UNIVERSITÀ DEGLI STUDI DI MILANO

GRADUATE SCHOOL IN EXPERIMENTAL AND CLINICAL PHARMACOLOGICAL SCIENCES

DEPARTMENT OF PHARMACOLOGICAL AND BIOMOLECULAR SCIENCES

XXX CYCLE

ERα: THE CROSSROAD BETWEEN METABOLIC FITNESS AND METABOLIC ILLNESS

BIO/14

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A.A 2016-2017

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ABSTRACT

English

Oestrogens are important regulators of energy homeostasis and lipid metabolism, acting both centrally and peripherally and, indeed, the impairment of oestrogen signalling is associated with the development of several metabolic diseases. The alpha isoform of the oestrogen receptor (ERα) is predominant in the liver and studies carried out in several laboratories, including ours, have shown that hepatic ERα exerts a major role in the control of lipid metabolism. Recently, we demonstrated the pivotal role played by oestrogens and ERα in female liver physiology. Indeed, our studies have shown that in female mammals, hepatic ERα has a pivotal role in adapting energy metabolism to the needs of reproductive functions and that is transcriptionally regulated by estrogens and food intake.

On the other hand, in males, the expression of hepatic ERα is significantly lower than in females, yet its activity is highly regulated by food intake indicating that also in males hepatic ERα might have a role in the control of lipid metabolism. This led us to further investigate the activity of hepatic ERα in males and the aim of this study was to compare female and male liver metabolism and to unravel the role, if any, played by hepatic ERα in the regulation of energy metabolism in male mice.

We performed this study in homeostatic conditions and in a stressed metabolic status, caused by the administration of an unbalanced diet. We analysed, with different techniques, key metabolic and inflammatory pathways in female and male control (SYN) and LERKO (*Liver ERα knockout*) mice fed with control diet (ND) or high fat diet (HFD) and the LERKO mouse model, generated by our lab, allowed us to single out the activity of hepatic ERα.

This comprehensive study revealed that both metabolic and inflammatory pathways are different between females and males in homeostatic conditions and that the administration of HFD, *per se* or also in combination with the selective ablation of hepatic ERα, leads to different outcomes depending on sex. Moreover, the results of this work allowed us to identify the sex specific role of hepatic ERα in the regulation of liver metabolism and inflammation in normal or stressed metabolic conditions, and, interestingly, to highlight ERα possible opposite regulatory action in female and male metabolism.

We believe that this is very relevant from both a pharmacological and a pathophysiological point of view. Indeed, the identification of a sex dependent regulatory action of ERα on metabolism would not only provide the conceptual bases for future therapeutic interventions, able to mimic the beneficial effect of oestrogens and ERα on metabolic parameters, but also further stress the necessity to consider sex as an essential biological variable in the design of personalized pharmacological therapies.

Italian

E' ampiamente riconosciuto che gli estrogeni sono importanti regolatori dell'omeostasi energetica e del metabolismo lipidico agendo sia a livello centrale che periferico e un mal funzionamento del *signalling* estrogenico è, in effetti, associato allo sviluppo di numerosi disturbi metabolici. L'isoforma alpha (ERα) del recettore estrogenico è quella maggiormente espressa nel fegato e studi condotti in diversi laboratori, incluso il nostro, hanno dimostrato che il recettore ERα epatico ha un ruolo chiave nella regolazione del metabolismo lipidico.

Recentemente abbiamo dimostrato che gli estrogeni ed ERα rivestono un ruolo fondamentale nella fisiologia epatica femminile. I nostri studi hanno, infatti, provato che nelle femmine di mammifero il recettore ERα epatico ha un ruolo centrale nell'adattare il metabolismo energetico ai bisogni legati alle funzioni riproduttive e che è regolato trascrizionalmente dagli estrogeni e dall'assunzione di cibo.

Nei maschi, il recettore ERα epatico è significativamente meno espresso rispetto alle femmine, tuttavia la sua attività è fortemente regolata dall'assunzione di cibo; questo suggerisce che il recettore possa avere, anche nei maschi, un ruolo importante nella regolazione del metabolismo lipidico.

Queste evidenze ci hanno portati a investigare ulteriormente la funzione di ERα nel fegato del maschio, e, in effetti, lo scopo di questo studio è stato quello di comparare il metabolismo epatico femminile e maschile e chiarire il ruolo, se presente, rivestito da ERα nella regolazione del metabolismo energetico nel maschio.

Abbiamo eseguito questo studio in condizioni omeostatiche e di stress metabolico, causato dalla somministrazione di una dieta sbilanciata. Abbiamo analizzato, servendoci di differenti tecniche, importanti *pathways* metabolici e infiammatori in topi femmine e maschi controllo (SYN) e LERKO (*Liver ERα knockout*), alimentati con dieta normale (ND) o grassa (HFD). Il modello murino LERKO, generato dal nostro laboratorio, ci ha permesso di isolare l'attività del recettore ERα epatico.

Questo studio ha dimostrato come sia i processi metabolici che infiammatori siano differenti tra femmine e maschi in condizioni omeostatiche e che la dieta grassa, *per se* o in combinazione con la selettiva ablazione del recettore ERα epatico, che si ha nel LERKO, porti a risultati diversi a seconda del sesso.

I risultati di questo lavoro ci hanno inoltre permesso di identificare il ruolo sesso-specifico del recettore ERα epatico nella regolazione del metabolismo energetico e dell'infiammazione e, soprattutto, di scoprire l'opposta attività regolatoria esercitata da ERα nel metabolismo di femmine e maschi.

Crediamo fermamente che questo abbia una grande rilevanza dal punto di vista farmacologico ma anche patofisiologico. L'identificazione della dipendenza dal sesso, per quanto riguarda l'attività regolatoria di ERα sul metabolismo, non solo fornisce le basi concettuali per futuri interventi terapeutici innovativi, in grado di mimare gli effetti benefici degli estrogeni e di ERα sui parametri metabolici, ma, sottolinea anche la necessità di

considerare il sesso come una variabile biologica essenziale nel disegno delle terapie farmacologiche personalizzate.

LIST OF ABBREVIATIONS

AAs	Amino acids
ABCA1	ATP binding cassette subfamily A member 1
ABCG1	ATP binding cassette subfamily G member 1
ABCG5	ATP binding cassette subfamily G member 5
ACADL	Acyl-coA dehydrogenase, long chain
ACAT2	Acetyl-coA acetyltransferase 2
ACLY	ATP citrate lyase
ACOX1	Acyl-coA oxidase 1
AFs	Activation functions
AIF1	Allograft inflammatory factor 1
AKT	Alias PKB
AN	Anorexia nervosa
ARC	Arcuate nucleus
ARE	Androgen response element
ArKO	Aromatase-deficient mice
Ars	Androgen receptors
cAMP	Cyclic adenosine monophosphate
CCL2	C-C motif chemokine ligand 2
CCR2	C-C motif chemokine receptor 2
CNS	Central nervous system
CPT1α	Carnitine palmitoyltransferase 1A
CVDs	Cardiovascular diseases
CXCL2	C-X-C motif chemokine ligand 2
CYP	Cytochrome P450
CYP2E1	Cytochrome P450 family 2 subfamily E member 1
CYP7a1	Cholesterol 7-alpha-hydroxylase
CYP27A1	Sterol 27-hydroxylase
DBD	DNA binding domain
DHCR7	7-Dehydrocholesterol reductase
DHT	Dihydrotestosterone
DIO	Diet-induced obesity
DMN	Dorsomedial nucleus
DPN	2, 3-bis (4-hydroxyphenyl)-propionitrile
E2	Oestradiol
ELOVL6	Fatty acid elongase 6
eNOS	Endothelial nitric oxide synthase
ERα	Oestrogen receptor alpha
ERαKO	Oestrogen receptor α knockout mice
ERβ	Oestrogen receptor beta

ERβKO	Oestrogen receptor β knockout mice
EREs	Oestrogen-responsive elements
ERs	Oestrogen receptors
ERsKO	ER knockout mice
FABP4	Fatty acid binding protein 4
FAD	Fatty acid desaturase
FAS	Fatty acid synthase
FASN	Alias FAS
FDA	Food and drug administration
FSH	Follicle-stimulating hormone
GH	Growth hormone
GHR	Growth hormone receptor
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GLUT-4	Glucose transporter 4
GnRH	Gonadotropin releasing hormone
GPER1	G protein-coupled oestrogen receptor 1
HADHα	Mitochondrial trifunctional protein, alpha subunit
HDL	High-density lipoprotein
HDL-C	High density lipoprotein cholesterol
HFD	High fat diet
HMGR	3-Hydroxy-3-methyl-glutaryl-CoA reductase
HRT	Hormonal replacement therapy
HSL	Hormone-sensitive lipase
IDOL	Lipoprotein receptor
IGF-1	Insulin growth factor-1
IGT	Impaired glucose tolerance
IL-1	Interleukin 1
Il-10	Interleukin 10
Il-1β	Interleukin 1β
IL-4	Interleukin 4
IL-6	Interleukin 6
IL12-β	Interleukin 12-β
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
IRS	Insulin receptor substrate
LBD	Ligand binding domain
LCAT	Lecithin–cholesterol acyltransferase
LDL	Low-density lipoprotein

LDLR	Low density lipoprotein receptor
LDs	Lipid droplets
LPS	Lipopolysaccharide
LERKO	Liver ERα knockout
LH	Luteinizing hormone
LHA	Lateral hypothalamic region
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LXRα	Liver X Receptor α
MCP-1	Monocyte chemo-attractant protein-1
MTTP	Microsomal triglyceride transfer protein
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
ND	Normal diet
NK	Natural killer cells
OVX	Ovariectomized
PCOS	Polycystic ovarian syndrome
PCSK9	Proprotein convertase subtilisin kexin type 9
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PLIN2	Perilipin 2
PMVK	Phosphomevalonate kinase
PPARα	Peroxisome proliferator-activated receptor α
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PPT	Propyl pyrazole triol
PVN	Paraventricular nucleus
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
SERMs	Selective oestrogen receptor modulators
SERPINE	Endothelial plasminogen activator inhibitor
SHP	Orphan nuclear receptor SHP
SP-1	SP1 transcription factor
SQSTM1	Sequestosome 1
SR-1B	Scavenger receptor class B member 1
SREBP-1C	Sterol regulatory element-binding protein-1C
STAT5b	Signal transducer and activator of transcription 5B
STZ	Streptozocin
SYN	$ER\alpha^{FLOX/FLOX}$

TG	Triglycerides
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor
VIM	Vimentin
VLDL	Very low density lipoprotein
VMN	Ventromedial nucleus
VSMC	Vascular smooth muscle cells

INTRODUCTION

1. Oestrogens

Oestrogens are steroid hormones highly conserved and present in all vertebrates and some invertebrates; they are produced in different organs and their levels change throughout life in physiological and pathological conditions.

In mammals, there are four main forms of estrogens: oestrone, oestradiol (also named 17β-oestradiol), 17α-oestradiol and oestriol, all C18 steroids derived from cholesterol, structures reported in Figure 1.

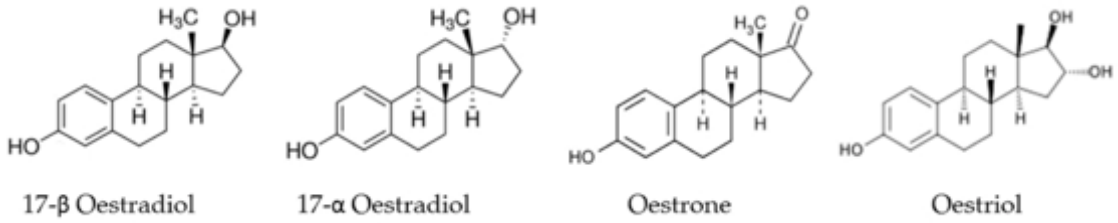


Figure 1: Molecular structures of natural oestrogens.

Oestradiol is the major product of the entire biosynthetic pathway and, in healthy premenopausal women, over 90% of oestradiol is synthetized by the ovaries where the precursor, androstenedione, is metabolized to oestrone and then converted to oestradiol[1].

The conversion of androstenedione to oestron and then to oestradiol is mediated by aromatase, an enzyme of the cytochrome P450 monooxigenase complex. Aromatase activity has been detected also in extragonadal tissues like brain, muscle, adipose, bone and, interestingly, in testis thus suggesting that estrogens play an important role also in male physiology[2].

Oestradiol has a natural isomer, 17α-oestradiol, which has been found at low concentrations in brain, adrenal glands, uterus, ovaries and testis[3]. 17α-oestradiol has a low ability to activate those signalling pathways normally induced by oestradiol and its physiological functions are still unclear[4].

Oestrone is secreted by the ovaries, but is also produced from conversion of androstenedione in peripheral tissues, like adipose tissue. The oestrogenic effects of oestron are weak and it acts like a paracrine and intracrine molecule; therefore, plasma concentrations of oestrone do not correlate with its biological effects.

Oestriol is the most abundant oestrogen in urine and is the metabolic product of oestradiol, oestrone, testosterone and androstenedione in different cell types in many non-ovarian tissues. This hormone is synthetized by the placenta and is the most abundant oestrogen in

maternal circulation during pregnancy[5]. Indeed, other than in pregnancy, plasma levels of oestriol are very low in women (before and after menopause) and in men.

The effects of oestrogens on reproduction are well known and have been extensively studied; however, they have also important effects in non-reproductive organs such as bone, metabolic tissues, brain, cardiovascular system and many others.

Oestrogens levels, mostly oestradiol, vary between men and women; they are affected by menopause and menstrual cycle and change across women life. The main differences in plasma oestradiol leves, based on reproductive status and sex, are summarized in Table 1.

Indeed, puberty is characterized by a raise in oestradiol concentrations resulting from low-amplitude nocturnal pulse of gonadotropins[6].

In the fertile period, across the menstrual cycle, the regulation of oestrogen synthesis is under the control of the hypothalamic-pituitary-gonadal axis: ovarian cycle is tightly regulated by pituitary gonadotropins, FSH (*follicle-stimulating hormone*) and LH (*luteinizing hormone*), whose synthesis depends upon the release of gonadotropin releasing hormone (GnRH).

In postmenopausal women, serum oestrogen concentrations drop and the most of estradiol is produced by conversion of testosterone in extra-gonadal tissues, mainly the white adipose tissue and adrenal cortex[7].

- Men: 20-55 pg/ml
- During the early follicular phase of menstruation: 21-72 pg/ml
- During the late follicular phase of menstruation: 53-312 pg/ml
- During the luteinizing hormone peak in menstruation: 131-388 pg/ml
- During the early luteal phase of menstruation. 48-154 pg/ml
- During the mid-luteal phase of menstruation: 75-207 pg/ml
- During the late luteal phase of menstruation: 27-214 pg/ml
- Following menopause, levels of oestradiol fall around 30 pg/ml

Table 1: Reference ranges for oestradiol. Modified from Morselli *et al.*,2017[8].

2. Oestrogen Receptors

The cellular effects of oestrogens are mostly mediated by two Oestrogen Receptors (ERs), belonging to the nuclear hormone-receptor superfamily: oestrogen receptor alpha and oestrogen receptor beta (ERα and ERβ). Apart from the ERαΔ3 isoform, all ER isoforms are composed of six functional domains, from A to F (Figure 2): *i)* the DNA binding domain (DBD), which is the most conserved domain and contains two zinc fingers involved in DNA binding and dimerization; *ii)* the ligand binding domain (LBD) in the C-terminal, responsible for the ligand binding and for the interaction with coregulatory proteins; *iii)* two AF (activation functions): AF-1, constitutively active, located at the N-terminal, represent the most variable region; AF-2 is ligand dependent and is situated at the C-terminal[9].

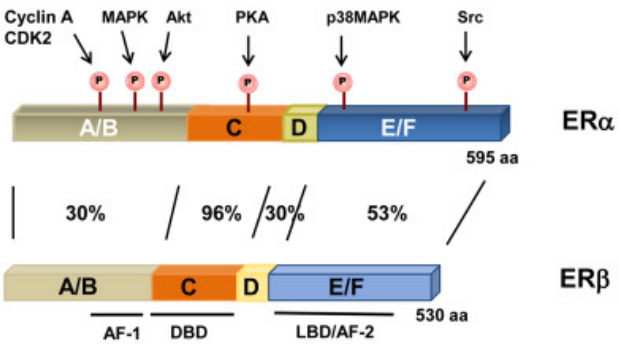


Figure 2: Structural composition of Estrogen Receptors (ER)α and ERβ. From the NH_2 terminus, the A/B domains contain the AF-1 site, the C/D domains contain the DNA binding domain, and the E/F domains contain the ligand binding site and the AF-2, at the COOH terminus. The % homology shared between ERα and ERβ in the different domains is shown and in figure are reported the main phosphorylation mediators and sites of ERα, which mediate its ligand-independent activation.

ERα and ERβ vary in structure and their encoding genes are on different chromosomes: ERα gene has been mapped on the long arm of chromosome 6, while ERβ gene is located on band q22-24 on chromosome 14.

Although the DNA binding domains of these receptors are highly similar (more than 90% amino acids identity), the overall degree of homology of the receptors is low and this is particularly true for the LBD, of which only 55% of the amino acids sequence is shared[10].

ERs act as ligand-mediated transcriptional factors. ER monomers in the cytosol form protein complexes with chaperone heat-shock proteins, which stabilize the receptor monomer in an inactivated state and mask the DBD of the receptor[11].

The ligand-mediated activation of the ER promotes the dissociation of the ER monomers from these protein complexes and their subsequent dimerization with other free monomers. Dimers can be in the form of ERα–ERα or ERβ–ERβ homodimers or in the form of ERα–ERβ heterodimers.

It has been demonstrated that homo-dimerization of ERα accelerates cellular proliferation[12] while the transcriptional activation of ERβ homo-dimer is thought to be protective against hormone-dependent diseases including breast and prostate cancers[13]. It seems that ERβ counteracts the stimulatory effects of ERα through the formation of functional hetero-dimer with ERα[14] and several experimental evidences suggest that hetero-dimer induces activation of target genes which are different from those induced by homo-dimers[15].

ER dimers enter the nucleus, where they bind, directly or indirectly through other transcription factors, to oestrogen-responsive elements (EREs) of target gene promoters, thus regulating the expression of the target genes[16]. The classical model for ER-mediated regulation of gene expression involves the direct binding of ER dimer to specific DNA sequences, known as EREs, which are inverted palindromic sequences repeated with a minimum of 3-base pair spacer: 5'- AGGTCAnnnTGACCT-3'.

The ER-ligand complex can also interact with other transcription factors, such as Fos/Jun or SP-1, thus influencing the transcription of those genes which do not contain EREs in their promoter[17,18].

Beyond the canonical model for estrogen-bound ER mediated effects, it has to be taken into account that most nuclear receptors are phosphoproteins and their activity can be altered by changes in their phosphorylation state in the absence of their cognate receptor: a mechanism referred as ligand-independent activation. The activators of protein kinases, such as growth factors, can evoke estrogen-independent activation of the receptor, inducing its phosphorylation at different sites, according to the activator[7]. Growth factors, protein kinase A (PKA) activators, neurotransmitters and cyclines are all molecules that were demonstrated to be able to activate ER-mediated transcription through the phosphorylation of the receptor[19].

In addition to being in the nucleus and cytosol, ERs can be found on the cell membrane, where they facilitate the activation of rapid intracellular cascades. The mechanism which has been proposed for the ER-membrane interaction involves the direct binding to caveolin 1 or, indirectly, the palmitoylation of ERα regulates its localization to the cell membrane[20,21].

At the cell membrane, ERs can activate the phosphoinositide 3-kinase (PI3K) signalling pathway, which activates other signalling pathways, such as rapid mobilization of intracellular calcium, generation of cAMP[22], nitric oxide production leading to the activation of tyrosine kinase receptors, epidermal growth factor receptor, insulin-like growth factor 1 receptor and protein kinase B (PKB)[23].

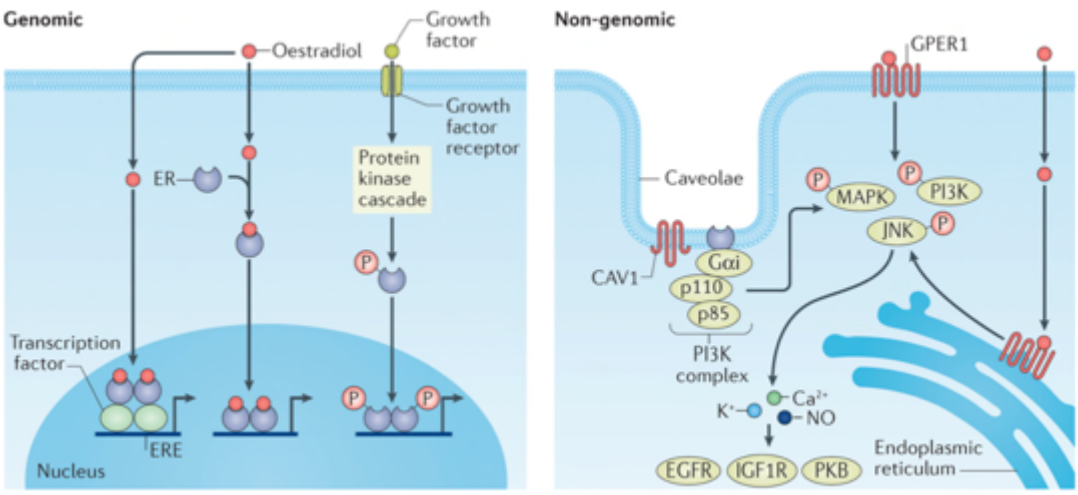


Figure 3: Oestrogen receptor signalling pathways. In the genomic signalling pathway, activated ERs can interact directly with ERE sequences or with other transcription factors; ER-mediated genomic actions can also occur independently from oestradiol, through ER phosphorylation. In non-genomic pathways, ERs located at the cell membrane and membrane-bound G protein-coupled oestrogen receptor 1 (GPER1) initiate rapid cytosolic signalling events. Modified from Morselli *et al.*, 2017[8].

Cell membrane oestrogenic signalling also occurs via the membrane-bound G protein-coupled oestrogen receptor 1 (GPER1, also known as GPER30), discovered in 1996 by Owman[24]; GPER1 has seven transmembrane domains and is responsible for the rapid modulation of cell-signalling pathways in response to oestradiol[25]. Oestradiol binds to GPER1 and activates multiple cellular effectors, such as JUN amino-terminal kinases (JNKs), mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K), as reported in Figure 3. GPER1 is involved in a wide range of different physiological and pathological conditions, and it has been demonstrated that *in vivo* is involved in many beneficial effects exerted by oestrogens at different levels (CNS, immune and cardiovascular system, pancreas)[26].

3. Physiological role of oestrogens

Oestrogens are primarily referred as females' sex hormones for their pivotal role in the regulation of women reproductive functions. Indeed, females' organogenesis, puberty, fertile age and menopause are strongly regulated by oestrogens; however, they also play a role in maintaining the male reproductive system.

Besides the effects on female and male reproductive systems, oestrogens have profound effects on other tissues like central nervous system (CNS), bone, immune and cardiovascular systems[27].

3.1 Oestrogens in reproductive tissues

Both oestrogen receptors, ERα and ERβ, are expressed in reproductive tissues and have different biological functions. Not surprisingly, deleting the gene of either receptor results in different phenotypes in mice and the use of transgenic mice, lacking one or both receptors (ER$\alpha^{-/-}$, ER$\beta^{-/-}$), has been useful to elucidate ERs functions.

Both male and female ER$\alpha^{-/-}$ mice are infertile, whereas ER$\beta^{-/-}$ mice show a sex bias: only ER$\beta^{-/-}$ female have reduced fertility. The lack of ERα does not prevent normal uterine development, but disrupts post-pubertal growth while ER$\beta^{-/-}$ females, even though they have decreased fertility, are able to carry pregnancies to term, indicating a normal uterine function[28].

Indeed, infertility in ER$\alpha^{-/-}$ female mice is due to the inability of the uterus, a central organ for reproduction and pregnancy, to respond to oestrogen[29] and also to defects of ovulation: these mice are anovulatory.

Taking into account that a proper expression of pituitary gonadotropins is necessary to stimulate ovulation successfully and that ER$\alpha^{-/-}$ females are unable to regulate pituitary gonadotropin's concentrations, it is not surprising that in ER$\alpha^{-/-}$ females' ovaries there are not fully developed follicles and no apparent corpora lutea. In addition, the ovaries contain many blood-filled cystic structures[30]. Since this phenotype has also been described in transgenic mice over-expressing LH, it seems that ER$\alpha^{-/-}$ females' ovaries are the result of the exposure to increased levels of LH. On the other hand, the ovaries of ER$\beta^{-/-}$ females appear to contain normal follicles at all stages of development as well as corpora lutea[31].

Another important reproductive tissue is the mammary gland, which in mammals is necessary to feeding the offspring, through the production of milk. At birth, the mouse mammary gland consists of a primitive ductal tree, which develops and fills the stroma of the gland in response to the increased levels of ovarian steroids at puberty. Since ER$\alpha^{-/-}$ females do not ovulate, their mammary glands do not develop beyond an immature state and only contain elementary ducts, characteristic of embryonic and fetal stages. In these animals, the release of prolactin from the anterior pituitary gland is reduced and lactation is

impaired. The mammary gland of ERβ$^{-/-}$ females develops and functions normally, indicating that ERβ is not required for this tissue[28,30].

As mentioned above, oestrogen signalling is also important for male reproductive system. ERα is expressed in prostatic tissue during fetal development, while ERβ expression starts in adulthood and an appropriate balance between the two receptors is required for a normal development of male reproductive tissue[32]. However, it seems that ERα is more relevant than ERβ for male reproduction system development and function since ERα$^{-/-}$ males have smaller testes, reduced sperm counts and quality and lower fertility rates as a result[33].

3.2 Oestrogens effects in non-reproductive tissues

As highlighted previously, oestrogens have important effects not only in reproductive tissues but also all over the body: they have a role in the maintenance of homeostasis in numerous tissues including the CNS, the immune and cardiovascular system.

3.2.1 Oestrogens and Central Nervous System

ERs were first localized in the brain of female rats in those areas involved in the control and regulation of reproductive functions (neuroendocrine system, hypothalamus and preoptic area). Moreover, the two ER isoforms are expressed in all the cellular types of the CNS (neurons, glia and neuronal stem cells) and they are transcriptionally active in the brain of male mice despite low levels of circulating estrogens, possibly because of aromatase activity within the brain or because of ligand independent transcriptional activation[34,35].

This evidence suggests that oestrogens play a pivotal role in brain development and function; actually, they influence numerous cellular functions including neuronal and glial plasticity, dendritic growth, synaptogenesis, differentiation, neurogenesis and cell migration[36].

During fetal development, oestradiol plays a critical role in brain sexual differentiation. It is well known that, in rodents, exposure to oestrogen on day 1 of life is needed for sexual imprinting of the brain[29]. Oestrogen is synthesized in the brain from testosterone secreted by the neonatal testis at very specific stages during development[37] and this reaction is catalyzed by aromatase. Exposure of females to oestrogen during this crucial period masculinizes the brain[38]. The organizational effect of neonatal exposure to oestradiol, leads to sex-specific neuroanatomy and patterns of neurotransmitter synthesis and release, resulting in sex-specific sexual behaviors, secretion patterns of gonadotropin-releasing-hormone and gonadotropins[36].

But oestradiol is not only necessary for the organization and sexual differentiation of the brain, it also has an essential role in maintaining normal brain function and protecting the brain from various neurodegenerative diseases and injuries, such as Alzheimer's disease and injury associated to cerebrovascular stroke[39]. Interestingly, using both ER$\alpha^{-/-}$ and ER$\beta^{-/-}$ mice, it has been demonstrated that the presence of ERα, and not ERβ, is necessary for oestradiol to exert protection against ischemic injury[40].

Many neurological disorders such as stroke, multiple sclerosis, Alzheimer and Parkinson's diseases are characterized by a common inflammatory component. In numerous *in vitro* and *in vivo* studies, it has been demonstrated that low, physiological concentrations of oestradiol have anti-inflammatory properties in the brain by suppressing the brain's innate immune response.

In order to mediate this anti-inflammatory response, oestradiol suppresses many pro-inflammatory factors such as free radicals, cytokines and chemokines, and the enzyme inducible nitric oxide synthase (iNOS), and enhances the synthesis of anti-inflammatory cytokines, chemokines, and other growth factors[41].

Finally, both ERα and ERβ are expressed in all hypothalamic nuclei[42] and it is well known that hypothalamic neurocircuits are essential for the regulation of energy balance. This tight regulation, which integrates multiple endocrine signals from the periphery, involves different neuronal areas such as the arcuate nucleus (ARC), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN), the paraventricular nucleus (PVN), and the lateral hypothalamic (LHA) region[43].

Among the ER isoforms, ERα is the major regulator of central energy homeostasis and its activation results in a reduction of food intake and increased energy expenditure, contributing to the overall decrease of body weight mediated by estrogens.

3.2.2 Oestrogens and Immune System

Oestrogens exert a wide range of actions within the immune system. Indeed, it is well documented that they are involved in auto-immune diseases and also in the immune response against parasitic and bacterial infections[44].

In females, during the fertile period, oestrogens have an anti-inflammatory role; with the onset of menopause, indeed, the incidence of those disorders characterized by a strong inflammatory component like osteoporosis, atherosclerosis and diabetes, approaches or exceeds that observed in males (Figure 4).

There is a large body of clinical and preclinical lines of evidence that the decline in ovarian function, occurring with menopause, is associated with increase in pro-inflammatory cytokines, like interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α[45].

Before the onset of menopause, there are many sex-specific immunologic differences regarding whether the innate or the adaptive component of the immune response and both these components are stronger in females than in males[46].

Several data show that sex steroids are able to influence immune cells development in primary lymphoid tissues, such as thymus[47] and have immunomodulatory effects on both peripheral T and B cells subsets in adult life[48,49].

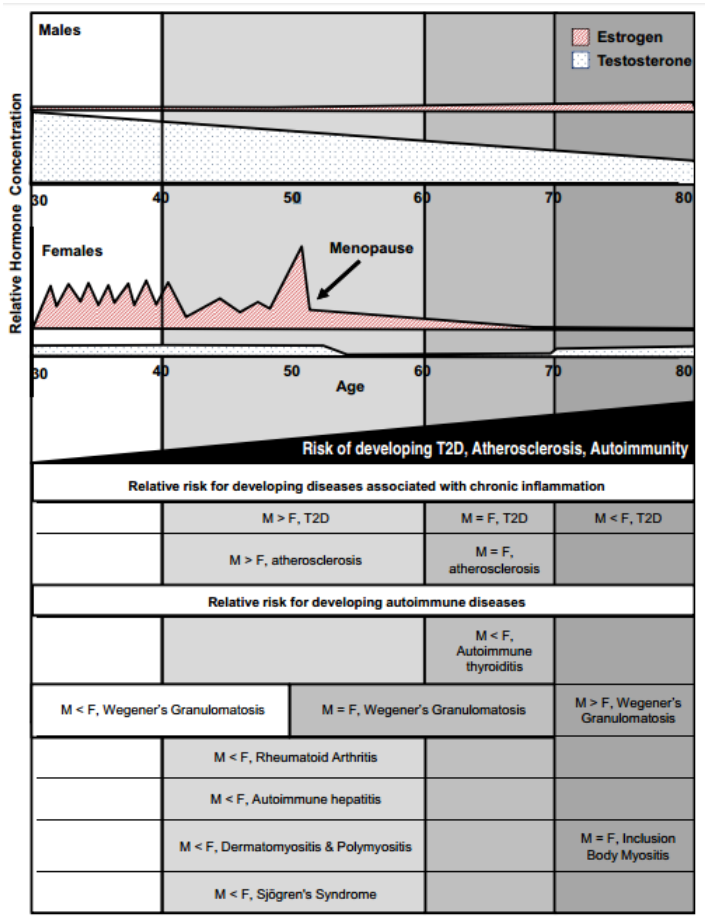


Figure 4: Correlations between the relative levels of sex steroids and the incidence of diseases associated with chronic inflammation. Type 2 diabetes (T2D) atherosclerosis, and autoimmunity in men and women between the ages of 30 and 80 years. White and shaded columns demarcate periods in the lifespan during which chronic inflammatory and autoimmune diseases manifest clinically. A comparison of the incidence of each disease in men and women for each time period and disease is indicated. Modified from Gubbels Bupp., 2015[50].

We know that oestrogens exert their functions by binding their own receptors and both ERs are expressed in T cells, B cells, dendritic cells, neutrophils, macrophages, natural killer (NK) cells, thymic stomal cells, and bone marrow[51]. Recent studies have shown that also the membrane-bound GPER1 may be involved in estrogen anti-inflammatory action; indeed, it is reported that GPER1 specific agonists can regulate the expression of the cell-surface Toll-like receptor 4 (TLR4) in macrophages[52].

In isolated macrophages, central effector cells of innate and adaptive immunity, Ribas et al. found that ERα is necessary for suppression of inflammation, IL-4 mediated induction of alternative activation (M2) of macrophages, and full phagocytic capacity in response to LPS (*lipopolysaccharide*)[53].

In immune cells, ERα plays a critical role in mediating a lot of cellular responses necessary for innate and adaptive immunity and when the levels of oestradiol decrease or ERα action is impaired, disease susceptibility increases as the functionality of critical immune cell types become compromised[44].

3.2.3 Oestrogens and the Cardiovascular System

Cardiovascular diseases (CVDs) are the leading cause of death in women and men in developed countries and the incidence of CVDs differs significantly between the two sexes.

Indeed, premenopausal women are protected from CVDs compared to age-matched males[54] and, additionally, low levels of oestrogens in young women (18-40 years) definitely increase their risk of CVDs as well as the early menopause is associated with atherosclerosis and with a greater risk in CVDs and CVD-related mortality[55,56].

In spite of this evidence, oestrogen supplementation therapy in postmenopausal women led to contradicting results with some reports suggesting that women who receive supplemental oestrogen therapy have an increased risk of CVDs[57].

For many years, the cardio protective effects of oestradiol were thought to be due to its ability to reduce LDL (*low-density lipoprotein*) and increase HDL (*high-density lipoprotein*) resulting in a positive effect on total serum cholesterol and lipoprotein profile. However, recent trials have failed to confirm this association in postmenopausal women receiving hormonal replacement therapy (HRT)[58].

Both ERs are expressed in the neonatal and adult hearts[59] and have been identified in the vascular endothelium, vascular smooth muscle (VSMC), adventitial cells and macrophages of blood vessels; additionally, GPER1 is expressed in endothelial cells and cardiomyocytes[58] and mediates the induction of intracellular signals that facilitate the cardiovascular protective actions of oestrogens.

The use of ER knockout mice (ERsKO) allowed delineating the specific functions of the two receptors. ERα has protective effects on vascular injury and is required to maintain glucose utilization in the mouse heart[60], whereas ERβ ameliorates reperfusion and, after ischemic injury, activates the antiapoptotic (PI3K-PKB) signalling pathway which prevents the generation of reactive oxygen species (ROS), thus improving myocardial function in the heart of female, but not male, mice[61].

Oestrogens play an important function also in the maintenance and restoring of the endothelium. Indeed, in endothelial cells, the interaction of non-nuclear ERα with specific G

proteins leads to the activation of endothelial NO synthase (eNOS), stimulating proliferation and migration of endothelial cells and promoting artery re-endothelialization, ending up in cardiovascular protection[20].

Finally, oestrogens can affect cardiovascular system by indirect systemic effects. They regulate the levels and activity of ion channels modulating cardiac repolarization. Studies conducted in rats have shown that female and male have functional differences in their calcium and potassium channels and these differences are due, at least in part, to oestrogens[62].

4. Oestrogens effects on Energy Metabolism

Oestrogens are recognized as important regulators of energy metabolism, acting both centrally and in the periphery, as shown in Figure 5. It is not surprising that these sex hormones, which control primarily reproductive functions, are also involved in the control of energy homeostasis; indeed, as will be discussed next, energy metabolism and reproductive functions are strongly coupled[63].

Oestrogens' decline which occurs in menopause leads to an increase of the prevalence of obesity and insulin resistance (IR), and aromatase-deficient mice (ArKO) of both sexes, in which the endogenous production of oestrogens is decreased, develop obesity in the absence of hyperphagia[44].

Oestrogens' effects on metabolism are largely mediated by ERα. Mice of both sexes lacking ERα have an increased body weight and food intake, show IR, impaired glucose tolerance and adipocyte hypertrophy[64–66]; additionally, the expression of glucose transporter 4 (GLUT-4) is strongly reduced in the skeletal muscles of these mice[67].

On the other hand, the role of ERβ in the regulation of metabolism and obesity is less clear. Mice lacking ERβ have a comparable body weight and fat distribution, as well as lipid and insulin levels when compared to their wild-type littermates[68].

Pharmacological oestrogens can reverse the progression of metabolic disorders and indeed, ostrogens have been approved by FDA for postmenopausal therapy. However, due to ubiquitous expression of ERs, the metabolic benefits provided by HRT are too often associated with side effects like increased risk of heart disease, gynecological and breast cancer.

One of the possible strategies to make oestrogens therapeutically more efficient and to avoid, as much as possible, the side effects is to develop novel tissue selective SERMs (*selective oestrogen receptor modulators*), without the effects of general oestrogen therapy. Recent studies focusing on the development of a glucagon-like peptide-1 (GLP-1)-oestrogen conjugate, which uses a peptide carrier to selectively deliver oestrogen to specific tissues, show high efficacy and significant less side effects[69].

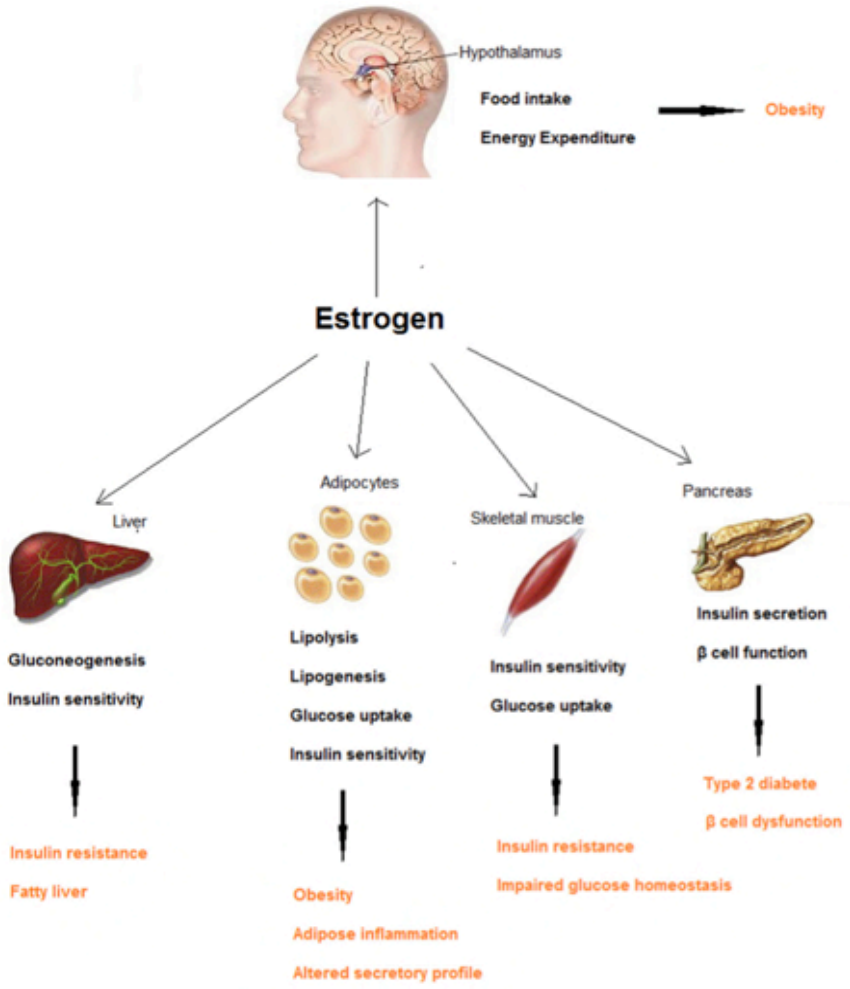


Figure 5: Oestrogen actions in the CNS and in the periphery. Modified from Jia *et al.*,2015[23].

4.1 Oestrogens and adipose tissue

Adipose tissue plays a major role in the regulation of lipid, glucose homeostasis and insulin sensitivity.

There is a strong evidence of sex differences in the pathophysiology of obesity and metabolic disorders[70,71]; indeed, women have a higher percentage of body fat than men and tend to accumulate more subcutaneous fat whereas men accumulate more visceral fat[72]. Furthermore, the increased abdominal obesity and visceral fat observed in postmenopausal women, which are associated with insulin resistance, can be reversed by HRT[73]. Overall, these data underline the central role of oestrogens in adipose tissue biology.

Oestrogens affect adipose tissue by induction of lipolysis, through the activation of hormone-sensitive lipase (HSL) and reduction of lipogenesis, mostly by acting on lipoprotein lipase (LPL), fatty acid synthase (FAS), fatty acid desaturase (FAD) and peroxisome

proliferator-activated receptor-γ (PPAR-γ)[74,75] (Figure 6). Moreover, oestrogens increase the expression of insulin receptors in adipocytes, thus enhancing insulin sensitivity.

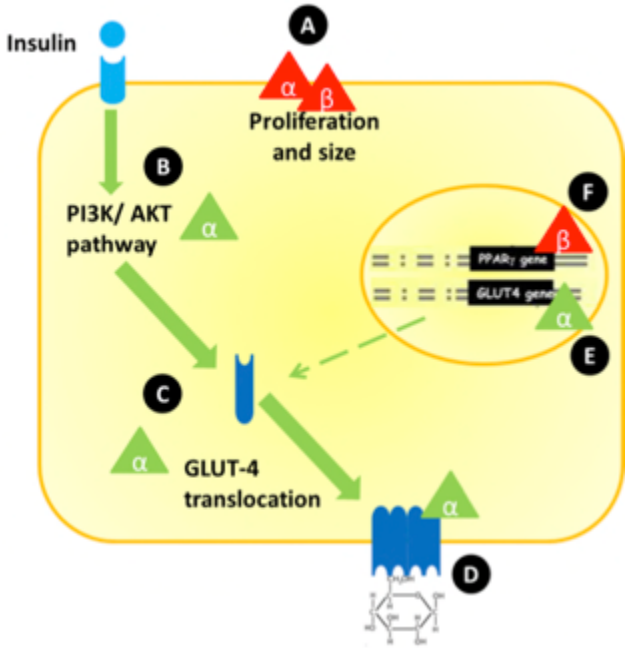


Figure 6: ERα and ERβ in adipose tissue. ERα decreases proliferation and size of adipocytes (A) and increases insulin signalling (B), GLUT4 translocation (C), glucose uptake (D), and the expression of GLUT-4 (E), whereas ERβ increases adipocyte size (A) and reduces PPARγ expression (F). Modified from Barros & Gustafsson.,2011[76]

Both ER isoforms are expressed in adipose tissue and whether the above-mentioned actions are mediated by ERα or ERβ is currently unknown. Female and male ERαKO mice have increased adipose tissue mass, without differences in energy intake, IR, impaired glucose tolerance (IGT) and also adipocyte hyperplasia and hypertrophy[65,77]. This evidence was confirmed by studies in 3T3-L1 pre-/adipocytes in which cells stably transfected with ERα showed attenuated triglyceride accumulation and reduced LPL expression[78].

On the other hand, the role of ERβ in adipose tissue biology is less clear. ERβKO male mice have a comparable body weight, fat distribution as well as lipid and insulin levels to control animals. However, ERβKO female mice, under high fat diet (HFD) feeding, have a higher weight gain than their wild-type littermates[23].

The higher body weight in ERβKO female is the result of enhanced adipogenesis and subsequent increased adipose tissue mass.

Recent studies on ovariectomized (OVX) Wistar rats under HFD feeding have shown that ERβ selective agonists can decrease the expression of lipogenic (sterol regulatory element-binding protein-1C (SREBP-1C), FAS) and adipogenic genes (LPL, PPAR-γ) in adipose tissue[79]. All these findings, together with the knowledge that PPAR-γ, a key adipogenic and lipogenic factor, is negatively regulated by ERβ suggest the anti-lipogenic actions of this

isoform. Finally, it is clear that both ER isoforms participate in the anti-lipogenic actions of oestrogens in adipose tissue.

Recently, several studies support the thesis that inflammation is the first step towards the development of IR in adipose tissue[80–82]. Actually, in OVX mice the obesity and IR observed correlate with an increased inflammation in adipose tissue, higher infiltration of immune cells, elevated expression of TNFα, IL-6, monocyte chemo-attractant protein-1 (MCP-1) and macrophage-specific markers in abdominal fat when compared to control animals[83]. On the contrary, a study performed by Riant *et al*[84] demonstrated that the administration of oestradiol in diet-induced obesity (DIO) mouse model lead to an increase in adipose tissue inflammation, and that this process is mediated by ERα.

Summarizing, the anti-inflammatory action of oestradiol in adipose tissue is a crucial point and can, at least in part, specify the anti-diabetogenic actions of oestradiol.

4.2 Oestrogens and skeletal muscle

Skeletal muscle is a central player in the maintenance of total body energy homeostasis; indeed, 75% of glucose clearance in response to postprandial insulin secretion is mediated by skeletal muscle[76].

Insulin, upon binding to its receptor, activates a phosphorylation cascade that involves several proteins, such as insulin receptor substrate (IRS), phosphatidylinositol-3 kinase and AKT kinase, leading to subsequent translocation of the cytoplasmic glucose transporter 4 (GLUT-4) to the cell membrane where is facilitates transport of glucose into the cell. GLUT-4 is highly expressed in muscle and represents a rate-limiting step in the insulin-induced glucose uptake[85].

Oestrogens modulate glucose homeostasis in the muscle mainly acting on several proteins of the insulin-signalling pathway, and on the expression and translocation of GLUT-4. Interestingly, during perimenopausal and postmenopausal periods, there is a clear decline in muscle strength that can be reversed by hormonal replacement therapy[86]. Furthermore, premenopausal women's muscles show enhanced insulin sensitivity despite 47% higher triglyceride content compared with age-matched men[87] and these observations are in line with a reduced respiratory quotient and a greater dependence on the fatty acids oxidation as an energy source in women[88].

Skeletal muscle expresses both ERs, and in mice ERβ is the predominant isoform[89]. The two receptors seem to have opposite effects on the expression of GLUT-4: ERα induces, whereas ERβ seems to inhibit GLUT-4 expression in this tissue[67,90]. ERs participate in almost every step of insulin-induced and in some steps of insulin-independent glucose uptake (Figure 7).

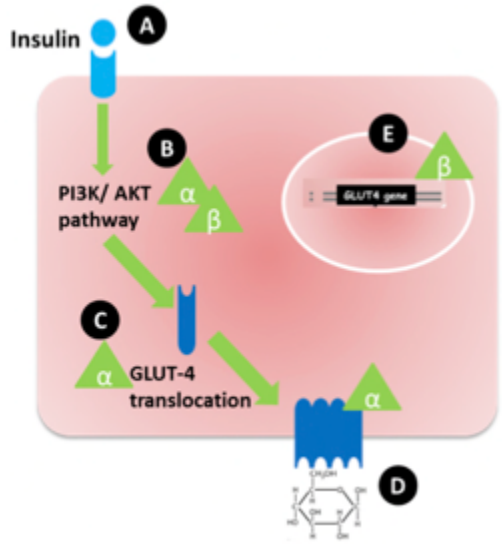


Figure 7: ERα and ERβ in skeletal muscle. (A–E) Upon binding to insulin receptors on the cell membrane (A), insulin signalling is triggered and several proteins are phosphorylated (B), leading to the translocation of GLUT4 to the cell membrane (C), where permits the influx of glucose by facilitated diffusion (D). Both ERs seem to activate the insulin-signalling cascade; ERα modulates GLUT4 translocation to the cell membrane and glucose uptake (D), whereas ERβ is a repressor of GLUT4 expression (E). Modified from Barros & Gustafsson.,2011[76].

The distinct role of each receptor has been partially gained from knockout mice models and from studies with selective estrogen receptor modulators (SERMs). ER$\alpha^{-/-}$ mice are glucose intolerant, insulin resistant[65] and display a reduced glucose uptake in muscle[67], while in ER$\beta^{-/-}$ glucose tolerance and insulin release are similar or even better than in wild-type mice[66,91]. Interestingly, tamoxifen, an ER antagonist, has no effect on glucose tolerance or insulin sensitivity in muscle of wild-type or ER$\beta^{-/-}$ mice, but in ER$\alpha^{-/-}$ mice, it increases GLUT4 expression and improves insulin sensitivity[90]. Furthermore, treatment with the ERα-selective agonist, PPT, increases GLUT4 translocation to the cell membrane of L6 myoblasts, while silencing ERα results in a decreased translocation[89].

Treatment of OVX rats with PPT increases GLUT4 expression and glucose uptake in skeletal muscle[92]. In contrast, treatment with the ERβ-selective agonist DPN decreases GLUT4 expression in muscle of ArKO male mice[67].

Concluding, it is evident that oestrogens modulate muscle metabolism acting on several pathways, either insulin-dependent or independent but more research is needed to unravel the specific function of each receptor isoform in this tissue.

4.3 Oestrogens and pancreas

Oestrogens are known regulators of pancreatic β cells function since 1940s, when it was demonstrated that oestradiol administration to rats increases insulin content in the pancreas. After partial pancreatectomy, oestradiol shows a strong association with islet hypertrophy and regeneration; moreover it influences not only islet sixe but also the release of insulin from the β cells[76].

Islets isolated from OVX mice respond differently to glucose than those isolated from intact mice: the ones from OVX have a smaller release of insulin and replacement of oestradiol normalizes this response.

In women, after menopause, insulin secretion is not different from that of premenopausal women. However, glucose tolerance tests showed that in the postmenopausal period, there is reduced insulin secretion and also elimination, which results in the maintenance of insulin levels similar to that in premenopausal women[93].

Lower doses of oestradiol, such as during HRT or hormone contraceptives therapies, increase insulin secretion, sensitivity and elimination while higher doses seem to have a time-dependent effect on insulin secretion. In the short term, hyperoestrogenism is associated to reduced insulin secretion, whereas in the long term, is observed an increased secretion[76]. Indeed, the increased serum levels of oestradiol occurring during pregnancy, may have a role in pancreatic islet adaptation to the new and augmented metabolic demand[94].

Oestrogens exert rapid effects on β cells, regulating membrane depolarization, Ca^{2+} influx, insulin secretion, and then overall glycemia. In addition, they are important regulators of β cells inflammation and apoptosis[95].

Both ERs have been identified in the nucleus and cell membrane of β cells and ERα is the functional predominant receptor isoform in the murine pancreas. ERα$^{-/-}$ mice display islet dysfunction, hyperinsulinemia and the oestradiol-dependent insulin release in cultured pancreatic islets derived from these animals is lower than that observed in either ERβ$^{-/-}$ or wild-type mice[96].

Moreover, oestrogens have also protective effects against apoptosis, one of the key features in both types 1 and 2 diabetes mellitus and these effects are mainly mediated by ERα; indeed, treatment with oestradiol can rescue β cells from streptozocin (STZ)-induced apoptosis, can increase insulin production, improve insulin resistance and glucose intolerance and all these protective effects are lost in ERα$^{-/-}$ female mice[97]. However, these protective ERα-mediated effects on islets survival are independent of the classical ERE activation and, instead, involve the G protein-coupled estrogen membrane receptor (GPER1)[98].

Several studies focused also on the oestrogenic regulation of the ATP-sensitive potassium (K_{ATP}) channels in β cells. Closure of K_{ATP} channels is a central event in the glucose-induced insulin release: when the channels are closed, membrane depolarizes and insulin is released. It has been demonstrated that oestradiol reduces the activity of K_{ATP} channels in β cells from wild-type and ERα$^{-/-}$ mice, but not in those from ERβ$^{-/-}$ mice[99].

All these studies demonstrate that oestrogens are key players in the physiopathology of pancreas and mediate protective actions involving β cells proliferation, differentiation, survival and maintenance of insulin secretion. The oestrogens-ER regulation of pancreatic function involves both genomic and rapid non-genomic phenomena (Figure 8).

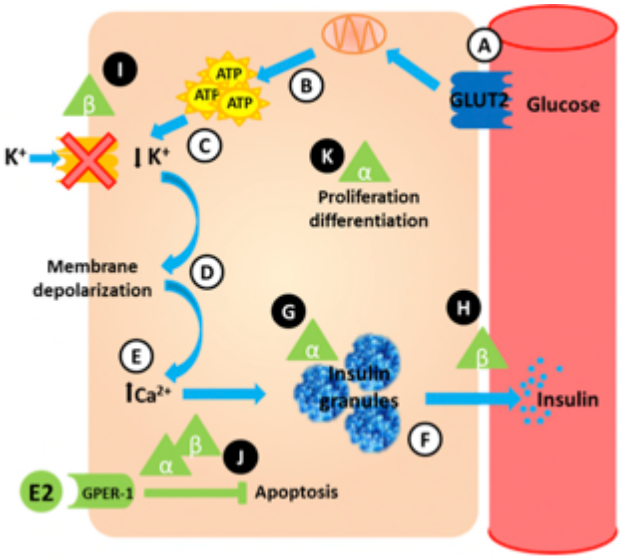


Figure 8: ERα and ERβ in pancreas. (A–K) Influx of glucose into the β cell, (A) increases ATP release from the mitochondria (B) and closes the KATP channels (C), leading to membrane depolarization (D). Ca2+ channels open and the intracellular Ca2+ content increases (E), promoting the release of insulin from granules into the circulation (F). ERα stimulates insulin synthesis (G) and ERβ participates in insulin release (H) and the closure of KATP channels (I). ERα and ERβ also participate in the E2-induced protective effects on apoptosis, via the membrane receptor GPER1, preventing the decrease of insulin release (J). Additionally, ERα regulates β cell proliferation and differentiation (K). Modified from Barros & Gustafsson.,[76]

4.4 Oestrogens and liver

Liver is a crucial peripheral integrator of different signals, including nutrients and it is able to tightly coordinate metabolism and reproductive needs. Indeed, it is a key visceral organ, which controls energy storage, regulating lipid transport, *de novo* lipogenesis, lipid oxidation and lipolysis. Liver steatosis, as occurs in nonalcoholic fatty liver disease (NAFLD), is due to an increased accumulation of triglycerides (TG) within the hepatocytes and its incidence is frequently associated with circulating low levels of high density lipoprotein cholesterol (HDL-C) and high levels of low density lipoprotein cholesterol (LDL-C)[100].

The incidence of steatosis is higher in men[101], whose lipid profile is characterized by lower HDL, higher LDL and TG levels as observed also in postmenopausal women, suggesting that lower circulating oestrogens levels may promote fat deposition in the liver[102]. This has been demonstrated also by using OVX mouse model; ovaries removal, and thus the majority of endogenous oestrogens, results in increased fat proportion in the liver even when OVX mice are pair-fed with the same amount of calories as intact females highlighting the direct role of oestrogens in hepatic lipogenesis inhibition, rather than the secondary effects of OVX induced overfeeding[103]. Moreover, the oestrogen deficient ArKO mouse model, displays spontaneous obesity and hepatic steatosis resulting from impaired fatty acid *β*-oxidation and increased expression of FAS in the liver of both female and male mice[104].

ERα is the predominant ER isoform in hepatocytes[105], while ERβ is expressed only in human fetal liver[106]. Several studies in knockout mice have tried to clarify the specific role of ERα in the regulation of hepatic energy metabolism.

Male and female ERα$^{-/-}$ mice have hepatic steatosis and this is due to an increased expression of lipogenic transcription factors, such as sterol regulatory element binding protein 1c (SREBP-1c), and a decreased expression of lipid transport genes[65,66]. On the other hand, liver-specific ERα and GPER1 knockout mice[107] have an increased hepatic fat deposition and display a compromised insulin signalling when fed with HFD. These lines of evidence suggest that, although it is widely recognized that oestrogens regulate liver lipid metabolism mainly by ERα activity[108,109], both ERα and GPER1 are needed to maintain lipid homeostasis.

Although ERα is the predominant receptor isoform in the liver, where it exerts its antilipogenic action, also ERβ has different functions in this tissue. ERβ$^{-/-}$ mice, fed with HFD, have higher body weight but decreased liver weight due to reduced hepatic TG accumulation[91]; additionally, they display increased hepatic insulin sensitivity combined with augmented insulin-stimulated AKT phosphorylation, thus indicating that ERβ might be lipogenic and diabetogenic in the liver[76].

Besides their crucial role in the regulation of hepatic lipid metabolism, oestrogens are also key regulators of cholesterol homeostasis in the liver, which is the main organ for cholesterol *de novo* biosynthesis, catalyzed by the rate limiting enzyme 3-hidroxy-3-methyl-glutaryl-CoA reductase (HMGR). An initial *in vitro* study demonstrated that HMGR promoter is induced by oestrogen treatment in breast cancer cell line MCF-7, but not in hepatic cell lines[110], suggesting a different oestrogens-mediated regulation of HMGR depending on tissues.

Other lines of evidence of oestrogen regulatory effects come from *in vivo* studies: total cholesterol and LDL are increased in ArKO mice[111], while in OVX rats there is an increased hepatic HMGR activity with a consequent increase in cholesterol and LDL levels[112]. Interestingly, oestrogen replacement in both ArKO mice and OVX rats normalizes LDL and cholesterol levels.

ArKO mice, with oestrogens deficiency, develop obesity and dyslipidemia[111,113], ERα knockout mice, in the whole body, have an increased expression of genes involved in lipid biosynthesis and lipid metabolism[66] and ERα and ERβ double knockout mice display increased body fat and circulating cholesterol levels, however these changes are not observed in ERβ$^{-/-}$ mice[68].

Also GPER30 seems to play a role in cholesterol regulation: indeed, in GPER1 knockout mice, LDL levels increase by about 200% while there is no change in HDL levels suggesting that GPER1 mainly regulates LDL metabolism[114]. In line with these observations, a recent study showed that, in human, the hypofunctional P16L genetic variant of GPER1 leads to increased plasma LDL concentrations[115,116]. On the contrary, the activation of GPER1

upregulates the expression of hepatic LDL receptor, by downregulating proprotein convertase subtilisin kexin type 9 (PSCK9) and enhancing LDL metabolism[115].
All these lines of evidence are in agreement with the observed increase in LDL and VLDL levels, and decreased HDL, which occurs during menopause, an event that can be reversed by HRT, which also promotes cholesterol secretion into bile[117].

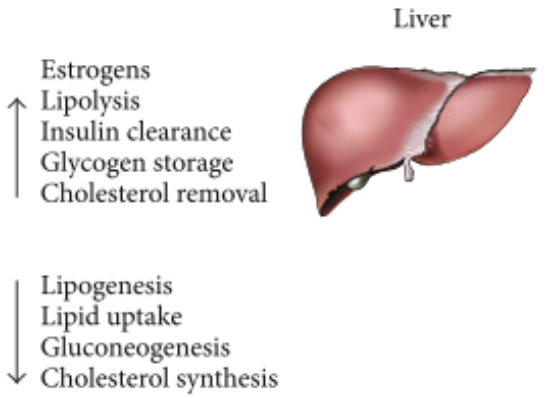


Figure 9: Metabolic effects of oestrogens on regulation of lipid, glucose, and cholesterol in the liver. Modified from Shen & Shi.,2015[100].

The great majority of the above mentioned studies were conducted using total knockout mice and the main problem associated with the use of this mouse model or also oestrogens deficient models, like ArKO or OVX mice, is the impossibility to study and unravel the role of each ER in a physiological context. In order to address this question our laboratory created the ERE-Luc mouse. Indeed, the design first and then the use of this transgenic mouse expressing the firefly luciferase gene under the control of ERE sequence, allowed us to demonstrate that ER transcriptional activity is high in liver and that its activity, looking at female mice in different phases of the estrous cycle, is strictly correlated with all reproductive organs, and this is not true for all other oestrogen target organs[34]. In addition, another work performed in our laboratory, clearly demonstrated that hepatic oestrogen receptor transcriptional activity is strongly associated with food intake[118] and this observation led to the hypothesis that liver is a crucial peripheral organ and may act as the principal coordinator between reproductive functions and energy metabolism.
This hypothesis was confirmed in a study conducted by Della Torre *et al.*,[119] where it was demonstrated that caloric restriction is able to induce a decreased hepatic ERα activity, associated with the disruption of the estrous cycle, while the administration of dietary amino acids (AAs) can prevent this effect, promoting ERα activity in the liver, inducing liver IGF-1 synthesis and enabling estrous cycle progression[119].

All together these studies allowed us to conclude that hepatic ERα acts as a sensor of nutrients availability and that it is able to block estrous cycle progression, and thus reproduction, in case of nutrients scarcity. Using the ERE-Luc mouse, we showed that ERα has an oscillatory pattern of transcriptional activity; this oscillatory pattern recurs every 4-5 day, as the estrous cycle, but it is not synchronous all over the body, apart from liver and reproductive tissues. Interestingly, in OVX mice the oscillatory pattern is maintained while the amplitude is reduced, ending up in an altered synchronicity, always except for reproductive and hepatic tissues[119].

These oscillations of ERα activity play a pivotal role in the regulation of female energy metabolism: indeed, in a study published by our group last year, we demonstrated that hepatic ERα is indispensable, at proestrus, for the synthesis of HDL able to elicit cholesterol efflux from macrophages and we proposed that these oscillations are necessary for the clearance of the excess of cholesterol, a mechanism which, probably, was selected during evolution to ensure that the excess of cholesterol synthetized under the pressure of the reproductive system could be re-utilized efficiently[120].

Hepatic ERα acts as a sensor able to integrate and translate the changes associated with the different reproductive phases, such as puberty, pregnancy and lactation, with energy metabolism, specifically by modulating the expression of genes involved in lipid and cholesterol metabolism. We found out the mechanism behind these events: a tight cross coupling between hepatic ERα and liver X receptor α (LXRα), one of the major hepatic nutritional sensors and transcriptional modulators of lipid and carbohydrate metabolism[121,122].

With the onset of menopause, this tightly coordinated sequence of events is disturbed, enabling the formation of unhealthy fat deposits in both liver and periphery.

Indeed, the deregulation of cholesterol metabolism is strongly associated with the severity of fatty liver diseases such as NAFLD[123] and it is well known that during menopause there is an increased prevalence of fatty liver diseases and the associated pathologies[124].

4.5 Energy metabolism and fertility

The reproductive system is extremely sensitive to influences coming from the environment[125] and the majority of animals adjust their reproductive pattern in order to increase and maximize the survival chances of their offspring. One of the most common strategies adopted is the timing of conception according to photoperiod and/or rainfall, which usually ensures that birth takes place in a favorable season for food and climatic conditions[126].

Reproduction needs greater energy expenditure in females than in males: the nourishing of the offspring during pregnancy and lactation and its subsequent raising are the biggest expense of energy for a female mammal during her lifetime and therefore the female reproductive system is substantially more sensitive to any perturbation than the male.

As a result, the female reproductive axis activity is influenced by the metabolic environment of the organism, both at the whole-body and cellular level. The central regulation of metabolism, via the interplay between hormones and several signals coming from specific neurons in the hypothalamus, influences both the amount and type of metabolic substrates available to peripheral organs, including the liver[127].

As expected there are different consequences of metabolic disturbances on reproductive physiology. For example, in anorexia nervosa (AN), the associated caloric restriction results in the central suppression of the female reproductive axis while, on the contrary, the caloric excesses linked to diabetes and obesity impairs reproduction at oocyte level and in early embryonic development[127].

In addition, women with ovarian dysfunction pathologies, such as polycystic ovarian syndrome (PCOS) and Turner syndrome, are more likely to develop metabolic disorders[128,129] and, physiologically, with the onset of menopause and so the cessation of ovarian functions, there is an increased risk for women to develop metabolic and cardiovascular disorders[130].

This strong link between energy balance and reproduction find its explanation in the central principle of biology, the synthetic theory of evolution. Indeed, those regulatory mechanisms, which control energy intake, storage and expenditure, exist because they are to a certain extent heritable, allowing animals to survive until reproductive maturity, and conferring also a reproductive advantage[131].

So, throughout evolution, the mechanisms, which ensure energy storage in case of food abundance and a strict control over fertility in a nutrient poor environment, have been developed and conserved. Indeed, reproduction in case of food scarcity would lead to the competition for food between the mother and the offspring and this, at the end, might result in the extinction of the species[63].

In oviparous species, the most important yolk proteins are vitellogenins, a large glycoproteins family that provides nutrients, such as amino acids, carbohydrates, phosphates and sulphates, but also lipids and hormones to the embryo[63].

These glycoprotein have also a key role in fat storage and mobilization and are synthetized in metabolic organs that are functionally comparable to liver[132] and interestingly, the mechanisms regulating vitellogenins synthesis and vitellogenins themselves are well conserved across all oviparous species, from invertebrates to vertebrates[133].

Several stimuli from local sources and the CNS, such as insulin-like peptides[134], amino acids and nutritional factors, are integrated in tissues which resemble liver[135], that control vitellogenins production in concert with gonadal hormones.

Looking at mammals, it seems that oestrogen-regulated synthesis of apolipoproteins, which occurs in liver[136], plays a role in the maintenance of reproductive capacity, since defective hepatic production of VLDL leads to female sterility[137]. Additionally, the intake of amino acids can regulate fertility, as the hepatic synthesis of insulin growth factor-1 (IGF-1), essential for the reproductive cycle, is regulated by amino acids-dependent activation of hepatic ERα[119].

The mammalian peripheral tissues communicate nutritional status to the CNS, specifically in the brain stem and arcuate nucleus, which, together with other hypothalamic nuclei, integrate and product the specific efferent signals; this central regulation of energy homeostasis and reproduction took precedence, in mammals, over the more ancestral mechanisms of fertility control[63].

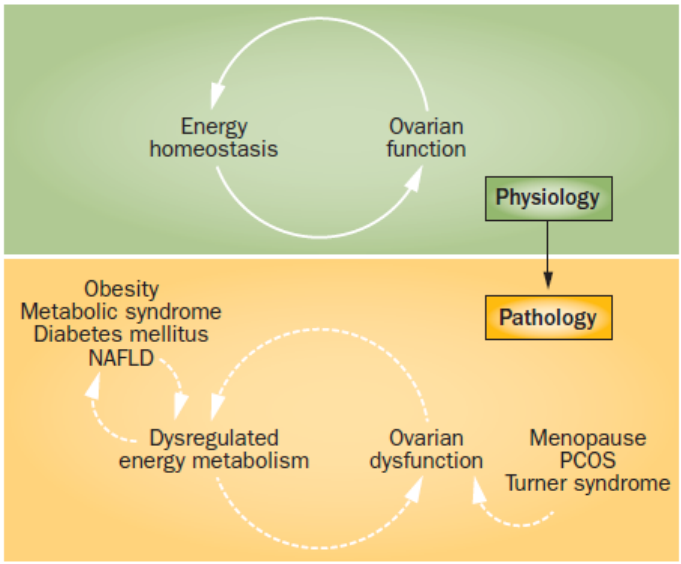


Figure 10: Reciprocal regulation of energy metabolism and reproduction. Physiologically, energy metabolism and reproduction are strict controlled to maintain a metabolic status finely tuned to reproductive needs. Alterations of ovarian functions change these metabolic pathways and might lead to obesity, metabolic syndrome, diabetes mellitus or NAFLD. Also changes in energy metabolism might impair reproductive activity. Modified from Della Torre *et al.*, 2014[63].

We have to take into account that in placental mammals, the nutritional load associated with the development and growth of the offspring has been transferred to the mother and so, the mechanisms regulating the strict association between energy availability and reproduction have been adapted to take into account the different energy demands of each stage of the reproductive cycle[138]. It is not hard to believe that the need for a reciprocal control of both energy homeostasis and reproduction might have promoted the evolutionary selection of ERα and ERβ as the link between these two functions. Indeed, these two receptors are sensory and regulatory effectors widely expressed in mammalian tissues[30], which can be activated by nutrients, nutritional signalling molecules and hormones and can regulate the expression of several genes[138].

5. Hepatic sexual dimorphism

The notion of 'liver sexuality' dates back to the observation, made in the 1930s, that female rats only required half the dose of amobarbital to be anesthetized compared with males while the first evidence of a sex-related difference in rat hepatic steroid metabolism was published in 1953 in a study conducted by Hübener and Amelung[139].

5.1 GH: a determinant of liver sex

Growth hormone (GH) is synthetized in the CNS pituitary glands and its secretion from the pituitary is controlled by neuroendocrine factors, which regulate pituitary GH secretion in a sexually dimorphic manner in different species, including rats, mice and humans. Indeed, adult male rats display a highly pulsatile GH signalling while in females the secretion of GH is continuous[140]. This different adult pattern, observed in GH secretion and in the expression of its receptor (GHR), is set during the neonatal period by exposure to gonadal steroids and is a major factor in settling and maintaining sexual dimorphism in the transcription of hepatic genes involved in endo- and xenobiotic metabolism and also in relevant metabolic pathways such as metabolism of lipids, bile acids, steroids and drugs[141]. Most of these hepatic sex differences are explained by the female specific GH secretion pattern, through the induction of female-predominant transcripts and suppression of male-predominant[142].

Oestradiol may indirectly affect the expression of GHR, by increasing or feminizing GH levels and so, while the sexual dimorphism in GH expression is driven by testosterone, the one related to GHR seems to be indirectly under the control of oestrogens[143].

The GH secretion pattern is especially pronounced in rats[144], while in mouse is less marked despite evidences of sex differences in hepatic gene expression[145,146], and in human even less[147].

A key difference between male and female GH profiles, both in rats and mice, is the sustained interpulse interval (Figure 11), characteristic of adult males, which is required for the expression of male-specific liver enzymes, such as cytochrome P450 (CYP) 2C11[148], and can also reflects the need to reset a GH-activated intracellular signalling pathway, such as the activation of signal transducer and activator of transcription 5b (STAT5b)[149].

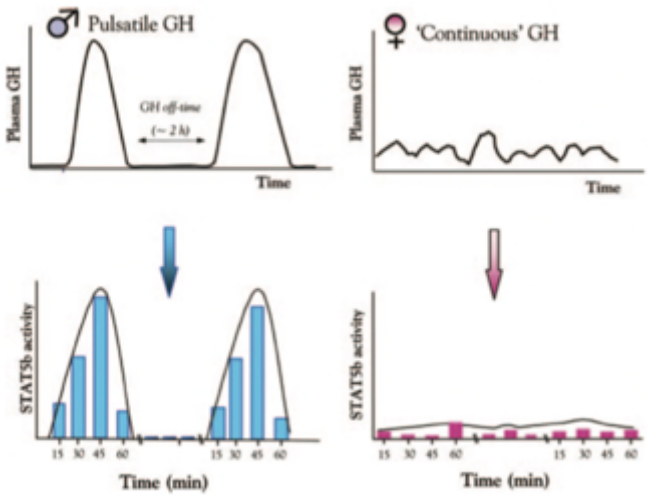


Figure 11: Activation of STAT5b by sex-specific plasma GH patterns. Modified from Waxman & O'Connor.,2006[149]

STAT5b is a signal transducer, which mediates many of the transcriptional responses of GH and some other hormones and cytokines. GH induces phosphorylation of STAT5b on Tyr 699, allowing the translocation of STAT5b from the cytosol to the nucleus, the DNA binding and then the transcriptional activity. This reaction of tyrosine phosphorylation is much more efficient in livers of adult male rats, stimulated by intermittent GH pulses, than in livers of female rats, which are exposed to plasma GH in a more continuous manner (Figure 11). Therefore, high levels of the nuclear and tyrosine-phosphorylated STAT5b protein can generally be found in males but not females rat liver nuclear extracts[150].

Initial studies on STAT5b null mice[151,152] revealed its essential role in sexually dimorphic liver gene expression. Indeed, in STAT5b deficient male mice approximately 90% of male-specific liver genes[153] are decreased, the pubertal and adult male body growth pattern is lost and some female-specific liver proteins are increased to the levels of normal adult females[151]. Interestingly, the surgical disruption of the GH axis causes, in rats, an impaired hepatic expression of some nuclear receptors including the oestrogen receptor alpha (ERα, NR3A1)[143].

As discussed here and in the previous sections, GH and oestradiol are key regulators of body growth and composition, metabolism and sex dimorphism (Figure 12). The signalling pathways of both GH and oestradiol play a pivotal role in liver physiopathology in both females and males and indeed there are several interactions between oestradiol and GH-regulated endocrine, metabolic and sex-differentiated functions in liver.

Oestrogens act at two different levels: *i)* the secretion of GH from the pituitary and *ii)* the regulation of GHR-JAK2-STAT5b signalling pathway highlighting, once again, the relevance of the interplay between these two players.

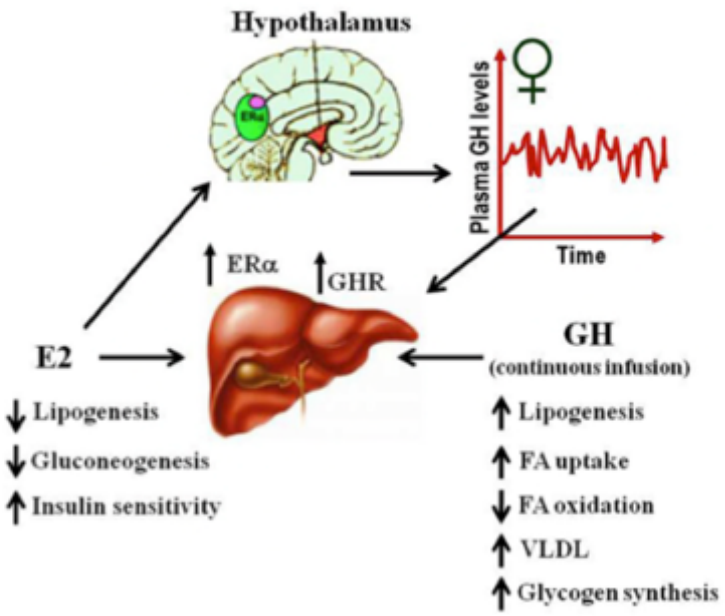


Figure 12: Physiological effects of oestrogens and GH on lipid and glucose metabolism in liver. Modified from Fernandez-Perez *et al.*, 2013[142].

The biological significance of oestrogens and GH-mediated regulation of hepatic metabolism, according to Mode & Gustaffson[139], might be linked to pregnancy. Indeed, pregnancy is characterized by high levels of circulating steroid hormones, which have to be metabolized and excreted requiring, for this reason, high levels of specific steroid metabolizing enzymes. In female rats this need is filled with the female-specific secretion pattern of GH, the master regulator while in women the normal GH secretion from the pituitary is replaced by a high and continuous levels of GH secreted by the placenta into the maternal compartment[154,155]. This demonstrates that, during pregnancy, the GH pattern is not so different between rats and men.

5.2 Sex differences in liver diseases

Steatosis is a common hallmark of different hepatic diseases, is characterized by an abnormal accumulation of lipids in the hepatocytes and displays a sexual dimorphism. Since some nuclear receptors (NRs), such as PPARs are master regulator of genes involved in lipid metabolism, their genetic deletion affects lipid homeostasis and, consequently, hepatic steatosis. In PPARα KO mice, hepatic abnormalities, such as fat accumulation and hepatomegaly, were found in males, but not females[156], which have a higher capacity to secrete triglycerides in VLDL compared to male mice[157].

It has also been demonstrated that ArKO male mice, which lack the intrinsic ability to synthetize oestrogens, spontaneously develop hepatic steatosis because of impaired

hepatocellular fatty acids β-oxidation and, interestingly, the replacement of oestradiol reduces the disease and restores both the mitochondrial and peroxisomal β-oxidation to normal levels[113,158]. In line with these findings, tamoxifen, a potent oestrogen receptor antagonist, is able to cause severe forms of steatosis that progress towards nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver disease (NAFLD)[159].

NAFLD is a chronic liver disease characterized by excessive lipid deposition within lipid droplets (LDs) in hepatocytes, in the absence of relevant alcohol consumption, hepatitis or other liver diseases[160] and its spectrum ranges from simple steatosis, through NASH and cirrhosis, to liver failure and hepatocellular carcinoma. Alterations in lipid metabolism, like impaired fatty acids oxidation, synthesis of lipotoxic lipid intermediates, increased levels of reactive oxygen species (ROS) and then the induction of a proinflammatory response further contribute to the progression of NAFLD[161,162].

Currently, it is the leading cause of chronic liver disease in developed countries, affecting ~25–30% of the US population and it is rapidly becoming the most common disease worldwide because of the increasing prevalence of obesity and its associated complications, which are the main risk factors for NAFLD development.

In a recent report, Younossi et al., model the projected clinical and economic burden of NAFLD in the USA and four European Union countries (France, UK, Germany and Italy): the economic burden is enormous, US$103 billion in USA and about €35 billion for the European countries annually[163].

Oestrogens also influence the incidence and progression of NAFLD. The incidence of this disorder displays a clear sexual dimorphism: in men, it is 2.0-3.5 fold higher than fertile age women[164,165] but after menopause, women have the same incidence than men.

However, HRT is able to reduce the risk of hepatic steatosis and the prevalence of NAFLD in post-menopausal women[166].

It is still non completely elucidated the exact etiology of NAFLD in postmenopausal women but it is well known that estrogen deficiency contributes to the loss of inhibition of *de novo* fatty acid synthesis, decreases VLDL-mediated lipids export and reduces hepatic fatty acid oxidation[138,167]. These observations are supported by studies on OVX rodents, in which it has been demonstrated that oestrogen administration prevents the deposition of lipids in the liver by inhibiting the expression of genes involved in lipogenesis and facilitating VLDL-mediated export of lipids from the liver and β-oxidation.

In a recent report, Wahli suggested that male sensitivity to liver disease depends on an imbalance in the nuclear receptors (NR) network as shown in Figure 13; thus, in males, the NR interactome is less stable and more susceptible to perturbations than that of females and this instability results in higher incidence of liver steatosis and cancer[143]. Interestingly, also the crosstalk between different NRs is sex dimorphic.

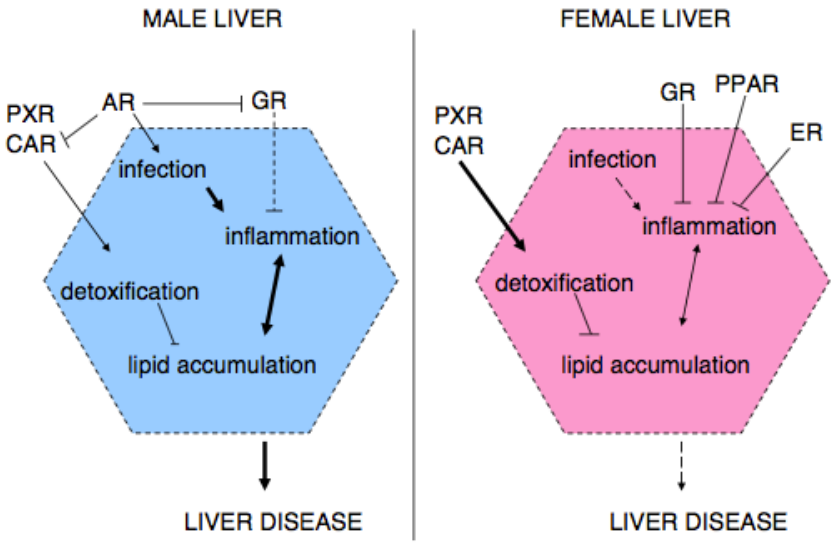


Figure 13: Nuclear receptors sex-dimorphic actions contribute to female resiliency to liver disease. The stimulatory (arrows) and inhibitory (blunt ends) signalling (dashed line=weak, heavy line=strong interaction) supports the hypothesis that female liver is more resilient to diseases due to a more robust nuclear receptor network than that of males. AR (Androgen receptor), CAR (Constitutive androstan receptor), ER (Oestrogen receptor), GR (Glucocorticoid receptor), PPAR (Peroxisome proliferator-activated receptor), PXR (Pregnane X receptor). Modified from Rando & Wahli.,2011[143].

AIM OF THE STUDY

In female mammals, oestrogens and liver ERα are able to regulate the hepatic expression of metabolic genes and ERα, the main isoform expressed in liver, acts as a nutrient sensor able to adapt energy metabolism according to the specific phase of the estrous cycle, as demonstrated in previous work of our laboratory. Indeed, in cycling females, it has been observed[138] that the expression of genes involved in lipid and carbohydrate metabolism changes significantly across the estrous cycle: it is minimum at proetrus, which is characterized by high levels of circulating oestrogens, and maximum at metestrus which, instead, is characterized by low levels of circulating oestrogens. This regulation is oestrogen and ERα-mediated since it is lost in both ovariectomized (OVX) and LERKO (*Liver ERα Knockout*) female mice.

We further explored the extent of the involvement of hepatic ERα in lipid metabolism in LERKO mice[168] and we demonstrated that in female, liver ERα exerts a major control on lipid synthesis.

At this point we wondered if this tight modulation of energy metabolism exerted by oestrogens and hepatic ERα is specific to females or if it is present also in males. We know that in males the expression of hepatic ERα is significantly lower than in females but it has been demonstrated, using the ERE-Luc mouse model, that ER transcriptional activity is very high in the hepatic area[34].

Thus, the aim of this study was to compare female and male hepatic metabolism and to unravel the role, if any, played by hepatic ERα in the regulation of energy metabolism in male mice. We decided to perform this study in basal condition and also in a stressed metabolic status, caused by the administration of an unbalanced diet, which allows us to evaluate also how the nutritional challenge could affect ERα activity in female liver.

To this purpose we analysed, using different techniques, key metabolic and inflammatory pathways in female and male control (SYN) or LERKO mice, in order to single out the activity of hepatic ERα, fed with control diet or high fat diet.

This comprehensive study allowed us to identify the sex specific role of hepatic ERα in the regulation of liver metabolism and inflammation in normal or stressed metabolic conditions, and, interestingly, to highlight its possible opposite regulatory action in the two sexes.

We firmly believe that this is very relevant from both a pharmacological and a pathophysiological point of view. Indeed, the identification of a sex dependent regulatory action of ERα on metabolism would not only help in the optimal design of new HRTs, able to mimic the beneficial effect of oestrogens and ERα on metabolic parameters, but would also help to understand whether ERα could be considered as a target of innovative sex-specific therapies for metabolic disorders.

MATERIAL & METHODS

1.1 Animals

To single out the activity of estrogens and cognate ERα in the liver I used a mouse model, generated by our laboratory, in which the ERα had been selectively deleted in the liver, the LERKO mouse (*Liver ERα Knockout*). LERKO was obtained crossing a mouse mutant in which the ERα had been floxed (SYN) with the Albumin-CRE mouse, in which the CRE recombinase is expressed selectively in the liver.

Floxed (ER$\alpha^{FLOX/FLOX}$)[169] and LERKO (Liver ERα knockout)[119] mice were housed in plastic cages with hardwood chips bedding. The animal room was maintained within a temperature range of 22– 25°C, a relative humidity of 50%±10% and there was a cycle of 12 hours light/dark (lights on, 07:00 a.m.).

Unless otherwise stated, mice were 8-9 months of age at euthanasia. Vaginal smears were performed at 9:00–10:00 a.m. Mice were euthanized after 6 hours of fasting between 2:00 and 5:00 p.m.to avoid any possible confounding effect due to the circadian rhythm or feeding status[170] and female mice were euthanized in the phase of metestrus. After euthanasia, tissues were rapidly collected, snap frozen on dry ice and stored at -80°C.

Body weight and food intake were measured once a week and the food intake was calculated as the amount of food consumed by the single cage averaged by the number of mice and the number of days, then the Kcal consumed were calculated for each diet. The control diet (ND) consisted of 4.3% fat, 67.3% carbohydrate, and 19.2% protein with 10% Kcal derived from fat, 70% Kcal derived from carbohydrates and 20% Kcal derived from proteins (D12450B, Research Diets, NJ, USA). The HFD used in these experiments consisted of 34.9% fat, 26.3% carbohydrate, and 26.2% protein with 60% of Kcal derived from fat, 20% Kcal from carbohydrates and 20% Kcal from proteins (D12492, Research Diets, NJ, USA).

Mice had free access to the diets and to filtered water and they were given ND and HFD from 2 to 8 months of age.

They were euthanized by anaesthesia overdosing and cervical dislocation.

All animal experimentations were performed in accordance with the ethical standards, the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the US National Institute of Health, and in accordance with the European Guidelines for Animal Care and Use of Experimental Animals. Experiments were approved by the Italian Ministry of Research and University and by the Ethical Committee of the University.

1.2 Vaginal smears

The phase of the estrous cycle was established by vaginal smears using about 20 µL of fluid collected by water vaginal flush smeared on a glass microscope slide. The smear was air-dried and stained using May-Grünwald-Giemsa method (MGG Quick Stain Kit, Bio-Optica) according to the manufacturer's protocol. Cytological assessment was done with a Microscope Axioscop2 mot plus (Zeiss, Germany) at the magnification of 200x.

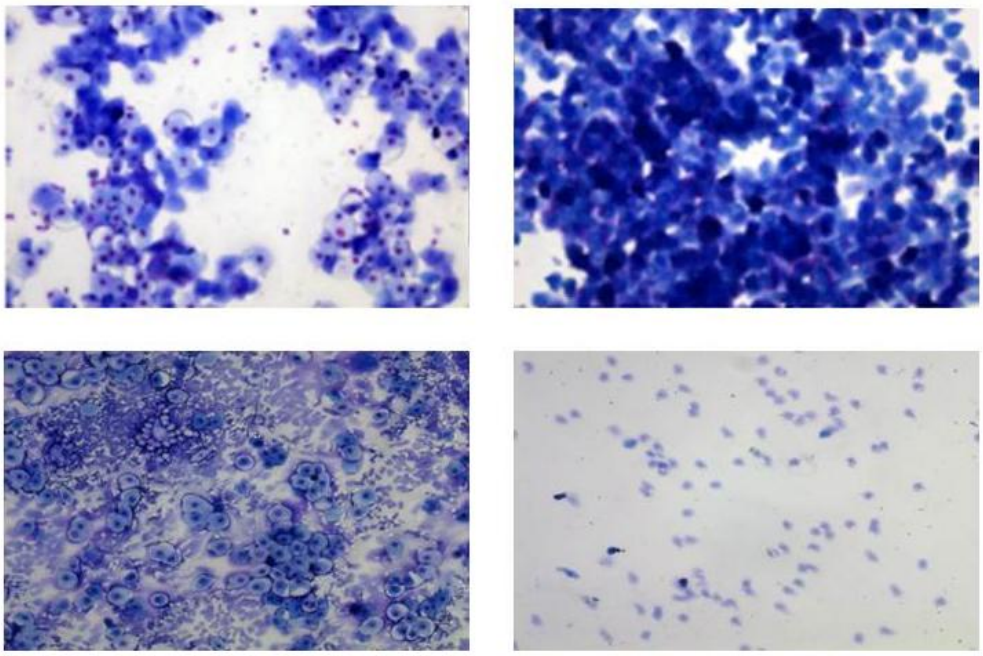


Figure 14: Histological images of the different phases of the estrous cycle. Cytological assessment of proestrus (top, left), estrus (top, right), metestrus (bottom, left) and diestrus (bottom, right).

1.3 Anesthesia

Mice were anesthetized with subcutaneous injection of 60 µL of a ketamine-xylazine water solution: 78% ketamine (Ketavet 50 mg/mL, Intervet) and 15% xylazine (Rompun 20 mg/mL, Bayer). This amount corresponds to a dose of 93.6 mg/kg of ketamine and 7.2 mg/kg of xylazine.

1.4 Liver histology

Biopses of mouse liver (from the left lobe) were fixed in 10% neutral formalin solution (Sigma-Aldrich) overnight at 4°C, cryopreserved in a 30% (w/v) sucrose solution for 24 hours at 4°C and then stored at -80°C. 7μm-thick liver sections were cut with a refrigerated microtome (Leica), collected on slides and stored at -80°C until staining.
Hematoxylin–eosin (H&E) staining was performed on frozen slides with Mayer Hematoxylin (Bio-Optica) for 1 min and, after water washing, with 1% Eosin Aqueous Solution (Bio-Optica) for 4 minutes. Oil Red O staining was performed as previously described[138] while Masson's Trichrome staining was performed by using the Accustain Trichrome stain kit (Masson; Sigma-Aldrich). After specific staining, slides were cleared in xylene and cover slipped with xylene-based mounting medium (Eukitt, Bio-Optica). Liver sections were evaluated in blind under a light microscope. Images of the stained sections were captured using Microscope Axioscop2 mot plus (Zeiss).

1.5 Western Blot Analysis

Samples of frozen mouse liver were homogenized in ice-cold buffer (20 mM HEPES, 5mM MgCl2, 420 mM NaCl, 0.1 mM EDTA, 20% Glycerol) containing protease and phosphatase inhibitors according to the manufacturer's protocols (Phosphatase and Protease Inhibitor Mini Tablets, Pierce). After 3 repeated cycles of freezing and thawing, the homogenate was centrifuged at 16100g for 15 min at 4°C and the supernatant was collected in a new tube. After appropriate quantitative analysis (Bradford assay, Pierce), equal amounts of protein samples (25 μg) were suspended in Laemmli sample buffer and separated in an 8-10% SDS polyacrylamide gel system (Biorad). After transfer, nitrocellulose membranes were incubated with specific antibodies overnight at 4°C and then with the secondary antibody conjugated with peroxidase for 1 h, at room temperature. The primary antibodies used were: anti-ERα and anti-PPARα (Santa Cruz), anti-LXRα (R&D systems), anti-β-actin (Sigma). Immunoreactivity was detected with an ECL Western Blotting Analysis System (Amersham) and acquired and analyzed using an Odissey Fc Imaging system and the Image StudioTM software (LiCorBiosciences).

1.6 RNA preparation and Real-Time PCR Gene Expression Analysis

Livers were homogenized in TRIzol® (Life Technologies, Carlsbad, CA) 6% (w/v) using a TissueLyser (QIAGEN, Milan, Italy). Total hepatic RNA was purified using RNeasy Mini Kit (QIAGEN, Milan, Italy), following the manufacturer's instructions and then was reverse transcribed to cDNA according to this procedure: 1 μg RNA was denatured at 75°C for 5 min in the presence of 1.5 μg of random primers (Promega) in 15 μl final volume. Deoxynucleotide triphosphate (GE Healthcare) and Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT, Promega) were added at 0.5 mM and 8 U/μl final concentrations respectively, in a final volume of 25 μl. The reverse transcriptase reaction was performed at 37°C for 1 h and the enzyme was inactivated at 75°C for 5 min.
Real-Time PCR experiments on liver were performed using TaqMan technology (Thermo Fisher) or SYBR Select Master Mix (Thermo Fisher) and we used 36b4 (Ribosomal protein, large, P0) as the reference for all the assays.
The reactions were carried out according the manufacturer's protocol using 7900HT standard real-time PCR system (Applied Biosystems) and the thermal profiles used were: 2 min at 50°C; 10 min at 95°C; 40 cycles (15 sec at 95°C, 1 min at 60°C) for TaqMan technology and 2 min 50°C, 2 min 95°C, 40 cycles (15 sec at 95°C, 30 sec 55-60°C, 1 min 72°C) for SYBR green mix. Data were analyzed using the Sequence Detection System Software v2.3 (Applied Biosystems) and the 2-ΔΔCt method[171].

The pre-made TaqMan Gene Expression assays, all from Thermo Fisher, used for gene expression analysis were: ERα (Mm00433147_m1), CPT1α (Mm01231183_m1), HADHα (Mm00805228_m1), PLIN2 (Mm00475794_m1), ACADL (Mm00599660_m1), CYP2E1 (Mm00491127_m1), FABP4 (Mm00445878_m1), PCSK9 (Mm01263610_m1), LCAT (Mm01247340_m1), ACAT2 (Mm00782408_s1), SR-B1 (Mm00450234_m1), MTTP (Mm00435015_m1), HMGCR (Mm01282499_m1), ABCG1 (Mm00437390_m1), IDOL (Mm00464140_m1), LDLR (Mm01177349_m1), ACOX1 (Mm01246831_m1), ABCG5 (Mm00446241_m1), ABCA1 (Mm00442646_m1), CYP7a1 (Mm00484150_m1), CYP27a1 (Mm00470430_m1), SHP (Mm00442278_m1), TNFα (Mm00443258_m1), Il-1β (Mm00434228_m1), Il-12β (Mm00434174_m1), Il-10 (Mm00439614_m1), CCL2 (Mm00441242_m1), CXCL2 (Mm00436450_m1), CCR2 (Mm00438270_m1), SQSTM1 (Mm00448091_m1), VIM (Mm01333430_m1), SERPINE (Mm00435860_m1), AIF1 (Mm00479862_g1), IL-6 (Mm00446190_m1).

The primers used for the analysis with SYBR green chemistry are reported in Table 2.

Gene	Forward sequence	Reverse sequence
FASN	5'-CCTCTGATCAGTGGCCTCCTC-3'	5'- GGATTCGGGAATACAAGTGGC-3'
ACLY	5'- GAAGCTGACCTTGCTGAACCC-3'	5'- CCGTAATTCGCCAGTTCATTG -3'
ELOVL6	5′- TCACCTTGTCCCAGATCACTC-3′	5′-CTGAGGTACATGAGCGAGGAC-3′
PMVK	5'- ATGGGGCTGTGATACAGACAG-3'	5'- CAAAGTTCCCAAAGTTGTCCA-3′
DHCR7	5'- ACTGTATGCTCTTGTGGGTCAG-3'	5'- TGGAGTAATGGCACCTTCTTG-3′
36B4	5′-GGCGACCTGGAAGTCCAACT-3	5′-CCATCAGCACCACGGCCTTC-3′

Table 2: Primers used in RT-PCRs analysis with SYBR green chemistry.

1.7 Statistical Analysis

Unless otherwise stated, statistical significance was assessed by one-way or two-way ANOVA using Bonferroni's multiple comparison post hoc tests that were performed with GraphPad Prism 5 (GraphPad Software).

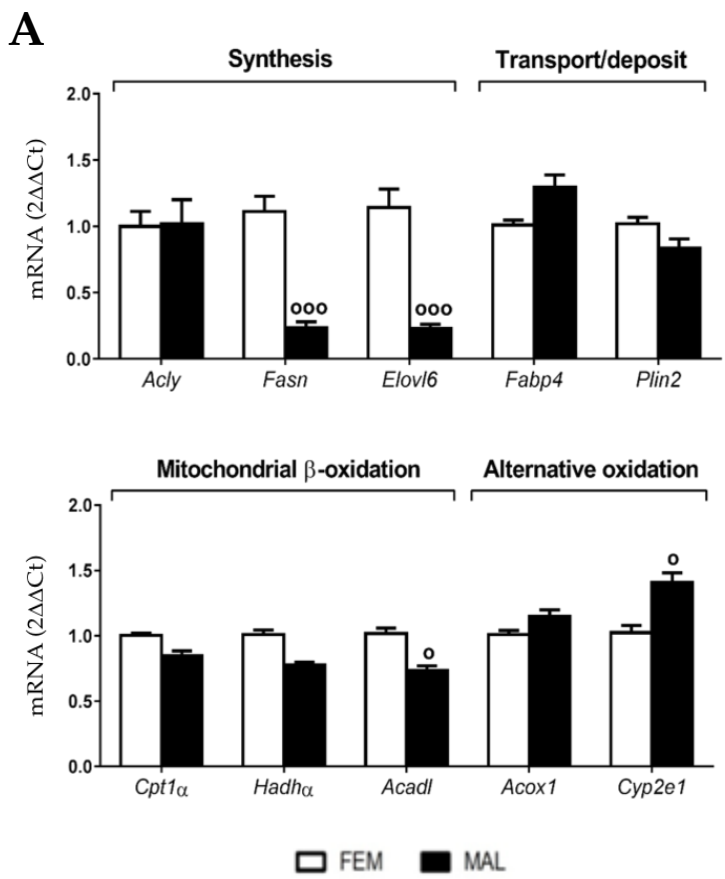
For matrix analysis, I performed a Pearson correlation of genes in pairs, on their expression values (Ct), obtained with the RT-PCR analysis. The statistical significance of the correlation was calculated using a two-tailed P value with GraphPad Prism 5 (GraphPad Software). Data has been represented using the Genesis software[172].

RESULTS

1.1 Fatty acids and cholesterol metabolism in SYN females and males

We know that female hepatic metabolism is strongly linked and regulated by the reproductive activity and, as discussed above, the expression of hepatic metabolic enzymes changes across the progression of the estrous cycle. On these premises, I decided to sacrifice the females at metestrus, in order to avoid the effects of circulating estrogens, and to compare them to intact males. Indeed, the liver of male mice does not express aromatase, therefore this organ, in males, is an unlikely target for sex hormones activity and so we did not need to orchidectomize them.

Firstly, I compared the hepatic lipid and cholesterol metabolism in both female and male control animals (SYN) fed with ND, analysing the expression of those hepatic metabolic enzymes, which are modulated in females during the progression of the estrous cycle. Looking at lipid metabolism (Figure 1A) we observed that in males there is a lower expression of enzymes involved in lipid synthesis compared to females, such as Fasn (*Fatty Acid Synthase*) and Elovl6 (*Fatty Acid Elongase 6*), enzymes involved, respectively, in fatty acids biosynthesis and elongation. We did not find major differences between females and males for the enzymes involved in fatty acids oxidation.



The analysis of the cholesterol metabolic pathways (Figure 1B) also showed differences between the two sexes. Indeed, in males there is a lower cholesterol synthesis compared to

females, as suggested by the basal lower expression of Hmgcr (*3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A Reductase*), the rate-limiting enzyme in cholesterol synthesis pathway, and Pmvk (*Phosphomevalonate Kinase*), also involved in cholesterol synthesis. On the other hand, males display a higher esterification, efflux, uptake and catabolism of cholesterol than females; indeed, in males, we can observe a significantly higher expression of Lcat (*Lecithin–Cholesterol Acyltransferase*) which is important for the esterification of cholesterol and then its removal from the circulation, and also Abcg1 (*ATP Binding Cassette Subfamily G Member 1*), which mediates the efflux of free cholesterol, and Sr-b1 (*Scavenger Receptor class B, type I*), involved in the reverse cholesterol transport (RCT) pathway, are more expressed in males than in females. Finally, there is also a significantly higher expression in males of Cyp27a1 (*Sterol 27-Hydroxylase*), which participates in the degradation of cholesterol into bile acids in both the classic and acidic pathway. Overall these data suggest that in homeostasis female and male hepatic metabolism may differ and that this sex difference involves several pathways of both lipid and cholesterol metabolism.

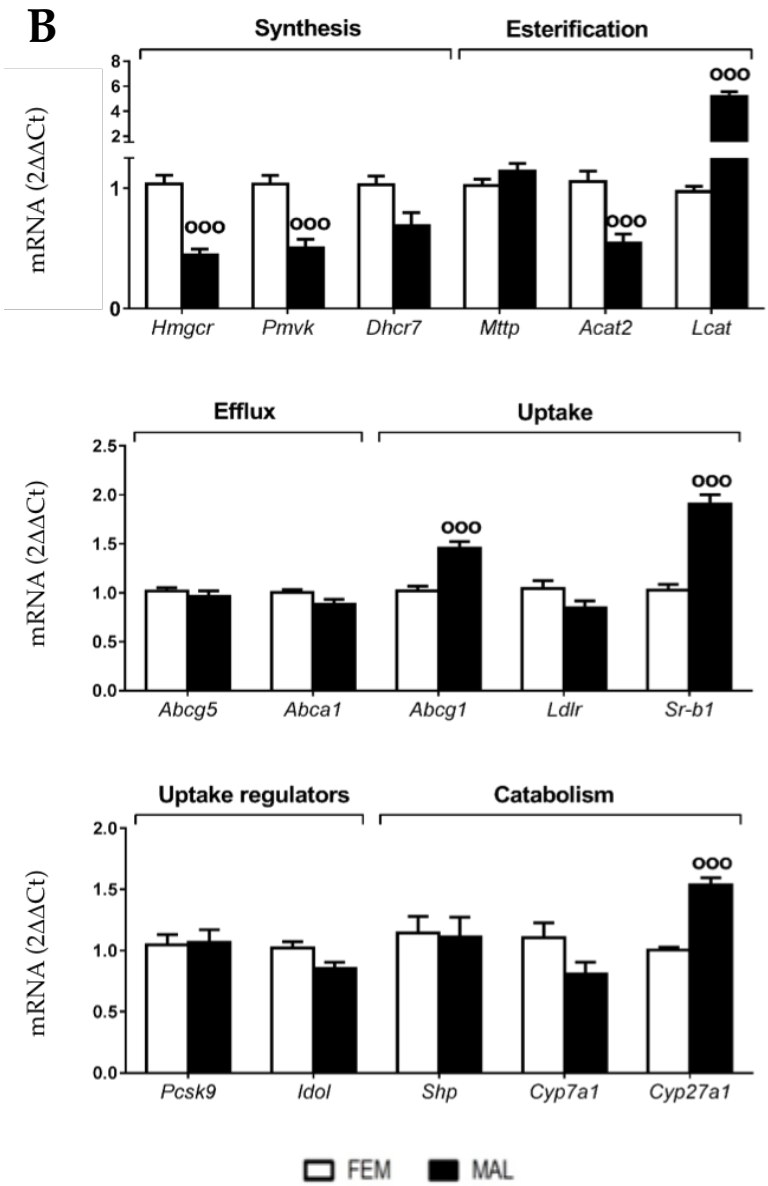


Figure 1: Hepatic expression of metabolic enzymes in SYN females and males in ND condition. Real-time PCR expression analysis of key hepatic enzymes involved in lipid (A) and cholesterol (B) metabolism on females (n=20) and males (n=14) in ND condition. Data are here represented as mean +/- SEM. °p <0.05 and °°°p <0.001 compared to females. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

1.2 HFD effects on Body Weight, Food Intake and Liver Histology in females and males

In our previous work it has been demonstrated that, in female mammals, oestrogens are able to regulate the hepatic expression of metabolic genes, especially those involved in lipid metabolism[138] and we know that, in females, an unbalanced diet enriched in fatty acids, like HFD, has detrimental effects on liver leading to hepatic fat deposition. So, at this point I decided to stress the hepatic metabolism with the administration of HFD, according to the protocol described in methods, and then evaluate the metabolic response in both female and male SYN and LERKO mice, in order to single out the activity of hepatic ERα in the modulation of this response in both physiological and stressed metabolic conditions.
Firstly, I analysed general parameters such as body weight and food intake (Figure 2) in SYN female and male mice fed with ND or HFD.

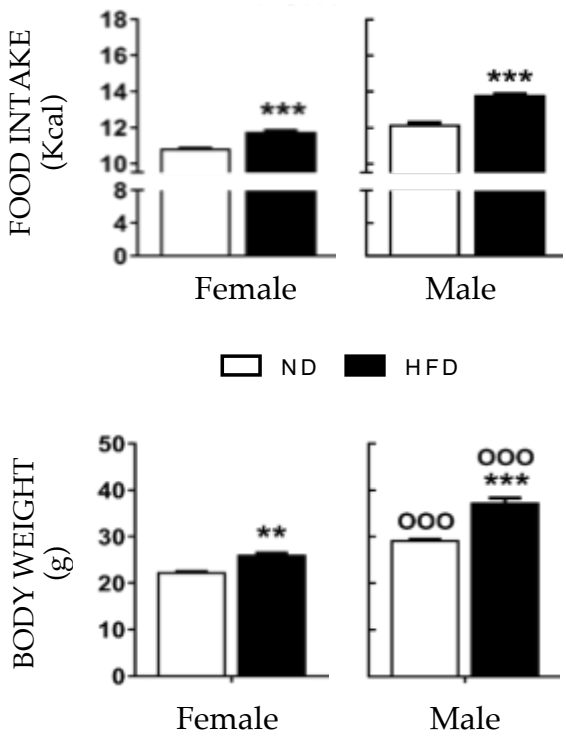


Figure 2: Effects of HFD on metabolic parameters. Food intake (top) and body weight (bottom) of SYN female and male mice fed with ND and HFD. Data are represented as mean +/- SEM. **p<0.01, ***p<0.001 versus ND, °°°p<0.001 versus females SYN ND. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test for body weight and with one-way ANOVA followed by Bonferroni post hoc test for food intake.

What emerges from this analysis is that in both females and males there is an increase in food intake, following the HFD administration, reflected by an increase in body weight after HFD. However, with regard to these two parameters, females and males behave similarly and there are no major sex differences.

At this point we focused, more specifically, on the diet effect on liver since it is known that HFD has detrimental effect on this organ[173]. Therefore, I evaluated, by a combination of immunohistochemical analysis, the hepatic morphology of female and male SYN mice fed with ND and HFD (Figure 3).

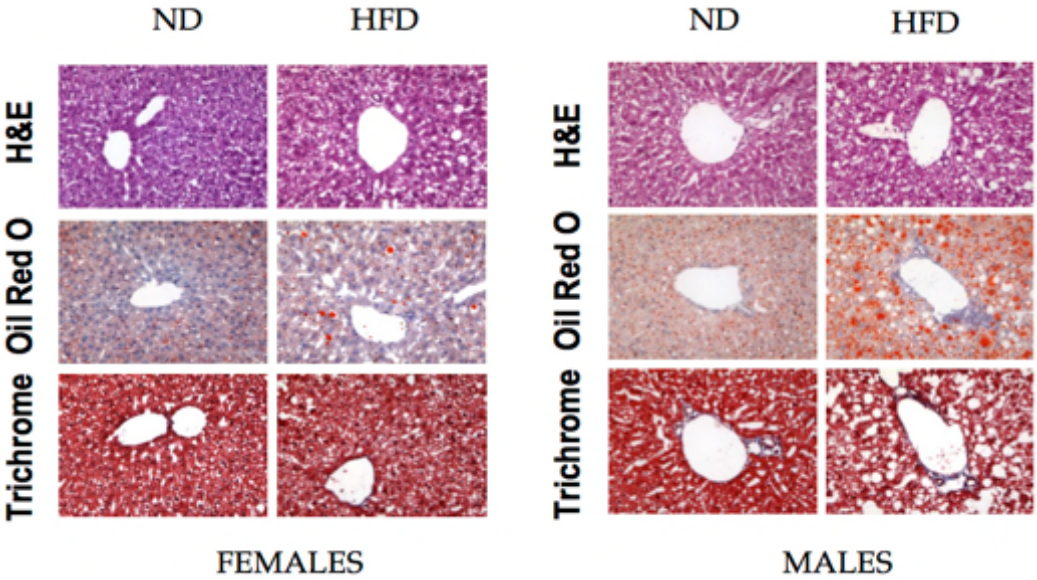


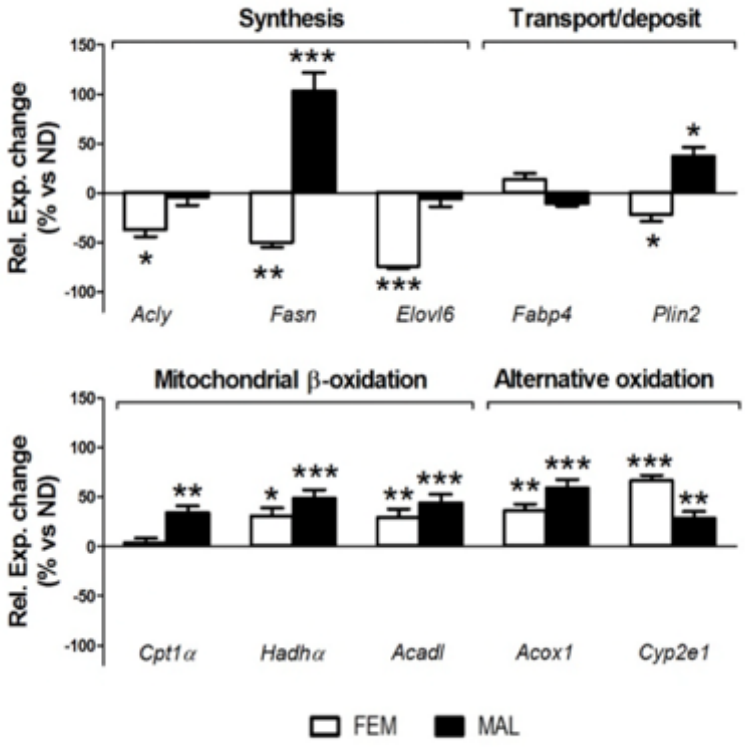
Figure 3: Effects of HFD on liver histology. Liver histology in SYN female, left, and male, right, mice. Top: Hematoxilyn & Eosin staining (H&E); Center: oil red O staining plus H&E (neutral fats are stained orange red, and the nuclei are shown in blue). Bottom: Masson's trichrome staining with aberrant collagen deposits (blue); the hepatocyte cytoplasm is red and the nuclei are dark red-black structures within cells.

The hepatic hematoxylin and eosin staining, first lane, clearly shows an augmented tissue degeneration following the administration of the HFD in both sexes, yet, on closer inspection, it becomes clear that the diet had affected males much more than females. Indeed, the Oil Red O staining, second lane, displays larger stained lipid droplets in males than in females and also the Masson's trichrome staining, third lane, reveals more collagen deposition (blue) and vacuolar degeneration in males than females. Overall, based on this analysis, we can point out that HFD has a stronger effect on male liver, which seems less able to deal with the overload of fat than female's.

1.3 Fatty acids and Cholesterol metabolism: effects of HFD in SYN and LERKO mice

In order to explain the metabolic alterations, observed in the histological analysis, induced by the diet in the two sexes, I measured by RT-PCR analysis the hepatic expression of those key metabolic enzymes involved in lipid and cholesterol metabolism, previously mentioned, following HFD administration in SYN females and males. Data are represented (Figure 4A) as percentage of increase or decrease *versus* ND, for a more immediate visual evaluation. The analysis of the two pathways, interestingly, shows differences between the two sexes in response to HFD. In particular, with regard to lipid metabolism (Figure 4A) females respond to HFD with a clear reduction in the expression of genes involved in lipid synthesis and deposit such as Acly (*ATP Citrate Lyase*), Fasn, Elovl6 and Plin2 (*Perilipin 2*) and with a slight increase in the expression of genes involved in fatty acids oxidation. On the contrary, males are unable to down-regulate lipid synthesis and transport in response to HFD like females and, indeed, in males there is a significant increase in the expression of Fasn and Plin2, while they display the same slight increase of fatty acids oxidation enzymes observed in females.

A



Since both Fasn and Plin2 displayed an interesting opposite trend in females and males, I wondered if there could be a significant difference between the two sexes in the expression of these genes in HFD condition, so I analyzed data considering females in ND as control in order to highlight these possible sex differences. However, as reported in Figure 4B, there are no differences between males and females in HFD in the expression of these two genes.

B

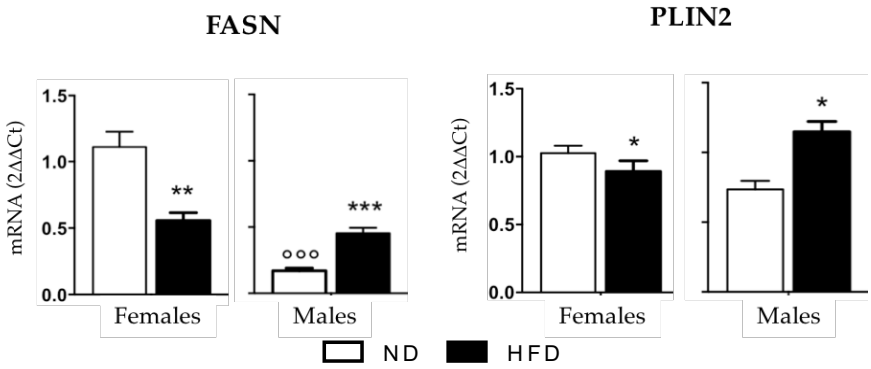


Figure 4B: Hepatic expression of FASN and PLIN2 in SYN females and males in HFD condition. Real-time PCR expression analysis of FASN and PLIN2 on female (n=20) and male (n=14) SYN mice in ND and HFD condition, females (n=21), males (n=23). Data are here represented as mean +/- SEM. *p <0.05, **p<0.01 and ***p <0.001 compared to ND; °°°p<0.001 compared to females. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

Looking at cholesterol metabolism (Figure 5), females respond to HFD with a slight increase in the expression of genes involved in cholesterol synthesis, esterification and efflux as suggested by the increase observed, respectively, in Dhcr7 (*7-Dehydrocholesterol Reductase*), Lcat (*Lecithin-Cholesterol Acyltransferase*) and Abca1 (*ATP Binding Cassette Subfamily A Member 1*). Interestingly, in females there is a significant increase in the expression of Cyp7a1 (*Cholesterol 7-alpha-Hydroxylase*), involved in cholesterol catabolism, which is the first and rate-limiting enzyme in bile acids synthesis. On the other hand, in males the administration of HFD leads to a higher increase in the expression of genes involved in cholesterol synthesis, such as Hmgcr, Pmvk, Dhcr7, compared to females and to a strong increase of Ldlr (*Low Density Lipoprotein Receptor*), which mediates the cholesterol uptake by the liver. In summary, in case of excess intake of lipids, female and male liver adopts different strategies to deal with the overload of fat. Furthermore, looking at the specific metabolic pathways modulated by the administration of HFD it seems that males respond to lipids overload ending up with a greater accumulation, since they are not able to down-regulate lipid synthesis and they have a greater cholesterol uptake by the liver: a result which reflects very well the observations made with the previous hepatic histological analysis.

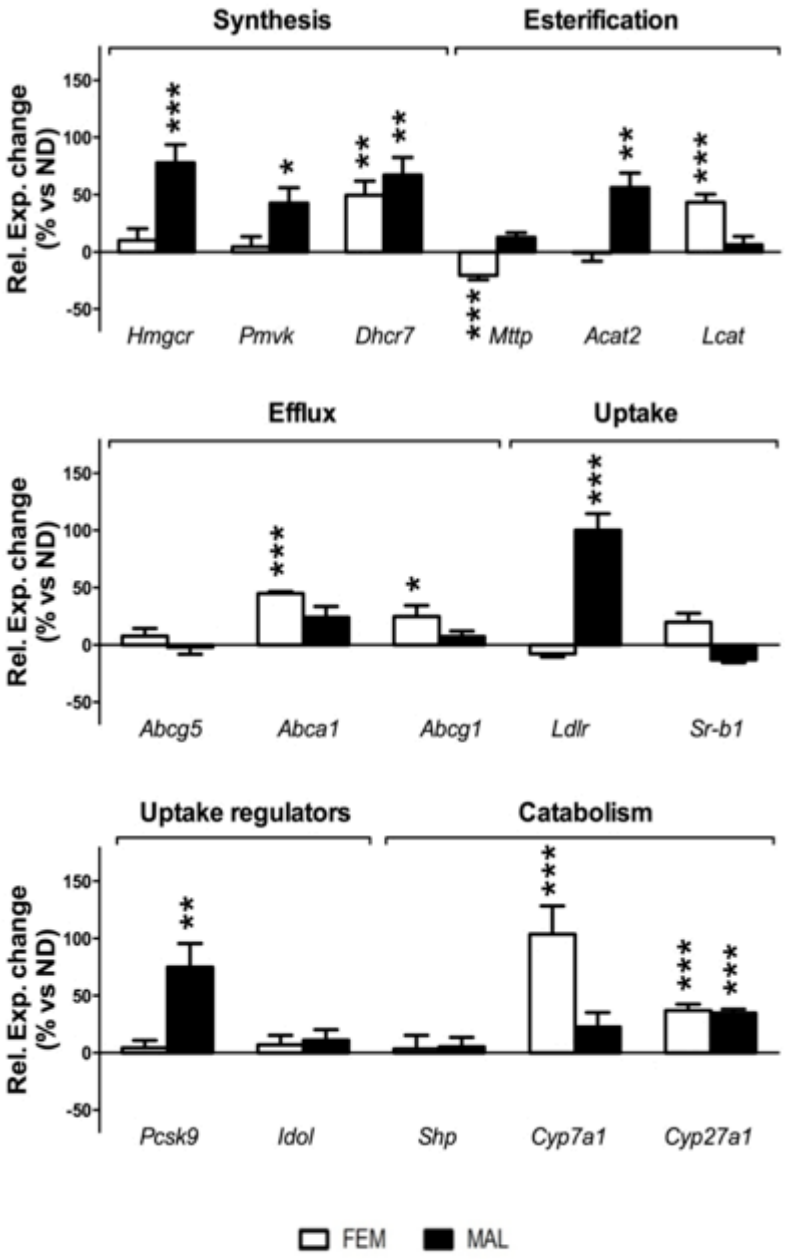


Figure 5: Hepatic expression of metabolic enzymes in SYN females and males in HFD condition. Real-time PCR expression analysis of key hepatic enzymes involved in lipid (A) and cholesterol (B) metabolism on females (n=21) and males (n=23) in HFD condition. Data are here represented as mean +/- SEM of the percentage of increase or decrease versus ND. *p <0.05, *p<0.01 and ***p <0.001 compared to ND. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

At this point, we wondered if hepatic ERα had a role in the modulation of the metabolic sex differences observed in both physiological condition and after HFD administration. To answer this question, I evaluated the metabolic profile of both female and male LERKO mice before and after HFD administration. Firstly, I looked at general parameters such as body weight and food intake (Figure 6). What emerges from this first evaluation is that in ND condition LERKO do not seem to be significantly different in body weight from SYN, even in females than in males; however, some metabolic alterations are present since there is a significant difference in food intake. With the administration of the HFD, there is in both groups, females and males, an expected and comparable increase in body weight and we have also an increase in food intake; however, the increased food intake, following the HFD, observed in LERKO is significantly higher than one in SYN. Overall, we can affirm that, with regard to these two parameters, LERKO males and females behave similarly.

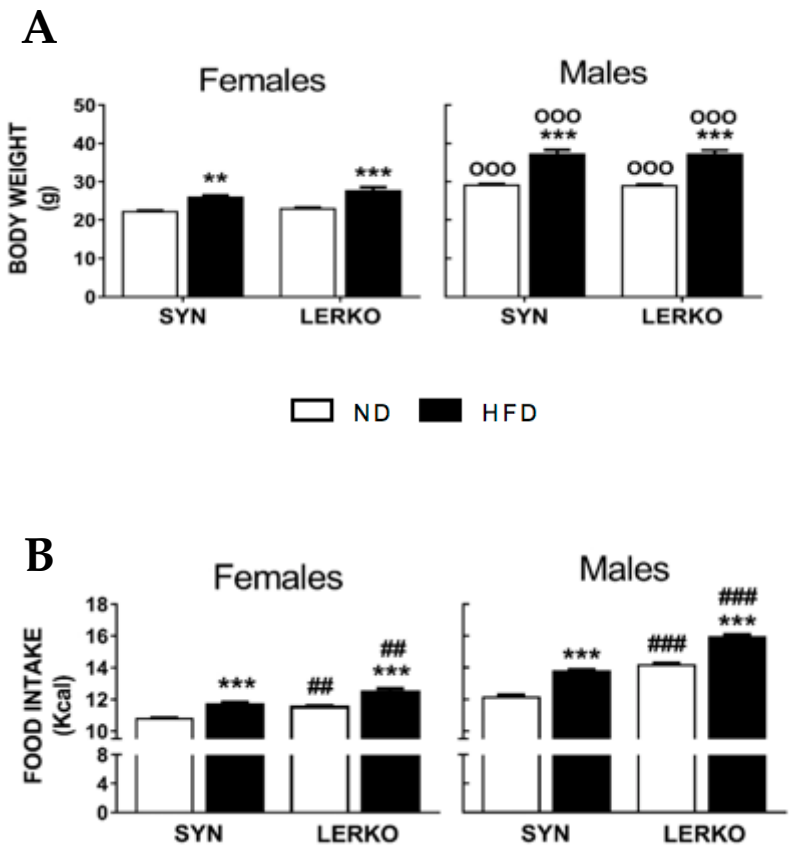


Figure 6: Effects of HFD on metabolic parameters. (A) Body weight of SYN and LERKO female and male mice, measured once a week. Data are represented as mean +/- SEM. **p<0.01, ***p<0.001 versus ND, °°°p<0.001 versus females SYN ND. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test. (B) Food intake evaluation in the same animal cohort. Data are represented as mean +/- SEM. ***p<0.001 versus ND, ##p<0.01, ###p<0.001 versus own control. P values were calculated with one-way ANOVA followed by Bonferroni post hoc test.

Moving forward with the analysis, first I evaluated the expression of the same genes seen before in LERKO female and male mice in ND condition, but I did not see differences between the two sexes in LERKO mice (data not shown), suggesting that hepatic ERα is not the main culprit of those sex differences observed in both lipid and cholesterol metabolism in ND. Then I performed the RT-PCR analysis on the same metabolic genes in LERKO mice following the HFD administration (Figure 8).

Interestingly, looking at both lipid and cholesterol key metabolic genes, in LERKO females there are not significant differences when compared to SYN, with the only exception of Hadhα (*Mitochondrial trifunctional protein, alpha subunit*), a gene involved in the mitochondrial beta-oxidation of long chain fatty acids, which is significantly lower in LERKO females compared to SYN, in HFD. On the other hand, in males, which express very low levels of hepatic ERα compared to females in the different phases of the estrous cycle, as reported in Figure 7, its selective ablation in liver, combined with the administration of HFD, has several effects on both lipid and cholesterol metabolism.

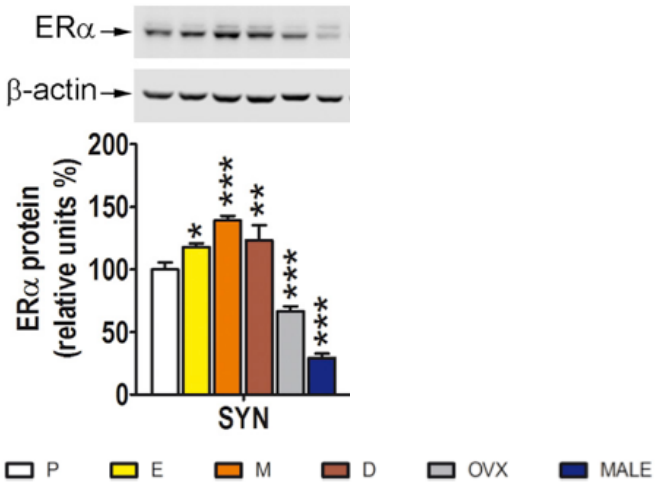
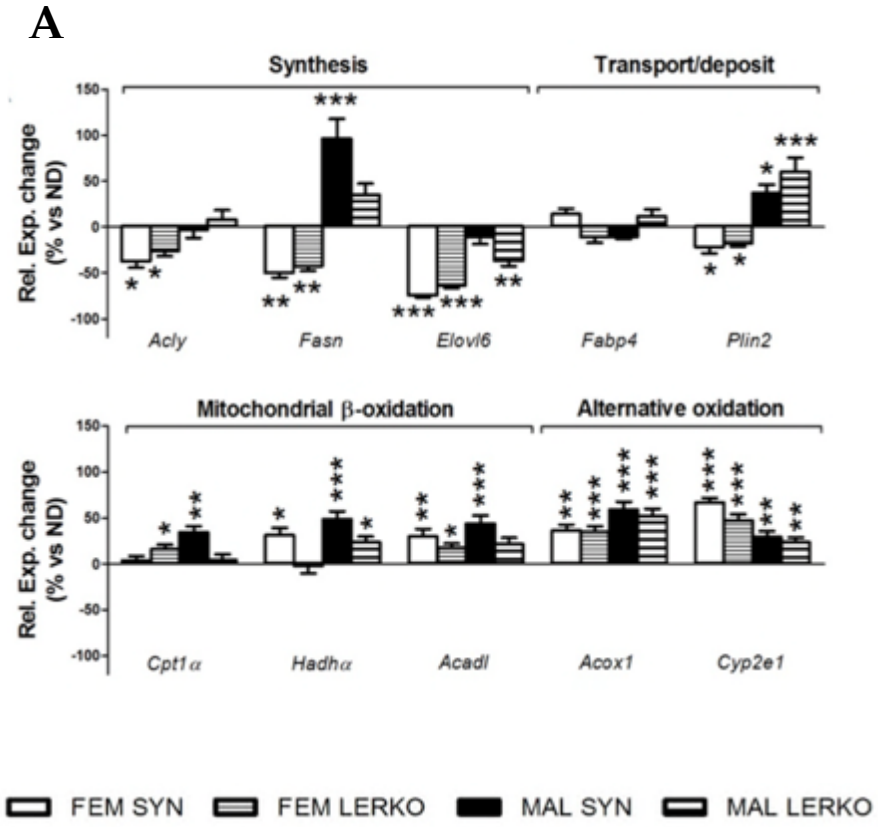


Figure 7: Hepatic ERα expression in SYN mice. Representative western blot and semi-quantitative analysis of ERα protein in liver extracts of 3-month-old cycling females, Ovariectomized (OVX) mice for 30 days and age-matched males. P (Proetrus), E (Estrus), M (Metestrus), D (Diestrus). The data indicate mean ± SEM; n = 5. The experiment was repeated three times. *p < 0.05, **p < 0.01, and ***p < 0.001 versus SYN at P. Modified from Della Torre *et al*.,2016.

Looking at lipid metabolism (Figure 8A), LERKO males display a decrease in the expression of enzymes involved in lipid synthesis, such as Fasn and Elovl6, a slight increase in lipid transport pathway and a scattered trend to decrease the expression of fatty acids β-oxidation enzymes, such as Cpt1α, Hadhα and Acadl (*Acyl-CoA Dehydrogenase, Long Chain*). So, it seems that arises a positive metabolic profile for LERKO males, unlike females, from this first panel of genes.


A
Synthesis
Transport/deposit
Rel. Exp. change (% vs ND)
Acly
Fasn
Elovl6
Fabp4
Plin2
Mitochondrial β-oxidation
Alternative oxidation
Cpt1α
Hadhα
Acadl
Acox1
Cyp2e1
FEM SYN
FEM LERKO
MAL SYN
MAL LERKO

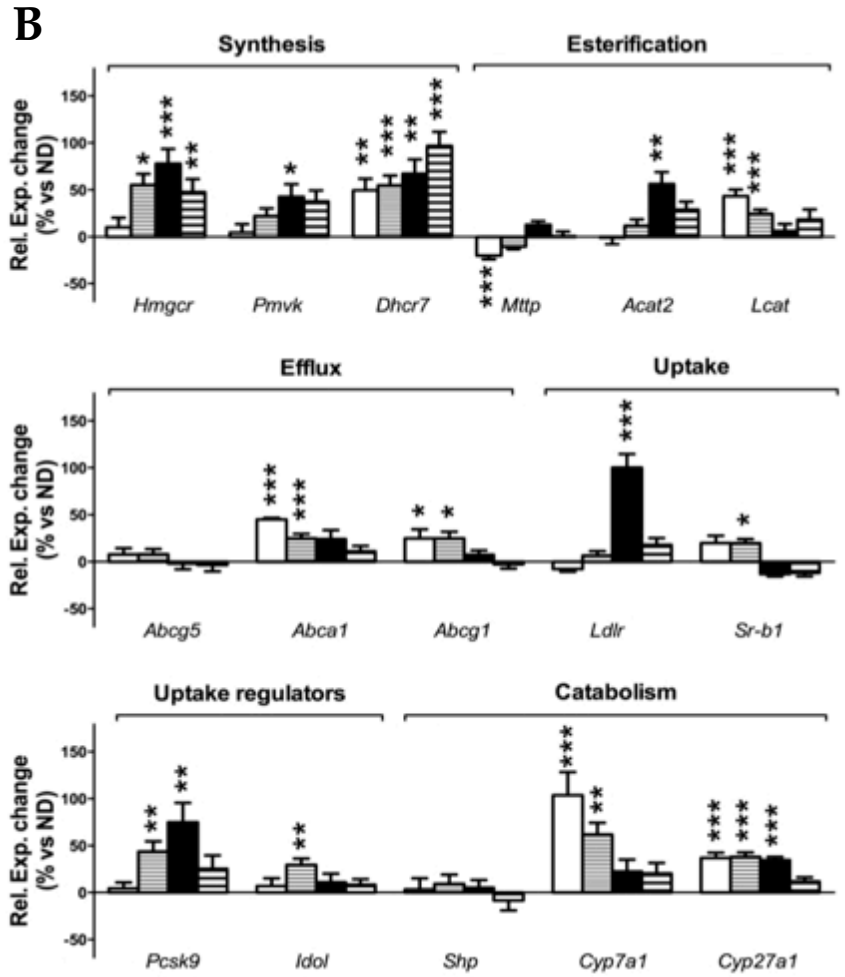

B
Synthesis
Esterification
Rel. Exp. change (% vs ND)
Hmgcr
Pmvk
Dhcr7
Mttp
Acat2
Lcat
Efflux
Uptake
Abcg5
Abca1
Abcg1
Ldlr
Sr-b1
Uptake regulators
Catabolism
Pcsk9
Idol
Shp
Cyp7a1
Cyp27a1

Figure 8: Hepatic expression of metabolic enzymes in SYN and LERKO females and males in HFD condition. Real-time PCR expression analysis of key hepatic enzymes involved in lipid (A) and cholesterol (B) metabolism on SYN female (n=21) and male (n=23) and LERKO female (n=21) and male (n=17) mice in HFD condition. Data are here represented as mean +/- SEM of the percentage of increase or decrease versus ND. *p <0.05, *p<0.01 and ***p <0.001 compared to ND. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

Moving forward with the metabolic profile analysis and looking at cholesterol metabolism (Figure 8B), we can note that, also in this metabolic pathway, LERKO males behave differently from SYN.

Proceeding from the top to the bottom panel, cholesterol esterification, uptake and catabolism pathways are affected. Indeed, LERKO males show a significant decrease in the expression of Acat2 (*Acetyl-CoA Acetyltransferase 2*), an enzyme that catalyzes the synthesis of cholesterol esters, which can be incorporated into apolipoprotein B (*apoB-containing lipoproteins*) for secretion from the cell. This decrease in the formation of cholesterol esters is in line with the observed slight decrease in Hmgcr expression, the key regulatory enzyme for cholesterol synthesis. Also, the expression of Ldlr, which mediates, through the recognition of apoprotein B100, the endocytosis of cholesterol-rich LDL removing them from the circulation, is strongly decreased in LERKO males. This means that the cholesterol uptake in the liver of LERKO males is significantly lower than SYN.

Finally, also the expression of Cyp27a1, responsible for the catabolism of cholesterol into bile acids is significant decreased in LERKO males, but taking into account the slight decrease in cholesterol synthesis this result is not surprising.

Summarizing, these data seem to suggest that the selective ablation of hepatic ERα in males leads to a positive, healthier metabolic profile.

1.4 Matrix analysis: a new approach to study fatty acids and cholesterol metabolism

Since liver metabolism is the result of a complex regulatory mechanism and is guaranteed by the continuous integration of multiple signals we decided to apply to our data another kind of analysis which could take into account more events at once, looking at whole pathways rather than at single genes.

The need to observe simultaneously more variables, led me to apply, for the analysis of our data, a system biology kind of approach, which could enable us to evaluate similarities and dissimilarities between the expressions of couples of genes in each animal, a matrix analysis. To do that, I compared, for each experimental group, the expression of genes in pairs and for each couple of genes I reported the statistical significance of the correlation of their expression with a color code according to the level of statistical significance.

I applied this analysis first on key lipid metabolic genes, most of which were analysed by RT-PCR, in female and male SYN mice in ND condition, Figure 9.

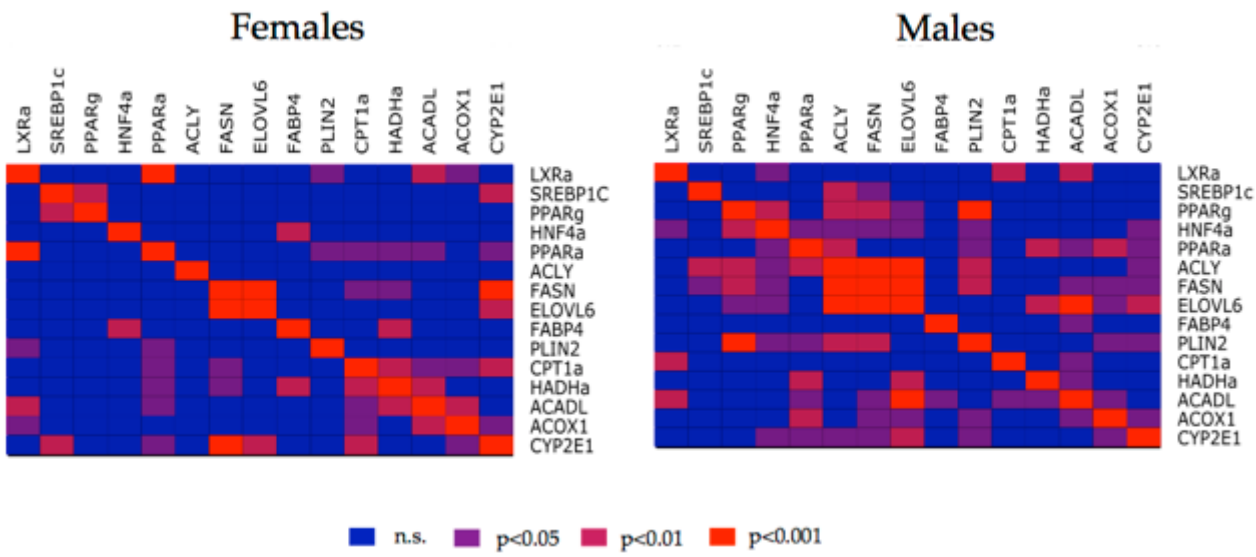


Figure 9: Matrix analysis of fatty acids metabolic genes in female and male SYN mice in ND condition. Pearson correlation analysis of key hepatic enzymes involved in fatty acids metabolism in female (left) and male (right) SYN mice in ND condition. *p <0.05, **p<0.01 and ***p <0.001. P values were calculated with two-tailed.

This first analysis in female and male SYN mice in ND clearly shows that there are significant sex differences. Indeed, the correlation among the key regulatory genes of fatty acids metabolism is greater in males than in females suggesting that this metabolic pathway is differently regulated in the two sexes even in physiological conditions.

As seen before, I carried out the analysis taking into account the effect of HFD administration and also in LERKO mice (Figure 10).

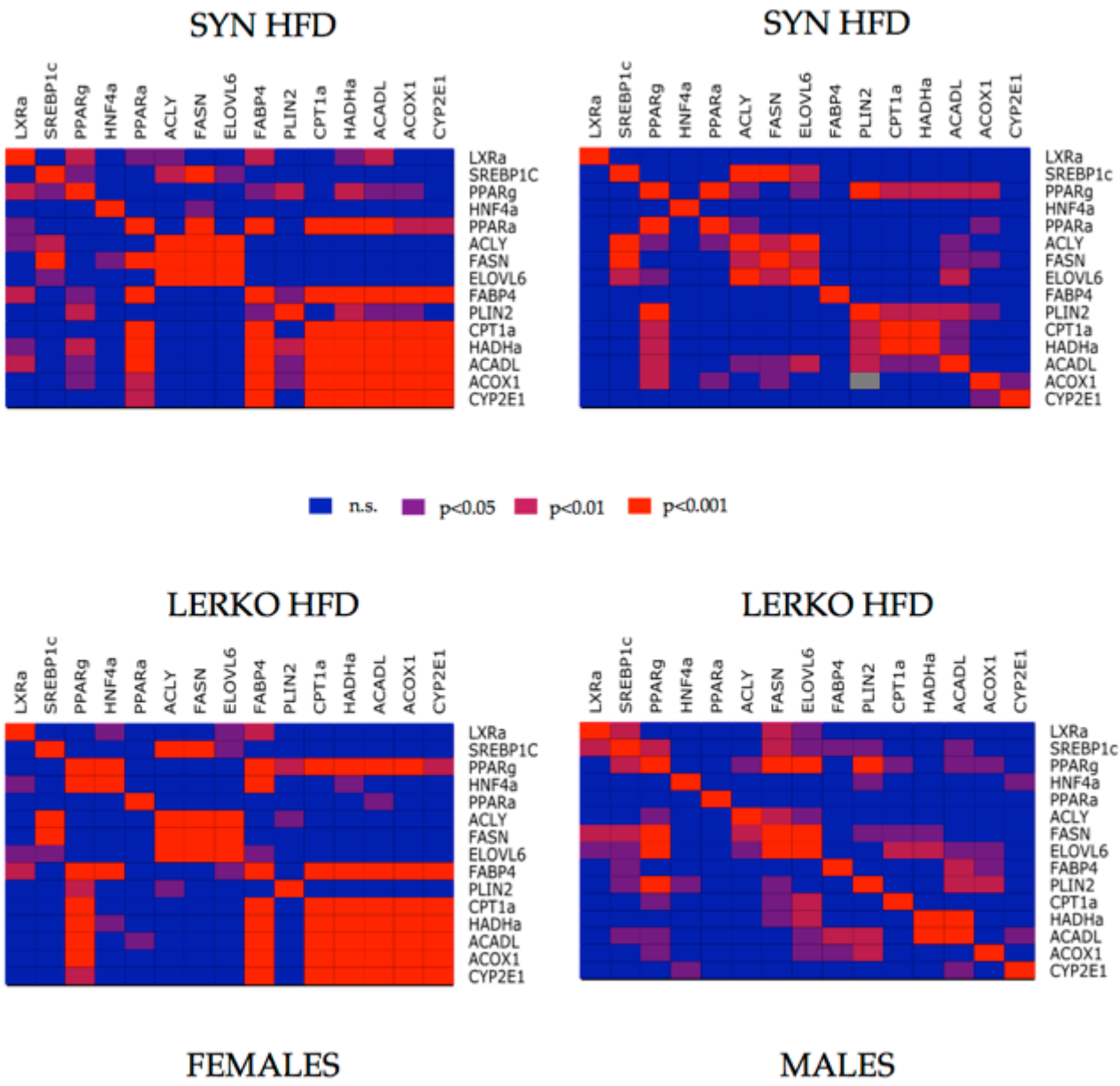


Figure 10: Matrix analysis of fatty acids metabolic genes in female and male SYN and LERKO mice in HFD condition. Pearson correlation analysis of key hepatic enzymes involved in fatty acids metabolism in female (left) and male (right) SYN (top) and LERKO (bottom) mice in HFD condition. *p <0.05, **p<0.01 and ***p <0.001. P values were calculated with two-tailed.

The administration of HFD leads, in SYN females, to a significant increase in the correlation of fatty acids metabolic genes compared to ND, while in males it seems that there is a slight decrease in the correlation and also a different spatial distribution of the correlated genes.
Moving to LERKO mice, in females, the strong increase of correlation observed in SYN females in HFD condition, is almost maintained while in males there is a very slight increase and spatial redistribution of the correlated genes.
So, this different approach to data analysis confirmed what it has been seen before with the RT-PCR: lipid metabolism is different between females and males in physiological conditions and the administration of HFD, *per se* or also in combination with the selective ablation of hepatic ERα, leads to different outcomes depending on sex.
I decided to move forward with the correlation and to analyse also the cholesterol metabolism, first in female and male SYN mice (Figure 11).

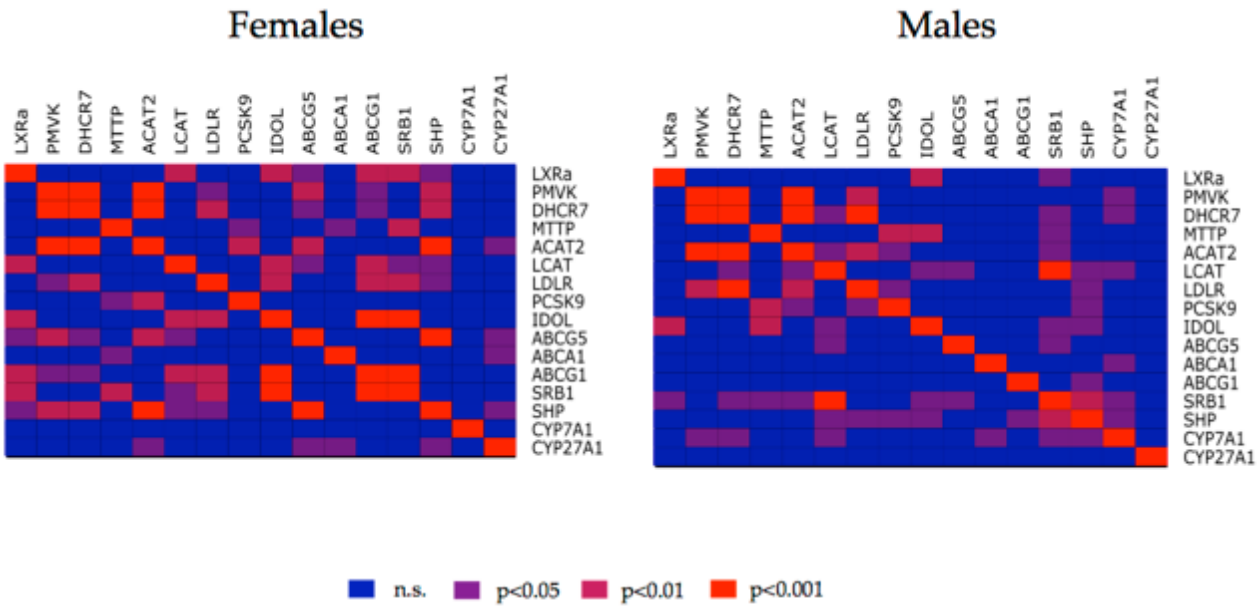


Figure 11: Matrix analysis of cholesterol metabolic genes in SYN female and male mice in ND condition. Pearson correlation analysis of key hepatic enzymes involved in cholesterol metabolism in SYN females and males in ND condition. *p <0.05, **p<0.01 and ***p <0.001. P values were calculated with two-tailed.

As depicted in these first two panels, female and male control mice have a different pattern of correlation among cholesterol metabolic genes. This analysis first and then this image shows us the correlation between these genes, a measure of their coordinated action and we can assert that in females, the action of cholesterol metabolic genes is more coordinated than in males. At this point, following the RT-PCR analysis, I decided to evaluate the effect of the

HFD on the correlation pattern in SYN mice and to perform this analysis also in LERKO, in order to single out the action of hepatic ERα (Figure 12).

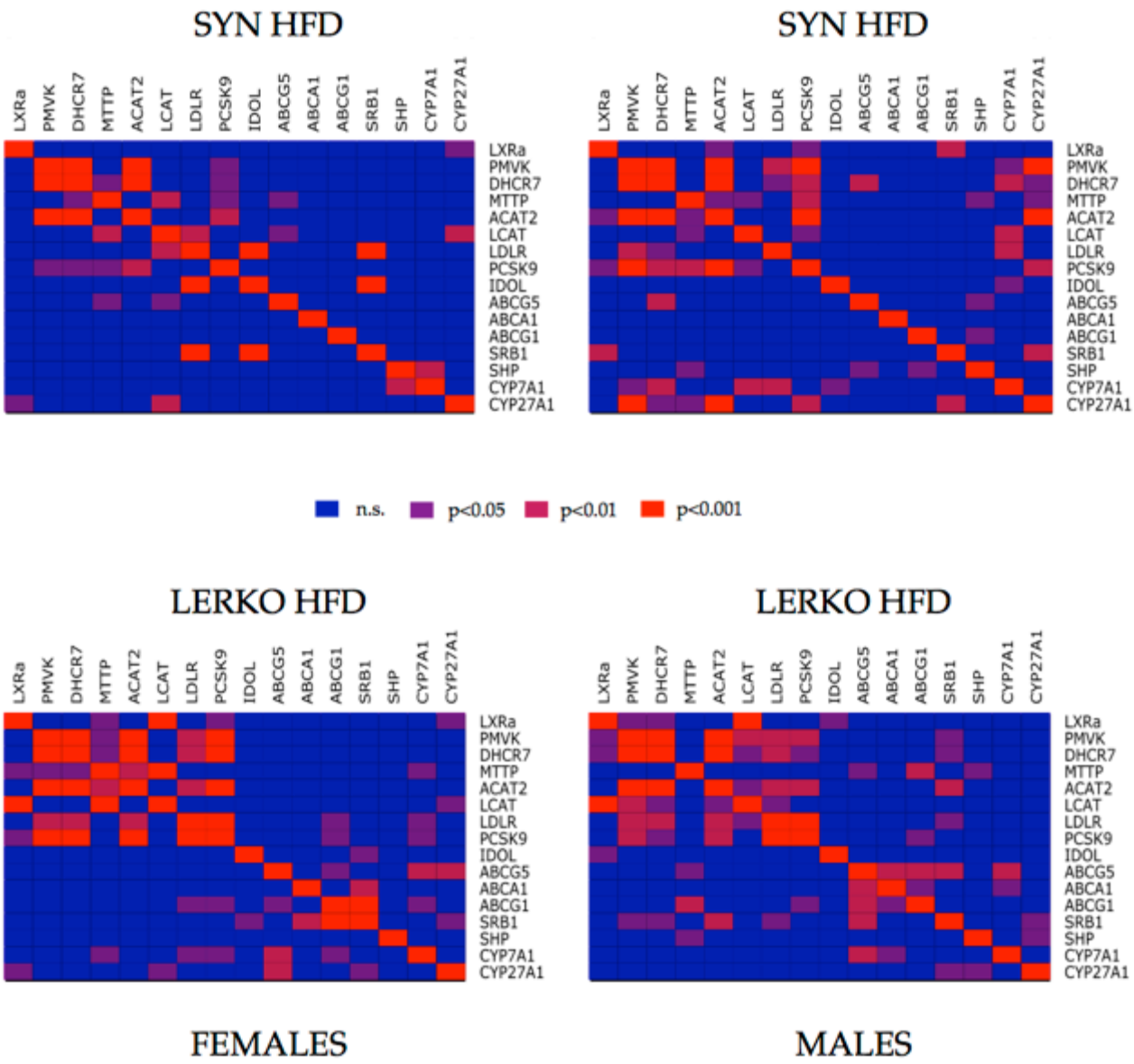


Figure 12: Matrix analysis of cholesterol metabolic genes in female and male SYN and LERKO mice in HFD condition. Pearson correlation analysis of key hepatic enzymes involved in cholesterol metabolism in female (left) and male (right) SYN (top) and LERKO (bottom) mice in HFD condition. *p <0.05, **p<0.01 and ***p <0.001. P values were calculated with two-tailed.

Looking at the effect of HFD in SYN mice, we can note that there are differences between the two sexes. Indeed, there is not only a different spatial distribution of the correlated genes but also males display a slightly greater correlation than females. Interestingly, comparing this panel with Figure 11, it turns out that in females the administration of the HFD leads to a significant decrease in the correlation of these genes and this is not true for males.
On the other hand, in both female and male LERKO mice there is an increase in the correlation of cholesterol genes compared to SYN and no major differences between the two sexes. Overall this correlation analysis on both fatty acids and cholesterol metabolism confirmed what we have seen before: these two important metabolic pathways are different between males and females even in physiological conditions. Interestingly, both the response to a nutritional challenge, like the HFD, and the selective ablation of hepatic ERα are sex dependent and they have a major role in lipids metabolism. Indeed, we have the stronger evidence of sex differences in lipids metabolism rather than cholesterol metabolism and it seems also that ERα is mostly involved in this metabolic pathway.

1.5 HFD effects on Liver Histology in female and male LERKO mice

In order to demonstrate these results, to verify the observed changes in both lipid and cholesterol hepatic metabolism and evaluate the resulting effects, I decided to perform the hepatic immunohistochemical analysis also on LERKO females and males.
Looking at histological data (Figure 13) it is clear that LERKO females were certainly less able than SYN to deal with the overload of fat deriving from HFD administration and indeed, they ended up depositing a lot of fat in the liver. Compared to SYN, LERKO females display an increased tissue degeneration as highlighted by the H&E staining, while the Oil Red O and the Masson's trichrome staining, respectively, show more and larger lipid droplets, portal infiltration of mononuclear leukocytes, more collagen deposition and aberrant vacuolar degeneration in LERKO females compared to SYN, in HFD.
Interestingly, LERKO males in HFD condition have a healthier liver compared to SYN and so it seems that, in males, the hepatic selective ablation of ERα has a beneficial effect on liver metabolism, a result, this, partially disclosed with the RT-PCR data previously analysed.
Summarizing, the specific ablation of hepatic ERα in females has detrimental effects on liver, even in ND but especially in HFD, while in males has a positive, beneficial effect since the liver of LERKO appears healthier than that of SYN, even in HFD. Overall this observation suggests that hepatic ERα might have an opposite function in the regulation of hepatic metabolism in the two sexes.

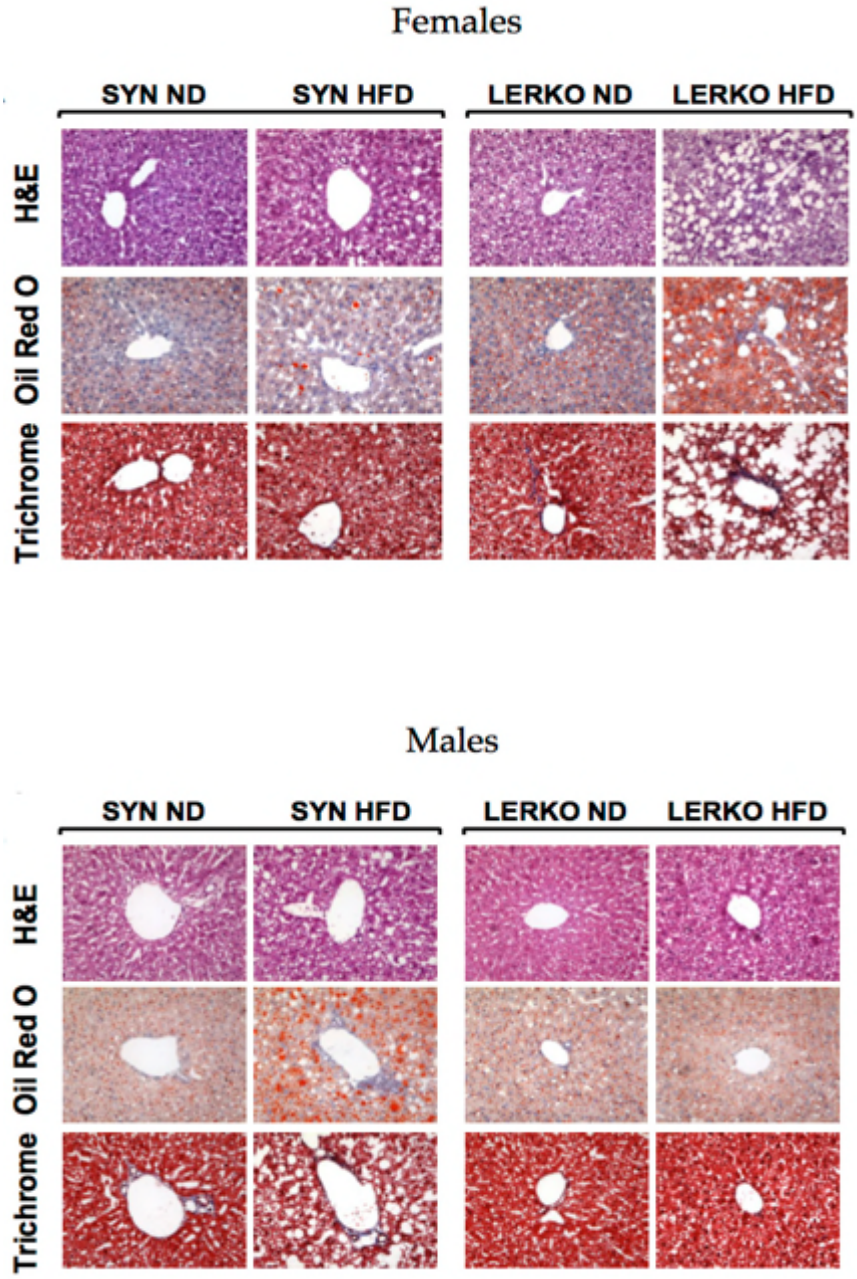


Figure 13: Effects of HFD on liver histology in SYN and LERKO. Liver histology in SYN and LERKO females and males. Top: Hematoxilyn & Eosin staining (H&E); Centre: oil red O staining plus H&E (neutral fats are stained orange red, and the nuclei are shown in blue). Bottom: Masson's trichrome staining with aberrant collagen deposits (blue); the hepatocyte cytoplasm is red and the nuclei are dark red-black structures within cells.

1.6 PPARα and LXRα: two major players in hepatic metabolic regulation

Looking at the opposite metabolic regulatory function of ERα in female and male livers, we wondered if this could be due to a sex-differentiated involvement of other metabolic regulators. Indeed, besides ERα there are two other nuclear receptors, PPARα (*Peroxisome Proliferator-activated Receptor α*) and LXRα (*Liver X Receptor α*), which are major players in the regulation of hepatic metabolism. PPARα is the major inductor of fatty acids oxidation while LXRα is the main hepatic regulator of cholesterol metabolism, stimulating both bile acids synthesis and cholesterol efflux.

We wondered if they could have played a role in the results so far obtained, in the sex differences observed in both fatty acids and cholesterol metabolic pathways.

So, I performed RT-PCR analysis on these two nuclear receptors to evaluate their expression in our experimental conditions, and also a Western Blot for their protein content.

Looking at PPARα expression (Figure 14A), we can note that there are no differences between the two sexes whether in physiological conditions or after HFD administration, both in SYN and LERKO and we have the same result also for its protein content (Figure 14B).

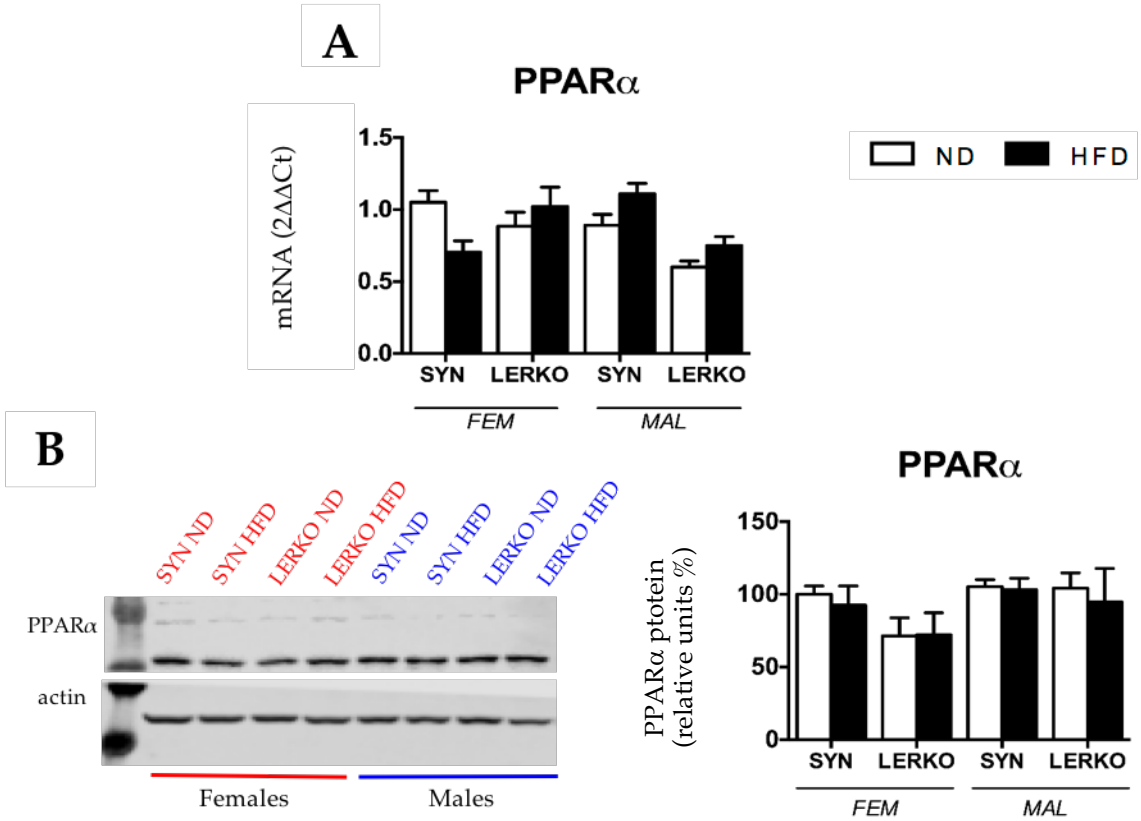


Figure 14: Hepatic expression of PPARα in SYN and LERKO females and males in ND and HFD condition. Real-time PCR expression analysis (A) and representative western blot and semi-quantitative analysis (B) of PPARα on female and male mice in ND and HFD condition. Data are here represented as mean +/- SEM. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

Moving to LXRα, the RT-PCR analysis (Figure 15A) reveals some differences: indeed, after HFD, in females, both SYN and LERKO, there is a significant increase of LXRα expression while males have a significant lower expression of the nuclear receptor compared to females and, interestingly, this result is in line with the decreased expression of its target genes, CYP7A1 and ABCG5, observed in both SYN and LERKO.

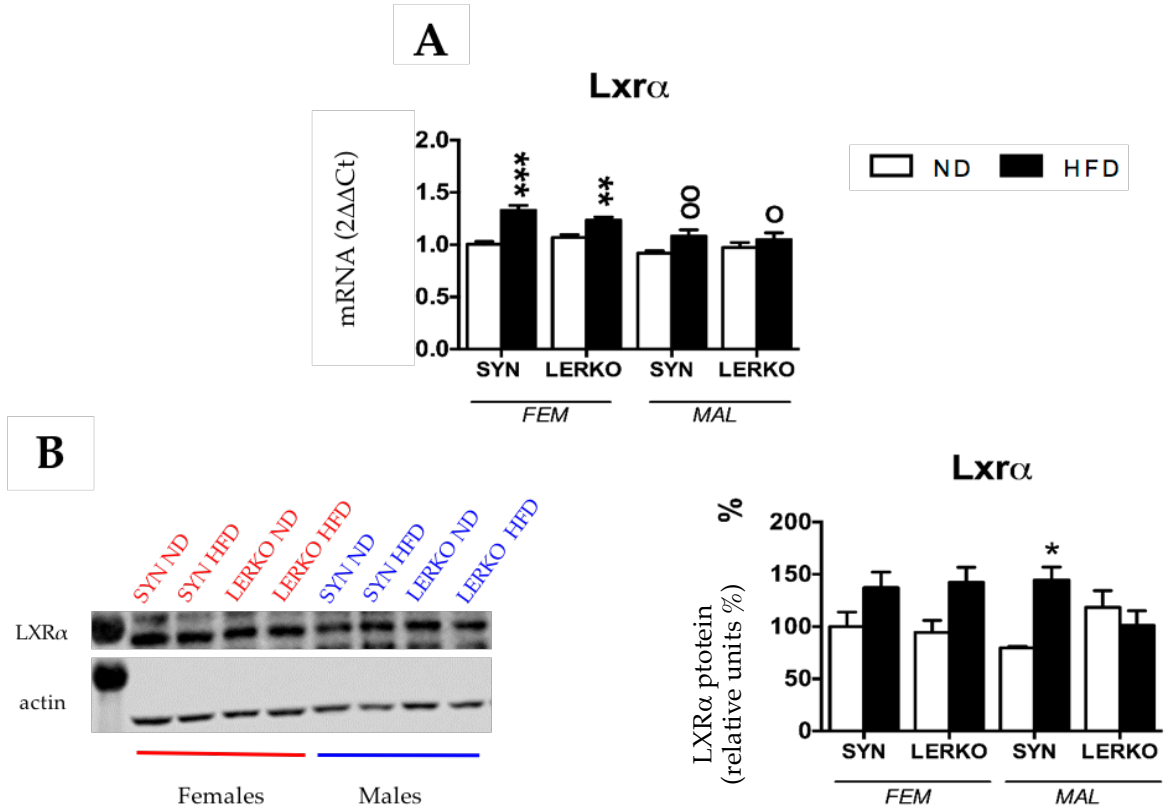


Figure 15: Hepatic expression of LXRα in SYN and LERKO females and males in ND and HFD condition. Real-time PCR expression analysis (A) and representative western blot and semi-quantitative analysis (B) of LXRα on female and male mice in ND and HFD condition. Data are here represented as mean +/- SEM. $p<0.05$,**$p<0.01$,***$p<0.001$ versus ND; °$p<0.05$,°°$p<0.01$ versus females. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

However, looking at the western blot analysis (Figure 15B), we did not see any differences in the protein content of LXRα between females and males whether in physiological conditions or after the administration of HFD in both SYN and LERKO mice. So, it seems that these two major players in the regulation of hepatic metabolism are not directly involved in those metabolic sex differences that we observed in our results suggesting that these effects are mainly mediated by ERα, which, as our results highlight, might exerts an opposite regulatory action in females and males metabolism.

1.7 Inflammation in female and male SYN and LERKO mice in ND and HFD

Lipid accumulation is a major cause of inflammation and chronic low-grade generalized inflammation, often called metainflammation, meaning metabolically-triggered inflammation, is associated with metabolic disorders.

Taking into account this, we decided to analyze also the inflammatory pathway and I began with the RT-PCR analysis of key inflammatory genes. First, I carried out the analysis on female and male SYN mice in ND condition, Figure 16.

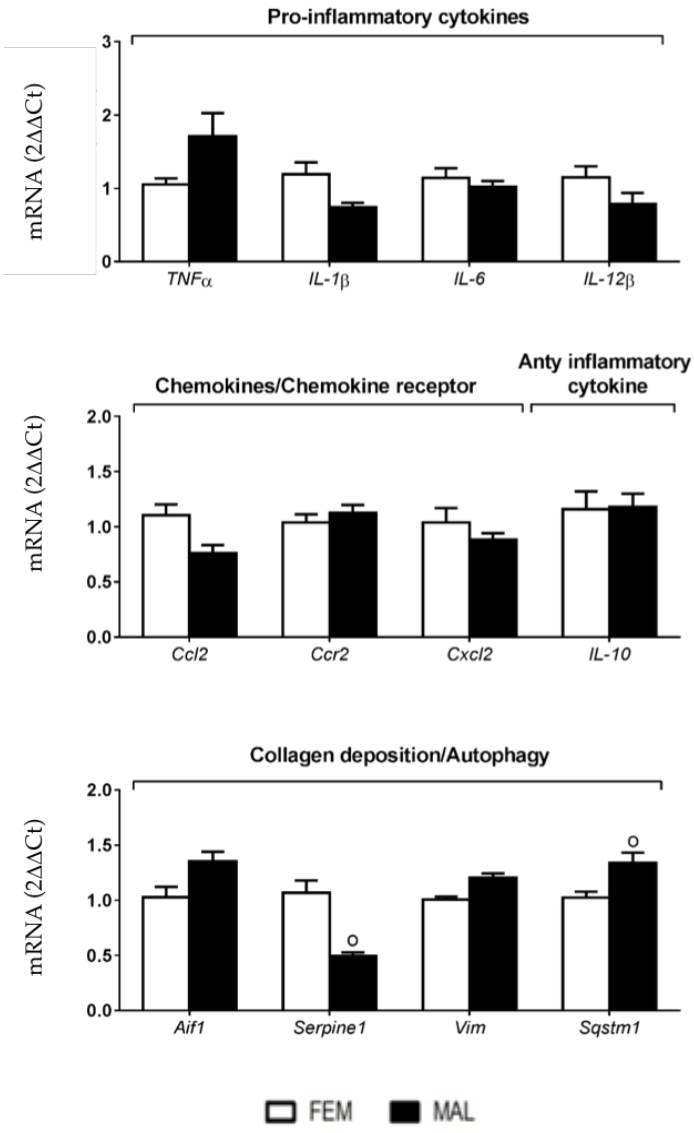


Figure 16: Hepatic expression of inflammatory genes in SYN female and male in ND condition. Real-time PCR expression analysis of key inflammatory genes on females (n=20) and males (n=14) in ND condition. Data are here represented as mean +/- SEM. °p <0.05 compared to females. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

Looking at the RT-PCR analysis of inflammatory genes in female and male SYN mice in ND we were unable to see any significant differences between the two sexes. So, we moved forward with the analysis of SYN mice after HFD administration, Figure 17.

We can note that there is a generalized increase in the expression of the inflammatory genes following the HFD and this increase involves both the pro-inflammatory and anti-inflammatory cytokines as well as chemokines and those genes involved in collagen deposition. This is a clear response to the overload of fat caused by the HFD, but once again we were unable to see sex differences in this response.

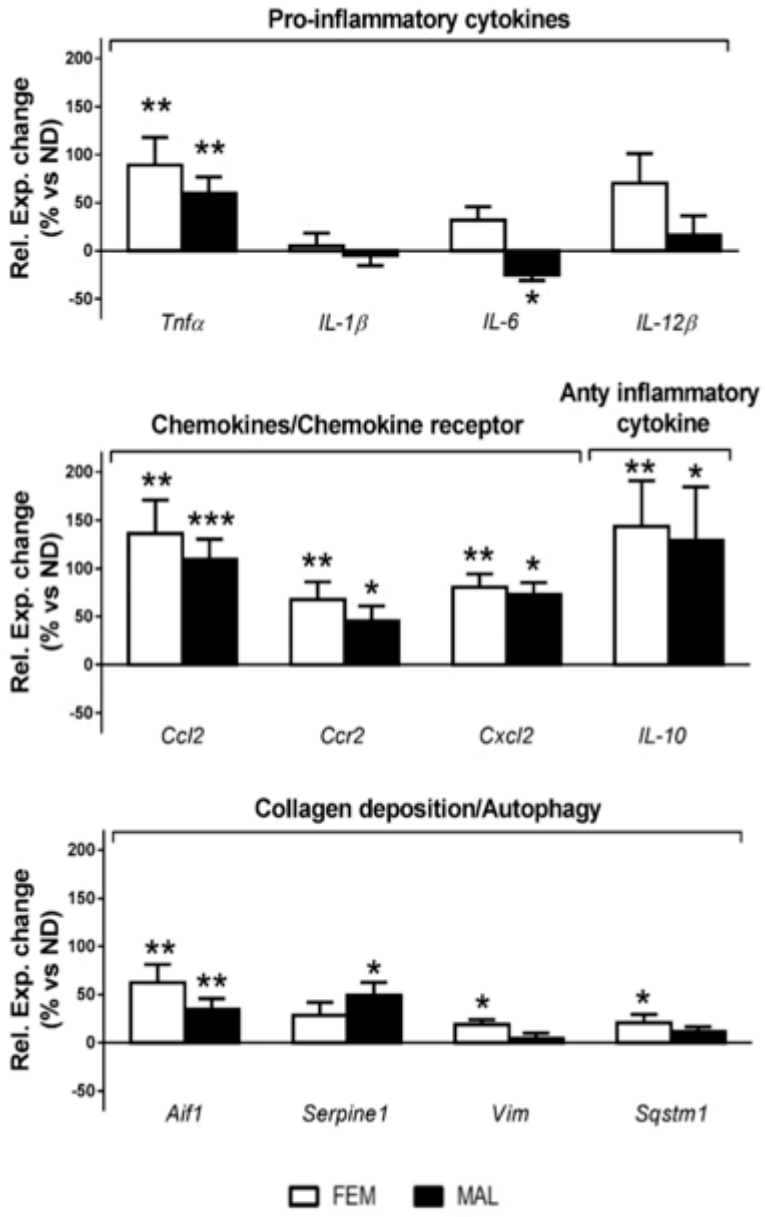


Figure 17: Hepatic expression of inflammatory genes in SYN females and males in HFD condition. Real-time PCR expression analysis of key inflammatory genes on females (n=21) and males (n=23) in HFD condition. Data are here represented as mean +/- SEM of the percentage of increase or decrease versus ND. *p <0.05, *p<0.01 and ***p <0.001 compared to ND. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

At this point we wondered whether and how the HFD administration combined with hepatic ERα ablation could affect the inflammatory pathway and then I analysed these genes also in LERKO mice following the HFD, Figure 18.

Interestingly it seems that the selective ablation of hepatic ERα, in both females and males, cancels, or rather mitigates, that effect of generalized increase in the expression of almost all the inflammatory genes, caused by the HFD. Indeed, in LERKO, both females and males, there is, for all the inflammatory genes analysed, a decrease in the expression levels compared to SYN. However, also in LERKO we do not have sex differences in the inflammatory pathway.

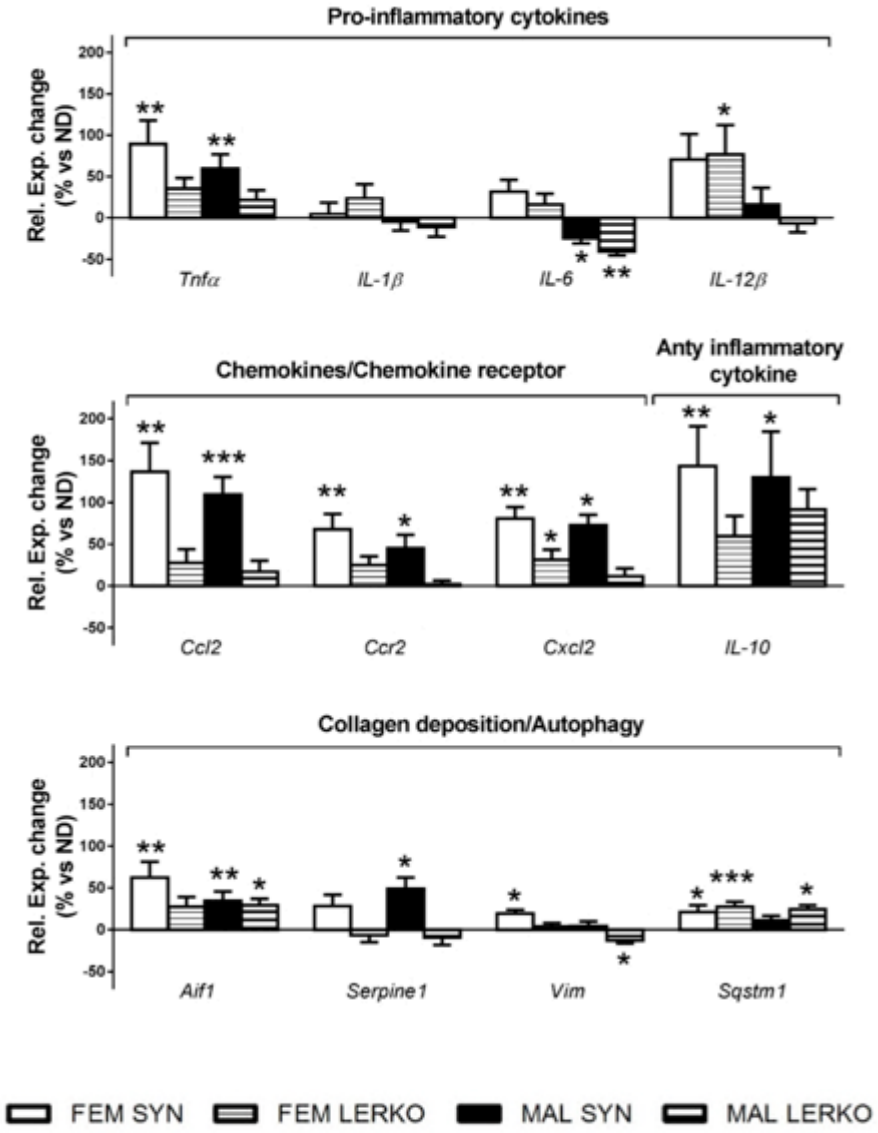


Figure 18: Hepatic expression of inflammatory genes in SYN and LERKO females and males in HFD condition. Real-time PCR expression analysis of key inflammatory genes on SYN female (n=21) and male (n=23) and LERKO female (n=21) and male (n=17) mice in HFD condition. Data are here represented as mean +/- SEM of the percentage of increase or decrease versus ND. *p <0.05, *p<0.01 and ***p <0.001 compared to ND. P values were calculated with two-way ANOVA followed by Bonferroni post hoc test.

Here we did not see those sex differences previously observed both in hepatic histology and in RT-PCR analysis of the cholesterol and fatty acids metabolic pathways. However, we expected to see sex differences also in the inflammatory processes analysed, since, as previously mentioned, inflammation in tightly linked to dysmetabolism.
I reasoned that probably RT-PCR analysis might not be sufficient to gather these differences and so I decided to apply the correlation analysis also on the inflammatory pathway, starting with female and male SYN mice in ND condition, Figure 19.

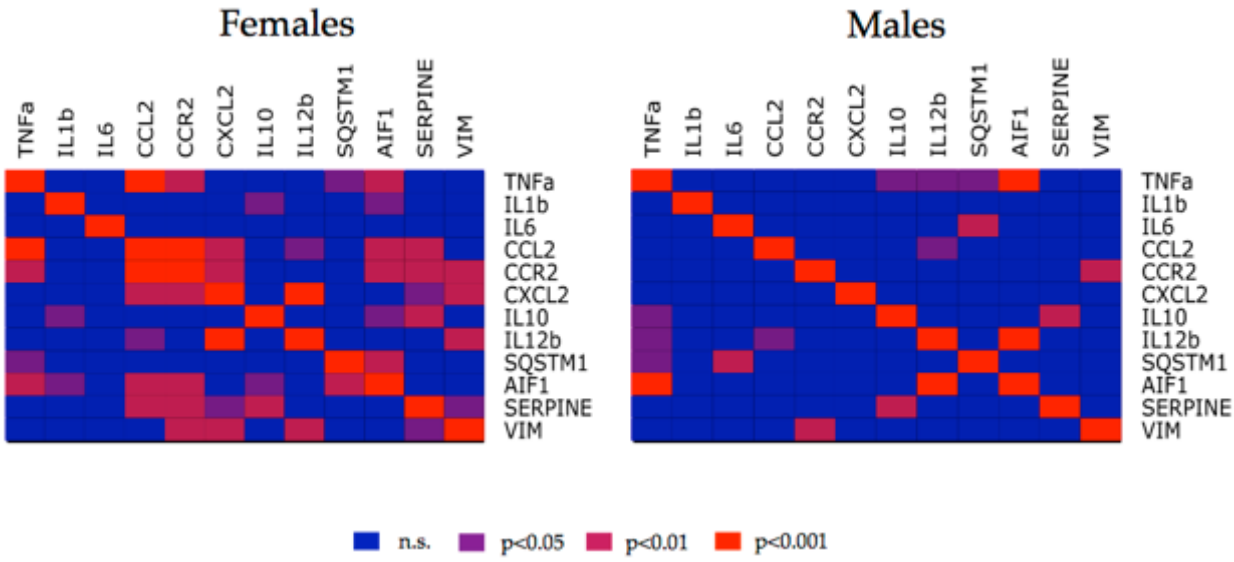


Figure 19: Matrix analysis of inflammatory genes in SYN females and males mice in ND condition. Pearson correlation analysis of key hepatic enzymes involved in cholesterol metabolism in SYN females and males in ND condition. *p <0.05, **p<0.01 and ***p <0.001. P values were calculated with two-tailed.

Interestingly, this type of approach to the analysis of the inflammatory genes revealed sex differences even in physiological conditions, differences that we did not see with the RT-PCR. Indeed, in females there is a significant greater correlation than in males suggesting that in females more genes act in a coordinated manner, and also the extent of this correlation is greater compared to males. I moved forward with the investigation and I analysed also LERKO mice and SYN after HFD administration, Figure 20.

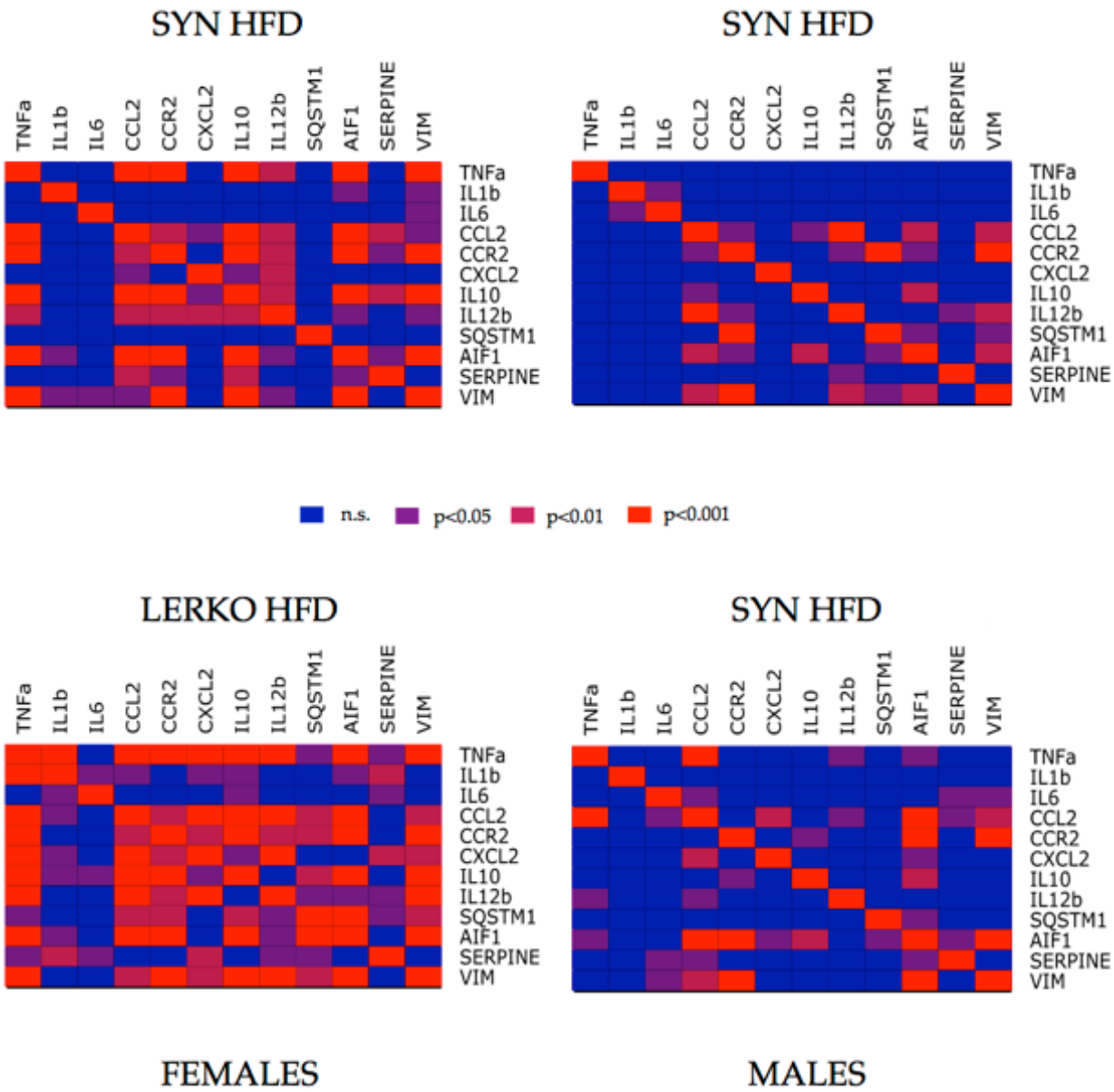


Figure 20: Matrix analysis of inflammatory genes in female and male SYN and LERKO mice in HFD condition. Pearson correlation analysis of key inflammatory genes in female (left) and male (right) SYN (top) and LERKO (bottom) mice in HFD condition. *p <0.05, **p<0.01 and ***p <0.001. P values were calculated with two-tailed.

We can note that the administration of HFD leads, in both SYN females and males to an increase in the correlation of inflammatory genes compared to ND, even if the extent of the total correlation is higher in females than in males. Moving to LERKO mice, in females, the already high correlation observed in SYN females in HFD condition still increases with, interestingly, a very high degree of correlation between the pro-inflammatory cytokine Il12-β (*Interleukin 12-β*), the chemokines Ccl2 (*C-C Motif Chemokine Ligand 2*), Cxcl2 (*C-X-C Motif Chemokine Ligand 2*), the autophagosome cargo protein Sqstm1 (*Sequestosome 1*) and Aif1 (*Allograft inflammatory factor 1*) which is found in activated macrophages and positively correlates with metabolic indicators. On the other hand, in males there is a very slight increase and spatial redistribution of the correlated genes.

This approach allowed us to see those sex differences that we did not see with the RT-PCR analysis and, interestingly, this result confirms what we have seen before with the expression analysis of the metabolic pathways and above all with the hepatic immunohistological analysis. Indeed, it confirms that the selective ablation of hepatic ERα has a detrimental effect on the female liver, and, apparently, a beneficial effect on the liver of males. So, it seems that hepatic ERα exerts an opposite function in the metabolic and in the associated inflammatory regulation in females and males, even if, as previously discussed, is very low expressed in male liver.

The table in figure 21 summarizes the main differences between females and males observed in this study.

	PHYSIOLOGICAL CONDITION	HFD CHALLENGE	LERKO & HFD CHALLENGE
♀		↓ Lipid synthesis ↑ Lipid β- oxidation ↑ Cholesterol catabolism ↑ Cholesterol synthesis- esterification efflux ↑ Inflammatory correlation	↑ Inflammatory correlation
♂	↓ Lipid & cholesterol synthesis ↑ Cholesterol esterification- efflux- uptake- catabolism ↓ Inflammatory correlation	↑ Lipid synthesis & deposit ↑ Lipid β-oxidation ↑ Cholesterol synthesis ↑ Cholesterol uptake ↑ Inflammatory correlation	↓ Lipid synthesis ↑ Lipid transport ↑ Lipid β- oxidation ↓ Cholesterol esterification- uptake ↑ Cholesterol synthesis ↑ Inflammatory correlation

Figure 21: Differences between females and males in the three experimental conditions. This table summarizes the main differences observed in the two sexes in this study. In physiological condition data are represented versus females; in HFD condition data are represented for both females and males versus their own control in physiological conditions; LERKO are reported versus their SYN control in HFD challenge.

DISCUSSION

Oestrogens are important regulators of energy metabolism, acting both centrally and in the periphery.

Indeed, the decline in oestrogens levels, which occurs with the onset of menopause, is associated with different metabolic impairments, such as increased body weight and visceral adiposity, decreased insulin sensitivity and increased IR. Interestingly, all these metabolic alterations, associated with menopause, play a role, at different extents, in the increased risk of cardiovascular diseases observed in post-menopausal women[174]. Actually, different gender studies pointed out that females are more protected from CVDs during fertile period[175] and that several metabolic syndrome features, like abdominal adiposity, dyslipidemia and IR, emerge with menopause, suggesting that oestrogens are the main mediators of female metabolic protection[176].

Pharmacological treatment with oestrogens reverts the progression of metabolic disorders and indeed, ostrogens have been approved by FDA for postmenopausal therapy. However, due to the ubiquitous expression of ERs, the metabolic benefits provided by HRTs are often associated with side effects involving heart diseases and breast cancer.

Oestrogens' effects on metabolism are mainly mediated by ERα; indeed, both female and male mice lacking ERα have an increased body weight and food intake, show IR, impaired glucose tolerance and adipocyte hypertrophy[64–66].

ERα is the predominant ER isoform in hepatocytes[105] and we demonstrated that, in female liver, it has an oscillatory activity which is responsible for the regulation of lipid synthesis and is also necessary to preserve an healthy hepatic metabolism[138]. In liver of female mice ERα acts as a nutrient sensor able to adapt energy metabolism to the specific phase of the estrous cycle: the expression of metabolic genes changes significantly across the progression of the estrous cycle, and this modulation is oestrogens and ERα-mediated since is lost in both OVX and LERKO female mice.

Interestingly, males have significantly lower levels of hepatic ERα than females; however, by utilizing the ERE-Luc mouse model, it has been possible to demonstrate that food intake has a strong effect on ER activity also in male liver[34].

On these premises, the aim of this work was to perform a comparative study to unravel the role played by hepatic ERα in the regulation of energy metabolism also in male mice. The influence of ERα on energy metabolism was assessed in two different conditions: a physiological condition (ND) and a state of obesity and metabolic dysfunction (HFD).

The administration of a lipid-enriched diet allowed us to investigate the role of ERα in different metabolic settings, specifically also in a stressed metabolic condition.

Firstly, we demonstrated, by gene expression analysis of key hepatic metabolic enzymes, that in homeostatic conditions male and female hepatic metabolism is quite different: indeed, males displayed lower lipid and cholesterol synthesis and a higher cholesterol efflux, esterification and catabolism compared to females.

So, hepatic metabolic profile of females and males seemed to be different in homeostatic conditions and when we looked at general metabolic parameters, like body weight and food intake, we did not see major differences between the two sexes, even after HFD administration, except the natural occurring higher body weight of males compared to females.

However, the more specific immunohistochemical analysis on liver revealed that the HFD had affected males much more than females, as indicated by the higher hepatic fat accumulation and collagen deposition displayed by males compared to females, following the HFD administration.

Once revealed this sex different effect of HFD on liver, we focused on the expression of key hepatic metabolic enzymes, after HFD, in order to understand the causes on which depends the different histological effect observed in the two sexes. Females replied to HFD with a clear reduction in lipid synthesis and with the increase of cholesterol catabolism while, on the contrary, males were unable to down-regulate lipid synthesis and displayed an increase in cholesterol synthesis and uptake after HFD. So, the excess intake of lipids, caused by the HFD, leads female and male liver to adopt different strategies in order to deal with the overload of fat and males respond ending up with a greater accumulation of fat compared to females.

However, the main focus of my PhD project regards the role played by hepatic ERα in the regulation of energy metabolism in males compared to females and therefore we performed the expression analysis of the key hepatic metabolic enzymes also in LERKO mice, after HFD, in order to single out the activity of the hepatic ERα.

Even though males express very low levels of ERα in liver compared to females, its selective hepatic ablation in LERKO male mice, combined with HFD, had many effects on both lipid and cholesterol metabolic pathways. Indeed, we observed, in LERKO males, a decrease in lipid synthesis and an increase in lipid transport, while regarding cholesterol they displayed a strong decrease in liver cholesterol uptake thus suggesting that the selective ablation of hepatic ERα in males led to a positive, healthier metabolic profile than control animals.

However liver metabolism is the resultant of a complex regulatory mechanism and is guaranteed by the continuous integration of multiple signals: this concept led us to apply for our analysis a system biology kind of approach, which could enable us to take into account more events at once. The matrix analysis applied for both lipid and cholesterol pathways in order to better address the complexity of the hepatic metabolic system, confirmed what we saw with the expression analysis. These two important metabolic pathways are different between females and males in physiological conditions, and the administration of HFD, *per se* or also in combination with the selective ablation of hepatic ERα, leads to different outcomes depending on sex.

A further demonstration of these results and the evaluation of the consequent effects of these changes came with the immunohistochemical analysis performed on LERKO female and

male livers. LERKO females replied to the overload of fat with a clear hepatic lipid accumulation while males, on the contrary, after HFD administration had a healthier liver compared to control animals suggesting that, in males, the ablation of hepatic ERα had a beneficial effect on liver metabolism, a result partially disclosed with the expression analysis. At this point, we reasoned that, besides ERα, there are two others major players in the regulation of hepatic metabolism: PPARα and LXRα. We wondered if they could have played a role in the sex differences observed in fatty acids and cholesterol pathways, but both the protein content and the RT-PCR analyses for PPARα and LXRα did not revealed sex differences in none of the experimental conditions, suggesting that they are not directly involved and that these effects are mainly mediated by ERα, which seems to exert an opposite regulatory function in females and males metabolism.

Immunity is strongly interrelated to energy metabolism. Indeed, lipid accumulation is a major cause of inflammation and chronic low-grade generalized inflammation, often called metainflammation, is associated with metabolic disorders.
On the other hand, it is well recognized that oestrogens exert a wide range of actions within the immune system. Indeed, in females, during the fertile period, oestrogens have an anti-inflammatory role and with the onset of menopause the incidence of those disorders characterized by a strong inflammatory component like osteoporosis, atherosclerosis and diabetes, approaches or exceeds the levels observed in males[50].
Before the onset of menopause, there are many sex-specific immunologic differences regarding whether the innate or the adaptive component of the immune response and both these components are stronger in females than in males[46].
Interestingly, in immune cells, ERα plays a critical role in mediating a lot of cellular responses necessary for innate and adaptive immunity and when the levels of oestradiol decrease or ERα action is impaired, disease susceptibility increases[44].
On these premises, we decided to analyze also the inflammatory pathway. The expression analysis of the major inflammatory genes did not reveal sex differences in none of the experimental condition analysed even if inflammation is tightly linked to dysmetabolism; however, the matrix analysis allowed us to see sex differences in the inflammatory pathways even in physiological conditions and interestingly, the obtained result was in line with the metabolic results: the selective ablation of hepatic ERα had a detrimental effect on female liver, and, apparently, a beneficial effect on the liver of males, suggesting that hepatic ERα could exert an opposite function in the regulation of the metabolic and inflammatory pathways in females and males.
This evidence of the possible opposite action of ERα in the regulation of females and males metabolism is not surprising if we take into account that in female organisms, the reproductive needs are guaranteed by adequate energy supplies[63] and that the majority of

animals adjust their reproductive pattern in order to increase and maximize the survival chances of their offspring.

The nourishing of the offspring during pregnancy and lactation is the biggest expenditure of energy for a female mammal during her lifetime and so the female reproductive system has to be substantially more sensitive to any perturbation than the male.
Therefore in mammals, the reproductive duties must have required a substantial adaptation of the mechanisms linking fertility to nutrition and so, during evolution, have been developed and conserved those mechanisms, which ensure energy storage in case of food abundance and a strict control over fertility in a nutrient poor environment in order to avoid the competition for food between the mother and the offspring thus preventing the extinction of the species[63]. It is not hard to believe that the need for a reciprocal control of both energy homeostasis and reproduction might have promoted the evolutionary selection of ERα as the link between these two functions.
Several experimental evidences in both animals and humans showed that female reproductive stages induce significant changes in liver metabolism and highlighted the key role played by liver in the modulation of the energetic needs according to the gestational phase, specifically in the switch from anabolic to catabolic metabolism reported at mid-end gestation in most species[177].
In order to ensure the perfect integration between reproductive functions and hepatic metabolism, the liver of female mammals must have evolved and specialized significantly during evolution and this could explain the strong hepatic sexual differentiation well documented by pharmacological and clinical studies.
Speaking of hepatic sexual differentiation, we have to mention the pituitary growth hormone (GH). GH is synthetized in the CNS pituitary glands and its secretion from the pituitary depends to testosterone exposure, which imprints pulsatile GH signaling in males from neonatal life to adulthood, with pulses of 3-4 hours od intervals, while in females the secretion of GH is continuous[140].
This sex specific pattern, observed in GH secretion and in the expression of its receptor (GHR), is a major factor in settling and maintaining sexual dimorphism in the transcription of hepatic genes involved in relevant metabolic pathways such as metabolism of lipids, bile acids, steroids and drugs[141].
The finding that hepatic ERα is directly involved in the regulation of lipid and cholesterol metabolism in both females and males and that exerts an opposite function in the two sexes, as highlighted in this work, dictates a revision of the widely held theory on the central role of GH in the sexual differentiated hepatic metabolism and suggests to verify whether, in our experimental conditions, also GH could be involved.
Summarizing, the results presented in this work allowed us to identify the sex specific role of hepatic ERα in the regulation of liver metabolism and inflammation in normal or stressed

metabolic conditions, and, interestingly, to highlight ERα possible opposite regulatory action in female and male metabolism.

This is very relevant from both a pharmacological and a pathophysiological point of view; indeed, the identification of a sex dependent regulatory action of ERα on metabolism not only provide the conceptual bases for future therapeutic interventions able to mimic the beneficial effect of oestrogens and ERα on metabolic parameters, but also further stress the necessity to consider sex as an essential biological variable in the design of personalized pharmacological therapies.

REFERENCES

1. Boulware, M. I. & Mermelstein, P. G. The influence of estradiol on nervous system function. *Drug News Perspect* **18,** 631–637 (2005).
2. De Ronde, W., Pols, H. A. P., Van Leeuwen, J. P. T. M. & De Jong, F. H. The importance of oestrogens in males. **58,** 529–542 (2003).
3. Toran-Allerand, C. D., Tinnikov, A. A., Singh, R. J. & Nethrapalli, I. S. 17α-Estradiol: A brain active estrogen? *Endocrinology* **146,** 3843–3850 (2005).
4. Ikeda, T., Makino, Y. & Yamada, M. K. 17α-Estradiol is generated locally in the male rat brain and can regulate GAD65 expression and anxiety. *Neuropharmacology* **90,** 9–14 (2015).
5. Strauss, J. F. & Barbieri, R. L. *Yen and Jaffe's Reproductive Endocrinology: Seventh Edition*. *Yen and Jaffe's Reproductive Endocrinology: Seventh Edition* (2013). doi:10.1016/C2011-0-04643-4
6. Goji, K. Twenty-four-hour concentration profiles of gonadotropin and estradiol (E2) in prepubertal and early pubertal girls: The diurnal rise of E2 is opposite the nocturnal rise of gonadotropin. *J. Clin. Endocrinol. Metab.* **77,** 1629–1635 (1993).
7. Huber, C. J. G. T. C. S. J. C. Production and actions of estrogens. *N. Engl. J. Med.* **346,** 340–352 (2002).
8. Morselli, E. *et al.* The effects of oestrogens and their receptors on cardiometabolic health. *Nat. Rev. Endocrinol.* **13,** 352–364 (2017).
9. Nilsson, S. *et al.* Mechanisms of estrogen action. *Physiol. Rev.* **81,** 1535–65 (2001).
10. Witkowska, H. E. *et al.* Characterization of bacterially expressed rat estrogen receptor beta ligand binding domain by mass spectrometry: structural comparison with estrogen receptor alpha. *Steroids* **62,** 621–31
11. Kuiper, G. G. *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138,** 863–70 (1997).
12. Powell, E. *et al.* Identification of estrogen receptor dimer selective ligands reveals growth-inhibitory effects on cells that co-express ERα and ERβ. *PLoS One* **7,** (2012).
13. Chang, E. C., Frasor, J., Komm, B. & Katzenellenbogen, B. S. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. *Endocrinology* **147,** 4831–4842 (2006).
14. Saji, S., Hirose, M. & Toi, M. Clinical significance of estrogen receptor beta in breast cancer. *Cancer Chemother. Pharmacol.* **56,** 21–26 (2005).
15. Chakraborty, S., Willett, H. & Biswas, P. K. Insight into estrogen receptor beta-beta and alpha-beta homo- and heterodimerization: A combined molecular dynamics and sequence analysis study. *Biophys. Chem.* **170,** 42–50 (2012).
16. Foryst-Ludwig, A. & Kintscher, U. Metabolic impact of estrogen signalling through ERalpha and ERbeta. *J. Steroid Biochem. Mol. Biol.* **122,** 74–81 (2010).
17. Kushner, P. J. *et al.* Estrogen receptor pathways to AP-1. [Review] [39 refs]. *J Steroid Biochem Mol Biol* **74,** 311–317 (2000).
18. Saville, B. *et al.* Activation at GC-rich (Sp1) Promoter Elements *. *J. Biol. Chem.* **275,** 5379–5387 (2000).
19. Stellato, C. *et al.* The 'busy life' of unliganded estrogen receptors. *Proteomics* **16,** 288–300 (2016).
20. Chambliss, K. *et al.* Non-nuclear estrogen receptor α signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. *J. Clin. Invest.* **120,** 2319–2330 (2010).
21. Levin, E. R. Plasma membrane estrogen receptors. *Trends Endocrinol. Metab.* **20,** 477–482 (2009).
22. Kelly, M. J. & Levin, E. R. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol. Metab.* **12,** 152–156 (2001).
23. Jia, M., Dahlman-Wright, K. & Gustafsson, J.-Å. Estrogen receptor alpha and beta in health and disease. *Best Pract. Res. Clin. Endocrinol. Metab.* **29,** 557–568 (2015).
24. Owman, C., Blay, P., Nilsson, C. & Lolait, S. J. Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues. *Biochem. Biophys. Res. Commun.* **228,** 285–92 (1996).
25. Filardo, E. J. & Thomas, P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: Its mechanism of action and role in female reproductive cancer, renal and vascular physiology. *Endocrinology* **153,** 2953–

2962 (2012).
26. Prossnitz, E. R. & Barton, M. The G protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol.* **7,** 715–726 (2012).
27. Barkhem, T. *et al.* Differential Response of Estrogen Receptor a and Estrogen Receptor b to Partial Estrogen Agonists/Antagonists. *Mol. Pharmacol.* **54,** 105–112 (1998).
28. Hewitt, S. C. & Korach, K. S. Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction* **125,** 143–149 (2003).
29. Gustafsson, J. Å. What pharmacologists can learn from recent advances in estrogen signalling. *Trends Pharmacol. Sci.* **24,** 479–485 (2003).
30. Couse, J. F., Korach, K. S. & Korach, K. S. Estrogen Receptor Null Mice : What Have We Learned and Where Will They Lead Estrogen Receptor Null Mice : What Have We Learned and Where Will They Lead Us ? " O. **20,** 358–417 (1999).
31. Krege, J. H. *et al.* Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15677–15682 (1998).
32. McPherson, S. J., Ellem, S. J. & Risbridger, G. P. Estrogen-regulated development and differentiation of the prostate. *Differentiation* **76,** 660–670 (2008).
33. Lee, H.-R., Kim, T.-H. & Choi, K.-C. Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse. *Lab. Anim. Res.* **28,** 71–6 (2012).
34. Ciana, P. *et al.* In vivo imaging of transcriptionally active estrogen receptors. *Nat. Med.* **9,** 82–86 (2003).
35. McCaffrey, A., Kay, M. A. & Contag, C. H. Advancing molecular therapies through in vivo bioluminescent imaging. *Molecular Imaging* **2,** 75–86 (2003).
36. Suzuki, S., Brown, C. M. & Wise, P. M. Mechanisms of neuroprotection by estrogen. *Endocrine* **29,** 209–215 (2006).
37. Tena-Sempere, M. *et al.* Neonatal exposure to estrogen differentially alters estrogen receptor alpha and beta mRNA expression in rat testis during postnatal development. *J. Endocrinol.* **165,** 345–357 (2000).
38. Vinader-Caerols, C., Collado, P., Segovia, S. & Guillamón, A. Estradiol masculinizes the posteromedial cortical nucleus of the amygdala in the rat. *Brain Res. Bull.* **53,** 269–273 (2000).
39. Wise, P. M. Estrogens and neuroprotection. *Trends Endocrinol. Metab.* **13,** 229–230 (2002).
40. Dubal, D. B. *et al.* Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1952–7 (2001).
41. Maggi, A., Ciana, P., Belcredito, S. & Vegeto, E. Estrogens in the nervous system: mechanisms and nonreproductive functions. *Annu. Rev. Physiol.* **66,** 291–313 (2004).
42. Mitra, S. W. *et al.* Immunolocalization of estrogen receptor ?? in the mouse brain: Comparison with estrogen receptor ?? *Endocrinology* **144,** 2055–2067 (2003).
43. Morton, G. & Schwartz, M. Central nervous system control of food intake and body weight. *Nat. Rev.* **443,** 289–95 (2006).
44. Hevener, A. L., Clegg, D. J. & Mauvais-Jarvis, F. Impaired estrogen receptor action in the pathogenesis of the metabolic syndrome. *Mol. Cell. Endocrinol.* **418,** 306–321 (2015).
45. Pfeilschifter, J., Köditz, R., Pfohl, M. & Schatz, H. Changes in proinflammatory cytokine activity after menopause. *Endocr. Rev.* **23,** 90–119 (2002).
46. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294,** 102–110 (2015).
47. Erlandsson, M. C., Ohlsson, C., Gustafsson, J. Å. & Carlsten, H. Role of oestrogen receptors ?? and ?? in immune organ development and in oestrogen-mediated effects on thymus. *Immunology* **103,** 17–25 (2001).
48. Bynoe, M. S., Grimaldi, C. M. & Diamond, B. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2703–2708 (2000).
49. Screpanti, I. *et al.* Steroid sensitivity of thymocyte subpopulations during intrathymic differentiation. Effects of 17 beta-estradiol and dexamethasone on subsets expressing T cell antigen receptor or IL-2

receptor. *J. Immunol.* **142,** 3378–83 (1989).
50. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294,** 102–110 (2015).
51. Bouman, A., Schipper, M., Heineman, M. J. & Faas, M. M. Gender difference in the non-specific and specific immune response in humans. *Am. J. Reprod. Immunol.* **52,** 19–26 (2004).
52. Rettew, J. A., McCall, S. H. & Marriott, I. GPR30/GPER-1 mediates rapid decreases in TLR4 expression on murine macrophages. *Mol. Cell. Endocrinol.* **328,** 87–92 (2010).
53. Ribas, V. *et al.* Myeloid-specific estrogen receptor alpha deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 16457–62 (2011).
54. Anand, S. S. *et al.* Risk factors for myocardial infarction in women and men: Insights from the INTERHEART study. *Eur. Heart J.* **29,** 932–940 (2008).
55. Cooper, G. S. & Sandler, D. P. Age at natural menopause and mortality. *Ann. Epidemiol.* **8,** 229–235 (1998).
56. Jacobsen, B. K., Nilssen, S., Heuch, I. & Kvåle, G. Does age at natural menopause affect mortality from ischemic heart disease? *J. Clin. Epidemiol.* **50,** 475–479 (1997).
57. Phillips, G. B., Pinkernell, B. H. & Jing, T. Y. The association of hypotestosteronemia with coronary artery disease in men. *Arter. Thromb* **14,** 701–706 (1994).
58. Villablanca, A. C., Jayachandran, M. & Banka, C. Atherosclerosis and sex hormones: current concepts. *Clin. Sci. (Lond).* **119,** 493–513 (2010).
59. Taylor, A. H. & Al-Azzawi, F. Immunolocalisation of oestrogen receptor beta in human tissues. *J. Mol. Endocrinol.* **24,** 145–155 (2000).
60. Arias-Loza, P.-A. *et al.* HHS Public Access. **510,** 84–91 (2015).
61. Wang, M. *et al.* Estrogen receptor beta mediates increased activation of PI3K / Akt signaling and improved myocardial function in female hearts following acute PubMed Commons. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **296,** R972-8 (2009).
62. Murphy, E. Estrogen signaling and cardiovascular disease. *Circulation Research* **109,** 687–696 (2011).
63. Della Torre, S., Benedusi, V., Fontana, R. & Maggi, A. Energy metabolism and fertility: a balance preserved for female health. *Nat. Rev. Endocrinol.* **10,** 13–23 (2014).
64. Cooke, P. S., Heine, P. A., Taylor, J. A. & Lubahn, D. B. The role of estrogen and estrogen receptor-alpha in male adipose tissue. *Mol. Cell. Endocrinol.* **178,** 147–154 (2001).
65. Heine, P. a, Taylor, J. a, Iwamoto, G. a, Lubahn, D. B. & Cooke, P. S. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12729–12734 (2000).
66. Bryzgalova, G. *et al.* Evidence that oestrogen receptor-α plays an important role in the regulation of glucose homeostasis in mice: Insulin sensitivity in the liver. *Diabetologia* **49,** 588–597 (2006).
67. Barros, R. P. A., Machado, U. F., Warner, M. & Gustafsson, J.-A. Muscle GLUT4 regulation by estrogen receptors ERbeta and ER . *Proc. Natl. Acad. Sci.* **103,** 1605–1608 (2006).
68. Ohlsson, C. *et al.* Obesity and disturbed lipoprotein profile in estrogen receptor-alpha-deficient male mice. *Biochem. Biophys. Res. Commun.* **278,** 640–5 (2000).
69. Finan, B. *et al.* Targeted estrogen delivery reverses the metabolic syndrome. *Nat. Med.* **18,** 1847–56 (2012).
70. Barros, R. P. a, Machado, U. F. & Gustafsson, J.-A. Estrogen receptors: new players in diabetes mellitus. *Trends Mol. Med.* **12,** 425–31 (2006).
71. Regitz-Zagrosek, V., Lehmkuhl, E. & Weickert, M. O. Gender differences in the metabolic syndrome and their role for cardiovascular disease. *Clin. Res. Cardiol.* **95,** 136–47 (2006).
72. Nuutila, P. *et al.* Gender and insulin sensitivity in the heart and in skeletal muscles: Studies using positron emission tomography. *Diabetes* **44,** 31–36 (1995).
73. Bonds, D. E. *et al.* The effect of conjugated equine oestrogen on diabetes incidence: The Women's Health Initiative randomised trial. *Diabetologia* **49,** 459–468 (2006).
74. Lundholm, L. *et al.* Key lipogenic gene expression can be decreased by estrogen in human adipose tissue. *Fertil. Steril.* **90,** 44–48 (2008).

75. Price, T. M. *et al.* Estrogen regulation of adipose tissue lipoprotein lipase--possible mechanism of body fat distribution. *Am. J. Obstet. Gynecol.* **178,** 101–7 (1998).
76. Barros, R. P. A. & Gustafsson, J. Å. Estrogen receptors and the metabolic network. *Cell Metab.* **14,** 289–299 (2011).
77. Naaz, a *et al.* Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). *Horm. Metab. Res.* **34,** 758–763 (2002).
78. Homma, H. *et al.* Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter. *J. Biol. Chem.* **275,** 11404–11411 (2000).
79. Weigt, C. *et al.* Molecular effects of ER alpha- and beta-selective agonists on regulation of energy homeostasis in obese female Wistar rats. *Mol. Cell. Endocrinol.* **377,** 147–158 (2013).
80. Wellen, K. E. & Hotamisligil, G. S. Obesity-induced inflammatory changes in adipose tissue. *Journal of Clinical Investigation* **112,** 1785–1788 (2003).
81. Xu, H. *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* **112,** 1821–30 (2003).
82. Kintscher, U. *et al.* T-lymphocyte infiltration in visceral adipose tissue: A primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler. Thromb. Vasc. Biol.* **28,** 1304–1310 (2008).
83. Rogers, N. H., Li, J. W. P., Strissel, K. J., Obin, M. S. & Greenberg, A. S. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology* **150,** 2161–2168 (2009).
84. Riant, E. *et al.* Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. *Endocrinology* **150,** 2109–2117 (2009).
85. Bjornholm, M. & Zierath, J. R. Insulin signal transduction in human skeletal muscle: identifying the defects in Type II diabetes. *Biochem Soc Trans* **33,** 354–357 (2005).
86. Phillips, S. K., Rook, K. M., Siddle, N. C., Bruce, S. a & Woledge, R. C. Muscle weakness in women occurs at an earlier age than in men, but strength is preserved by hormone replacement therapy. *Clin. Sci. (Lond).* **84,** 95–98 (1993).
87. Høeg, L. *et al.* Higher intramuscular triacylglycerol in women does not impair insulin sensitivity and proximal insulin signaling. *J. Appl. Physiol.* **107,** 824–31 (2009).
88. Cortright, R. N. & Koves, T. R. Sex differences in substrate metabolism and energy homeostasis. *Can J Appl Physiol* **25,** 288–311 (2000).
89. Galluzzo, P. *et al.* 17beta-Estradiol regulates the first steps of skeletal muscle cell differentiation via ER-alpha-mediated signals. *Am. J. Physiol. Cell Physiol.* **297,** C1249-62 (2009).
90. Barros, R. P. a, Gabbi, C., Morani, A., Warner, M. & Gustafsson, J.-A. A. Participation of ERalpha and ERbeta in glucose homeostasis in skeletal muscle and white adipose tissue. *Am. J. Physiol. Endocrinol. Metab.* **297,** E124-33 (2009).
91. Foryst-Ludwig, A. *et al.* Metabolic actions of estrogen receptor beta (ERβ) are mediated by a negative cross-talk with PPARγ. *PLoS Genet.* **4,** (2008).
92. Gorres, B. K., Bomhoff, G. L., Morris, J. K. & Geiger, P. C. In vivo stimulation of oestrogen receptor α increases insulin-stimulated skeletal muscle glucose uptake. *J. Physiol.* **589,** 2041–54 (2011).
93. Godsland, I. F. Oestrogens and insulin secretion. *Diabetologia* **48,** 2213–2220 (2005).
94. Liu, S. & Mauvais-Jarvis, F. Minireview: Estrogenic protection of β-cell failure in metabolic diseases. *Endocrinology* **151,** 859–864 (2010).
95. Yamabe, N., Kang, K. S. & Zhu, B. T. Beneficial effect of 17β-estradiol on hyperglycemia and islet beta-cell functions in a streptozotocin-induced diabetic rat model. *Toxicol. Appl. Pharmacol.* **249,** 76–85 (2010).
96. Alonso-Magdalena, P. *et al.* Pancreatic insulin content regulation by the Estrogen receptor ERα. *PLoS One* **3,** (2008).
97. Le May, C. *et al.* Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient

diabetes mellitus in mice. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 9232–7 (2006).

98. Liu, S. *et al.* Importance of extranuclear estrogen receptor-α and membrane G protein-coupled estrogen receptor in pancreatic islet survival. *Diabetes* **58,** 2292–2302 (2009).
99. Soriano, S. *et al.* Rapid regulation of K(ATP) channel activity by 17{beta}-estradiol in pancreatic {beta}-cells involves the estrogen receptor {beta} and the atrial natriuretic peptide receptor. *Mol. Endocrinol.* **23,** 1973–1982 (2009).
100. Shen, M. & Shi, H. Sex Hormones and Their Receptors Regulate Liver Energy Homeostasis. *Int. J. Endocrinol.* **2015,** (2015).
101. Kolovou, G., Damaskos, D., Anagnostopoulou, K. & Cokkinos, D. V. Apolipoprotein E gene polymorphism and gender. *Ann Clin Lab Sci* **39,** 120–133 (2009).
102. Trapani, L., Segatto, M. & Pallottini, V. Regulation and deregulation of cholesterol homeostasis: The liver as a metabolic 'power station'. *World J. Hepatol.* **4,** 184–190 (2012).
103. D'Eon, T. M. *et al.* Estrogen regulation of adiposity and fuel partitioning: Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J. Biol. Chem.* **280,** 35983–35991 (2005).
104. Fisher, C. R., Graves, K. H., Parlow, A. F. & Simpson, E. R. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Med. Sci.* **95,** 6965–6970 (1998).
105. Gao, H., Fält, S., Sandelin, A., Gustafsson, J.-A. & Dahlman-Wright, K. Genome-wide identification of estrogen receptor alpha-binding sites in mouse liver. *Mol. Endocrinol.* **22,** 10–22 (2008).
106. Brandenberger, A. W., Tee, M. K., Lee, J. Y., Chao, V. & Jaffe, R. B. Tissue distribution of estrogen receptors alpha (ER-alpha) and beta (ER-beta) mRNA in the midgestational human fetus. *J. Clin. Endocrinol. Metab.* **82,** 3509–3512 (1997).
107. Meoli, L. *et al.* Sex- and age-dependent effects of Gpr30 genetic deletion on the metabolic and cardiovascular profiles of diet-induced obese mice. *Gene* **540,** 210–216 (2014).
108. Faulds, M. H., Zhao, C., Dahlman-Wright, K. & Gustafsson, J. Å. The diversity of sex steroid action: Regulation of metabolism by estrogen signaling. *J. Endocrinol.* **212,** 3–12 (2012).
109. Lemieux, C. *et al.* Estrogen receptor alpha-mediated adiposity-lowering and hypocholesterolemic actions of the selective estrogen receptor modulator acolbifene. *Int. J. Obes.* **29,** 1236–1244 (2005).
110. Di Croce, L. *et al.* The promoter of the rat 3-hydroxy-3-methylglutaryl coenzyme A reductase gene contains a tissue-specific estrogen-responsive region. *Mol. Endocrinol.* **13,** 1225–1236 (1999).
111. Jones, M. E. *et al.* Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12735–12740 (2000).
112. Nigro, M. *et al.* A change in liver metabolism but not in brown adipose tissue thermogenesis is an early event in ovariectomy-induced obesity in rats. *Endocrinology* **155,** 2881–2891 (2014).
113. Nemoto, Y. *et al.* Altered expression of fatty acid-metabolizing enzymes in aromatase- deficient mice. *J. Clin. Invest.* **105,** 1819–1825 (2000).
114. Sharma, G. *et al.* GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. *Endocrinology* **154,** 4136–4145 (2013).
115. Hussain, Y. *et al.* G-protein estrogen receptor as a regulator of low-density lipoprotein cholesterol metabolism: Cellular and population genetic studies. *Arterioscler. Thromb. Vasc. Biol.* **35,** 213–221 (2015).
116. Feldman, R. D. *et al.* A common hypofunctional genetic variant of GPER is associated with increased blood pressure in women. *Br. J. Clin. Pharmacol.* **78,** 1441–1452 (2014).
117. Darabi, M. *et al.* Increased leukocyte ABCA1 gene expression in post-menopausal women on hormone replacement therapy. *Gynecol. Endocrinol.* **27,** 701–705 (2011).
118. Ciana, P. *et al.* Estrogenic activities in rodent estrogen-free diets. *Endocrinology* **146,** 5144–5150 (2005).
119. Della Torre, S. *et al.* Amino acid-dependent activation of liver estrogen receptor alpha integrates metabolic and reproductive functions via IGF-1. *Cell Metab.* **13,** 205–214 (2011).
120. Della Torre, S. *et al.* An Essential Role for Liver ERα in Coupling Hepatic Metabolism to the Reproductive Cycle. *Cell Rep.* **15,** 360–371 (2016).

121. Li, A. C. & Glass, C. K. PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. *J. Lipid Res.* **45,** 2161–2173 (2004).
122. Zhang, Y. *et al.* Liver LXRα expression is crucial for whole body cholesterol homeostasis and reverse cholesterol transport in mice. *J. Clin. Invest.* **122,** 1688–1699 (2012).
123. Min, H. *et al.* Increased Hepatic Synthesis and Dysregulation of Cholesterol Metabolism Associated with the Severityof Nonalcoholic Fatty Liver Disease. *Cell Metab.* **15,** 665–674 (2012).
124. Gutierrez-Grobe, Y., Ponciano-Rodríguez, G., Ramos, M. H., Uribe, M. & Méndez-Sánchez, N. Prevalence of non alcoholic fatty liver disease in premenopausal, posmenopausal and polycystic ovary syndrome women. The role of estrogens. *Ann. Hepatol.* **9,** 402–409 (2010).
125. Martin, G. B., Rodger, J. & Blache, D. Nutritional and environmental effects on reproduction in small ruminants. *Reprod. Fertil. Dev.* **16,** 491–501 (2004).
126. Baird, D. T. *et al.* Nutrition and reproduction in women. *Hum. Reprod. Update* **12,** 193–207 (2006).
127. Seli, E., Babayev, E., Collins, S. C., Nemeth, G. & Horvath, T. L. Minireview: Metabolism of Female Reproduction: Regulatory Mechanisms and Clinical Implications. *Mol. Endocrinol.* **28,** me20131413 (2014).
128. Essah, P. A. & Nestler, J. E. The metabolic syndrome in polycystic ovary syndrome. *Journal of Endocrinological Investigation* **29,** 270–280 (2006).
129. Gravholt, C. H. Epidemiological, endocrine and metabolic features in Turner syndrome. *European Journal of Endocrinology* **151,** 657–687 (2004).
130. WING, R., MATTHEWS, K., KULLER, L., MEILAHN, E. & PLANTINGA, P. WEIGHT-GAIN AT THE TIME OF MENOPAUSE. *Arch. Intern. Med.* **151,** 97–102 (1991).
131. Schneider, J. E. Energy balance and reproduction. *Physiol. Behav.* **81,** 289–317 (2004).
132. Hagedorn, H. H., Fallon, A. M. & Laufer, H. Vitellogenin synthesis by the fat body of the mosquito Aedes aegypti. Evidence for transcriptional control. *Dev. Biol.* **31,** 285–294 (1973).
133. Chen, J. S., Sappington, T. W. & Raikhel, A. S. Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *J. Mol. Evol.* **44,** 440–451 (1997).
134. DePina, A. S. *et al.* Regulation of Caenorhabditis elegans vitellogenesis by DAF-2/IIS through separable transcriptional and posttranscriptional mechanisms. *BMC Physiol.* **11,** 11 (2011).
135. H.H., H. & A.M., F. Ovarian control of vitellogenin synthesis by the fat body in Aedes aegypti. *Nature* **244,** 103–105 (1973).
136. Tam, S. P., Archer, T. K. & Deeley, R. G. Biphasic effects of estrogen on apolipoprotein synthesis in human hepatoma cells: mechanism of antagonism by testosterone. *Proc Natl Acad Sci U S A* **83,** 3111–3115 (1986).
137. Frykman, P. K., Brown, M. S., Yamamoto, T., Goldstein, J. L. & Herz, J. Normal plasma lipoproteins and fertility in gene-targeted mice homozygous for a disruption in the gene encoding very low density lipoprotein receptor. *Proc. Natl. Acad. Sci.* **92,** 8453–8457 (1995).
138. Villa, A. *et al.* Tetradian oscillation of estrogen receptor α is necessary to prevent liver lipid deposition. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 11806–11 (2012).
139. Mode, A. & Gustafsson, J.-A. Sex and the liver - a journey through five decades. *Drug Metab. Rev.* **38,** 197–207 (2006).
140. Jansson, J. O., Ekberg, S., Isaksson, O., Mode, A. & Gustafsson, J. Å. Imprinting of growth hormone secretion, body growth, and hepatic steroid metabolism by neonatal testosterone. *Endocrinology* **117,** 1881–1889 (1985).
141. Baik, M., Yu, J. H. & Hennighausen, L. Growth hormone-STAT5 regulation of growth, hepatocellular carcinoma, and liver metabolism. *Ann. N. Y. Acad. Sci.* **1229,** 29–37 (2011).
142. Fernández-Pérez, L., Guerra, B., Díaz-Chico, J. C. & Flores-Morales, A. Estrogens regulate the hepatic effects of growth hormone, a hormonal interplay with multiple fates. *Front. Endocrinol. (Lausanne).* **4,** 1–12 (2013).
143. Rando, G. & Wahli, W. Sex differences in nuclear receptor-regulated liver metabolic pathways. *Biochim.*

Biophys. Acta - Mol. Basis Dis. **1812,** 964–973 (2011).

144. Edén, S. Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology* **105,** 555–60 (1979).

145. Isensee, J. & Noppinger, P. R. Sexually Dimorphic Gene Expression in Mammalian Somatic Tissue. *Gend. Med.* **4,** (2007).

146. MacLeod, J. N., Pampori, N. A. & Shapiro, B. H. Sex differences in the ultradian pattern of plasma growth hormone concentrations in mice. *J. Endocrinol.* **131,** 395–399 (1991).

147. Veldhuis, J. D. Neuroendocrine control of pulsatile growth hormone release in the human: relationship with gender. *Growth Horm. IGF Res.* **8 Suppl B,** 49–59 (1998).

148. Waxman, D. J., Pampori, N. A., Ram, P. A., Agrawal, A. K. & Shapiro, B. H. Interpulse interval in circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450. *PNAS* **88,** 6868–72 (1991).

149. Waxman, D. J. & O'Connor, C. Growth Hormone Regulation of Sex dependent Liver Gene Expression. *Molecular Endocrinology* **.,** 2613–2629 (2006).

150. Choi, H. K. & Waxman, D. J. Growth hormone, but not prolactin, maintains low-level activation of STAT5a and STAT5b in female rat liver. *Endocrinology* **140,** 5126–5135 (1999).

151. Udy, G. B. *et al.* Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7239–44 (1997).

152. Teglund, S. *et al.* Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* **93,** 841–850 (1998).

153. Holloway, M. G., Laz, E. V & Waxman, D. J. Codependence of growth hormone-responsive, sexually dimorphic hepatic gene expression on signal transducer and activator of transcription 5b and hepatic nuclear factor 4alpha. *Mol. Endocrinol.* **20,** 647–60 (2006).

154. Alsat, E., Guibourdenche, J., Luton, D., Frankenne, F. & Evain-Brion, D. Human placental growth hormone. *American Journal of Obstetrics and Gynecology* **177,** 1526–1534 (1997).

155. Frankenne, F. *et al.* The physiology of growth hormones (GHs) in pregnant women and partial characterization of the placental GH variant. *J. Clin. Endocrinol. Metab.* **66,** 1171–1180 (1988).

156. Costet, P. *et al.* Peroxisome proliferator-activated receptor alpha-isoform deficiency leads to progressive dyslipidemia with sexually dimorphic obesity and steatosis. *J. Biol. Chem.* **273,** 29577–85 (1998).

157. Lindén, D. *et al.* PPARalpha deficiency increases secretion and serum levels of apolipoprotein B-containing lipoproteins. *J. Lipid Res.* **42,** 1831–1840 (2001).

158. Hewitt, K. N., Pratis, K., Jones, M. E. E. & Simpson, E. R. Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout mouse. *Endocrinology* **145,** 1842–1848 (2004).

159. Cole, L. K., Jacobs, R. L. & Vance, D. E. Tamoxifen induces triacylglycerol accumulation in the mouse liver by activation of fatty acid synthesis. *Hepatology* **52,** 1258–1265 (2010).

160. Chalasani, N., Younossi, Z. & Lavine, J. disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological. … **55,** 2005–23 (2012).

161. Kohjima, M. *et al.* Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int. J. Mol. Med.* **20,** 351–358 (2007).

162. Malaguarnera, M., Di Rosa, M., Nicoletti, F. & Malaguarnera, L. Molecular mechanisms involved in NAFLD progression. *Journal of Molecular Medicine* **87,** 679–695 (2009).

163. Younossi, Z. M. *et al.* The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology* **64,** 1577–1586 (2016).

164. Omagari, K. *et al.* Fatty liver in non-alcoholic non-overweight Japanese adults: Incidence and clinical characteristics. *J. Gastroenterol. Hepatol.* **17,** 1098–1105 (2002).

165. Weston, S. R. *et al.* Racial and ethnic distribution of nonalcoholic fatty liver in persons with newly diagnosed chronic liver disease. *Hepatology* **41,** 372–379 (2005).

166. Clark, J., Brancati, F. & Diehl, A. Non-alcoholic fatty liver disease. *J. Gastroenterol. Hepatol.* **17 Suppl,** S186–S190 (2002).
167. Paquette, A., Wang, D., Jankowski, M., Gutkowska, J. & Lavoie, J.-M. Effects of ovariectomy on PPAR alpha, SREBP-1c, and SCD-1 gene expression in the rat liver. *Menopause* **15,** 1169–1175 (2008).
168. Torre, S. Della *et al.* An Essential Role for Liver ER a in Coupling Hepatic Metabolism to the Reproductive Cycle Article An Essential Role for Liver ER a in Coupling Hepatic Metabolism to the Reproductive Cycle. *Cell Rep.* **15,** 1–12 (2016).
169. Dupont, S. *et al.* Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127,** 4277–4291 (2000).
170. Della Torre, S. *et al.* The conundrum of estrogen receptor oscillatory activity in the search for an appropriate hormone replacement therapy. *Endocrinology* **152,** 2256–2265 (2011).
171. Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real- Time Quantitative PCR and the 2 X○○C T Method. *METHODS* **25,** 402–408 (2001).
172. Sturn, A., Quackenbush, J. & Trajanoski, Z. Genesis: cluster analysis of microarray data. *Bioinformatics* **18,** 207–208 (2002).
173. Benard, O. *et al.* Impact of high-fat diet on the proteome of mouse liver. *J. Nutr. Biochem.* **31,** 10–19 (2016).
174. Carr, M. C. The emergence of the metabolic syndrome with menopause. *Journal of Clinical Endocrinology and Metabolism* **88,** 2404–2411 (2003).
175. Maxwell, S. R. Women and heart disease. *Basic Res. Cardiol.* **93 Suppl 2,** 79–84 (1998).
176. Farhat, M. Y., Lavigne, M. C. & Ramwell, P. W. The vascular protective effects of estrogen. *FASEB J.* **10,** 615–624 (1996).
177. Ghio, A., Bertolotto, A., Resi, V., Volpe, L. & Di Cianni, G. Triglyceride metabolism in pregnancy. *Adv. Clin. Chem.* **55,** 133–153 (2011).