From THE DEPARTMENT MOLECULAR MEDICINE AND SURGERY
Karolinska Institutet, Stockholm, Sweden

ESTROGEN: MOLECULAR MECHANISMS OF ANTIDIABETIC ACTION

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Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by E-Print AB 2016

ISBN 978-91-7676-530-2

Estrogen: Molecular Mechanisms of Antidiabetic Action

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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الحمدلله والشكرعدد خلقه ورضا نفسه وزنة عرشه ومداد كلماته

إلى والدتي و والدي رحمهما الله تعالى

ABSTRACT

The pathogenesis of metabolic syndrome is multifactorial and hormones play an essential role in its development. Estrogen loss in postmenopausal women has been linked to several features of metabolic syndrome, while estrogen replacement therapy reverses these pathological and metabolic features. However, estrogen replacement therapy is associated with adverse effects including breast cancer and coagulopathy. Therefore, identification of the molecular mechanisms of estrogen's beneficial effects would give the possibility to target them directly and to prevent the undesirable side effects. In this study, administration of 17β-estradiol (E2) in a perimenopausal mouse model maintained on a high fat diet (HFD) for 10 months results in improved glycemic control and body weight reduction, in association with multiple molecular mechanisms in the visceral adipose tissue (**Paper 1**) and liver (**Paper 2**) without suppression of inflammatory mediators in these organs (**Paper 3**).

In visceral adipose tissue, E2 administration results in an anti-obesogenic effect and a reduction of adipocyte size in parallel with molecular mechanisms, including the activation of the lipolytic enzyme *pnpla2*, the suppression of lipogenic gene expression possibly through downregulating the expression levels of the nuclear receptor *nr2c2/tr4*, and the induction of brown adipose tissue-specific gene expression via E2-dependent alterations in methylation levels (**Paper 1**). In liver, E2 treatment induces activation of hepatic AMPK, which results in the suppression of hepatic lipogenesis via inhibition of NR2C2/TR4, and in the inhibition of gluconeogenesis through suppression of transcript levels of the gluconeogenic *g6pc*; E2 treatment also normalizes hepatic triglycerides through the suppression of lipogenesis and the activation of triglyceride mobilization; the summative effect of these events results in improvements in hepatic insulin signaling (**Paper 2**).

E2 induces increased expression levels of pro-inflammatory mediators in the visceral adipose, which does not impact upon the positive actions of E2 on body weight, fasting blood glucose levels, and glycemic control (**Paper 3**). The reductions induced by E2 in the plasma levels of adiponectin, and in its mRNA levels in adipose tissue, could explain the continued elevated expression levels of these mediators. Tracing the events of metabolic pathogenesis due to ovariectomy-related estrogen loss in an ovariectomized mouse model shows that estrogen signaling appears to suppress features of hepatic insulin resistance resulting from short-term HFD exposure by opposing the continuous accumulation of hepatic triglycerides, and via reduced expression levels of gluconeogenic genes (**Paper 4**). Ovariectomy resulted in weight gain, elevation of fasting blood glucose levels, increased hepatic triglycerides and increases in expression levels of *nr2r2/tr4* and the pro-inflammatory *tlr2*, independently of dietary intervention.

In conclusion, the current study highlights significant molecular mechanisms responsible for the effects of estrogen in relation to features of metabolic syndrome, giving the possibility to target them directly and to prevent the undesirable side effects of systemic E2 treatment.

LIST OF SCIENTIFIC PAPERS

I. **Saad Misfer Al-Qahtani**, Galyna Bryzgalova, Ismael Valladolid-Acebes, Marion Korach-André, Karin Dahlman-Wright, Suad Efendić, Per-Olof Berggren, Neil Portwood (2016). 17β-Estradiol Suppresses Visceral Adipogenesis and Activates Brown Adipose Tissue-Specific Gene Expression. Horm Mol Biol Clin Investig. 10.1515/hmbci-2016-0031

II. **Saad Misfer Al-Qahtani**, Galyna Bryzgalova, Suad Efendić, Per-Olof Berggren, Neil Portwood (2016). Activation of hepatic AMPK by 17β-estradiol suppresses both nuclear receptor NR2C2/TR4 and its downstream lipogenic targets, reduces gluconeogenic genes and improves insulin signaling. Dis Mol Med, 4(4), 55-67.

III. **Saad Misfer Al-Qahtani**, Galyna Bryzgalova, Per-Olof Berggren, Neil Portwood. 17β-estradiol reduces body weight and improves glycemic control without suppression of inflammatory mediators in plasma, adipose tissue and liver. Manuscript.

IV. **Saad Misfer Al-Qahtani**, Galyna Bryzgalova, Per-Olof Berggren, Neil Portwood. Ovariectomy results in a continuous accumulation of hepatic triglycerides and the upregulation of inflammatory mediators following short-term exposure to high-fat diet. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ACC1	Acetyl-CoA carboxylase
AdipoQ	Adiponectin
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ADRβ3	Beta-3 adrenergic receptor
AMPK	5' adenosine monophosphate-activated protein kinase
ARKO	Aromatase knockout
BERKO	Estrogen receptor β knockout
CD36	Fatty acid translocase
CD68	Cluster differentiation 68
CD74	HLA class II histocompatibility antigen gamma chain
CIDEA	Cell death-inducing DNA fragmentation factor
Cox8b	Cytochrome c oxidase, subunit VIIIb
CPT1b	Carnitine palmitoyltransferase 1b
CPT2	Carnitine palmitoyltransferase 2
CRP	C-reactive protein
CYP7α1	Cholesterol 7a-hydroxylase (cytochrome P450 7A1)
DIO-2	Type II iodothyronine deiodinase
E2	17β-estradiol
ERKO	Estrogen receptor α knockout
ERs	Estrogen receptors
ERT	Estrogen replacement therapy
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FAS	Fatty acid synthase
FOXO	Forkhead box protein
G6PC	Glucose 6 phosphatase catalytic
G6PC3	Glucose 6 phosphatase catalytic 3
G6PT1	Glucose 6 phosphatase transport 1

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HFD	High fat diet
IHC	Immunohistochemistry
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit β
IL	Interleukin
IRS	Insulin receptor substrate
LDLR	Low density lipoprotein receptor
Lep	Leptin
LepR	Leptin receptor
LFD	Low fat diet
LIPE	lipase, hormone sensitive
LXRα	Liver X receptor
MCP1	Monocyte chemoattractant protein 1
MHC2	Histocompatibility 2, class II antigen A, beta 1
MRI	Magnetic resonance imaging
NR2C2/TR4	Nuclear receptor subfamily 2, group C, member 2
OVX	ovariecmized
PC	Pyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1α	Peroxisome proliferator receptor gamma coactivator 1-alpha
Pnpla2	Patatin-like phospholipase domain containing 2
Ppar	Proliferator-activated receptor
SCD1	Stearoyl-CoA desatturase 1
SERM	Selective ER modulators
SHAM	Sham-operated
SREBP1	Sterol regulatory element binding protein 1c
SS	Sodium salicylate
Stat3	Signal transducer and activator receptor 3
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
UCP-1	Uncoupling protein 1

1 INTRODUCTION

1.1 HORMONES AND HORMONE RECEPTORS

Integration between the endocrine and nervous systems is essential for regulation and coordination of biological process such as growth, differentiation, metabolism and reproduction [1]. Via neurotransmitters, the nervous system controls various rapid and localized effects, while the endocrine system acts through hormones to produce generalized effects on the whole body. Disruption of the endocrine system due to loss of hormones results in pathological conditions [1], and replacement therapy is one of the main pharmacological strategies to reverse the pathogenesis of these pathological conditions.

Hormones are chemical messengers which are synthesized and produced by glands, and which are carried through blood to distant tissues to exert specific biological functions. There are four chemical categories of hormones: amino acid derivatives, fatty acid derivatives, polypeptides and steroid hormones. Hormones exert their actions through binding to specific receptors in the target tissue. The receptors are expressed either on membranes, in which case they are known as membrane receptors, or intracellularly, and known as intracellular receptor [2].

Hormones with a hydrophilic (water-soluble) nature, such as peptide hormones, react with membrane-bound receptors and act through the activation of second messengers which regulate the function of proteins and transcription factors (Figure 1). Hydrophobic (lipid-soluble) hormones, unlike hydrophilic hormones, can cross the phospholipid layer of cell membranes and interact with intracellular receptors. Hydrophobic hormones, such as steroids, bind to intracellular receptors, often resulting in the regulation of transcription of specific genes (Figure 2).

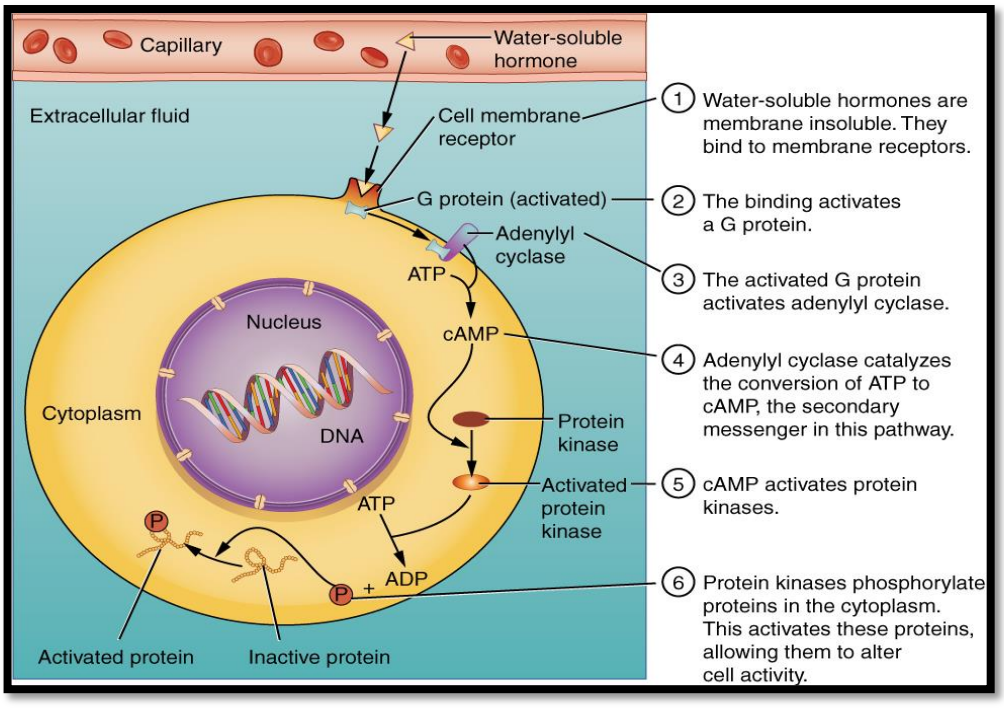

Figure 1: Hydrophilic hormones (CC BY 3.0 by OpenStax CNX, https://courses.lumenlearning.com/ap2/chapter/hormones/)

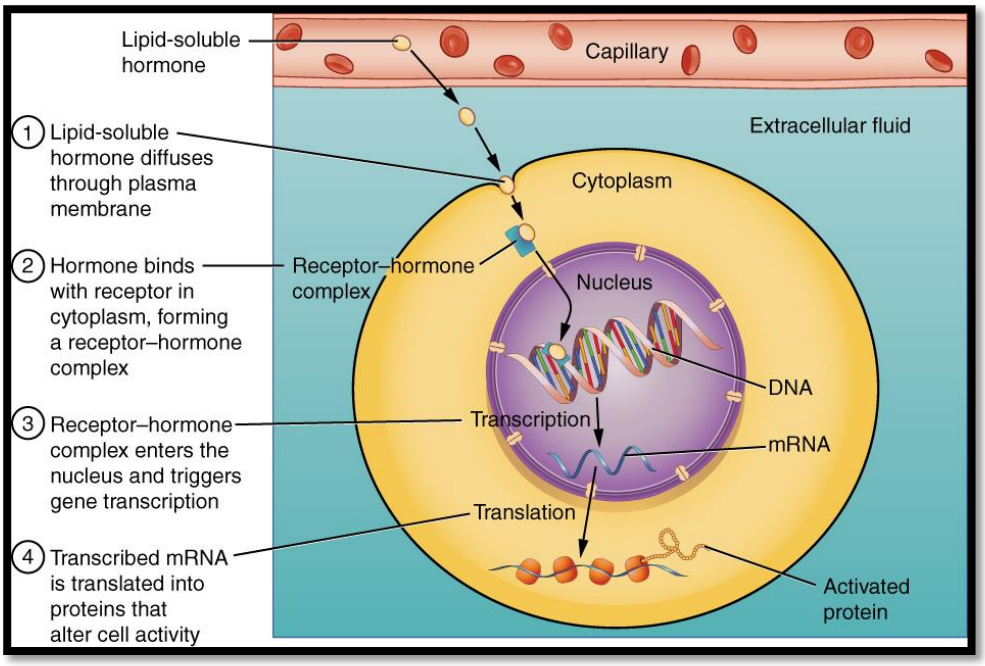

Figure 2: Hydrophobic hormones (CC BY 3.0 by OpenStax CNX, https://courses.lumenlearning.com/ap2/chapter/hormones/)

1.2 ESTROGENS

Estrogens are a steroid compounds derived from cholesterol which function as a primary sex hormone in females. However, estrogens are found also in males and control sexual behaviors. Moreover, during the few last decades, estrogens have been linked to non-gender effects beyond the induction of female secondary sexual characteristics and the control of male sexual behaviors. Estrogens are reported to exert direct or indirect regulatory roles on growth, differentiation and function in different tissues, including the brain, bone, adipose tissue, glands and cardiovascular systems [3]. There are three major types of estrogens: estrone (E1), 17β-estradiol (E2) and estriol (E3). Biochemically, aromatase cytochrome P450 is the enzyme which converts androstenedione to E1, after which E1 is converted to E2. As a precursor for sexual steroids, androstenedione is converted to testosterone which is ultimately aromatized to E2 [4].

1.2.1 Estrogen receptors

Estrogen receptors (ER) exist as two isoforms: ERα and ERβ. They are nuclear receptors which are known as ligand-activated transcription factors. ERs have a structure typical of nuclear receptors, consisting of five domains. Each domain represents a specific function, many of which are known [5] (Figure 3): an N-terminal A/B domain, a DNA binding C domain, domain D, domain E, and a C-terminal F domain. The A/B domain activates the transcription of genes independently of ligand binding, while the E domain is the ligand-binding domain. The D domain is a hinge region between the C DNA-binding domain and the E domain, which is responsible for ligand binding. The F domain is highly variable between nuclear receptors, and has been reported to stimulate and suppress transcription [6]. Transcriptional activity of ERα is mediated by both the A/B domain, which is also known as activation function 1 (AF-1), and the E domain, also called activation function 2

(AF-2), while that ERβ-mediated transactivation is mediated to a greater degree through AF-2, with less involvement of AF-1 [7].

ERα and ERβ are expressed in many tissues in males and females, including the ovaries, testes, prostate, liver, adipose tissue, hypothalamus, brain, muscle, pituitary gland, vascular endothelium and bone marrow. However, the ERα/ERβ ratio is variable in different tissues and usually localized in specific cell type or histological layer when they are expressed in the same tissue [8].

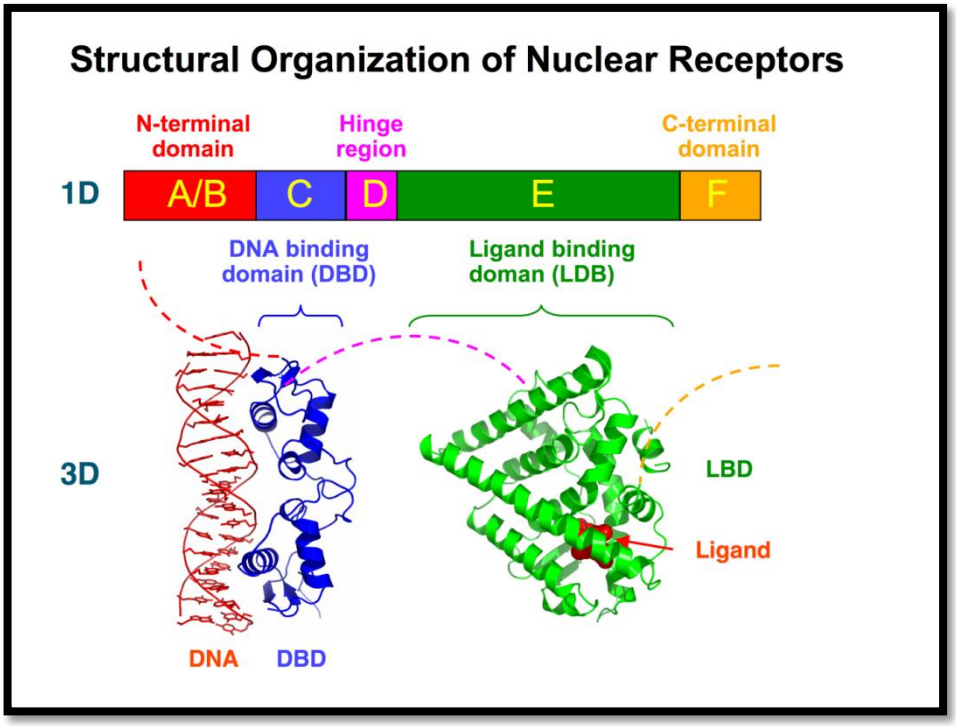

Figure 3: Nuclear receptor (CC BY-SA 3.0 by Boghog2, https://commons.wikimedia.org/wiki/File:Nuclear_Receptor_Structure.png)

1.2.2 Mechanisms of estrogen actions

E2 exerts its action via genomic and non-genomic mechanisms with different pathways [9, 10].

Genomic mechanisms:

The first genomic mechanism of estrogen action is a conventional mechanism in which activation of ER by estrogen leads to its dissociate from heat-shock protein, which normally keeps ER in an inactive form [11]. Consequently, ER is translocated into the nucleus and binds to estrogen response elements (EREs) in the promoters of target genes, leading to subsequent activation or inhibition of transcription [9, 10] .

The second genomic mechanism is a ligand-independent mechanism during which intracellular kinase pathways are activated, leading to the phosphorylation and activation of ER at EREs in gene promoters in a ligand-independent style [10].

The third genomic mechanism is ERE-independent, in which estrogen-ER complexes mediate the transcription of genes containing alternative response elements through

combination with other DNA-bound transcription factors which can associate with the activated ER, leading to an up-regulation of gene expression [10].

Non-genomic mechanism:

In this mechanism, no mRNA or protein is synthesized, and estrogens induce cellular effects independently of the transcriptional activity of ERs [12]. These effects involve rapid signaling which leads to different signal transduction pathways exclusively in estrogen-sensitive cells [13].

1.2.3 Approaches to study estrogen/estrogen receptor signaling

There are different investigative approaches to study estrogen/estrogen receptor signaling in E2 target tissues in both physiological and pathological conditions. These approaches are classified as transgenic experimental models, non-transgenic experimental models and selective ERs modulators.

Transgenic models are represented by genetically modified mice with disrupted ERα and/or ERβ, and are powerful tools for the assessment of estrogen/estrogen receptor signaling. ERα knockout (ERKO) mice were generated firstly in 1993 [14], while ERβ knockout (BERKO) mice were first described in 1998 [15, 16]. Furthermore, in 1998, another knockout model was created by disruption of the aromatase gene (ARKO mice) [17]. This mouse model expresses ERs, but cannot synthesize estrogens. Finally, organ-selective ER knockout mice have been created, such as liver-selective ERα knockout (LERKO) mice [18].

Examples of non-transgenic models for the study of estrogen/estrogen receptor signaling include a long-term high fat diet (HFD) feeding model, which is a model of perimenopause and mimics aging-related menopause [19]. A second model is the ovariectomized (OVX) mice mouse, which mimics surgical menopausal hormone loss; sham-operated mice (SHAM) used as controls for this model, and are subjected to all of the surgical procedures as in OVX mice, except for ovariectomy [20].

Selective ER modulators (SERM) are a group of agonists and antagonists that bind to the ERs as ligands and lead to the activation or inhibition of transcription of target genes [21]. Tamoxifen is a SERM which is used as an antagonist treatment for breast cancer and as an agonist in the uterus and bone [22, 23]. Raloxifen is another SERM with a highly selective action which is used in the prevention of postmenopausal osteoporosis [24].

1.3 ESTROGENS IN DIAGNOSTIC PATHOLOGY

In clinical and anatomic pathology, estrogens are investigated to diagnose and follow up hypo- and hyper-estrogenism - associated diseases/conditions.

1.3.1 Estrogen in diagnostic clinical pathology

In clinical pathology, assessment of serum levels of E1, E2 or both is needed in a variety of lab diagnostic tests in males and females. The reference values are determined in correspondence with age and sex (Table 1) [25, 26].

Table1: References values of Estrone and Estradiol [25]

Estrogen	Age	Sex	Reference Value
E1	2 weeks-18 years	M	Undetectable up to 10 pg/mL -60 pg/mL
E1	2 weeks-14.5 years	F	29 pg/mL-200 pg/mL
E1	Adult > 18 years	M	10-60 pg/mL
E1	Adult > 14 years	F	Premenopausal: 17-200 pg/mL Postmenopausal: 7-40 pg/mL
E2	2 weeks-18 years	M	Undetectable up to 10 pg/mL-40 pg/mL
E2	2 weeks-14.5 years	F	24 pg/mL-350 pg/mL
E2	Adult > 18 years	M	10-40 pg/mL
E2	Adult > 14 years	F	Premenopausal: 15-350 pg/mL** Postmenopausal: <10 pg/mL
			*Estrone and estradiol levels in newborns (1-14 days) are very high at birth but will drop to prepubertal levels

The clinical significance of serum E1 and E2 assays includes but is not limited to [26-30]: (1) diagnosis and follow up of early and delayed puberty in females; (2) diagnosis and follow up of suspected disorders of sex steroid metabolism such as 17-alpha-hydroxylase deficiency and aromatase deficiency; (3) monitoring hormone replacement therapy in postmenopausal women or anti-estrogen therapy in cancers; (4) knowledge of serum E2 levels is important to evaluate hypogonadism and oligomenorrhea; (5) E2 levels are crucial during *in vitro* fertilization to assess ovary and follicle development and ovulation; (6) E2 levels are useful in evaluation of feminization in males and in diagnosis of estrogen-producing tumors.

Irregular menstrual cycles with high E2 levels are indicative of possible polycystic ovarian syndrome, or of the presence of androgen or estrogen producing tumors, while gynecomastia and high E1 and E2 levels in adult males are signs of liver disease or estrogen-androgen producing tumors e.g. prostate carcinoma [25].

1.3.2 Estrogen in histopathology

In anatomic pathology and histopathology, steroid hormone receptor states in breast cancer are assessed mainly by semi-quantitative immunohistochemistry (IHC) and molecular expression profiling [31]. The clinical significance of assessment of ER levels is the substantial benefit of anti-estrogenic hormone therapy for ER-positive cancers, since more than 2/3 of ER-positive tumors are responsive to hormonal manipulation with good prognosis, compared to veer-negative tumors [25, 31]. ER nuclear staining is interpreted as negative if reactive cells constitute less than 1% of the total, as focally positive if reactive cells constitute 1-10% of the total, and as positive if the reactive cells constitute more than 10% of the total [25].

1.4 METABOLIC SYNDROME: MOLECULAR PATHOGENESIS AND ESTROGEN'S ROLE

Metabolic syndrome is characterized by group of clinical manifestations include obesity, dyslipidemia, hypertension, insulin resistance and a pro-inflammatory state; affected patients with metabolic syndrome are at a high risk to develop type 2 diabetes (T2D) [32]. The pathogenesis of metabolic syndrome is multifactorial and hormones play an essential role in its development. Many clinical and basic research studies have reported a correlation between E2 and features of metabolic syndrome [33]. The pathogenesis of metabolic syndrome involves multiple mechanisms in different tissues that express ERs (Figure 4), including the brain, adipose tissue, liver, skeletal muscle and pancreas [34].

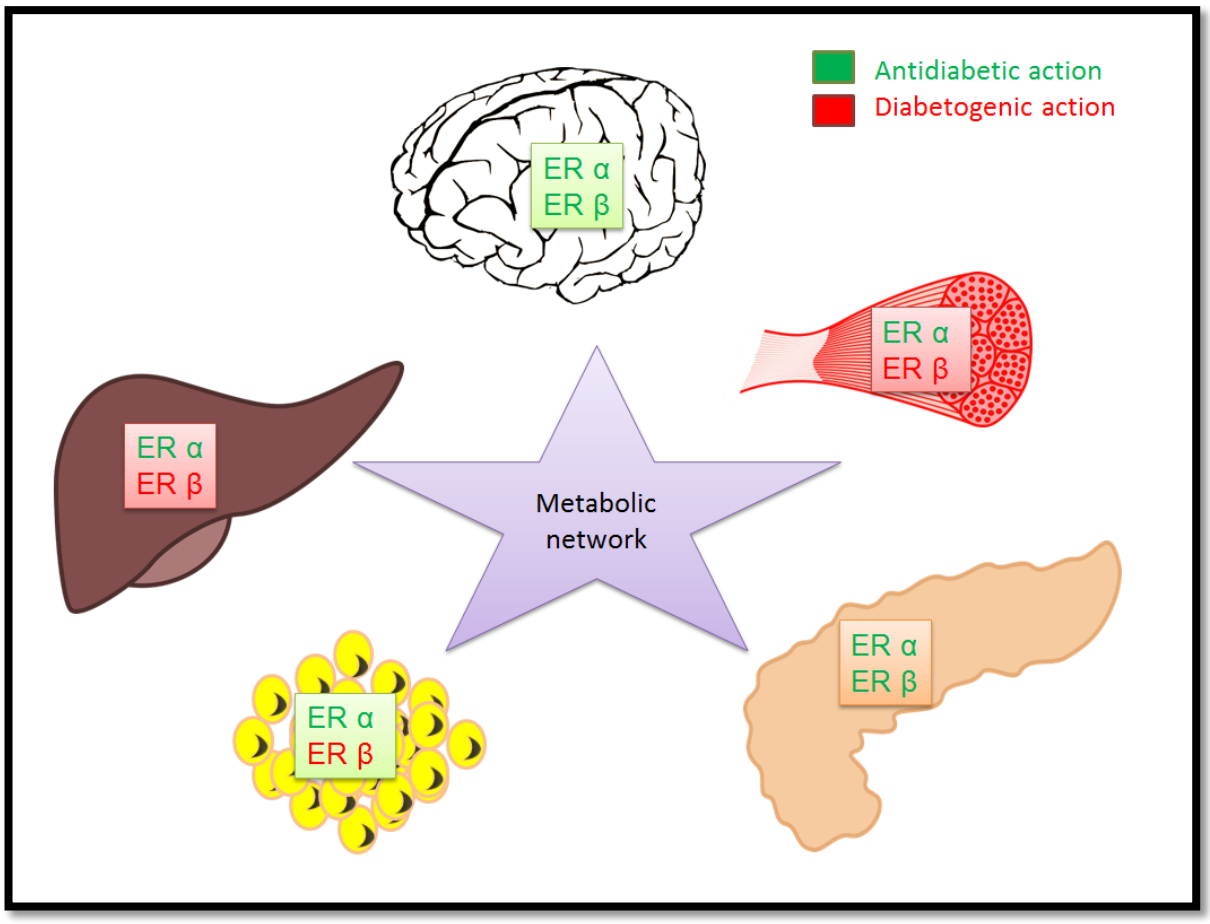

Figure 4: Metabolic network and estrogen receptors

1.4.1 Estrogen and the brain

The brain has a role in regulation of metabolism through signals including hormones, cytokines and nutrients. In women, higher levels of E2 due to physiological or pathological conditions lead to decreased food intake and suppressed fat accumulation. Conversely, surgical or age-related menopause leads to an increase in food intake, an effect that is reversed by estrogen replacement therapy (ERT). Such observations show that E2 has an appetite-regulatory function in the central nervous system. Experimentally, intracranial injection of E2 in rats leads to loss of appetite. It is now known that these effects demonstrate an interactive axis between E2 and the hypothalamus [35]

E2 interacts with the hypothalamus through direct central mechanisms and indirect mechanisms. The central mechanisms involve activation of the pro-opiomelanocortin (POM) neurons, which express ERα and have anorexigenic effects through release of α-melanocyte-stimulating hormone and cocaine & amphetamine-regulated transcript which target receptors of the melanocortin system, mainly isoforms 3 and 4 [36]. In ERKO mice, there is no upregulation of POM, which results in increased food intake [37]. Another central mechanism involves the activation of polypeptide neurons which express both ERα and ERβ and oppose the role of POM through release of polypeptides which prevent binding between α-melanocyte-stimulating hormone and receptors of the melanocortin system resulting in increased food intake. Both of POM and polypeptide neurons are part of the arcuate nucleus in the hypothalamus. ERα and/or ERβ are expressed in other hypothalamic areas involved in hunger and satiety, such as the lateral, ventromedial and dorsomedial hypothalamic regions. It is thought that the central actions of E2 on metabolism result from changes in feeding behavior rather than modulation of the metabolism of carbohydrates and lipids [34].

In addition to the central actions mentioned above, E2 also regulates peripheral hormones which regulate appetite and feeding through their actions in the brain. The regulation of these hormones by E2 is considered as an indirect mechanism through which E2 interacts with hypothalamus. These hormones include leptin, insulin, glucocorticoids and ghrelin. For instance, ERα and leptin receptor colocalize in the hypothalamus mainly in the arcuate nucleus [38], and E2 affects expression of leptin receptor, which results in increased hypothalamic sensitivity to leptin and ultimately affects body fat content [39]. E2 also affects body fat distribution by modulating insulin sensitivity and mRNA levels of both insulin and glucocorticoid receptors in the hypothalamus [40].

1.4.2 Estrogen and adipose tissue

Adipose tissue is a loose connective tissue which participates in metabolism, and has endocrine and immune roles. The size of the adipose tissue is considered as a mirror of the balance between intake and expenditure of energy [41, 42]. Central obesity and increased visceral adiposity are linked to pathogenesis and features of metabolic syndrome including insulin resistance, hypertension, and dyslipidemia. Clinically, the incidence of obesity and obesity-related disorders are higher in men, compared with women. However, after menopause, there is increased visceral fat mass and an increased risk for developing cardiovascular diseases and insulin resistance [43]

In animal models, E2 treatment of female mice results in a reduction of body weight in association with decreased expression of lipogenic genes in adipose tissue [19]. Another study links E2-induced reductions in body fat in ovariectomized rats to activation of 5'AMP-activated protein kinase (AMPK), increased uncoupling protein (UCP) and decreased the expression of resistin [44]. Moreover, the administration of tamoxifen as an anti-estrogen to ERKO and BERKO mice resulted in decreased GLUT4 expression in BERKO mice, but not in ERKO mice, suggesting that ERα regulates GLUT4 expression in adipose tissue [45]. Finally, ovariectomy of ERKO mice reduced body fat significantly, giving a conclusion that ERα and ERβ have opposite functions on fat metabolism [46].

1.4.3 Estrogen and the liver

The liver plays a major role in metabolism, possessing many functions including glycogen storage, plasma protein & clotting factor synthesis, hormone production and detoxification.

Disturbances in liver function are related to insulin resistance, hyperglycemia and dyslipidemia. Liver function tests and lipid profiles reflect the activity of the liver. Abnormal lipid profiles, characterized by lower HDL levels and higher LDL and triglyceride levels, are more common in males, compared with females. In addition, studies reveal that the incidence of fatty liver is higher in males than in females. However, after menopause, the frequency of abnormal lipid profiles and fatty liver disease increases in females, compared with males [47, 48]. In postmenopausal women, the consequences of ERT in postmenopausal women and E2 specifically increase HDL, decreased LDL, reduced triglycerides and lowered fasting glucose levels [49]. In contrast, anti-estrogen therapy leads to an abnormal lipid profile and steatosis, increasing the risk of diabetes and metabolic syndrome [50].

Experimentally, it has been reported that ERα is the main ER isoform in liver [51], and many studies on mice models have attempted to elucidate the participation of ERs in hepatic metabolism. Previously, we have shown that in the livers of ERKO mice, there is increased glucose production and elevated expression of genes involved in hepatic triglyceride synthesis [52]. Studies using the ERα agonist, propyl pyrazole triol (PPT), showed increased expression of signal transducer and activator of transcription-3 (*stat3*) in the livers of wild-type mice [51], and the activation of hepatic Stat3 has been reported to have an antidiabetic effect [53]. A study on ob/ob mice showed that these mice respond to PPT with improved glucose tolerance and insulin sensitivity in association with suppression of mRNA levels of glucose-6-phosphatase (*g6p*) and vLDL receptor [54]. Similarly, these ERα-mediated effects of E2 are also shown in HFD mice [55].

Hepatic inflammation is considered as a pathogenic feature of insulin resistance. Treatment of WT, ERKO and BERKO mice with an inflammatory inducer, interleukin (IL) 1β and E2, showed that E2 suppressed the expression of cytokines and transcription factors in WT and BERKO mice, but not in ERKO mice, suggesting that ERα mediates E2-induced anti-inflammatory effect in the liver [56].

Finally, ERβ has metabolic functions in the liver also. BERKO mice fed HFD had lower triglyceride levels and improved hepatic insulin sensitivity in association with increased insulin mediated-Akt phosphorylation, suggesting that ERβ has a diabetogenic role [57].

1.4.4 Estrogen and skeletal muscle

Skeletal muscle is the main organ responsible for glucose uptake in response to binding of insulin to its receptor in the skeletal muscle. Insulin activates a phosphorylation cascade that involves several proteins and leads to the translocation of GLUT4 from the cytoplasm to the cell membrane and allows transport of glucose into the cell. In the physiological range, E2 is beneficial for muscular insulin sensitivity, but low or high levels of E2 are linked to insulin resistance [58].

Skeletal muscle expresses both ERs, and in mice ERβ is the main isoform [59, 60]. Previously, it has shown that in BERKO mice, both glucose tolerance and insulin release

are normal or better than in wild-type mice, and in the absence of ERα there is decreased glucose uptake in muscle [52]. When ARKO male mice are treated with a selective ERβ agonist, diarylpropionitrile, there is a decrease in GLUT4 expression in muscle [59]. Tamoxifen has no effect on glucose tolerance in muscle in wild-type or BERKO mice, but in ERKO mice, tamoxifen increased GLUT4 expression and improved insulin sensitivity [45]. In the same context, treatment of a myoblast cell line with PPT increased GLUT4 translocation to the cell membrane [61]. Treatment of ovariectomized rats with PPT also increased glucose uptake due to high GLUT4 expression in skeletal muscle [62]. In summary, these data suggest an inhibitory action of ERβ and stimulatory role for ERα on GLUT4 expression.

1.4.5 Estrogen and the pancreas

ERs are key players in the physiology and pathology of the pancreas. They have essential and important roles in the proliferation, differentiation and survival of β cells, in addition to the regulation of insulin synthesis and release. Both ERs have been demonstrated in the nucleus and cell membrane of β cells [63]. A study performed to confirm the role of ERs in the regulation of pancreatic β cell function showed that E2 increased insulin secretion and reduced glucose plasma levels through both classical transcriptional activation, and via a non-classical pathway that involved membrane ERs; the classical pathway was suppressed by ER antagonists, whilst the non-classical pathway was not [63]

In BERKO mice, no changes have been observed in insulin or glucose levels, but increased islet size has been reported, suggesting the importance of ERβ in regulating islet size *in vivo* [45]. We have shown previously that ERKO mice have increased fasting insulin and glucose levels which may link the absence of ERα to islet dysfunction [52]. Furthermore, the importance of ERα is illustrated in studies of islets isolated from wild-type, ERKO, and BERKO mice. When islets were treated with E2, a strong insulin secretion was reported in cells from wild type and BERKO mice, but not in those from ERKO mice.

E2 exerts anti-apoptotic effects on β cells [64]. In ARKO mice, there is increased apoptosis of β cells, and treatment of these mice with E2 prevented apoptosis and avoided the development of diabetes. Similarly, in ERKO mice, the protective effects of E2 were reduced, and based on this, ERα appears to protect β cells against apoptosis [64].

1.5 INFLAMMATION

1.5.1 Definition and classification

Inflammation is a vascular and cellular response which protects against stimuli of cell injury. It is classified as either acute or chronic (Table 2), and is mediated through chemical mediators which are released by host inflammatory cells [65].

Table 2: Acute vs. chronic inflammation

Inflammation	**Acute**	**Chronic**
Duration	Hours	Days (prolonged)
Cellular events mediated by	Neutrophils	Monocytes and macrophages Eosinophils: parasitic infection Lymphocytes: viral disease
Vascular events	Classical vascular response without angiogenesis	Classical vascular response with angiogenesis
Outcome and fate	Resolution and repair, or progress to chronic	Resolution in association with tissue remodeling and repair, or it persists and associated with scarring, systemic dysfunctions and homeostasis loss

1.5.2 Inflammatory cells and mediators

Inflammation is mediated by cellular infiltration, consisting of mainly neutrophils in acute inflammation, and by macrophages in chronic inflammation. These cellular events are triggered by chemical mediators, and they are classified into two main categories (Figure 5): cell-derived mediators and plasma-derived mediators [65]. Cell-derived mediators are released by leukocytes, macrophages, lymphocytes, endothelial and mast cells and include: vasoactive amines, arachidonic acid derivatives, cytokines and nitric oxide. Plasma-derived proteins are released mainly by liver and include: kinin, complements, coagulation protein and fibrinolytic protein [65]. All of these different mediators are responsible for the cardinal/external features of inflammation: heat, redness, swelling, pain, and loss of function.

Cytokines include polypeptides such as interleukin (IL) and tumor necrosis factor (TNF), and play an essential role as pro-inflammatory (IL1, IL6) and anti-inflammatory (IL-10) mediators. Arachidonic acids are metabolized by two metabolic pathways: cyclooxygenase

and lipoxygenase [65]. As a result of cyclooxygenase, different prostaglandins and thromboxane A2 are formed and lead to different vascular/platelets events [65]. The coagulation factor XII (Hageman factor) typically initiates the actions of plasma-derived mediators through the activation of four cascades: fibrinolysis, coagulation, kinin and complement system [65].

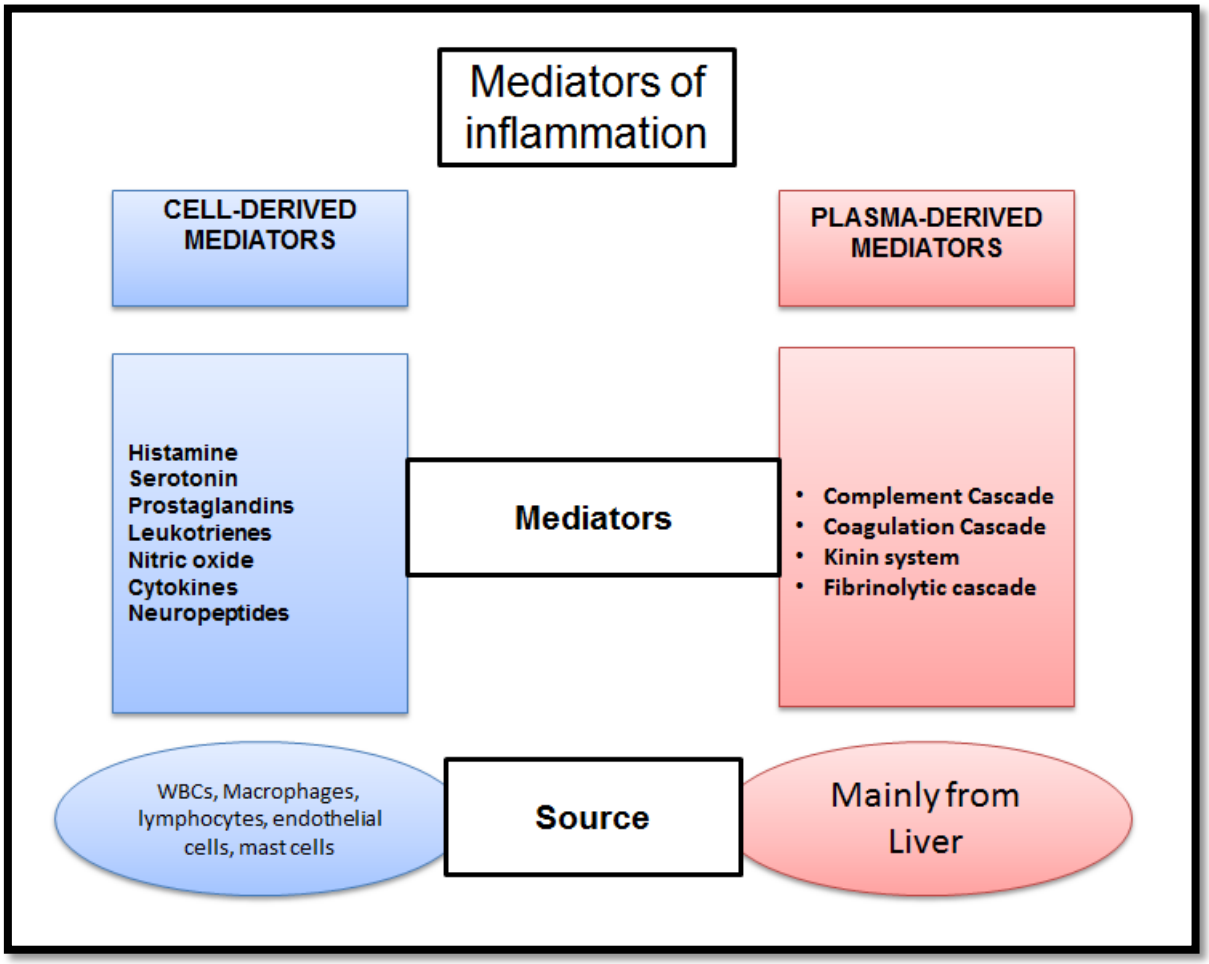

Figure 5: inflammatory mediators

1.5.3 Anti-inflammatory mechanisms

Although inflammation is a defense mechanism against detrimental stimuli and initiates repair, the inflammatory process and subsequent repair/remodeling events can cause harm and complications. Thus, mediators of inflammation and their molecular mechanisms of its action are the focus of studies with the goal of preventing and treating chronic diseases associated with inflammation [65]. Glucocorticoids/steroids and non-steroidal anti-inflammatory drugs are the most frequent used treatments (Figure 6).

The anti-inflammatory effects of glucocorticoids involve molecular mechanisms which mainly result in the suppression of phospholipase [65]: these include direct effects on gene expression, or indirect effects through interaction with transcription factors. However, since the anti-inflammatory mechanisms of glucocorticoids involve interruptions in other

physiological-molecular cascades, this treatment modality is accompanied by severe systemic side effects [66].

Non-steroidal anti-inflammatory drugs suppress cyclooxygenase 1and 2 selectively and non-selectively with less complications, compared to steroids [67]. Using of non-steroidal treatments has been reported to show positive effects on some chronic diseases associated with mild degrees of inflammation, such as improved glycemic control and reduced HbA1c in diabetic patients [68].

Finally, physical exercise has been reported to suppress inflammation through myokines, for example IL15, which is released by muscles [69] . In addition, the coagulation factor XII has been suggested to be a target to reduce thrombosis and inflammation [70].

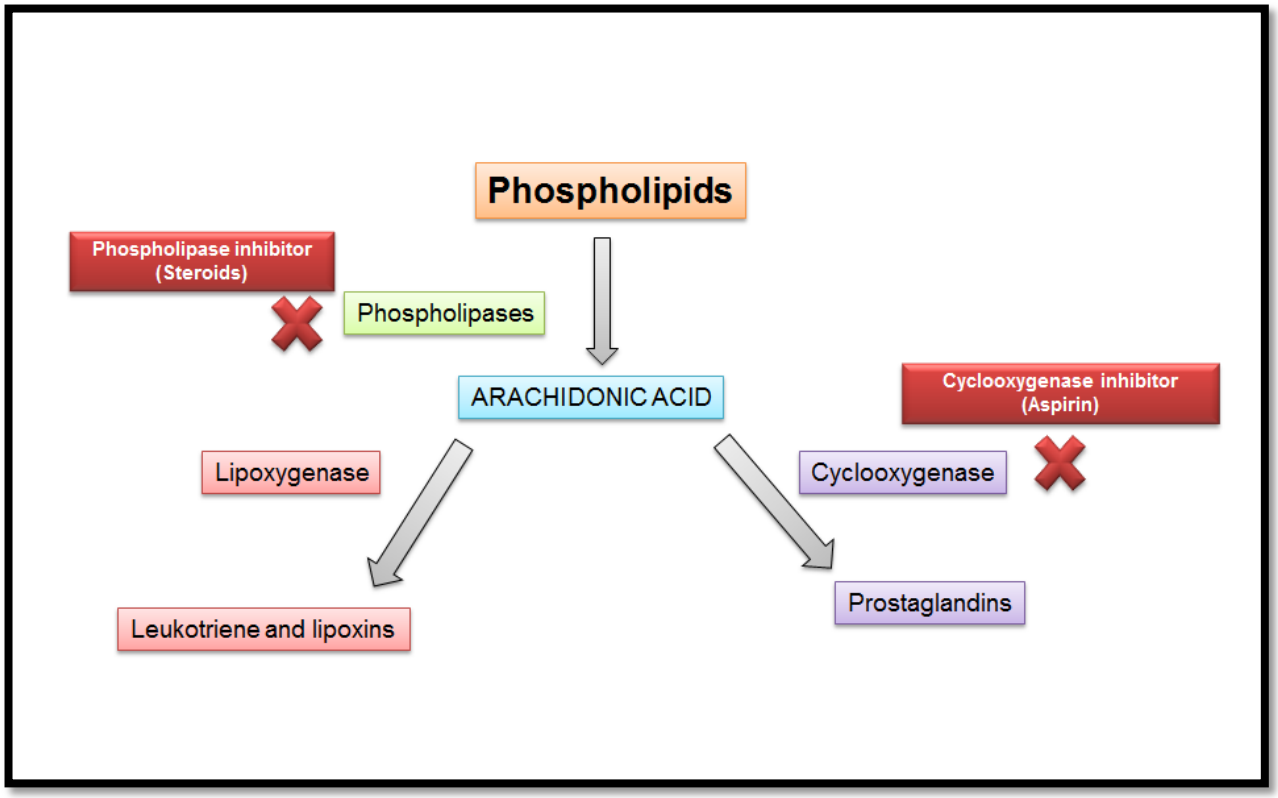

Figure 6: Anti-inflammatory mechanisms

1.5.4 Inflammation and the role of estrogen

Obesity is one of the clinical manifestations of metabolic syndrome, and is associated with chronic low-grade inflammation in the liver and adipose tissue, features which are recognized as an important link between obesity and insulin resistance [71]. Menopause is considered to be an additional risk factor for obesity in females, compared with males [72]. A deficiency of circulating estrogen levels is associated with a shift towards an abdominal fat distribution which predisposes women to metabolic syndrome and T2D [73, 74]. The antidiabetic effects of E2 and ERT have been demonstrated by various clinical studies [75-77], and also by experimental studies [19, 78, 79]. However, the changes in the immune system which occur as result of estrogen deficiency and during subsequent ERT are not fully clear [80, 81]. For example, clinical trials [82, 83] have reported that ERT results in increases of serum (c-reactive protein) CRP levels in postmenopausal women. Similarly,

studies showed that E2 treatment led to increases in the levels of CRP, together with concomitant increases in the levels of IL6, in healthy postmenopausal women [84]. In contrast, it has been shown that E2 suppresses circulating CRP levels in postmenopausal women with T2D [85]. Experimentally, elevated levels of plasma IL6 have been reported previously in ovariectomized mice due to fatty feeding, while plasma levels of TNFα were unchanged [86]. One of the suggested explanations, the aging process and the onset of menopause, may be relevant to the determination of the exact role of E2 on inflammation, since it has been shown E2 inhibits inflammation in cells derived from young mice, but not in cells from aged mice, supporting an age-specific action for E2 [87, 88].

1.6 TYPE 2 DIABETES

1.6.1 Background

Generally, about 10% of all diagnosed adult diabetics are categorized as having type l diabetes (T1D), while T2D accounts for 80% of all diagnosed diabetes [2]. In addition, there are other types of diabetes (10%) such as mitochondrial diabetes and gestational diabetes [2].

T1D develops because of a loss of insulin production due to the autoimmune destruction of pancreatic beta cells. Included in the T1D category is latent autoimmune diabetes of the adult, which is a slowly progressing autoimmune form of diabetes. In the other hand, development of T2D is due to insulin resistance and β-cell dysfunction [2].

According to the 2006 WHO recommendations for the diagnostic criteria for diabetes, diabetes is diagnosed by any of the following [89]: (1) fasting blood glucose level ≥7.0 mmol/l (≥126 mg/dl) with no caloric intake for eight hours, (2) Two-hours glucose level ≥11.1 mmol/l (≥200 mg/dl), (3) HgA1c is ≥6.5 %.

1.6.2 Pathogenesis of type 2 diabetes

T2D is a heterogeneous disease with complex etiological factors that are caused by both genetic inheritance and environmental factors. T2D is characterized by an impairment of insulin secretion due to β-cell dysfunction, and by resistance to the actions of insulin in target tissues [2].

During the development of the disease, as a compensatory mechanism, the β-cell population responds to decreased tissue sensitivity to insulin by increasing its function to maintain glucose homeostasis. However, this occurs only up to a certain limit, after which the β-cells fail and a hyperglycemic state develops. There is thus a progressive deterioration of both function and mass of the β-cell population. Several acquired factors, in parallel with a genetic predisposition, can affect β-cell function and survival. Among the acquired factors, both glucotoxicity and lipotoxicity can lead to increased apoptosis and a number of molecular changes [2].

Insulin resistance is defined as the inability of insulin to produce its known biological actions at normal circulating concentrations. Along with T2D, insulin resistance is also a common feature of several disorders including obesity, metabolic syndrome and

nonalcoholic fatty liver disease (NAFLD). Insulin resistance leads to an impaired inhibition of the hepatic production of glucose and release of vLDL, decreases glucose uptake by adipose tissue and muscle, and increased lipolysis and circulating levels of free fatty acids [2].

2 AIMS OF THE STUDY

Estrogen loss in perimenopausal and postmenopausal women has been linked to several features of metabolic syndrome [73, 90], while ERT reverses these pathological and metabolic features [91]. However, ERT has been reported in basic and clinical research to associate with adverse effects including breast cancer and coagulopathy [92, 93]. Therefore, identification of the molecular mechanisms of estrogen's beneficial effects would give the possibility to target them directly and to prevent the undesirable side effects.

The overall aim of this study is to elucidate the molecular mechanisms underlying the beneficial antidiabetic effects of E2, and to investigate the molecular pathogenesis of metabolic disease that results due to loss of estrogen signaling.

Specific aims were as follows:

To identify the molecular mechanisms of body weight reduction and suppression of visceral adipogenesis by E2 (Paper 1)

To investigate the molecular effects of E2 on hepatic AMPK and insulin signaling (Paper 2)

To assess the effects of E2 on inflammatory processes which may be involved in the pathogenesis of metabolic disease (Paper 3)

To trace the hepatic molecular pathogenesis of metabolic disorders associated with the interruption of estrogen signaling by ovariectomy (Paper 4)

3 MATERIAL AND METHODS

3.1 EXPERIMENTAL MODELS AND ETHICAL CONSIDERATIONS

Ethical permission for all of the work in this study has been approved by the local ethical committee at Karolinska Institute. Two models were employed in this study. The first is a long-term HFD feeding model of perimenopausal obesity which involves impaired glucose tolerance, elevated fasting blood glucose levels and insulin resistance. These features are accompanied by reduced plasma E2 levels, and E2 administration was shown to reconstitute E2 levels to those comparable with 4 month-old mice[19]. The second model is an OVX/SHAM-mouse model which mimics human menopausal ovarian hormone loss [20].

3.1.1 Perimenopausal model

This model was employed in the first three papers: paper 1, paper 2 and paper 3. Female C57BL/6J mice of seven weeks of age were acclimatized for one week, and then housed at 22–23°C in a 12 hr-12 hr light-dark cycle with free access to water and food. The mice were maintained until 12 months of age on either a low fat diet (LFD) containing 4.5 g fat/100 g (Lactamin, Sweden), or on an HFD containing 34.9 g fat/100 g (D12492, Research Diets, USA).

3.1.2 Ovariectomized/SHAM model

This model was used in the fourth paper. This model consists of ovariectomized (OVX) or sham-operated (SHAM) female C57BL/6JRj mice; the control SHAM mice underwent the same surgical procedure that was performed in OVX mice, except for ovariectomy. The mice were housed at 22–23°C in a 12 hr-12 hr light-dark cycle with open access to water and food. At the age of 11 weeks, the diet was changed from regular chow (Lactamin, Sweden) to either a control LFD containing 4.3 g fat/100 g (D12450, Research Diets, USA), or to an HFD containing 34.9 g fat/100 g (D12492, Research Diets, USA).

3.2 TREATMENT PROCEDURES AND TISSUE PROCESSING

Four different modalities of treatment, along with dietary intervention, were performed in the first three papers: paper 1, paper 2 and paper 3; whilst in paper 4, only a dietary intervention was performed. After nine months of housing with LFD or HFD feeding, mice were divided for treatment by one of four modalities, as follows:

A) The first modality of treatment consisted of the subcutaneous injection of 0.05 mg/kg body weight E2 (Sigma-Aldrich, Sweden; LFD+E2 and HFD+E2 mice) prepared in vehicle, or with the same volume of vehicle (sesame oil/ethanol, 9:1; LFD, HFD mice).

B) The second modality consisted of the subcutaneous injection 40 mg/kg body weight of sodium salicylate (SS) (Sigma-Aldrich, Sweden; LFD+SS, HFD+SS mice).

C) The third modality was a combination of E2 and SS (LFD+E2+SS, HFD+E2+SS) at the dosages employed above.

D) The fourth modality aimed to study hepatic insulin signaling, in which the first treatment mentioned earlier was performed with an additional step during which mice in the LFD, HFD and HFD+E2 groups were given an intraperitoneal injection of either insulin (2 mU/g; Actrapid, Novo-Nordisk), or a similar volume of normal saline, after which all mice were sacrificed 5 minutes later.

The first modality of treatment was applied in the studies which form the results in paper 1, paper 2 and paper 3. The second and third ones were used only in paper 3. The fourth modality, acute insulin treatment, was employed in paper 2 only. In all studies, mice were sacrificed by cervical dislocation after CO_2 inhalation, and tissues were snap-frozen in dry ice and stored at -80ºC.

3.3 INTRAPERITONEAL GLUCOSE TOLERANCE TEST, BLOOD GLUCOSE AND PLASMA INSULIN LEVELS

In all of the studies, mice were fasted either overnight or for 8 hours, after which blood glucose levels were determined by glucometer (MediSence, Abbott Scandinavia, Sweden). Intraperitoneal glucose tolerance tests (IPGTT) were performed by intraperitoneal injection of glucose (2 g/kg body weight), after which blood glucose levels were assessed at different time points ranging between zero and 120 minutes. IPGTT was performed in all studies, and included in all papers. Plasma insulin levels were assessed by either an AlphaLISA kit (Perkin-Elmer, Sweden) in paper 1, or by a RIA kit (Novo Nordisk) in paper 2, according to the manufacturers' protocols.

3.4 TRIGLYCERIDE ASSAY

Triglycerides were extracted from liver tissues and quantified using a Colorimetric Assay Kit (Cayman Chemical, BioNordika, Sweden). This assay was used in paper 2 and paper 4.

3.5 BIOCHEMICAL ASSAYS FOR PLASMA LEVELS OF ADIPOKINES AND CYTOKINES

In paper 3, the levels of cytokines, leptin and adiponectin were assayed. Plasma levels of inflammatory cytokines were measured using a BioPlex assay, according to the manufacturer's protocol (Bio-Rad, Sweden). Plasma leptin and adiponectin levels were assayed by double-antibody RIA techniques (Linco Research, St. Charles, MO).

3.6 MAGNETIC RESONANCE IMAGING (MRI)

Using the perimenopausal model, MRI was employed in paper 1 to calculate the volumes of the visceral and subcutaneous fat depots in relation to the total body weight at the end of the one month of E2 treatment. LFD, HFD and HFD+E2 mice were anesthetized with 1.5-2.0% isoflurane in O_2, with maintenance of body temperature. MRI was employed from the thoracic region down to pelvic region, and an average of 31 transverse slices was captured. Next, the image series were exported to ImageJ (1,47v, Wayne Rasband, NIH, USA) for analyses and calculations.

3.7 FROZEN SECTIONING, IMMUNOHISTOCHEMISTRY AND HISTOLOGY

The visualization of morphology using histology, and the detection of specific antigens by employing IHC, are powerful techniques in the diagnostic and research laboratory. In paper 1, both histology and IHC were used. For histology, sections were prepared from visceral adipose tissue by cryostat. The sections were stained with hematoxylin and eosin, and were visualized using a digital camera (10x and 40x magnifications, Leica DM 3000 LED, LAS Version 4.3.0). The purpose of this study was to assess the shape and size of adipocytes in the visceral adipose depot in different experimental groups.

For IHC, frozen sections of 20 µm thickness were prepared from acetone-fixed adipose tissue. After blocking with 5% rabbit serum in antibody diluent (Cell Signaling Technology), the sections were incubated with anti-uncoupling protein 1 (UCP1) antibody (Abcam, UK) overnight. After three washes with Tris-buffered saline (TBS), the sections were incubated with secondary antibody (Abcam, UK); immunostaining was enhanced by use of diaminobenzidine, after counterstaining with hematoxylin. Three images per section were collected using a digital camera (10x and 40X magnification, Leica DM 3000 LED, LAS Version 4.3.0).

3.8 PROTEIN EXTRACTION AND WESTERN BLOT

Using RIPA buffer, protein was extracted from frozen tissues and the protein concentrations were assayed according to a standard assay protocol (DC protein assay, Bio-Rad,USA). Protein samples were run on 10% ECL gels (Amersham, GE Healthcare, UK) and transferred to PVDF membranes. The membranes were probed with antibodies of interest to determine the presence and amount of the respective protein target.

Western blotting is a robust and frequently used technique in molecular studies, and was employed in paper 1 and paper 2. The technique involves the electrophoretic separation of proteins, followed by a transfer step to membranes; following blocking, the membranes are probed with antibodies targeting specific proteins.

3.9 NUCLEIC ACID EXTRACTION

In addition to protein extraction, the extraction of biomolecules such as DNA and RNA is a crucial methodology in current molecular biological applications for analytical, diagnostic or preparative purposes [94]. Total RNA was prepared from visceral adipose tissue and liver using RNeasy Lipid kits (Qiagen, Sweden) and TRIzol reagent (Life Technologies Europe, Sweden), respectively. Extracted liver RNA was further cleaned using RNeasy Mini kits, and RNA from both tissues was treated with DNase I (Qiagen, Sweden). Genomic DNA was extracted from adipose tissue using a PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific, Sweden), according to manufacturer's protocol for mammalian tissues.

3.10 REAL-TIME POLYMERASE CHAIN REACTION

The real-time polymerase chain reaction is a laboratory diagnostic and investigative technique in molecular biology/pathology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, not at its end-point as in conventional PCR [95]. The real-time PCR was employed in all papers in this thesis. Using a 7300 Real-Time PCR system, all target mRNAs (papers 1, 2, 3 and 4) were assayed in triplicate 25 µl reactions which included the target Assay-on-Demand and 1x Master Mix (Applied Biosystems, Sweden). The same system was also used to assess the amplification of genomic DNA templates (paper 1) in a duplicate 25 µl reactions containing 1x SYBR Green Master Mix (Applied Biosystems, Sweden) and 50nM of each primer of interest (ThermoFisher Scientific, Sweden).

3.11 EPIGENETIC STUDY

Alterations in methylation levels is an epigenetic mechanism that has been linked to genetic alterations associated with diseases due to environmental factors [96]. In paper 1, analysis of methylation was achieved using an OneStep qMethyl Kit (Zymo Research, Irvine, CA, USA). The primers employed were designed using the web-based primer design tool Primer3. Aliquots of 280 ng of each sample of DNA were placed into test and reference reactions containing 28 µl of 1x Test/Reference Reaction Premix in a final volume of 140 µl. The Test Reaction Premix contains methylation sensitive restriction enzymes, while the Reference Reaction Premix does not. The amplification of DNA templates was assessed by using a 7300 Real-Time PCR system as described above.

4 RESULT AND DISCUSSION

4.1 17B-ESTRADIOL SUPPRESSES VISCERAL ADIPOGENESIS AND ACTIVATES BROWN ADIPOSE TISSUE-SPECIFIC GENE EXPRESSION

The aim of this study was to identify the molecular mechanisms which underlie the glycemic control and the anti-obesogenic effects of E2 in a perimenopausal mouse model fed on either LFD or HFD. Menopause is associated with an abnormal fat distribution and a central obesity which predisposes women to metabolic syndrome and T2D [73, 74]. ERT reduces T2D incidence [97], and the risk of T2D development in premenopausal women is less than men [98]. In experimental models, disruption of estrogen signaling by ovariectomy or by transgenic modification results in increased adiposity and weight gain [55, 99, 100]. Administration of E2 to these models promotes weight loss in association with the regulation of food intake [101] and increased energy expenditure [102]. In our study, HFD mice gained body weight and developed features of insulin resistance, including elevated levels of fasting blood glucose and plasma insulin and glucose intolerance due to HFD feeding for 10 months. Although E2 had no significant effects in LFD mice, E2 reduced body weight significantly in HFD+E2 mice in association with the improvement of the glycemic control. The reduction of body weight in HFD+E2 mice was observed from the day 10, which is consistent with previous reports [103, 104]. However, the E2-induced body weight effect was preceded by a reduction in food intake from the day 4.

MRI studies showed that E2-induced weight loss is paralleled with significant reductions in the visceral adipose depot, without significant changes in the subcutaneous depots. These observations occurred in parallel with morphological changes in the adipocyte population form this depot, such that hypertrophied adipocytes were observed in HFD mice, whilst adipocytes in HFD+E2 mice were smaller and comparable in size to those of LFD mice. These observations support what has been concluded previously in E2-treated ovariectomized mice [105], and indicate that E2 induces a restoration of the insulin-sensitive phenotype in the visceral adipose tissue, since adipocyte size correlates inversely with insulin sensitivity in humans [106] and rodents [107]. In our study, the results of the IPGTT are consistent with these changes in visceral adipocyte morphology.

Since hypertrophic changes of adipocytes have been linked to altered lipid metabolism [108], the mRNA levels of lipogenic and lipolytic genes in the visceral adipose depot were investigated. The results show that E2-induced reductions in adipocyte size involved the reduced expression of the lipogenic genes *scd1* and *fas*, and increased expression of adipose triglyceride lipase *pnpla2*. Moreover, E2 suppressed the expression levels of the nuclear receptor *nr2c2/tr4*, which has been identified as a transcriptional regulator of lipogenic gene expression [109]. Low E2 levels have been linked to leptin insensitivity [110], and E2 treatment of ovariectomized mice has been shown to promote leptin sensitivity [111]. Consistent with this, in the current report, E2 administration led to the downregulation of leptin transcript levels, whilst it upregulated that of leptin receptors. These results are considered as features consistent with the promotion of leptin sensitivity by E2 in HFD+E2,

and this is supported by the observed increases in *stat3* mRNA levels, which is activated by leptin [112]. From another angle, *stat3* has a role in the regulation of brown adipocyte differentiation and function [113], a fact which reflects the possibility of increased energy expenditure as a mechanism of E2-induced the loss of adiposity. Therefore, the expression of brown adipose -specific markers was assessed. The results show that the transcript of *ucp-1* and its protein levels were upregulated by E2. The expression of UCP-1 is known to be involved in the beiging of white adipose depots, including the visceral depot [114]. Additionally, the mRNA levels of the thermogenic gene *dio-2*, along with the brown adipose-specific gene *cpt1b* [115], were increased significantly. Consistent with the upregulation of *ucp-1* and *dio-2* expressions, the expression of adrenergic receptor β3 (*adrβ3)*, which is known to regulate UCP-1 during the thermogenic response [116] , was increased in the HFD+E2 group.

It was observable that the effects of E2 were specific to chronic HFD exposure, and HFD feeding has been linked to epigenetic changes in methylation [117]. Thus, analysis of methylation was relevant to understanding the molecular events in HFD+E2 mice, and the results revealed that the up-regulation of expression of *adrβ3* and *dio-2* by E2 treatment was associated with the suppression of methylation levels. These results are supported by a previous report showing that methylation at specific sites within these genes results in decreased transcriptional activity [96].

In conclusion, the anti-obesogenic effect of E2 on visceral adipose tissue results in improvement of glucose homeostasis in this mouse model of perimenopausal obesity. The anti-obesogenic effects include the suppression of visceral adipogenesis and the reduction of adipocyte size. This study provided a molecular explanation for these anti-obesogenic effects, including the activation of the lipolytic enzyme *pnpla2*, the suppression of lipogenic gene expression possibly through downregulating the expression levels of the transcriptional nuclear receptor *nr2c2/tr4*, and the induction of brown adipose tissue-specific gene expression via E2-dependent alterations in methylation levels (Figure 7).

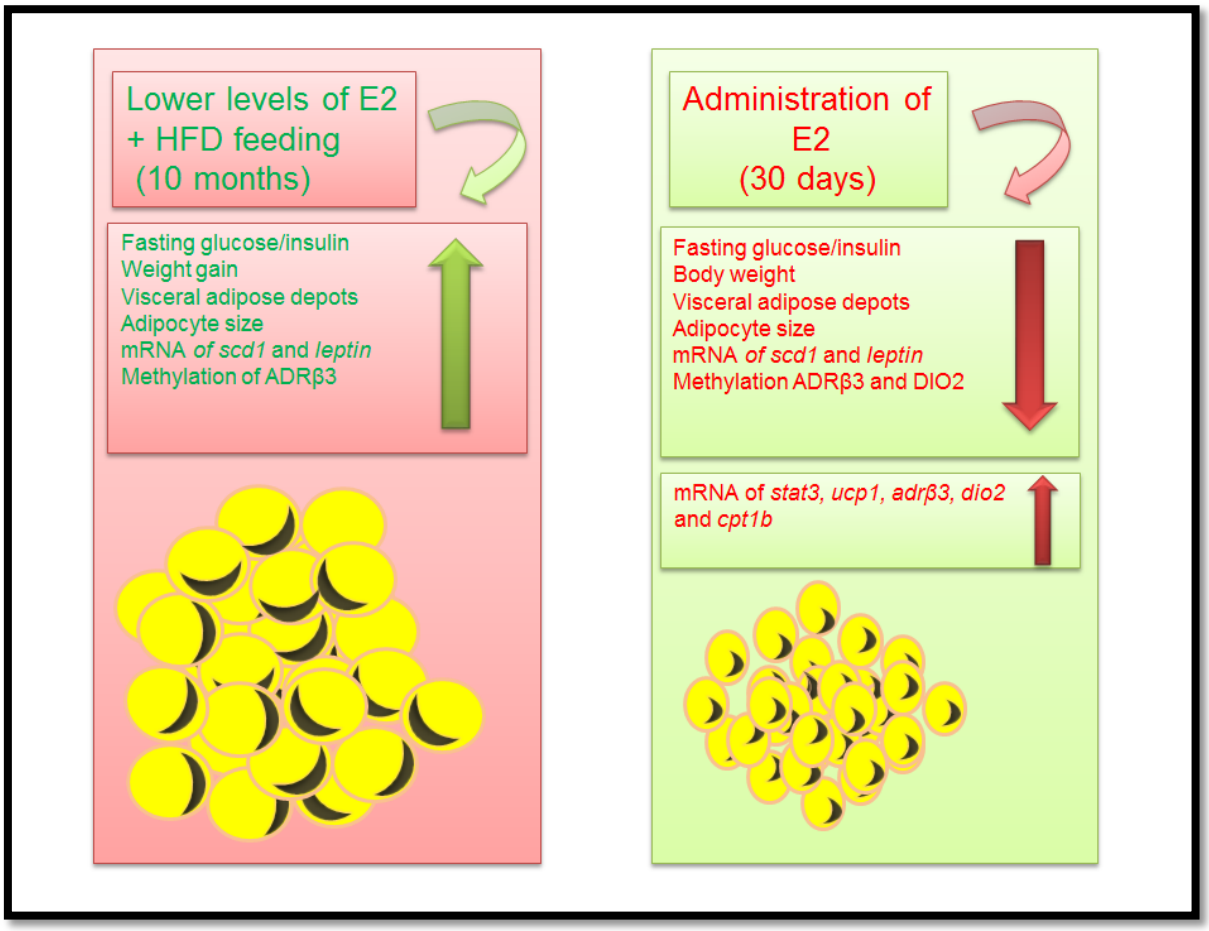

Figure 7: Molecular mechanisms of antidiabetic effects of E2 on visceral adipose tissue (**Paper 1**)

4.2 ACTIVATION OF HEPATIC AMPK BY 17B-ESTRADIOL SUPPRESSES BOTH NUCLEAR RECEPTOR NR2C2/TR4 AND ITS DOWNSTREAM LIPOGENIC TARGETS, REDUCES GLUCONEOGENIC GENES AND IMPROVES INSULIN SIGNALING

This study aimed to identify the hepatic mechanisms involved in the beneficial effects of E2 on insulin sensitivity in the perimenopausal mouse model exposed to 10 months HFD feeding. Activation of hepatic AMPK is a known therapeutic mechanism for the improvement of hepatic insulin resistance [118, 119] resulting mainly from both lipid accumulation and increased hepatic gluconeogenesis [120, 121]. Therefore, it was of interest to assess whether the beneficial effects of E2 on glycemic control involve hepatic AMPK activation. In this study, phosphorylation of hepatic AMPK at Thr172 was significantly reduced in HFD mice, whilst E2 activated that in HFD+E2. In addition, activated AMPK suppresses hepatic lipogenesis via the inactivation of the ACC1 [118]. Consistent with this, the results of our report showed that the activation of AMPK is accompanied by a subsequent increase in the phosphorylation of ACC1 on Ser79, which ultimately inactivates this lipogenic enzyme. Another mechanism through which activated hepatic AMPK reduced lipogenesis has been reported recently [109]. This study shows that inactivation of the nuclear receptor NR2C2/TR4 due to activation of hepatic AMPK by metformin suppresses

downstream lipogenic gene expression. In paper 2 presented here, E2 treatment of HFD+E2 mice led to reductions in NR2C2/TR4 expression at both the mRNA & protein levels. This effect of E2 treatment has been reported previously by our group in adipose tissue (paper 1) [122]. These observations suggested that the expression of further downstream lipogenic genes, including *scd1, fas* and *acc1*, may also be reduced. Consistent with this, E2 treatment in HFD+E2 mice reduced the mRNA levels of these genes, and in addition, protein levels of ACC1 were reduced significantly. Since lipid accumulation in the liver is described as a strong pathogenic factor which results in insulin resistance in this organ [120], our results suggest that the beneficial effects of E2 involve the hepatic AMPK-dependent inhibition of lipogenic gene expression via the inhibition of NR2C2/TR4 levels. This conclusion was supported by further assessment of hepatic triglyceride contents; as expected these molecular events induced by E2 treatment in HFD+E2 mice were accompanied by normalization of hepatic triglycerides which were increased significantly due to HFD feeding. Finally, the mRNA levels of hormone sensitive lipase (*lipe*) were suppressed in HFD mice, and E2 treatment in HFD+E2 mice resulted in upregulated *lipe* expression; this suggests that E2-induced reductions in hepatic triglyceride levels may not only be due to suppression of lipogenesis, but also with increased triglyceride mobilization via stimulation of *lipe* expression.

Assessment of fasting blood glucose levels showed that HFD feeding resulted in elevated fasting blood glucose levels, and the beneficial effects of E2 in HFD mice involved normalization of this parameter. These observations indicate an involvement of hepatic gluconeogenesis, and therefore the transcript levels of some gluconeogenic genes were assayed. The results showed that E2 treatment of HFD+E2 mice resulted in suppression of the expression levels of glucose-6-phosphatase catalytic (*g6pc*) subunit, *g6pt1,* and pyruvate carboxylase. Expression levels of the latter two were upregulated in HFD mice and correlated with fasting blood glucose levels, whilst the expression levels of *g6pc* did not change and so did not correlate with elevated fasting blood glucose levels in HFD mice. These observations are supported by available studies in the literature. For example, it has been shown that the protein levels of the pyruvate carboxylase correlate significantly with plasma glucose levels [123]. Moreover, a lack of correlation between fasting blood glucose levels and the expression levels of *g6pc* has been reported formerly in patients with T2D [124]. However, it has been shown, firstly, that *g6pc* expression levels are suppressed by activated hepatic AMPK [125], and secondly, that *g6pc* is a direct gene for estrogen receptor α [54]. Our current results are supported by these two reports, since, firstly, the expression levels of *g6pc* were suppressed by E2 treatment in both LFD+E2 and HFD+E2 mice, and secondly, E2 treatment activated hepatic AMPK, which may give a mechanistic explanation for the suppression of *g6pc* by E2 treatment.

In fact, the beneficial effects of activated hepatic AMPK extend beyond the suppression of hepatic lipogenesis and gluconeogenesis. It has been demonstrated that the positive role of adiponectin on metabolic homeostasis is medicated through AMPK signaling via adiponectin receptors [126-128]. However, in this report, the results show no correlation between E2-

induced activation of hepatic AMPK and the expression levels of adiponectin and its receptors. It has also been reported previously that E2 suppressed plasma levels of leptin in HFD+E2 mice [19], and in the current study, hepatic leptin receptor expression in HFD+E2 was significantly increased.

Since E2 treatment significantly normalized glucose levels before and during the IPGTT in HFD+E2 mice, these results together with other studies show that E2 treatment affects insulin tolerance positively [19, 129], suggesting that E2 improves insulin signaling. Thus changes in insulin-dependent phosphorylation of IRS-AKT-FOXO protein intermediates in the hepatic insulin signaling pathway were investigated. Insulin promotes the synthesis of glycogen, lipids and protein in the liver, and suppresses hepatic gluconeogenesis. Thus the IRS-AKT-FOXO cascade has been investigated to study the pathogenesis of hepatic insulin resistance. For example, the double knockout of both hepatic *Irs1* and *Irs2* (L-DKO mice) interrupts insulin/food-induced phosphorylation of Akt and Foxo1, and results in hyperglycemia due to increased endogenous glucose production [130]. Similarly, knockout of both *akt1* and *akt2* in the liver (Akt-DLKO) results in the same diabetic phenotype as observed in L-DKO [131]. L-DKO mice were employed in other studies involving further *foxo1* gene knockout (L-TKO), which resulted in reversal of the diabetic and growth retardation features observed previously in L-DKO [132], suggesting FOXO1 activation has a role in development of diabetes. In our study, insulin-resistant HFD mice showed a reduced insulin-dependent phosphorylation of AKT2 and FOXO1. However, E2 treatment in HFD+E2 mice was associated with increased insulin-dependent phosphorylation of the signaling intermediates, in parallel with normalized hepatic triglyceride levels, and suppressed expression of gluconeogenic genes.

In summary, improvements of glycemic control induced by E2 treatment in HFD+E2 mice are associated with multiple hepatic molecular mechanisms: E2 treatment induces activation of hepatic AMPK, which results in the suppression of hepatic lipogenesis via inhibition of NR2C2/TR4, and in the inhibition of gluconeogenesis through suppression of the transcript level of *g6pc*; finally, E2 treatment normalizes hepatic triglycerides through the suppression of lipogenesis and the activation of triglyceride mobilization. The summative effect of these events is the improvement of hepatic insulin signaling (Figure 8).

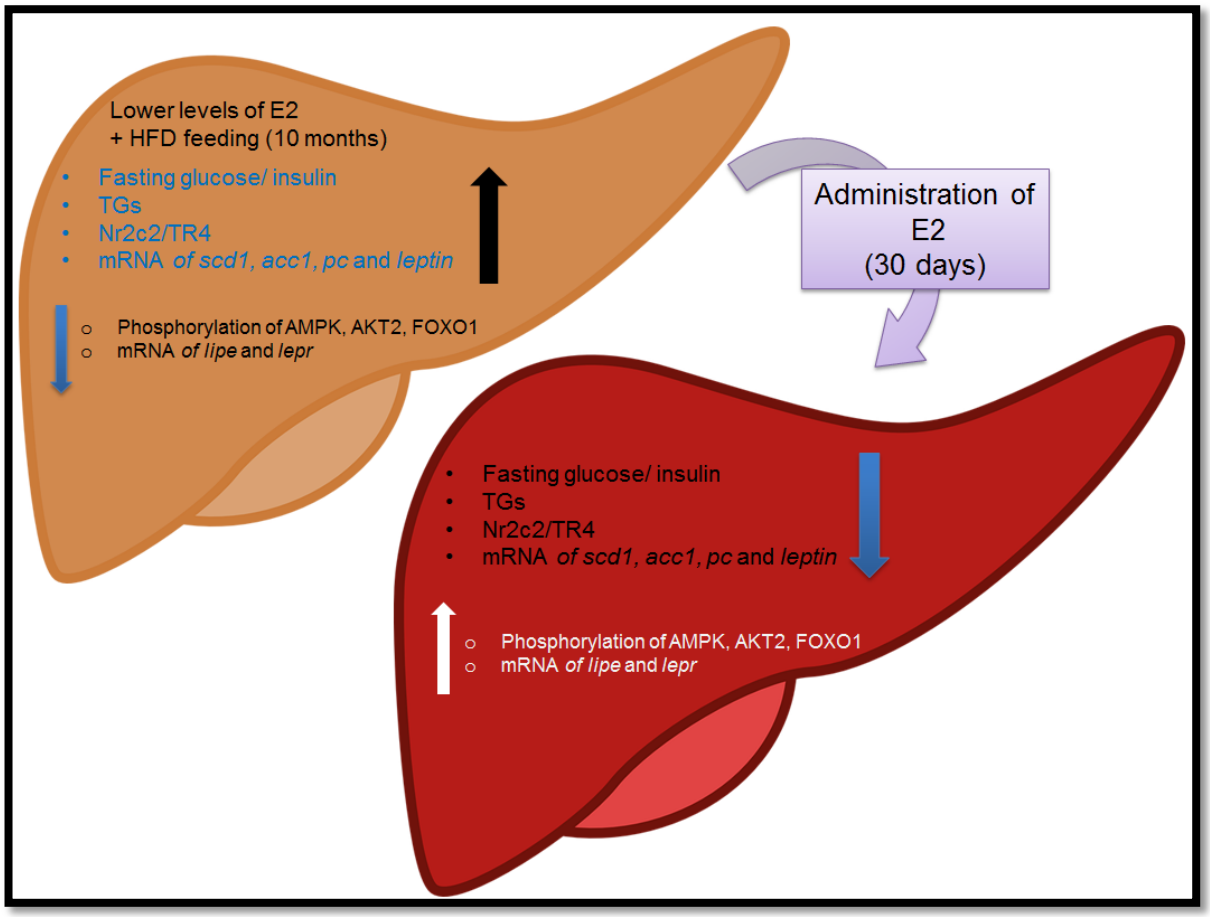

Figure 8: Hepatic molecular mechanisms of antidiabetic effects of E2 (**paper 2**)

4.3 17B-ESTRADIOL REDUCES BODY WEIGHT AND IMPROVES GLYCEMIC CONTROL WITHOUT SUPPRESSION OF INFLAMMATORY MEDIATORS IN PLASMA, ADIPOSE TISSUE AND LIVER

In the previous two studies, it was reported that administration of E2 in a perimenopausal mouse model resulted in reductions of body weight and improvements in glucose homeostasis. These effects were associated with various molecular mechanisms in the adipose tissue (paper 1) [122] and liver (paper 2) [133]. Furthermore, this model is mimics perimenopause-associated obesity and glucose intolerance, and it is known that obesity is associated with chronic low-grade inflammation in the liver and adipose tissue, a feature which is recognized as an important link between obesity and insulin resistance [71].

The current study was performed to assess the extent to which the beneficial effects of E2 involve alterations in inflammatory status. The antidiabetic effects of E2 and ERT have been demonstrated by various clinical studies [75-77], and also by experimental studies [19, 78, 79]. However, the changes in the immune system which occur as result of estrogen deficiency and during subsequent ERT are a source of debate [80, 81]. Observational studies [134, 135] and clinical trials [82, 83] have reported that ERT results in increases of serum CRP levels in postmenopausal women. Similarly, studies have shown that E2 treatment led to increases in

the levels of CRP, together with concomitant increases in the levels of IL6 in healthy postmenopausal women [84]. However, other studies report contradictory findings. For example, it has been shown that E2 suppresses circulating CRP levels in postmenopausal women with T2D [85]. There is a lack of experimental studies which are consistent with these observations. However, elevated levels of plasma IL6 have been reported previously in ovariectomized mice due to fatty feeding, while plasma levels of TNFα are unchanged [86].

In our study, no significant changes were observed with regard to the plasma levels of the cytokines IL1β, IL6, granulocyte-macrophage colony-stimulating factor (GM-CSF), or of the chemokine MCP1. In addition, the expression levels of various cytokines and pro-inflammatory mediators were unchanged in the liver, irrespective of dietary intervention or E2 treatment; however, in the visceral adipose tissue, E2 treatment led to an upregulation of the expression levels of *il1β, il6*, toll-like receptor 2 (*tlr2*), inhibitor of nuclear factor kappa-B kinase subunit β (*ikkβ*) and histocompatibility 2 class II antigen A beta 1 (*mhc2*). These findings reflect the possibility that the antidiabetic actions of E2 in mice subject to chronic HFD exposure do not involve an anti-inflammatory action in insulin target tissues. Accordingly, the effects of a combination treatment of E2 and SS were studied to determine whether such a combination could lead to additive glycemic effects.

SS is one of several nonsteroidal anti-inflammatory drugs which has been reported to suppress cyclooxygenase 2 non-selectively [67]. In addition, it has been shown clinically that high dose aspirin treatment improves glycemic parameters [136]. In the same context, use of the anti-inflammatory drug salsalate decreases HbA1c [68]. The results in paper 3 showed that SS treatment led to reductions in body weight and in fasting blood glucose levels, in parallel with the suppression of gluconeogenic gene expression. Conversely, SS treatment failed to improve glucose tolerance and, either alone or in combination with E2, failed to suppress the upregulation of pro-inflammatory mediators induced by E2 in visceral adipose tissue.

Diet-induced obesity and inflammation-related disorders have been linked to abnormal plasma levels and altered expression of the adipokines leptin and adiponectin. Circulating leptin levels directly correlate with adipose tissue mass, and leptin controls appetite [137]. Mutation in the genes encoding leptin or its receptors results in prominent obesity in mice and humans [138, 139]. Experimentally, HFD feeding induces leptin mRNA and increases serum leptin levels, in parallel with insulin resistance and glucose intolerance. However, in both the current report and previous studies [19, 122, 133], we have shown that E2 suppresses leptin plasma and expression levels, and increases the mRNA levels of leptin receptor.

The levels of adiponectin, unlike leptin, do not correlate with fat mass [140]. In the current report, E2 suppressed both plasma levels of adiponectin, and also its expression levels in the visceral adipose.The downregulation of adiponectin levels by E2 has been interpreted to be a detrimental effect of E2 treatment in a previous report [19]. Adiponectin has an anti-inflammatory role through its inhibition of TNFα [141], IL6 and nuclear factor κB [142]. These actions of adiponectin may occur via induction of the anti-inflammatory cytokine IL-

10 [143]. Consistent with these reports, the E2-induced suppression of adiponectin levels in the current study may underlie the apparent pro-inflammatory action of E2 via the suppression of *il10* expression and the upregulated expression levels of *il1β, il6, ikkβ* and *tlr2* in the visceral adipose tissue of HFD+E2 mice.

In conclusion, the local increased expression levels of pro-inflammatory mediators in visceral adipose, the unchanged plasma levels of these pro-inflammatory mediators, and their unaltered hepatic mRNA levels do not impact upon the positive actions of E2 on body weight, fasting blood glucose levels, and glycemic control. The reductions induced by E2 in plasma levels of adiponectin, and in its mRNA levels in adipose tissue, could explain the continued elevated expression levels of these mediators. The aging process and the onset of menopause may be relevant to the determination of the exact role of E2 on inflammation, since it has been shown E2 inhibits inflammation in cells derived from young mice, but not in cells from aged mice, supporting an age-specific action for E2 [87, 88]. Further studies employing mouse models in which chronic endotoxemia is induced at various ages will clarify these issues, and will provide the opportunity to study the promotion of insulin sensitivity by E2 in the presence of a chronic inflammatory state (Figure 9).

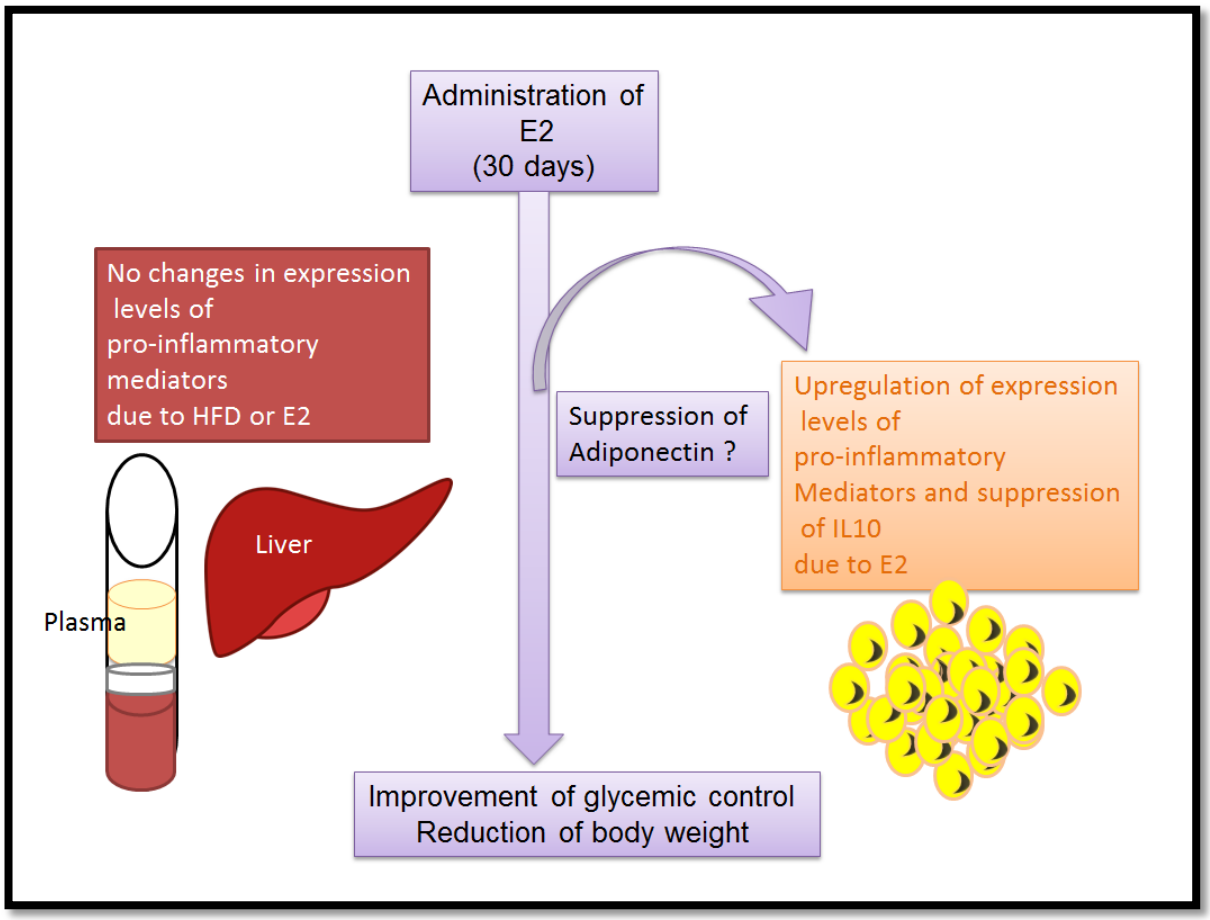

Figure 9: Antidiabetic effects of E2 and inflammatory mediators (**Paper 3**)

4.4 OVARIECTOMY RESULTS IN A CONTINUOUS ACCUMULATION OF HEPATIC TRIGLYCERIDES AND THE UPREGULATION OF INFLAMMATORY MEDIATORS FOLLOWING SHORT-TERM EXPOSURE TO HIGH-FAT DIET

The current study employs OVX and SHAM mice, which were fed on either LFD or HFD for 3 days or 5 days. As reported previously, this feeding regime leads to features of NAFLD which result in hepatic insulin resistance due to Kupffer cell activation [144]. The goal of this study was to assess whether estrogens were able to regulate the effects of short-term HFD exposure with regards to the features of NAFLD.

The continuous increase in the prevalence of obesity is associated with the rising incidence of NAFLD, which currently surpasses that of alcoholic liver disease [145, 146]. The pathogenesis of NAFLD is poorly understood, but the accumulation of hepatic triglycerides is considered as its main feature [147] since it may play a major role in the development of hepatic insulin resistance and T2D [120, 121]. Epidemiological studies have shown that the risk of NAFLD increases in postmenopausal women [47, 48], which is most likely due to the loss of the protective effects of E2, while ERT is associated with the reduced risk of NAFLD [48].

In animal models, treatment of OVX mice with E2 reverses hyperglycemic changes due to ovariectomy [79], and protects them from HFD-induced hepatic insulin resistance [148]. Both elevated hepatic triglyceride content (paper 2) [133] and increased levels of endogenous glucose production [52, 55] are observed in chronic HFD–exposed perimenopausal mice and in ERKO mice. The restoration of insulin sensitivity and improved hepatic insulin signaling by E2 treatment is associated with the suppression of lipogenic gene expression through suppression of NR2C2/TR4 (paper 2) [133], which controls *scd1* and *fas* expression [109], or via suppression of *lxrα* and its target *srebp1* [19]. These effects of E2 treatment occurred in parallel with reduced hepatic triglyceride content (paper 2) [133]. The results here show that short-term HFD feeding in both SHAM and OVX mice result in increased mRNA levels of the lipogenic genes *scd1, fas* and *acc1*. However, the results of our study also show a novel observation in SHAM mice fed on an HFD, since in presence of estrogen, there was a transient upregulation of these lipogenic genes and the transcription factors regulating their expression, *lxrα* and *srebp1*, after 3 days of HFD feeding, but then their suppression after 5 days of HFD feeding. This pattern of expression changes was not observed in OVX mice fed on an HFD, in which the upregulated expression of lipogenic genes and of *lxrα* and *srebp1* increased progressively from 3 to 5 days. Furthermore, the expression levels of *nr2c2/tr4* were upregulated in both OVX mice fed on an LFD, and in OVX mice fed on HFD, regardless of diet. This suggests that the expression of *nr2c2/tr4* is at least partly controlled through estrogen signaling.

In the present study, the mRNA levels of gluconeogenic genes, including *g6pc, g6pt1* and pyruvate carboxylase, were assessed. No correlation was found between *g6pc* and the elevated levels of fasting blood glucose levels among the different groups, and this finding is consistent with other studies which reveal the lack of correlation between hepatic G6PC

expression levels with fasting glucose levels [124, 133]. However, the expression levels of pyruvate carboxylase do correlate with increased hepatic glucose production in OVX mice fed on an HFD, as has been shown before [123]. Previously, expression levels of *g6pc*, *g6pt1* and pyruvate carboxylase were suppressed significantly by E2 treatment of HFD+E2 mice (paper 2) [133].

The pathogenesis of NAFLD is known to involve both lipid accumulation in the liver and hepatic inflammation [149]. NAFLD-induced hepatic inflammation is characterized by increased hepatic production of pro-inflammatory cytokines [150]. In our study, HFD feeding for 5 days resulted in increased mRNA levels of the macrophage marker *cd68*, regardless of whether the mice were OVX or SHAM, suggesting the lack of any interaction between estrogen signaling and the expression of this gene. Meanwhile, *tlr2* expression levels were upregulated in OVX mice fed on either an LFD or an HFD, suggesting the presence of an interaction between estrogen signaling and *tlr2* expression. TLR2 signaling has been reported to contribute to the adverse effects of HFD feeding in mice, and TLR2-deficient mice are protected from HFD-induced insulin resistance [151].

Collectively, estrogens appear to suppress features of hepatic insulin resistance resulting from short-term HFD exposure, firstly by counteracting the continuous accumulation of hepatic triglycerides, and secondly via the reduced expression levels of gluconeogenic genes. OVX mice gained weight, had elevated fasting blood glucose levels, increased hepatic triglycerides and increased expression levels of both *nr2r2/tr4* and the pro-inflammatory *tlr2*, independently of dietary intervention (Figure 10). These observations were paralleled in OVX mice fed on an HFD by a continuous and progressive upregulation of lipogenic genes and their regulatory transcription factors*, lxrα* and *srebp1*. In contrast, SHAM mice fed on an HFD exhibited levels of hepatic triglycerides which were higher than SHAM mice fed on an LFD, but which were lower than those of OVX fed on an HFD. The latter observation may be due to an estrogen-driven downregulation of these lipogenic genes and transcription factors in SHAM mice after 5 days of HFD feeding, following their previous upregulation after 3 days of HFD feeding. Finally, although HFD feeding for 5 days resulted in increased mRNA levels of the macrophage marker *cd68*, regardless of whether the mice were OVX or SHAM, OVX fed on an HFD for 5 days had significant increases in the expression levels of the gluconeogenic pyruvate carboxylase, and upregulation of the pro-inflammatory gene *il6* (Figure 11).

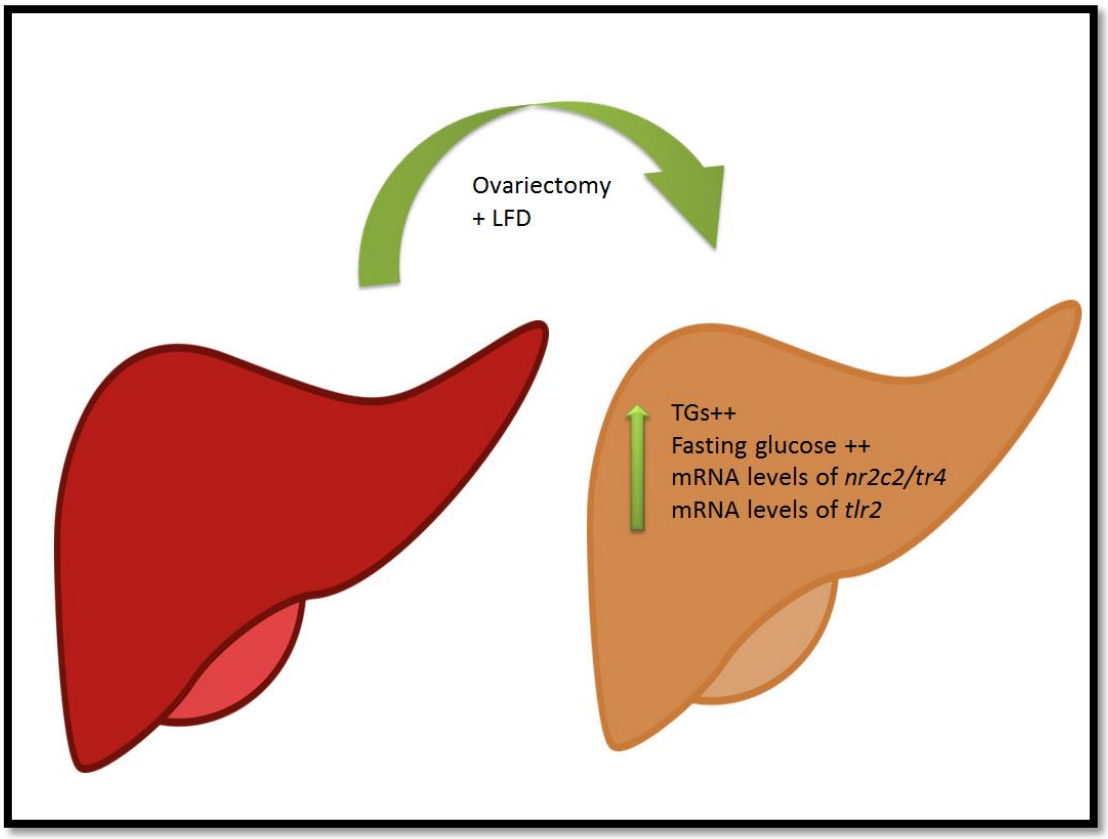

Figure 10: Effects of ovariectomy on hepatic metabolism (**Paper 4**)

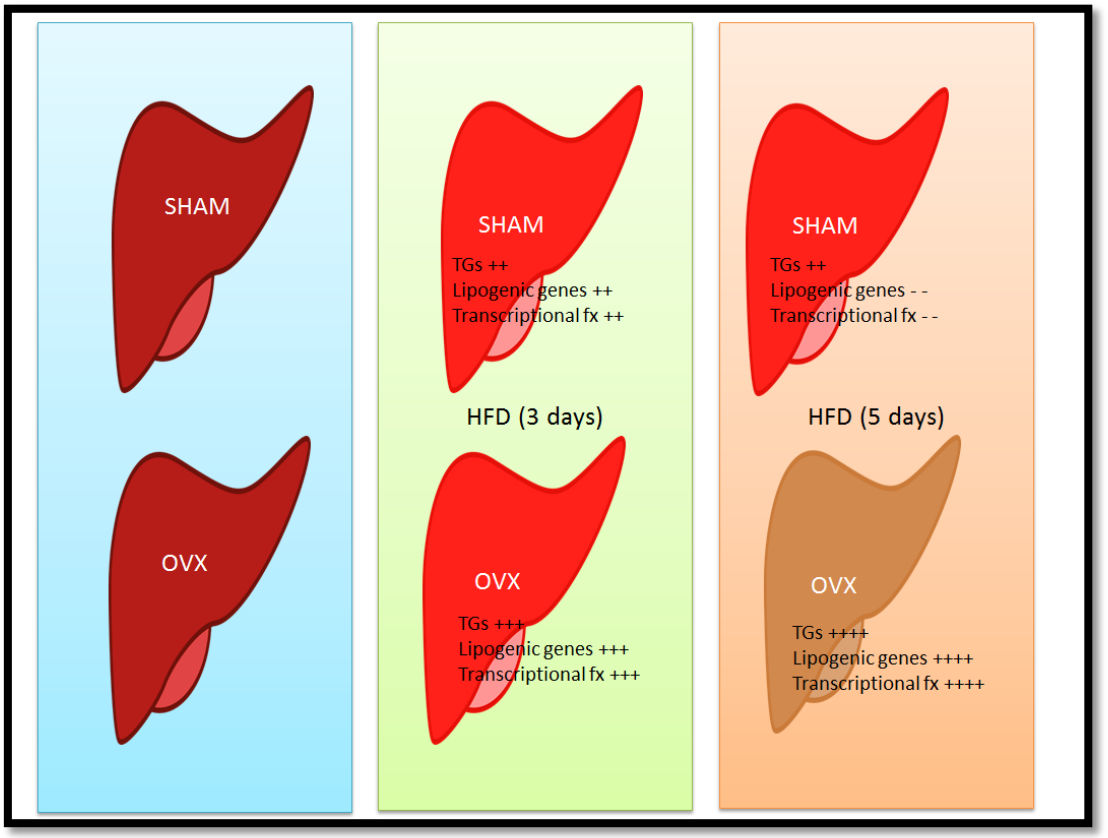

Figure 11: Effects of ovariectomy on hepatic metabolism (**Paper 4**)

5 CONCLUSIONS

This study aimed to elucidate the beneficial antidiabetic molecular mechanisms of E2, and the molecular pathogenesis of metabolic diseases that result due to estrogen signaling loss. The following points can be concluded:

- The anti-obesogenic effects of E2 on visceral adipose tissue are associated with improvements in glucose homeostasis in this mouse model of perimenopausal obesity and insulin resistance. The anti-obesogenic effects include the suppression of visceral adipogenesis and the reduction of adipocyte size. This study provided molecular explanations for the anti-obesogenic effects of E2; including the activation of the lipolytic enzyme *pnpla2*, the suppression of lipogenic gene expression possibly through downregulating the expression levels of the nuclear receptor *nr2c2/tr4*, and the induction of brown adipose tissue-specific gene expression via E2-dependent alterations in methylation levels (**Paper 1**).

- Improvements of glycemic control induced by E2 treatment in HFD mice are associated with multiple hepatic molecular mechanisms: E2 treatment induces activation of hepatic AMPK, which results in the suppression of hepatic lipogenesis via inhibition of NR2C2/TR4, and in the inhibition of gluconeogenesis through suppression of transcript levels of *g6pc*; E2 treatment also normalizes hepatic triglycerides through the suppression of lipogenesis and the activation of triglyceride mobilization; the summative effect of these events results in improvements in hepatic insulin signaling (**Paper 2**).

- The local increased expression levels of pro-inflammatory mediators in visceral adipose, the unchanged plasma levels of these pro-inflammatory mediators, and their unaltered hepatic mRNA levels, do not impact upon the positive actions of E2 on body weight, fasting blood glucose levels, and glycemic control. The reductions induced by E2 in the plasma levels of adiponectin, and in its mRNA levels in adipose tissue, could explain the continued elevated expression levels of these mediators, and the supression of the anti-inflammatory *il10*(**Paper 3**).

- Estrogen signaling appears to suppress features of hepatic insulin resistance resulting from short-term HFD exposure by opposing the continuous accumulation of hepatic triglycerides, and via reduced expression levels of gluconeogenic genes. Ovariectomy resulted in weight gain, elevation of fasting blood glucose levels, increased hepatic triglycerides and increases in expression levels of *nr2r2/tr4* and the pro-inflammatory *tlr2*, independently of dietary intervention (**Paper 4**).

6 ACKNOWLEDGEMENTS

First and foremost, I would like to praise and thank **ALLAH (God)** for my deepest faith and for his blessings, uncountable gifts and for everything in my life.

Second, I would like to follow by thanking my mother **Miseada Bint Daham ♥** (1963-2003), the greatest lovely mother in the whole history of humankind. I am really speechless to express my feelings but surely it has not been easy to live without you, mom. Your former little kid is on his way to get the second doctoral degree, one more part of other achievements, all of which are attributed to you. You will not be with me physically, but your memories escort me everywhere and forever.

My father, friend, teacher and pacemaker **Misfer Bin Ali ♥** (1939-2014): your generosity, valor in leading with charity, faith, wisdom and poetry will last and be an inspiration to me forever. I really miss you and wished you could see this.

Thanks **Saud Bin Saleh, Abdullah Daham** and **Khalid Daham** for your encouraging words and support since I was a student in the primary school.

I am especially thankful for the administrations at **the Department of Pathology, the College of Medicine, and the University Hospital at Najran University, Saudi Arabia;** in particular **Dr. Jubran Al-Qahtan**i and **Dr. Ahmad Zaker,** for providing me with total support during my training and studies.

Many thanks, **Dr. Neil Portwood**, for supervising my training and research. Despite the hard moments, I owe you for developing my skills and knowledge in pathology and mainly in the area of molecular, genetics and epigenetics. Thank you for involving me in the research project of estrogen and introducing me to the world of animal models in medical research. Your trainee is now prepared not only as a medical pathologist, but as a researcher too, with many tools he wouldn't have had without you.

Special thanks extend to my co-supervisor **Professor Per-Olof Berggren** for giving me the opportunity to work within his elegant laboratory during the conduct of my research, for all the fruitful brainstorming sessions, and for his invaluable advice and support

I am also thankful to **Katarina Breitholtz** and **Ann-Britt Wikström** for their professional administrative and fabulous help during my studies. I am particularly grateful to **Professor Anders Franco-Cereceda,** head of the department; to **Dr. Catharina Lavebratt,** director of Postgraduate Studies; and to all of the staff at the administration: **Therese Kindåker**, **Britt-Marie Witasp**, **Kerstin Florell**, **Karolina Jildenhed**, **Elinor Petersson**, **Lilian Pagrot** and **Ann-Mari Richardsson** for their administrative assistance, and to **Jan-Erik Kaarre** and **Thomas Westerberg** for timely IT support.

Many thanks **Agneta Hilding** for your help and support in biostatistics, also Dr. **Zuheng Ma** for training me in the area of proteomics, Dr. **Galyna Bryzgalova** for your support and help during my research, and all other former colleagues at M1, Karolinska University Hospital, Solna.

Special thanks **Professor Lisa Juntti-Berggren** for involving me in the project of APOCIII and for giving me the chance to use my background in diagnostic histopathology in our research. My warm thanks Dr. **Ismael Valladolid-Acebes** for your invaluable support in the area of histology and immunohistochemistry and for your helpful scientific discussions.

My special appreciation extends to my previous and present postgraduate mates **Karin Åvall**, **Amira Alkharusi, Fahad Zadjali**, **Mohammed Hamza**, **Tina**, **Fara**, **Shima**, **Senthil**, **Tianwei**, **Julien**, **Carole**, **Meike Paschen**, **Pim van Krieken**, **Subu Rajaserkaran**, **Cheng Xu**, **Muhammad Irfan**, **Robin Fröbom**, **Sympath Narayanan**, **Sofie Eliasson**, **Teresa Daraio, Fernando Tessaro** and **Yue Shi** for their exceptional support, scientifically and socially.

I also would like to thank all staff members at L1, Karolinska University Hospital, Solna; and in particular **Andrea Dicker**, **Anna Voznesenskaya**, **Barbara Leibiger**, **Christopher Barker**, **Concha F. Garcia-Prieto**, **Christina Bark**, **Elisabeth Noren-Krog**, **Eduardo Nolasco**, **Elisabetta Dare**, **Erwin Ilegems**, **Essam Refai**, **Ingo Leibiger**, **Irina Zaitseva**, **Ishrath Ansurudeen**, **Jacob Grünler**, **Jia Yu**, **Lina Yu**, **Martin Köhler**, **Massimiliano Ria**, **Monica Isaksson Strand**, **Montserrat Visa Majoral**, **Nancy Vargas**, **Sergei Zaitsev**, **Shao-Nian Yang**, **Stefan Jacob, Teresa Pereira**, **Tilo Moede**, **Yan Xiong**, **Yvonne Strömberg**, and the group members in **Miami** and in **Singapore** for their constructive discussions and recommendations.

I am particularly grateful to all staff at the Department of Pathology, University Pathology Building, Queen Mary Hospital, Hong Kong; and specifically, **Professor U.S. Khoo**, **Dr. Jason So** and **Ms. Irene MK** for their efforts and support.

I would like to take this opportunity also to thank all of the representatives at the **Embassy of Saudi Arabia** in Stockholm, and the **Cultural Bureau** in Germany for their endless support. The same is true for all former and present Saudi scholars here in Sweden working as medical doctors, nurses, PhD students and others.

I would like to express my appreciation and thankfulness to my uncle **Saleh Bin Ali** with my deepest prayers for him to recover and return to health, another reason for happiness and hope. I would like to thank my mother-in-law **Meajeba Bint Hadi**, my aunts **Hedaya Bint Jewerallah, Nora Bint Daham** and **Misfera Bint Daham** for their kindness, amenity and tenderness.

My beloved brothers and sisters **Moshabab, Saeed, Safar, Nasser, Faisal, Miseada, Nora, Mohammad, Azzla, Naif, Shahad and Raza;** and my nephews **Bader, Bassam,**

Mohammad and the latest lovely nephew Misfer, thank you all for your outstanding support and love.

Ali♥ (1984-2013) and Fahad♥ (1985-2013) bin Saleh: while I am sure you are in a better place, it has not been easy to let you go. Your memories are embedded in my heart forever.

My lovely queen **Fedda♥ Bint Saleh,** no words would express my gratefulness to you. My deepest gratitude and heartfelt love for your support, love, understanding, patience, vineyard, sacrifices and every single moment I have had and will have with you. You are the real success in my life and I wouldn't be here without you.

The last but not least, **Abdulmalik♥ (Malik)**, **Abdulmoeiz♥ (Moeiz)** and **Abdulmohaimin♥ (Mohaimin);** you are the unique gifts from Allah, my heartbeats and source of my happiness and hope. My sons, you are all partners in this and I am so proud of you as polite and sophisticated boys. May Allah bless you and give you the power of science and faith.

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