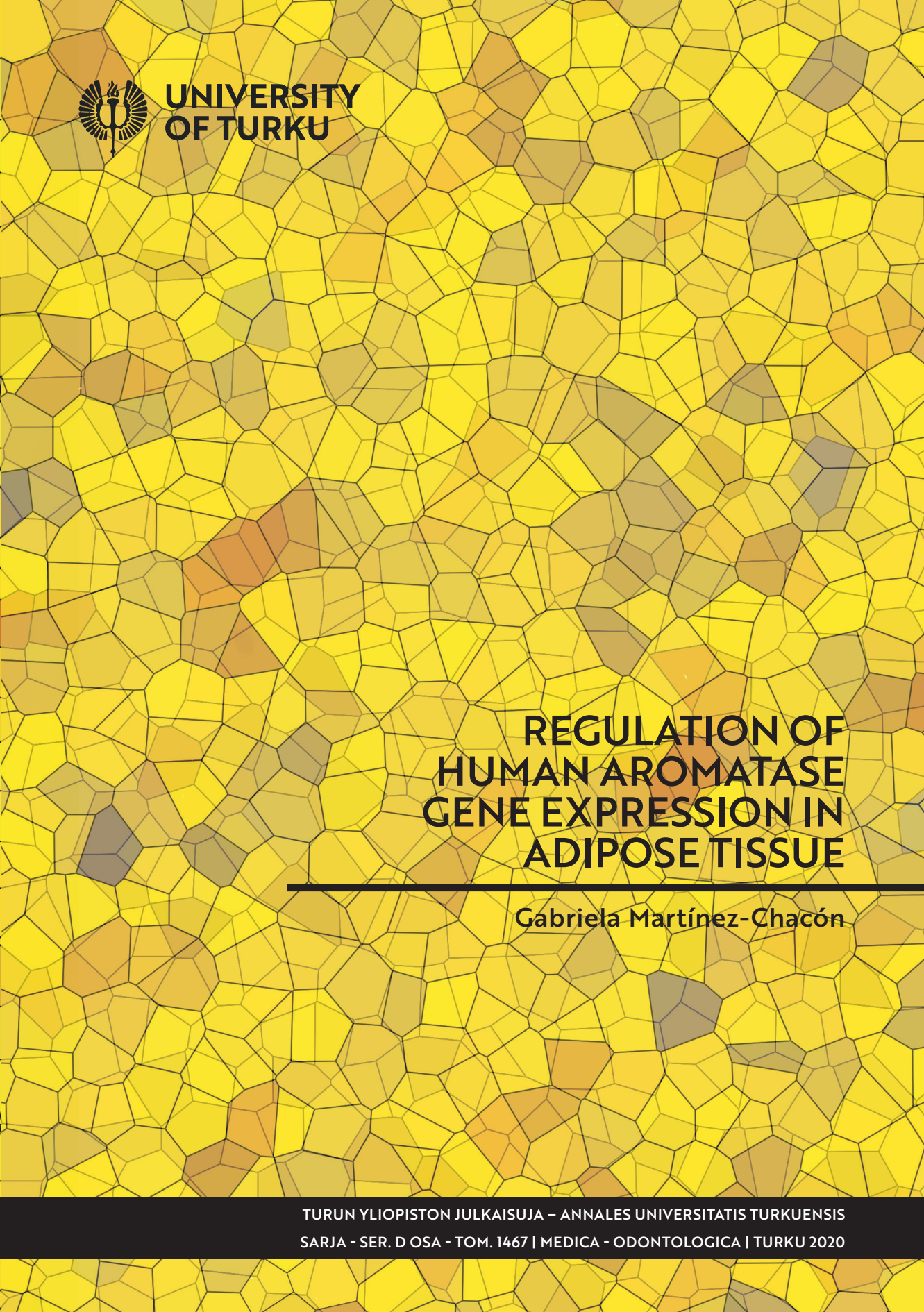

REGULATION OF HUMAN AROMATASE GENE EXPRESSION IN ADIPOSE TISSUE

Gabriela Martínez-Chacón

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University of Turku

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“I believe that nothing happens by chance. Deep down, things have their own secret plan, even though we don't understand it”

— Carlos Ruiz Zafón, The Shadow of the Wind

“Pa’ lante, Pa’ lante como un elefante”

— Battle cry of my grandma. “Keep going, keep going as an elephant”

UNIVERSITY OF TURKU
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ABSTRACT

The conversion of androgens to estrogens is catalyzed by the aromatase enzyme, the product of the *CYP19A1* gene, in gonads and peripheral tissues. Estrogens produced in the white adipose tissue (WAT) are postulated to play a central role in the development of postmenopausal breast cancer in women and male obesity-related secondary hypogonadism (MOSH). Obesity-related WAT inflammation is considered one of the main driving forces of excessive aromatization in WAT. Regulation of *CYP19A1* gene in WAT involves the activation of the glucocorticoid-dependent promoter I.4 and the balanced action of local inducers and repressors. This process is complex, and the mechanism behind the link between adiposity, WAT inflammation and excessive aromatization are not well understood. By using the aromatase reporter (hARO-Luc) mouse model and primary adipose stromal cells (ASCs) and adipose tissue samples, two factors, interleukin 10 (IL-10), an anti-inflammatory cytokine, and CC chemokine ligand 2 (CCL2), a proinflammatory chemokine, were found to modulate aromatase gene expression. While IL-10 acts as a suppressor of *CYP19A1* via PI.4, CCL2 stimulates this process in ASCs. This was confirmed *in vivo* in hARO-Luc mouse model, where obesity-related increase in the expression of aromatase reporter in WAT associated with lower IL-10 and/or higher CCL2 levels. Moreover, subcutaneous fat from obese women expresses significantly higher CCL2 and *CYP19A1* mRNA levels. As a further proof-of-concept, we showed that attenuation of WAT inflammation by anti-oxidative plant polyphenols in diet is associated with decrease in aromatase reporter expression in hARO-Luc males. *In vitro* studies confirmed that polyphenols modulate the expression of *CYP19A1* in stromal cells. Overall, these results may bring valuable insights into the mechanisms driving aromatase gene expression in WAT in postmenopausal women and men, as well as new approaches for the prevention of breast cancer.

KEYWORDS: Obesity, CYP19A1, white adipose tissue, inflammation, IL-10, CCL2, dietary polyphenols

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TIIVISTELMÄ

Aromataasientsyymi katalysoi estrogeenituotannon viimeistä vaihetta, jossa androgeenit muuntuvat estrogeeneiksi. Aromataasigeeni (*CYP19A1*-geeni) ilmentyy erityisesti sukupuolirauhasissa, mutta myös lukuisissa muissa kudoksissa, kuten rasvassa ja aivoissa. Valkoisessa rasvakudoksessa tuotettujen estrogeenien oletetaan vaikuttavan keskeisesti rintasyövän kehitykseen vaihdevuosi-iän ohittaneilla naisilla ja miehillä lihavuuteen liittyvän sekundaarisen hypogonadismin kehittymiseen. Lihavuuteen liittyvää valkoisen rasvakudoksen tulehdusta pidetään merkittävänä paikallista aromatisaatiota lisäävänä tekijänä. Muutokset tulehdusta edistävien ja hillitsevien tekijöiden pitoisuuksissa lisäävät aromataasientsyymin tuotantoa valkoisessa rasvakudoksessa aktivoimalla *CYP19A1*-geenin ilmentymistä. Ihmisen aromataasigeenin ilmentymistä säätelevät hyvin monet eri tekijät lukuisten kudosspesifisten promoottereiden välityksellä. Rasvakudoksessa aromaasigeenin ilmentymistä säädellään mm. glukokortikoidiriippuvaisen promotterin I.4 kautta. Säätelymekanismi on hyvin monimutkainen ja huonosti tunnettu.

Tässä työssä käytettiin naisten rinnan rasvan välikudoksesta eristettyjä mesenkymaalisia soluja, rasvakudosnäytteitä, sekä siirtogeenistä ihmisen aromataasigeenin säätelyalueen raportoijahiirimallia (hARO-Luc). Työssä osoitettiin, että tulehdusta hillitsevä sytokiini interleukiini 10 (IL-10) sekä tulehdusta edistävä CC kemokiini ligandi 2 (CCL2) muokkaavat *CYP19A1*-geenin ilmentymistä rasvakudoksessa. Rinnan välikudoksen soluissa IL-10 vähensi I.4. promoottorin kautta välittyvää *CYP19A1*-ilmentymistä kun taas CCL2 lisäsi sitä. Tulos vahvistettiin *in vivo* hARO-Luc hiirissä osoittaen, että lihavuuteen liittyvä lisääntynyt aromataasiraportoijan ilmentyminen rasvakudoksessa oli yhteydessä kudoksen vähentyneeseen IL-10-pitoisuuteen ja lisääntyneeseen CCL2 pitoisuuteen. Myös lihavien naisten ihonalaisen rasvan näytteissä *CYP19A1* ja *CCL2* ilmentyivät enemmän kuin vastaavissa normaalipainoisten naisten näytteissä. Lisäksi osoitimme, että rehun antioksidatiiviset kasvipolyfenolit vähensivät valkoisen rasvan matala-asteista tulehdusta ja aromataasiraportoijan ilmentymistä hARO-Luc koirashiirissä, mikä vahvistaa havaintoa rasvakudoksen tulehduksen ja *CYP19A1*-ilmentymisen välisestä yhteydestä. *In vitro* tutkimuksemme osoittivat, että kasvien polyphenolit muokkaavat *CYP19A1*-geenin ilmentymistä rasvan välikudoksen soluissa. Tulokset auttavat ymmärtämään aromataasigeenin ilmentymisen säätelymekanismeja miesten ja vaihdevuosi-iän ohittaneiden naisten rasvakudoksessa ja saattavat tarjota uusia keinoja rintasyövän ehkäisyyn.

AVAINSANAT: Lihavuus, *CYP19A1*, valkoinen rasvakudos, tulehdus, IL-10, CCL2, ravinnon polyfenolit

Table of Contents

Abbreviations

ACTB	β-actin
AI	Aromatase inhibitor
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
$Arom^{hum}$	Humanized aromatase mouse model
ATF-2	Activating transcription factor 2
ASCs	Adipose stromal cells
A4	Androstenedione
BAF	Breast adipose fibroblast
BrAT	Breast adipose tissue
BAY 11-7082	NFκB inhibitor
BMI	Body mass index
CAFs	Cancer associated fibroblast
cAMP	Cyclic adenosine monophosphate
CCL2	CC chemokine ligand 2
CCR2	CCL2 receptor
cDNA	Complementary DNA
CLS	Crown like structures
COX-2	Cyclooxygenase-2
CREB	cAMP-response element-binding protein
CYP19A1	Aromatase protein
CYP19A1	Human aromatase gene
Cyp19a1	Mouse aromatase gene
DEX	Dexamethasone
DHE	Dehydroepiandrostenedione
DHEA-S	Dehydroepiandrostenedione sulfate
DMSO	Dimethylsulphoxide
EGR2	Epidermal growth factor receptor
END	Enterodiol
EN	Enterolactone

EP	Prostaglandin E2 receptor
ER	Estrogen receptor
ERK1/2	Extracellular signal-regulated protein kinases
E1	Estrone
E1S	Estrone sulfate
E2	Estradiol
E3	Estriol
FFA	Free fatty acids
FIAF	Fasting-induced adipose factor
FSK	Forskolin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma interferon activation site
GC-MS	Gas chromatography–mass spectrometry
GNS	Genistein
GLP-1	Glucagon-like peptide 1
GPCR	G protein couple receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
G6PD	Glucose-6-phosphate dehydrogenase
hARO-Luc	Human aromatase Luc reporter mouse model
HER-2	Human epidermal growth factor receptor 2
HFD	High fat diet
HIF-1α	Hypoxia inducible factor 1α
HRT	Hormone replacement therapy
HSDs	Hydrodysteroid dehydrogenases
IC50	Half maximal inhibitory concentration
IDC	Invasive ductal carcinoma
IFNγ	Interferon γ
IGF-1	Insulin like growth factor 1
IKK	IκB kinase
IL	Interleukin
JAK	Janus kinase
LDL	Low-density lipoprotein
LFD	Low fat diet
LKB1	Serine–threonine liver kinase B1
LPL	Lipoprotein lipase
LRH-1	Liver receptor homolog 1
LSmix	Stilbenoid mixture
L19	Ribosomal protein L19
MAPK	Mitogen-activated protein kinase
MePS	Pinosylvin monomethylether

Min	Minutes
MOSH	Male obesity-associated secondary hypogonadism
MR	Mateiresinol
MSCs	Mesenchymal stromal stem cells
M1	Activated macrophages
M2	Alternative activated macrophages
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor-κB
NR5A	Nuclear receptor 5A
NSAIDs	Non-steroidal anti-inflammatory drugs
NTG	Nortrachelogenin
OSM	Oncostatin
OVX	Ovariectomized / ovariectomy
PAI-1	Plasminogen activator inhibitor-1
PGE2	Prostaglandin E2
PII	Aromatase promoter II
PI.4	Aromatase promoter I.4
PI.3	Aromatase promoter I.3
PI3K	Phosphatidylinositol 3-phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKE	Polyphenol rich pine knot extract
PMA	Phorbol 12- myristate 13-acetate
Ppia	Peptidylprolyl isomerase A
PS	Pinosylvin
PYY	Peptide YY
P38/JNK1	p38 mitogen-MAPK/Jun N-terminal kinase
qPCR	Quantitative polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSV	Resveratrol
RS504303	CCR2 inhibitor
SB 239063	p38-MAPK inhibitor
SCFA	Short chain fatty acids
Sec	Seconds
SECO	Secoisolarecinol
SEM	Standard error of mean
SERMs	Selective estrogen receptor modulators
SF-1	Steroidogenic factor 1
SHBG	Sex hormone-binding globulin

Slug/SnaH	Zinc-finger Proteins
Sp1	Specificity protein 1
STAT3	Signal transducer and activator of transcription 3
S1	Silencer 1
T	Testosterone
TNFα	Tumor necrosis factor α
U0126	ERK1/2 inhibitor
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WT	Wild type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I L. Polari, E. Yatkin, **M.G. Martínez Chacón,** M. Ahotupac, A. Smeds, L. Strauss, F. Zhang, M. Poutanen, N. Saarinen, S.I. Mäkelä. Weight gain and inflammation regulate aromatase expression in male adipose tissue, as evidenced by reporter gene activity. *Molecular and Cellular Endocrinology,* 2015; 412: 123-130

II **Martínez-Chacón G**, Brown KA, Docanto MM, Kumar H, Salminen S, Saarinen N, Mäkelä S. IL-10 suppresses TNF-α-induced expression of human aromatase gene in mammary adipose tissue. *The FASEB Journal*, 2018; 32(6):3361-3370

III **Martínez-Chacón G**, Yatkin E, Polari L, Peuhu E, Hartiala P, Saarinen N, Mäkelä S. CC chemokine ligand 2 (CCL2) stimulates aromatase gene expression in mammary adipose tissue via ERK1/2 signaling pathway. *Manuscript*

In addition, some **unpublished data** is included in this thesis.

1 Introduction

Estrogens are sex steroid hormones that are critical to a wide range of physiological functions. Apart from their major role in reproduction, estrogens have also important roles in energy metabolism and body fat distribution in females and males [3-6]. Estrogens have strong positive effects on cell proliferation and differentiation while also regulate energy intake, storage, and expenditure, as well as feeding behavior [7,8]. Excessive estrogen levels associate, however, with malignancy and metabolic disorders. The link between obesity and elevated estrogen levels is particularly important as it may contribute to the development of postmenopausal breast cancer in women and secondary hypogonadism in men [9-14].

Estrogen biosynthesis from androgens is driven by the action of the aromatase enzyme in a process that takes place mostly in the gonads and placenta, but also in white adipose tissue (WAT), skin, brain and bone [15-18]. In humans, aromatase enzyme is encoded by a single gene, *CYP19A1*. Transcription of *CYP19A1* is regulated by the use of multiple tissue-specific promoters and the action of distinct transcriptional factors, signaling pathways, and cellular mediators, which are also regulated in a tissue-specific manner [18]. Regulation of *CYP19A1* in healthy WAT, including that of the breast, involves the alternative use of at least three promoters: promoter I.4, and the almost unused I.3 and II [19]. Transcriptional activation of PI.4 in cultured adipose stromal cells (ASCs) requires the combined effect of glucocorticoids and proinflammatory cytokines, including interleukin (IL)-6, IL-1β, IL-8, and tumor necrosis factor (TNF)-α. PII and PI.3, in turn, can be stimulated by factors that activate the cyclic adenosine monophosphate (cAMP) signaling pathway in the tissue, such as prostaglandin E2 (PGE2) [20,21]. As such, the use and the activity of these promoters are closely depended on the inflammatory status of the WAT. It is then logical to assume that in a healthy WAT, the presence of a balanced interplay between pro- and anti-inflammatory factors and signaling cascades may be partially responsible for the low levels of aromatase and estrogen in the tissue. However, although much is known about the factors that induce *CYP19A1* transcription in WAT, there is almost no information on the possible tissue-derived factors suppressing this process.

Obesity has been associated with increased *CYP19A1* expression and elevated estrogen production in WAT [22-25]. Excessive fat accumulation and its subsequent inflammation are proposed to be the leading causes of this such increase. It is held that the high levels of proinflammatory factors, IL-6, TNFα, CCL2, and PGE2, secreted by the hypertrophic adipocytes and resident macrophages may upregulate all local *CYP19A1*-related promoters and with this the production of the enzyme [26-28]. After menopause, being obese or overweight is considered the most important modifiable risk factor for developing breast cancer [29-31]. Studies confirm that in morbid obesity and in breast cancer, levels of *CYP19A1* expression in the breast adipose tissue are often three- to four-fold higher than normal [19,24,26]. Increased *CYP19A1* in these two extreme conditions are thought be partly driven by a shift in tissue promoter usage, where the augmented local levels of PGE2 favor the use of aberrant and more potent promoters, PII/PI.3, over PI.4. The regulation of *CYP19A1* transcription in WAT is, however, rather complex, and the mechanisms and factors underlying the link between obesity and dysregulation of aromatase expression are not fully understood. In fact, it is likely that other endogenous inflammatory factors, which levels are altered during obesity, may also play a direct role in the local regulation of aromatase expression.

The use of aromatase inhibitors (AI) has been established as more effective approach than tamoxifen for the treatment of breast cancer. But despite proven efficacy, their long-term use for breast cancer prevention is limited because of the significant side effects caused by the systemic depletion of estrogens. Therefore, there is a strong need for improving our understanding of the mechanisms and molecular players that regulate the production of aromatase in the breast adipose tissue as this may facilitate the development of more safe and effective strategies aimed at breast cancer prevention and treatment.

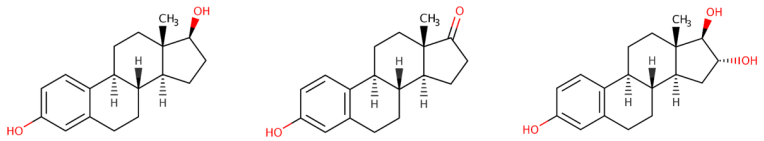
Consumption of seeds, berries, and soy, which contain dietary polyphenols such as lignans, stilbenoids, and isoflavones, are associated with reduced risk of breast cancer in postmenopausal women [32-34]. According to several *in vitro* studies, the chemopreventive effects of polyphenols are mainly attributed to their powerful anti-inflammatory and antioxidant effects, but also to the downregulation of aromatase transcription and activity, which have been proved only in cancer cells [35]. However, not much is known on the transcriptional mechanisms they exert to modulate *CYP19A1* expression in primary stromal cells.

This thesis work focuses on identifying novel obesity-related factors that regulate aromatase gene *CYP19A1* expression in the WAT of females and males, particularly in the mammary/breast adipose tissue. Furthermore, this study provides *in vivo* evidence for the role of obesity and ovariectomy on inflammation and aromatase gene expression in different WAT depots, as well as the role of dietary polyphenols on the regulation of inflammation and *CYP19A1* transcription in WAT and breast ASCs.

2 Review of the Literature

2.1 Estrogen Biosynthesis and Action

Estrogen actions are not limited to fertility and sexual functions in females; estrogens also exert important actions on non-reproductive organs in women and men, including brain, liver, adipose tissue, and skin. A substantial amount of evidence supports the critical role of estrogen in adipose tissue metabolism and inflammation and systemic energy homeostasis [8,17,36]. However, questions remain regarding the mechanisms by which these metabolic functions are displayed.



Estradiol (E2)
17-β estradiol

Estrone (E1)

Estriol (E3)

Figure 1. The three estrogens: estradiol, estrone, estriol. Reprinted with permission of Creative Commons License (CC BY 4.0). https://www.ebi.ac.uk/chebi/init.do

Estrogen hormones have a characteristic aromatic A-ring with a phenolic hydroxyl at C3, which distinguishes them from other sex steroids, e.g. C19 androgens. Natural estrogens (C18 steroids) include estradiol, estrone, and estriol (Fig. 1). 17β-estradiol (E2) is the most potent estrogen in this group, followed by estrone (E1), and the weaker estriol (E3) [37,38]. Although all three estrogens are essential for development and reproduction in females, the levels at which they are produced vary according to different periods of life. In fertile non-pregnant women, ovarian E2 is the predominant bioactive estrogen in the circulation with concentrations ranging 1.5 to 4 times higher than E1. These concentrations of E2, E1, and E3, however, are highly variable among individual women. During pregnancy, E3, synthesized in the fetus and placenta, exceeds the production rates of E2, and E3 becomes the dominant estrogen. Finally, when ovaries stop functioning after menopause, the E2:E1 ratio

reverses and circulating E2 levels become approximately one-tenth of those observed in premenopausal women [38-40].

During the reproductive years of women, sex steroid hormones are mainly produced in the ovaries from cholesterol through a complex chain of reactions catalyzed by the enzymes steroid cytochrome P450 (CYP) hydroxylases and hydroxysteroid dehydrogenases (HSDs). Aromatase CYP19A1 catalyzes the rate-limiting step in the estrogen biosynthesis, converting C19 androgens to C18 estrogens. In the ovary, both C19 substrates, androstenedione (A4) and testosterone (T), are converted into E1 and E2, respectively (Fig. 2).

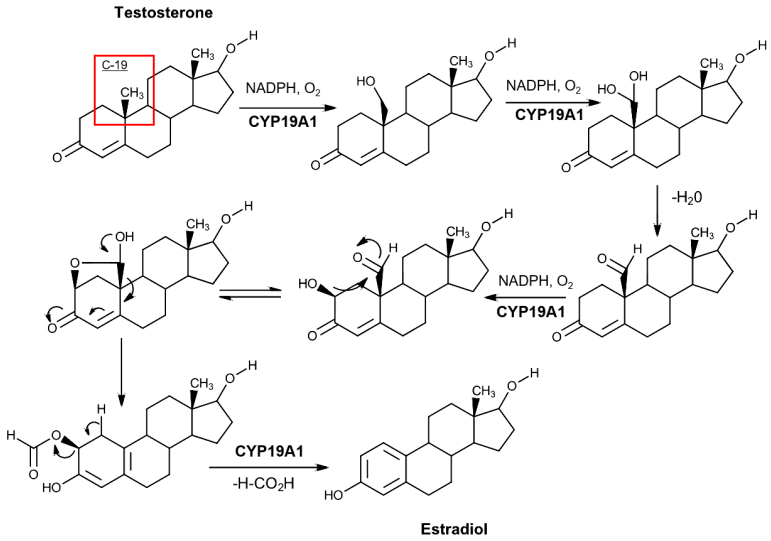


Figure 2. Aromatization of Testosterone. Modified from [41]

Peripheral tissues, including adipose tissue, brain, bone, and skin, are also capable of producing estrogens, specially E1, through local aromatization of C19 sources delivered from the circulation [17]. Then, E1 can be converted to E2 by the action of 17β-hydroxysteroid dehydrogenases (17β-HSDs) in the same tissue (Fig. 3A). Furthermore, estrogens can be also found as conjugated forms in the circulation. Estrone sulfate (E1S) serves as a stable reservoir for the formation of active forms E1 and E2 in different peripheral tissues. Estrogen sulfotransferase and steroid sulfatase enzymes catalyze the interconversion between E1 and E1S [42,43].

2.1.1 Menopause and estrogen production

Fluctuations in estrogen levels are characteristic in women throughout life and delineate the different stages of their reproductive life cycle, from puberty to menopause. Whereas in premenopausal women, estrogens act as a circulating hormone produced mainly by the ovaries, in postmenopausal women, after cessation of ovarian function, estrogens are produced in extragonadal sites restricted to paracrine or intracrine functions. Gradual but significant changes in women's metabolism start to manifest as a result of a different regulation in the estrogen synthesis at peripheral places, and the subsequent and massive decline in the total circulating levels of estrogens. At menopause, ovarian estrogen synthesis ceases and circulating E2 levels drop by up to 95% (Fig. 3A).

Peripheral estrogen production in adipose tissue and skin mesenchymal stromal cells, bone osteoblasts and chondrocytes, vascular endothelia, smooth muscle, and brain, are the most important sources of estrogens in postmenopausal women and men [44,45]. Unlike the ovaries, peripheral tissues are unable to synthesize C19 precursors from cholesterol, but, instead, circulating A4 and dehydroepiandrosterone sulfate (DHEA) of adrenals and/or ovarian origins are locally aromatized to E1 (Fig. 3B). To reach full biological activity, E1 is further converted to E2 within the same tissue in a reaction catalyzed by the 17β-HSDs [18]. It is known that the majority of the peripheral estrogen production acts in an intracrine or paracrine manner [46]. Maintaining the production of estrogens in these tissues is important after menopause. For instance, in bone, estrogens drive growth and maintain proper mineralization, thus preventing osteoporosis and fractures, while in the brain, estrogens influence sexual behavior, maintain normal cognitive function, and control appetite [17,47].

Peripheral conversion of adrenal androgens into E1, particularly in the white adipose tissue (WAT), becomes the primary source of estrogens after menopause[8,48]. Both E1 and E2 levels in WAT are several folds higher than in the circulation [49,50]. Although the amount of E2 in peripheral sites is low compared to that in blood before menopause, it is enough for the hormone to exert its biological functions within the tissues. Because of this, it is thought that circulating levels of estrogens in postmenopausal women reflect only the metabolism of estrogens in peripheral sites.

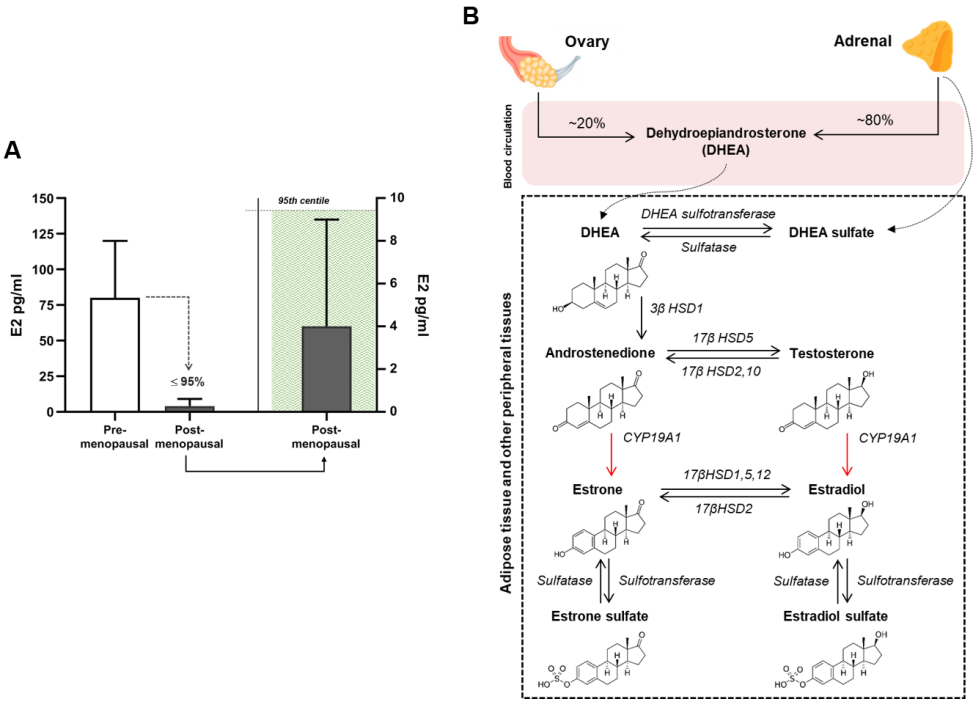


Figure 3. Menopausal changes in estrogen synthesis and activity. A. Natural decline in circulating estradiol levels and activity in postmenopausal women. On the left, the dramatic fall (~95%) in E2 circulating levels between pre- and post-menopausal women. On the right, E2 circulating levels in postmenopausal women. Levels are often under the 95th centile among normal postmenopausal women. **B.** WAT becomes the major estrogen-producing site after menopause. Estrogen synthesis in extragonadal sites is dependent on circulating C19 precursors, dehydroepiandrostenedione (DHEA) and dehydroepiandrostenedione sulfate (DHEA-S). Only 20% of the total circulating DHEA has an ovarian origin, the remainder is secreted by the adrenal glands. The predominant estrogen produced in extragonadal sites is estrone (E1), which is converted into E2 in the same tissue or inactivated to estrone sulfate before being released in the circulation. Modified from [51]

Ovaries of postmenopausal women maintain the production of T, which means that after menopause levels of T are around one-fold higher than E2 in the circulation [52]. Ovarian production of T, however, only represents about 25% of the total circulating levels. The other 75% is originated from circulating precursors, DHEA, secreted by both the ovaries and adrenals, and DHEA-S, which is released exclusively from adrenals. Moreover, DHEA and DHEA-S are present in the circulation of postmenopausal women at concentrations far above those of A4 and T [52,53]. It is important to note that sex steroids produced in tissue are rapidly inactivated into their conjugated forms before being released in the circulation [46].

Due to the fact that WAT becomes the principal site of estrogen biosynthesis after menopause, estrogen production rates in elderly women positively correlates with their weight and adiposity [54]. Furthermore, the peripheral aromatization of androgens gradually increases with age. WAT expression of CYP19A1 increases at rates that can even quadruple by the age of 60 [55]. These changes in estrogen production and aromatase expression after menopause may explain the increased susceptibility of elderly women to develop obesity and breast cancer.

2.1.2 CYP19A1 (Aromatase)

CYP19A1 is a member of the cytochrome (CYP) P450 superfamily, heme-containing mono-oxygenase enzymes widely found across nature [56]. With more than 74 families and 504 individual members, these proteins catalyze a large array of reactions, including steroid synthesis and metabolism [57]. Overall, the CYP450 family is associated with phase 1 reactions, hydrolysis, oxidation, and reduction of organic compounds, both endogenous and exogenous (e.g. sex steroids, eicosanoids, fatty acids, and drug compounds) [58,59]. Most of these reactions are originated by both the insertion of one atom of oxygen into the substrate bound close to the heme group and electron transfer from nicotinamide adenine dinucleotide phosphate (NADPH) to the heme catalytic site by NADPH-cytochrome P450 reductase [59]. With some exceptions, each member can metabolize several substrates due to the low specificity of their substrate-binding sites. However, CYP19A1 has a high specificity for its substrate, C19 androgens [60].

CYP19A1 (CYP450 family 19, subfamily A, member 1) is a membrane protein localized in the endoplasmic reticulum of estrogen-producing cells [57,60]. It is the only known enzyme that catalyzes the rate-limiting and irreversible conversion of C19 androgens into C18 estrogen. The general pathway of estrogen biosynthesis originates from cholesterol in the gonads, followed by a series of successive enzymatic reactions that end in the conversion of C19 precursors into C18 steroids by the CYP19A1 enzyme, e.g. E1 and E2 are synthesized by aromatization of C19 androgens, A4, and T, respectively [61,62] (Fig. 2). This last step is unique and rate-limiting for the estrogen biosynthesis so that its blockade fully inhibits estrogen production without affecting other steroids [63,64]. The enzymatic biosynthesis of estrogens involves the CYP19A1 and one of the cofactors, flavoprotein nicotinamide adenine dinucleotide (NADH) or NADPH (used to deliver electrons). However, NADPH is the most preferred cofactor. Together, CYP19A1 and NAPDH catalyze three consecutive hydroxylation reactions, each requiring one mole of oxygen and one mole of the cofactor. The first two steps entail the insertion of two hydroxyl groups at methyl groups at C-19_1 and C-19_2, with the second position dehydrated to

aldehyde. The last oxidative step leads to the phenolization of the steroid A-ring by the loss of one molecule of formic acid [42,65] (Fig. 2).

In humans, CYP19A1 is encoded by the *CYP19A1* gene (Fig. 4). This gene located on chromosome 15q 21.2, spans over 123 kb of DNA and produces tissue-specific transcripts from different promoters through alternative splicing. It is composed of 10 exons (I-X) and 9 introns, but only 30 kb of the 3'-end, containing exons II-X, encodes the actual protein. The remaining 93kb upstream of the gene (5'-flanking end), contains multiple exons I or alternative untranslated promoters that differently regulate aromatase expression in target tissues [66,67]. Currently, at least nine tissue-specific promoters have been recognized in humans: promoter/exon I.1 (placenta major), 2.a (placenta minor), I.8 (placenta, brain and prostate), I.4 (adipose tissue and skin), I.5 (fetal tissues), I.7 (endothelium), I.f (brain), I.2 (placenta and testis), I.6 (bone), I.3 (adipose tissue) and promoter/exon II (PII, gonads and adipose tissue) [68-77]. In other mammalian species, the aromatase gene also contains several tissue specific promoters in the first exon. However, there are significant differences between species regarding the number and localization of these promoters [15,78]. This issue is described in greater detail in section 2.4.

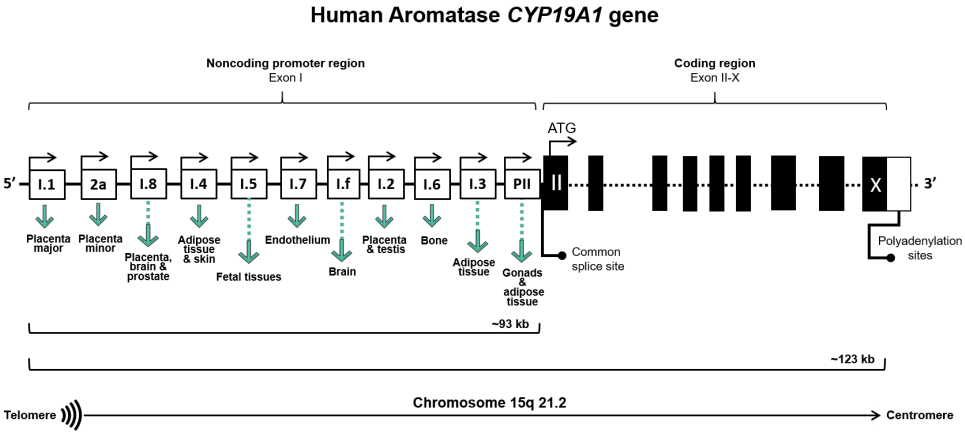


Figure 4. Structure of the human aromatase *CYP19A1* gene. The *CYP19A1* gene is located on chromosome 15 from where it is transcribed from telomere to centromere. Through 123 kb of length, the human aromatase gene is composed of a coding region of nine exons (exon II-X, approximately 30 kb) followed by two polyadenylation sites at the 3'end, and a noncoding region at the 5' with several tissue-specific promoters (exon I, about 93 kb). This upstream region regulates the transcription of the gene in a tissue-specific manner through the use of alternative promoters and generates unique 5'uncoding-exon-I transcripts in each aromatase expressing tissue. Each promoter transcript is then spliced alternatively onto a common splice acceptor site at exon II; thus, the final mature RNA only contains the coding region of the gene. This results in the translation of the same aromatase protein independently of the tissue promoter used. Modified from [79].

Transcription of *CYP19A1* proceeds from telomere (5') to centromere (3'), starting with the activation of any particular promoter and the following generation of unique transcripts carrying the 5'-untranslated region of the activated promoter and the protein encoding sequence. Each promoter transcript is then spliced onto a common junction 38 bp upstream the ATG translational start site at exon II. Thus, the reading frame to translate the protein is always the same regardless of the promoter being used, giving rise to an identical protein in all expressing tissues [18,72]. Furthermore, aromatase expression in every tissue is not only regulated by a specific promoter usage, but transcriptional activity of each promoter is also regulated by an interplay of transcription factors, signaling pathways, inducers, and repressors, in a tissue specific fashion. Each promoter region also contains specific initiation sites and protein complexes mediating transcription. For example, with the exception of PI.4 and PI.7, most I exon sequences have a TATA box, which requires a pre-initiation complex binding with the TATA box-binding protein. Instead, PI.4 has a GC box, a classic binding site for members of the specificity protein 1 (Sp1), and PI.7 contains a GATA box, a binding site of hematopoietic transcription factors [18,80]. This complex regulation makes the expression of aromatase a unique process in each tissue, as clearly proven by different transcriptional rates of *CYP19A1* depending on the tissue and its specific requirements.

2.1.2.1 Expression of aromatase *CYP19A1* gene in adipose tissue

In adipose tissue, including that from the breast, *CYP19A1* expression occurs primarily in adipose stromal cells (ASCs) or breast adipose fibroblasts (BAFs), accounting for more than 85% of the total local production. The rest is thought to be produced by the mature adipocytes [81,82]. Transcripts from three different aromatase promoters, promoter I.4 (PI.4), PII, and PI.3, are known to be expressed in ASCs [72,83]. Of all three, transcription depends almost exclusively on the use of PI.4 in disease-free breast adipose tissue, while the other two, PII and PI.3, remain practically inactive [72,84,85]. This particular order in promoter usage, combined with the balance between transcriptional inducers and repressors in tissue, maintains a low level of *CYP19A1* expression in the breast, whilst ensuring adequate/basal levels of aromatase production and activity [18,83].

The 5′-region of exon PI.4 contains a putative silencer and several transcriptional element sites, including an AP1 site, an interferon-γ element (GAS) responsive site, a glucocorticoid-responsive site (GRE), and a Sp1-binding site (Fig. 5). Transcription of *CYP19A1* via PI.4 requires the strict activation of both the GRE responsive site by the glucocorticoid receptor (GR) and the Sp1 site by the Sp1 protein[86,87]. In cultured ASCs, cortisol and its synthetic analog, dexamethasone (DEX), stimulate PI.4-related transcription in a dose-dependent effect [86]. Such effect

is potentiated by the addition of serum [20,88]. Sources of glucocorticoids for adipose tissue are primarily circulating cortisol and cortisone with the latter being converted to the more potent cortisol by the action of 11β-HSD1within the adipose tissue [89,90]. Furthermore, in serum-free conditions, activation of PI.4 can be enhanced by the synergistic action of glucocorticoids and type I cytokines, oncostatin M (OSM) and TNFα [84]. Thus far, type I cytokines, including IL-6, IL-8, IL-11, IL-1β, and OSM, are known to activate the GAS transcriptional site of PI.4 via the JAK1/STAT3 signaling pathway. TNFα, on the other hand, is the only known factor able to induce the AP-1 responsive site via nuclear factor-κB (NFκB) and mitogen-activated protein kinase (MAPK) signaling pathways [20,84,91-93].

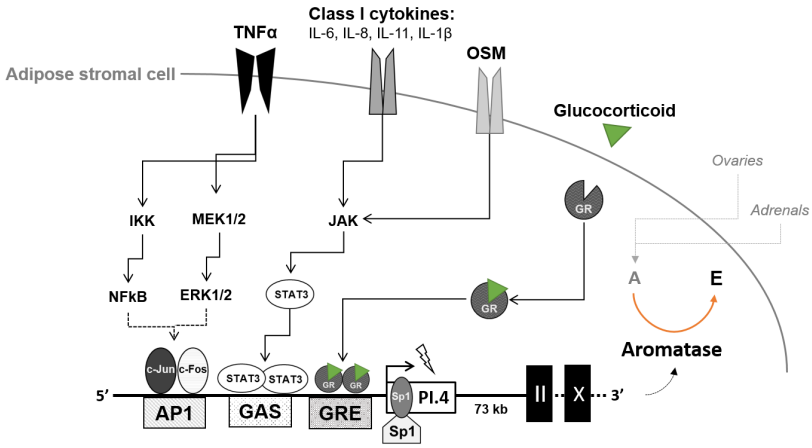


Figure 5. Factors involved in the activation of PI.4. In cultured adipose stromal cells, the activity of PI.4 is strictly dependent upon the activation of its GRE responsive site by activated glucocorticoid receptor complex. Glucocorticoids and serum-containing medium are usually enough to stimulate transcription via PI.4. Without serum, PI.4 can be activated by the combined action of glucocorticoids and class I cytokines, oncostatin M (OSM) or TNFα. Class I cytokines and OSM selectively activate receptor tyrosine kinases and JAK/STAT3 intracellular signaling cascade, resulting in the phosphorylation of STAT3 and its interaction with the GAS responsive site at PI.4. On the other hand, TNFα activates tumor necrosis-receptors, triggering both IKK/NFκB and MAPK/ERK1/2 pathways while stimulating the expression of c-Jun and c-Fos, which then form a complex that binds the AP-1 site within the promoter. Activation of the Sp1 responsive site is also required for aromatase PI.4 transcription. Modified from [15]

In addition to I.4 transcripts, *CYP19A1* in adipose tissue is also transcribed by the gonadal promoters I.3 and II. These are the two most proximal promoters to the ATG common splice site, and because they are only separated by 215 bp, both promoters share response element sites and regulatory mechanisms [20]. As in the gonads, the transcriptional activity of these two promoters is stimulated by cyclic adenosine monophosphate (cAMP) in the adipose tissue [94] (Fig. 6).

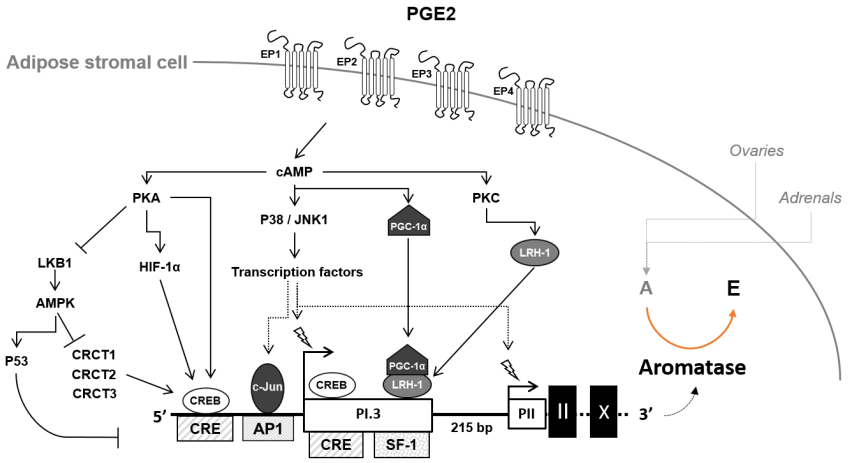


Figure 6. Proposed regulation of promoter I.3/II in adipose tissue. Treatment of cultured adipose stromal cells with PGE2 activates promoter I.3 and II via any of its four EP-GPCR receptors on the cellular membrane. Due to the close proximity to each other, and also to the common splicing site, these two promoters share similar regulatory mechanisms and responsive elements. EP receptors stimulate the cAMP signaling pathway and related mediators, including PKC, PKA, and P38/JNK1. PKC/PKA activates CREB and the nuclear receptor LRH-1 with its coactivator PGC1-α, which then activates CRE and SF-1 responsive elements, respectively. In addition, increased levels of cAMP lead to the inhibition of CRCT coactivators by the LKB1/AMPK cascade, allowing them to interact with CREB and enhance its stimulatory effect on aromatase transcription. PGE2 also activates p38 and JNK signaling pathways related to AP1 and CRE activity on both promoters. Modified from [79].

Transcriptional activation of PII/PI.3 requires stimulation by the cAMP-response element-binding protein (CREB) and the activity of an orphan nuclear receptor, either the NR5A subfamily liver receptor homolog 1 (LRH-1/NR5A2) or the steroidogenic factor 1 (SF-1/NR5A1), on the SF-1 responsive site along the promoter's 5'- region [95-97].

Transcription via PII in adipose tissue involves protein kinase C (PKC) and protein kinase A (PKA) activation of two MAPK family members, p38 and JNK, and their related transcription factors ATF-2 and c-Jun [79,98-100]. Once activated, these two transcription factors bind and activate CREB at the responsive site [94,101]. In addition, activation of the CREB-regulated transcription co-activators (CRCT)-1, 2, and 3 proteins and the hypoxia-inducible factor (HIF)-1α, further stimulate PII activity[102]. CRCT proteins stimulate CREB following PKA activation, but without stimulation, the action of the serine–threonine liver kinase B1/AMP-activated protein kinase complex (LKB1/AMPK) on CRCT proteins keep these neutralized [103]. Additionally, LKB1/AMPK induces P53 transcriptional activity, which in turn inhibits PII activation at the transcriptional site. PGE2 and leptin stimulation of ASCs has been shown to downregulate P53[104,105].

Furthermore, PI.3 and PII transcription are thought to be suppressed by an inhibitory transcriptional complex made of non-phosphorylated ATF-2 and zinc-finger DNA-binding, SnaH, and Slug proteins. This complex binds near the CREB transcriptional site, and it is highly active in non-cancerous conditions, limiting *CYP19A1* transcription via PII/PI.3[106]. Moreover, it was found that the silencer S1 blocks the SF-1 responsive site at the PI.3 transcriptional site in normal breast epithelial cells and adipose fibroblasts, thus further repressing the activation of these two gonadal promoters [107,108].

PI.3/II transcriptional activity in breast ASCs can be efficiently induced *in vitro* by phorbol esters (PMA) and forskolin (FSK), activators of PKC and adenylate cyclase, respectively [109]. In cultured breast ASCs exposed to breast-cancer-cells conditioned media or in direct co-culture with breast cancer cells, *CYP19A1* expression via PII activity is increased; however, the factors and mechanisms behind this aberrant activity are still unclear [110,111]. Several studies suggest that prostaglandin E2 (PGE2) is the key endogenous regulator of PII in the adipose tissue since it is known to be a strong inducer of cAMP [92,110,112,113]. PGE2 induction of aromatase in ASCs is initiated by the stimulation of any of its G-protein receptors EP-1, EP-2, EP-3 or EP-4, followed by the activation of PKA and PKC signaling pathways [114-117]. Additionally, the PGE2 effect on cAMP mediates the inhibition of LKB1 activity so that CRCT proteins can translocate to the nucleus and stimulate aromatase transcription via PII [19,102,118].

Interestingly, *CYP19A1* expression and activity in subcutaneous WAT depots appears to be different from that in visceral depots. In women, the activity of aromatase is found to be higher in the fat around the hips, followed by that in the thigh and abdomen [55,85,119,120].

2.1.3 Estrogens mediate gender differences in body composition and energy metabolism

Numerous studies provide evidence on the role of estrogens in regulating energy intake, expenditure, and storage in women and men, and it is particularly important in controlling body fat distribution and body composition changes during aging. These actions are considered to be driven by both central and systemic mechanisms [36,121-124].

Overall, estrogen effects are mediated by activation of the estrogen receptors (ER)-α and -β in responsive organs. Although both isoforms have a similar binding affinity for E2, they are functionally different, having a distinct pattern of expression and tissue distribution as well as a different ligand activation. ERα and ERβ often behave differently when interacting with the same estrogen ligand, sometimes triggering opposite effects [125-127]. Estrogen signaling, therefore, is selectively modulated depending on the balance between ERα and ERβ actions in each target tissue.

Central actions of estrogens are mediated via ERα and ERβ within the hypothalamus, ventromedial nucleus, arcuate nucleus, medial preoptic area, and paraventricular nuclei of the brain [123,128]. E2 actions in the brain regulate food intake and satiety by complex and less well-defined mechanisms that include the stimulation of neuropeptide-Y (NPY) and agouti-related protein, the excitation of anorexigenic neurons, and the inhibition of orexigenic factors, such as leptin. At the same time, E2 modulates energy expenditure by central action at the ventricular nucleus, from where it induces thermogenesis in brown adipose tissue [123,129,130]. One of the most interesting metabolic effects of estrogens is their potential to decrease food intake and to favor energy expenditure in animal models [36,131,132].

On average, males have less total body fat with more central or abdominal fat deposition (apple shape), whereas females, commonly with higher body fat content, have prominent subcutaneous deposition, especially in the gluteal/femoral area, pelvis, buttocks, and thighs (pear shape). Higher levels of estrogens in females confer them with a greater capacity for storing energy as fat, but also with a more efficient way to utilize, transport, and expand it where necessary [122,123]. Consequently, women are less prone to develop cardiovascular or metabolic diseases during reproductive age [133,134].

Estrogens coordinate sex-specific actions in peripheral organs that regulate fat storage and distribution, insulin sensitivity, and immune response [5,135,136]. Adipose tissue distribution in females and males are differently mediated by estrogens through a sex and adipose tissue depot-specific expression of ERα and ERβ. Compared to males, females exhibit a lower ratio ERα/ERβ in subcutaneous fat depots, which tilts the balance in favor of greater accumulation of fat within gluteal and femoral areas. Conversely, a visceral fat deposition is favoured in males due to a lower ratio ERα/ERβ in the abdominal area and by higher levels of testosterone and reduced LPL activity in subcutaneous depots [137-139].

As women enter menopause, circulating levels of E2 decline, and with this, metabolic and histological changes begin to occur. Adipose tissue distribution in postmenopausal women changes towards a more androgenic pattern, favouring the abdominal or visceral deposition of excessive fat over subcutaneous sites. In addition, low estrogen levels affect the central control of appetite and satiety, predisposing women to weight gain [140-142]. Therefore, the risk of developing obesity dramatically increases in elderly women, reaching around 65-70% in women over 50 [141,143]. Accordingly, menopause is associated with a higher incidence of metabolic-related diseases, including metabolic syndrome, type II diabetes, cardiovascular diseases, and cancer; reaching the men's incidence rates at same age [144-147]. In parallel, fluctuations in ovarian hormones during the menopausal transition lead to a gradual involution of the breast parenchymal tissue [148]. At this stage, the glandular tissue reduces from 35% in a premenopausal stage to less than 5% after menopause. Following this, the

proportion of subcutaneous fat within the breast increases making fat the most abundant component inside the postmenopausal breast. While the specific mechanisms by which this occurs are not yet well recognized, it is known that involution of the breast parenchyma occurs gradually as ovarian estrogen production declines and the endocrine stimulation of the tissue ceases.

Ovariectomized (OVX) rodents exhibit greater body weight and fat accumulation as well as increased food intake and lower physical activity. Estrogen replacement therapy in these animals successfully attenuates some of the metabolic impairments, decreases body weight and visceral fat, and improves their insulin sensitivity [129,149,150]. In women, however, the efficacy of hormone replacement therapy (HRT) to lower the metabolic consequences of menopause is still controversial. Numerous randomized trials have failed to find evidence to support the claim that HRT reduces energy intake. Nevertheless, in some cases, HRT in postmenopausal women has shown to diminish weight gain and body fat while reducing abdominal fat and enhancing systemic insulin actions [130,151]. Regarding the glandular involution, although some studies have reported a small increase in breast density in women taking HRT, several more have shown no significant differences in breast morphology [63,152,153]. Despite the potential benefits of HRT in reducing the burden of menopause-associated changes and symptoms [154], the use of HRT after menopause is linked to the increased risk of breast and endometrial cancer [155]. Postmenopausal women who use the combined HRT with estrogen and progestin face a greater risk of breast cancer than those taking estrogen alone [156,157]. However, women who use only estrogen have a greater risk of endometrial cancer [158].

2.1.4 Gut microbiota and estrogens, a link regulating energy homeostasis

E2 displays both central and peripheral signals to drive metabolic changes and to control weight gain[159]. However, the interrelationships between sex steroid balance and body metabolism are complex, and the exact mechanisms underlying sex steroid–associated obesity and/or metabolic dysregulation in women and men are still unclear.

Gut microbiota is now considered a major factor in health and disease, as changes in gut bacterial composition and diversity are associated with certain metabolic, immune, and behavioral states [160-163]. It is generally recognized that gut microbiota regulates important metabolic functions related mostly with energy homeostasis, activating local and systemic molecular responses affecting appetite, body weight, and adipose tissue functioning [160,164,165]. As mentioned in the previous sections, low levels of E2 after menopause predispose women to obesity. We propose here that the sex steroid balance may mediate obesity and WAT inflammation through changes in the gut microbiota (Fig.7).

In humans, gut microbiota consists of about 100 trillion microbes, mostly bacteria, but it is also composed of viruses, fungi, and protozoa. A range of 1000 bacterial species is thought to live in the human intestine, encoding over three million genes and producing, in turn, thousands of essential metabolites [162,166]. The composition of the gut bacterial ecology coevolves with its host throughout life, reaching its highest complexity at adulthood. At this age, around 50 different bacterial phyla are known to live in the human intestine, of which some 80% belong to only three phylum, listed according to their abundance: *Bacteroidetes* → *Firmicutes* → *Actinobacteria* [167,168]. Although these taxonomic features of gut bacteria apply to most humans, substantial individual variability is noted in terms of abundance and diversity of species, making our gut microbiome a unique fingerprint. Many studies support a tight and bilateral partnership between the gut microbiota and the human host. The gut microbial composition is specific to individuals, but it can be affected by host factors, such as age, gender, and ethnicity, as well as by diet, antibiotics, and physical activity [169-172]. Common physiological and pathological conditions also play roles in shaping the composition of this bacterial ecology. Importantly, changes in the abundance of specific taxa of bacteria could be relevant in the pathogenesis of several chronic diseases. These include autoimmune diseases, asthma, obesity, type 1 and 2 diabetes, inflammatory bowel disease, and colorectal cancer [173-175].

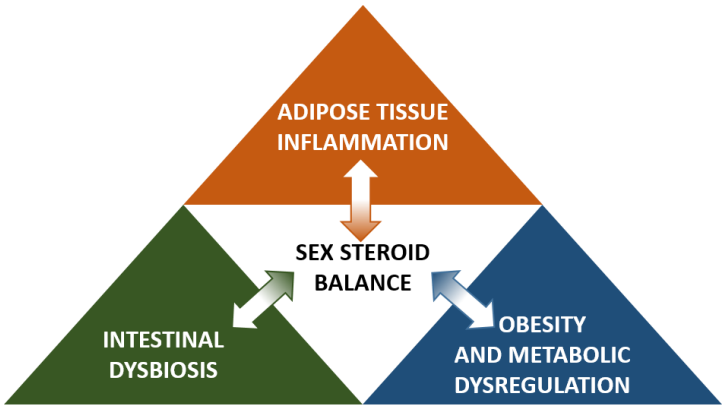


Figure. 7. Potential interrelationship between sex steroids, obesity, and gut microbiota.

Numerous animal and human studies have demonstrated that an increased *Firmicutes-Bacteroidetes* ratio influences weight gain and obesity [170,172,176,177]. While in humans the evidence supporting these observations remains controversial, by using germ-free and obese animal models researchers have consistently identified this particular bacterial shift as the initial trigger of local and systemic metabolic alterations linked to higher energy intake, impaired insulin sensitivity, and greater fat accumulation. As shown in

figure 8, the "obese-type gut microbiota", as it is usually called, is associated with several detrimental local responses, particularly intestinal inflammation, enhanced food energy extraction, gut barrier dysfunction, and altered secretion of gut hormones and factors involved in metabolism, including peptide YY (PYY), glucagon-like peptide (GLP), angiopoietin-like protein 4/fasting-induced adipose factor (FIAF) and short-chain fatty acids (SCFA) [165,176,178]. Moreover, these local bacterial and metabolic changes correlate with higher body weight and adiposity, liver fat accumulation, and hyperphagia, suggesting these local changes may stimulate further systemic alterations on distant metabolic organs, which induce an obesogenic metabolic state [178,179].

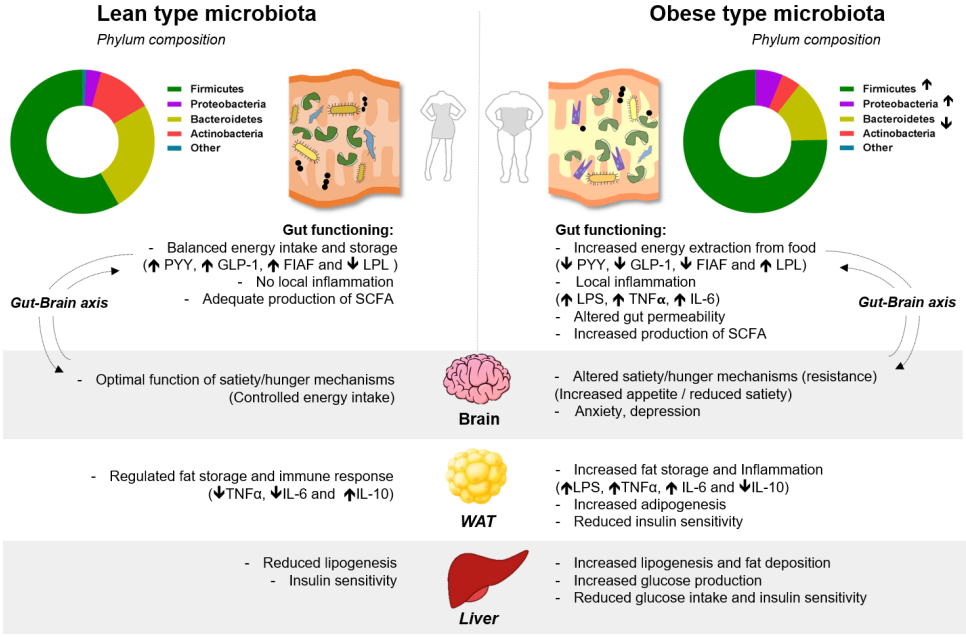


Figure 8. Gut microbiota regulates energy balance. Obese subjects are associated with less diverse gut bacterial ecology and an increased *Firmicutes / Bacteroidetes* ratio. The obese bacterial profile promotes functional alterations in the intestine towards improving its capacity to harvest calories from food by altering the expression of gut hormones and factors regulating gut motility and glucose and lipid metabolism. Moreover, it also associates with a local low-grade inflammatory state and impaired gut permeability, leading to an increased release of pro-inflammatory factors and free fatty acids into the circulation. Obese gut microbiota produces more short-chain fatty acids (SCFA) that can induce lipogenesis and increase triglyceride deposition. In addition to these local disturbances, gut microbiota appears to affect whole-body metabolism by inducing responses via other endocrine organs, including the brain, adipose tissue, and liver. Bacterial metabolites appear to exert their metabolic effects by interacting with enteroendocrine L cells in the intestine, altering the secretion of anorexigenic and hunger hormones and affecting central appetite pathways (gut-brain axis). Furthermore, increased pro-inflammatory factors and SCFA in circulation may stimulate triglyceride accumulation and adipogenesis in fat adipose tissue depots and the liver, thereby reducing the insulin sensitivity in these organs.

It is already recognized that gut microbiota plays an important role in women's estrogen metabolism [161]. Estrogens are metabolized in the intestine by the bacterial secretion of β-glucuronidase, an enzyme that deconjugates estrogens into their active forms [180]. It was known already in 1970 that gut bacterial ablation induced by broad-spectrum antibiotics is associated with reduced deconjugation of estrogens and increased excretion of conjugated forms in women, which suggested protection against breast cancer [180,181]. Current epidemiological studies, however, demonstrate a positive association between antibiotic intake and breast cancer risk [182]. Although the mechanisms of these associations are unclear, the studies prove that gut dysbiosis could have a direct or indirect impact on circulating estrogen levels. On the other hand, Bertozzini and coworkers reported that in healthy women, the fecal bacterial composition remains relatively stable throughout life but tends to change after menopause [183]. They also reported important differences in gut bacterial composition between genders, which they suggested is caused by the different levels of sex steroids in females and males.

Furthermore, the recently introduced sequencing technologies have provided more detailed and consistent evidence that support a dynamic link between estrogen and gut microbiota regarding regulation of body weight, metabolism and immunity. While a significant portion of these data is derived from animal models, several *in vivo* studies have demonstrated that gut microbiota composition is, indeed, different between females and males, which may explain why females and males show distinct susceptibilities to infections and autoimmune diseases. Compared to male mice, females have a more diverse gut bacterial ecology and a greater tendency to fluctuations in terms of abundance, which coincide with the pulsatile secretion of sex steroids during the estrous cycle [184-187]. Moreover, by using OVX mice, researchers have been able to assess the impact of sex hormone levels on the composition of the gut microbiome and to partly restore and prevent the gut disturbances and metabolic consequences by using HRT [187-189]. However, it is still not clear from these animal studies whether a mechanistic link exist between sex steroid imbalance, gut dysbiosis, and metabolic disorders.

In humans, on the other hand, gut microbiota studies are extremely challenging because of the significant variability in composition between subjects, the numerous external modifiers, and the different laboratory techniques used during the collection, storage, and analysis of stool samples[190]. Although the data remains inconclusive, it still supports important differences in the bacterial gut composition between women and men [191-193]. Moreover, according to intervention studies in postmenopausal women, adopting a diet rich in fruits, vegetables, and seeds may increase the abundance of beneficial bacteria while reducing the levels of certain communities of bacteria associated with inflammation [194-196]. Recent studies on the gut estrobolome (bacterial genes metabolizing estrogens in the gut) also confirm the

role of gut microbiota in controlling the systemic levels of estrogens in women [161,186,197]. The most interesting result in this regard is perhaps that changes in the estrobolome during obesity and menopause may contribute to breast carcinogenesis, altering the levels of circulating estrogens [9,198]. Overall, despite the limitations, the current evidence may support the existence of a reciprocal interaction between estrogens and the gut microbiota regulating energy metabolism.

2.2 Postmenopausal Breast Cancer and Aromatase

Breast cancer is the most common cancer in women worldwide with over two million new diagnoses in 2018 [199]. Almost 8 out of 10 breast cancer cases occur around the age of 50, when women are likely to be at menopause [200]. Based on current estimates, breast cancer is still the leading cause of cancer-related deaths among women in developed and developing countries, while it is ranked as the second cause of cancer-related deaths in the USA and the EU, after lung cancer [201]. Despite the marked improvement in survival rates (up to almost 90%) in many developed countries since the nineties, it is estimated that 627000 women died in 2018 from breast cancer around the world [199,202]. Worldwide incidence rates of breast cancer have continued increasing over the last years parallel to the population aging, increased industrialization, and adoption of unhealthy lifestyles [199,202-204]. These trends have forced a rethink of the current approaches among the medical and scientific community and a closer examination of preventive strategies to reduce breast cancer incidence.

Breast cancer is a complex and heterogeneous disease that is composed of a spectrum of many subtypes, each of which expresses different pathologic and histological features, and different clinical outcomes, treatment responses, and prognoses [205-209]. Invasive ductal carcinoma (IDC), the most common form of breast cancer, commonly arises within the glandular tissue, adjacent to or from the terminal duct or lobular units of the mammary gland. Based on the cellular features, most breast cancers are adenocarcinomas and are generally classified as ductal or lobular carcinomas. Invasive ductal carcinoma accounts for 70-80% of all breast cancer cases, followed by the less aggressive lobular carcinoma [206,210]. Moreover, five main molecular subtypes of breast cancer have been identified and characterized by the pattern of expression of various biomarkers, including the status of steroid hormone receptors and human epidermal growth factor receptor (HER)-2 and proliferation: Luminal A, Luminal B, HER-2 enriched, and triple-negative/basal-like breast cancers [205,206,210]. More than 80% of diagnosed breast cancers in postmenopausal women are estrogen receptor positive (ER+), highlighting the importance of estrogens in the development and progression of these tumors [211]. Compared with

other subtypes, ER+ tumors are better differentiated, less aggressive, and are associated with lower mortality rates. Although primary ER+ tumors are highly responsive to estrogen ablation therapy, it has been reported that one-third of these breast cancer patients become resistant to treatment [205,211,212].

Many important risk factors for postmenopausal breast cancer have been identified through years of epidemiological studies. The most important risk factors for breast cancer are factors and conditions that increase tissue- or circulating-estrogen levels [198,213]. Some of these are well defined as non-modifiable factors, including family history, age at menarche, and menopause. However, many other factors related to lifestyle can certainly be modified [214,215]. Indeed, it has been estimated that over 40% of the total breast cancer cases in the USA [216,217], UK [218,219], and Australia [220] could be prevented by addressing the most important modifiable risk factors for postmenopausal breast cancer: obesity, physical inactivity, and alcohol consumption [221]. It has been proposed that obesity-related increased aromatization of androgens in breast adipose tissue is a key driver of breast carcinogenesis after menopause.

2.2.1 The role of estrogens in breast carcinogenesis

At least three distinct mechanisms have been proposed to explain the role of E2 in carcinogenesis: stimulation of cell proliferation via activation of its receptor, DNA damage through production of estrogen metabolites, and neoplastic transformation via aneuploidy (Fig. 9) [222,223].

Supported by a substantial amount of experimental and epidemiological evidence, E2 stimulation of cellular proliferation through ERα is the most widely acknowledged E2-mediated mechanism for carcinogenesis [10,223-225]. It is thought that increased frequency of cell division rises the chance of DNA copying errors or mutations, leading to genetic and/or epigenetic aberrations, which in turn, accumulate and suddenly initiate neoplastic transformation [63].

As proliferating cells need substantial energy resources, they often exhibit an increased mitochondrial activity that associates with elevated production of reactive oxygen species (ROS). Increased ROS has been reported in breast tumors and different cancer cell lines [226,227]. These secondary messengers are known to exert deleterious effects on the DNA (genotoxicity), increasing genomic instability, and to mediate cell proliferation via redox-associated signaling pathways [213]. Estrogens can be metabolized within tissues to catechol estrogen metabolites, followed by the subsequent formation of ROS and unstable secondary intermediates, such as semiquinones and quinones. When combined, these two sub-products may influence genotoxicity by promoting the formation of covalent adducts with DNA [228-231].

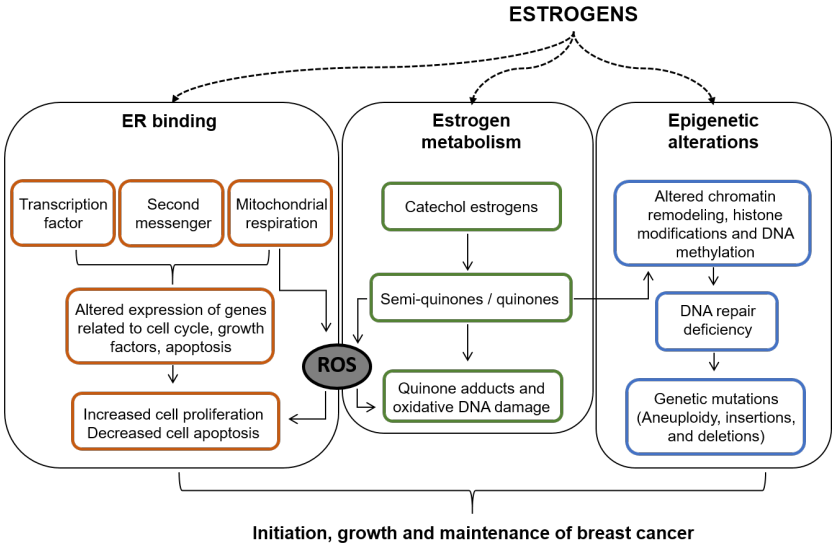


Figure 9. Mechanisms of estrogen-induced carcinogesis. The role of estrogens in the initiation, growth, and maintenance of breast cancer is well established through preclinical data, epidemiological studies, and clinical trials. Thus far, three different mechanisms are thought to be responsible for the malignant actions. The first, and most widely documented, involves the activation of both estrogen receptors α and β, and their subsequent transcriptional activity or related signaling pathways, which induce aberrant cell proliferation and mutagenesis. The second involves the oxidative metabolism of estrogens, the formation of quinones and ROS, and the potential oxidative damage these estrogen metabolites can initiate on the DNA. The third mechanism entails altered estrogen levels that may lead to a deficient DNA repair control through progressive disruption of elements implicated in the cell cycle and epigenetic regulation, thus increasing the chance of mutagenesis. Modified from [228]

A third mechanism is that of E2 functioning as an inducer of aneuploidy (gain or loss of chromosomes). *In vitro*, E2 has been shown to disrupt microtubule organization in epithelial cells, which affects cell shape and polarity while promoting the formation of abnormal centrosomes during cell division [222,232]. Consequently, these changes may facilitate the amplification of aberrant chromosomes and alter cell cycle progression and checkpoints, thus promoting the proper genetic instability that enables carcinogenesis [233].

Over the years, several studies in postmenopausal women demonstrated that women in the highest quintile of plasma free E2 have almost a three-fold higher risk for breast cancer [234,235]. Given that estrogen formation after menopause takes place in extragonadal tissues, such as the skin and subcutaneous adipose tissue, it is not surprising that adiposity and BMI correlate positively with levels of circulating estrogens E1 and E2 in these women [234,236-238]. However, circulating levels of estrogens often do not reflect the levels in peripheral tissues [239]. In breast tumors, levels of E1, E1-S, and E2 are shown to be up to 10-50-fold higher than those in

blood [240-242]. This highlights the importance of both the local aromatization and the paracrine action of estrogens as the major endocrine mechanisms that stimulates cancer development in the breast adipose tissue.

2.2.2 Aromatase expression and regulation in breast cancer

Postmenopausal breast tumors are characterized by elevated estrogen production due to the increased aromatase expression and activity levels within the tumor itself, but mostly on the surrounding breast stroma [18,113,243,244]. The so-called desmoplastic reaction in breast tumors involves a dense layer of undifferentiated adipose fibroblasts that encapsulate the malignant cells, giving them the perfect niche to support cancer cell growth [245]. Essentially, a dynamic interaction takes place between cancer cells and their surrounding stroma to produce and maintain high levels of aromatase and estrogens, growth factors, collagen, and many other essential factors [246]. During this interaction, malignant epithelial cells produce large quantities of TNFα, IL-11 and IL-6, which may promote local aromatase expression while also maintaining stromal cells in an undifferentiated state, as they are also antiadipogenic factors [15,245,247]. As a response to the highly inflammatory environment, cancer-associated fibroblast (CAFs) respond by efficiently expressing aberrant levels of aromatase. However, it should be noted that regulation of aromatase expression in these cells differs from that in the healthy breast stroma. Furthermore, aromatase expression in the fat tissue adjacent to the tumor is also elevated at levels close to those within the tumor [15,107,242]. This maintains a gradient of aromatase expression in the affected breast tissue while providing optimal and continuous estrogen fuel to support cancer progression.

Aberrant aromatase mRNA levels in CAF are maintained through a switch in aromatase promoter use, from the weak PI.4 to the more potent PI.3 and PII [83,113]. Thus, the activity of these two promoters is the major driver of aromatase within the tumor environment (Fig. 10). However, the transcriptional activity of PI.4 is also upregulated in breast cancer, thereby contributing significantly to the total aromatase being produced [242,248]. This malignant shift to use aberrant promoters is believed to be partly caused by the excessive levels of PGE2 secreted by the malignant epithelial cells and the abundant immune cells surrounding the tumor. In fact, it has been demonstrated that the levels of TNFα, IL-6, and cyclooxygenase-2 (COX-2) are positively correlated with *CYP19A1* transcripts in cancerous breast tissues [249]. Additionally, altered levels of transcription factors LHR-1, ATF-2, and C/EBPβ in the breast cancerous milieu lead to the phosphorylation of ATF-2 into the PI.3/PII inhibitory transcriptional complex, thus allowing an increase in their transcriptional activity [15,18,250]. Other important factors thought to induce PII in breast cancer are HIF-1α and leptin, whose concentrations are significantly elevated in cancer and

obese conditions, and the reduced levels or absence of PI.3/PII related repressors, S1 and SnaH and Slug proteins [106,251,252]. In addition to the tumor stroma, aromatase is also overexpressed in the vascular endothelial cells of breast cancer tissues via aromatase PI.7 via not fully understood mechanisms [18,80,249].

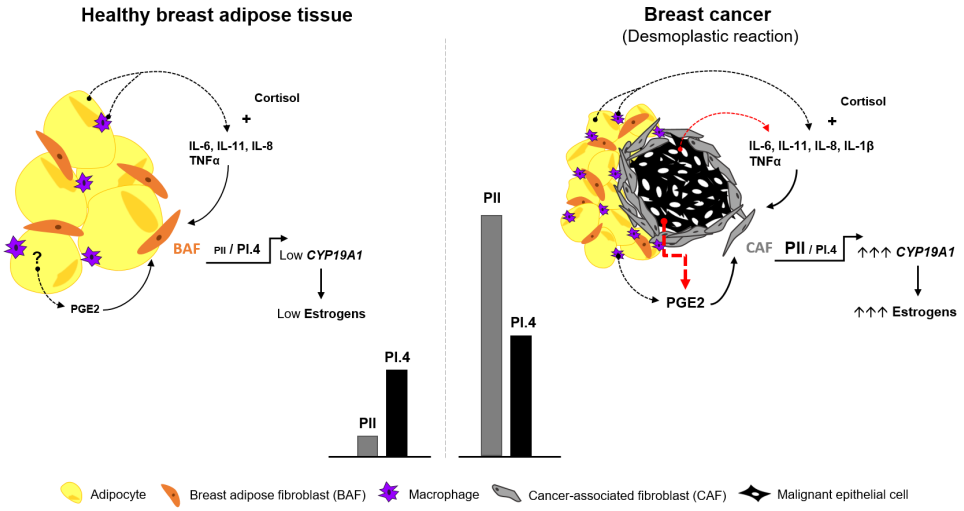


Figure 10. Aromatase promoter use in the healthy and cancerous breast. In a healthy breast, low levels of aromatase expression are maintained through the coordinated and balanced action of local inducers and repressors of PI.4 transcriptional activity in adipose tissue. Class I cytokines and TNFα produced by the adipocytes and macrophages stimulate PI.4-related aromatase gene expression in breast adipose fibroblast (BAF) in the presence of glucocorticoids. Transcriptional activity of promoter II and I.3 in adipose tissue remains very low in non-cancerous conditions. This pattern in promoter usage, however, changes in breast cancer where promoter I.3 and PII activity increase well above that of PI.4. This results in an aberrant production and activity of aromatase inside the tumor, but mainly in the surrounding stroma (desmoplastic reaction) consisting of cancer-associated fibroblasts (CAF). The driving cause of this shift is thought to be the increased production of proinflammatory factors, particularly PGE2, by the malignant epithelial cells, adipocytes and resident macrophages.

Currently, aromatase inhibitors (AI, anastrozole, and letrozole) are the most effective endocrine therapy against ER+ breast cancer, proving that excessive aromatization is an important factor in breast carcinogenesis. Several randomized trials have proved the therapeutic superiority of AI over selective estrogen receptor modulators (SERMs, e.g. tamoxifen) in the treatment of locally advanced or metastatic breast cancer [253-256] . Moreover, tamoxifen has shown to increase the expression of ERα within the tumor, while AI may promote the expression of ERβ [257]. This last issue may explain the elevated resistance observed in patients using SERMs and the superior efficacy of AI [258].

2.2.3 Obesity-inflammation-aromatase axis

Obesity, defined as having a body mass index (BMI) greater than 30, is considered a global health challenge that affects over 600 million adults worldwide [259]. In the last decades, the prevalence of obesity has significantly increased, and it is expected to further grow over the next decades. In the USA, almost 40% of the total adult population are obese, followed by Europe with a prevalence of around 23% [260-264]. Although prevalence rates may differ significantly among men and women within and between countries, on a global scale, more women are obese than men [259,264-266]. Rates of obesity dramatically increase in women after the age of 40, reaching almost 65% between 40-59 years, and 73% in women over 60 [141].

Strong associations between obesity and several metabolism-related diseases are well established, including hypertension, cardiovascular diseases, type 2 diabetes and metabolic syndrome [267-270]. Obesity is also associated with increased risk of postmenopausal breast cancer and with poorer survival rates and increased recurrence [1,29,271]. A substantial amount of evidence indicates that serum estrogen levels are associated with increasing BMI in postmenopausal women [30,237,272,273]. Importantly, by losing weight and increasing physical activity, women could reverse the implication of this relationship and reduce their risk of breast cancer [236,274,275]. Excessive energy intake forces adipose tissue to adjust to meet the new metabolic circumstances, thus promoting the stock of lipids and the expansion of the tissue via hyperplasia and/or adipocyte hypertrophy. At the same time, a pro-inflammatory milieu arises in the obese WAT due to the presence of cellular stress, fibrosis, hypoxia and adipocyte dysfunction or death [276-278].

In the early stages of obesity, hyperplasic adipose tissue and hypertrophic adipocytes secrete increased levels of pro-inflammatory factors, including TNFα, IL-6, IL-1β, adipokines, and chemokines, particularly leptin and CCL2, respectively. This, in turn, is associated with immune activation, characterized by increased recruitment and invasion of immune cells, especially macrophages, and increased lipolysis, which result in the release of free fatty acids (FFA) by the adipocytes [267,279-281]. This inflammatory condition is able to activate the I.4 promoter in BrAT while leading to the local overstimulation of aromatase gene transcription.

As this process progresses and adiposity reaches critical levels, the obesity-mediated inflammatory, metabolic, and hormonal disturbances intensify. Increased number of activated M1 macrophages within the BrAT exacerbates the local level of pro-inflammatory factors (TNFα, IL-1β, IL-8, IL-6, and PGE2) and, importantly, the secretion of CCL2 [267,279,282]. The crosstalk between hypertrophic adipocytes and M1 macrophages provokes the initiation of a vicious circle of additional macrophages and pro-inflammatory immune responses [283]. Hypertrophic adipocytes often undergo cell death, which contribute then to the formation of crown-like structures (CLS). These are defined as clusters of lipid-scavenging macrophages surrounding free lipid

droplets from dead adipocytes [282,284]. The presence and abundance of CLS in obese WAT are often used as a marker of chronic inflammation, but also as a marker to indicate severity of obesity, as they are associated with altered adipose tissue gene expression, cytokine overproduction, systemic insulin resistance and vascular endothelial dysfunction [262,285,286]. Moreover, due to limited vascularization and restricted oxygen availability in the obese WAT, a hypoxic environment ensues with increased levels of HIF-1 and vascular endothelial growth factor (VEGF) [277,287]. The lipid-laden adipocytes, on the other hand, release large amounts of saturated FFAs that are ready to activate inflammasome complexes, which results in the production of NFκB and subsequent activation of its related pro-inflammatory pathways, as well as in the over-secretion of PGE2 by macrophages [27,281,283,288,289].

Ultimately, the combination of tissue inflammation and hypoxia during obesity is associated with a stage of insulin resistance, increased lipolysis and excess leptin production in the adipose tissue that may promptly promote systemic metabolic dysfunctions, such as diabetes [290-292]. More importantly, at this stage, elevated levels of TNFα, IL-6, IL-11, IL-1β, and PGE2 in BrAT might already promote carcinogesis either by a direct action on cell proliferation or by increasing aggregation of aromatase expressing stromal cells within the tissue. In addition to the increased levels of PGE2, elevated levels of other aromatase PII inducer factors, including leptin and HIF-1, are also found in the obese BrAT [27,105,251,293].

Studies have demonstrated that circulating and WAT levels of estrogens are higher in obese postmenopausal women [22,82,236,237,294-297]. The causes of this are partly attributed, firstly, to an obesity-mediated decrease in SHBG (sex hormone-binding globulin) concentration, thus enhancing the androgen and estrogen delivery to peripheral tissues, and secondly, to an increased expression of aromatase in WAT via upregulation of all local promoters, PI.4, PI.3 and PII [120,297-299]. Adiposity directly correlates with the expression levels of aromatase gene in adipose tissue, where levels can reach up to three-four-fold higher in the breast of obese postmenopausal women [26,27,295,300]. While increased levels of androgens in the obese breast adipose tissue may offer more substrate for estrogen synthesis, the increased local aromatase expression may support a greater conversion of androgens to estrogens within the tissue.

Taken together, obesity-mediated inflammatory, hormonal, and metabolic alterations support the increased activity of PI.4 and aberrant use of PI.3/PII in WAT, thereby enhancing aromatase expression and estrogen production, and the risk of developing breast cancer in postmenopausal women. While the mechanisms behind this complex interplay are not fully defined, the obesity→ inflammation→ aromatase axis is regarded as a significant driver of breast carcinogenesis after menopause and is thus a valuable target for cancer prevention (Fig. 11) [24,301-303].

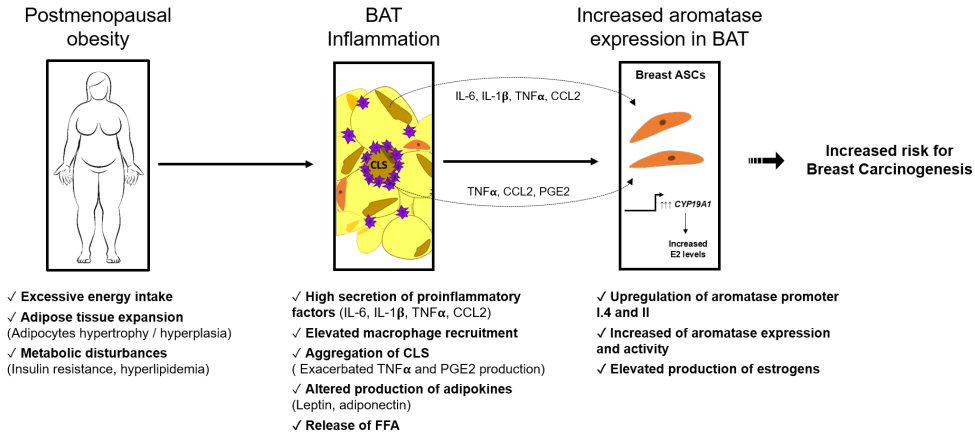


Figure 11. The obesity-inflammation-aromatase axis driving breast growth in postmenopausal women. The prevalence of obesity and overweight is higher in postmenopausal women, as well as is the risk of metabolic syndrome and ER+ breast cancer. Adiposity and associated low-grade inflammation in the breast adipose tissue (BrAT) modulate susceptibility to carcinogenesis. In an attempt to store the excessive caloric energy, inflamed and hypertrophic adipocytes secrete elevated levels of proinflammatory factors that, in addition, alter the local metabolic function, stimulate the recruitment of macrophages and subsequent formation of CLS. Combined, these three highly inflammatory entities support and maintain high levels of TNFα, IL-1β, IL-6 and PGE2 that overstimulate the expression of aromatase in BrAT through the action of all local promoters I.4, I.3 and II. Breast adipose tissue of obese women exhibits higher levels of testosterone, which may result in greater availability of C19 substrates for aromatization. Consequently, high levels of estrogens in the breast of obese postmenopausal women may prompt epithelial cells to proliferate, which increases the risk of local carcinogenesis.

2.3 Dietary Polyphenols and Regulation of Adipose Tissue Function

Polyphenols are secondary plant metabolites with a variety of essential intrinsic functions, including protection against UV light, herbivores, and pathogens. Moreover, polyphenols are implicated in the formation of bitter taste and pigmentation in plants that attracts pollinating insects [304,305]. These chemical compounds characterize for a very strong antioxidant activity as a result of the aromatic rings and hydroxyl groups in their structure [306].

To date, several thousand molecules having a polyphenol structure have been identified, and these are the most abundant antioxidants in the human diet [307]. Nevertheless, depending on the number of phenolic rings and the position of their hydroxyl groups and other structural elements, most of polyphenols can be classified into only four groups: phenolic acids, flavonoids, stilbenoids, and lignans (Fig. 12).

The structure of polyphenols is also important with regard to their biological activity, efficacy, bioavailability, and pharmacokinetics [308-310]. Phytoestrogens, including

isoflavones, stilbenes, and lignans, are considered nonsteroidal estrogens with a similar structure and function to E2 [311-313]. The dietary isoflavone daidzein and the lignan secoisolariciresinol are metabolized by the gut microbiota to equol and enterolignans, respectively, the estrogenic and antioxidant potential of which are even higher than their initial precursors [314]. However, consumption of polyphenol-rich food usually provides only a low concentration of these compounds in blood and urine, sometimes due to poor absorption or because of extensive metabolism and rapid excretion [308,310].

Nevertheless, the consumption of fruits, seeds, vegetables, and soy, which contain dietary polyphenols, are associated with a reduced risk of several cancers [32,315]. While the mechanisms by which polyphenols interfere in cancer development and progression are still controversial, there is a clear indication that chemopreventive actions of these compounds result from improving energy metabolism and inflammation, as well as, directly suppressing tumor development and progression [315].

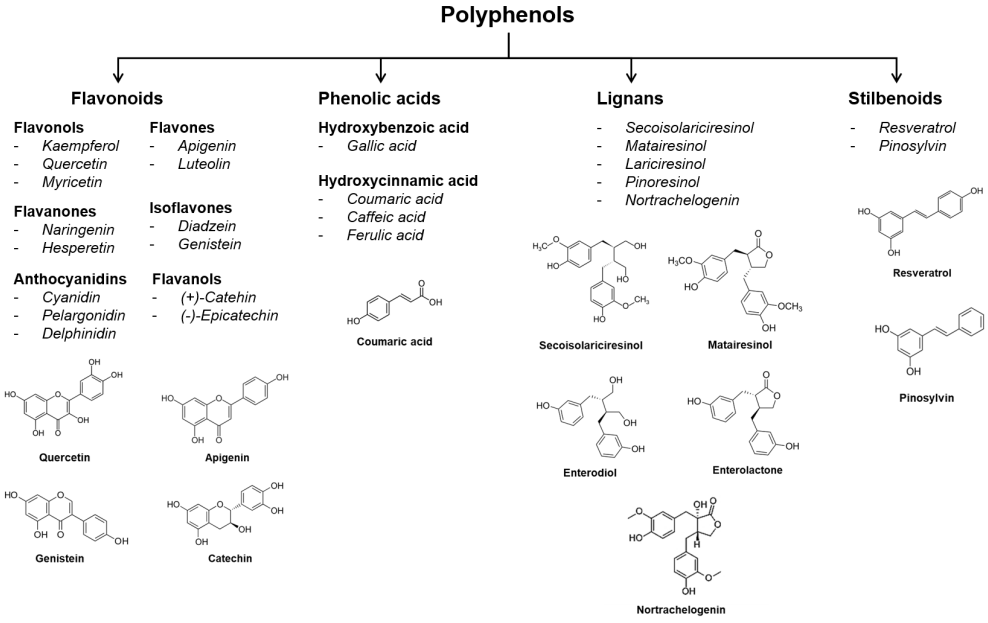


Figure 12. Classification of dietary polyphenols and the chemical structure of selected examples.

2.3.1 Role of polyphenols in obesity and obesity-related inflammation

Numerous studies in cells and obese animal models suggest that polyphenols, especially catechins (flavonoids, very abundant in green and black tea), resveratrol

(RSV, a stilbenoid common in red grape skin and wine), and curcumin (a hydroxycinnamic acid) have significant effects on obesity [316,317]. Whether these interventions were based on polyphenol-rich diets or pure isolated compounds, long-term interventions in obese mice and rats have shown to improve energy metabolism and reduce body weight and triglycerides, making them a valuable alternative to prevent obesity and other metabolic related diseases in humans, such as diabetes, hypertension, cardiovascular diseases, and cancer.

The anti-obesity effects of dietary polyphenols are thought to be driven by several mechanisms: 1. Reducing food intake, 2. Decreasing lipogenesis, 3. Increasing lipolysis, 4. Inducing fatty acid β-oxidation, 5. Preventing adipocyte differentiation, 6. Suppressing inflammatory responses and oxidative stress and, 7. Altering the composition of the gut microbiota [316]. Interestingly, dietary polyphenols appear to modulate signaling pathways related to adipogenesis, lipogenesis, and lipolysis in cells and animal studies [318,319].

Cathechins and RSV have been shown to activate AMPK/sirtuin-1/ peroxisome proliferator-activated receptor gamma coactivator 1-alpha cascade, therefore reducing cholesterol, fatty acid and triglyceride formation in adipose tissue [316,320-322]. Polyphenols may also reduce body fat and fat storage by increasing lipolysis, fatty acid β-oxidation and thermogenesis via stimulation of key metabolic factors such as hormone-sensitive lipase, carnitine palmitoyltransferase-1, and uncoupling proteins [323-326]. In cultured preadipocytes, catechin, RSV, and curcumin have been shown to inhibit expression of genes that participate in the differentiation of these cells into mature adipocytes, such as CCAAT/enhancer-binding protein-β proteins, peroxisome proliferator-activated receptor gamma and adipocyte fatty acid-binding protein 2 [316]. In the case of inflammation, catechins, RSV, curcumin, and lignans act as anti-inflammatory agents blocking the production of obesity-related cytokines, resistin, IL-6, TNFα and CCL2, while suppressing the recruitment of macrophages into the adipose tissue. This last feature is attributed to a direct effect on NF-κB and MAPK signaling pathways [316,327-332]. Furthermore, recent evidence suggests that the ability of polyphenols to modulate energy metabolism may involve modulation of the gut-brain axis [333,334]. In fact, polyphenols are shown to alter the gut microbiota by reducing the growth of inflammation-related bacteria or to favor the growth of beneficial bacteria and potentially shift the microbiota composition into a non-obesogenic type [335-339]. On the other hand, the intestinal microbiota is a key metabolic site for many phenolic compounds, generating the bioactive metabolite compounds that will ultimately produce the effects in the body [340].

The current evidence on the consumption of polyphenols and the reduction of obesity is limited and still quite inconsistent. One of the main reasons for this is the huge variation in absorption and metabolism between individuals during a dietary intervention [310,341]. Nevertheless, some human studies have reported significant

effects on polyphenols reducing body weight and preventing obesity. For example, catechins from tea have been shown to reduce body weight gain, fat mass, and waist circumference in obese humans [342,343]. Higher consumption of flavonoids, especially soy isoflavones, are associated with lower BMI and lower plasma lipids levels in obese subjects, although the potency of such effects may vary when using soy or the pure compounds [344-346]. Flaxseed lignans, on the other hand, have been shown to reduce central obesity and to improve the metabolic profile of obese postmenopausal women, particularly in improving insulin sensitivity[347-350].

2.3.2 Role of dietary polyphenols in the prevention of breast cancer

The impact of polyphenols on breast cancer risk is thought to be largely influenced by their ability to modulate oxidative stress and inflammation, but also by their actions on the molecular targets and protein cascades critical for breast cancer development and progression. These include cell survival, proliferation, migration and differentiation, production, and action of sex hormones, immune responses, to name a few. However, as for other disease conditions, the beneficial effects of dietary polyphenols on breast cancer remain limited and controversial since most of the evidence still comes from cells and animal studies.

Among all dietary polyphenols, the most studied compounds in epidemiological trials are the isoflavones, genistein and daidzein. Numerous studies evaluating the association between high consumption of soy and soy-derived isoflavones and breast cancer risk, particularly among Asian population, have reported positive effects in reducing breast cancer incidence [351-357]. Most of these preventive actions appeared to be the result of reducing important risk factors, including circulating ovarian hormones and adrenal androgens levels, obesity, and inflammation. However, several other epidemiological studies in pre- and postmenopausal women have not identified an association between soy intake and breast cancer risk [358-363].

Resveratrol has demonstrated potential breast cancer-preventive effects in recent preclinical studies [364]. However, few epidemiological trials have investigated these actions in humans. For example, Levi and coworkers analyzed the impact of resveratrol from three different dietary sources, red wine, white wine, and grapes, on postmenopausal breast cancer risk [365]. The study suggested an inverse relationship between breast cancer risk and dietary resveratrol intake, but only when it was obtained from grapes since alcohol intake was positively associated with breast cancer risk. Resveratrol consumption also increased the concentration of the sex hormone-binding globulin (SHBG) in postmenopausal women [366], which inversely associated with the risk of breast cancer.

Furthermore, a high intake of flaxseed containing dietary lignans, secoisolarecinol (SECO), a precursor of the enterolignans, enterodiol (END), and enterolactone (ENT) is associated with reduced breast cancer risk in both pre- and postmenopausal women [34,367,368]. In postmenopausal women, daily consumption of around 32 g of flaxseeds was associated with 20% lower risk of breast carcinogenesis and breast cancer mortality [367,369]. In these studies, authors also suggest that such preventive effects may be due to a strong anti-inflammatory effect by lignans. Moreover, consumption of phytoestrogens (isoflavones and lignans) was associated with a reduced breast cancer risk in premenopausal women [356,368]. The risk was also lower among overweight women, which indicates that modulation of BMI and other obesity-related disturbances could be their possible mechanism of action. In postmenopausal women with primary breast cancer, the study conducted by Thompson et al. showed that daily intake of muffins containing 25 g of flaxseeds reduced markers of tumor growth [370]. In combination with the aromatase inhibitor, anastrozole, 25 g of flaxseeds/day did not show any change in tumor markers of proliferation, apoptosis, or circulating sex hormone levels in postmenopausal women with ER+ breast cancer [371].

Multiple questions remain unanswered regarding the specific molecular mechanisms of action of dietary polyphenols to reduce breast cancer risk. Nevertheless, an increasing amount of data indicates that consumption a polyphenol-rich diet could prevent postmenopausal breast cancer. However, further studies need to investigate whether these compounds could improve current drug preventive strategies.

2.4 Nonclinical Models to Study the Regulation of Human Aromatase Gene

Mechanisms by which obesity, inflammation, and breast cancer regulate expression of *CYP19A1* in extragonadal tissues are difficult to study in human subjects. Therefore, several *in vitro* and *in vivo* models have been developed to study the regulation of *CYP19A1* expression.

An important finding regarding aromatase regulation in WAT emerged with the discovery of the specific cell-site expressing this gene within the tissue. In 1992, it was found that aromatase mRNA expression in the adipose tissue primarily occurs in the stroma rather than in the mature adipocytes [81,288]. While the debate is still ongoing, it is widely accepted that the epithelial fraction is also an important source of aromatase in the breast, especially in cancer conditions [244,372,373]. Since then, an *in vitro* model of primary cultured adipose fibroblasts has been successfully used to study the regulation of estrogen biosynthesis in physiological and disease conditions [374]. However, there are important limitations with translating the *in vitro* data to *in*

vivo conditions. For example, while it is known that glucocorticoid induces aromatase in cultured ASCs, no association has been found regarding the levels of glucocorticoids and the aromatase expression in women's WAT *in vivo* [19,375].

Although, aromatase is widely expressed in most vertebrate species, particularly in the gonads and brain, humans and higher primates are the only species with a strong extragonadal expression [15,376]. Furthermore, there are also important regulatory and structural differences in the aromatase gene between species. Compared to that of humans, the mouse aromatase gene (*Cyp19a1*) covers only 104 kb, but equally it contains IX translated exons (Fig. 13). Transcription of *Cyp19a1* is also regulated in a tissue-specific manner by the use of tissue-specific exons I or tissue promoters. However, its regulation appears to be significantly simpler as it contains fewer promoters. Only five aromatase promoters have been identified in mice: P2 (ovaries), I.f (brain), E_{tes} (testis), I.3 (ovaries and testis), and I.4 (visceral adipose tissue), but their regulation remains unknown (Fig. 13) [377-379].

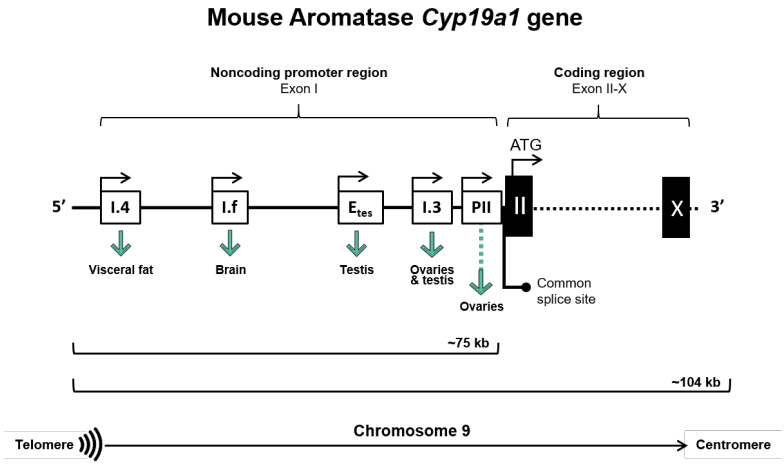


Figure 13. Structure of the *Cyp19a1* gene. Similar to the human aromatase gene, *Cyp19a1* in mice is also regulated by several tissue-specific promoters situated upstream the actual coding region containing nine exons (II-X). However, only five promoters have been identified in mouse tissues: PI.4 in gonadal fat, I.f in brain, E_{tes} in testis, I.3 in gonads and PII in ovaries. Modified from [379]

Nonetheless, due to the relatively high sequence homology that some of these promoters have to their human counterpart, researchers have used WT mice, especially C57BL/6J, to study the impact of distinct obesogenic conditions on mouse aromatase expression in the adipose tissue. Initially, Zhao and coworkers [377] demonstrated the presence of one promoter driving aromatase in visceral adipose tissue of male mice in a similar way as the I.4 promoter in humans. While they could

not find expression of this promoter in subcutaneous adipose tissue or in females by using primary mouse adipose fibroblasts, they found that local *Cyp19a1* mRNA expression was induced by DEX, similar to the I.4 human promoter. Later, it was shown that in both OVX and *ob/ob* obese C57BL/6J females, mammary gland aromatase mRNA expression is increased [302]. However, due to the existing differences in aromatase gene regulation between humans and mice, humanized aromatase mouse models are preferred. Several humanized aromatase mouse models have been generated over the past years. Most of these have utilized only specific fragments or promoter regions of the *CYP19A1* [380-383]. However, at least two transgenic mouse models have been successfully designed to express almost all promoters along the exon I of *CYP19A1*. The humanized aromatase ($Arom^{hum}$) mouse model created by Bulun's group in 2012 [119] and the human aromatase reporter (hARO-Luc) mouse model created in 2013, by Poutanen's group [384].

Transgenic $Arom^{hum}$ mice (FVB/N) express human aromatase gene in several mouse tissues driven by its native promoters [119]. Importantly, the aromatase tissue expression pattern in these animals mimics the expression distribution in women. In the case of mRNA expression in mammary adipose tissue, it seems that the local aromatase transcripts are derived from proximal promoters II and I.3 and distal I.4. Therefore, and because the aromatase protein is also produced, this model has been particularly used to study the regulation of aromatase in mammary adipose tissue and its implications in breast diseases. These animals show higher estrogen levels in mammary gland tissue compared to the WT females, and therefore they exhibit increased mammary duct elongation by the age of puberty and, after 24 weeks of age, higher incidence of breast hyperplasia and carcinogenesis [119]. In the same study, researchers used $Arom^{hum}$ females on a high-fat diet (HFD) to study the effects of weight gain on mammary aromatase expression. Their results support the idea that diet-induced weight gain stimulates aromatase and estrogen production in the breast, as total aromatase mRNA expression and PI.4 and PII specific transcript expression were found elevated in the mammary adipose tissue of these animals [119].

While the sequence of the aromatase gene inserted in $Arom^{hum}$ mice contains all translated exons, the hARO-Luc reporter mouse model contains only three translated exons (II-IX). This allowed the inclusion of 100 kb of the 5' region or exon I regulatory region, containing promoters I.1, I.8, I.4, I.5, I.7, I.f, I.2, I.6, I.3 and II (Fig. 14) 384. This shorter sequence allowed researchers to introduce also a luciferase reporter at the common splice site, providing thus an easy way to measure or track gene transcription within tissues and cells. Nevertheless, it is important to point out that the human aromatase protein is not produced in this model. Thus far, the correct function and tissue distribution of promoters I.1, I.4, I.7, I.f, I.3, and II have been clearly proved in male and female hARO-Luc mice [384,385.] An important limitation of this model is, of course, its inability to correlate the expression of

aromatase with the tissue level of estrogens, as the inserted sequence translating the gene generates a truncated protein.

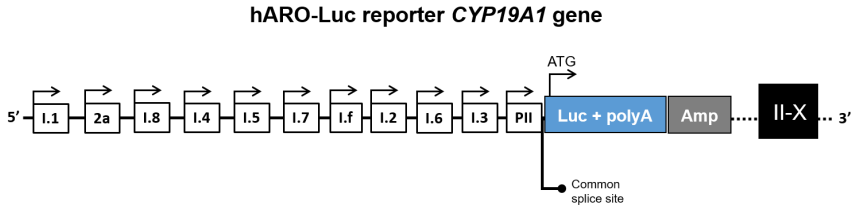


Figure 14. Reporter gene structure used in the hARO-Luc mouse model. Modified from [384]

3 Aims

The main objective of this work was to identify novel obesity-related factors that regulate aromatase expression in white adipose tissue (WAT) depots, with emphasis on those in the mammary/breast adipose tissue, as these may represent new targets for breast cancer therapy and prevention. We hypothesized that inflammatory cytokine profile in the adipose tissue determines the local aromatase level, and consequently, the local estrogen production. Results were obtained from hARO-Luc reporter mice as a tool to characterize the impact of weight gain and adiposity on *CYP19A1* gene expression in WAT depots, and from primary human cells and tissue samples as models for validation and clinical translation.

The specific aims were as follows:

1. To confirm the suitability of the hARO-Luc mouse as a model to study obesity-mediated disturbances in *CYP19A1* regulation in WAT **(I)**
2. To study the role of endogenous anti-inflammatory and proinflammatory factors, particularly IL-10 and CCL2, in the regulation of *CYP19A1* expression in bone marrow-derived mesenchymal stromal cells (MSCs) from hARO-Luc mice and human breast adipose stromal cells (ASCs) **(II, III)**
3. To evaluate the effect of weight gain and adiposity on promoting inflammation and *CYP19A1* expression in different WAT depots *in vivo* by using HFD-fed male and female hARO-Luc mice and subcutaneous fat tissue samples from obese women **(I, III)**
4. To investigate the impact of ovariectomy (OVX) and its related weight gain on *CYP19A1* gene expression in WAT and gut bacterial composition *in vivo* using OVX female hARO-Luc mice **(II)**
5. To study the potential effect of dietary polyphenols on obesity-related inflammation and *CYP19A1* reporter activity in WAT *in vivo* using HFD-fed hARO-Luc males, as well as their effects on *CYP19A1* transcription *in vitro* using cultured hARO-Luc MSCs and human ASCs **(I, unpublished data)**

4 Materials and Methods

4.1 Animal Experiments

Animal care and use were conducted in accordance with the Finnish Act on Animal Experimentation and EU laws, guidelines, and recommendations. The studies were approved by the national Animal Experiment Board in Finland (ESAVI/7471/04.10.03/ 2012). Mouse line maintenance and genotyping was done in collaboration with the Turku Center for Disease Modeling (TCDM).

hARO-Luc mice [384] were housed under standard conditions in the Central Animal Laboratory at the University of Turku. The mice were maintained with 12 hours light/dark cycle in constant temperature (21 ± 3 °C) and humidity (55 ± 15 %), fed with soy-free RM3 chow (SDS, Whitham, Essex, UK) and tap water *ad libitum.* Mice were euthanized by CO_2 inhalation followed by cervical dislocation.

4.1.1 Primary bone marrow derived MSCs **(I, II)**

Mesenchymal stromal cells (MSCs) were collected from 4-6-weeks-old hARO-Luc female mice. Cells were isolated from femurs and tibias by flushing bone marrow from the diaphysis with stromal medium: Minimum Essential Medium alpha (MEM, Life Technologies Ltd, NY, USA) medium supplemented with 15% heat inactivated fetal bovine serum (USA origin, Gibco, Carlsbad, CA, USA), 10 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 mM L-glutamine, 10 nM of DEX (SERVA, Heidelberg, Germany) and 0.25 µg/ml amphotericin B (Fungizone, Life Technologies Ltd). After centrifugation at 200 x g for 8 min, the cell pellet was resuspended in stromal medium, plated at density of $1x10^6$ cells/cm^2 into T75 flasks, and cultured at 37 °C humidified atmosphere with 5 % CO_2. After 2 days, non-adherent cells were removed by washing the culture with phosphate buffered saline (PBS) and fresh stromal medium was changed every 48 hours until the culture was 60-90% confluent. For every assay, cells from 2-3 animals were pooled.

MSCs were plated into 12-wells culture plates with DEX-free stromal medium (**II**) or DEX and serum-free SC medium (**I**) at a density of 40.000 cells per well. To study the expression of aromatase gene (Luc reporter activity) via PI.4 or PII, 80-90% confluent MSCs were exposed to 250 nM DEX + 5 ng/ml TNFα (Sigma-Aldrich, Saint Louis, MO, USA) or 25 µM forskolin (FSK, Sigma-Aldrich) + 4 nM

phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), respectively. For cells in publication **I,** medium was supplemented with 10 mg/L polyphenol rich pine knot extract (PKE) or one of the following polyphenols (10 μM): pinosylvin monomethylether (MePS), pinosylvin (PS), nortrachelogenin (NTG) or stilbenoid mixture (LSmix). For cells in publication **II**, 20 ng/ml IL-10 (PeproTech, Rocky Hill, NJ, USA) was additionally added into the culture medium.

After 24 h incubation, cells were washed with PBS and lysed with Tropix lysis solution (Applied Biosystems, Foster City, CA) supplemented with 0.5 mM dithiotretiol (Sigma Aldrich) for protein concentration and luciferase activity analyses, or with RLT buffer supplemented with β-mercaptoethanol for RNA isolation.

4.1.2 High-fat diet (HFD)-induced obesity: *In Vivo* study **(I)**

The experimental design followed is shown in figure 15. A total of 44 hARO-Luc males were used for this study. At 6 weeks of age, animals were distributed according to their body weight and adiposity into 3 different dietary intervention groups: 1. LFD group (n=17) fed with purified low-fat diet providing 10 % of calories from fat (D12450 - Research Diets Ltd, New Brunswick, NJ, USA), 2. HFD group (n=17) fed with HFD providing 60% of calories from fat (D12492 - Research Diets Ltd), and 3. HFD-PKE group (n=10) fed with HFD supplemented with polyphenol-rich extract (PKE, 1600 mg/kg/diet). The preparation, analysis of the composition and bioavailability of PKE, obtained from *Pinus sylvestris* was carried out as described in [386]. Dietary interventions lasted for 8 weeks.

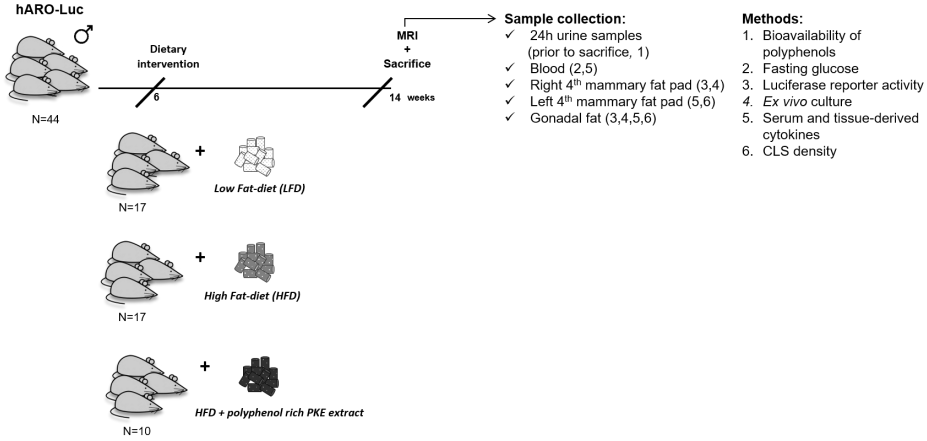


Figure 15. Experimental design for the dietary intervention study in I. In sample collection, the numbers in parentheses specify the method for which it was used.

Mice were weighed weekly and their body adiposity was measured by EchoMRI (EchoMRI LCC, Houston, TX, USA) in the beginning, middle and end of dietary intervention (at 6, 10 and 14 weeks of age). Food intake per cage was measured twice a week. To analyze the bioavailability of PKE-derived polyphenols in the animals, 24 h urine samples were collected in metabolic cages, and the concentration of MePS, PS, NTG and matairesinol (MR) were determined as previously described [386].

Prior to sacrifice, mice were fasted for 4 hours and blood samples were collected from tail vein for fasting glucose measurements. For serum measurements, blood samples were collected by intracardiac puncture and immediately stored at -70 °C. Subcutaneous (inguinal fad pad) and gonadal fat tissue samples were collected, weighed and snap frozen to liquid nitrogen and stored at -70°C for Luc activity measurements, *ex vivo* cultures and histological analyses.

4.1.3 Ovariectomy (OVX)-induced weight gain: *In vivo* study **(II)**

For this experiment, a total of 11 hARO-Luc female mice were used. Each animal was housed individually and maintained on soy-free chow diet (RM3, SDS). The experimental design followed is shown in figure 16. Starting at 4 weeks of age, mice were separated and placed into individual cages. At the age of 8 weeks, 6 females were OVX whilw 5 remained intact. Mice were weighed weekly and their body fat composition was measured by EchoMRI one week before OVX and later, just before the end of the experiment. The two 4th inguinal mammary fad pads and gonadal fat samples were collected and weighed. The right mammary fat pads and gonadal fat were snap frozen in liquid nitrogen and stored at -70 °C for Luc activity measurements. The left mammary fat pads were divided in two pieces. One was used for measuring crown like structures (CLS) density, and the second one, along with a gonadal fat sample, were placed into a 12-well plate and maintained with 1 ml modified Eagle Medium (DMEM/F-12, Life Technologies Ltd) containing 100 IU/ml penicillin and 100 µg/ml streptomycin for 24 h. Conditioned media were collected and stored at -70 °C for *ex vivo* tissue-derived IL-10 measurement. Additionally, colon tissue and fecal samples were collected and immediately frozen in liquid nitrogen and stored at -70 °C for qPCR, Luc activity and microbiota sequencing analyses.

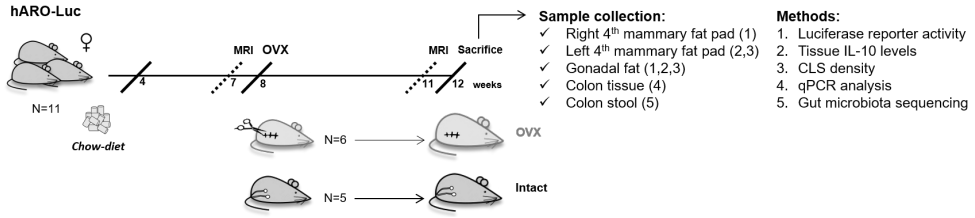


Figure 16. Experimental design for the OVX-induced weight gain study in II. In sample collection, the numbers in parentheses specify the method for which it was used.

4.1.4 High-fat diet (HFD)-induced obesity: *In Vivo* study **(III)**

The experimental design followed is shown in figure 17. 6-week-old hARO-Luc female mice were weighed and their whole-body fat content was measured with EchoMRI. After this, mice were allocated into two groups with similar body weights and adiposity, and fed with purified low-fat D12450B diet (LFD, 10% of calories from fat) or high-fat D12492 diet (HFD, 60% of calories from fat) obtained from Research Diets Inc. Experiments were done in three parts with 4 to 6 animals per group for a final number of 16 mice on LFD and 17 mice on HFD. After 8 weeks, whole body fat content was measured, four hours fasting blood was collected from tail vein samples for fasting plasma blood glucose, and mice were sacrificed. Cytokine concentrations were measured in serum samples obtained from cardiac puncture blood. Right inguinoabdominal mammary fat pad and samples from gonadal fat and retroperitoneal fat were snap frozen in liquid nitrogen and stored at -70 °C for Luc activity measurements. Furthermore, mammary fat pad (left side), gonadal fat and retroperitoneal fat were collected and equally divided. One part was placed into a 12-well plate for *ex vivo* tissue-derived cytokines measurement, and another one was exposed to 10% neutral buffered formalin. Formalin fixed tissues were sectioned and stained with hematoxylin (HE), and CLSs were counted from HE stained sections.

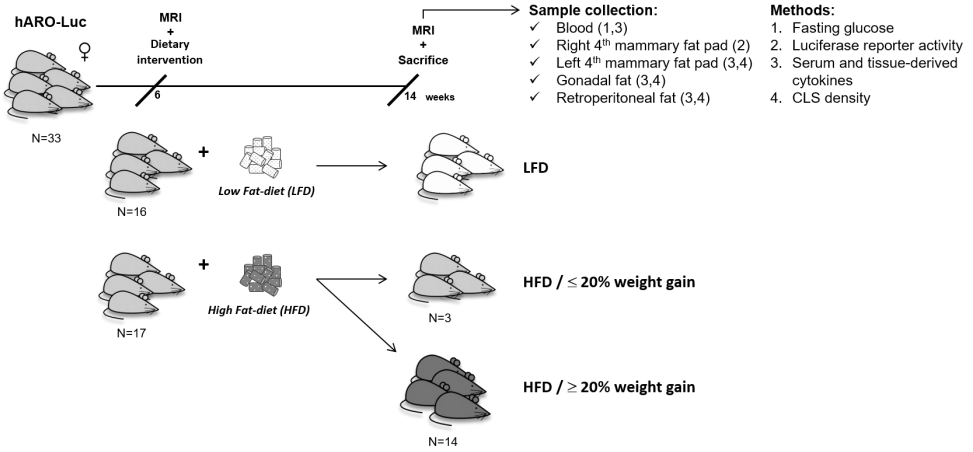


Figure 17. Experimental design for the HFD-induced obesity study in III. In sample collection, the numbers in parentheses specify the method for which it was used.

4.1.5 White adipose tissue (WAT) explants: *Ex vivo* cultures **(I, III)**

Inguinoabdominal subcutaneous and gonadal adipose tissue samples were collected from 4-month-old hARO-Luc males on HFD (**I**) and from 4-6-month-old hARO-Luc females (**III**). Following a previously described method [387], tissue samples were divided into equal explants of approximately 100 mg of weight, and placed separately on 24-well culture plates with 1ml of DMEM/F12, supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. In every experiment, tissues from 4 animals were used. Luc aromatase reporter activity via PI.4 was induced with 10 nM and 250 nM DEX in gonadal and subcutaneous fat tissue samples, respectively. To examine the effect of other common proinflammatory factors on WAT aromatase expression, tissue samples in **I** were treated also with 100 or 1000 ng/ml lipopolysaccharide (LPS, Sigma Aldrich) from E. coli 0111: B4. To investigate the potential effect of CCL2 on regulation of local aromatase gene expression, tissues in publication **III** were additionally treated with 100 ng/ml CCL2.

After 24 h incubation, tissue samples were collected and analyzed for Luc activity.

4.2 Human Tissues and Cells

4.2.1 Primary human breast ASCs **(II, III)**

In publication **II**, to examine the potential effect of IL-10 on modulation of aromatase and PI.4 transcripts expression, primary adipose stromal cells (ASCs)

were isolated from breast adipose tissue of two women undergoing reduction mammoplasty (average age 45 y, average BMI 30 kg/m^2). The collection of these samples was done at the Monash Medical Centre hospital in Melbourne, Australia; and was approved by the Monash Health Human Research Ethics Committee B (00109B). In the manuscript **(III),** to examine the potential activation of MAPK-ERK1/2 signaling pathway by CCL2 on breast ASCs, were obtained from Turku University Hospital (Ethical committee approval ETKM 23/2018). Isolation was done by collagenase and hyaluronidase digestion as previously described [374]. Cells were maintained in Waymouth's medium (Life Technologies Ltd) or DMEM/F-12 containing 5-15% calf serum until they reached 70% confluency. ASCs in **I** were then treated with 10 nM DEX alone or in combination with 5 ng/ml TNFα, and 10 μM IL-10, while cells in **III**, were treated as described later in 4.7.

To investigate the impact of the ERK1/2 signaling pathway on TNFα-mediated *CYP19A1* expression (**II**) and the role of CCL2 and dietary polyphenols in the regulation *CYP19A1* transcription (**III** and **unpublished data,** respectively) in the breast adipose tissue, cryopreserved breast ASCs isolated from women undergoing elective breast reduction were purchased from ZenBIO, Inc (Research Triangle Park, NC, USA). For each experiment, three replicates using cells from three different donors (aged 20 – 66 y, BMI: 25.1 – 29.1 kg/m2) were performed. At first, ASCs were plated at a density of 2x10^4/cm^2 in T75 flasks containing DMEM/F-12 supplemented with 15% heat inactivated fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin; and were maintained at 37°C humidified atmosphere with 5% CO2. Once they reached approximately 70% confluency, cells were serum starved overnight in medium containing 0.1% BSA (serum-free medium). For publication **II**, ASCs were treated with 10 nM DEX alone or in combination with 5 ng/ml TNFα and U0126 (ERK1/2 inhibitor, Sigma-Aldrich). In **III**, ASCs were treated with 10 nM DEX alone or in combination with 100 ng/ml CCL2 and one of the following inhibitors (10 μM): U0126, BAY 11-7082 (NFκB inhibitor, Sigma-Aldrich) or RS 504393 (CCR2 inhibitor, Tocris Bioscience, Bristol, UK). Lastly, for the **unpublished data**, ASCs were treated with 10 nM DEX alone or in combination with 5 ng/ml TNFα to induce PI.4-related *CYP19A1* expression, and with 25 μM FSK + 4 nM PMA to induce PII-related *CYP19A1* expression. Additionally, cells were treated with one of the following polyphenols (10 μm): PS, genistein (GNS), resveratrol (RSV, Sigma-Aldrich), enterodiol (END), enterolactone (ENT) or secoisolarecinol (SECO). PS and SECO were isolated from wood knot materials at Åbo Akademi (Laboratory of Forest -Products Chemistry). ENT and END were synthesized at Åbo Akademi (Department of Organic Chemistry). GNS was obtained from Prof. William Helferich, University of Illinois.

After 24 h treatment, all ASCs were washed with PBS and lysed with TRIsure for RNA isolation.

4.2.2 Subcutaneous adipose tissue **(III)**

Snap-frozen adipose tissue samples from abdominal subcutaneous depot were purchased from ZenBIO, Inc. Tissue samples were collected from non-obese BMI (≤25) or obese (≥30) women aged 32 - 47 years.

4.3 Determination of CLS Density **(I-III)**

To assess the inflammatory status of different adipose tissue samples collected from **I**, **II** and **III** *in vivo* studies, formalin fixed tissue samples were processed for histological analysis of CLS. Paraffin embedded sections were cut at 5 µm thickness and stained with HE. To determine the average adipocyte area (1/number of adipocytes per 1 mm^2) and the CLS density (number of CLS structures per 1 mm^2 of WAT, stained fat tissue sections were scanned with Olympus BH2 virtual microscope (Digital Virtual Microscope, Soft Imaging System, Olympus, Germany) or Pannoramic 250 slide scanner (3DHISTECH Ltd, Hungary), and values were calculated from 4 randomly selected areas.

4.4 Luciferase Reporter Activity Assay **(I-III)**

Adipose tissue samples were homogenized in 300 µL of lysis buffer containing 25 mM Tris acetate (pH 7,8), 1.5 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM dithiothreitol, and Complete Miniproteinase inhibitor tablets (Roche Diagnostics, Penzberg, Germany). The homogenates were then centrifuged at +4°C, 800 x g for 30 min. Luc activity was measured from supernatants with the Luciferase assay kit (BioThema AB, Handen, Sweden) according to manufacturer's instructions by using the Victor2 Multilabel counter (PerkinElmer, Turku, Finland). The results were related to the sample weight, or protein content measured by using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) as instructed by the manufacturer.

4.5 Analysis of Serum and Tissue-derived Cytokines **(I-III)**

For the analysis in **I**, the concentration of leptin, insulin, CCL2, IL-6, and TNFα were quantified in serum and adipose tissue explant media with multiplex assays (Merck Millipore) according to manufacturer's instructions using Luminex 200 reader (Luminex corporation). For calculations, first the amount of each factor per adipocyte (m) was estimated by using the average adipocyte volume determined from the sample histological sections. Next, the relative values of CCL-2, IL-6 and TNFα ($index_{analyte}$) was determined with the formula: $index_{analyte} = m_{analyte}(99/(m_{max}$-

m_{min}))- m_{min}(99(m_{max}-m_{min}))+1 with the highest amount (m_{max}) being adjusted to 100 and the lowest (m_{min}) to 1. Finally, the total cytokine production in each adipose tissue sample (*cytokine index*) was calculated with the formula: *cytokine index*= $index_{CCL-2}$ + $index_{IL-6}$ + $index_{TNF\alpha}$.

To study the potential changes in adipose tissue IL-10 production prompted by OVX in **II**, IL-10 levels were measured from mammary and gonadal adipose tissue conditioned media by using the IL-10 Mouse ELISA Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. The colorimetric reaction was read at 450 nm with EnSight Multimode Plate Reader (PerkinElmer). Each sample was measured in duplicate and IL-10 levels were normalized to tissue protein.

For experiments in **III**, CCL2, leptin, IL-6, and TNFα levels were quantified from tissue exposed media and serum samples with the MILLIPLEX MAP Mouse Adipocyte Magnetic Panel (#MADCYMAG-72K; Merck Millipore, Billerica, MA, USA) according to manufacturer's instructions by using the Luminex 200 analyzer (Luminex Corporation, Austin, TX). The results were related either to the sample weight or protein content.

4.6 RNA Isolation, cDNA Synthesis and quantitative (q)-PCR **(II, III, unpublished data)**

In **II**, total RNA was isolated from human and mouse cells/tissues by using RNAeasy Mini Kit (Qiagen, Hilden, Germany), and treated with deoxyribonuclease I (Amplification Grade kit, Invitrogen, Paisley, UK) as instructed by the manufacturer. For *in vitro* studies using hARO-Luc-derived MSCs, 0.5 µg of total RNA was converted into cDNA by using DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) and the primer *LucAs1* (Table 1). qPCR reactions were then performed with the primers luciferase antisense *LucAs2* and the complementary sense for *aromatase PI.4* using DyNAmo flash SYBR green qPCR kit (Finnzymes). Cycling conditions were 95°C for 7 min, 40 cycles at 95°C for 10 sec, 60°C for 40 sec and 72°C for 15 sec. For hARO-Luc-derived tissue samples, cDNA synthesis was carried out by using DyNAmo cDNA synthesis kit (Finnzymes) and random hexamers primers. qPCR reactions were done using DyNAmo flash SYBR green qPCR kit with mouse primers: *Il-10, Il-6, Pyy, Gcg-1, Tnfα, Fiaf, Lpl*, under the following conditions: 10 min at 95°C and variable number of cycles at 95°C for 10 sec, specified annealing temperature for 30 sec, and 72°C for 15 sec. Transcript levels were then normalized to expression levels of mouse *Ppia* and mouse *L19* genes (Table 1). For human ASCs, 0.5 µg of total RNA was reverse transcribed using Super-Script III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, US). qPCR was performed with the primers: *hArom* and *PI.4* by using FastStart DNA Master SYBR Green Kit (Roche, Pleasanton, CA, USA). Cycling conditions were 95°C for 10 min and

variable number of cycles at 95°C for 10 sec, specified annealing temperature for 30 sec, and 72°C for 15 sec. Expression levels were normalized to housekeeping gene human *ACTB*.

For transcriptional studies in **III** and **unpublished data**, total RNA from human cells/tissues was isolated by using TRIsure (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. Isolated RNA (1 µg) was then digested with deoxyribonuclease I (Amplification Grade kit, Invitrogen, Paisley, UK) and converted to cDNA by using SensiFAST cDNA Synthesis Kit (Bioline, London, UK), as instructed by the manufacturer. qPCR was performed with the primers *hArom* and *PI.4* by using the Dynamo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific). The cycling conditions were 95°C for 10 min and variable cycles of 95°C for 10 sec, 59°C for 30 sec and 72°C for 15 sec. Expression levels of the final products were normalized to one or two of these human housekeeping genes *ACTB*, *GAPDH*, *RPL19* or *G6PD* (Table 1).

Table 1. Genes analyzed by qPCR

Gene	Forward 5´-3´	Reverse 5´-3´
Mouse cells / tissues		
LucAs1		AATAACGCGCCCAACACCGG
LucAs2	ACTGCATACGACGATTCTGT	
Aromatase PI.4	GACCAACTGGAGCCTGACAG	
Il10	TGAGGCGCTGTCGTCATCGATTTCTCCC	ACCTGCTCCACTGCCTTGCT
Il6	GGCCTTCCCTACTTCACAAG	ATTTCCACGATTTCCCAGAG
Pyy	AGCTCTGTTCTCCAAACTGC	TGCAAGTGAAGTCGGTGTAG
Gcg1	TGGCAGCACGCCCTTC	GCGCTTCTGTCTGGGA
Tnf	GAACTGGCAGAAGAGGCACT	AGGGTCTGGGCCATAGAACT
Angptl4 (Fiaf)	GATAGGTATCTCTGCTGCTGGG	GGAGGTTGCCGACATAAAGC
Lpl	CTGCTGGCGTAGCAGGAAGT	GCTGGAAAGTGCCTCCATTG
Ppia	CATCCTAAAGCATACAGGTCCTG	TCCATGGCTTCCACAATGTT
Rpl19 (L19)	GGACAGAGTCTTGATGATCTC	CTGAAGGTCAAAGGGAATGTG
Human cells / tissues		
CYP19A1	TTGGAAATGCTGACCCGAT	CAGGAATCTGCCGTGGGAGA
Aromatase PI.4	GTAGAACGTGACCAACTGG	CACCCGGTTGTAGTAGTTGCAGGCACTGCC
CCL2	AATGGTCTTGAAGATCACAGCTTC	TAGCAGCCACCTTCATTCCCCAAG
ACTB (ß-actin)	TGCGTGACATTAAGGAGAAG	GCTCGTAGCTCTTCTCCA
GAPDH	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC
RPL19 (L19)	AGGCACATGGGCATAGGT	CCATGAGAATCCGCTTGT
G6PD	GGCAACAGATACAAGAACGTGAA	CCCTCATACTGGAAACCCACT

4.7 NFκB, p38- and ERK1/2-MAPK Activity **(II, III)**

To identify the underlying mechanisms behind the IL-10 inhibition of TNFα-induced aromatase expression **(II)** and the stimulatory action of CCL2 on aromatase expression **(III)**, functional assays of the canonical TNFα and CCL2-related pathways were performed. Primary human breast ASCs were plated in 24 well plates at the density of 20,000 cells/well. At 80-90% confluency, cells were treated with 20 ng/ml IL-10 or 10 μM of the following inhibitors, BAY 11-7082 (NFkB inhibitor), SB 239063 (p38-MAPK inhibitor, Tocris Biosciences) or U0126 (ERK1/2 inhibitor). After 15 min incubation, 5 ng/ml of TNFα or 100ng/ml CCL2 was added, and 15 min later, endogenous levels of the active proteins were measured from the cell lysates (containing 10-15 μg of protein per well) by using NFkB p65 (pS536) ELISA SimpleStep Kit (Abcam), p38 MAPK alpha (pT180/Y182 + Total) ELISA Kit (Abcam) and ERK1/2 (pT202/Y204) SimpleStep ELISA Kit (Abcam), respectively. The assay was performed according to the manufacturer's instructions and analyzed at 450 nm using EnSight Multimode Plate Reader (Perkin-Elmer, Norwalk, CT, USA)

4.8 Gut Microbiota Sequencing Analysis **(II)**

DNA isolation from 0.1 mg of colon fecal samples was carried out using PowerFecal DNA isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) following manufacturer's instructions. The V4-V5 region of 16S rRNA gene was amplified using KAPA HiFi PCR kit (KAPA Biosystems, Wilmington, MA, USA) with 515F and 926R fusion primers containing identification indices as well as sequences required for Illumina sequencing [388]. The PCR conditions comprised an initial denaturation step at 98 °C for 4 min, followed by 25 cycles at 98 °C for 20 sec, 65 °C for 20 sec, and 72 °C for 35 sec, and ended with an extension step at 72 °C for 10 min. The PCR products were purified with Agencourt AMPure XP Magnetic beads (Beckman Coulter Inc., Indianapolis, IN, USA). Length and integrity of the amplicons were checked with TapeStation (Agilent Technologies Inc., Santa Clara, CA, USA), and the final DNA concentrations was measured with Qubit 2.0 fluorometer (Life Technologies). Sequencing was performed at Turku Clinical Sequencing laboratory by Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA). QIIME (v 1.8) pipeline was used with default parameters for identifying representative sequences for each operational taxonomic unit (OTU) generated from complete linkage clustering with a 97 % similarity and aligned to the GreenGenes 13_8 database. OTU table for these samples was further processed at different taxonomic levels.

4.9 Preparation of PKE Extract and Polyphenol Compounds **(I)**

Knots from Finnish Scots pine (*pinus sylvestris*) were obtained from an industrial pulp mill process (UPM Tervasaari, Valkeakoski, Finland). The knots were freeze-dried, ground, and extracted through multiple cycles of hexane at 90°C (5 min) and ethanol water at 100°C (5 min) using a Dionex Accelerate Solvent Extractor. The chemical composition of the PKE extract was determined using a GC-flame ionization detector (FID), GC-MS and high-performance size-exclusion chromatography [389]. The major chemical compounds in the PKE extract were lignans (16%), stilbenoids (17%), oxidized resin acids (20%), resin acids (24%), and higher-molar-mass compounds (550–4000 Da, 18%). The main polyphenols in the PKE extract were (wt-% of dry extract): pinosylvin 4-monomethyl ether (MePS, 10.2%), nortrachelogenin (NTG, 7.0%), pinosylvin (PS, 4.0%), mateiresinol (MR, 1.5%), abietic acid (1.5%), and pinostilbene (0.4%).

NTG, MR, PS and MePS were isolated (purity of >95%) in the Laboratory of Wood and Paper Chemistry at Åbo Akademi (Turku, Finland) as described previously [390]. A mix (LSmix) of the four main PKE-derived stilbenoids (PS, MePS, NTG and MR) was prepared using the approximately same concentrations to these in the PKE extract (10 mg/l).

4.10 Antioxidant Properties of PKE and PKE-derived Phenolic Compounds **(I)**

The antioxidant activity of the extract and isolated polyphenols was measured by using three different biochemical methods already described in [391,392]. Trolox (vitamin E) was used as an antioxidant reference compound. Briefly, the antioxidant activity of the compounds against peroxyl radicals generated from the thermal decomposition of 2,2`-azobis-amidinopropane in phosphate buffer was determined using rat liver microsomes and the Bio-Orbit 1251 Luminometer (Bio-Orbit. Turku, Finland). The reaction was initiated by the addition of *tert*-butylhydroperoxide, followed by the assessment of the chemiluminescence by the Bio-Orbit. Additionally, the ability of the compounds to inhibit low-density lipoprotein (LDL) oxidation was examined after incubation with phosphate buffer, LDL and Cu^{2+} at 37°C. To assess lipid oxidation, conjugated dienes were measured with PerkinElmer Lambda 2 spectrophotometer (PerkinElmer Corp., Norwalk, CT).

4.11 Statistical Analyses **(I-III)**

Statistical analyses were performed by using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). To assess the statistical significance between two

groups, unpaired parametric t-test was used. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used. Pearson correlation test was used to evaluate the relationship between two variables. Differences were considered statistically significant at $P \leq 0.05$. Data is expressed as mean ± standard error of mean (SEM).

5 Results

5.1 Obesity and OVX-related Increased Adiposity Upregulates hARO-Luc Reporter Gene in Male and Female Mice **(I-III)**.

As shown in the *in vivo* studies carried out in I, II and III, increased adiposity, either as a consequence of long HFD intake or OVX, upregulated the activity of the aromatase Luc reporter in WAT in male and female hARO-Luc mice (more detailed results in the following sections). These results confirm the suitability of this mouse model to investigate *in vivo* the role of obesity in the regulation of *CYP19A1* in WAT.

5.2 IL-10 Inhibits Aromatase Gene Expression in Breast Adipose Tissue **(II)**

5.2.1 IL-10 blocks TNFα-induced total aromatase reporter and PI.4 transcripts expression in hARO-Luc MSCs

The potential effect of IL-10 on aromatase Luc reporter was initially investigated *in vitro* using hARO-Luc MSCs under conditions favorable to study PI.4 transcriptional activity. Accordingly, MSCs were exposed to DEX alone or with the combination of DEX + TNFα and/or IL-10 in serum-containing medium. IL-10 inhibited TNFα-mediated induction of aromatase gene expression in these cells. Suppressive action of IL-10 altered the synergistic effect between TNFα and DEX while no changes in expression were observed in cells treated with DEX alone (**II**, Fig. 1A). A more detailed analysis on the distinct mesenchymal promoter transcripts revealed that IL-10 blocked specifically PI.4 transcript expression in MSCs treated with the combination DEX/TNFα (**II**, Fig. 1B).

Combined, these results indicate that IL-10 acts as a suppressor of aromatase gene expression in MSCs, an action that may prevent TNFα induction of aromatase expression via distal promoter I.4 in the breast adipose tissue.

5.2.2 IL-10 suppresses TNFα-induced *CYP19A1* expression in human breast ASCs via down-regulation of PI.4

In order to translate the previous results into a human breast tissue setting, primary breast ASCs were treated with the same treatments used for hARO-Luc mouse MSCs. Similar to MSCs, IL-10 was able to fully suppress both TNFα-induced *CYP19A1* and PI.4 transcripts expression in cultured breast ASCs (**II**, Fig. 2A and B).

5.2.3 IL-10 mediates the suppression of *CYP19A1* expression through inhibition of TNFα-related ERK1/2 signaling pathway

To identify the molecular mechanism by which IL-10 suppresses TNFα action on aromatase gene transcription, functional activity of TNFα-related signaling pathways was examined. Stimulation of breast ASCs by TNFα activates two critical inflammatory pathways, NFκB, and the MAPKs-related signaling involving p38, JNK, and ERK1/2. Out of these, IL-10 showed to mediate the downregulation of aromatase gene expression by specifically blocking phosphorylation of the ERK1/2 signaling, upon treatment of breast ASCs with TNFα (**II**, Fig. 3B). No effect on aromatase expression was observed in cells treated with either NFκB (**II**, Fig. 3A) or P38 specific inhibitors (data not shown). Moreover, to evaluate the potential role of ERK1/2 pathway in the transcriptional activation of aromatase by TNFα, *CYP19A1* transcripts expression was measured in breast ASCs treated with U0126, a specific inhibitor of ERK1/2. Together, these data shown that, indeed, TNFα-induced aromatase expression is inhibited when blocking its inflammatory action through the ERK1/2 pathway (**II**, Fig. 3C).

5.3 CCL2 Upregulates Aromatase Gene Expression in Breast Adipose Tissue **(III)**

5.3.1 CCL2 regulates glucocorticoid-mediated *CYP19A1* transcription through activation of promoter I.4

To investigate whether CCL2 could act as a direct regulator of aromatase gene transcription in the breast adipose tissue, possible changes in transcription rates were assessed *in vitro* using CCL2-treated hARO-Luc-derived mammary fat explants and primary breast ASCs.

Firstly, hARO-Luc-derived mammary fat explants were treated with CCL2 and DEX to evaluate a potential action of this chemokine via PI.4. In effect, CCL2 enhanced glucocorticoid mediated Luc reporter activity through a further stimulation in the mammary fat pad (**III**, Fig. 4). Thereafter, to confirm similar responses in

human cells, cultured primary breast ASCs were treated with CCL2 alone or in combination with DEX. As expected, the combination DEX/CCL2 caused a higher induction of *CYP19A1* gene expression when compared to that induced by DEX alone (**III**, Fig. 5A). Furthermore, the direct implication of CCL2 on the regulation of aromatase transcription was further confirmed by the inhibition of its effect on *CYP19A1* expression when using the combination CCL2 and the inhibitor RS504303, a specific CCR2 receptor antagonist (**III**, Fig. 5A). To further examine whether CCL2 action on aromatase expression implicates upregulation of PI.4, transcript expression of this specific promoter was measured. Similar expression patterns as with total aromatase were obtained, and the combination DEX/CCL2 induced PI.4 transcription up to levels three-fold higher compared to the stimulation of DEX on PI.4 activity (**III**, Fig. 5B). Apart from this, no induction of PII-driven aromatase expression was detected in human ASCs (**III**, supplementary Fig. 1).

5.3.2 CCL2 induction of *CYP19A1* gene expression in breast ASCs involves the activation of the MAPK-ERK1/2 signaling pathway

As described before, the interaction of CCL2 with its membrane receptor, CCR2, activates NFκB and MAPKs (especially, ERK1/2) inflammatory cellular pathways, both equally associated with upregulation of aromatase promoter I.4 activity in breast ASCs [393-395]. To identify the possible intracellular pathway driving CCL2 induction of PI.4 transcriptional activity, cultured breast ASCs were treated with specific inhibitors of NFκB (BAY 11-7082) and MAPK-ERK1/2 (U0126) cascades. While the treatment with the NFκB inhibitor did not alter CCL2-mediated *CYP19A1* expression, blocking the ERK1/2 cascade by U0126 inhibitor led to a significant reduction in CCL2-mediated gene transcription (**III**, Fig. 5C). This indicates that the ERK1/2 signaling pathway could be the main molecular mechanism driving CCL2 actions on adipose tissue aromatase expression.

5.4 Obesity is Associated with Inflammation and Elevated Aromatase Expression in Different WAT Depots of hARO-Luc Mice and Women **(I, III)**

5.4.1 High-fat diet promotes weight gain, adiposity, and metabolic dysfunction in female and male hARO-Luc mice

In both male and female hARO-Luc mice, eight-week-long HFD feeding resulted in significant weight gain and higher body fat mass when compared with control mice

fed with LFD (**I** and **III**, Fig. 1A and B). Along with the increased adiposity, HFD also primes important metabolic impairments evidenced by increased serum levels of leptin (**I**, Fig. 1C), glucose and insulin in males, and higher circulating leptin and glucose levels in females (**III**, Fig. 1C and D). Furthermore, particularly in males, HFD-fed mice exhibited heavier subcutaneous and gonadal adipose depots in addition to larger adipocyte sizes than those from LFD-fed mice (**I**, Fig. 1D, E, F, and G).

5.4.2 Excessive fat accumulation in WAT depots promotes tissue proinflammatory milieu and upregulates aromatase Luc expression in hARO-Luc mice

In males, HDF-fed hARO-Luc mice exhibited higher aromatase Luc reporter activity in subcutaneous and gonadal adipose depots compared to respective tissues in LFD-fed mice (**I**, Fig. 3A and B). Higher aromatase Luc reporter correlated with both gonadal and mammary adipose tissue weight (**I**, Fig. 3C and D). Remarkably, obese HFD-fed animals demonstrated a low-grade inflammatory state evidenced by higher levels of CCL2, TNFα, and IL-6 (calculated as "total cytokine index") in serum and tissue subcutaneous and gonadal fat (**I**, Fig. 2A, B, and C), and by a higher CLS density in both WAT depots, but especially in gonadal fat (**I**, Fig. 2D and E). Accordingly, there was a correlation between adiposity in the HFD fed male mice and the levels of pro-inflammatory cytokines in serum, subcutaneous, and gonadal tissues (**I**, Fig. 2F, G, and H).

The direct effects of some aromatase PI.4-related inducers in the regulation of aromatase gene expression in male WAT were examined by using fat tissue explants cultures from hARO-Luc mice. Treatment of subcutaneous and gonadal fat explant with DEX promoted a significant increase in aromatase Luc reporter activity (**I**, Fig. 3E and F). Interestingly, to achieve similar levels of Luc induction in the tissue, 10 nM DEX was used for gonadal fat explants, while for subcutaneous fat, 250 nM DEX was required, which represents a dose difference of more than 20-fold. Moreover, the addition of lipopolysaccharides (LPS) to the culture media of both WAT depot explants further stimulated the DEX-induced activity of the Luc reporter in a dose-dependent manner (**I**, Fig. 3E and F).

As previously reported, several mice strains, including FVB/N, do not gain weight similarly when fed with HFD [396-398]. In female hARO-Luc mice (**III**), this difference in response to an HFD was more evident than in males. Of all 17 females on HDF, 14 gained weight by the end of the intervention while 3 did not gain weight or gained less than 20%. Nevertheless, as shown in section 5.4.1, the majority of HFD-fed females exhibited evident metabolic disturbances with higher circulating leptin and glucose levels. However, an increased aromatase Luc reporter activity was found only in the mammary fat pad from those animals that gained weight by more

than 20% (**III**, Fig. 2A). A positive correlation was noted between the levels of Luc activity in mammary fat pad and both adiposity and weight gain of HFD-fed female mice (**III**, Fig. 2B and C).

On the other hand, aromatase Luc reporter activity in the mammary fat pad of HFD-female mice was also found to be positively associated with the tissue levels of leptin (**III**, supplementary table 1), and pro-inflammatory factors, CCL2 (**III**, Fig. 2D) and IL-6 (**III**, supplementary table 1). However, as shown in the previous study with OVX- hARO-Luc females, despite the increased weight gain and adiposity in the HFD group, very low numbers of CLS (≤2 per mm^2) were observed in the mammary fat pad of these females. Such CLS density, moreover, did not correlate with the mammary gland tissue aromatase Luc reporter activity (data not shown). Furthermore, in the case of gonadal and retroperitoneal fat tissues, no significant correlations were found between the local aromatase Luc reporter activity and the tissue CCL2 or IL-6 secretion level. The same applied to the other analyzed adipose tissue-secreted factors, TNFα, IL-1β, IL-1α, VEGF, PAI-1, resistin, and adiponectin, with no significant changes in gonadal nor retroperitoneal fat either (**III**, supplementary table 1).

5.4.3 Obesity is associated with increased *CCL2* and *CYP19A1* mRNA levels in the subcutaneous adipose tissue of women

Transcription levels of *CCL2* and *CYP19A1* in subcutaneous adipose tissue of obese and lean women were measured and compared in order to test whether obesity induces similar alterations in human adipose tissues as in hARO-Luc mouse adipose tissues. In women with clear obesity, *CCL2* and *CYP19A1* transcripts levels were significantly higher in subcutaneous fat tissues compared to those of lean women (**III**, Fig. 3A and B). These differences were particularly evident for CCL2, with nearly 6-fold higher mRNA expression in obese than in lean tissues. These results indicate that excess weight and adiposity in women promote a proinflammatory status in subcutaneous adipose tissue that may upregulate the local expression and activity of aromatase, and the resultant production of estrogen in the breast.

5.5 Obese OVX hARO-Luc Females Gain Weight and Exhibit Increased Aromatase Luc Reporter and Reduced IL-10 Levels in Mammary Fat Pad, and Altered Gut Microbiota **(II)**

In hARO-Luc females, ovariectomy led to greater weight gain and body fat mass (**II**, Fig. 4A and B). Increased fat accumulation in OVX mice resulted in mammary fat

pad weights almost 3-folds higher compared to those from intact mice (**II**. Fig. 4C). Parallel to the increased adiposity, OVX females also exhibited increased aromatase Luc reporter activity and reduced IL-10 levels in mammary fat pad when compared to their intact and lean littermates (**II**, Fig. 4D and E). To evaluate the potential influence of reduced levels of IL-10 on aromatase reporter activity after OVX, the values were correlated. The results (**II**, Fig. 4F) showed a significant negative correlation between IL-10 and aromatase reporter levels in the OVX mammary fat pads. Interestingly, no difference was noted in the mammary CLS density between OVX and intact females; in fact, a very low density was observed in both groups (absence or a maximum of 0.03 CLS per mm^2). This association in aromatase reporter activity and IL-10 expression, however, was observed only in mammary fat tissue, in other words, no significant association was observed in gonadal fat samples at the end of the experiment.

5.5.1 Loss of ovarian hormones promotes a gut bacterial composition that facilitates obesity

Bacterial composition, aromatase Luc reporter expression, and the expression of several genes related to energy metabolism were analyzed in fecal or colon samples collected from OVX and intact hARO-Luc females. As expected, the colon bacterial profile in all the animals was dominated by *Firmicutes* and *Bacteroidetes* at the phylum level, with a total of 98.5% bacteria belonging these two groups. The rest was composed of bacteria from phyla *Verrucomicrobia* (~0.36%), *Proteobacteria* (~0.36%), *Deferrebacteres* (~0.30%), and *Actinobacteria* (~0.30%). No significant differences were noted in bacterial diversity between samples from OVX and intact mice, but the *Bacteroidetes/Firmicutes* ratio was found to be lower in samples from OVX females (**II**, Fig. 5A). While OVX reduced the number of *Bacteroidetes* by almost 10% (OVX 71% vs intact 81%), *Firmicutes* content increased at almost similar proportions (OVX 27% vs. intact 18%). Despite these changes after OVX, aromatase Luc reporter expression remained unaltered in colon tissue samples (**II**, Fig. 5B). In addition, our results indicated that OVX-mediated changes in gut bacterial composition might prompt altered gut expression levels of genes related to gut motility, appetite, and inflammation. More specifically, the colon tissue of OVX mice demonstrated lower transcript levels of proglucagon (Gcg-1) and higher levels of peptide YY (Pyy) (**II**, Fig. 5C and D). No significant changes in mRNA expression levels were detected among genes regulating adipose tissue metabolism, fasting-induced adipose factor (Fiaf), or lipoprotein lipase (Lpl) (data not shown). Moreover, colon mRNA expression of IL-6 was elevated in OVX compared to intact mice; however, there were no changes in the local expression of TNFα and IL-10 (**II**, Fig. 5E-G).

5.6 Dietary Polyphenols as Potential Suppressors of Obesity-related Inflammation and as Regulators of Aromatase Gene Expression in WAT **(I, unpublished data)**

5.6.1 Dietary intervention with PKE extract attenuates inflammation and aromatase Luc reporter activity in male HFD-fed hARO-Luc mice activity *in vivo*

By feeding the mice with HFD or HFD supplemented with polyphenol-rich pine knot extract (PKE) for eight weeks, we demonstrated that the consumption of a diet supplemented with polyphenol compounds diminished CLS density (i.e. marker of WAT inflammation) in subcutaneous and gonadal fat depots (**I**, Fig. 4A and B). PKE-supplemented HFD suppressed the levels of aromatase Luc reporter activity in subcutaneous WAT depot when compared to the levels expressed in non-supplemented HFD-fed mice (**I**, Fig. 4C and D). However, PKE-supplemented HFD did not show any effect on body weight, which partly indicates the good tolerability of PKE, but neither did it demonstrate an effect on adiposity or the levels of proinflammatory cytokines in WAT (data not shown). Nevertheless, PKE reduced fasting blood glucose and the levels of leptin and insulin in serum (data not shown).

In a previous study from our group, the tolerability and bioavailability of orally administered PKE polyphenols were demonstrated *in vivo* in immunocompromised mice [386]. Given the amount of PKE within the diet (1.6 g /Kg diet), the calculated average consumption of PKE per animal was 5 mg/mouse/day (120 mg/kg of body weight). Similar to this previous study, micromolar concentrations of all PKE-derived polyphenols were found in the urine of hARO-Luc males (data not shown), confirming thus the absorption of ingested PKE polyphenols. Furthermore, by applying three different biochemical methods, the antioxidant ability of each of the isolated polyphenolic compounds in the PKE extract was determined and subsequently compared to one of the most potent antioxidants, vitamin E. The results showed that PKE extract alone and its main polyphenol compounds (pinosylvin, pinosylvin 4-monomethyl ether, nortrachelogenin, and matairesinol) have antioxidant effects *in vitro* that are comparable with vitamin E (**I**, table 1). In particular, the capacity of PKE (89 g/mol), nortrachelogenin (NTG, 62 g/mol) and matairesinol (MR, 120 g/mol) compounds to scavenge peroxyl radicals was significantly greater than that of vitamin E (110 g/mol). Although the IC50 concentrations of PKE were slightly lower than that of vitamin E, PKE was able to inhibit both tert-butylhydroperoxide-induced lipid peroxidation in rat liver microsomes (IC50 16 ng/ml) and the oxidation of human LDL (IC50 3.8 μg/mg of LDL). PS was the most potent phenolic compound from the PKE extract to inhibit

LDL oxidation (IC50 2.5 μg/mg of LDL), while MR and nortrachelogenin (NTG) were the second- and third-most potent compounds to suppress the tert-butylhydroperoxide-induced lipid peroxidation (IC50 17 ng/ml and 26 ng/ml, respectively). These results are in line with previously published data on the antioxidative potential of wood-derived stilbenoids and lignans [399].

5.6.2 Effects of dietary polyphenols on aromatase Luc reporter expression in hARO-Luc-derived MSCs

The effect of PKE and its derived phenolic compounds on human aromatase gene expression was examined *in vitro* using hARO-Luc (male) bone-marrow-derived MSCs. To investigate their potential effect on modulating either of the two-aromatase promoters expressed in the human adipose tissue, PI.4 and PII, cultured MSCs were exposed to specific conditions to induce expression of these promoters. DEX + TNFα was used to induce PI.4, while FSK + PMA was used to activate PII-related gene transcription. As expected, aromatase Luc reporter activity in MSCs was significantly upregulated by PI.4 and PII stimulating conditions (**I**, Fig. 4E and F). In MSCs with PI.4-induced aromatase Luc reporter activity, only NTG and LSmix were able to downregulate the level of aromatase reporter. Conversely, the other tested compounds, PKE, pinosylvin 4-monomethyl ether (MePS) and PS, enhanced aromatase reporter gene expression in MSCs (**I**, Fig. 4E). Regarding the effect of polyphenols on PII-induced aromatase expression, all tested phenolic compounds and mixtures, except MePS, inhibited the activity of PII-mediated aromatase Luc reporter in hARO-Luc-derived MSCs (**I**, Fig. 4F). These findings proposed that the chemopreventive effects of polyphenols could be partly attributed to a modulation of aromatase gene transcription in breast adipose stroma.

5.6.3 Effects of dietary phytoestrogens on *CYP19A1* gene transcription in human breast ASCs: *In vitro* studies **(unpublished data)**

This research project also investigated whether dietary polyphenols could regulate breast adipose aromatase at the transcriptional level. Cultured primary breast ASCs were treated with known local aromatase-PI.4 and -PII transcription inducers, and several phytoestrogens, including the isoflavone genistein (GNS), the stilbenoids resveratrol (RSV), and PS, and the lignans secoisolariciresinol (SECO), enterodiol (END) and enterolactone (ENT).

With regard to the potential regulatory action of the selected flavonoids and stilbenoids on PI.4-mediated *CYP19A1* expression, the results demonstrated that

only RSV and GNS (*P*= 0,09) were able to downregulate the glucocorticoids mediated transcriptional *CYP19A1* activity in ASCs (Fig. 18).

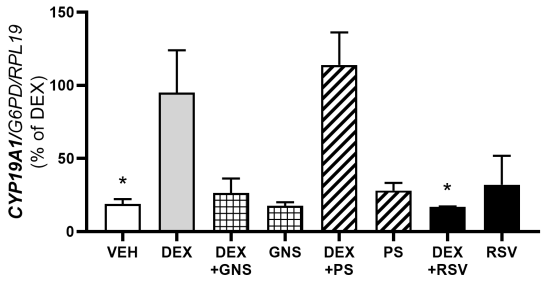


Figure 18. Effect of genistein, pinosylvin, and resveratrol on glucocorticoid mediated *CYP19A1* expression in breast ASCs. Data (mean ± SEM) are shown as relative to dexamethasone (DEX, set at 100%). **P*<0.05. Treatments: 10 nM DEX and 10 µM polyphenols, genistein (GNS), pinosylvin (PS) and resveratrol (RSV).

Because lignans have shown strong anti-inflammatory effects in animal and clinical studies [316,400] and to downregulate aromatase expression (e.g. previous results on hARO-Luc MSCs, **I**), we studied the potential action of several lignans as inhibitors of *CYP19A1* expression in human ASCs. However, none of the tested lignans was able to downregulate aromatase gene expression, neither via PI.4 nor PII-transcriptional activity in these cells (Fig. 19A and B). Instead, ENT further upregulated DEX-induced *CYP19A1* expression levels (Fig. 19A), while END did the same but on FSK/PMA-induced transcription (Fig. 19B). This suggests that lignans particularly ENT and END may promote the expression of aromatase gene in the breast.

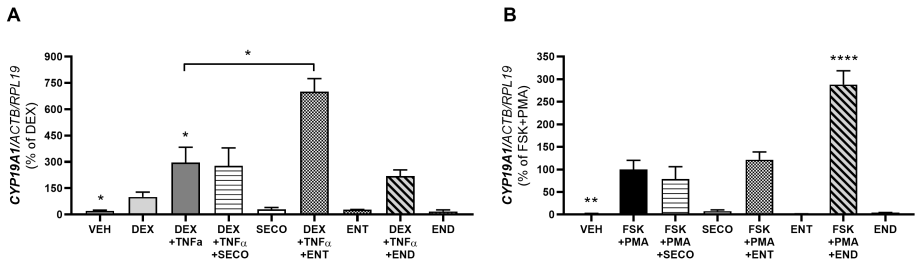


Figure 19. Effect of lignans, secoisolariciresinol, enterodiol, and enterolactone on PI.4 and PII-related *CYP19A1* expression in breast ASCs. A. Effect of dietary lignans on DEX/TNFα-induced *CYP19A1* expression. Data (mean ± SEM) are shown as relative to dexamethasone (DEX, set at 100%). B. Effect of dietary lignans on FSK/PMA-induced *CYP19A1* expression. Data (mean ± SEM) are shown as relative to forskolin + phorbol 12-myristate 13-acetate (FSK+PMA, set at 100%). **P*<0.05, ***P*≤0.01, *****P*≤0.0001. Treatments: 10 nM DEX, 25 µM FSK, 4 nM PMA and 10 µM polyphenols, secoisolariciresinol (SECO), enterolactone (ENT) and enterodiol (END).

6 Discussion

6.1 IL-10 as Part of the Endogenous "Brake" Mechanisms Regulating Aromatase Expression in Breast Adipose Tissue

While much is known about the regulatory effect of proinflammatory factors on aromatase gene expression in the breast adipose tissue, there is limited published information on the role of local anti-inflammatory factors in this transcriptional control. The data in **II**, proves the importance of IL-10, an anti-inflammatory cytokine, in the regulation of *CYP19A1* expression in the breast adipose stroma where it acts as a local transcriptional inhibitor.

Considered as the quintessential anti-inflammatory cytokine, IL-10 plays a critical role in maintaining tissue homeostasis and regulating inflammation, thanks largely to its ability to inhibit excessive proinflammatory responses of T-cells and macrophages. More precisely, IL-10 suppresses the production of proinflammatory cytokines, TNF-α, IL-1β, and IL-8, while it can also prevent their inflammatory signaling cascades on other immune and non-immune cells [401]. The inhibitory action of IL-10 on TNFα, is perhaps one of the most relevant and studied effect of this cytokine as TNFα is a key factor in the development of several chronic diseases [402]. In obesity, TNFα levels in WAT are increased by almost 7.5 fold compared to lean subjects [403]. Furthermore, higher activity of TNFα-induced NFκB and MAPKs signaling is associated with impaired metabolism and increased local aromatase expression in WAT [26,280,404]. Therefore, we investigated the potential effect of IL-10 on the modulation of TNF-mediated *CYP19A1* expression in the mammary/breast adipose stroma. In this way, we discovered that IL-10 inhibits the levels of aromatase Luc reporter activity in hARO-Luc-derived MSCs and the *CYP19A1* expression in primary breast ASCs induced by TNFα in the presence of glucocorticoids.

Although the molecular mechanisms driving the effect of TNFα on *CYP19A1* expression are still not well defined, it is known that activation of both NFκB and MAPK signaling cascades by TNFα stimulate promoter I.4 transcription [395]. Here, we also demonstrated that IL-10 suppresses specifically TNFα-induced aromatase PI.4 in cultured breast ASCs, a molecular action that potentially involves the inhibition of the TNFα signal transduction via the MAPK-ERK1/2 cascade. To our knowledge, IL-10

is the first adipose tissue-related factor known to downregulate aromatase in WAT via PI.4. Previously, ghrelin, a gut-related hormone, was shown to suppress aromatase expression in breast ASCs via the proximal PII[405]. Overall, our findings suggest that high tissue IL-10 may help maintain normal basal levels of aromatase and estrogens in the healthy breast adipose tissue. Altered circulating and adipose tissue levels of IL-10 have been reported in obese subjects [406-409]. In this regard, our data could predict that reduced levels of IL-10 in obese postmenopausal women may contribute to the excessive aromatase and estrogen levels in their breasts.

6.2 CCL2, an Inducer of Aromatase Gene Transcription in the Breast Adipose Stroma

In obese postmenopausal women, WAT inflammation is accompanied by increased aromatase expression in the breast adipose tissue [23,27,295,299]. While it is true that proinflammatory factors, including TNFα [93], IL-6 [92], PGE2[114], and leptin [410], directly or indirectly contribute to the abundance of *CYP19A1* transcripts in the breast adipose tissue, it is not yet known whether other immune mediators, such as chemokines, could have similar implications in this process. On this basis, study III demonstrates that CCL2, a well-known proinflammatory chemokine, may also regulate the expression of aromatase in breast adipose tissue through stimulation of the glucocorticoid-mediated promoter I.4 transcription.

CCL2, also known as monocyte chemotactic protein (MCP)-1, is one of the key chemokines driving the migration and infiltration of macrophages into inflammatory sites. It is produced primarily by monocytes/macrophages, but also by several non-immune cells, such as fibroblasts and endothelial and epithelial cells [411,412]. In the adipose tissue, CCL2 actions are primarily mediated via the GPCR receptor, CC-receptor 2 (CCR2), expressed in macrophages, adipocytes and stromal cells [412-414]. During the first stages of obesity, the growing need for fat storage prompts the already hypertrophic adipocytes to release large amounts of CCL2 to recruit macrophages and enhance tissue expansion [277,280,281]. CCL2 level, on the other hand, is then further increased at later stages, when adipose tissue macrophages start to accumulate and the number of adipose tissue CLSs grow [267,279,280,415]. In addition to a very powerful chemo-attractive effect, CCL2 also stimulates inflammation via activation of proinflammatory signaling pathways, particularly the PI3K/Akt/NFκB and Ras/MEK/ERK signaling cascades [393,416,417]. The latter assumes even greater significance since both signaling pathways are known to also upregulate aromatase expression via PI.4 in cultured breast ASCs [395]. Importantly, CCL2 expression in breast cancer has shown to be upregulated and strongly modulated by estrogens [418,419]. Given the prominent role of CCL2 in the development and progression of obesity-mediated WAT inflammation, it was pertinent to hypothesize that CCL2 may have direct effects on *CYP19A1* transcription in the breast adipose tissue.

We also discovered that the CCL2 stimulating effect on aromatase involves the activation of CCR2 at the cell membrane and the downstream activation of the MAPK-ERK1/2 signaling pathway in cultured breast ASCs. While no previous studies address a direct effect of CCL2 on human aromatase expression, our results indicate that CCL2 and TNFα share similar regulatory mechanisms in the induction of aromatase transcription in WAT. Recent studies have provided new evidence for TNFα on its downstream mechanisms, suggesting these involves the activation of the NFκB and MAPK signaling pathways, the transactivation of early transcription factors 2 and 3 (EGR2 and EGR3), and the indirect induction of the AP-1 transcriptional site within promoter I.4 [395]. Further studies are needed to elucidate the specific molecular mechanisms involved in the induction of aromatase expression by CCL2 in the breast adipose stroma.

6.3 Mechanisms behind the Obesity → Inflammation → Aromatase axis in WAT

6.3.1 Adipose tissue expansion-induced inflammation as the primary cause of the increased aromatase expression in obese female and male WAT. ***The role of CCL2***

In lean subjects, the balance between energy intake and expenditure supports an anti-inflammatory environment in WAT, favoring the secretion of anti-inflammatory cytokines, such as IL-10, IL-4, IL-13, and adiponectin [420], and limiting the levels of proinflammatory mediators, thus maintaining at low the local transcription levels of aromatase. Conversely, in obese and overweight status, the unhealthy WAT expansion is often associated with the onset of hypertrophic adipocytes, macrophages, and crown-like structures (CLS) [267,276,282]. As a result, the obese adipose tissue preferentially releases proinflammatory cytokines, including TNF-α, IL-6, resistin and leptin, and chemokines, especially CCL2 [420]. In this study, an attempt was made to relate the expression of aromatase in WAT to the local production of pro and anti-inflammatory factors in obesity conditions. By using male and female hARO-Luc mice on HFD as well as subcutaneous adipose tissue samples from obese women, we demonstrated that elevated levels of CCL2 in WAT might contribute to the increased local aromatase expression.

In hARO-Luc males (I), exposure to HFD associated with higher body weight and adiposity, systemic metabolic dysregulation, and WAT inflammation. For instance, in addition to higher serum leptin and glucose levels, all males on HFD exhibited a prominent increase in body weight and body fat composition. The presence of inflammatory markers was clearer in the obese WAT depots, evidenced by elevated CLS density and increased cytokine index in subcutaneous and gonadal

adipose tissue. As in previous studies using aromatase mouse models [302], obesity in male hARO-Luc correlated with increased Luc activity and cytokine index in gonadal and subcutaneous WAT. Furthermore, the *ex vivo* stimulation of these distinct WAT depots with LPS (bacteria-derived lipopolysaccharide, an inducer of TNFα secretion) was shown to upregulate aromatase Luc reporter activity in the presence of distinct concentrations of DEX. Gonadal fat required a far lower concentration of DEX than subcutaneous fat to activate the local aromatase transcription. This difference in DEX response could be explained by a different tissue composition, metabolism, or expression of glucocorticoid receptors. However, more research is needed to clarify the possible gender-specific mechanisms that regulate the expression of aromatase in visceral and subcutaneous fat depots.

Obesity is associated with elevated circulating estrogen in men as a result of increased aromatization in WAT and the inhibition of the hypothalamic-pituitary-gonadal axis [421-424]. In combination, these conditions can lead to the development of the so-called Male Obesity-associated Secondary Hypogonadism (MOSH), characterized by impaired fertility, bone mineralization, body fat metabolism/composition, and cognitive function. Increased estrogen production in men can also lead to the development of gynecomastia and male ER+ breast cancer [13,425,426]. Treatment with aromatase inhibitors has shown to effectively reverse MOSH [427]. In our diet-induced obesity model, the observed metabolic and inflammatory disturbances in the WAT may mimic those in obese males, which indicates that limiting inflammation could form the basis of new approaches aimed at prevention or more efficient treatments for these pathological conditions.

While the metabolic consequences of HFD in male FVB/N mice have been reported [397], our results may provide important knowledge on the role of obesity and its related WAT inflammation in the male WAT aromatase expression.

In hARO-Luc females (III), HFD-induced weight gain also associated with increased mammary fat aromatase Luc reporter activity. However, conversely to males, 15% of HFD-fed female mice did not shown increase in body weight. Higher aromatase expression was then only observed in HFD-fed animals who gained more than 20% weight. This latter result highlights the significant role of adiposity in influencing inflammation and aromatase production in WAT. Interestingly, expression of aromatase in gonadal and retroperitoneal WAT depots was not affected by the increased weight and adiposity in females. Similar results were obtained in Arom^{hum} FVB/N by Chen and coworkers [119]. HFD-fed Arom^{hum} females demonstrated an increased expression of *CYP19A1* only in the mammary fat pad. Furthermore, increased *Cyp19a1* expression was reported in the mammary fat of HFD-fed OVX and *ob/ob* C57BL/6J females, but, unlike our results, they also showed upregulated aromatase expression in visceral fat [302]. These differences in response are likely to be strain specific. However, it is known that the inflammatory

milieu and the regulation of *CYP19A1* transcription in the subcutaneous adipose tissue differ from visceral fat depots [120,296,428]. In fact, in obese women, changes in aromatase gene expression are found to be more evident in subcutaneous fat depots than in central visceral fat [85,120,296,429]. Particularly, obese postmenopausal women exhibit an almost three-four-fold higher aromatase expression in the breast adipose tissue than that of healthy women [24,295,300].

Overall, the upregulation of *CYP19A1* in these previous human and mouse studies was attributed to the augmented expression of TNFα, IL-6, and IL-1β in the WAT depots of obese subjects. However, in OVX and *ob/ob* C57BL/6J mice and obese women, it was also attributed to an elevated tissue number of CLS [119,285,295,302]. Similar to these studies, obese hARO-Luc females exhibited mammary adipose tissue inflammation with higher levels of IL-6 and CCL2, which also correlated with the tissue Luc reporter activity. Moreover, as it is already the case for IL-6, we have now shown that CCL2 positively regulates aromatase gene expression in cultured breast stromal cells. Thus, it is evident that CCL2 not only plays a critical role in obesity-mediated inflammation and disturbed WAT metabolism, but it also contributes to the overexpression of aromatase within the mammary adipose tissue.

Furthermore, significant differences were not noted in CLS density in any WAT depot between obese and lean hARO-Luc females, which differs from the previous mouse and human studies. Increased CLS density in WAT is considered an important marker of local inflammation since it is associated with higher levels of PGE2, CCL2, and TNFα [23,267,302,430]. Accordingly, higher CLS numbers are associated with elevated activity of aromatase promoters I.4 and II [27,119]. In our study, the absence of CLS may rather indicate a more modest proinflammatory condition that is sufficient to upregulate aromatase PI.4 and there to increase the transcription of the aromatase gene in the mammary fat pad. While the increased activity of PII is considered the main driving force boosting the aberrant production of aromatase in the severely obese and/or cancerous breast, upregulation of PI.4 is also found in the early and the advanced phases of obesity as well as in breast cancer [14,19]. Therefore, it is vital to understand the regulation of this promoter and its role in limiting production of estrogen in high-risk obese postmenopausal women and preventing further carcinogenesis (Fig. 20).

In women, obesity-mediated inflammation is considered one of the most important drivers of upregulated aromatase expression and estrogen levels in the breast adipose tissue after menopause and one of the major risk factors for ER+ breast cancer [23,26,431]. While the exact mechanisms and factors behind these associations *in vivo* are unknown, it is thought that the increased production of proinflammatory factors in WAT is the missing link in the obesity, aromatase, and postmenopausal breast cancer triad [21,28]. Identifying these factors and understanding their role in the estrogen biosynthesis is then central to preventing breast carcinogenesis.

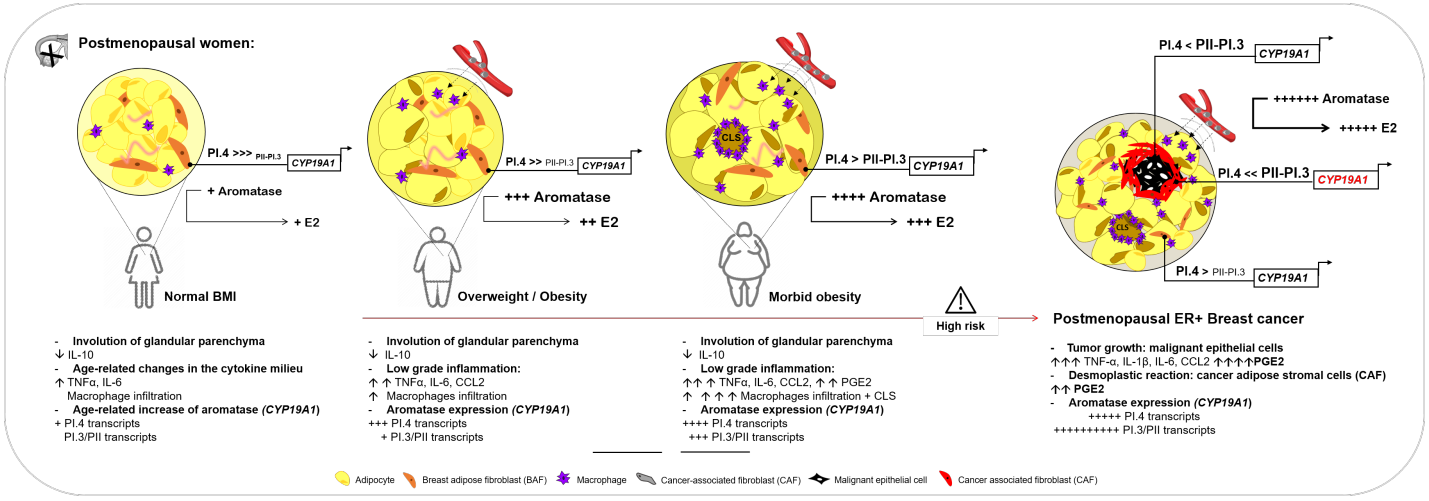


Figure 20. Menopause- and cancer-related changes in the regulation of aromatase expression in breast adipose stroma. Menopause is associated with inflammation and increased expression and activity of *CYP19A1* in breast adipose tissue (BrAT). This situation may be attributable to the upregulation of PI.4 by an imbalance in the local cytokine milieu, accompanied by increased proinflammatory inducers, including TNFα, IL-6, and CCL2 levels, and reduced availability of the endogenous inhibitor, IL-10. As the weight and adiposity increases in postmenopausal women, the already existing inflammatory conditions in BrAT exacerbate, as does the transcriptional activity of PI.4. Initially, the enlarged and inflamed adipocytes secrete greater amounts of TNFα, IL-6, IL-1β, and CCL2, which intensify the local inflammation, increase the number of resident macrophages, and overstimulate the transcription of *CYP19A1* mostly via PI.4. However, the physiological difference in activity between PI.4 and the other local promoter, PII, is progressively reduced as the hypertrophic adipocytes begin to die, the recruitment of macrophages increases and the number of crown-like structures (CLS) becomes more evident. Together, these three elements raise the levels of PGE2 within the tissue, which in turn, upregulates the activity of the more potent PII. Postmenopausal women with severe or morbid obesity often present with 3-4 fold higher expression of *CYP19A1* in BrAT, attributed partly to an overstimulated local activity of both PI.4 and PII. At this point, women are at elevated risk for ER+ breast cancer, with a more than 80% higher risk compared with women with a healthy BMI [1]. In ER+ breast cancer, aberrant expression of *CYP19A1*, particularly in the layer of cancer-associated fibroblasts surrounding the tumor, can reach four-five-fold higher compared with non-cancerous adipose tissue in the breast. Such levels of transcription are driven by PII to a greater extent than PI.4 [2]. Nevertheless, while the contribution of PI.4 is not as high as PI.3/PII, dysregulation of PI.4 appears to be highly important in the earlier stages of the disease, contributing to the initial steps leading to carcinogenesis.

Compared to lean women, obese subjects have significantly higher CCL2 levels in WAT, which is significantly associated with elevated tissue macrophages and insulin resistance [432-434]. In postmenopausal women, on the other hand, circulating levels of CCL2 are also higher, regardless of their BMI [435-437]. In this study, we provided additional data on the role of obesity in promoting inflammation and aromatase expression in the subcutaneous adipose tissue. By comparing the mRNA expression of *CYP19A1* and *CCL2* in subcutaneous adipose tissue samples from obese and lean women, we found that obese adipose tissue expresses significantly higher levels of both aromatase and CCL2, providing thus additional support to our previous *in vitro* and *in vivo* results. Our findings may propose new mechanisms that explain the higher levels of aromatase in the postmenopausal breast, and how weight gain and excessive adiposity could lead this altered condition.

6.4 Estrogen Deficiency, Obesity, and WAT Inflammation in Females

6.4.1 Ovariectomy upregulates aromatase reporter expression in the mammary adipose tissue of hARO-Luc mice. ***Role of local IL-10 and adiposity***

There is a dynamic and reciprocal interplay between estrogen levels and WAT metabolism and inflammatory response, which controls body weight and systemic metabolism, but it is still not fully defined. In study **II**, we demonstrated that loss of ovarian hormones might increase the expression of aromatase in the mammary adipose tissue by altering the local cytokine profile, while promoting a gut bacterial ecology that supports obesity.

OVX and intact hARO-Luc females were compared in terms of body composition, glucose metabolism, and adipose tissue IL-10 levels and aromatase Luc reporter activity. Additionally, we also investigated whether changes in estrogen levels after OVX may affect the bacterial composition and function of the intestine. As other researchers have already reported [149,302,438], in our study, increased body weight and adiposity were the most evident metabolic changes caused by the loss of ovarian hormone production in OVX hARO-Luc mice. Although, OVX females exhibited a significant increase in gonadal and subcutaneous fat, no other metabolic disturbances were detected, for example, altered glucose or insulin levels. Nevertheless, we found that the ongoing estrogen deficiency particularly affect the expression of aromatase Luc reporter and the levels of IL-10 in subcutaneous WAT. OVX hARO-Luc mice exhibited increased activity of aromatase reporter and reduced levels of IL-10 in the mammary fat pad, while levels remained unaltered in gonadal fat. Furthermore, supporting our *in vitro* findings, tissue levels of IL-10

correlated inversely with the local aromatase reporter activity in mammary fat tissue. These results are consistent with previous studies in OVX animals and postmenopausal women, showing that increased adiposity is associated with increased *CYP19A1* expression in the mammary and breast adipose tissue, respectively [119,295]. In addition to adiposity, higher aromatase expression in these previous studies was related to an elevated production of proinflammatory factors and a high density of CLS. However, our results showed that the increased aromatase reporter expression in the OVX hARO-Luc females was not driven by the presence of CLS, which is evidenced by a very low number of CLS in the mammary fat tissue of OVX and intact mice. Combined with the lack of profound metabolic disturbances, the absence of CLS in these animals may reflect a modest stage of inflammation, during which, aromatase PI.4, may still be the main promoter driving the transcription of aromatase reporter in the mammary tissue.

Regarding the changes in IL-10, almost no information exists about the physiological levels of this anti-inflammatory cytokine in mammary/breast adipose tissue, neither on the role of menopause or obesity in controlling these local levels. What is known is that circulating IL-10 levels are altered in women with obesity, particularly among those with metabolic syndrome [439-441]. Interestingly, it is also known that in the healthy breast tissue, IL-10 is mainly produced by glandular cells, followed by adipocytes, macrophages, and stromal cells [280,408,442]. Involution of the parenchymal (glandular) fraction of the breast is a well-established process that initiates in women around the age of menopause [148]. Less glandular tissue producing IL-10 may explain, at least in part, why aromatase expression in the breast adipose tissue increases after menopause, as it is not caused solely by an increased BMI. These results may propose new mechanisms for the pathogenesis of postmenopausal breast cancer. Further clinical studies are needed to assess whether the local cytokine profile in WAT, particularly in the breast, changes in women throughout menopause.

6.4.2 Altered gut microbiota: a factor contributing to the link between ovariectomy and increased adiposity?

Increasing evidence exist to substantiate the connection between gut bacterial composition and host metabolism and inflammatory status. This is particularly so as specific changes in the gut bacterial ecology are associated with the development of metabolism-related disorders, including obesity, diabetes, and cancer [161,163,443,444]. This connection, however, has shown to be far more complex and dynamic than initially thought, as obesity and other metabolic diseases are known also to alter the gut microbiota composition [172,176,177].

Compared to lean individuals, obese subjects present a less diverse bacterial ecology followed by an increased *Firmicutes/Bacteroidetes* ratio [170,172,176]. Such

microbial profile is considered a key driver promoting and maintaining an obesogenic environment characterized by increased energy intake, higher fat accumulation, and inflammation [445,446]. The fact that gut function and systemic metabolism are interconnected made us consider whether estrogens could play a role in this interplay, and if so, how these responses are affected by the dramatic and systemic estrogen deprivation after menopause.

In study **II**, we demonstrated that OVX in hARO-Luc mice changes their gut microbiota composition and favors an obesogenic type bacterial ecology. More specifically, colon bacterial samples from OVX females presented an increased *Firmicutes/Bacteroidetes* ratio compared to these from the intact group. These results also concur with previous data obtained in OVX rats [189]. In humans, age-mediated changes in women's microbiota were also observed by Minelli and coworkers in 1993 [183]. The authors reported fluctuations in the gut bacterial ecology of healthy women throughout life, with menopause enhancing the proliferation of proinflammatory bacteria, including *Escherichia coli* and *Enterobacter cloacae*. They concluded that such bacterial changes between pre- and postmenopausal women might result from the physiological sex steroid imbalance after menopause.

Currently, at least three molecular mechanisms have been proposed to explain the link between specific gut bacterial changes and modulation of the host metabolism: **1.** Increased energy intake and appetite by altering the fecal concentrations of short-chain fatty acids (SCFAs) and the secretion of intestinal hormones, particularly glucagon-like-peptide (GLP)-1, and peptide-YY; **2.** Higher adipocyte triglyceride deposition by blocking the inhibitory activity of fasting-induced adipose factor (FIAF) on the lipoprotein lipase (LPL) enzyme; and **3.** Local and systemic inflammation, which promotes an increased secretion of proinflammatory factors (IFNγ, IL-6, TNF-α, and LPS) by the intestine and their subsequent release into the circulation [165,178,445]. Colon tissue samples from OVX hARO-Luc females expressed lower GLP-1 and higher PYY mRNA levels compared to the intact group. No significant differences in expression were found in LPL and FIAF genes. However, OVX females exhibited signs of gut inflammation, evidenced by higher mRNA levels of IL-6. Expression levels of TNFα and IL-10 genes were altered but did not reach statistical significance.

Overall, these results may offer new information on the role of estrogens in metabolism and the potential mechanisms through which these sex hormones controls body weight and energy homeostasis. Reduction of systemic estrogens during menopause may support an intestinal microbiota that influences adiposity and weight gain. Together, altered gut microbiota, menopausal weight gain, breast involution, and WAT inflammation, may account for the increased aromatase expression in the breast of postmenopausal women (Fig. 21). Nevertheless, further *in vivo* animal and human studies are needed to confirm and define the mechanisms

that drive this complex interplay, its role in the development of postmenopausal breast cancer, and the potential action of HRT on improving or preventing these gut bacterial changes.

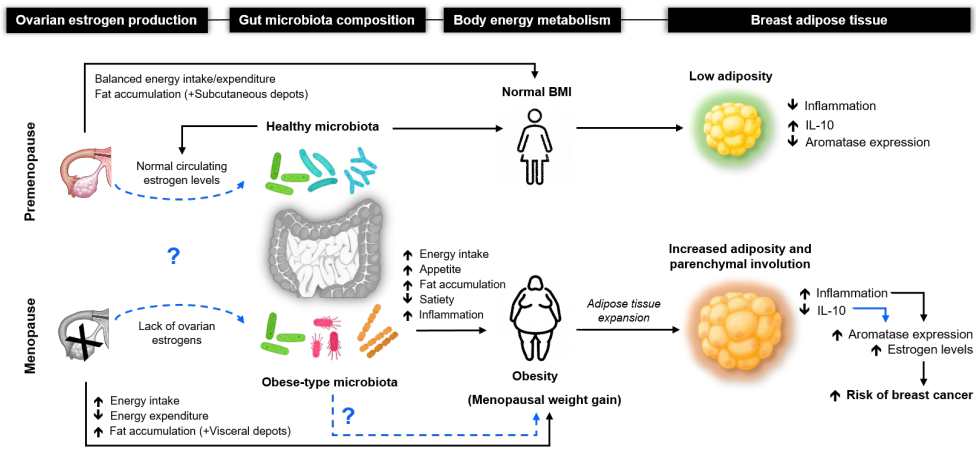


Figure 21. Uncovering new factors behind the link between menopause and increased risk of breast cancer. Loss of ovarian estrogens after menopause is associated with weight gain, augmented fat accumulation (particularly in visceral depots), and increased inflammation and aromatization in the breast adipose tissue (BrAT). Therefore, obese postmenopausal women often exhibit increased levels of aromatase in BrAT, which directly associates with enhanced local estrogens production. Exposure to cumulative high estrogen levels within the menopausal breast lead to aberrant cell proliferation and subsequent greater likelihood for cancer development. Among the interplay of mechanisms linking the menopause, obesity and breast cancer triad, it is likely that other obesity and menopause-related factors, including gut dysbiosis and breast parenchymal involution, respectively, may also contribute to the increased breast aromatase expression. We propose that reduced circulating levels of estrogens must promote changes in the gut microbiota that favor weight gain and higher fat accumulation, predisposing postmenopausal women to obesity. Parallel to this, the already-initiated involution of the breast during menopause might result in lower local IL-10 levels, which in return, could translate into a higher production and activity of proinflammatory cytokines, and consequently, to an overstimulated transcription of aromatase.

6.5 Dietary Phytochemicals as Potential Anti-inflammatory and Cancer Chemopreventive Agents

6.5.1 Dietary polyphenol supplementation attenuates obesity-mediated WAT inflammation and aromatase expression

Dietary polyphenols such as stilbenoids, flavonoids, and lignans have shown preventive effect on obesity and its metabolic complications. Their effects are

believed to be through the suppression of adipocyte differentiation, preadipocytes proliferation, triglyceride accumulation, lipogenesis, hyperglycemia, and inflammation, while stimulating fatty acid β-oxidation, lipolysis, insulin sensitivity, and adiponectin secretion [316,317,447]. In study **I**, we demonstrated that dietary supplementation with a polyphenol-rich extract alleviates WAT inflammation and inhibits aromatase expression in the subcutaneous fat of HFD-fed hARO-Luc males. Despite the fact that supplementation with pine knot extract (PKE) did not reduce body weight or adiposity in these obese mice, it improved some important markers of metabolic dysregulation, lowering circulating leptin, insulin, and fasting glucose levels. Subbaramaiah and colleagues reported similar effects *in vivo* using OVX C57BL/6J mice on HFD and a dietary supplement containing phenolic antioxidants, particularly resveratrol, curcumin, and epigallocatechin gallate [448]. In their study, polyphenol supplementation inhibited mammary fat pad inflammation in the OVX females, reduced CLS density, and the local production of proinflammatory factors and related signaling pathways. Additionally, they observed reduced aromatase gene expression and activity in mammary gland.

Our results indicate a good bioavailability of the PKE-formed polyphenolic compounds in the mice and their significant antioxidant activity *in vitro*. Such results support our previous study [386]and other research data [449] on the bioavailability and antioxidative effect of wood-derived stilbenoids and lignans. The anti-inflammatory properties of PKE *in vivo* could thus be the result of a strong antioxidant effect and an inhibitory action of macrophage recruitment in the WAT.

6.5.2 Stilbenoids, flavonoids, and lignans as modulators of *CYP19A1* gene transcription in stromal cells.

It has been suggested that a polyphenol-rich diet may contribute to the prevention of estrogen-dependent cancers [32,33]. Therefore, great interest is shown in identifying polyphenols, to evaluate their chemopreventive potential and to define the mechanisms through which these effects are displayed. Some dietary polyphenols have been shown to modulate aromatase gene expression and activity in breast cancer cells and placental microsomes [450-453], nonetheless, their effect on primary stromal cells and their mechanisms to regulate aromatase at the transcriptional level remain largely unknown.

As already stated, in cultured breast ASCs, treatment with DEX+TNFα or FSK+PMA stimulates total *CYP19A1* expression via PI.4 and PII, respectively [18]. In study **I**, male hARO-Luc-derived MSCs exposed to the two combinations exhibited a significant increase in aromatase Luc reporter activity. We also proved that the lignan, NTG, and the stilbenoid, PS are capable suppressors of aromatase expression in stromal cells. NTG inhibited DEX/TNFα- and FSK/PMA-induced Luc reporter

activity in hARO-Luc MSCs, thus indicating it has the ability to modulate PI.4 and PII transcriptional activity. To support such effects, it has been demonstrated that NTG can inhibit the production of proinflammatory factors, specifically reactive nitrogen species (RNS), PGE2, IL-6, and CCL2 [454]. However, it is still unknown whether it can also block their intracellular signaling cascades. On the other hand, PS showed different promoter agonist/antagonist effects, inducing DEX/TNFα-mediated aromatase Luc activity while inhibiting FSK/PMA-induced Luc activity. PS, thus, may modulate pathways that are specifically involved in the transcriptional activation of PII in stromal cells. Similar to NTG, the stilbenoid PS has demonstrated the ability to decrease the production of RNS, IL-6, and CCL2 in M1 macrophages [455]. NTG and PS appear to share similar anti-inflammatory mechanisms. However, further studies are needed to elucidate the mechanisms involved in the modulation of aromatase in the adipose stroma.

Genestein (GNS), resveratrol (RSV), PS, and the lignans, enterodiol (ENT) and enterolactone (ENL), are efficient modulators of aromatase expression and activity *in vitro* [456,457]. Studies converge on the same result that the chemopreventive actions of polyphenols are partly caused by a direct inhibition of aromatase and estrogen production, particularly in breast cancer cells. However, only three studies have been conducted under these conditions. Wang et al. [458], demonstrated that ENL and ENT may suppress aromatase enzyme activity in primary preadipocytes derived from visceral depots. Campbell and Kurzer [459], reported no effect of GNS on aromatase activity in human preadipocytes from subcutaneous and visceral depots. And van Meeuwen et al. [460], reported a potent inhibition of aromatase activity by GNS in breast fibroblasts.

In our study, 10 μM GNS and RSV were able to suppress the glucocorticoid mediated total *CYP19A1* expression in primary breast ASCs, thus showing potential ability to modulate PI.4 transcriptional activity. Results with GNS, however, contradict those obtained previously in breast cancer cells and visceral fat preadipocytes [14,55,120,300], but it is important to emphasize that aromatase expression has shown to be differently regulated in cancer and adipose stromal cells from visceral and subcutaneous depots, so as in breast adipose tissue. Accordingly, our findings concur with previous GNS data in breast fibroblasts [460]. On the other hand, our results with RSV are in line with already reported studies, more specifically with the one from Wang Y [450], where it showed to inhibit aromatase both mRNA and protein expression in breast cancer cells. However, in this previous study, RSV blocked the activation of aromatase via PII/PI.3. Instead, uur results suggest that RSV could also repress PI.4 in healthy breast stromal cells. Regarding PS, mouse and human *in vitro* experiments indicate that it might not be able to modulate aromatase expression via PI.4.

Further studies are warranted to evaluate whether these polyphenols could also affect PII transcriptional activity in non-cancerous breast ASCs and in PI.4 and/or PII in cancer-related adipose fibroblasts (CAF).

Furthermore, the potential modulatory effect of the lignan secoisolariciresinol (SECO) and its mammalian-lignan metabolites, ENT and END, on *CYP19A1* expression was examined in primary breast ASCs (unpublished data). Only END and ENT, but not SECO, were shown to affect the breast stroma *CYP19A1* expression. However, these effects contradicted those previously reported. ENT was shown to further upregulate DEX/TNFα-induced *CYP19A1* transcription, while END did the same but on FSK/PMA-induced *CYP19A1* transcription. The reasons for these differences in response may relate to the fact that mRNA levels do not always coincide with the protein levels and, furthermore, as explained before, regulation of aromatase might not be the same in all WAT depots. While the promoters driving *CYP19A1* transcription in WAT depots are the same, the responsiveness of the surrounding stromal cells to the local inducers appears to differ between subcutaneous and visceral fat, but also between breast adipose tissue and other subcutaneous depots [55,85,120,295]. Most preclinical models used for testing the lignans for breast cancer prevention lack information about their potential effects on aromatase expression. However, it is possible that downregulation of aromatase activity in healthy stromal cells derives from an indirect action of lignans on the local production of proinflammatory factors. These results may provide new mechanistic evidence for a potential regulatory effect of polyphenols on aromatase expression. Ultimately, this information may aid to understand how dietary polyphenols affect breast cancer development.

6.6 Strategies for Prevention of Breast Cancer

There is an urgent need to develop new and more efficient approaches to reduce postmenopausal breast cancer risk, as it has become a major health problem worldwide. In parallel with the dramatic increase in the incidence of obesity, particularly in developed countries, it has been found that increased frequency, morbidity, and mortality for breast cancer among postmenopausal women correlate with excessive BMI and adiposity [259,271,461]. Current strategies for primary breast cancer prevention aim at decreasing risk factors, thus weight loss and disrupting or interfering with the obesity-inflammation-estrogen axis represents one of the most efficient approaches for obese postmenopausal women [28,462].

Caloric restriction has shown to reduce mammary tumor growth and metastasis in a variety of animal models [463]. A 30% caloric restriction in obese mice was associated with lower inflammation in the mammary fat pad, evidenced by reduced CCL2 levels and infiltrated macrophages, lower production of IL-6 and TNFα, and inhibited NF-

κB and AP-1 inflammatory activities. At the same time, caloric restriction restored the correct metabolic function of these animals, increased insulin sensitivity, lowered circulating glucose and IGF-1 levels, and promoted adiponectin secretion [464]. Remarkably, weight loss in obese postmenopausal women, either by improving physical activity or by undergoing bariatric surgery, was associated with a significant reduction in breast cancer incidence and mortality [274,465]. The Iowa Women's Health Study based on 34000 women demonstrated that a tendency towards maintaining 5% weight loss can reduce the risk of breast cancer by up to 25%, even in postmenopausal women with normal BMI [466]. Recent data suggest that caloric-restricted diet-mediated weight loss in postmenopausal women, with or without exercise, can lead to significant reductions in serum estrogens and free testosterone, as well as in C-reactive protein, leptin, and insulin, and increased adiponectin [467].

In addition to weight loss and physical activity, diet is considered one of the most important and most modifiable risk factors for breast cancer [468]. Several studies have proved an inverse association between breast cancer risk and a low-fat diet with high vegetables, fruits, whole grain, soy, and fish, consumption [468,469]. Plant rich diets contain numerous polyphenols, which, as discussed above, have been linked to reduced breast cancer risk, progression, or recurrence [33]. While the chemopreventive effects of dietary polyphenols may be based on strong anti-oxidant and anti-inflammatory properties, it is possible that some compounds could also exert direct actions on estrogen production [35,456]. However, there are still discrepancies between the clinical studies and the research in cells and animal models with more controlled conditions. Better-designed preclinical studies could address these differences.

Current preventive pharmacological therapies for postmenopausal women at increased risk include selective the estrogen receptor modulators (SERMs), tamoxifen and raloxifene, and the aromatase inhibitors (AI), anastrozole and exemestane [462,470]. Several randomized trials of SERMs demonstrated the overall reduction of all breast cancer incidence with tamoxifen, including ductal carcinoma *in situ*, by 38% [471]. However, most studies have not been able to report a decrease in breast cancer mortality rates with SERMs. The efficacy between tamoxifen and raloxifene was compared in the Study of Tamoxifen and Raloxifene, or STAR, trial [472]. Tamoxifen appeared to be more effective than raloxifene in preventing invasive breast cancer. However, raloxifene exhibited fewer side effects than tamoxifen did, particularly a lower incidence of endometrial cancer and venous thromboembolism. At least two studies have demonstrated the efficacy of anastrozole in reducing breast cancer, one showing a 65% reduction after a five-year treatment, while in the second study, the incidence was reduced by 53% [473,474]. Compared with SERMs, AI has proved to be superior in reducing the incidence of hormone-dependent breast cancers in postmenopausal women [475,476]. When comparing the adverse profiles of these therapies, AIs are associated with a higher incidence of osteoporosis, stroke,

hypercholesterolemia, and vaginal dryness, but a lower incidence of venous thrombosis, pulmonary emboli, and vaginal discharge than tamoxifen [470]. Nevertheless, despite their beneficial effect in high-risk women, long-term antiestrogen therapies are associated with important side effects that may reduce their feasibility as preventive agents in women without a history of breast cancer or premalignant lesions.

Other pharmacological interventions proposed for breast cancer prevention include non-steroidal anti-inflammatory drugs (NSAIDs) and metformin. The use of NSAIDs is based on blockage of PGE2 biosynthesis by inhibiting COX-2 activity, thus reducing its bioavailability in WAT and the local production of aromatase and estrogens [477]. Compared with women who never used aspirin, regular intake was associated with a reduction of breast cancer risk by 20% [478]. Studies have shown that aspirin use (6-7 days/week) after a breast cancer diagnosis may significantly reduce the risk of recurrence and mortality [479]. Furthermore, daily use of NSAIDs is associated with 52% reduced ER+ breast cancer recurrence in obese women [480]. However, no studies have addressed whether long-term NSAIDs could reduce breast cancer risk among obese postmenopausal women with breast tissue inflammatory markers. Additional use of TNFα and CCR2/CCL2 inhibitors may offer a broader strategy for reducing obesity-related inflammation in postmenopausal women and, therefore, their breast cancer risk. The current data on TNFα, however, is very limited and controversial, especially because it is regarded as a tumor-killing cytokine and therefore essential for suppressing carcinogesis. TNFα-antagonist therapy has shown to promote carcinogenesis in some patients with rheumatoid arthritis [481,482]. However, a larger study in Sweden contradicted these results, reporting no change in breast cancer recurrence in patients with rheumatoid arthritis and a prior breast cancer diagnosis [483]. In the case of CCL2, several anti- CCR2 antibodies are already on the market but very few clinical trials are being conducted in cancer patients. Currently, only two CCR2 antagonists are in clinical trials for bone metastasis and pancreatic cancer [484,485].

The use of metformin, an antidiabetic drug, is also associated with a reduced incidence of cancer [486]. Interestingly, it is known that in cultured breast ASCs, metformin treatment reduces aromatase expression under FSK/PMA conditions via activation of AMPK activity [487]. Several ongoing clinical trials are expected to produce more detailed information on its potential chemopreventive effect [488,489]. Though, recent results from the randomized trial [490] using aromatase inhibitors and metformin on ER+ metastatic breast cancer patients has failed to show positive effects for the addition of metformin.

In summary, approaches that aim to reduce weight and adiposity, increase vegetable and fruit consumption, and decrease inflammation and estrogen production may offer the most promising strategy to prevent breast cancer development in obese postmenopausal women.

7 Conclusions

Based on the evidence obtained in this research work, the following conclusions can be drawn:

1. The hARO-Luc reporter mouse model is a valuable tool for studying *in vivo* the impact of obesity on the regulation of *CYP19A1* gene expression in the WAT of females and males.
2. Two novel adipose tissue cytokines, IL-10, an anti-inflammatory factor, and CCL2, a proinflammatory chemokine, were identified as novel regulators of *CYP19A1* gene expression in the breast adipose tissue (Fig. 22). IL-10 inhibited TNFα-mediated transcription of *CYP19A1* in cultured breast ASCs, thus acting as a negative regulator. Thus far, IL-10 is the first known adipose tissue secreted factor to downregulate local aromatase expression, since only ghrelin, a gut hormone, has shown similar effects in breast ASCs [405]. CCL2, on the other hand, further induced DEX-mediated *CYP19A1* gene transcription also in breast ASCs, making it a potential stimulator of aromatase in WAT. The mechanism that may drive the IL-10 inhibitory effect on TNFα was found to be the suppression of ERK1/2-induced aromatase PI.4 transcriptional activity, while, in the case of CCL2, it may be the activation of the CCR2/ERK1/2 signaling pathway, and the subsequent synergic stimulation of PI.4 transcriptional activity.

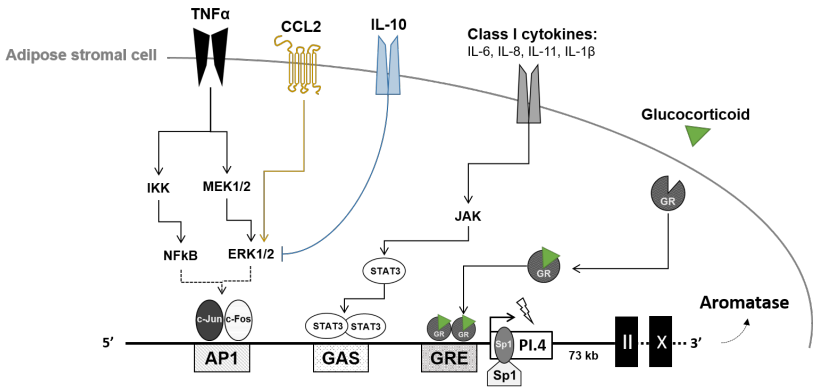


Figure 22. New factors involved in the regulation of PI.4 in the breast stroma.

3. In hARO-Luc females and males, long-term HFD feeding promoted weight gain and increased adiposity, which in turn was associated with increased aromatase Luc reporter activity and altered expression of proinflammatory factors in WAT. In females, mammary fat pad expression of aromatase and proinflammatory factors were particularly affected, more than in other WAT depots. Interestingly, mammary fat CCL2 level in obese HFD females was increased and positively correlated with Luc reporter activity. In males, subcutaneous and gonadal fat depots showed increased Luc activity and clear markers of inflammation, including a higher proinflammatory cytokine index, high CLS density, and adipose tissue hypertrophy. Furthermore, we found that *CYP19A1* and *CCL2* mRNA levels were higher in the subcutaneous adipose tissue of obese women. These results further confirm that obesity favors the establishment of a proinflammatory environment in WAT, which may overstimulate the local production and activity of aromatase.
4. Ovariectomy (OVX) in hARO-Luc mice is associated with weight gain and elevated adiposity, as well as with increased aromatase Luc reporter activity and reduced IL-10 levels in mammary fat pad. Furthermore, lower IL-10 levels in the mammary tissue, perhaps as a consequence of parenchymal involution, correlated with higher local Luc reporter activity. This indicates that loss of ovarian estrogen after menopause may promote a proinflammatory milieu that drives increased aromatase expression in the breast adipose tissue of postmenopausal women. Additionally, reduced levels of estrogens in female mice showed to induce changes in gut microbiota that are associated with increased energy intake and obesity in human subjects. This particularly evidence provide new insights into the mechanisms by which estrogen controls systemic metabolism and the underlying causes of menopausal weight gain.
5. Polyphenol-rich intervention with pine knot extract (PKE) on HFD-fed male hARO-Luc mice attenuated WAT inflammation while preventing the upregulation of aromatase Luc reporter activity in subcutaneous fat. *In vitro*, of all the polyphenols in PKE, only nortrachelogenin was shown to have a potential inhibitory effect on aromatase Luc activity in hARO-Luc MSCs via PI.4 and PII. Furthermore, in human ASCs, resveratrol and genistein inhibited *CYP19A1* mRNA levels, while enterolactone and enterodiol were shown to enhance the gene transcription levels. It is possible that polyphenols, especially lignans, may reduce estrogen levels and breast cancer risk by suppressing inflammation, rather than directly inhibiting aromatase at the transcriptional level.

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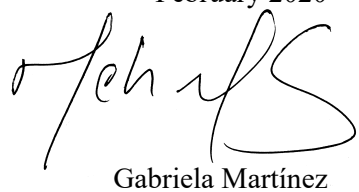

Gabriela Martínez

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