma, circular myometrium and longitudinal myometrium, respectively.

3.9.3. Determination of the glandular density

We evaluated the density of normal and altered glands: glands with cellular anomalies, cystic glands, glands with daughter glands, glands with squamous metaplasia and conglomerate of glands. To determine gland density, the images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope with a Dplan 20× focusing eyepiece. 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 (glands), Pi is the number of incident points over glands, and P is the number of incident points over the stroma. To obtain the data for the point-counting procedure (Figure 5), a square grid of Image Pro-Plus was used on at least 10 randomly selected fields per section and in two sections per animal (separated 50 µm from each other). The results were expressed as Vv x 1000 for each type of gland.

3.9.4. Determination of the stromal nuclei density

The images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope with a Dplan 20× focusing eyepiece. Stromal nuclei density was determined by calculating the ratio between the area occupied by stromal nuclei and the total area of SS, defined as a 200 µm wide area adjacent to the epithelium, from the basement membrane toward the outer layer. As shown in Figure 6, areas were quantified using the Fiji software of

Image Pro-Plus (Schindelin et al., 2012), excluding uterine glands, on at least 15 randomly selected fields per animal (Guerrero Schimpf et al., 2018).

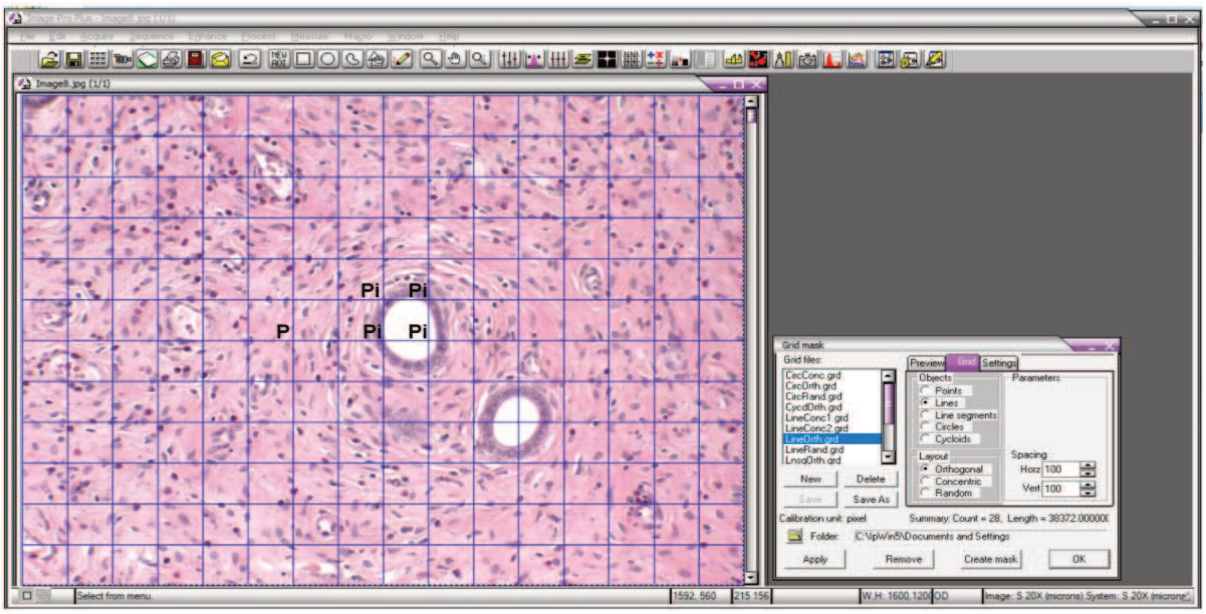

Figure 5. Determination of glandular density by using the Fiji software of Image Pro-Plus in a longitudinal section of the uterus stained with hematoxylin and eosin. Pi indicates the incident points over glands, and P indicates the incident points over the stroma.

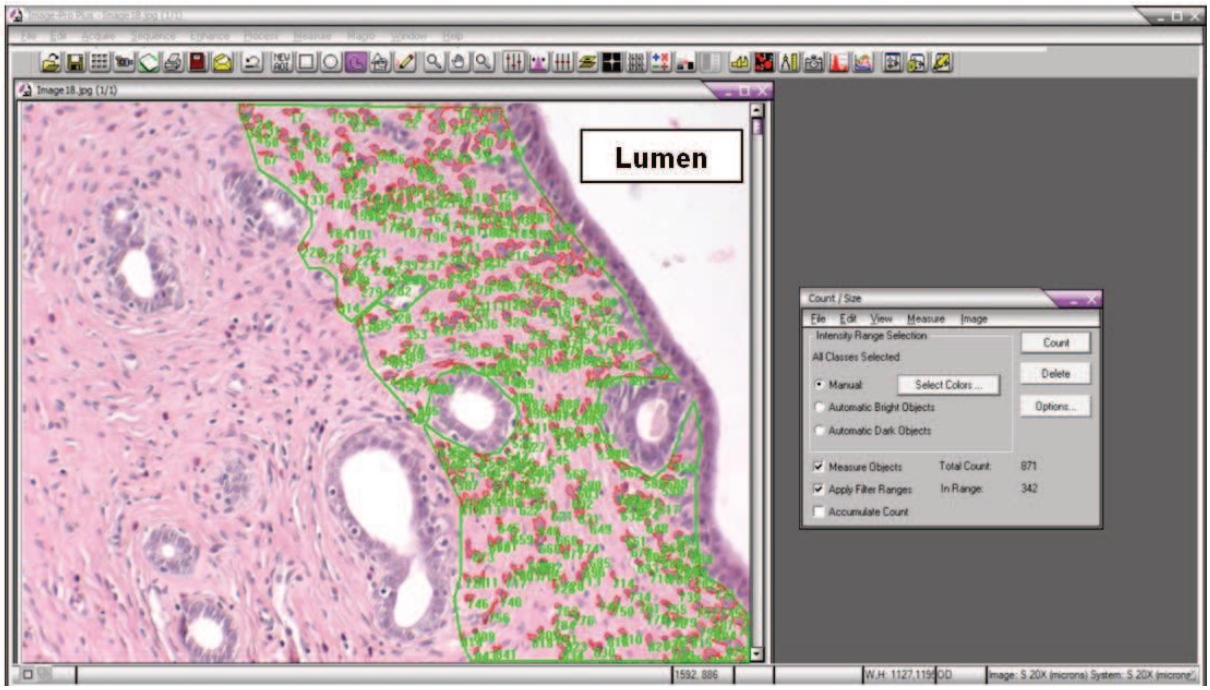

Figure 6. Determination of stromal nuclei density in a longitudinal section of the uterus stained with hematoxylin and eosin. Following spatial calibration of the Image Pro-Plus software, the area of nuclei (green numbers) was evaluated respect to the total area in the stroma (inside the area delimited by the green line).

3.10. Quantification of protein expression

3.10.1. Quantification of protein expression by image analysis

The protein expression in the uterine cells was evaluated by image analysis using the Fiji software and expressed as integrated optical density (IOD), which is a dimensionless parameter that combines the average 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. Images were recorded with a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (Olympus). At least 15 fields of each histological compartment per animal were analyzed, covering similar areas in each experimental group.

EXPERIMENT I. The protein expression of ESR1 and PR in the GE of each type of gland was evaluated using a Dplan 20× focusing eyepiece.

EXPERIMENT II. The protein expression of ESR1, PR and PTEN in the LE was evaluated using a Dplan 40× focusing eyepiece, whereas that in the SS and that in the GE were evaluated using a Dplan 20× focusing eyepiece.

3.10.2. Quantification of Ki67 and p27 expression

EXPERIMENT I. The expression of Ki67 in the GE of each type of gland was evaluated using the Olympus BH2 microscope with a Dplan 40× focusing eyepiece, as a percentage of Ki67-positive cells over the total number of cells present in each type of gland.

EXPERIMENT II. The expression of Ki67 and p27 in the LE and GE was evaluated using the Olympus BH2 microscope with a Dplan 40× focusing eyepiece, as a percentage of Ki67- or p27-positive cells over a total of 1500 cells per compartment. The p27 expression in the SS was evaluated using a Dplan 100× focusing eyepiece (numerical aperture = 1.25) on at least 25 fields of each sample. Results were obtained considering the Vv of the p27-positive cells following the formula Vv =Pi/P, where Vv is the estimated volume fraction of the object under study; Pi is the number of incident points over p27-positive cells (brown cells), and P is the number of incident points over the stroma.

3.11. Statistical analysis

All data are shown as the mean ± SEM. Body weight was statistically analyzed by Student's *t* test.

For ***EXPERIMENT I***, a Mann-Whitney *U* test was performed.

For ***EXPERIMENT II***, the data of incidence of luminal epithelial cell hyperplasia were analyzed using Fisher's exact test. The energy intake and bw were statistically analyzed by

Student's *t* test. An exploratory analysis was first conducted to evaluate whether the data were normally distributed (Shapiro–Wilk test) and variances were homogeneous (Bartlett's test). Then, the appropriate statistical analysis was selected. The followed parameters were evaluated using ANOVA to obtain the overall significance, followed by Tukey's test for multiple comparisons:

- Adiposity index,
- glucose levels,
- the thickness of SS and circular myometrium,
- the densities of stromal nuclei and normal glands,
- the expression of ESR1 in LE,
- the expression of PR in LE and SS, in normal and altered glands,
- the expression of Ki67 in all evaluated compartments (LE and GE, including normal and altered glands),
- the expression of PTEN in all evaluated compartments (LE, SS and GE),
- the expression of p27 in LE and SS.

The analysis of the rest of the variables was conducted using Kruskal-Wallis test followed by Dunn's method for multiple comparisons:

- The serum levels of E2 and P4,
- the thickness of longitudinal myometrium,
- the density of altered glands,
- the expression of PR and p27 in GE,
- the expression of ESR1 and p27 in normal and altered glands.

Statistical analyses of data were carried out using R statistical software (The R Foundation for Statistical Computing version 3.6.1, https://www.r-project.org/). Differences with $p < 0.05$ were considered as significant.

4. Results

4.1. EXPERIMENT I: Long-term effects of neonatal exposure to a low dose of GBH on the uterus of aging rats

4.1.1. Neonatal exposure to GBH did not alter the body weight

Taking into account that our experimental model aimed to evaluate the long-term consequences of GBH exposure, we first determined whether GBH exposure altered the bw of the animals. We found no significant differences in bw between Control and GBH-treated females from birth to adulthood (Figure 7).

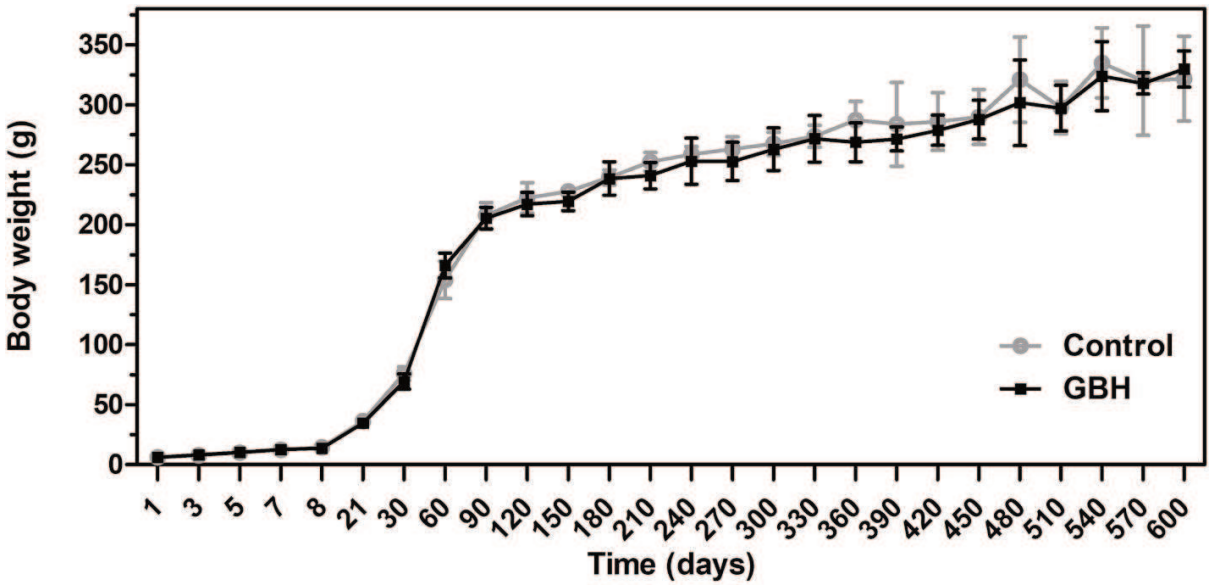

Figure 7. Body weight of rats exposed to saline solution (Control) or glyphosate-based herbicide (GBH) from postnatal day (PND) 1 to 600 (n = 10-11 rats per group). The values are expressed as the mean ± SEM (Mann-Whitney *U* test).

4.1.2. Long-term effects of neonatal GBH exposure

All Control animals survived until the end of the study, whereas three animals of GBH group died early. One of them died for unknown reasons at PND 540 and the others were sacrificed at PND 510 due to either vaginal bleeding or the presence of macroscopic/palpable tumors.

4.1.3. GBH did not alter the ovarian steroid levels

The serum levels of E2 and P4 were similar between Control and GBH-exposed rats (E2: Control: 38.97 ± 2.06 pg/ml vs GBH: 33.26 ± 4.10 pg/ml, p = 0.24; P4: Control: 10.09 ± 2.49 ng/ml vs GBH: 10.61 ± 3.61 ng/ml, p = 0.89).

4.1.4. GBH increased the ESR1 expression in the epithelium of glands with squamous metaplasia

Considering that GBH induced morphological changes in the uterine glands (Guerrero Schimpf et al., 2022), we aimed to assess whether those alterations could be associated

with changes in steroid hormone receptors and if cells were proliferating. Thus, in the present PhD thesis we evaluated the protein expression of Ki67 as marker of cell proliferation, ESR1 and PR in normal and altered glands (namely: glands with cellular anomalies, glands with daughter glands, glands with squamous metaplasia and cystic glands). Animals exposed to GBH increased the expression of ESR1 in glands with squamous metaplasia respect to animals exposed to the vehicle (Figure 8 and Table 4). The expression of ESR1 in normal glands, glands with cellular anomalies, glands with daughter glands and cystic glands was similar between Control and GBH animals. The expression of PR and Ki67 was not affected by GBH treatment in all glandular epithelial cells (Table 4).

Table 4. Protein expression and its distribution in different types of uterine glands from Control and GBH-treated rats.

Type of gland	Control	GBH	p-value
	ESR1 (IOD)		
Normal glands	3.48 ± 0.56	3.89 ± 0.93	0.83
Glands with cellular anomalies	1.63 ± 0.31	2.15 ± 0.81	0.78
Glands with daughter glands	0.02 ± 0.01	0.48 ± 0.47	0.38
Glands with squamous metaplasia	0.57 ± 0.24	1.67 ± 0.40	0.02 *
Cystic glands	2.86 ± 1.06	1.24 ± 0.38	0.19
	PR (IOD)		
Normal glands	2.84 ± 0.51	2.80 ± 0.73	0.85
Glands with cellular anomalies	1.23 ± 0.30	1.84 ± 0.50	0.30
Glands with daughter glands	1.41 ± 0.28	1.24 ± 0.32	0.72
Glands with squamous metaplasia	1.43 ± 0.31	0.74 ± 0.16	0.08
Cystic glands	0.56 ± 0.17	0.96 ± 0.49	0.95
	Ki67 (%)		
Normal glands	13.82 ± 1.62	15.17 ± 2.37	0.95
Glands with cellular anomalies	13.63 ± 4.30	22.85 ± 3.38	0.17
Glands with daughter glands	36.12 ± 10.27	21.74 ±6.51	0.8
Glands with squamous metaplasia	16.63 ± 4.48	9.23 ± 3.97	0.29
Cystic glands	25.99 ± 7.66	10.79 ± 1.21	0.33

Control, rats exposed to vehicle; GBH, rats exposed to glyphosate-based herbicide. Values are expressed as integrated optical density (IOD) or percentage (%) and showed as the mean ± SEM (Mann-Whitney *U* test). * $p < 0.05$ (n = 10-11 rats per group).

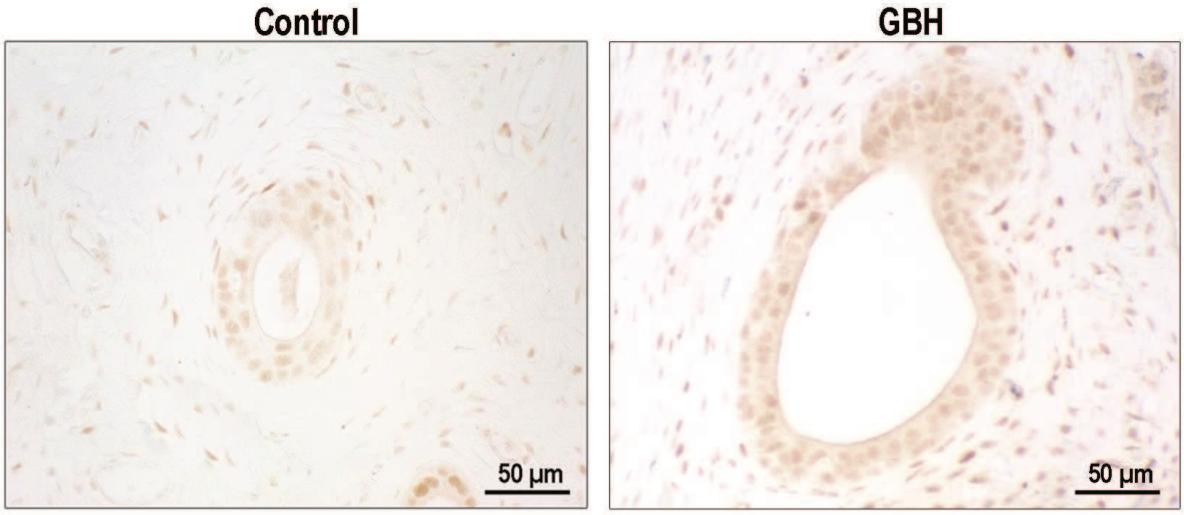

Figure 8. Effect of GBH on the uterine expression of estrogen receptor α (ESR1). Representative photomicrographs of ESR1 immunoreactions on the glandular epithelium of glands with squamous metaplasia.

With these results, we have finished a work, which previous results were published in Guerrero Schimpf, Milesi, **Zanardi** and Varayoud (Food and Chemical Toxicology 2022; 159:112695).

4.2. *EXPERIMENT II:* Effect of the subchronic exposure to a GBH on the uterus of adult rats fed with CAF diet

4.2.1. The addition of GBH maintained the high adiposity index induced by CAF diet

On PND 21, when rats were assigned to either the control diet (standard chow, Control group) or the CAF diet (CAF group), the average bw was similar across groups (Figure 9A). In contrast, on PND 140, the bw of CAF animals was higher than that of Control animals (Figure 9A). Finally, on PND 240, all experimental groups showed similar bw (Figure 9A), despite the energy intake in the CAF group was higher than that of the Control group (Figure 9B). However, CAF and CAF+GBH animals showed an increased adiposity index, expressed as the sum of perigonadal, retroperitoneal, and subcutaneous fat pads (Figure 9C).

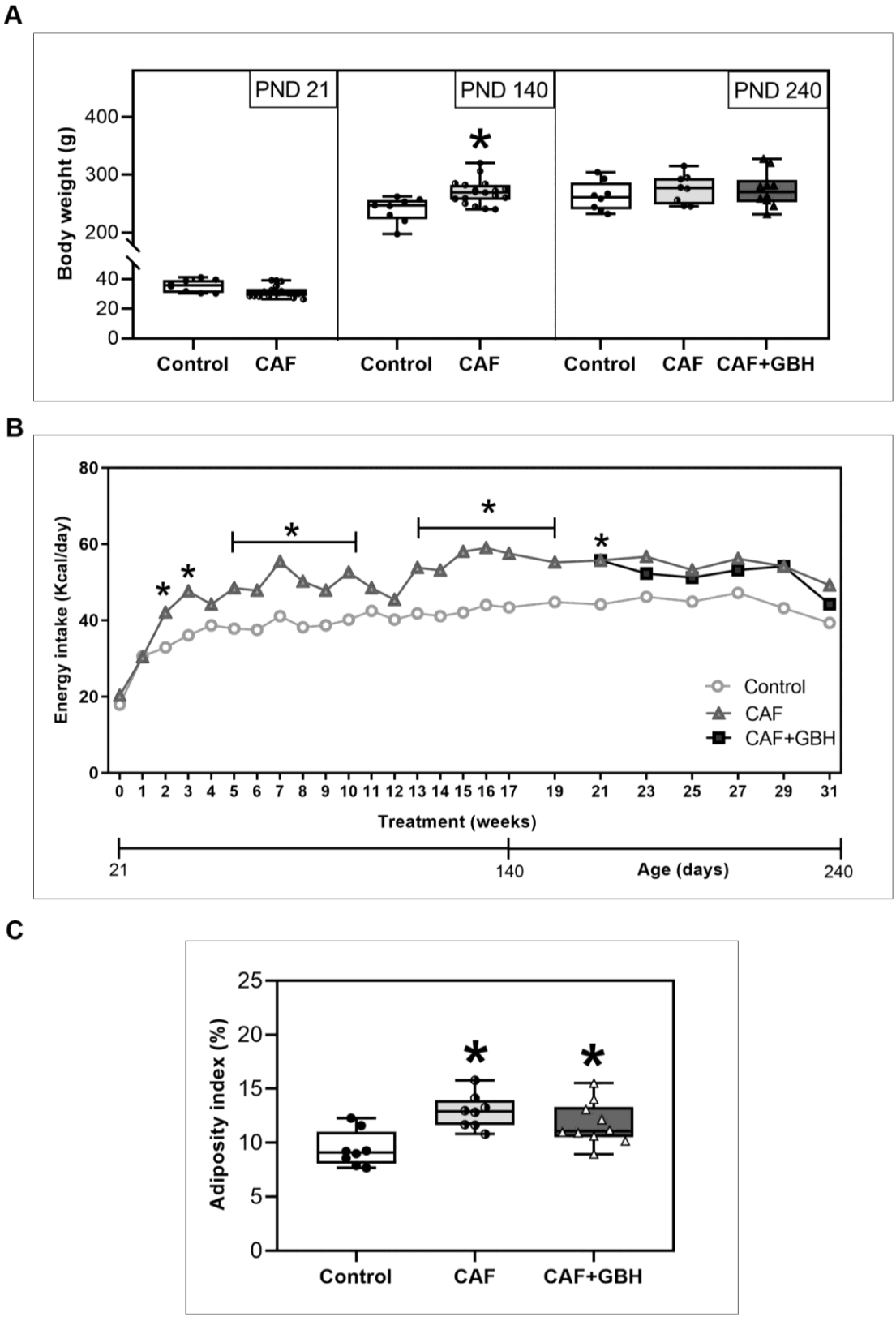

Figure 9. (A) Body weight of female rats on postnatal day (PND) 21, 140 and 240. (B) Average energy intake during the dietary treatment of Control, CAF and CAF+GBH rats. * indicates significant differences at $p < 0.05$, by Student's *t* test. (C) Quantification of adiposity index (expressed as the sum of perigonadal, retroperitoneal and subcutaneous fat

pads). Data are presented as median and interquartile range (n = 8-10 rats per group). *, $p < 0.05$ vs. the Control group (ANOVA followed by Tukey's post-hoc test).

4.2.2. The addition of GBH increased the progesterone levels

Among all parameters measured in serum samples, the levels of glucose were similar between Control, CAF and CAF+GBH rats (Control: 7.05 ± 0.20 mmol/L, CAF: 6.92 ± 0.12 mmol/L, CAF+GBH: 7.45 ± 0.18 mmol/L, $p = 0.08$; ANOVA followed by Tukey's post-hoc test). Regarding steroid hormones, no differences were detected in E2 and P4 levels from CAF and CAF+GBH animals compared to those in Control animals (Table 5). However, the serum levels of P4 in CAF+GBH animals were higher than those in the CAF group (Table 5).

Table 5. Hormonal serum levels of Control, CAF and CAF+GBH rats.

	Control	CAF	CAF+GBH	p-value
E2 (pg/ml)	13.50 ± 2.49	7.00 ± 1.00	14.13 ± 3.98	0.21
P4 (ng/ml)	5.75 ± 1.16	4.16 ± 0.94	13.32 ± 2.87$^{\#}$	0.02

Control, rats fed with standard chow diet; CAF, rats fed with cafeteria diet; CAF+GBH, rats fed with cafeteria diet plus glyphosate-based herbicide. The values are expressed as the mean ± SEM (n = 8-10 rats per group). $^{\#}$ $p < 0.05$ vs. the CAF group (Kruskal-Wallis followed by Dunn's post-hoc test).

4.2.3. The addition of GBH altered the glandular morphology inducing preneoplastic lesions

The thickness of SS was increased in the CAF+GBH group respect to the Control group (Table 6). To determine whether this change was associated with changes in the number of cells, we evaluated the uterine cell density. The density of stromal nuclei was increased not only in the CAF+GBH group but also in the CAF group, compared to the Control group (Table 6). The incidence of luminal epithelial hyperplasia, longitudinal myometrium thickness and the circular myometrium thickness were not different between groups (Table 6). However, an interesting observation was that the density of normal glands was higher in both CAF and CAF+GBH animals than in Control animals (Figure 10A and D).

Table 6. Uterine histomorphological parameters of Control, CAF and GBH-treated rats.

	Control	CAF	CAF+GBH	p-value
Incidence of luminal epithelial hyperplasia (%)	1/8	2/8	4/10	0.31
Subepithelial stroma (SS) thickness (µm)	320.4 ± 39.02	347.2 ± 48.55	518.8 ± 55.69*	0.03
Longitudinal myometrium thickness (µm)	66.6 ± 6.85	92.9 ± 10.71	101.6 ± 19.91	0.14
Circular myometrium thickness (µm)	116.7 ± 11.23	125.4 ± 12.42	152.5 ± 20.12	0.32
Nuclear density	25.96 ± 2.67	37.27 ± 3.04*	36.48 ± 2.69*	0.01

Control, rats fed with standard chow diet; CAF, rats fed with cafeteria diet; CAF+GBH, rats fed with cafeteria diet plus glyphosate-based herbicide. The values are expressed as the mean ± SEM (n = 8-10 rats per group). *, $p < 0.05$ vs. the Control group. The incidence of luminal epithelial cell hyperplasia was analyzed using Fisher's exact test; the thickness of SS and circular myometrium and the nuclear density were analyzed using ANOVA followed by Tukey's post-hoc test; the thickness of longitudinal myometrium was analyzed using Kruskal-Wallis followed by Dunn's post-hoc test.

Then, the analysis of altered glands (Figure 10A to D) showed that the density of glands with cellular anomalies, glands with daughter glands plus conglomerate of glands was increased in the CAF+GBH group compared to the Control group (Figure 10D). These results indicate that the combination of CAF and GBH exposure enhances the presence of these preneoplastic lesions.

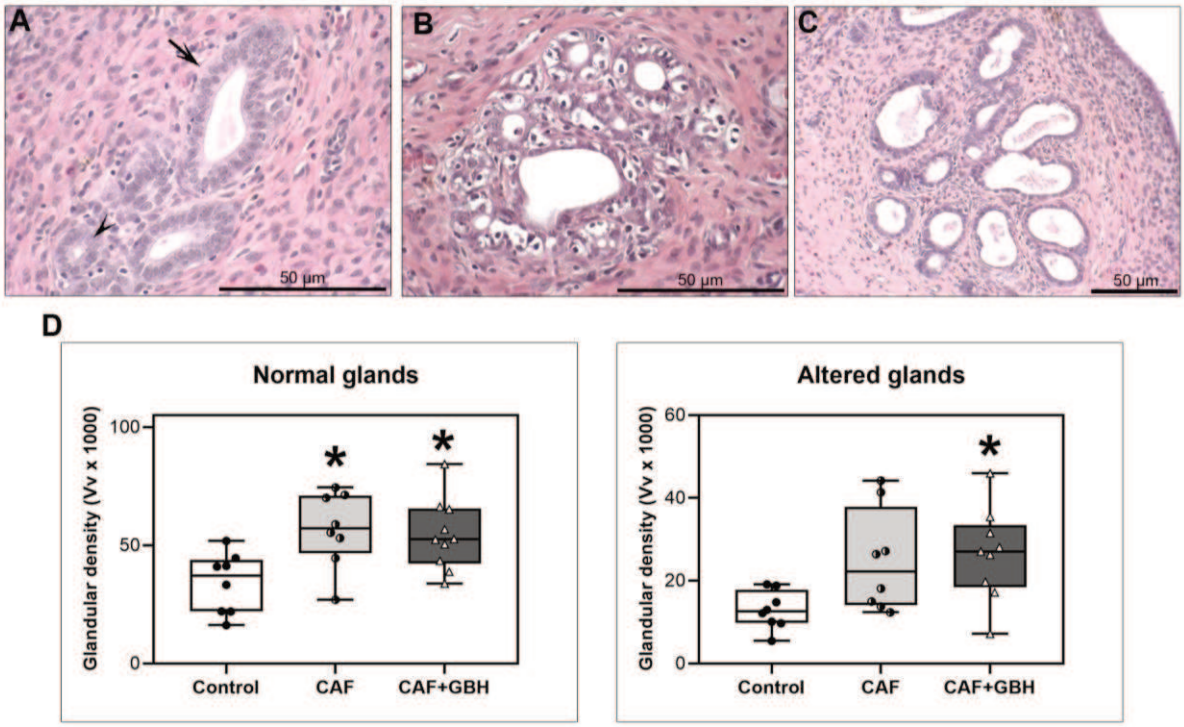

Figure 10. Effects of CAF and CAF+GBH on the uterine glandular epithelium. Representative photomicrographs showing different types of endometrial glands observed in adult rats. (A) Glands with cellular anomalies (arrow) and normal glands (arrowhead), (B) gland with daughter glands and (C) glands forming conglomerates. (D) Quantification of the density of normal and altered glands, expressed as Vv x 1000. Altered glands include glands with cellular anomalies, glands with daughter glands plus conglomerate of glands. Data are presented as median and interquartile range (n = 8-10 rats per group). *, $p < 0.05$ vs. the Control group. The density of normal glands was analyzed using ANOVA followed by Tukey's post-hoc test; the density of altered glands was analyzed using Kruskal-Wallis followed by Dunn's post-hoc test.

4.2.4. The addition of GBH did not alter the cell proliferation nor the ESR1 expression induced by CAF diet

To assess whether morphological alterations prompted by GBH and/or CAF diet were associated with changes in steroid hormone receptors, we evaluated the protein expression of ESR1 and PR. The CAF+GBH group showed an increase in the ESR1 expression in LE, SS and total glands (normal plus altered) compared to the Control group, whereas the CAF group only showed an increase in the SS and total glands (Figure 11). The expression of PR was not affected by CAF nor CAF+GBH in LE, SS and GE (Figure 11).

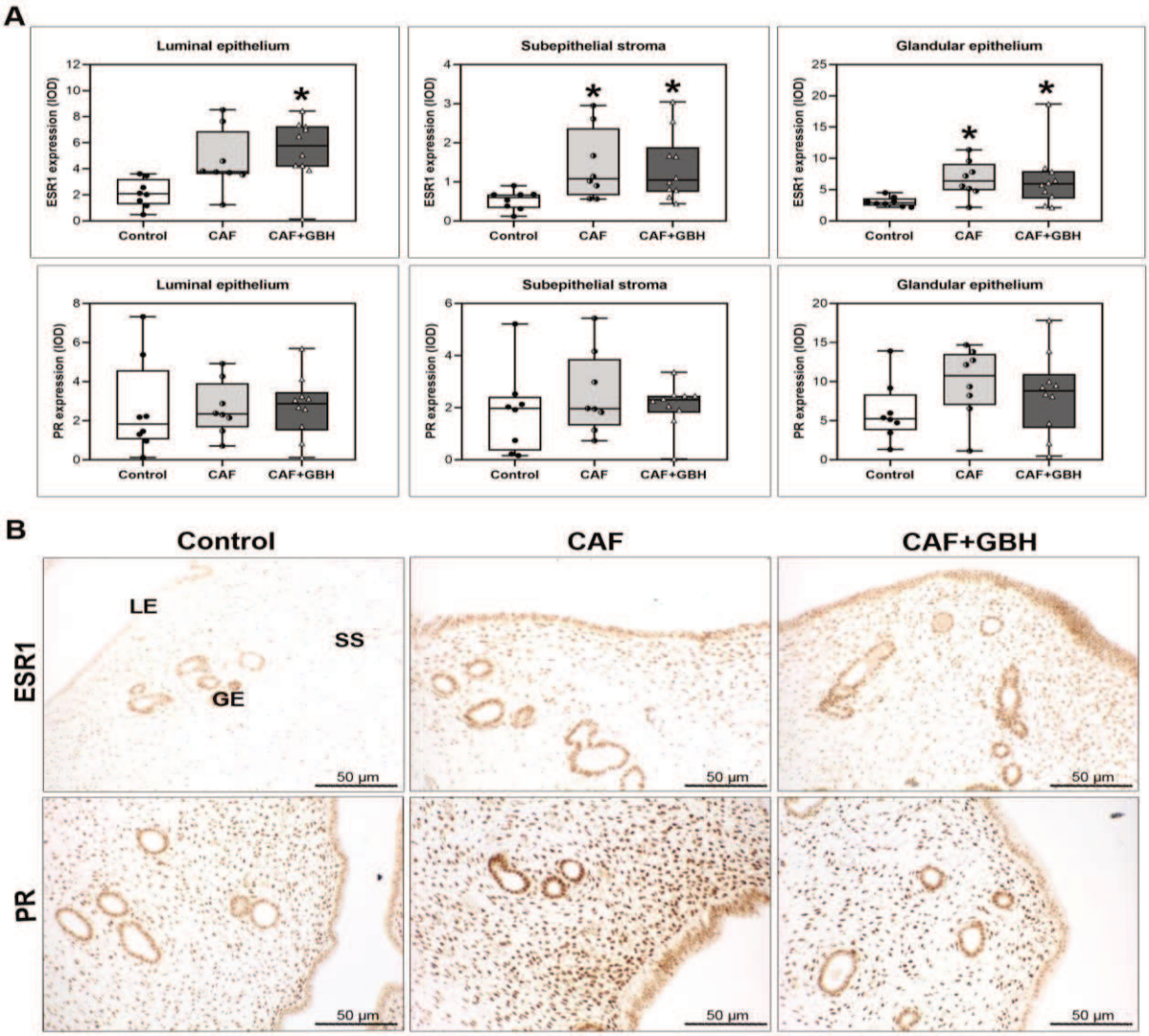

Figure 11. Effect of CAF and CAF+GBH on the uterine expression of estrogen receptor α (ESR1) and progesterone receptor (PR). (A) Quantification of ESR1 and PR expression in luminal epithelium, glandular epithelium of total glands (normal plus altered), and subepithelial stroma, expressed as integrated optical density (IOD). Data are presented as median and interquartile range (n = 8-10 rats per group). *, $p < 0.05$ vs. the Control group. The expression of ESR1 in LE, and the expression of PR in LE and SS were analyzed using ANOVA followed by Tukey's post-hoc test; the expression of ESR1 in GE and SS and that of PR in GE were analyzed using Kruskal-Wallis followed by Dunn's post-hoc test. (B) Representative photomicrographs of ESR1 and PR immunoreaction on uterine sections. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma.

Then, we evaluated if the treatments alter the cell proliferation. Both CAF and CAF+GBH groups showed an increased expression of Ki67 in the LE compared to the Control group (Figure 12). Furthermore, the proliferation index in the GE was higher in CAF group than in the Control group.

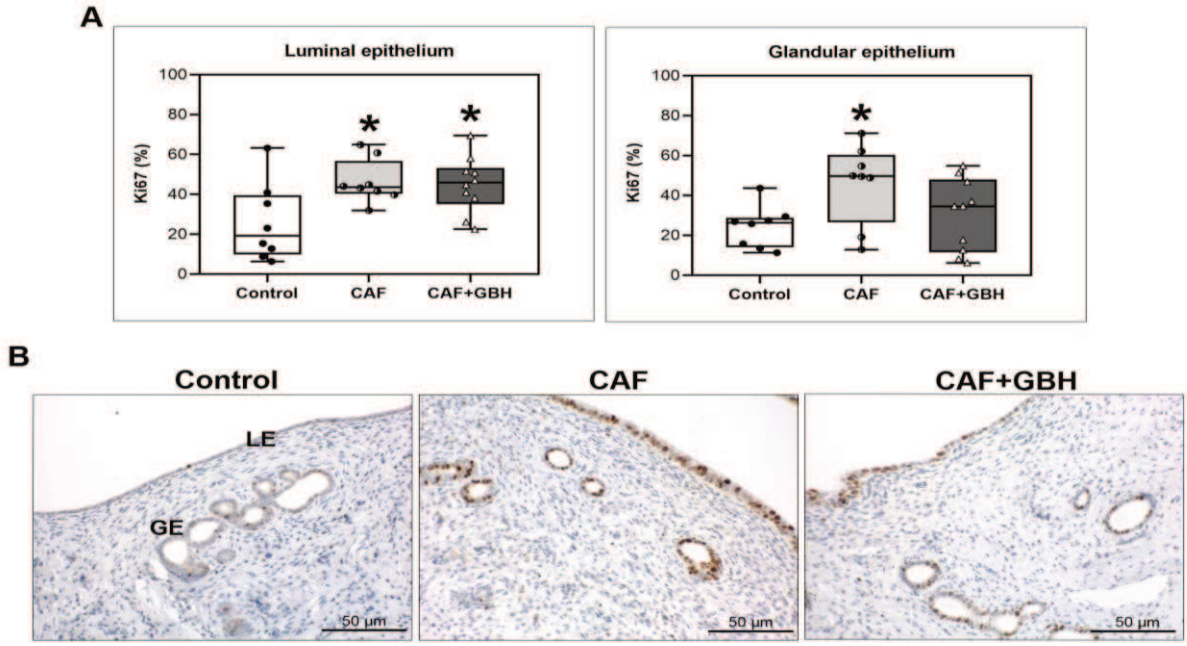

Figure 12. Cell proliferation by Ki67 immunodetection in the uteri from Control, CAF and CAF+GBH-treated rats. (A) Quantification of Ki67 expression in luminal epithelium and glandular epithelium of total glands (normal plus altered), expressed as percentage (%). Data are presented as median and interquartile range (n = 8-10 rats per group). *, $p < 0.05$ vs. the Control rats (ANOVA followed by Tukey's post-hoc test). (B) Representative images of immunohistochemical detection of Ki67 on uterine sections. LE, luminal epithelium; GE, glandular epithelium.

4.2.5. The treatment with CAF+GBH reduced the expression of PTEN and p27

The expression of both p27 and PTEN was lower in the SS of CAF+GBH animals than in that of Control animals (Figure 13). No differences were detected in the SS of CAF animals and Control animals. The expression of p27 and PTEN in the LE and GE was similar between all experimental groups (Figure 13).

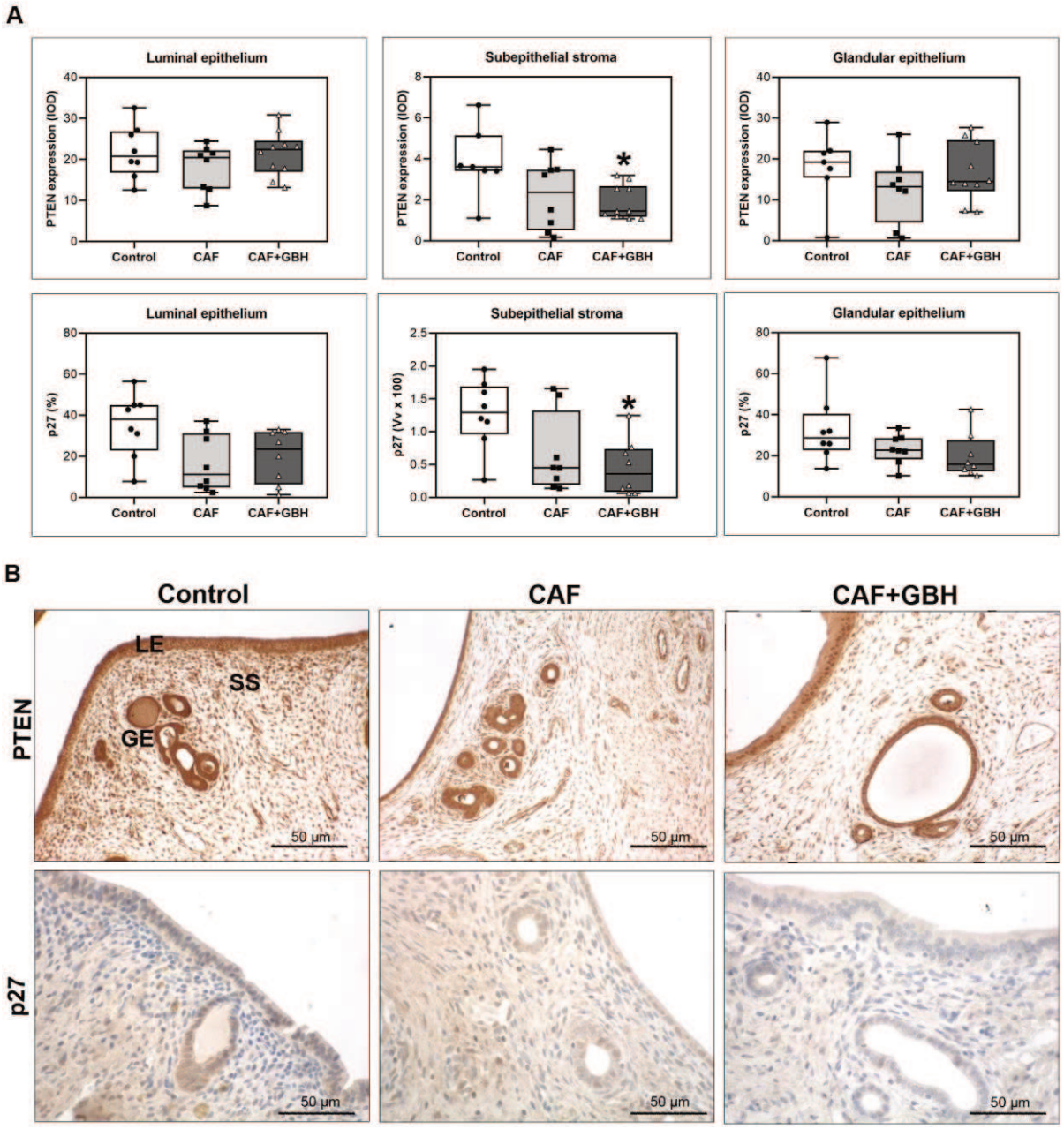

Figure 13. PTEN and p27 protein expression in the uteri from Control, CAF and CAF+GBH-treated rats. (A) Quantification of PTEN and p27 expression in luminal epithelium, glandular epithelium of total glands (normal plus altered) and subepithelial stroma. Values are expressed as integrated optical density (IOD), percentage (%) or Vv x 100. Data are presented as median and interquartile range (n = 8-10 rats per group). *, $p < 0.05$ vs. the Control rats (ANOVA followed by Tukey's post-hoc test for PTEN in all evaluated compartments and for p27 in LE and SS; Kruskal-Wallis followed by Dunn's post-hoc test for p27 in GE). (B) Representative images of immunohistochemical detection of PTEN and p27 on uterine sections. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma.

4.2.6. Protein expresion in normal and altered glands

Then, we evaluated if the morphological alterations in the uterine glands prompted by CAF and GBH were associated with changes at a protein level. In normal glands, the cell proliferation was higher in the CAF group than in the Control group (Table 7). In altered glands, the expression of ESR1 was higher in CAF and CAF+GBH animals than in Control animals (Table 7).

Table 7. Protein expression and its distribution in different types of uterine glands from Control, CAF and CAF+GBH-treated rats.

Type of gland	Control	CAF	CAF+GBH	p-value
	ESR1 (IOD)			
Normal glands	3.47 ± 0.41	6.40 ± 1.09	7.51 ± 1.62	0.06
Altered glands	2.95 ± 0.39	7.22 ± 1.31**	6.36 ± 1.38*	0.01
	PR (IOD)			
Normal glands	5.34 ± 0.95	11.06 ± 1.86	8.33 ± 1.48	0.05
Altered glands	7.26 ± 2.08	9.30 ± 1.59	8.68 ± 1.90	0.76
	Ki67 (%)			
Normal glands	20.26 ± 2.76	45.06 ± 7.12*	29.92 ± 5.59	0.02
Altered glands	31.65 ± 6.24	52.77 ± 6.52	31.03 ± 6.10	0.05
	p27 (%)			
Normal glands	36.51 ± 6.76	23.07 ± 2.77	19.80 ± 2.39	0.06
Altered glands	29.5 ± 5.89	25.55 ± 2.92	20.18 ± 3.88	0.19

Control, rats fed with standard chow diet (n=8); CAF, rats fed with cafeteria diet (n=8); CAF+GBH (n=10), rats fed with cafeteria diet plus glyphosate-based herbicide, ESR1, estrogen receptor α; PR, progesterone receptor. Altered glands include glands with cellular anomalies, glands with daughter glands and conglomerate of glands. Values are expressed as integrated optical density (IOD) or percentage (%). *, $p < 0.05$ and **, $p < 0.01$ vs. the Control group (ANOVA followed by Tukey's post-hoc test for PR and Ki67 expression and Kruskal-Wallis followed by Dunn's post-hoc test for ESR1 and p27 expression).

5. Discussion

In the present Chapter, we aimed to evaluate the uterine effects of a low dose of GBH in rats, by using different times and routes of exposure. We were focused on growing and endometrial carcinogenesis-related process. In the *EXPERIMENT I*, we determined the long-term effects of GBH on the uterus, at molecular level, of aging rats exposed during the first week of life. In the *EXPERIMENT II,* we evaluated whether the subchronic administration of a GBH during adulthood impacts on the uterine effects induced by CAF diet. The route of administration selected was different between *EXPERIMENT I* and *II*. We chose the subcutaneous via for *EXPERIMENT I* because it is the only administration route that warrants the incorporation of a chemical compound in newborn pups (Guerrero Schimpf et al., 2017; Milesi et al., 2012). In *EXPERIMENT II*, we selected the oral via because it is the most representative of natural exposure in humans. Indeed, a previous work proved that the effect of GBH on the uterus does not depend on the route of administration since similar responses were observed between the oral and subcutaneous via, at least in the same organ of study (Alarcón et al., 2019).

For ***EXPERIMENT I,*** we selected a model of exposure during the first week of life, a period which has already been proved to be highly susceptibility to hormonal and chemical challenge for reproductive organs (Altamirano et al., 2018; Guerrero Schimpf et al., 2017; Ingaramo et al., 2020; Monje et al., 2009; Guerrero Schimpf et al., 2018). During the first week of life, the uterus continues with its development and differentiation processes, being a time "sensitive window" to EDC exposure (Ingaramo et al., 2016, 2020; Luque et al., 2018; Newbold et al., 2009; Varayoud et al., 2014). The exposure to certain chemicals during this critical period of differentiation can cause uterine disorders later in life, including endometrial hyperplasia and even endometrial cancer (Milesi et al., 2021; Rochester, 2013; Varayoud et al., 2014; Walker, 2011).

The idea that disease in adulthood may have an etiology that arises in prenatal and early neonatal life is not exclusive to the field of hormone-dependent cancer (González-Casanova et al., 2020; Newbold et al., 2009). Many researchers have demonstrated that perinatal exposure to certain environmental chemicals may provoke the global epidemic of obesity (Darbre, 2017; González-Casanova et al., 2020; Plagemann, 2005; Yilmaz et al., 2020). Concerning glyphosate, a previous work found induction of obesity in the second and the third generation of female rats exposed during gestation, showing a transgenerational obese phenotype in almost a half of the glyphosate lineage (Kubsad et al., 2019). However, we failed to find changes in the bw between animals exposed to vehicle and GBH, thus our current results do not support the hypothesis that glyphosate could act as environmental

obesogen. Other works also found no changes (Çağlar & Kolankaya, 2008; Milesi et al., 2018) in the bw of animals exposed to glyphosate or GBH, or even found a reduced bw gain (Beuret et al., 2005; Ren et al., 2018). Thus, it would be interesting to perform more studies evaluating the role of GBH as an environmental obesogen.

The development of the uterus is tightly regulated by fluctuations in the levels of the ovarian steroid hormones (Dixon et al., 2014; Freeman, 2006) and an imbalance in their actions may cause uterine lesions, including endometrial hyperplasia and cancer (Dixon et al., 2014; Sanderson et al., 2016). In rodents and humans, the increased serum E2/P4 ratio is considered a risk factor for endometrial carcinogenesis (Dixon et al., 2014; Yoshida et al., 2015). In relation to glyphosate effects, previous works reported that perinatal exposure induces steroidogenesis disruption (Lorenz et al., 2020; Ren et al., 2018). Thus, we planted to evaluate whether neonatal exposure to GBH impacts on steroidogenesis in aging rats. We found no changes in the E2 and P4 levels at the moment of the sacrifice of animals. However, GBH treatment induced an almost two-fold increase in the E2/P4 ratio earlier in life (PND 120) in these animals (Guerrero Schimpf et al., 2022). This result could be a possible mechanism underlying the long-term adverse effects detected in the uterus of GBH-exposed rats (Guerrero Schimpf et al., 2022).

We have demonstrated that our model of short exposure to GBH induces preneoplastic lesions in aging rats, reflected by an increased incidence of glands with daughter glands (Guerrero Schimpf et al., 2022). Other alterations in the glandular morphology of these animals were observed, including glands with cellular anomalies, glands with squamous metaplasia and cystic glands (Guerrero Schimpf, 2018). The presence of these alterations in the morphology of glands is key factors defining the risk for progression to carcinoma (Chandra et al., 2015; Travaglino et al., 2020). Prompted by the alterations found in the glandular morphology, we decided to evaluate if these alterations were accompanied by molecular changes involved in endometrial carcinogenesis. We evaluated the protein expression of Ki67 as a proliferative marker and that of steroid hormone receptors in the epithelium of altered glands. Despite no changes in the incidence of glands with squamous metaplasia were found between Control and GBH-exposed rats, we observed an increased expression of ESR1 without changes in cell proliferation in this type of altered gland. According to this, in the mammary glands of these animals, we also observed an increased ESR1 expression without changes in cell proliferation, in moderate plus florid hyperplastic ducts (Zanardi et al., 2020). Both glands with squamous metaplasia and hyperplastic ducts are considered preneoplastic lesions (Gunin et al., 2001; Singh et al., 2000). In accordance with us, female rats exposed neonatally to diethylstilbestrol, a well-known xenoestrogen, developed endometrial hyperplasia without changes in cell proliferation between normal

endometrium and hyperplastic endometrium (McCampbell et al., 2008). In addition, in aging rats, the uterine ESR1 expression was similar between animals developing various proliferative lesions and even endometrial cancer (Yoshida et al., 2012). As it is evident by the mentioned results, some preneoplastic lesions can be detected without a simultaneous increase in the proliferation. Considering that animals are aging, we can speculate that the glandular cells have proliferated before the moment of sacrifice leading to these preneoplastic lesions.

Overall, with the *EXPERIMENT I* we could demonstrate the involvement of ESR1 in uterine preneoplastic lesions of animals exposed to a low dose of GBH. The disruption of uterine ESR1 expression due to the glyphosate exposure was previously evaluated by using different experimental models, associated or not with fertility failures or endometrial carcinogenesis (Alarcón et al., 2020; Guerrero Schimpf et al., 2017; Ingaramo et al., 2016, 2017; Lorenz et al., 2019, 2020; Varayoud & Durando et al., 2017). In addition, Fu et al. (2021) demonstrated that GBH alters the hypothalamic-pituitary-ovarian axis hormones and produces damage in the uterus of piglets with altered oxidative stress and antioxidant system, both processes involved in carcinogenesis. Despite the aforementioned studies showing a potential endocrine disruptive effect related to uterine carcinogenesis, GBH is still the most widely used herbicide in the world.

Although many studies proposed to evaluate the effects of glyphosate on uterine carcinogenesis, the potential interaction with other environmental factors could not be considered. Given the prevalence of GBH in our environment, interactions between GBH and other exogenous exposures are highly possible (Barnett & Gibson, 2020). Thus, understanding GBH co-exposure effects with other factors is critical for understanding the real world risks associated with GBH exposure. In such context, CAF diet reflects the variety of highly palatable and energy-dense foods prevalent in Western society (Lalanza & Snoeren, 2021), where the incidence of endometrial hyperplasia is about 200,000 new cases per year (Chandra et al., 2015). Since endometrial hyperplasia might be related to environmental factors, including unhealthy diet and chemical exposure (Moore & Brewer, 2017; Sanderson et al., 2016; Si et al., 2017; Yoshida et al., 2015), we consider that, to improve our understanding of the etiology of uterine disorders, it is necessary to evaluate the combination of risk factors (Moore & Brewer, 2017; Pronk et al., 2004; Si et al., 2017). In a previous study, we have detected that CAF diet induces endometrial hyperplasia in female Wistar rats fed from weaning to adulthood (Gastiazoro et al., 2018). Thus, the aim of ***EXPERIMENT II*** was to define whether the exposure to GBH added to CAF may exacerbate the effects induced by CAF alone on the rat uterus. Our model of CAF diet increased the bw of adult female rats, in agreement with that previously reported (Chen et al., 2014; da Costa

Estrela et al., 2015; Gastiazoro et al., 2018; Lalanza et al., 2014; Palframan & Myers, 2016). This result on its own is unsurprising given the high energy density and the palatability of the foods included in the CAF diet. The increased bw induced by the CAF diet was found on PND 140, but later, at PND 240, neither the CAF nor the CAF+GBH group showed differences in the bw in comparison with the Control group. These results related to the CAF diet are similar to those of Palframan & Myers (2016), who found an increased bw after three months of treatment with CAF diet, and a later dissipation of this effect. Regarding GBH exposure, this result is not correlated with the hypothesis that environmental compounds could act as obesogens (Darbre, 2017; González-Casanova et al., 2020; Yilmaz et al., 2020). The lack of effect on bw has also been observed in rats co-treated with the EDC bisphenol A and a low-protein diet, at similar age (Varuzza et al., 2019). Perhaps the two factors here studied in combination (i.e. unhealthy diets and possible obesogens) behave like antagonists in this endpoint. Despite the similar bw found at PND 240 between all groups, CAF and CAF+GBH animals exhibited higher adiposity index than Control animals. Similar results have been reported in rodents fed with CAF diet (Andreoli et al., 2016; da Costa Estrela et al., 2015; Gastiazoro et al., 2018; Lalanza et al., 2014; Lazzarino et al., 2017, 2019) and co-treated with high fat diet and bisphenol A or the pesticide imidacloprid (Stoker et al., 2020; Sun et al., 2017). Our data indicate that CAF diet is the main responsible for the increased adiposity index both in CAF and CAF+GBH animals, since no additional differences are raised after GBH exposure. The importance of focusing the study not only in bw but also in the adiposity index is based on the link between adipose tissue and endometrial carcinogenesis. Adiposity increases the peripheral conversion of androgens, resulting in high levels of estrogens. This estrogenic microenvironment is considered a risk factor for hormonal carcinogenesis (Dixon et al., 2014; Michalczyk et al., 2021).

Prompted by these results, we next focused on the secretion of steroid hormones. The levels of P4 were higher in CAF+GBH animals than in CAF ones. Thus, the addition of GBH affected the P4 level or the combination of both factors had an impact on this parameter. Following the first of the mentioned theories, a previous study has shown that GBH interferes with the ovarian function by stimulating the production of P4 and inhibiting that of E2 in granulosa cells, a fact that could result in follicular atresia (Gigante et al., 2018). The altered steroidogenesis induced by GBH is also associated with altered aromatase activity (Richard et al., 2005). However, we cannot rule out that the addition of GBH in animals fed with CAF diet can alter steroidogenesis by directly affecting the ovarian tissue or indirectly interfering with the ovarian function, by acting on the hypothalamic-pituitary-gonadal axis (Serra et al., 2021), which is central for uterine function. On the other hand, since the coordinated actions of E2 and P4 regulate the proliferation and differentiation of uterine cells,

an imbalance in the E2 and P4 actions may cause uterine disorders (Dixon et al., 2014). In this sense, P4 is involved in the development of uterine fibroid (Kim et al., 2013a) and an increased E2/P4 ratio has been associated with the pathogenesis of endometrial carcinogenesis (Sanderson et al., 2016). In this PhD thesis, we did not detect changes in the E2 levels, at least at the moment of sacrifice of animals. We could propose that alterations in the E2/P4 ratio could occur before the end of the experiment, affecting the normal endocrine response of uterine cells.

In the uterus, epithelial-stroma interactions are responsible for physiological functions and the emergence of several lesions. Endometrial hyperplasia is an uterine lesion representing a spectrum of morphological and molecular endometrial alterations (Chandra et al., 2015; Travaglino et al., 2020). This pathology is characterized by abundant cellular stroma and abnormal cell proliferation accompanied by high ESR1-expressing cells in the epithelium and stroma (Kreizman-Shefer et al., 2014; Masjeed et al., 2017; Sanderson et al., 2016; Travaglino et al., 2020; Yoshida et al., 2012). Such changes were found in both CAF and CAF+GBH animals. Specifically, we observed that the densities of stromal nuclei and normal glands were increased and that the cell proliferation in the epithelium and ESR1 expression in the epithelium and stroma were also increased. Similar results were observed by us in adult rats treated with the CAF diet (Gastiazoro et al., 2018). Based on our present and previous results, the CAF diet was apparently responsible for inducing endometrial hyperplasia in CAF and CAF+GBH animals. In addition, the co-treatment with CAF+GBH increased the SS thickness. Thus, the addition of GBH could be the responsible for this morphological alteration, as was previously observed in animals treated with this herbicide alone (Guerrero Schimpf et al., 2017; Ingaramo et al., 2019). Overall, these alterations detected here might promote the development of endometrial cancer (Sanderson et al., 2016).

The importance of glands in relation to preneoplastic and neoplastic lesions has been extensively described. All forms of hyperplasia share certain morphological features, showing an irregularity in both gland shape and size (Gunin et al., 2001; Sobczuk & Sobczuk, 2017). These glandular alterations are key factors defining the risk for progression to carcinoma (Chandra et al., 2015; Travaglino et al., 2020). Previous works have reported the presence of glandular abnormalities in rodents exposed to environmental chemicals, including GBH (Bosquiazzo et al., 2013; Ferreira et al. 2020; Guerrero Schimpf et al., 2022; Vigezzi et al., 2016, 2015), as well as in rodents fed with different types of unhealthy diet (Nakai et al., 2005; Shang et al., 2017). Therefore, we evaluated whether the CAF diet alone and in combination with GBH could alter the morphology of glands, a histological feature of endometrial hyperplasia. We observed alterations in the uterine gland morphology of all

experimental groups. Thus, these altered structures could be a consequence of the normal female aging process in rats (Bosquiazzo et al., 2013; Vigezzi et al., 2015, 2016). However, we also detected that CAF+GBH increased the altered gland area, specifically in glands with cellular anomalies, glands with daughter glands plus conglomerate of glands, in comparison with the Control animals. This allowed us to conclude that the combination of the CAF diet and GBH increases the development of preneoplastic lesions. In agreement with this observation, although in a different tissue, it has been shown that GBH added to a high fat diet had worse adverse effects associated with jejunum inflammation than the single treatments (Panza et al., 2021). Other studies have also found that the co-treatment with bisphenol A and unhealthy diet increases the susceptibility to mammary carcinogenesis (Leung et al., 2017; Varuzza et al., 2019) and alters the male reproductive function (Tarapore et al., 2017). In summary, our results and the mentioned studies indicate that the exposure to environmental compounds coupled with a non-healthy lifestyle worsens the effects of the threats to which we are habitually exposed.

Some endometrial hyperplasias can progress to malignancy (Sanderson et al., 2016). A known molecule used as a prognostic marker to determine the risk of endometrial hyperplasia progression is the tumor suppressor gene PTEN (Mutter, 2000b). The loss of PTEN function represents an early event in endometrial carcinogenesis (Prat et al., 2007). Here, CAF+GBH animals, but not CAF animals, showed a reduction in PTEN expression, specifically in the SS of the endometrium. The involvement of PTEN in carcinogenic process has been observed not only in the uterus but also in other hormone-dependent tissues (Gao et al., 2019; Mutter et al., 2000a,b; Wise et al., 2017; Zhang et al., 2022). In this regards, in a previous study in female rats, bisphenol A predisposed to thyroid tumors and the addition of di-(2-ethylhexyl) phthalate, a probable human carcinogen, enhanced the effects of bisphenol A on cancer promotion by inhibiting PTEN (Zhang et al., 2022). In contrast, some natural compounds, such as curcumin and ginseng, have protective effects against bisphenol A or phthalate by reversing the reduced PTEN expression (Saadeldin et al., 2018; Sonavane & Gassman, 2019).

Several studies have reported that PTEN blocks the cell proliferation by inhibiting the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway (Kim et al., 2013b; Pieczyńska et al., 2011; Sun et al., 2001; Vivanco & Sawyers, 2002). The loss of PTEN and subsequent AKT activation results in the activation of ESR1-dependent pathways associated with a possible induction of endometrial carcinogenicity. Interestingly, the PTEN/PI3K/AKT signaling pathway can also be activated by estrogen, suggesting a complex interaction between these two signaling pathways (Joshi & Ellenson, 2017; Kim et al., 2013b; Mutter, 2000b; Pieczyńska et al., 2011; Prat et al., 2007; Saito et al., 2011). In agreement with this,

the decreased expression of PTEN in CAF+GBH animals was coupled with an increased cell proliferation and ESR1 expression. Therefore, we can speculate that the proliferative event found in these animals may be a consequence of PTEN loss, whereas PTEN loss may lead or be a consequence of ESR1 increases. However, the loss of PTEN expression would explain the increased cell proliferation and ESR1 expression in CAF+GBH animals but not in CAF animals, suggesting the involvement of other pathways in CAF animals.

The PTEN gene may act as a suppressor of tumor activity by the activation and increased expression of p27 (An et al., 2002). A positive correlation between PTEN and p27 protein expression has been observed in endometrial hyperplasia and endometrial cancer, supporting the idea that p27 might be the downstream target of PTEN in these pathologies (Erkanli et al., 2006). p27 is a down-regulator of cell proliferation (Zheng et al., 2018). Thus, the reduction of p27 indicates that, in CAF+GBH animals, one of the brakes of proliferation is down-regulated. Previous studies have demonstrated that p27 could act as a tumor suppressor and that its protein expression decreases as the severity of the endometrial lesion progresses from endometrial hyperplasia to cancer (Kacar Özkara & Corakci, 2004). Thus, the loss of p27 expression is an important step in the process of endometrial carcinogenesis (Erkanli et al., 2006). Finally, as we observed with PTEN protein expression, the endometrial hyperplasia found in CAF+GBH animals was accompanied by a reduced p27 expression, indicating the exacerbated effect of both treatments. Thus, the addition of GBH reduced the p27 and PTEN protein levels, which might indicate a poor prognosis of the endometrial hyperplasia. However, further studies are needed to evaluate the molecular mechanistic effects of the interaction between these factors on the uterine pathologies.

Taking together all the results of *EXPERIMENT II*, we reinforced the findings that CAF diet induces endometrial hyperplasia (Gastiazoro et al., 2018), and provided evidence that the addition of GBH increases the effects of CAF diet alone. The co-treatment with CAF+GBH increased glandular abnormalities, SS thickness and reduced the expression of key proteins such as PTEN and p27. These results indicate that the interaction between unhealthy diet and environmental chemicals such as GBH could predispose to uterine pathological disorders.

CHAPTER II: Assessment of estrogenic effect of hops extract: *in vivo* and *in vitro* studies

1. Introduction

1.1. Botanical dietary supplements for women's health

Menopause is the time of life when menstrual cycles cease, and it marks the end of the woman's reproductive capacity (Dunneram et al., 2019; Minkin, 2019). Menopause is caused by reduced secretion of the ovarian hormones E2 and P4, which occurs when the pool of ovarian follicles becomes depleted (Bruce & Rymer, 2009; Nelson et al., 2005).

Many symptoms are attributed to menopause, as for example hot flashes, night sweats, vaginal dryness and/or insomnia (Dennerstein et al., 2000). To alleviate these symptoms and to protect women against estrogen-deficiency, the HRT used to be the primary treatment option (Angioli et al., 2018). However, its use is highly debated after the WHI study, which found associations between the use of HRT during a long time and an increased risk of developing hormone-dependent cancer (D'Alonzo et al., 2019). The update of WHI trial outcomes with extended post-intervention follow-up of 13 years to those postmenopausal women found that combined therapy (estrogen plus progestin) increases the risk of developing breast cancer (Chlebowski et al., 2013; Manson et al., 2013) and that estrogens alone use increases the risk of developing endometrial cancer (Beral et al., 2005). Thus, the risk of endometrial cancer increases with enhanced exposure to estrogen, which often results from hormone therapy (Beral et al., 2005). Because of its potential carcinogenic effect, many women have turned to herbal remedies (Moore et al., 2017), making these botanical dietary supplements (BDS) increasingly popular (Dietz et al., 2016; Smith et al., 2018). Among the BDS traditionally used to treat menopausal symptoms, we can mention black cohosh, valerian, isoflavons containing in soy and red clover, hops extracts, locorice, rhubarb, chasteberry, and alfalfa (Dietz et al., 2016).

1.1.1. Hops extract

Many herbal treatment protocols for menopausal symptoms contain hops (Humulus lupulus L.), an herbaceous climbing plant in the family of Cannabaceae (Aghamiri et al., 2016). Hops is native to central Europe, but today it is naturalized throughout the northern temperate regions, such as Asia and North America (Bocquet et al., 2018). The use of hops for the production of dietary supplements to treat menopausal symptoms is obtained from the female hop cones and is currently marketed, either as a single botanical or in combination with other botanicals (Krause et al., 2014; Tronina et al., 2020). In addition to its use as a "natural" alternative to HRT, hops is mainly used in beer brewing as preservative and

flavoring agent and as one of the ingredients of herbal sedative/sleeping pills (Aghamiri et al., 2016; Bolton et al., 2019; Keiler et al., 2013).

The use of hops as an alternative to HRT is due to the presence of the estrogenic compound 8-prenylnaringenin (8-PN), which is responsible for the reduction of menopausal symptoms (Bolton et al., 2019; Dietz et al., 2017). One of the most commonly experienced menopausal symptoms is hot flashes (Nelson, 2008). In a rat model of menopausal hot flashes, the administration of 8-PN, similar to E2, was able to restore the temperature into the normal range (Bowe et al., 2006). The effectiveness to reduce hot flashes was also observed in estrogen-deficient ovariectomized rats treated with a hops extract having a high content of 8-PN (Ban et al., 2018) and with a combination of hops and red clover extracts (Kim et al., 2020). In addition to the mentioned studies, hops extract alleviated menopausal symptoms in menopausal women by randomized, placebo-controlled clinical trials (Aghamiri et al., 2016; Erkkola et al., 2010; Heyerick et al., 2006).

- **Metabolism of hops extract**

Extracts from spent hops are composed of 8-PN, xanthohumol (XH), isoxanthohumol (IX), and 6-prenylnaringenin (6-PN). The proportion of the compounds in hop cones is very variable and depends on many factors including variety, climatic conditions, cultivation area, and storage conditions (Jelínek et al., 2010). Also, the amount of each compound can change due to the conversions during drying and brewing in the extract, and then in the organism (Bolton et al., 2019). By using ovariectomized rats treated with hops extract for 8 and 20 weeks, Keiler et al. (2017a,b) have shown that despite the amount of 8-PN in the extract was 100 times lower than that of XH, 8-PN had the highest serum concentration, followed by XH.

The major component of hops extract is XH, which has several anti-carcinogenic properties, shown *in vitro* at key stages of the carcinogenic process including initiation, promotion and progression phases (Cho et al., 2008; Gerhauser et al., 2002). Like the other hops compounds, XH is biochemically convertible in the organism. As shown in Figure 14, the pharmacokinetics of pure XH, as well as XH containing the extract, have been previously studied (Bai et al., 2022; Jirásko et al., 2010; Keiler et al., 2017b; Legette et al., 2012, 2014; Nookandeh et al., 2004; Nowak et al., 2020). In rats, the bioavailability of XH is low and dose-dependently with rapid absorption, metabolism and elimination (Bai et al., 2022; Legette et al., 2012). The concentration-time curve of XH exhibits a double-peak phenomenon with high concentrations at 0.5-2 h and 8-12 h postprandially (Bai et al., 2022; Legette et al., 2012). Similar response was observed in rats treated with XH containing the extract (Nowak et al., 2020). The double-peak response evidences both small and large

intestinal absorption as well as a possible enterohepatic recirculation (Bai et al., 2022; Legette et al., 2012). Xanthohumol is converted into IX, 8-PN, 6-PN and desmethylxanthohumol (DMX), which evidences that XH undergo phase I and II metabolism (Bai et al., 2022; Legette et al., 2012). The major metabolite of XH is IX and, as for XH, the concentration-time curve of IX exhibits a double-peak phenomenon (Bai et al., 2022; Legette et al., 2012). The concentration-time curve of 8-PN shows high plasma concentrations at later time point. The concentration-time curves of XH, IX, and 8-PN analyzed in rats (Legette et al., 2012), in addition to studies performed in cell lines, suggest that XH is converted into its flavanone isomer IX in the stomach and then, IX can be O-demethylated by hepatic CYP1A2 or by gut microflora enzymes to form 8-PN (Legette et al., 2012; Nikolic et al., 2005; Possemiers et al., 2005, 2008). On the other hand, XH can be directly metabolized to DMX, which is later converted into either 6-PN or into 8-PN (Bai et al., 2022; Legette et al., 2012). Thus, some XH effects may be trigger by 8-PN exposure or vice versa (Liu et al., 2015). Due to the multiple biotransformation of XH, the biological effect of 8-PN depends on its dose in the extract and its conversion from XH. Therefore, it is necessary to consider the amount of XH consumed, which is the main source of phytoestrogens in the diet (Liu et al., 2015; Tronina et al., 2020).

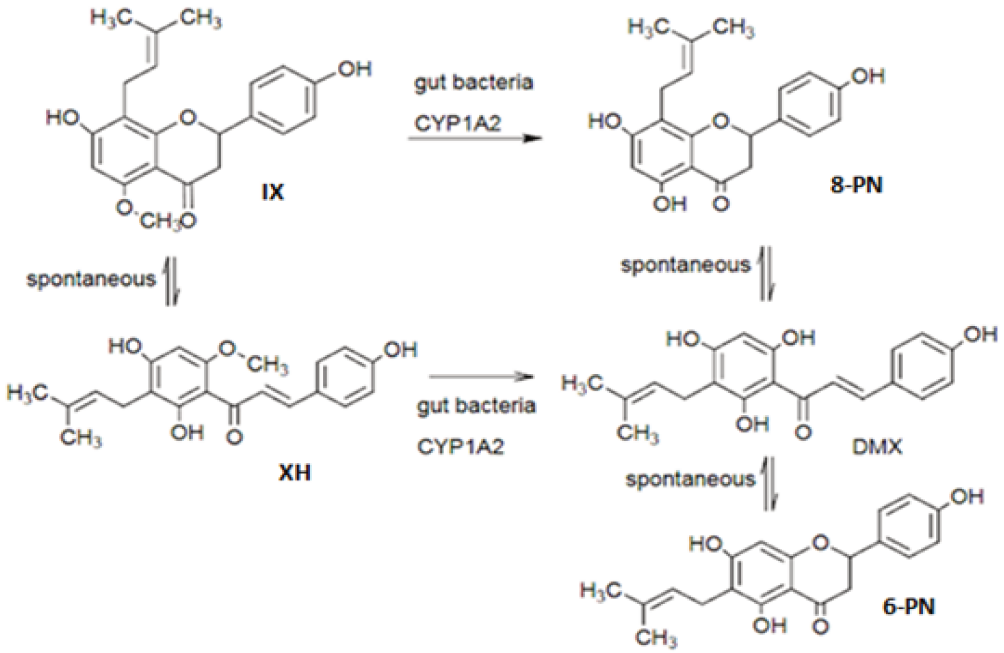

Figure 14. Pathway for xanthohumol (XH) metabolism and production of its metabolites: isoxanthohumol (IX), 6-prenylnaringenin (6-PN), 8-prenylnaringenin (8-PN), and desmethylxanthohumol (DMX). The figure was taken and modified from Legette et al. (2012).

Xanthohumol is mainly excreted as XH followed by IX, 8-PN and 6-PN, and the main excretion route of XH and its metabolites is through the feces and less through the urine (Avula et al., 2004; Bai et al., 2022; Nookandeh et al., 2004). The metabolism of XH and IX and the formation of 8-PN by metabolism were confirmed in the feces of rats fed with hops extracts (Jirásko et al., 2010). Similar to the pharmacokinetic in rats, the absorption pattern of XH in human is low, biphasic that involves an enterohepatic recirculation (Legette et al., 2014; van Breemen et al., 2014), and XH and IX conjugates are the major circulating metabolites (Legette et al., 2014). The similarity in the metabolism between rats and humans, allows for the translation of animal study findings to future clinical studies (Liu et al., 2015).

As mentioned, hops extracts consist of a variety of components that affect a wide range of biological targets, leading to various pharmacological actions (Bolton et al., 2019). Once the individual pharmacological effects have been attributed to certain phytocomponents and the interactions of the compounds are known, specialized extracts can be developed (Bolton et al., 2019). Depending on the biological purpose, the extract can be created by eliminating ("knocking-out/-down") compounds that interfere with the desired bioactivity or are responsible for undesirable effects, or by enriching ("knock-edin") compounds with the desired activities for an optimum efficiency (Bolton et al., 2019). In such context, Dietz et al. (2017) and Ramos Alvarenga et al. (2014) generated DESIGNER extracts (Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources). These extracts were created to maximize beneficial results and limit possible adverse effects (Dietz et al., 2017; Ramos Alvarenga et al., 2014). In this sense, combined estrogenic and chemopreventive properties would be beneficial for postmenopausal women, whereas chemoprevention without estrogenic activities would be useful for premenopausal women. Thus, it is thought only postmenopausal women would likely benefit from the 8-PN estrogenic component (Bolton et al., 2019).

1.2. Estrogen receptor and aryl hydrocarbon receptor signaling pathways

1.2.1. Structural properties and mechanisms of signaling of estrogen receptors

The ER is a ligand-induced nuclear transcription factor that is a member of the nuclear receptor family (Klinge, 2001; Swedenborg & Pongratz, 2010; Tarnow et al., 2019). There are two ER isoforms that share significant sequence homology: ERα and ERß. The structure of ERα and ERβ is composed of three main domains (Figure 15): the N-terminal domain (NTD), the DNA-binding domain (DBD), and the ligand-binding domain (LBD) (Fuentes & Silveyra, 2019; Klinge, 2001). The NTD or A/B domain, which contains activation function 1 (AF-1), is responsible for protein-protein interactions and transactivation, and functions

independently of ligands (Ellmann et al., 2009). The DBD contributes to ER dimerization and binding to specific sequences in the chromatin. These canonical sequences are known collectively as estrogen response elements (ERE) (Ellmann et al., 2009; Fuentes & Silveyra, 2019). The carboxy terminal domain, LBD (E domain), which contains activation function 2 (AF-2), is a ligand-dependent domain that represents an interaction site for co-activators and co-repressors (Ellmann et al., 2009). The DBDs of both receptors share 97% sequence similarity, while the LBDs share only 60% of their sequences. However, both receptors have similar affinities for E2 (Hewitt & Korach, 2008).

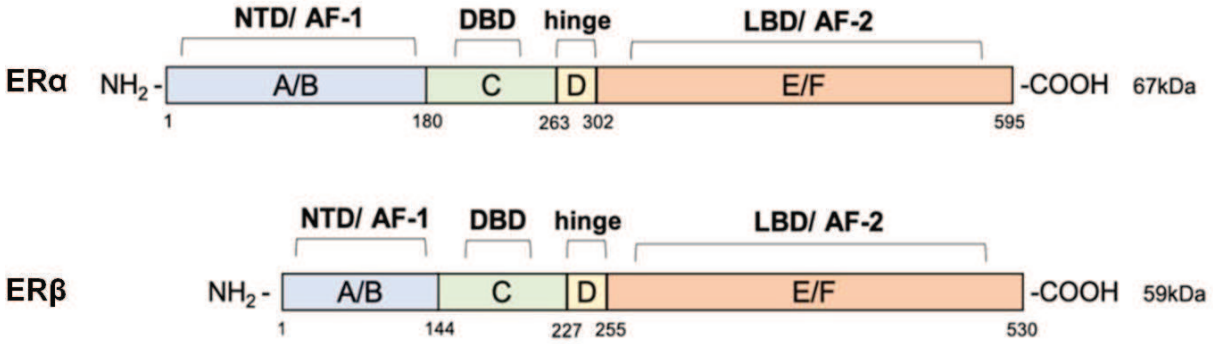

Figure 15. Structural organization of estrogen receptors (ERα and ERß). Figure taked and modified from Fuentes & Silveyra (2019).

The mechanism of ER action involves different pathways, based on the outcome of cellular events, the so-called classical and non-classical pathways. In the classical or genomic pathway (Figure 16, 1), the binding of ligands with ERs in the cytoplasm of target cells induces a conformal change in the receptor that promote dimerization (Ellmann et al., 2009; Fuentes & Silveyra, 2019; Kumar & Thompson, 1999). The ligand bound ER dimer is translocated to the nucleus, where it binds to the chromatin at EREs sequences located in the promoters of target genes (Klinge, 2001). The ligand–receptor complex interacts with co-activator and co-repressor molecules and modulates the transcription rates of target genes (Fuentes & Silveyra, 2019; Klinge, 2001).

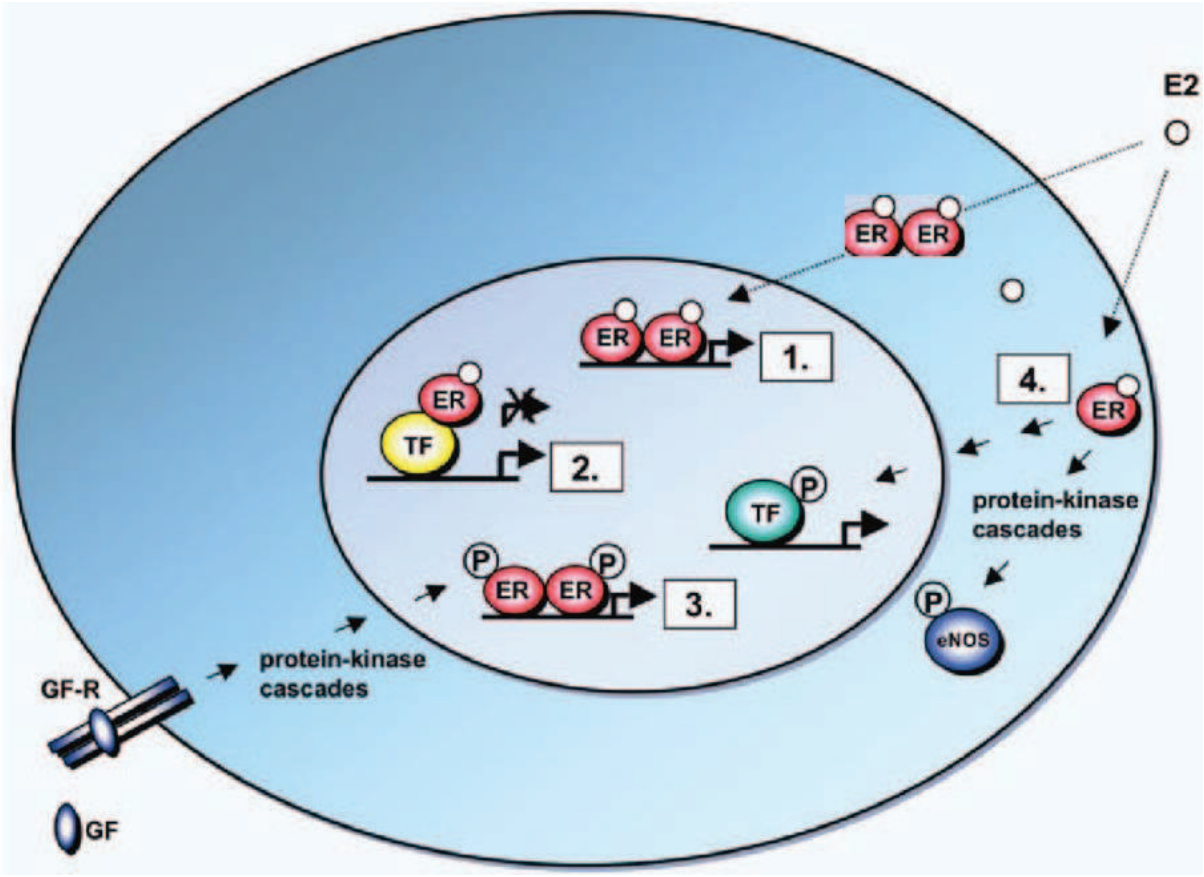

Figure 16. Schematic representation of estrogen receptors genomic and non-genomic pathways. Figure taked and modified from Björnström & Sjöberg (2005).

In the non-classical (non-genomic) pathway (Figure 16, 2 to 4), the ERs do not operate as transcription factors in the nucleus and involve three different mechanisms:

2. ERE independent genomic signaling: this mechanism involves a "tethering" of the ligand-activated receptor to other transcription factors that are directly bound to DNA via their respective response elements. One of the best-described examples includes the interaction of ER with Fos and Jun proteins at the activator protein 1 (AP-1) binding sites in genes encoding ovalbumin, insulin-like growth factor 1 (IGF1), collagenase, cyclin D1 and choline acetyltransferase. Other transcription factors include Sp1 transcription factor, nuclear factor κB (NFκB), CCAAT/enhancer binding protein β (C/EBPβ), GATA binding protein 1 (GATA1) and signal transducer and activator of transcription 5 (STAT5) (Björnström & Sjöberg, 2005).

3. Ligand-independent signaling: this mechanism causes ER activation and target gene transcription through phosphorylation of ERs or their associated co-regulators (Binder et al., 2015; Björnström & Sjöberg, 2005; Vrtačnik et al., 2014).

4. Non-genomic signaling: this pathway starts with the binding of ligands with ERs located at the plasma membrane resulting in the activation of various protein-kinase cascades (Vrtačnik et al., 2014). The ligand–receptor binding complex can cause not only activation of kinase signaling cascades, but also mobilization of intracellular calcium, stimulation of adenylate cyclase activity and cyclic adenosine monophosphate (cAMP) production (Binder et al., 2015; Björnström & Sjöberg, 2005; Vrtačnik et al., 2014).

1.2.2. Structural properties and mechanisms of signaling of aryl hydrocarbon receptor

Aryl hydrocarbon receptor (AHR) is a ligand-induced nuclear transcription factor that is member of the basic helix-loop-helix (bHLH) receptor family (Klinge, 2001; Swedenborg & Pongratz, 2010; Tarnow et al., 2019). The structure of AHR, AHR nuclear translocator (ARNT) and its regulator, AHR repressor (AHRR) is shown in Figure 17. The AHR is composed of three main domains: the bHLH domain at the N terminal region, PER-ARNT-SIM (PAS) domain and the carboxy-terminal domain. The bHLH, which consists of two α-helices separated by a non-helical loop, allows the dimerization with ARNT, the binding of DNA and the interactions with chaperones such as Hsp90 (Heat Shock Protein 90). The PAS domain consists of two structural repeats A and B. PAS A is involved in the dimerization with ARNT and PAS B also allows ligand binding (Larigot et al., 2018; Monostory & jean marc, 2008). The carboxy-terminal domain, which contains transcriptional activation domains, allows interaction with co-activators and co-repressors. The structure of ARNT is similar to that of AHR in terms of the bHLH and PAS A domains, which are involved in the dimerization with AHR and in DNA-binding. However, in spite of the presence of a PAS B domain, ARNT is not able to bind ligands (Larigot et al., 2018; Monostory & jean marc, 2008). The structure of AHRR is similar to the AHR and ARNT structures but lacks a ligand-binding domain (PAS B) and a transactivation domain. The AHRR binds to co-repressors, which are involved in a negative regulatory loop for AHR (Larigot et al., 2018).

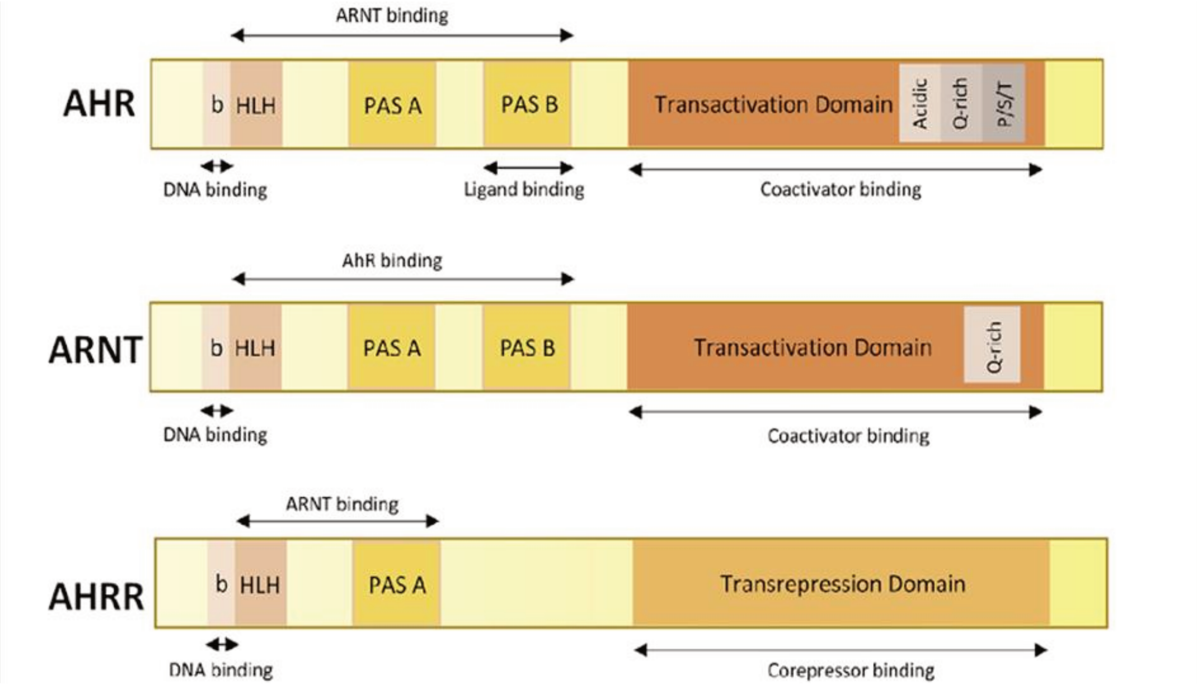

Figure 17. Structural organization of aryl hydrocarbon receptor (AHR), AHR nuclear translocator (ARNT) and AHR repressor (AHRR). Figure taked and modified from Larigot et al. (2018).

As shown in Figure 18, upon binding with an AHR ligand in the cytoplasm, the AHR undergoes conformational changes and dissociates from its chaperone proteins such as Hsp90. The AHR translocates into the nucleus, forms a dimer structure with ARNT, and binds the dioxin/xenobiotic response element (DRE/XRE) in the enhancer/promoter of target genes to induce transcription (Safe & Wormke, 2003; Tarnow et al., 2019). The AHR regulates a variety of phase I and II enzymes, being the cytochrome enzymes P450 1A1 (CYP1A1) and 1B1 (CYP1B1) the two major enzymes in its gene battery (Tijet et al., 2006). The mechanism by which AHR regulates its target genes includes negative feedback regulation via the AHRR (Larigot et al., 2018). The AHRR suppresses AHR activity by binding to ARNT and XRE/DRE (AhRR-ARNT complex) and is able to modulate the transcription of AHR-dependent genes (Yang et al., 2018).

The AHR can also mediate signals via non-classical pathways independent of the ARNT activation. In the non-canonical genomic pathway, AHR affects genes via other transcription factors, such as NF-κB and ER (Figure 18) (Holme et al., 2019; Larigot et al., 2018). In the non-genomic pathway, the activation of AHR may imply an increase of focal adhesion and Src kinases, and increased concentrations of Ca2+ (Holme et al., 2019; Larigot et al., 2018). The Ca2+ signaling is central to AHR-induced gene expression, including CYP enzymes and pro-inflammatory cytokines (Holme et al., 2019; Larigot et al., 2018). Based on the AHR target genes encoded, AHR is involved in estrogen metabolism, regulation of cell

proliferation, apoptosis, tumor suppressor functions and reproductive functions (Hernández-Ochoa et al., 2009; Puga et al., 2009).

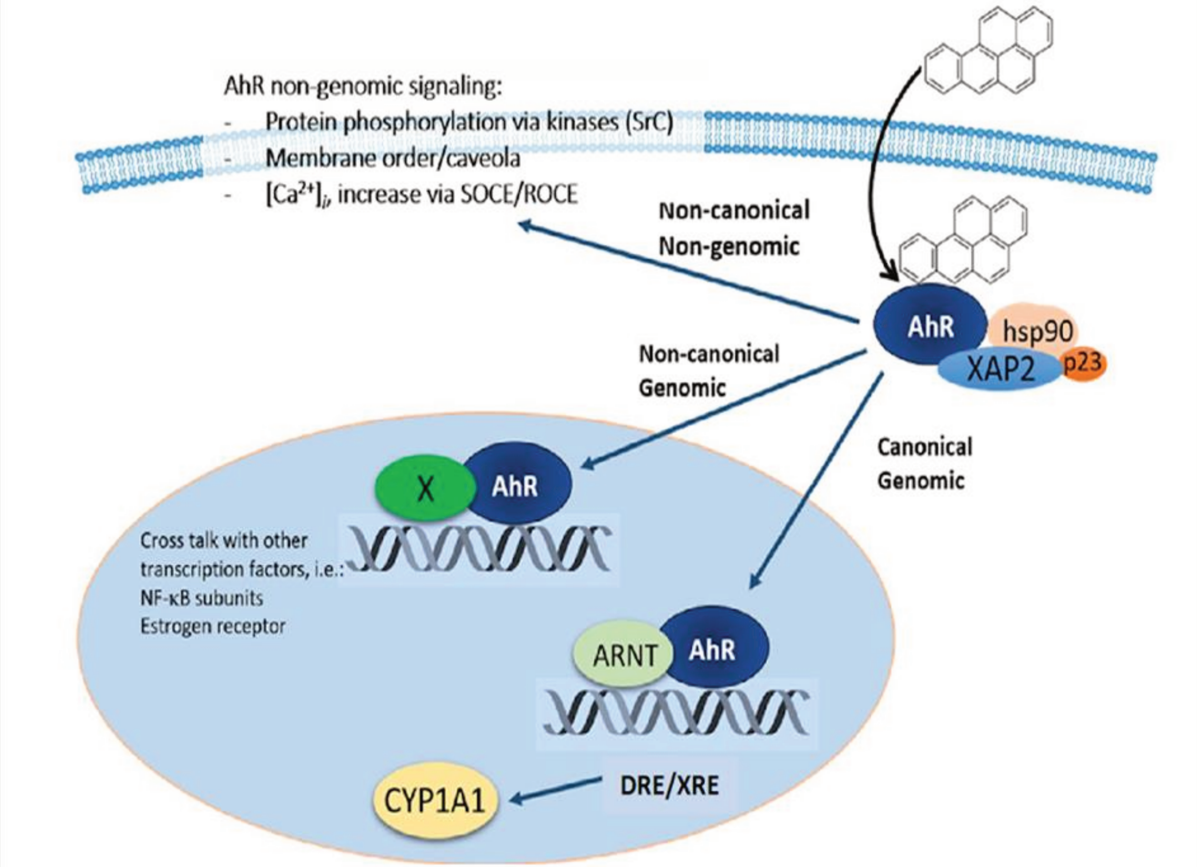

Figure 18. Schematic representation of aryl hydrocarbon receptor (AHR) genomic and non-genomic pathways. Figure was taken and modified from Holme et al. (2019).

1.2.3. Crosstalk between estrogen receptor and aryl hydrocarbon receptor

Several studies have investigated interactions between ER and AHR (reviewed in Safe & Wormke, 2003). The AHR may inhibit the ER activity through different mechanisms (Figure 19): A) competition for shared coactivators, including ARNT; B) altered estrogen synthesis/metabolism by the regulation of the CYPs gene expression; C) increased proteasomal degradation of ER; D) direct inhibition by the activated AHR/ARNT heterodimer by binding to inhibitory XRE (iXRE) present in ER target genes (Hitzman et al., 2020; Safe & Wormke, 2003; Shanle & Xu, 2011).

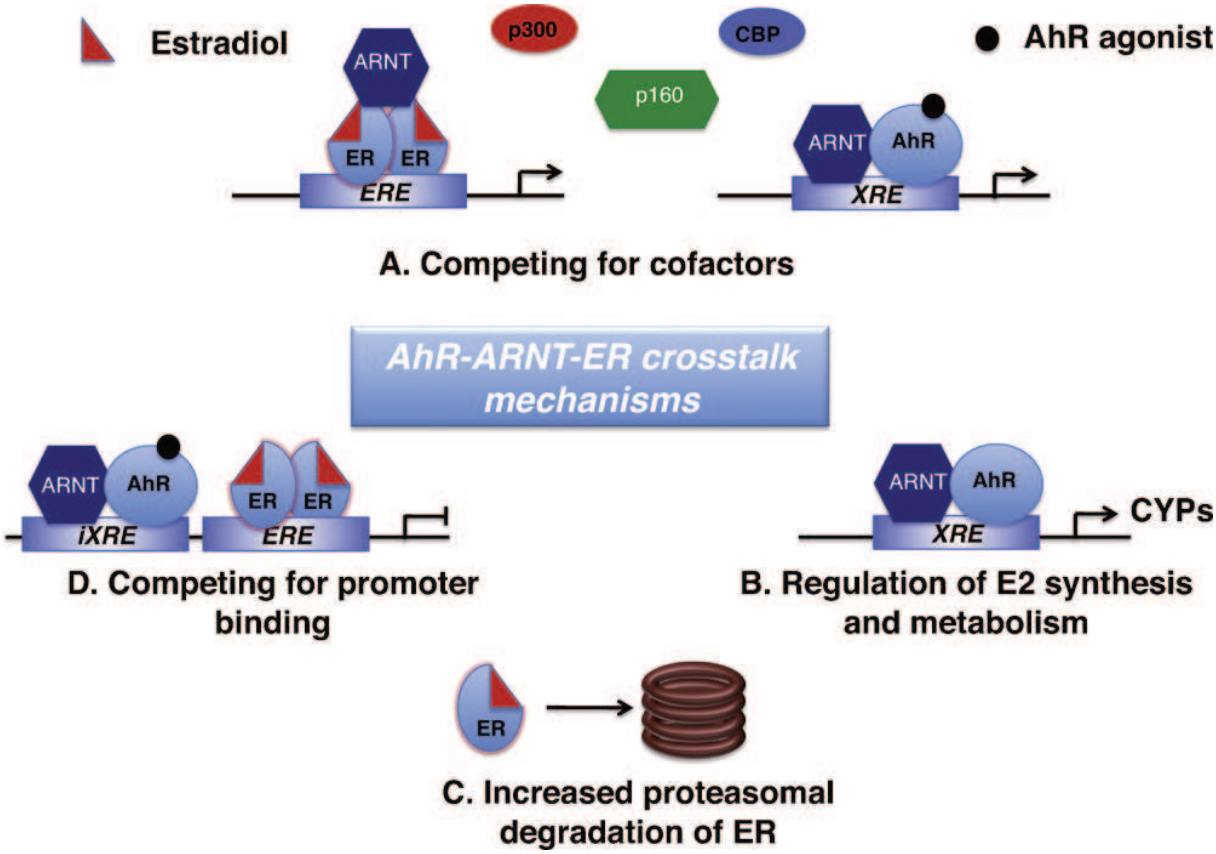

Figure 19. Proposed mechanisms of crosstalk between aryl hydrocarbon receptor (AHR) and estrogen receptor (ER) signaling pathways. Figure taked from Swedenborg & Pongratz (2010).

1.2.4. Modulation of estrogen receptor and aryl hydrocarbon receptor by hops compounds

Different *in vivo* and *in vitro* studies have demonstrated that 8-PN is the most estrogenic compound in the extract (Krause et al., 2014; Milligan et al., 1999; Nasri & Pohjanvirta, 2021; Overk et al., 2005, 2008; Zierau et al., 2002). On the molecular level, 8-PN preferentially functions through the ERα (Helle et al., 2014; Schaefer et al., 2003). Studies performed in rats observed that 8-PN increases the uterine wet weight, further demonstrating its estrogenic effects, but also raises questions concerning its safety with respect to the promotion of E2-dependent tumor growth (Diel et al., 2004; Zierau et al., 2008). However, 8-PN containing hops extract was not shown to increase uterine wet weight (Keiler et al., 2017a). This different responsiveness between pure compound and extract could be explained by the presence of other bioactive compounds like XH or 6-PN (Bolton et al., 2019; Dietz et al., 2017). By the one hand, XH antagonizes the estrogenic effect of 8-PN in Ishikawa cells (Dietz et al., 2017). The antiestrogenic effect of XH could be through inhibiting aromatase activity and thus, estrogen formation (Monteiro et al., 2007). Another reason could be due to the fact that XH inhibits estrogen-induced tumor growth in breast cancer by

inducing G1-arrest (Yoshimaru et al., 2014). Thus, since the concentration of XH in hops extracts is higher than that of 8-PN (van Breemen et al., 2014), XH might reduce the proliferative activities of 8-PN in the whole hop extract. By the other hand, 6-PN is an AHR agonist that induces the metabolism of estrogens in breast cancer cells by the increase of CYPA1 and CYP1B1 expressions (Hitzman et al., 2020; Wang et al., 2016).

1.3. Models for estrogen carcinogenesis and chemopreventive study

It is well-known that estrogens stimulate the proliferation of reproductive tissues, leading to pathological processes such as endometrial carcinogenesis (Santen et al., 2010). One chemopreventive mechanism involved in estrogen carcinogenesis is explained by the key role of CYP1s on estrogen metabolism. The CYP1A1 and CYP1B1 metabolize estrogens into 2-hydroxylated and 4-hydroxylated catechols, respectively (Cavalieri et al., 1997; Lakhani et al., 2003). The estrogen metabolism catalyzed by CYP1A1 is considered a detoxification pathway and the metabolite, 2-Methoxyestradiol, has antiproliferative/ anticancer activity (Gong et al., 2011; Lakhani et al., 2003; Tarnow et al., 2019). In contrast, the estrogen metabolism catalyzed by CYP1B1 is considered a genotoxic pathway due to the formation of reactive quinones, which causes DNA damage (Cavalieri et al., 1997). Thus, the estrogen metabolism catalyzed by CYP1B1 in the endometrium is thought to be an important event in endometrial cancer etiology (Rylander-Rudqvist et al., 2004).

The expression of both CYP1A1 and CYP1B1 are controlled by AHR. Thus, exposure to AHR ligands leads to increased metabolic elimination of estrogens (Shanle & Xu, 2011). As mentioned before, the AHR can control estrogen action through the induction of ER degradation and inhibition of estrogen signaling (Wormke et al., 2000, 2003). It has been demonstrated that AHR agonists exert significant antiestrogenic activity in both endometrial cancer cell lines and rat uterus (reviewed in Safe & Wormke, 2003; Helle et al., 2014). Thus, since endometrial cancer is associated with estrogen exposure (Santen et al., 2010), such antiestrogenic activity mediated by AHR might prevent endometrial carcinogenesis. The protective effect of AHR on endometrial carcinogenesis was demonstrated in aging female rats treated with 2,3,7,8-tetraclorodibenzo-p-dioxina (TCDD), a well-known AHR agonist, with a reduction of spontaneous uterine tumors (Kociba et al., 1978). In addition, epidemiological studies have shown that cigarette smoke, which contains AHR active compounds, could protect against uterine cancer (reviewed in Safe & Wormke, 2003).

Another key target gene activated in the AHR genomic pathway related to anti-carcinogenic activity is the AHRR (Vogel & Haarmann-Stemmann, 2017), whose expression is inversely correlated with tumor cell growth (Zudaire et al., 2008). The activity of AHRR as a tumor

suppressor gene was demonstrated in several types of cancer, including colon, breast, lung, stomach, cervix, and ovary (Vogel & Haarmann-Stemmann, 2017; Zudaire et al., 2008).

1.3.1. *In vitro* and *in vivo* models for the study of estrogen carcinogenesis

The possibility of working under standardized conditions makes cell lines extremely useful for the discovery of molecular mechanisms and biological pathways related to an observed phenotype, while also allowing for cost-effective high-throughput screenings (Van Nyen et al., 2018). The most commonly known endometrial cancer cell lines (AN3-CA, ECC-1, HEC1A, HEC1B, Ishikawa, and KLE) are endometrial cancer type I (Skok et al., 2020; Van Nyen et al., 2018). Among these cell lines, the Ishikawa cells line was first described by Nishida et al. (1985) and Nishida (2002). This cell line was established from an endometrial adenocarcinoma G1 from a 39-year-old woman. This cell line is characterized to form a monolayer in a mosaic fashion with piling. Although Ishikawa cells express both ER and PR, the expression of both receptors disappears after long-term culture, thus cells tend to transform into undifferentiated ones (Nishida, 2002).

Despite *in vitro* assays can identify substances with estrogenic activity; endocrine metabolism pharmacokinetics indicates the need to use *in vivo* assays in the overall evaluation of potential estrogenic compounds (Clode, 2006; Kleinstreuer et al., 2015). In this context, the uterotrophic assay emerges as a robust test to evaluate compounds suspected of being estrogenic and it was validated by the Organization for Economic Cooperation and Development (OECD) (Kanno et al., 2003; Owens & Koëter, 2003). Since then, the uterotrophic assay is considered the “gold standard” test to identify ER agonists (U.S. EPA, 2011). The uterotrophic response is mediated via ERα, thus only compounds that are able to bind, activate or modulate ERα elicits a positive response (Gibson & Saunders, 2014).

The uterotrophic assay uses either sexually mature ovariectomized or sexually immature female rats. The intact female rats are used because do not produce endogenous estrogens and consequently the uterus becomes sensitive to external estrogenic substances (U.S. EPA, 2011). The uterotrophic assay is based on the classical physiological response of the uterus following E2 treatment: increased uterine weight (Gibson & Saunders, 2014). The treatment with E2 increases electrolytes and water imbibition and induces cell division as part of uterine growth. The peak of mitotic division occurs approximately 24 h after E2 administration with high response in the epithelium, followed by the stroma, and the myometrium (U.S. EPA, 2011). Therefore, a compound is considered estrogenic if it increases the uterine weight or alters other endpoints such as epithelial proliferation, epithelial cell height, or the expression of estrogen-response genes (Gibson & Saunders, 2014).

Based on the strong associations found between ERα and AHR signaling in experimental models of breast cancer and on the link between estrogen signaling and endometrial cancer (Rodriguez et al., 2019), we hypothesize that:

a) The difference in the effect of 8-PN and hops extract on the endometrium could be explained by looking for potential interactions of the far less studied 6-PN with ERα and AHR pathways to better understand its mode of action;

b) The reduction of XH amount in hops extract and consequently on the 8-PN bioavailability in the organism could have an impact on the estrogenic response of the extract in ovariectomized rats.

2. Goals

2.1. Main goal

To determine the estrogenic effects of hops extract by using *in vivo* and *in vitro* studies and to evaluate the molecular mechanisms of anti-carcinogenic process in order to propose hops extract as a safe compound.

2.2. Specific goals

EXPERIMENT I. To evaluate the effects of hops extract and its bioactive compounds (6-PN and 8-PN) on ERα and AHR signaling pathways, in a human endometrial cancer cell line. To this end, we aimed to determine:

a) the estrogenic effects of hops extract and its bioactive compounds (6-PN and 8-PN),
b) the activation of ERα and AHR by hops and its bioactive compounds,
c) the potential interactions of 6-PN with both ERα and AHR pathways, to explain the difference in the estrogenic effect of 8-PN and hops extract on the endometrium,
d) the gene expression of some mediators and targets of ERα and AHR signaling pathways involved in chemopreventive processes.

EXPERIMENT II. To evaluate the safety of the use of a standardized hops extract and an extract reduced in XH (named knock-out Hops: KO-Hops) through a classical uterotrophic assay. To this end, we aimed to:

a) provide additional information regarding the estrogenic effect of a standardized hops extract and if the knock-out Hops has estrogenic effect,
b) determine if several uterine estrogen-dependent endpoints are affected by the treatment.

3. Materials and Methods

3.1. Substances and extract

The extracts were obtained from the Department of Medicinal Chemistry and Pharmacognosy at the University of Illinois, Chicago (UIC) in cooperation with the UIC's Center for Botanical Dietary Supplements Research. Hydroalcoholic extract (UIC Botanical Center voucher code #BC402) from spent hops was prepared using good manufacturing practice at Hopsteiner (New York, NY, USA). The preparation and standardization (chemical and biological) of the hops extract used has been previously described in detail (Krause et al., 2014; van Breemen et al., 2014). Briefly, hops with an optimum content of prenylated flavonoids were extracted with ethanol, and the bitter acids were removed by supercritical fluid carbon dioxide extraction of the dried crude extract. The resulting spent hops extract was provided as a dry, pale yellow powder. The details of the chemical standardization of the spent hops extract for four marker compounds (XH, IX, 8-PN, 6-PN) have been reported using LC-MS (Krause et al., 2014) as well as UHPLC and qHNMR (Ramos Alvarenga et al., 2014).

For ***EXPERIMENT I*****:** 8-PN was synthesized from naringenin as previously described by Gester et al. (2001). The purity of the compound was assessed to be > 99% by gas chromatography and HPLC. All solvents were HPLC grade and purchased from Thermo Fisher (Fair Lawn, NJ, USA). Purified water was prepared by using a Millipore Milli-Q purification system (Millipore, Billerica, MA, USA).

3-methylcholanthrene (3-MC), E2, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and 6-PN from Sigma-Aldrich (Prague, Czech Republic). All compounds were stored at −20°C until use and showed no detectable signs of degradation over time. Treatments were performed using serial dilutions of hops extract, test compounds, and controls in DMSO. As references, 10^{-8} M E2 and 10^{-6} M 3-MC were used as positive controls of ERα and AHR pathways, respectively. All test compounds were dissolved in DMSO and added in a way that the DMSO concentration in the test did not exceed 0.1%.

For ***EXPERIMENT II****:* E2 was purchased from Sigma-Aldrich. The composition of the standardized hops extract and the modified hops extract (KO-Hops) is detailed in Table 8. Hops extract has relatively high levels of 8-PN and XH, which exerts good estrogenic and chemopreventive properties (Dietz et al., 2017). The KO-Hops extract was reduced in XH levels and the levels of 8-PN were retained, yielding an extract with minimal chemopreventive property but significant estrogenicity provided by 8-PN. This extract was generated using countercurrent separation (CCS) in two steps, as described previously

(Ramos Alvarenga et al., 2014). The first step produced the initial level of DESIGNER extracts and the second one enhanced the "knock-out" selectivity. The CCS utilizes immiscible liquid−liquid two-phase solvent systems as chromatographic phases.

Table 8. Content of bioactive compounds in Hops and KO-Hops extracts.

Compoud	Hops	KO-Hops	Hops/KO-Hops
XH	33%	0.07%	471.43
IX	1.50%	1.34%	1.12
8-PN	0.33%	0.25%	1.32
6-PN	1.10%	0.08%	13.75
DMX	~1%	0.10%	10
XH/8-PN	100	2.8	

3.2. EXPERIMENT I *(in vitro)*

3.2.1. Cell line and culture conditions

The human endometrial adenocarcinoma cell line Ishikawa was provided by Masato Nishida, Department of Obstetrics and Gynecology, University of Tsukuba (Nishida et al., 1985).

Cells were cultured in Dulbecco's modified Eagle's medium F12/F12 1:1 (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.

3.2.2. Trypan blue dye exclusion assay

To determine the optimal concentrations of hops extract and its bioactive compounds for subsequent experiments in Ishikawa cells, different concentrations of hops extract (0.7 µg/ml, 7 µg/ml, 70 µg/ml), 6-PN (10^{-7} M, 10^{-6} M, 10^{-5} M) and 8-PN (10^{-7} M, 10^{-6} M, 10^{-5} M) were tested, as was previously evaluated (Hitzman et al., 2020; Overk et al., 2005; Wang et al., 2016). Cells were seeded in a total volume of 2 ml at a density of 200,000 cells per well in a 6 well plate until monolayer formation (24 h). The next day, the medium was removed and replaced by a new medium with the tested substances for 24 h 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. The 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).

Viability (%) was calculated as follows: viable cells count (unstained cells count)/ total cells count (stained cells count + unstained cells count) x 100. Results are expressed relative to the results of the vehicle control. The exclusion criterion was to reject the concentrations that caused a viability ≤ 80% and showed a statistically significant difference respect to the vehicle, for considering them toxic.

3.2.3. Luciferase assay for dioxin response element and estrogen response element activation

Ishikawa cells were cultured in DMEM/F12 medium without phenol red containing 5% dextran charcoal-treated FCS (DCC-FCS, Invitrogen, Karlsruhe) and 1% ITS for 48 to 72 h. For transfection, cells were transferred to a 24 well plate at the required density of 40,000 cells with 500 µl of DMEM/F12 - DCC-FCS 5% - ITS 1% medium per well. Cells were incubated for 24 h and transfected at 80% confluence with luciferase and renilla plasmids. The 2xERE-tk-luc and the DRE3 reporter plasmids were kindly provided by Dr. Luisella Toschi (Schering AG, Berlin) and Stephen Safe (Texas A&M University), respectively.

The 2xERE-tk-luc (400 ng) and the DRE3 (200 ng) reporter plasmids were mixed with 1.5 µg/ml or 0.75 µg/ml of Attractene Transfection Reagent (Qiagen), respectively, and incubated for 10-15 min at RT. The transfection complex was then added to each well and incubated for 24 h. At the end of this period, the medium was removed and replaced by DMEM/F12 medium containing 5% DCC-FCS and 1% ITS with hops extract or the compounds. After 24 h incubation, cells were harvested and luciferase activity was measured using a commercial kit (Promega, Mannheim, Germany) according to the manufacturer's instructions using the FLUOstar OPTIMA luminometer (BMG Labtechnologies, Germany).

Results were normalized against total protein content, which was determined using bicinchoninic acid. The results were plotted as fold induction respect of control (DMSO). E2 was used as a positive control for the ERα pathway and 3-MC as a positive control for the AHR pathway. All measurements were performed in triplicate for each tested concentration and analyzed from at least three independent experiments.

3.2.4. Alkaline phosphatase activity

The alkaline phosphatase (AlkP) induction assay was designed to evaluate the estrogen-induced production of the AlkP, an enzyme known to be regulated by ovarian hormones in

the uterus (Holinka et al., 1986). The induction of AlkP activity indicates an estrogenic response, whereas inhibition represents an antiestrogenic effect (Pisha & Pezzuto, 1997).

Forty-eight to 72 h before treating the cells, the growth medium was replaced with DMEM/F12 containing 5% DCC-FCS and 1% ITS. For experiments, Ishikawa cells were cultured into a 96 well plate at the required density of 11,000 cells in DMEM/F12 - DCC-FCS 5% - ITS 1% medium with DMSO, E2, or different concentrations of hops extract, 8-PN or 6-PN per well for 72 h, as previously described (Wober et al., 2003). After 72 h incubation with hops extract or the compounds, cells were lysed by freezing at -80°C after washing twice with PBS and resuspended in reaction buffer (274 mM mannitol, 100 mM CAPS, 4 mM $MgCl_2$, pH 10.4) containing 5 mM p-nitrophenylphosphate, according to Littlefield et al. (1990) and following protocols previously described (Wober et al., 2003). The reaction was incubated for 60 minutes at 25ºC, protected from light.

AlkP activity was assayed by a method involving the hydrolysis of p-nitrophenylphosphate to p-nitrophenol at pH 10.4 and the spectrometric determination of the kinetic of the product formation at 405 nm. Results are expressed relative to the control (DMSO). E2 was used as a positive control for estrogenicity. All measurements were performed in triplicate for each tested concentration and analyzed from at least three independent experiments.

3.2.5. Determination of gene expression

Ishikawa cells were seeded in a plastic culture 25 cm^2 flask with DMEM/F12 - FBS 10% - ITS 1% medium with DMSO, E2, 3-MC or different concentrations of hops extract, 8-PN or 6-PN 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, USA) and checked by PCR. First-strand cDNA synthesis was performed by mixing 2 µg of digested RNA with MMLV reverse transcriptase (Promega, USA) and Oligo (dT) 12-18 primers (Eurofins MWG Operon, Germany). Quantitative real-time polymerase chain reaction (qPCR) was applied for cDNA amplification with SybrGreen I (Sigma-Aldrich, Taufkirchen, Germany) as the detection dye using the iCycler iQ™ Real-Time PCR Detection System (BioRad, USA). After initial denaturation at 95°C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95°C for 15 sec, annealing at 56-60°C for 15 sec, and extension for 15 sec at 72°C. Product purity was confirmed by melting curves analysis and random samples were subjected to agarose gel electrophoresis. In all assays, controls containing no template DNA were included, yielding no consistent amplification. Primer sequences are summarized in Table 9. The relative mRNA amounts of target genes were calculated after normalization to an endogenous reference gene (ribosomal protein

S18). Results are expressed as relative amounts of mRNA compared to the vehicle-treated cells using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001). All PCR reactions were conducted from at least three independent cell culture experiments.

Table 9. Primers* used for gene expression analysis by qPCR.

Gene name	Primer sequence (5′–3′)	Amplicon size (bp)
AHR	f: CGTGGGTCAGATGCAGTACAA	144
	r: AGTGGCTGAAGATGTGTGGT	
AHRR	f: GGCCCTGACCTTGTCCTT	150
	r: CTGTGCTTTCCCGCTGTC	
ARNT	f: CCAGCAGCCTCATCATCGTT	124
	r: GCTGTTGCTCTGATCTCCCA	
C3	f: TGCGGCTACCCTACTCTGTTGTTCG	250
	r: GACGGCAGCCTTGACTTCCACTTCC	
CLU	f: CCCCACCGGAGGCCTCACTTCTT	285
	r: CCCGGCACTTGTCACACTGGTCCT	
CYP1A1	f: CTTCCGACACTCTTCCTTCG	123
	r: GGTTGATCTGCCACTGGTTT	
CYP1B1	f: AACGTACCGGCCACTATCAC	140
	r: CCACGACCTGATCCAATTCT	
ESR1	f: CTGGCCCAGCTCCTCCTCATCCT	194
	r: CAAGTGGCTTTGGTCCGTCTCCTC	
PCNA	f: CCACTCCACTCTCTTCAACGG	128
	r: TCCTTCTTCATCCTCGATCTTGG	
RPS18	f: TCTAGTGATCCCTGAAAAGTTCC	152
	r: CGTGGATTCTGAATAATGGTG	

AHR, aryl hydrocarbon receptor; *AHRR*, aryl hydrocarbon receptor repressor; *ARNT*, aryl hydrocarbon receptor nuclear translocator protein; *C3,* complement component 3; *CLU*, clusterin, *CYP1A1*, cytochrome P450 1A1, *CYP1B1*, cytochrome P450 1B1; *ESR1*, estrogen receptor α; PCNA, proliferating cell nuclear antigen; RPS18, ribosomal protein S18.
*All primers were designed according to human sequences.

3.3. EXPERIMENT II *(in vivo)*

3.3.1. Animals

All usage of animals (housing, handling, and experimental techniques) were in accordance with the codes set out in the Declaration of Helsinki, as well as in agreement with the ethical

standards of the European as well as the German Animal Welfare legislation. Experiments were planned and conducted to follow closely to the 3R (replacement, reduction, refinement) principles of animal welfare. All procedures were licensed and carried out according to the institutional and state Animal Care and Use Committee guidelines, regulated by the German Federal laws for animal welfare.

Young adult female Wistar rats were maintained under controlled conditions of temperature (20 ± 1°C, relative humidity 50-80%) and illumination (12 h light/12 h darkness). Animals were housed in open cages (Tecniplast) with four to six animals/cage. All rats had free access to water and a standard phytoestrogen free rat diet (Teklad Global Diet, 2019 Harlan, Madison, WI, USA) since PND 21.

3.3.2. Experimental procedures

The timeline of experimental handling is shown in Figure 20. Seven weeks old rats were subjected to ovariectomy (n=40) or sham surgery (SHAM group; n=5). The surgery was performed under anesthesia by intramuscular injection of ketamine-hydrochloride (90 mg/kg bw, Zoetis Deutschland GmbH, Berlin, Germany) and xylazine (10 mg/kg bw, Xylariem, Pharma Partner, Hamburg, Germany). After 14 days of endogenous hormonal decline, the animals were exposed for 72 h with the following treatments (5 animals/group):

Vehicle: Control group;
Sham surgery + vehicle: SHAM group;
Ovariectomy + vehicle: Ovx group;
Ovariectomy + E2 (4 µg/kg bw/day): E2 group;
Ovariectomy + Hops high (200 mg/kg bw/day): Hops high group;
Ovariectomy + Hops middle (40 mg/kg bw/day): Hops middle group;
Ovariectomy + Hops low (8 mg/kg bw/day): Hops low group;
Ovariectomy + KO-Hops high (200 mg/kg bw/day): KO-Hops high group;
Ovariectomy + KO-Hops middle (40 mg/kg bw/day): KO-Hops middle group;
Ovariectomy + KO-Hops low (8 mg/kg bw/day): KO-Hops low group.

The animals of the Hops and KO-Hops groups were fed with a peanut butter mixture (vehicle) enriched with the plant extracts according to their group. The animals of the Control, SHAM and Ovx groups were fed with a peanut butter mixture (vehicle). The animals of the E2 group were exposed subcutaneously to E2, which was dissolved in DMSO and castor oil. All animals had free access to standard diet. The number of animals per group was determined according to previous works (Momesso et al., 2021; Overk et al., 2008; Stoker et al., 2020).

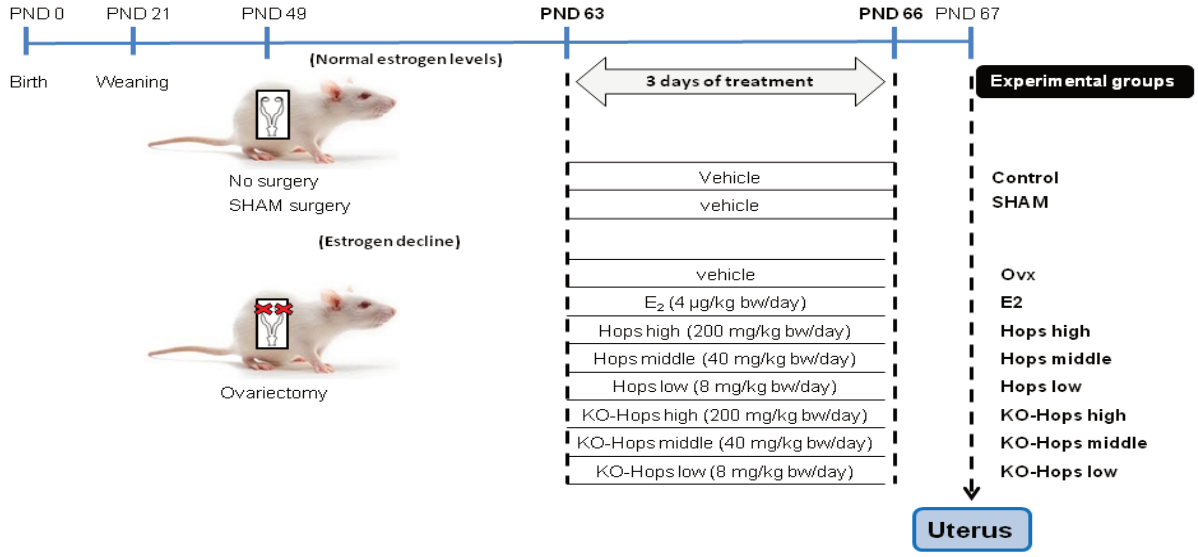

Figure 20. Schematic representation of the experimental protocol used in *EXPERIMENT II*, to study the estrogenic effects of Hops and KO-Hops extracts in the uterus of Wistar rats.

All animals were sacrificed 24 h after the last day of treatment using CO_2 inhalation subsequent to a light O_2/CO_2 anesthesia and the uterus was isolated. One uterine horn was snap frozen in liquid nitrogen to perform RT-qPCR. The other uterine horn (1.5 cm) was weighed and fixed in a 4% formaldehyde solution (Carl Roth, Karlsruhe/Germany) and embedded in paraffin for histological and immunohistochemical analyses, as described above (*Chapter I, section 3.7 and 3.8*).

3.3.3. Histological analysis

The **thickness of SS and myometrium (circular plus longitudinal)** layers were analyzed by image analysis, using Fiji software as described above in *Chapter I, section 3.9.2.*

The **luminal epithelial cell height** was evaluated following a similar procedure. Briefly, the images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope with a Dplan 40× focusing eyepiece, and analyzed by image analysis, using the Fiji software. All measurements were made in areas where luminal folds were not present, and care was taken to avoid measuring sections that were cut obliquely. To spatially calibrate the Image Pro-Plus analyzer, square grids from Neubauer's chamber images were captured. Image analysis was performed in at least 15 randomly selected fields per animal.

The **volume fraction of uterine glands** was calculated as described above (Chapter I, section 3.9.3).

3.3.4. Quantification of protein expression

The **expression of ESR1** in LE, GE, and SS was evaluated by image analysis, using Fiji of Image Pro-Plus, and expressed as IOD, as described above in *Chapter I, section 3.10.1*.

The **expression of Ki67** in LE and GE was evaluated as a percentage of Ki67-positive cells (see *Chapter I, section 3.10.2*).

3.3.5. Determination of gene expression

Total RNA was isolated from rat uterine horn (50 – 100 mg of sample), using the Micro-Dismembrator S (Sartorius) and peq-GOLD TriFast method according to the manufacturer's instructions (PEQLAB Biotechnology GmbH, Erlangen, Germany). Possible DNA contamination was eliminated by enzymatic digestion with RQ1 RNase-Free DNase, (M610A, Promega) and checked with PCR. After DNase-inactivation with 1.25 mM EDTA, cDNA synthesis was performed using 1 µg digested RNA with M-MLV Reverse Transcriptase (M170B, Promega), associated buffer, RNaseOut™ (Invitrogen), dNTPs (Invitrogen) and Oligo dT primers (Eurofins).

Quantitative real-time PCR was performed with SybrGreen I (Sigma) as detection dye using the CFX96TM Real-Time System with C1000TM Thermal Cycler (BioRad, Hercules, CA, USA). PCR reactions consist of a first denaturing cycle at 95°C 3 min, followed by 40 cycles of 15 sec at 95°C, 10 sec at 60°C and 20 sec at 72°C. Fluorescence was quantified at the end of the 72°C annealing step and the amplicon specificity was confirmed by a melting curve analysis (60-95°C).

Well-established genes for the evaluation of estrogen influence on the uterus were chosen and the associated primers used for RT-qPCR are shown in Table 10. Optimal combinations of magnesium chloride and primer concentrations were experimentally determined for every qPCR. The relative mRNA amounts of target genes were calculated after normalization to an endogenous reference gene (ribosomal protein S18). Results were expressed as relative amounts of mRNA compared to the Ovx animals using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Table 10. Primers* used for gene expression analysis by RT-qPCR.

Gene name	Primer sequence (5′–3′)
Esr1	f: TGAAGCACAAGCGTCAGA GAGAT
	r: AGACCAGACCAATCATCAGGAT
Esr2	f: CTACAGAGAGATGGTCAAAAGTGGA
	r: GGGCAAGGAGACAGAAAGTAAGT
Pr	f: CTACTCGCTGTGCCTTACCA
	r: GGACCACCCCTTTCTGTCTT
PCNA	f: GAG CAA CTT GGA ATC CCA GAA CAG G
	r: CCA AGC TCC CCA CTC GCA GAA AAC T
C3	f: ACA GCC TTC CCG GGA GCA TCA ACA
	r: AGC GCA CCA CAG GAG GCA CAG AGT C
Clu	f: CCC TCC AGT CCA AGA TGC TCA ACA C
	r: CCA TGC GGC TTT TCC TGC GGT ATT C
Rps18	f: CGT GAA GGA TGG GAA GTA TAG C
	r: TAT TAA CAG CAA AGG CCC AAA G

C3, complement component 3; *Clu*, clusterin; *Esr1*, estrogen receptor α, *Esr2*, estrogen receptor β; *PCNA*, proliferating cell nuclear antigen; *Pr*, progesterone receptor; *Rps18*, ribosomal protein S18.

*All primers were designed according to rat sequences.

3.4. Statistical analysis

EXPERIMENT I. Data are expressed as the mean ± SEM of pooled results obtained from at least three independent experiments. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett's test using the Graph-Pad Prism software Version 5.00 (San Diego, CA, USA). Values with $p < 0.05$ (*) were regarded as statistically significant.

EXPERIMENT II. Data are expressed as the mean ± SEM. A Kruskal–Wallis analysis was performed to obtain the overall significance (testing the hypothesis that the response was not homogeneous across treatments), followed by Dunn's method for multiple comparisons. Mann-Whitney U test was used to compare Hops and KO-Hops extract at each dose (Hops low vs KO-Hops low, Hops middle vs KO-Hops middle, and Hops high vs KO-Hops high). Values with $p < 0.05$ were accepted as significant.

4. Results

4.1. EXPERIMENT I

4.1.1. Effects of hops extract, 8-PN and 6-PN on the cell viability of Ishikawa cells

As shown in Figure 21, the extract at 70 µg/ml reduced the cell viability below 80%. Thus, this dose was excluded from the experiment. No changes in the cell viability were detected in any of the other tested doses of hops extract, or the individual compounds 8-PN or 6-PN.

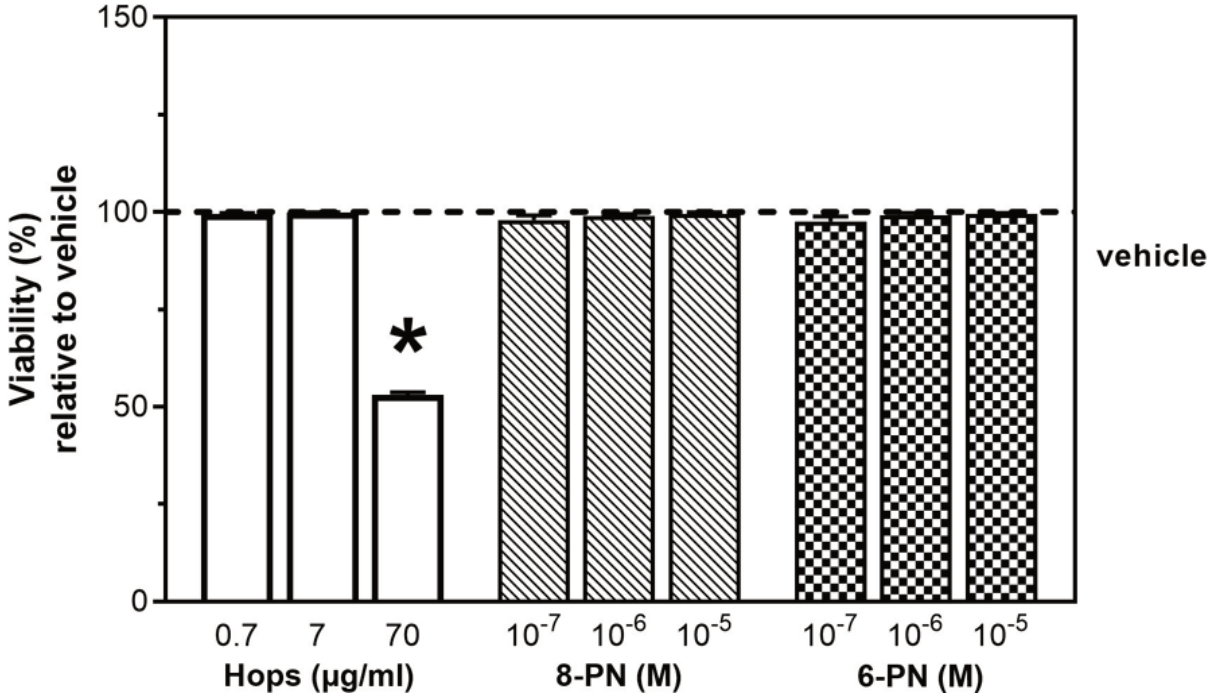

Figure 21. Effects of hops extract (Hops) and its bioactive compounds on cell viability. Ishikawa cells were treated with Hops (0.7 µg/ml, 7 µg/ml, 70 µg/ml), 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN) (10^{-7} M, 10^{-6} M, 10^{-5} M). Cell viability was evaluated by the trypan blue exclusion assay. Results are expressed as percentage of viability (% Viability) respect to the vehicle (DMSO: set to 100%). Data are expressed as the mean ± SEM of pooled results obtained from three independent experiments. Statistical significance: *$p < 0.05$ (ANOVA followed by Dunnet's post-hoc test).

However, the transfection with a 2xERE-tk-luc plasmid in combination with 8-PN at 10^{-5} M appeared to be cytotoxic for the cells. Therefore, we decided to exclude 8-PN at 10^{-5} M and to test an additional 8-PN dose at 10^{-8} M. Based on these results, we decided to use 0.07 µg/ml, 0.7 µg/ml and 7 µg/ml of hops extract, 10^{-8} M, 10^{-7} M and 10^{-6} M of 8-PN, and 10^{-7} M, 10^{-6} M and 10^{-5} M of 6-PN in all experiments to perform a dose-response analysis.

4.1.2. Activation of AHR and ERα pathways by positive controls: E2 and 3-MC

For AHR pathway activation, dose range finding studies from 10^{-9} M to 10^{-5} M showed induction of luciferase reporter gene activity for 3-MC at doses of 10^{-6} M and 10^{-5} M (Figure 22).

For ERα pathway activation, E2 at doses from 10^{-10} M to 10^{-7} M showed induction in a dose range finding studies from 10^{-11} M to 10^{-7} M (Figure 22).

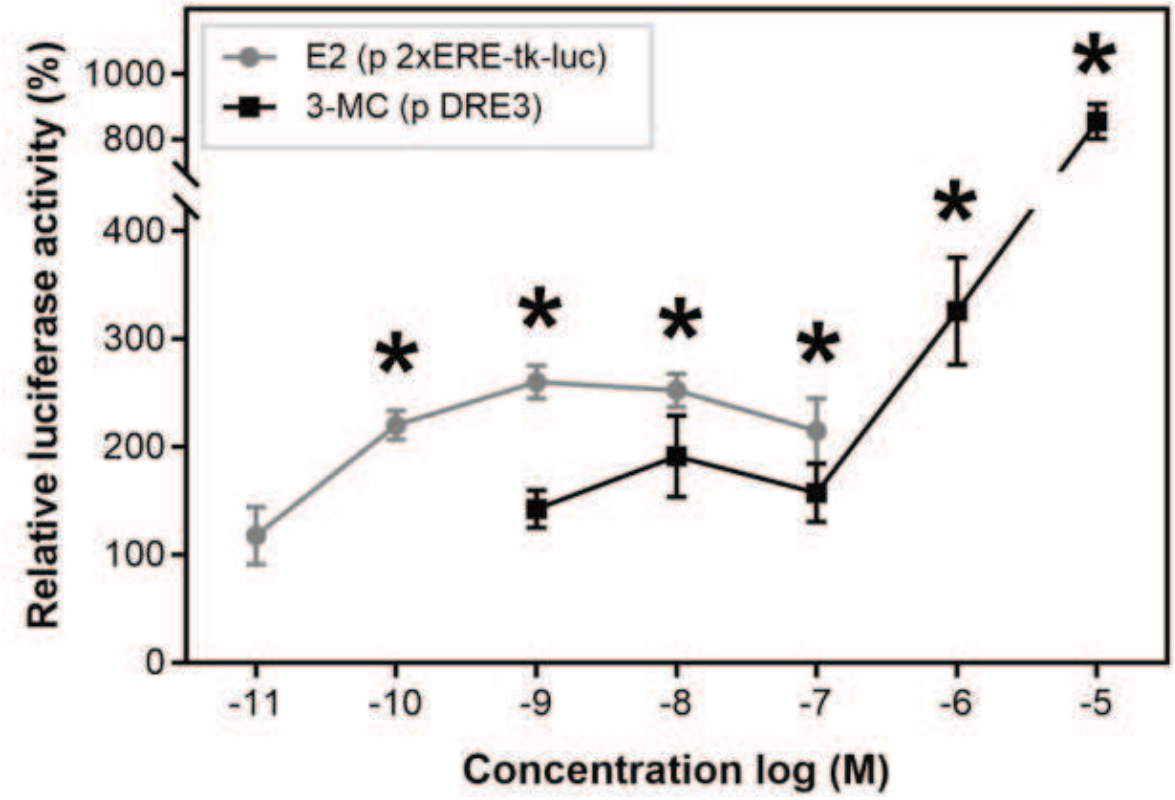

Figure 22. Dose-response curves of activation of ERα and AHR pathway by E2 or 3-MC treatment. Ishikawa cells were transfected with p 2xERE-tk-luc or p DRE3 for 24 h and then treated with E2 or 3-MC, respectively for 24 h. The luciferase activity was measured and data are expressed as the mean ± SEM of pooled results obtained from three independent experiments. For determination of significance (*p < 0.05), each treatment group was compared with the vehicle (DMSO set to 100%) using ANOVA followed by Dunnet's post-hoc test).

4.1.3. Hops extract and 6-PN acted as AHR agonists

Hops extract at a concentration of 7 µg/ml induced an activation of AHR leading to four-fold induction of luciferase activity, as did 10^{-6} M 3-MC (Figure 23). 6-PN at a concentration of 10^{-5} M increased the DRE-luciferase activity about three-fold compared to the vehicle, while 8-PN had not a significant effect at any concentration (Figure 23).

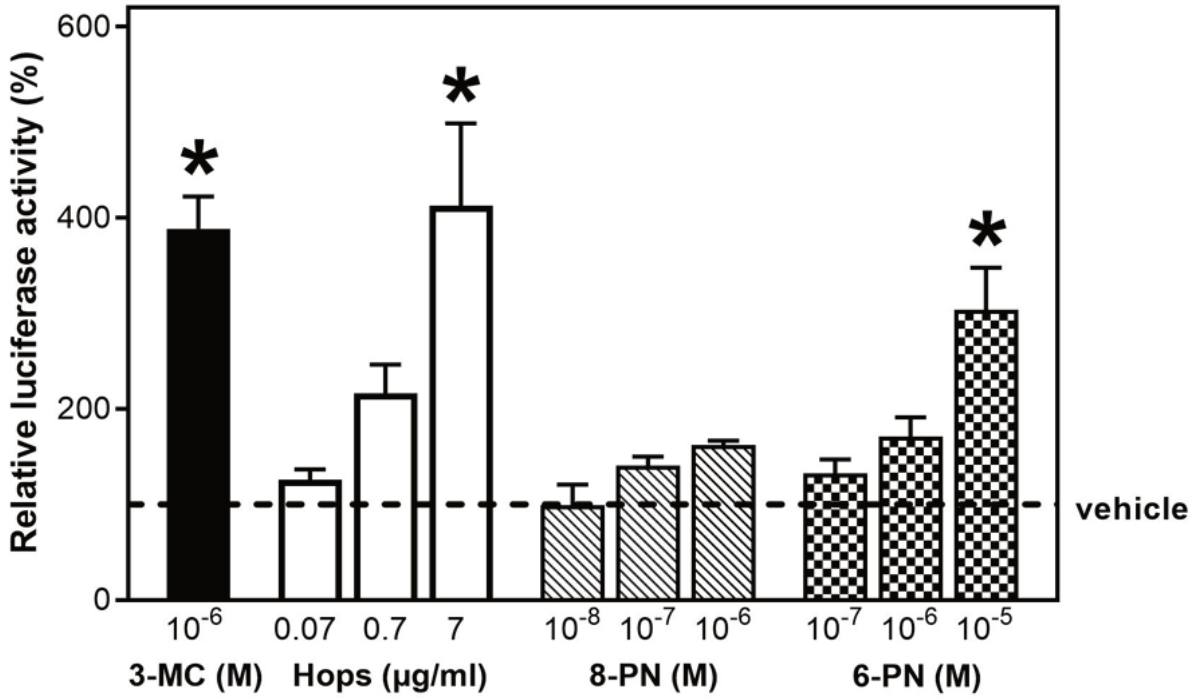

Figure 23. Effects of hops extract (Hops) and its bioactive compounds on AHR activation. Ishikawa cells were transfected with p DRE3 and then treated with Hops (0.07 µg/ml, 0.7 µg/ml, 7 µg/ml), 8-prenylnaringenin (8-PN; 10^{-8} M, 10^{-7} M, 10^{-6} M) and 6-prenylnaringenin (6-PN; 10^{-7} M, 10^{-6} M, 10^{-5} M) for 24 h. 3-MC (10^{-6} M) was used as positive control. The luciferase activity was measured and data are expressed as the mean ± SEM of pooled results obtained from three independent experiments. For determination of significance (*$p < 0.05$), each treatment group was compared with the vehicle (DMSO: set to 100%) using ANOVA followed by Dunnet's post-hoc test.

4.1.4. Hops extract, 8-PN and 6-PN acted as ERα agonists

Hops extract at concentrations of 0.7 µg/ml and 7 µg/ml, but not at a concentration of 0.07 µg/ml, increased the ERE-luciferase activity in Ishikawa cells (Figure 24). 8-PN induced a very strong activation of ERα with similar responses at all tested concentrations, as did 10^{-8} M E2 (Figure 24). 6-PN increased the ERE-luciferase activity in a dose-dependent manner (Figure 24).

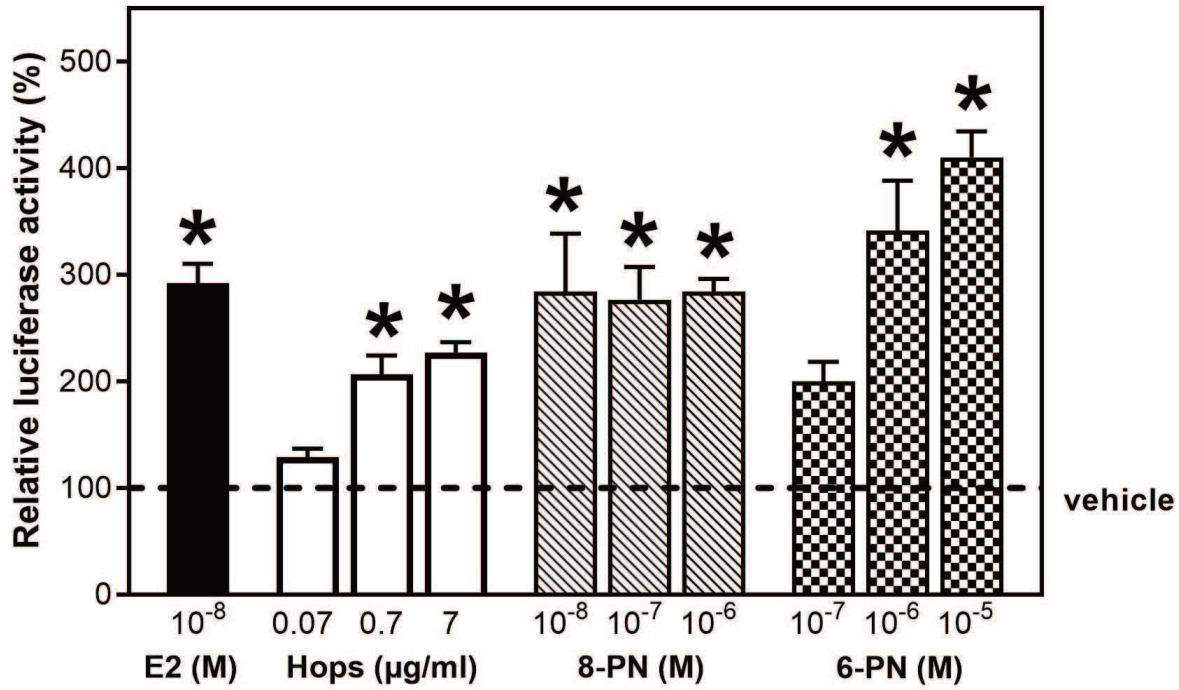

Figure 24. Effects of hops extract (Hops) and its bioactive compounds on ERα activation. Ishikawa cells were transfected with p 2xERE-tk-luc and then treated with Hops (0.07 µg/ml, 0.7 µg/ml, 7 µg/ml), 8-prenylnaringenin (8-PN; 10^{-8} M, 10^{-7} M, 10^{-6} M) and 6-prenylnaringenin (6-PN; 10^{-7} M, 10^{-6} M, 10^{-5} M) for 24 h. E2 (10^{-8} M) was used as positive control. The luciferase activity was measured and data are expressed as the mean ± SEM of pooled results obtained from three independent experiments. For determination of significance (*$p < 0.05$), each treatment group was compared with the vehicle (DMSO: set to 100%) using ANOVA followed by Dunnet's post-hoc test.

4.1.5. Hops extract, 8-PN and 6-PN acted as estrogenic compounds

For E2, the range of doses from 10^{-10} M to 10^{-7} M induced the AlkP activity (Figure 25A). Hops extract at a concentration of 0.7 µg/ml and 6-PN at a concentration of 10^{-6} M increased the AlkP activity (Figure 25B). All the concentrations of 8-PN revealed an increased AlkP activity around 400%, similar to the effect of 10^{-8} M E2 (Figure 25B).

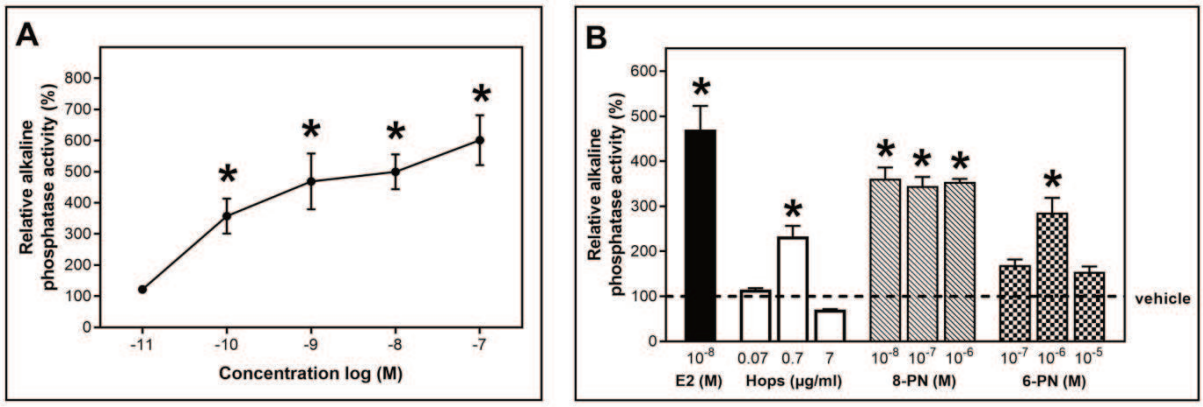

Figure 25. Estrogenic effects of E2, hops extract (Hops) and its bioactive compounds by the activation of the alkaline phosphatase induction assay. Ishikawa cells were treated with A) E2 (10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M), B) Hops (0.07 µg/ml, 0.7 µg/ml, 7 µg/ml), 8-prenylnaringenin (8-PN; 10^{-8} M, 10^{-7} M, 10^{-6} M) or 6-prenylnaringenin (6-PN; 10^{-7} M, 10^{-6} M, 10^{-5} M) for 72 h. Data are expressed as the mean ± SEM of pooled results obtained from three independent experiments. For determination of significance (*$p < 0.05$), each treatment group was compared with the vehicle (DMSO: set to 100%) using ANOVA followed by Dunnet's post-hoc test.

4.1.6. The mRNA expression of *ESR1, ARNT, AHRR, CYP1A1* and *CYP1B1* was up-regulated by 6-PN

We analyzed the mRNA expression of proliferating cell nuclear antigen (*PCNA*) and some members of ER signaling pathway: *ESR1, C3* and *CLU* (Parker et al., 2009; Toffanin et al., 2008; Ullah et al., 2017; Won et al., 2012). The gene expression of *PCNA, ESR1, C3* and *CLU* in Ishikawa cells were not different between vehicle and any compound; except that an increased expression of the *ESR1* mRNA was found after the exposure to 6-PN at 10^{-6} M (Table 11).

Respect to the AHR signaling pathway, no changes were detected between cells treated with any of the test compounds and the vehicle for *AHR* mRNA expression (Figure 26A). However, 6-PN increased the mRNA expression of A*RNT* and *AHRR* at 10^{-5} M (Figure 26B and C).

About genes involved in the metabolism of estrogens (*CYP1A1* and *CYP1B1*), hops extract at a concentration of 7 µg/ml induced *CYP1B1* mRNA expression and 6-PN at a concentration of 10^{-5} M induced both *CYP1B1* and *CYP1A1* mRNA expression, similar to the effect of 10^{-6} M 3-MC (Figure 26D and E). The treatment with 10^{-5} M 6-PN preferentially increased *CYP1A1* mRNA levels to around 20-fold compared to the 10-fold induction of *CYP1B1* (Figure 26D and E). Like 10^{-8} M E2, none of the concentrations of 8-PN had a significant effect compared to the vehicle-treated cells (Figure 26D and E).

Table 11. Relative gene expression of genes involved in ER pathway.

	ESR1 mRNA	*C3* mRNA	*CLU* mRNA	*PCNA* mRNA
E2 10^{-8}M	1.11 ± 0.1	0.66 ± 0.07	0.92 ± 0.08	0.97 ± 0.09
3-MC 10^{-6}M	0.84 ± 0.11	1.29 ± 0.1	0.87 ± 0.06	0.70 ± 0.04
Hops 0.07µg/ml	0.76 ± 0.11	0.85 ± 0.15	0.85 ± 0.18	0.91 ± 0.03
Hops 0.7µg/ml	1.15 ± 0.07	0.82 ± 0.09	1 ± 0.07	0.99 ± 0.12
Hops 7µg/ml	0.77 ± 0.08	0.44 ± 0.04	0.89 ± 0.1	0.97 ±0.13
8-PN 10^{-8}M	1.23 ± 0.28	0.64 ± 0.09	0.84 ± 0.03	0.83 ± 0.07
8-PN 10^{-7}M	1.25 ± 0.09	0.99 ±0.16	0.92 ± 0.07	0.94 ± 0.03
8-PN 10^{-6}M	1.38 ± 0.07	0.99 ± 0.11	1.03 ± 0.08	0.95 ±0.11
6-PN 10^{-7}M	0.72 ± 0.13	1.14 ± 0.37	1.33 ± 0.27	0.98 ± 0.15
6-PN 10^{-6}M	2.07 ±0.56*	1.21 ± 0.29	1.29 ± 0.2	1.15 ± 0.11
6-PN 10^{-5}M	1.23 ± 0.36	0.70 ± 0.14	0.86 ± 0.1	1.02 ± 0.14

6-PN, 6-prenylnaringenin; 8-PN, 8-prenylnaringenin; *C3*, complement component 3; *CLU*, clusterin; *ESR1*, estrogen receptor α; Hops, hops extract; *PCNA*, proliferating cell nuclear antigen.

mRNA expression was analyzed via RT-qPCR. The samples were normalized to the housekeeping gene *RPS18*. Values are expressed as the mean ± SEM of pooled results obtained from three independent experiments. For determination of significance (*$p < 0.05$), each treatment group was compared with the vehicle (DMSO; ANOVA followed by Dunnet's post-hoc test). Values greater than 1 represent an increased gene expression, whereas data less than 1 assessed as a decrease.

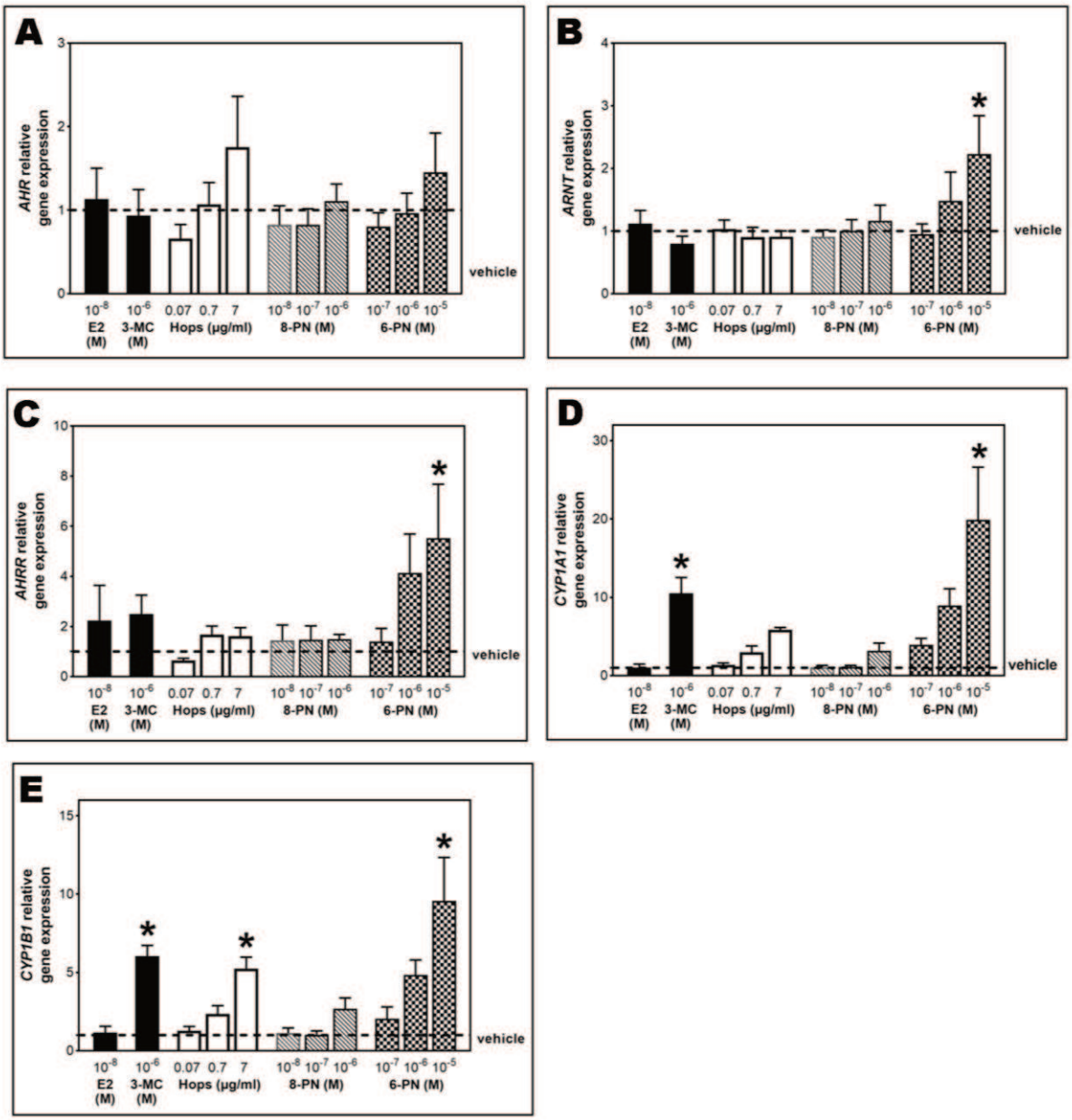

Figure 26. Effects of hops extract (Hops) and its compounds on *ARNT*, *AHRR*, *CYP1A1* and *CYP1B1* mRNA expression. Ishikawa cells were treated with E2 (10^{-8} M), 3-MC (10^{-6} M), Hops (0.07 µg/ml, 0.7 µg/ml, 7 µg/ml), 8-prenylnaringenin (8-PN; 10^{-8} M, 10^{-7} M, 10^{-6} M) and 6-prenylnaringenin (6-PN; 10^{-7} M, 10^{-6} M, 10^{-5} M). (A) *AHR* (B) *ARNT*, (C) *AHRR*, (D) *CYP1A1* and (E) *CYP1B1* mRNA expression were analyzed via RT-qPCR. The samples were normalized to the housekeeping gene RPS18. Data are expressed as the mean ± SEM of pooled results obtained from three independent experiments. For determination of significance (*$p < 0.05$), each treatment group was compared with the vehicle (DMSO: set to 1) using ANOVA followed by Dunnet's post-hoc test. Values greater than 1 represent an increased gene expression, whereas data less than 1 assessed as a decrease.

4.2. EXPERIMENT II

4.2.1. Validation of uterotrophic assay

As expected, the ovariectomy reduced the relative uterine wet weight (RUWW; Control: 2.44 ± 0.34 mg/g bw; SHAM: 2.26 ± 0.37 mg/g bw; Ovx: 0.42 ± 0.06 mg/g bw; Figure 27) and this effect was reversed by E2 treatment (E2: 3.31 ± 0.35 mg/g bw; Figure 27). These results allow us to continue the experimental protocol as shown in the following sub-sections.

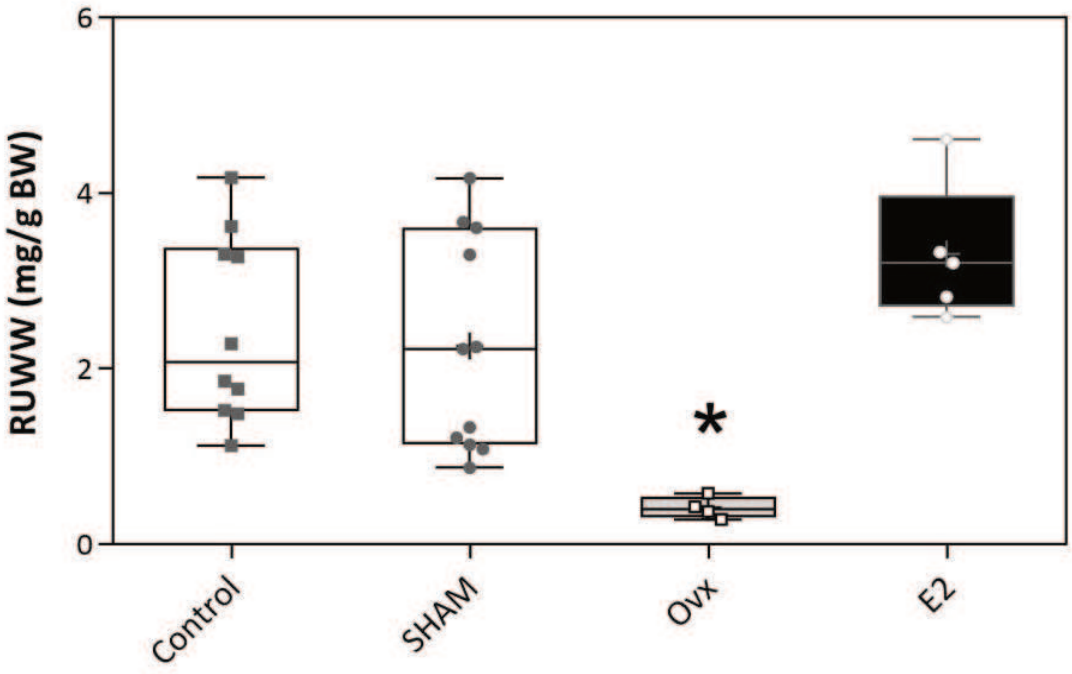

Figure 27. Relative uterine wet weight (RUWW) of Control, SHAM, Ovx and E2 groups. Data are presented as median and interquartile range (n= 5 rats per group). *, $p < 0.05$ (Kruskal-Wallis followed by Dunn's post-hoc test).

4.2.2. Effects of hops extracts

- **Hops and KO-Hops lack estrogenic effect**

On the organ level, neither Hops nor KO-Hops groups compensated for the uterine weight loss induced by the Ovx, therefore there were no differences between groups (Figure 28).

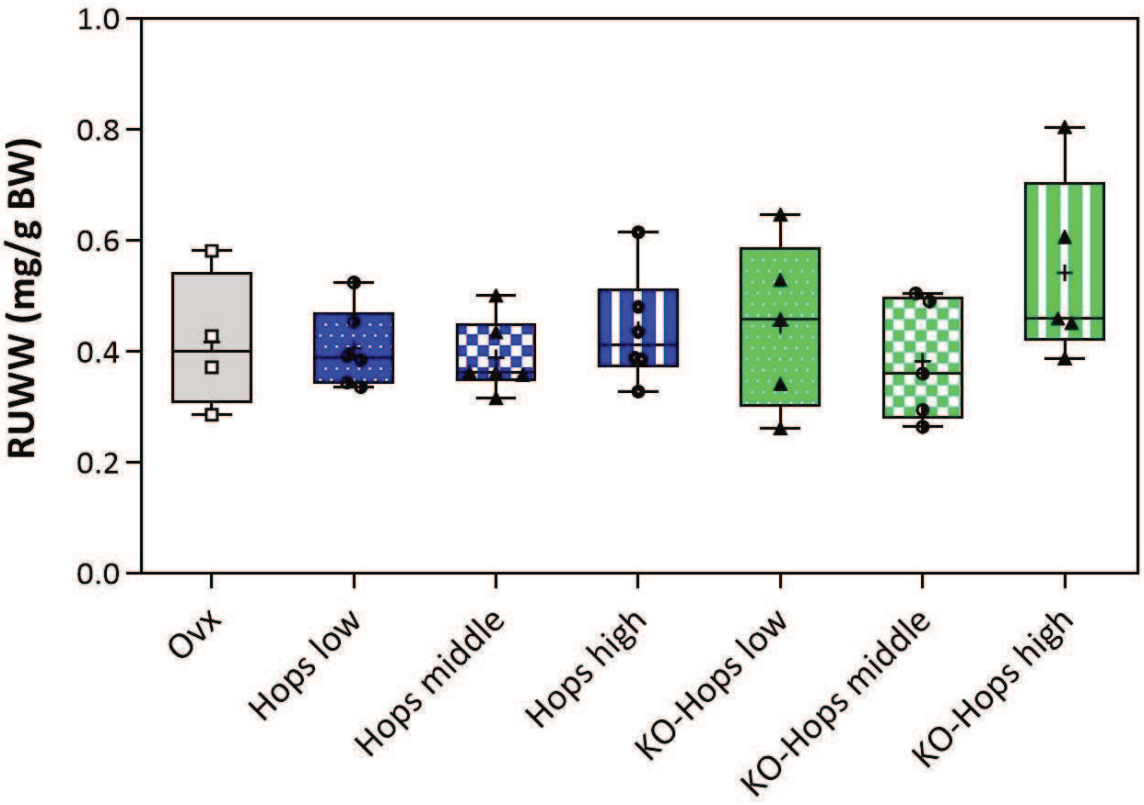

Figure 28. Effect of Hops and KO-Hops on the relative uterine wet weight (RUWW). Data are presented as median and interquartile range (n= 5 rats per group). For determination of significance, each treatment group was compared with the Ovx group (Kruskal-Wallis).

- **Hops and KO-Hops increased the glandular density**

We evaluated some estrogen-dependent endpoints such as luminal epithelial cell height, the thickness of SS and myometrium, and the glandular density. The luminal epithelial cell height and the thickness of SS and myometrium were similar between Hops and KO-Hops groups respect to the Ovx group. However, Hops middle group exhibited a higher luminal epithelial cell height respect to KO-Hops middle group (Figure 29).

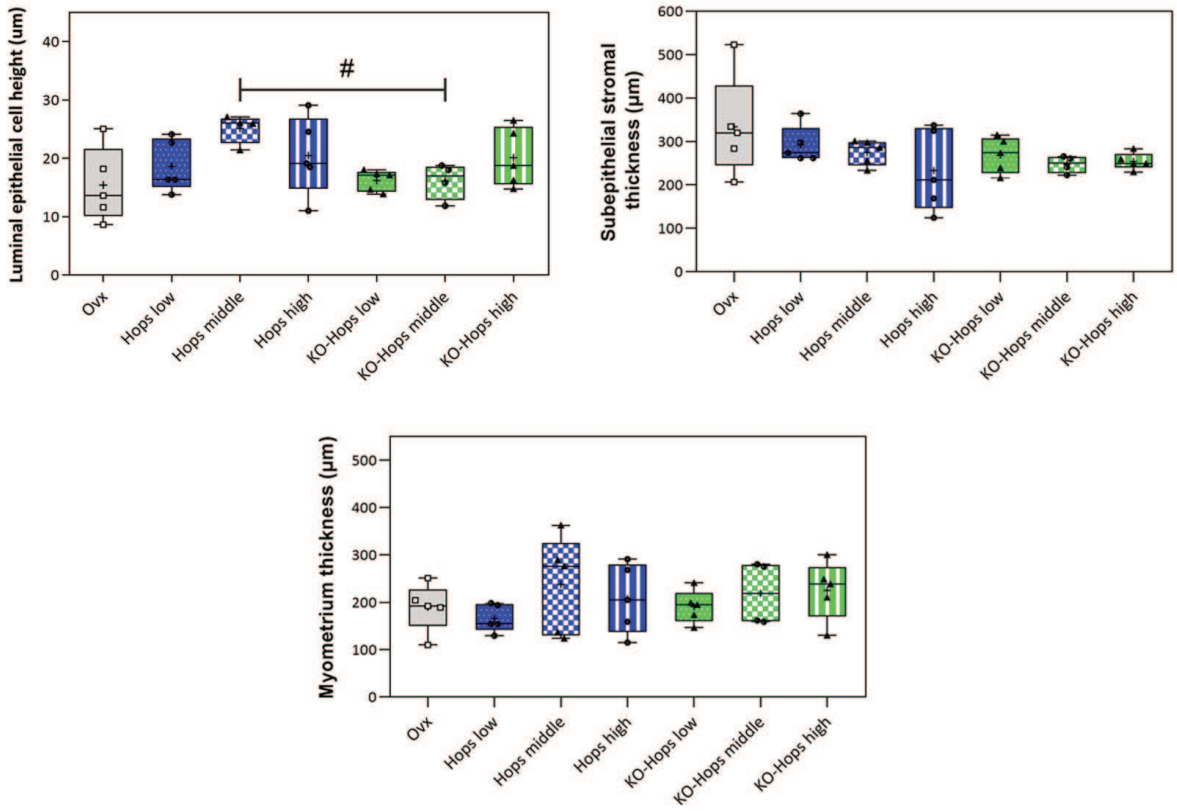

Figure 29. Quantification of luminal epithelial cell height and the thickness of subepithelial stroma and myometrium. Data are presented as median and interquartile range. (n= 5 rats per group). For determination of significance, each treatment group was compared with the Ovx group (Kruskal-Wallis). #, indicates difference between Hops middle and KO-Hops middle groups ($p < 0.05$, Mann-Whitney U test).

The uterine glandular density was higher in Hops middle and KO-Hops low groups than in Ovx group. Then, when we compared Hops and KO-Hops extract at each dose, KO-Hops low group exhibited a higher uterine glandular density than Hops low group (Figure 30).

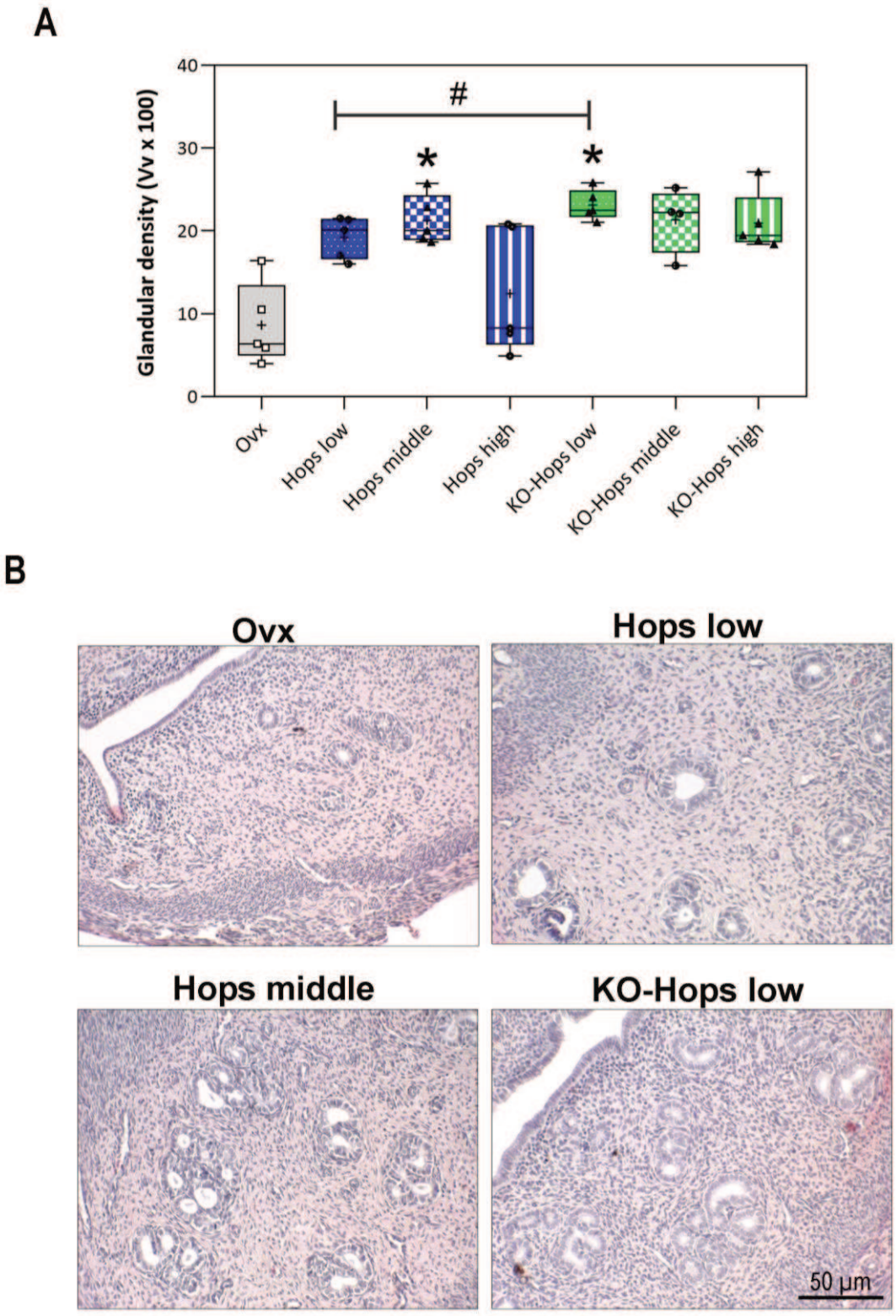

Figure 30. Effect of Hops and KO-Hops on the uterine glandular density. Data are expressed as Vv x 100 and presented as median and interquartile range (n= 5 rats per group). *, $p < 0.05$ vs. the Ovx group (Kruskal-Wallis followed by Dunn's post-hoc test). $^{\#}$, indicates difference between Hops low and KO-Hops low groups ($p < 0.05$; Mann-Whitney U

test). (B) Representative photomicrographs of the uterus stained with hematoxylin and eosin of the Ovx, Hops low, Hops middle and KO-Hops low groups.

➢ **Hops and KO-Hops did not alter the cell proliferation**

Hops and KO-Hops animals had similar cell proliferation respect to Ovx ones in LE and GE (Figure 31). However, the cell proliferation in LE of KO-Hops low group was higher than that of Hops low group (Figure 31).

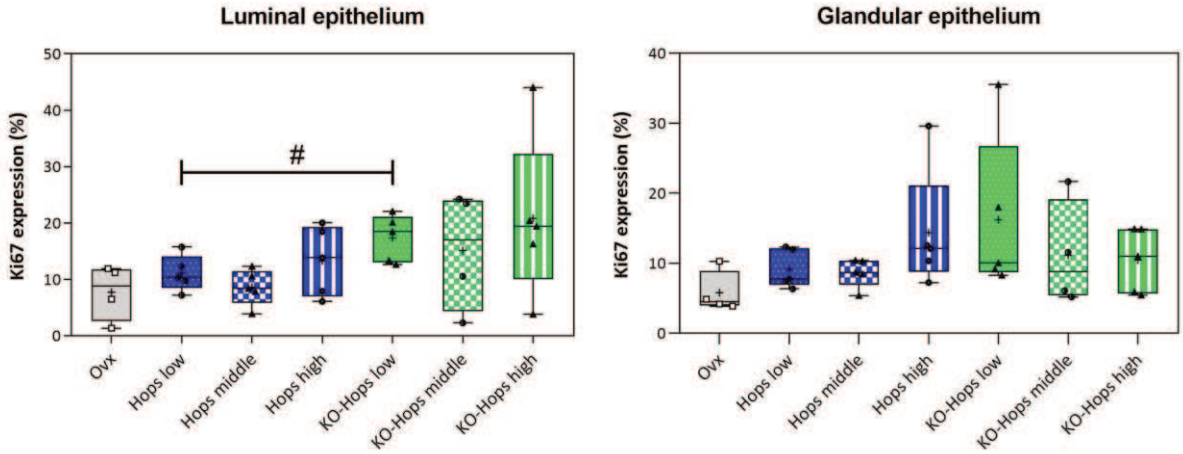

Figure 31. Quantification of cell proliferation in the luminal and glandular epithelium. Data are presented as median and interquartile range. For determination of significance, each treatment group was compared with the Ovx group (Kruskal-Wallis). #, indicates difference between Hops low and KO-Hops low ($p < 0.05$; Mann-Whitney U test).

➢ **Hops at middle concentration reduced the expression of ESR1**

The ESR1 expression was reduced in the LE of Hops middle group, respect to Ovx group (Figure 32).

➢ **Hops and KO-Hops did not modify the expression of estrogen-sensitive genes**

The expression of all estrogen-dependent genes evaluated here, namely *Esr1, Esr2, Pr, Pcna, C3* and *Clu* was similar between Hops and KO-Hops animals, respect to Ovx animals. However, KO-Hops low group increased the *C3* mRNA expression compared to Hops low group (Table 12).

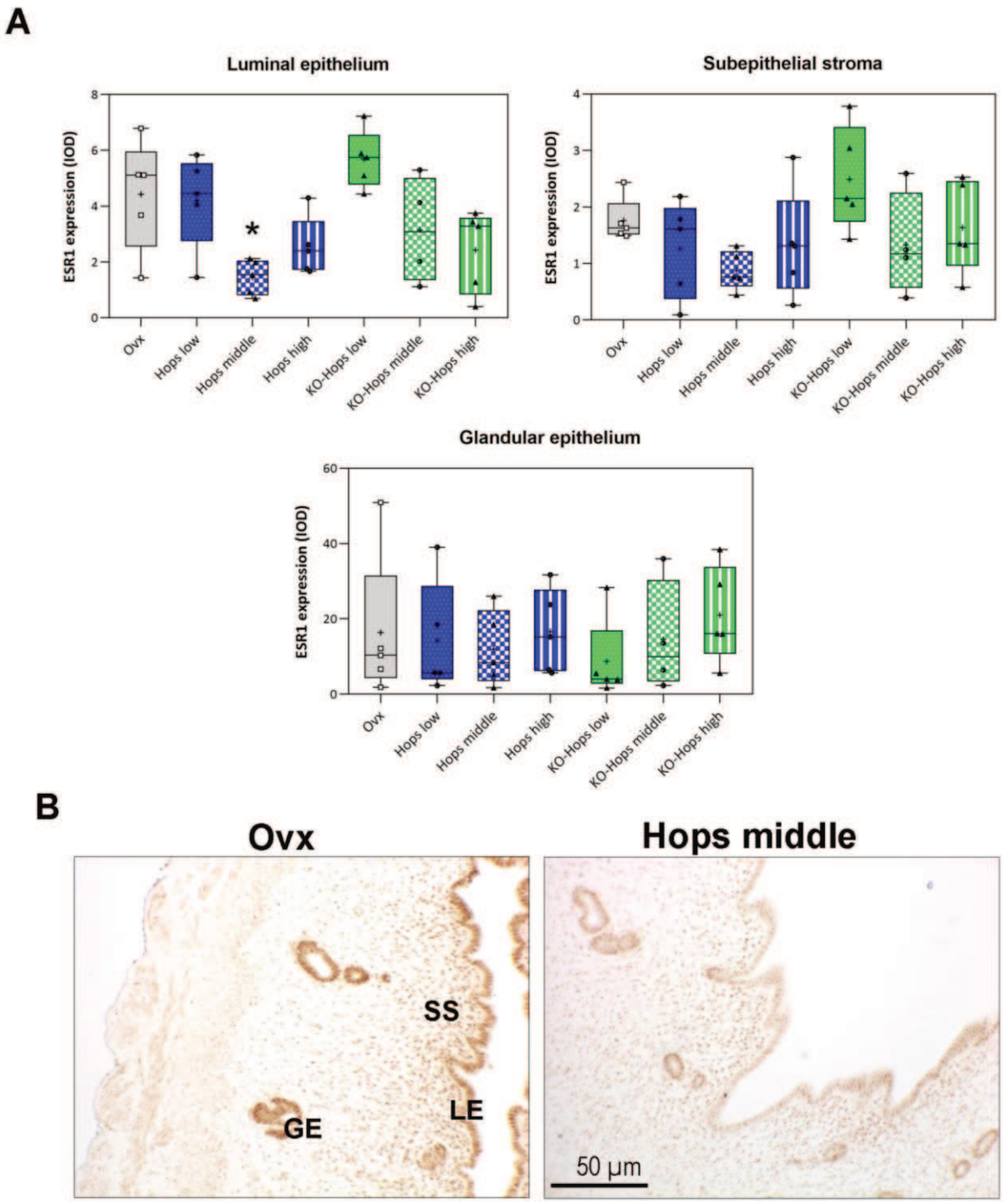

Figure 32. Effect of Hops and KO-Hops on the uterine expression of ESR1 (A) Quantification of ESR1 expression in luminal epithelium, subepithelial stroma and glandular epithelium (n= 5 rats per group), *p < 0.05 vs. the Ovx group (Kruskal-Wallis followed by Dunn's post-hoc test). (B) Representative photomicrographs of ESR1 immunoreaction on uterine sections of Ovx and Hops middle groups. LE, luminal epithelium; GE, glandular epithelium; SS, subepithelial stroma.

Table 12. Effects of Hops and KO-Hops extracts on *Esr1, Esr2, Pr, Pcna, C3* and *Clu* mRNA expression.

	Esr1 mRNA	*Esr2* mRNA	*Pr* mRNA	*Pcna* mRNA	*C3* mRNA	*Clu* mRNA
Ovx	1.06 ± 0.22	1.06 ± 0.19	1.12 ± 0.33	1.02 ± 0.10	0.42 ± 0.07	1.02 ± 0.13
Hops low	0.81 ± 0.11	0.74 ± 0.07	0.71 ± 0.17	1.03 ± 0.06	0.35 ± 0.07	1.15 ± 0.15
Hops middle	0.77 ± 0.10	1.05 ± 0.17	0.96 ± 0.20	0.86 ± 0.05	0.24 ± 0.04	0.97 ± 0.07
Hops high	0.66 ± 0.07	1.10 ± 0.15	1.15 ± 0.16	1.03 ± 0.08	0.77 ± 0.36	1.21 ± 0.09
KO-Hops low	0.76 ± 0.10	1.51 ± 0.27	1.71 ± 0.17	1.05 ± 0.05	9.60 ± 6.60 #	0.96 ± 0.22
KO-Hops middle	0.83 ± 0.16	0.77 ± 0.15	1.05 ± 0.41	1.17 ± 0.10	0.57 ± 0.23	1.09 ± 0.07
KO-Hops high	0.91 ± 0.07	0.86 ± 0.11	1.10 ± 0.06	1.03 ± 0.06	0.43 ± 0.10	1.23 ± 0.15

C3, complement component 3; *Clu*, clusterin; *Esr1*, estrogen receptor α; *Esr2*, estrogen receptor β; *Pcna*, proliferating cell nuclear antigen; *Pr*, progesterone receptor. mRNA expression were analyzed via RT-qPCR. The samples were normalized to the housekeeping gene RPS18. Values are expressed as the mean ± SEM. (n= 5 rats per group). For determination of significance, each treatment group was compared with the Ovx group (Kruskal-Wallis). #, $p < 0.05$ vs. the Hops low group (Mann-Whitney U test). Values greater than 1 represent an increased gene expression, whereas data less than 1 assessed as a decrease.

5. Discussion

The perception that herbal remedies are safer than conventional medications could explain why postmenopausal women have chosen some BDS as an alternative to traditional estrogen replacement therapies (Gong et al., 2016; Van Bremen, 2015). Therefore, the characterization of these BDS regarding potential health effects and risks has been on demand. In this Chapter, we aimed to evaluate the safety of hops extract in the uterus by using *in vitro* and *in vivo* studies. For *EXPERIMENT I*, we determined the roles of ERα and AHR signaling pathways in the actions of hops extract, 8-PN, and 6-PN in endometrial adenocarcinoma cell line Ishikawa. For *EXPERIMENT II,* we evaluated the estrogenic effect of hops extract and an extract reduced in XH in the uterus of rats by using a classical uterotrophic assay.

For ***EXPERIMENT I,*** we evaluated the estrogenic activity of hops extract, 8-PN, and 6-PN in the endometrial adenocarcinoma cell line Ishikawa. We determined their effects on the induction of a reporter gene activity through ERα activation and of AlkP activity, both assays target key points in the ER signaling pathway. Our results agree with others previously reported: hops extract (Hajirahimkhan et al., 2013; Liu et al., 2001; Overk et al., 2005) and 8-PN have estrogenic activity in Ishikawa cells (Aichinger et al., 2018; Hajirahimkhan et al., 2013; Kretzschmar et al., 2010; Overk et al., 2005; Wober et al., 2003). The estrogenic effect of 8-PN was shown to be inhibited by Fulvestrant, an ER antagonist, confirming that this response is a consequence of ER activation (Aichinger et al., 2018; Effenberger et al., 2005). Moreover, 6-PN at 10^{-6} M and 10^{-5} M had estrogenic activity by the ERE-luciferase assay, and 6-PN at 10^{-6} M had also estrogenic activity by the AlkP activity assay. Our results about the estrogenic activity of 6-PN disagree with others reported by using AlkP activity assay. 6-PN showed either a very weak estrogenic activity (Milligan et al., 1999) or even no estrogenic activity (Overk et al., 2005) by using 3 and 4 days of exposure, respectively. These apparent discrepancies about estrogenic or not estrogenic responses could be attributed to different exposure times, medium conditions, and the number of passages (Anzai et al., 1989; Holinka et al., 1986; Nishida, 2002; Pisha & Pezzuto, 1997; Wormke et al., 2000).

The principle of traditional toxicology “the dose makes the poison” meaning higher doses have a greater impact than lower ones (which might have no effect) does not apply to all compounds (Gross, 2007; Vandenberg et al., 2012). In this sense, we observed that hops extract and 6-PN exhibited monotonic dose-response curves of estrogenicity by using the ERE-luciferase assay and non-monotonic dose-response curves by using the AlkP activity assay. The type of non-monotonic curve observed by the exposure to hops extract and 6-PN

was inverted U-shaped, with maximal responses observed at intermediate doses (Vandenberg et al., 2012). Also, 8-PN exhibited a response that is neither monotonic, both for ERE-luciferase nor AlkP activity assays.

Some BDS for menopausal symptoms contain AHR agonists that can increase estrogen metabolism (Collins et al., 2009; Dunlap et al., 2017; Gaube et al., 2007; Hitzman et al., 2020; Wang et al., 2016). Here, hops extract and 6-PN acted as AHR agonists in Ishikawa cells. We therefore suggest that hops extract activated the AHR pathway through its constituent 6-PN. These results are in agreement with previous studies (Hitzman et al., 2020; Wang et al., 2016), who found that hops extract and 6-PN but not 8-PN activates the AHR pathway in MCF-7 breast cancer cells.

The AHR, upon binding of a ligand, forms a heterodimeric complex with ARNT, which induces estrogen-metabolizing enzymes CYP1A1 and CYP1B1 (Larigot et al., 2018; MacPherson et al., 2014; Mimura et al., 1999). The expression of both *CYP1A1* and *CYP1B1* depends on the AHR activation, as was previously confirmed by the use of siRNA-mediated AHR silencing and AHR antagonists (Kim et al., 2006; Kulas et al., 2021; Rasmussen et al., 2016; Roblin et al., 2004). As other AHR agonists, 3-MC is able to up-regulate the gene expression of *CYP1A1* and *CYP1B1* in Ishikawa cells. The expression of these genes is also modulated by ERα (Hitzman et al., 2020; Tsuchiya et al., 2004). Although E2 modulates their gene expression in ER-positive breast cancer cells (Hitzman et al., 2020; Tsuchiya et al., 2004) and in the rat uterus (Kretzschmar et al., 2010b; Rataj et al., 2012), no changes in the gene expression of *CYP1A1* and *CYP1B1* were found by E2 treatment. The lack of *CYP1B1* mRNA expression induction by E2 treatment was also reported in Ishikawa cells (Tsuchiya et al., 2004).

The estrogen metabolism catalyzed by CYP1A1 is considered a detoxification pathway, whereas the estrogen metabolism catalyzed by CYP1B1 is considered a genotoxic pathway (Cavalieri et al., 1997; Lakhani et al., 2003; Tarnow et al., 2019). The expression of both *CYP1A1* and *CYP1B1* is activated by some BDS, namely genistein, daidzein, and S-equol from soy, and liquiritigen from licorice root, and such effect is mediated through AHR pathway (Gong et al., 2016). In this thesis, hops extract at a concentration of 7 µg/ml up-regulated the expression of *CYP1B1* mRNA in Ishikawa cells. The induction or not of *CYP1B1* mRNA expression is dependent not only on the kind of BDS but also on the studied cell line. For example, other BDS, like red clover and *Prunella vulgaris* increased the *CYP1B1* mRNA expression in endometrial and breast cancer cells (Collins et al., 2009; Dunlap et al., 2017). However, hops extract did not increase the *CYP1B1* mRNA expression in breast cancer (Hitzman et al., 2020; Wang et al., 2016). The different response of hops

extract in our study and in those of Hitzman et al. (2020) and Wang et al. (2016) could be partially explained by the use of different cell lines obtained from different tissues. In addition, 6-PN at a concentration of 10^{-5} M increased the *CYP1A1* and *CYP1B1* mRNA expression. 6-PN might promote the induction of the detoxification pathway over the genotoxic pathway in the endometrium, reflected by a higher up-regulation of *CYP1A1* than the *CYP1B1* mRNA expression. However, we only evaluated the expression at the mRNA level but not the protein expression, leaving some uncertainty. Overall, the present outcomes agree with previous studies in breast cancer cells by showing that 6-PN preferentially affects the mechanism of detoxification of estrogens (Hitzman et al., 2020; Wang et al., 2016). The increased CYP1A1 dependent detoxification pathway may be due to the promotion of proteasomal degradation of ERα, which up-regulates *CYP1A1* transcription and also reduces ERα-mediated down-regulation of *CYP1A1* (Hitzman et al., 2020). The interaction between AHR and ERα was also demonstrated in the uterus and mammary gland of ovariectomized rats (Helle et al., 2016, 2017). The AHR activation by 3-MC inhibits E2-dependent regulation of gene expression demonstrating its antiestrogenic effect. The antiestrogenic effect in the mammary gland is partly due to lower protein levels of ESR1 in ductal epithelial cells (Helle et al., 2016), whereas the antiestrogenic effect in the uterus is due to higher ESR2 levels in the endometrial epithelium (Helle et al., 2017). Based on the antiestrogenic effect of AHR ligands by interaction with the ERα pathway, 6-PN could be utilized for the prevention of hormone-dependent cancers like breast and endometrial cancer. In relation to our results, 6-PN activates the AHR pathway and the expression of genes involved in the estrogen metabolism. These effects were observed with 6-PN at a concentration of 10^{-5} M, which is approximately 16 times higher than the maximum plasma concentrations (6×10^{-7} M) found in women after taking a single oral dose of 500 mg 6-PN (Calvo-Castro et al., 2018). Thus, further research is needed to define a prevention treatment with 6-PN for women.

The mechanism by which AHR regulates its target genes includes negative feedback loop via the AHRR. AHRR suppresses AHR activity by binding to ARNT and DRE (AHRR-ARNT complex), and is able to modulate the transcription of AHR-dependent genes (Larigot et al., 2018). As 6-PN at a concentration of 10^{-5} M up-regulated not only the *ARNT* but also the *AHRR* mRNA expression, the overexpression of *ARNT* by 6-PN does not affect the capacity of *AHRR* to repress AHR. In fact, the expression of *AHRR* gene is induced by the AHR/ARNT heterodimer (Mimura et al., 1999). The increased gene expression of *AHRR* was also observed in a number of cell lines exposed to other AHR agonists, including 3-MC (Baba et al., 2001; MacPherson et al., 2014; Tsuchiya et al., 2003; Vorrink et al., 2014). Since *AHRR* is a target molecule for therapeutic intervention against human cancer because

it functions as a tumor suppressor gene (Zudaire et al., 2008), we hypothesize that 6-PN may have health benefits on the endometrium.

With the *EXPERIMENT I* we could provide new insights into the actions of the ERα and AHR agonist 6-PN in an *in vitro* model. We demonstrated that 6-PN up-regulates the gene expression of *AHRR*, a known tumor suppressor gene, and that of *CYP1A1* and *CYP1B1*, genes involved in the metabolism of estrogens. However, hops extract only modulates the genotoxic pathway of estrogen metabolism by an up-regulation of *CYP1B1*. Based on the gene regulation pattern in response to 6-PN, a 6-PN-riched hop extract might contribute to antitumor activity on the endometrium. In addition, hops extract and 6-PN activated both ERα and AHR pathways and this implies that both receptors are regulators of the biology and pharmacology of hops preparations. Moreover, hops extract, 8-PN and 6-PN have estrogenic activity demonstrated by the activation of the ERE-luciferase and the AlkP induction assays. Keeping in mind that hops extracts are composed not only of 8-PN and 6-PN but also of other chemical compounds, we would like to highlight that it is essential to perform other studies with hops extract to give a detailed comprehension of the involved pathways.

In ***EXPERIMENT II,*** we tested the possible estrogenic effect of hops extract and an extract reduced in XH, using a 3-day uterotrophic assay in adult rats (a classical tool for the detection of estrogenicity). First, it was necessary to validate the uterotrophic assay in our animals. A substance is said to behave as an estrogenic compound when it can induce an increase in the uterine wet weight, being this result a hallmark of positive uterotrophic assay (Diel et al., 2004; Kanno et al., 2003; Newbold et al., 2001; Owens & Koëter, 2003). After 14 days of ovariectomy, there is an important reduction of endogenous estrogens that induces uterine atrophy, and the treatment with E2 for 3 days is able to reverse such atrophy (Zingue et al., 2017). Our results show that ovariectomy produced the characteristic reduction of uterine weight and that E2 group exhibited its typical uterotrophic response, by increasing the uterine weight. Thus, we could validate our experiment.

Different *in vivo* studies performed in ovariectomized rats have demonstrated that 8-PN increased the uterine wet weight (Diel et al., 2004; Overk et al., 2008; Rimoldi et al., 2006; Zierau et al., 2008). However, we found that 8-PN contained in both hops extracts did not increase the uterine wet weight of ovariectomized rats. These present results could be explained by the AHR agonistic activity of 6-PN that we demonstrated in the *EXPERIMENT I.* Since several publications described an AHR-ER cross-talk in the uterus (Helle et al., 2017; Shanle & Xu, 2011), a modulation of ER signaling by AHR activation could not be excluded. The lack of estrogen response of hops extract observed here agrees with previous

reports using hops extract at a concentration in the same order of magnitude (60 mg/kg bw/day) (Keiler et al., 2017a) or even at the same concentration (40 mg/kg bw/day) (Overk et al., 2008). However, our *in vivo* results are in contrast to our *in vitro* data on ERα transactivation and AlkP activity assays showing estrogenic activity. Based on our *in vitro* results and the mentioned *in vivo* studies (Diel et al., 2004; Overk et al., 2008; Rimoldi et al., 2006; Zierau et al., 2008), we can infer that the bioavailability of 8-PN concentrations in both hops extracts was not high enough to increase the uterine mass in these animals.

The uterotrophic assay has given negative results for several well-known estrogen-mimics compounds, such as bisphenol A, genistein, endosulfan, and kepone (Diel et al., 2000; Möller et al., 2010; Newbold et al., 2001). Thus, the sensitivity of this assay to test possible estrogen-mimics compounds at low doses has been questioned (Diel et al., 2000; Newbold et al., 2001). Studying only the RUWW (a macroscopic parameter) could give a false negative result. Thus, the morphological evaluation and molecular targets offer the opportunity to increase the likelihood of identifying estrogenic effects that could have been missed. A deep approach on the effects of both hops extracts was achieved by the histological study of uterine tissue. This approach allowed us to assess different cellular endpoints associated with uterine effects that could be associated with endometrial carcinogenesis (Sanderson et al., 2016). One of the best-recognized estrogenic uterine responses is the change of epithelial cells from cubic to columnar, increasing the luminal epithelial cell height (Padilla-Banks et al., 2001; Rimoldi et al., 2006). We found no changes in the luminal epithelial cell height after the exposure to both hops extracts. This result is in line with Keiler et al. (2017a), who found no estrogenic effect in response to the hops extract with regard to luminal epithelial cell height in ovariectomized rats. Another morphological parameter proposed to enhance the sensitivity of the uterotrophic assay is the determination of endometrial gland number (Newbold et al., 2001). We found that animals exposed to Hops at 40 mg/kg bw/day and KO-Hops at 8 mg/kg bw/day showed an increased glandular density, a feature of endometrial hyperplasia (Sanderson et al., 2016). As mentioned, this pathology is a central clinical topic, because it has the potential to transform into cancer.

The uterotrophic response has been attributed to at least two events: water imbibitions and/or cell proliferation. These trophic and proliferative effects on the uterus are mediated by the ESR1. We determined the expression of ESR1 on two independent experimental levels (mRNA and protein expression) and cell proliferation. No changes in cell proliferation were found between animals treated with both types of extracts respect to animals treated with the vehicle, as previously reported by Keiler et al. (2017a). Animals of the Hops middle group exhibited a reduced protein ESR1 expression in the LE. The reduced ESR1 expression is in agreement with those induced by pure 8-PN and E2 (Diel et al., 2004; Zierau et al., 2008). In

addition, we quantified the mRNA expression of *Pr*, *C3*, and *Clu*, reliable uterine response genes. Neither changes in *Pr, C3* nor *Clu* expression were found in Hops and KO-Hops animals, relative to Ovx one. The lack of effect at the mRNA level reinforces the no estrogenic property of these extracts.

The estrogenic effect of 8-PN depends not only on its dose in the extract but also on its conversion from XH, the main source of phytoestrogens in the diet (Liu et al., 2015; Tronina et al., 2020). In the extract, the amount of 8-PN is very low being around 100 times lower than that of XH. However, the metabolism of XH (Bolton et al., 2019) leads to a higher serum concentration of 8-PN than that of XH in the body of rats (Keiler 2017a,b). In the present study, animals of the Hops middle group exhibited a higher luminal epithelial cell height respect to KO-Hops middle ones. Since it is known that this histological estrogen-induced feature is increased by 8-PN (Diel et al., 2004; Overk et al., 2008), we can infer that the XH contained in the Hops extract was converted into 8-PN in the body of rats. However, the XH conversion and/or the bioavailability of 8-PN in the extract were not enough to induce a statistical increase respect to the Ovx group. In contrast, an induction of some estrogen endpoints was observed in KO-Hops low respect to Hops low, namely the glandular density, cell proliferation and *C3* gene expression. Such parameters are well known to be increased by the exposure to 8-PN and E2 (Christoffel et al., 2006; Diel et al., 2004; Newbold et al., 2001). The fact that the KO-Hops at 8 mg/kg bw/day extract induced these estrogenic parameters respect to Hops extract at the same dose is suggesting an inhibitory effect of XH on 8-PN activity, as was previously observed in a human endometrial cancer cell line (Dietz et al., 2017). We also can infer that the metabolism of XH to 8-PN was not enough to alter such parameters or that the effect of antagonism was higher than the effect of metabolism. However, these theories are frankly speculative because we did not determine the serum levels of hops compounds.

Overall, in *EXPERIMENT II*, we observed no estrogenic uterine effects in response to *Hops* extracts, independently of their composition (*Hops* and *KO-Hops,* did not show estrogenic response). We would like to highlight that we observed an increased glandular density by the exposure to Hops extract at 40 mg/kg bw/day accompanied by alteration in the ESR1 expression and an increased glandular density by the exposure to KO-Hops at 8 mg/kg bw/day. Thus, our data together with those in the literature (Keiler et al. 2017a; Overk et al., 2008) reinforce the hypothesis that hops extract has no estrogenic property in the uterus of rats. On the other hand, we are aware of a certain weakness of our study: the small number of animals used. This fact could weaken the robustness of the statistical analysis, since some results are shown as not having a significant difference despite the fact that they appear to be so at first glance.

In conclusion, our results demonstrating a weak estrogenic effect on the uterus suggest minimal probability to induce endometrial hyperplasia and cancer associated and could be a safer alternative to the conventional HRT.

Finally, as it is reflected in these studies, our lifestyle has a key role in our health and can influence the development of a number of diseases such as uterine pathologies, thus we encourage promoting a healthy lifestyle to avoid consequences on female human health.

Conclusions

The present PhD thesis provided evidence that GBH provoked molecular and morphological alterations involved in endometrial disorders, in both experimental models of exposure. It is important to highlight that these effects were observed at a concentration that is in the order of magnitude of RfD and that represents a realistic concentration to be found in everyday life.

Despite there being controversies regarding glyphosate effects on health, our findings call into question the safety of this herbicide, adding evidence as a possible carcinogen for humans, supporting the IARC's conclusion. At this time, we consider that experimental and epidemiological studies are needed to evaluate the safety of GBHs to provide recommendations and guidelines to regulate its use. Thus, additional studies are urgently needed to determine the potential health implications for animal and human populations exposed to this herbicide.

It is out of question that humans and animals must be better protected than now, especially through new rules and limitations regarding the usage of GBHs. We consider that it is the duty of the institutions to place the "precautionary principle" before economic interests, namely the protection of humans and animals from exposure to a substance whose side effects are not yet known.

Regarding the hops extract, we could demonstrate its uterine safety by *in vitro* and *in vivo* studies. By the *in vitro* study, we demonstrate the relevant role of 6-PN contained in the hops extract as a potential modulator of estrogen metabolism due to its ERα and AHR agonist activity. Thus, we proposed 6-PN as a beneficial compound on the endometrium. However, hops extract had estrogenic activity. In this sense, since hops supplements are widely used by women for menopausal symptom relief, we suggest the need for caution as well as more research about the use of hops preparations because of the role of estrogenic compounds on the endometrial cancer development.

By the *in vivo* study, we showed a weak estrogenic effect of two types of hops extracts on the uterus. Thus, no adverse side effects due to the application of the hops extract in menopausal applications can be delineated, being a safer alternative to the conventional HRT.

Since the "DESIGNER" (Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources) technology can modify the hops compound by selectively enriching compounds of interest (Ramos Alvarenga et al., 2014; Dietz et al., 2017), a 6-PN-riched hop extract might contribute to antitumor activity on the endometrium.

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