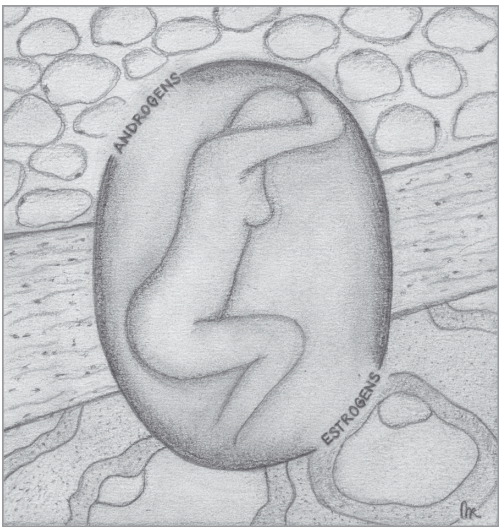
Metabolic and Ovarian Consequences of Perinatal Sex Steroid Programming



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Till min familj

ABSTRACT

Endocrine and metabolic disturbances in adulthood may stem from insults such as nutritional and hormonal alterations that occur at critical periods in pre- or postnatal life – a process known as programming. This means that suboptimal conditions *in utero* and early life may contribute to adult reproductive and metabolic impairments such as type 2 diabetes, insulin resistance, and dyslipidemia.

The aims of this thesis were 1) to identify the potential metabolic and ovarian programming effects of early postnatal sex steroid exposure in adult female rats, and 2) to utilize data collected by the Swedish Twin Registry to investigate, in a large cohort of dizygotic twins, the potential effects of prenatal androgen exposure on metabolism and anthropometry in adult women with a male twin.

The main findings of this thesis were:

A single early postnatal dose of testosterone or estradiol caused insulin resistance and an increase in mesenteric adipocyte size in adult female rats. Testosterone exposure also resulted in dyslipidemia and estradiol exposure in elevated triglyceride levels. Rats exposed to estradiol displayed more pronounced insulin resistance than rats exposed to testosterone or dihydrotestosterone. Testosterone-injected rats exhibited increased mesenteric adipose tissue. Dihydrotestosterone-injected rats exhibited reduced insulin sensitivity only. Estradiol administration directly after birth altered ovarian morphology and expression of genes involved in follicle development. Estradiol exposure also decreased the weight of parametrial adipose tissue, increased parametrial adipose tissue lipoprotein lipase activity, and altered parametrial adipose tissue expression of genes involved in adipose tissue metabolism. In addition, reduced insulin sensitivity in postnatal estradiol-exposed rats was accompanied by an increase in the serum levels of inflammatory markers, and skeletal muscle alterations in the expression of immune-related genes and genes involved in the regulation of glucose and lipid metabolism. Adult women with at twin brother exhibited increased weight and BMI, and a higher risk of being overweight compared to women from same-sex twin pairs. The differences in BMI and weight between the groups were observed in women of 60 years and older, but not in those below 60 years of age. Dyslipidemia, but not type 2 diabetes mellitus, was more common in women with a male twin.

In summary, perinatal exposure to sex steroids affected the developing organism, predisposing to reproductive and endocrine abnormalities and features of the metabolic syndrome at adult age. Changes in insulin sensitivity, lipid profile, adipose tissue distribution, cellularity and metabolism, as well as in ovarian morphology, are factors that can be programmed perinatally with health consequences in adulthood. Our observations of dyslipidemia and increased BMI and body weight in opposite-sex female twins are consistent with the results of animal experiments, indicating that the programming effects of early androgen exposure are of relevance also for humans.

POPULÄRVETENSKAPLIG SAMMANFATTNING

En ogynnsam påverkan, i form av till exempel över- eller underskott av vissa hormoner eller näringsämnen, under känsliga perioder i fosterlivet (prenatalt) eller efter födelsen (postnatalt) kan ge kvarstående effekter i det vuxna livet. Denna process, som kallas ”programmering”, kan bidra till störningar i fortplantningsförmåga och ämnesomsättning vilket bland annat kan leda till insulinokänslighet, typ 2 diabetes och blodfettsrubbningar i vuxen ålder. Dessa riskfaktorer ingår i ett allt mer vanligt förekommande tillstånd som kallas det metabola syndromet. Det finns med säkerhet flera orsaker bakom utveckling av denna välfärdssjukdom. Genetiska faktorer och livsstil i det vuxna livet är involverade, men påverkan av olika slag tidigt i livet kan också spela en viktig roll.

Syftet med denna avhandling var att 1) undersöka potentiella programmeringseffekter av tidig postnatal exponering för manliga könshormoner (testosteron och dihydrotestosteron) och kvinnligt könshormon (östradiol), på ämnesomsättning och äggstockar hos vuxna honråttor och 2) använda data insamlade av Svenska Tvillingregistret för att undersöka potentiella effekter av prenatal exponering för manliga könshormoner med avseende på blodfettsrubbning, vikt och body mass index (BMI) hos vuxna kvinnor med en tvillingbror.

Huvudfynden i den här avhandlingen är:

En enda injektion av testosteron eller östradiol till honråttor direkt efter födseln leder till utveckling av insulinokänslighet, stora fettceller och blodfettsrubbningar i vuxen ålder. Råttor injicerade med testosteron fick även en ökad mängd fett inuti buken. Råttor som fick dihydrotestosteron uppvisade endast insulinokänslighet. Honråttor injicerade med östradiol uppvisade kraftigare insulinokänslighet jämfört med de råttor som exponerats för testosteron eller dihydrotestosteron. Östradiolexponering resulterade även i mindre äggstockar med markanta strukturella förändringar samt i ägglossningsrubbningar. Fettvävnaden som omger äggstockarna minskade i vikt och uppvisade förhöjd aktivitet av enzymet lipoproteinlipas, vilket är involverat i fettsyreupptag. Dessa råttor hade även förhöjda nivåer av inflammationsmarkörer i blodcirkulationen och skelettmuskulaturen, samt förändringar i skelettmuskulaturens uttryck av gener involverade i socker- och fettmetabolismen. Vuxna kvinnor med en tvillingbror visade sig ha högre vikt och BMI jämfört med kvinnor med en tvillingsyster. Dessa skillnader observerades inte hos kvinnor som var under 60 år, men däremot hos de kvinnor som var 60 år och äldre. Både blodfettsrubbning så väl som övervikt (BMI>25) var vanligare hos kvinnor med en tvillingbror.

Sammanfattningsvis talar denna avhandling för att könshormonexponering tidigt i livet kan påverka hälsotillståndet i vuxen ålder genom att orsaka reproduktionsstörningar samt utveckling av riskfaktorer som ingår i det metabola syndromet. Våra observationer avseende högre vikt och BMI samt blodfettsrubbning hos kvinnor med en manlig tvilling överensstämmer med resultaten från våra djurexperiment. Detta indikerar att programmeringseffekterna observerade hos råtta efter tidig postnatal exponering för manligt könshormon är av relevans även för människa.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I **Postnatal testosterone exposure results in insulin resistance, enlarged mesenteric adipocytes, and an atherogenic lipid profile in adult female rats: comparisons with estradiol and dihydrotestosterone.**

Alexanderson C, Eriksson E, Stener-Victorin E, Lystig T, Gabrielsson B, Lönn M, Holmäng A.

Endocrinology. 2007 Nov; 148(11):5369-76.

II **One single early postnatal oestradiol injection results in profound effects on ovary and parametrial adipose tissue in adult female rats.**

Alexanderson C, Stener-Victorin E, Lönn L, Kullberg J, Levin M, Cajander S, Lönn M, Holmäng A.

Submitted.

III **Early postnatal estradiol exposure causes insulin resistance and signs of inflammation in circulation and skeletal muscle.**

Alexanderson C, Eriksson E, Stener-Victorin E, Lönn M, Holmäng A.

Submitted.

IV **Having a male twin is associated with body mass index and metabolism in middle-aged and old women.**

Alexanderson C, Henningsson S, Lichtenstein P, Holmäng A, Eriksson E.

Submitted.

TABLE OF CONTENTS

ABBREVIATIONS

AR	androgen receptor
BMI	body mass index
CL	corpora lutea
C3	complement component 3
CPT1b	carnitine-palmitoyl transferase 1b
CT	computed tomography
CV	coefficient of variation
DEXA	dual energy X-ray absorptiometry
DHT	dihydrotestosterone
E	estradiol
ER	estrogen receptor
FFA	free fatty acid
GIR	glucose infusion rate
Glut	glucose transporter
HDL-C	high density lipoprotein cholesterol
it.a.	inter-assay
i.a.	intra-assay
LDA	low density arrays
LDL-C	low density lipoprotein cholesterol
MRI	magnetic resonance imaging
NIDDM	non-insulin dependent diabetes mellitus
PCOS	polycystic ovary syndrome
PPARd	peroxisome proliferator-activated receptor delta
RBP4	retinol binding protein 4
SEM	standard error of the mean
STR	Swedish Twin Registry
T	testosterone
TC	total cholesterol
T2DM	type 2 diabetes mellitus
TG	triglycerides
TGFβ	transforming growth factor β
MCP-1	monocyte chemoattractant protein-1
sICAM-1	soluble intercellular adhesion molecule-1

INTRODUCTION

The etiology of conditions such as the metabolic syndrome in adult life is multifactorial, involving both genetic and environmental factors. Events occurring in early life may also predispose to adult disease. This thesis focuses on perinatal sex steroid exposure and its effects on adult ovarian and other endocrine traits, and on the features of the metabolic syndrome, insulin resistance in particular.

Programming

In 1991, Lucas defined the concept of programming as "a stimulus or insult operating at a critical or sensitive period of development, resulting in a long-lasting or lifelong effect on the structure and function of the organism".[1] These stimuli may include maternal infection or stress as well as an excess or deficiency of normally-occurring substances, e.g. hormones, nutrients and vitamins. Programming depends not only on the type of stimulus but also, critically, on the timing of exposure. Different tissues and cells have different "specific windows of sensitivity", each tissue having its own critical period of vulnerability to programming. The duration and magnitude of the stimulus are also significant to the outcome.[2-4] Another important principle of the programming concept is that males and females may display different responses to an identical stimulus.[5] Numerous programming studies in humans and animals have thus demonstrated different metabolic outcomes depending on gender.[6]

Several programming hypotheses based on Lucas's concept have been put forward. The "thrifty phenotype hypothesis" proposed by Hales and Barker in 1992[7] suggests that insulin resistance and type 2 diabetes mellitus (T2DM) in adults are the result of poor fetal nutrition. They suggested that undernourishment *in utero* causes developmental and growth maladaptations that can be described as a metabolic thrift. These adaptive changes are beneficial for survival in deprived conditions but detrimental when food is abundant. This thrifty phenotype becomes permanent and, combined with adiposity in later life, may lead to T2DM. Barker also put forward the "fetal origins hypothesis", proposing that coronary heart disease and related disorders such as hypertension and insulin resistance are the result of adaptations made by the undernourished fetus and infant.[8,9] As it is now known that growth during infancy and childhood is also linked to adult disease, the term "developmental origins hypothesis" is now preferred.[10]

The crux of these hypotheses is that when the fetal or early environment is poor, there is an adaptive response which promotes the growth of certain key organs to the disadvantage of others. This leads to altered postnatal metabolism, designed to enhance postnatal survival. Programming is therefore an adaptive response that has immediate advantages for the survival of the organism. However, if there is a mismatch between early life conditions and the conditions in adulthood, programming in combination with lifestyle factors and genetic predisposition may have unfavorable health effects such as the metabolic syndrome[11] (Fig. 1.). The metabolic syndrome is described in more detail below.

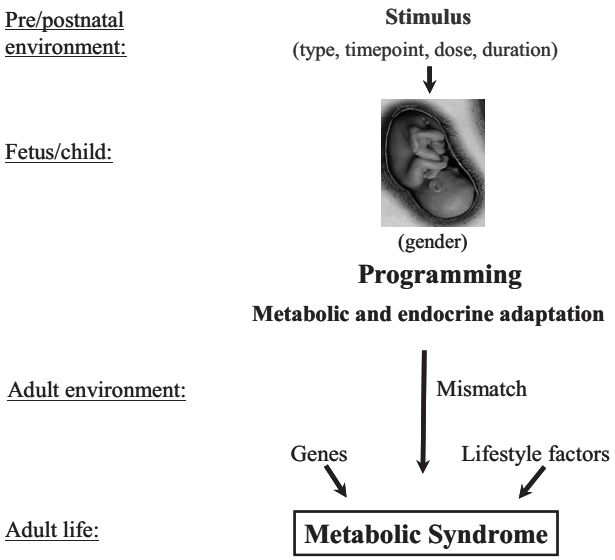

Figure 1. Schematic picture showing how early programming may lead to the development of the metabolic syndrome.

Epidemiological evidence

The "fetal origins hypothesis" is based upon a number of epidemiological findings as described in this section. One of the earliest reports of programming was made in 1964 by Rose,[12] who showed that individuals suffering from ischemic heart disease were twice as likely to have a sibling who was stillborn or who died in infancy. This indicated that poor conditions in early life may be associated with an increased risk of developing cardiovascular disease in adulthood. Later on, studies in England and Wales supported

these findings by showing a positive geographical correlation between neonatal and postneonatal mortality rates and ischemic heart disease rates in adults.[13] Low birth weight has also been shown to correlate with impaired glucose tolerance, non-insulin dependent diabetes (NIDDM), hypertension and death from cardiovascular disease, as shown in a Hertfordshire population.[14,15]

Sex steroid programming in humans

Fetal hormone transfer

Animal studies show that exposure to testosterone and its metabolites is influenced by the intrauterine position of the fetus. In litter-bearing mammals, a fetus situated between two male fetuses is exposed to higher levels of testosterone than a fetus situated between two female or one female and one male fetus.[16] Fetal hormone transfer may occur in different ways. A maternal-fetal transfer route has been suggested,[17] and the feto-fetal transfer route has also been offered as an explanation. The latter suggests that diffusion of testosterone between fetuses occurs across amniotic membranes.[18,19] Interestingly, in humans, testosterone levels in amniotic fluid have shown to be higher in male than in female fetuses[20].

An increase in the circulating levels of some androgens, including testosterone and androstenedione, has been described in normal pregnancy.[21-23] Normally the human fetus is protected from maternal androgens by increased placental aromatase activity[24] and by high levels of sex-hormone binding globulin, which interferes with the biological activity of androgens.[25,26] This protection may be impaired if placental aromatase activity is inhibited or the production of sex hormone binding globulin is reduced, e.g. by insulin.[27,28] This may be important in pregnant hyperinsulinemic subjects, such as pregnant women with polycystic ovary syndrome (PCOS).[29] Serum levels of sex hormone binding globulin are also often low in individuals with NIDDM and cardiovascular disease.[30,31]

Opposite-sex twins

Females from opposite-sex twins share a prenatal environment with a male co-twin and are thought to be exposed to increased levels of testosterone.[32] No studies have hitherto investigated the metabolic effects of the potential prenatal testosterone transfer from the male twin in these female opposite-sex twins. However, a small number of studies carried

out on human twins have produced interesting results on the effects on sexually dimorphic traits. For example, females from opposite-sex twin pairs exhibit more masculine personality characteristics, e.g. increased sensation seeking[33] and aggressive behavior[34] compared to females from same-sex twin pairs. Masculinization has also been observed for traits that are unlikely to be socially influenced, for example tooth size[35] and the second to fourth digit ratio.[36]

Programming with androgens

PCOS is the most common female endocrinopathy and is associated with hyperandrogenism (where ovarian-androgen excess is thought to be the primary source[37]), ovulatory dysfunction and polycystic ovaries.[38] Women with PCOS are also more prone to develop hyperinsulinemia, insulin resistance and T2DM.[39,40] These disturbances are the underlying reasons for PCOS being referred to as the "female metabolic syndrome". Development of PCOS probably requires the interaction of several environmental and genetic factors.[41] Experimental animal research and clinical observations have led to the developmental origins hypothesis of PCOS.[42] According to this hypothesis, fetal exposure to androgen excess can induce changes in differentiating tissues leading to the reproductive and metabolic characteristics of the PCOS phenotype in adult life.[42,43] Increased androgen concentrations are found in pregnant women with PCOS compared to controls, and this may act as a potential source of androgen excess in the fetus.[29] It has also been shown that androgen excess occurs during breastfeeding in women with PCOS – a possible maternal source of infant androgen excess.[44]

The developmental origins hypothesis of PCOS is consistent with the increased prevalence of this disorder in women with classical congenital adrenal hyperplasia. This disease is a disorder of the adrenal cortex that results in cortisol deficiency and androgen excess already evident in early fetal life.[45] Studies have suggested that anovulation, ovarian hyperandrogenism, luteineizing hormone hypersecretion and polycystic ovaries, common in these women, are due to prenatal androgenization.[46,47] Additional mechanisms that may contribute to excess androgen exposure of the female fetus, regardless of the source of androgens, could be reduced fetal binding capacity of androgens by sex hormone binding globulin or reduced aromatization of androgens leading to increased tissue androgen

availability.[48] Both maternal and fetal hyperandrogenism can therefore provide plausible mechanisms for female fetal androgen excess and programming of PCOS in humans.

Programming with estrogens

Endocrine-disrupting chemicals are natural or synthetic compounds that interfere with the normal function of an organism's endocrine system.[49] As prenatal and early postnatal life are very sensitive developmental periods, exposure to environmental endocrine disruptors at these times can disturb the development of the endocrine system and of the organs that respond to endocrine signals. Several known environmental endocrine disruptors are released into the environment in large quantities.[50] Many of these chemicals have estrogenic effects, such as phytoestrogens and polychlorinated biphenyls.[49] An area of interest is the possible hormonal effects on the developing fetus and infant of dietary phytoestrogens which exist in, e.g., soya-based infant formulas.[51] A crucial question posed in this context is whether phytoestrogen exposure in developing humans or animals could alter adipocyte development and/or adult adipocyte number.[52]

The use of diethylstilbestrol in pregnant women at the beginning of the 1940s represents the prime example of fetal exposure to estrogens. This agent is a synthetic estrogen that was used to prevent miscarriage. However, its use was banned when it became apparent that it had detrimental reproductive consequences in daughters exposed *in utero.*[53]

Animal models of sex steroid programming

A number of androgen-programming studies have been carried out in female animals to evaluate the possible effects on metabolism and on ovarian and endocrine status. These studies were predominantly done on rhesus monkeys, sheep and rats.[54-56] The early influence of androgens on the sexual differentiation of the brain in males, an area studied for several decades, is to a great extent exerted by the testosterone metabolite estradiol acting via estrogen receptors (ERs).[57,58] Hence, it is not unlikely that also the effects of testosterone on metabolism and ovaries observed in female animals have been due, at least in part, to the aromatization of testosterone to estradiol. However, we have not found any previous studies that have investigated the metabolic outcomes in adult female rats of early postnatal exposure to *estrogen*, or any that have compared the effects of testosterone, estradiol and dihydrotestosterone (DHT) exposure.

Metabolic effects

Early prenatal testosterone treatment of female rhesus monkeys induces selective deposition of intra-abdominal fat,[59] impaired pancreatic β-cell function,[60] insulin resistance, and increased rates of T2DM[61] and hyperlipidemia.[62,63] Late prenatal treatment induces increased total body fat and increased deposition of abdominal and non-visceral abdominal adipose tissue compared to control females and early testosterone-treated females,[64] as well as impaired insulin sensitivity with increased body mass index (BMI).[60] With increasing BMI, normal female rhesus monkeys preferentially accumulate non-visceral fat, while both early and late testosterone-treated female monkeys accumulate visceral adipose tissue.[64]

The metabolic outcomes in rhesus monkeys are similar to those seen in female sheep treated prenatally with testosterone. These animals display impaired insulin sensitivity in early postnatal life,[65] together with a tendency of higher total and low density lipoprotein cholesterol (LDL-C).[66]

Androgenization of newborn female rats with a single dose equivalent to the endogenous release in male pups directly after birth results in insulin resistance in adult age, as measured by hyperglycemic and euglycemic hyperinsulinemic clamp methods. In addition, these rats develop increased body weight and centralization of body fat.[67] Similarly, androgenization of female rats on the 5th day of life induces insulin resistance as well as increased visceral adipose tissue, body weight and plasma leptin in adult animals.[68] Furthermore, transient prenatal androgen treatment induces the features of the metabolic syndrome, including increased body weight, raised serum insulin, adiposity, dyslipidemia and hepatic steatosis in the adult female rat.[55]

Ovarian and endocrine effects

Female rhesus monkeys and sheep treated with testosterone prenatally exhibit ovulatory dysfunction, with enlarged and polyfollicular ovaries in adulthood.[54,69] The female monkeys are also hyperandrogenic.[70]

Female rats exposed to androgen or estrogen in the immediate postnatal period develop persistent estrus with anovulation as adults,[71,72] with smaller ovaries of polycystic appearance lacking corpora lutea (CL).[72-74]

It is generally believed that the treatment of female fetal/neonatal animals with testosterone or estrogen structurally and functionally defeminizes the neuroendocrine system, with impaired feedback regulation of hypothalamic gonadotropin releasing hormone and pituitary gonadotropins.[71,75] The mechanisms possibly responsible for this, and the effects of peripheral organs such as the ovaries, are the subject of some debate. However, the subject of the neuroendocrine axis is beyond the scope of this thesis.

Sex steroid production and function

Sex steroids include androgens, estrogens and progesterone. In females, their secretion is regulated by the hypothalamic-pituitary-ovarian axis, which is a complex and highly coordinated system sensitive to different inputs.[49] Androgens exert their effects either directly via the androgen receptor (AR) or indirectly by aromatization of testosterone to estradiol, which acts exclusively on ER.[76] Two different ERs have been identified – ERα and ERβ.[77] DHT, an androgen converted from testosterone primarily at peripheral sites, is a non-aromatizable androgen.[78] DHT has a higher affinity for AR than testosterone, as indicated by a slower dissociation rate.[79]

In females, the ovary and adrenal cortex produce androgens, but in rodents the existence of adrenal androgen production is the subject of some debate.[80-82] Estrogen synthesis occurs by aromatization of testosterone mainly in the female rat ovary, but also in other tissues such as adipose tissue, skin, placenta, bone and brain.[83]

In males, there are three phases of high testosterone production. The first occurs early in fetal development and acts to differentiate the male genital organs. The second phase takes place shortly after birth, and the third phase begins at puberty and continues throughout adulthood. Testosterone and estrogens act on most body systems, and their effects include the development of secondary sexual characteristics and the regulation of reproductive organs and the reproductive cycle.[83,84] The influences of testosterone and estradiol on adipose tissue and insulin sensitivity are central to this thesis. Testosterone plays a major role in determining the sex-dependent distribution and mass of adipose tissue in men and women,[85] and estradiol is a major regulator of adipose tissue during development and in adulthood.[86] These estrogen effects may either be direct effects on adipogenesis, lipogenesis and lipolysis, or indirect effects exerted e.g. via the central nervous system.[86] Testosterone and estradiol are also involved in regulating insulin

sensitivity, as shown by both human and experimental data.[87] In women, increased serum testosterone levels predispose to an unfavorable body fat distribution and to insulin resistance.[88]

Metabolic syndrome

According to the International Diabetes Foundation, the metabolic syndrome is defined as central obesity plus any two of the following four factors: raised triglycerides (TG) or specific treatment for this lipid abnormality, reduced high density lipoprotein cholesterol (HDL-C) or specific treatment for this lipid abnormality, raised blood pressure or treatment for previously diagnosed hypertension, and raised fasting plasma glucose or previously diagnosed T2DM.[89] The syndrome is also known as "Syndrome X"[90], "the insulin resistance syndrome"[91] and "the deadly quartet".[92] It has also been described as insulin resistance and visceral obesity in combination with a cluster of metabolic risk factors that create a predisposition to cardiovascular disease.[90] However, there is as yet no universally accepted term for the syndrome and several definitions of the condition have been published.[93-95] For example, as chronic low-grade inflammation has been associated with features of the metabolic syndrome,[96,97] it has been proposed that a measure of inflammatory status should be included in the definition.[98-100]

In recent years the syndrome has been more prevalent in men than in women. However, the prevalence has increased, in young women in particular.[101] The different components of the condition – impaired glucose tolerance, abdominal obesity, hypertension and dyslipidemia – also vary between the genders. Some of these differences may be attributable to the effects of sex hormones.[101]

Insulin resistance

As early as in the 1930s, Himsworth defined insulin resistance as a condition whereby a given insulin concentration fails to produce the expected magnitude of effects on target cells.[102] Blood glucose levels remain normal as long as the pancreatic β-cells manage to increase insulin secretion sufficiently to compensate for the dysfunctional insulin-response in peripheral tissues. Over time, the β-cells become however exhausted and the secretion of insulin fails, resulting in NIDDM.[103]

Insulin exerts its effects by binding to insulin receptors that stimulate glucose uptake in skeletal muscle and adipose tissue by increasing the number of intracellular glucose

transporters. Glucose transporter (Glut) 4 is the main insulin-responsive transporter and is located primarily in muscle cells and adipocytes.[104] Multiple mechanisms are responsible for insulin resistance in patients with NIDDM, in whom post-receptor defects are the most common.[103]

Skeletal muscle and liver are the most important insulin-sensitive organs, and the largest systemic effect is seen when insulin resistance is present in these organs.[103] Other tissues, such as ovaries and adipose tissue, may also act as insulin-resistant targets but this has a minor impact on the insulin sensitivity of the whole body. However, these organs are important as *mediators* of alterations in skeletal muscle and hepatic insulin sensitivity. The roles of different organs and secreted factors on insulin sensitivity are shown in Fig.2.

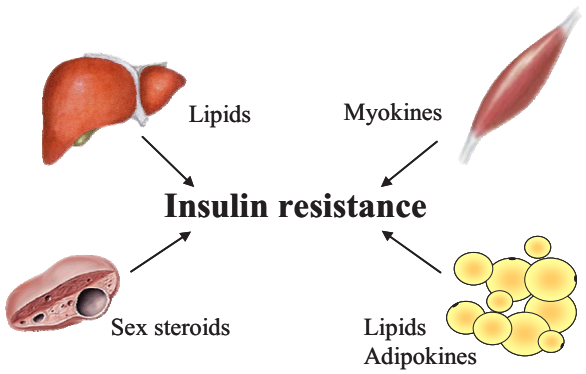

Figure 2. Secretory factors from different organs (liver, skeletal muscles, ovary, adipose tissues) influencing insulin sensitivity.

Skeletal muscle

In humans and rodents, skeletal muscle represents the largest insulin-sensitive tissue in the body and accounts for 75% of all insulin-mediated glucose disposal.[105] Consequently, any change in skeletal muscle mass, metabolic rate and/or response to hormones or other circulating factors would significantly affect the whole body glucose disposal rate.

Insulin resistance in skeletal muscle

Studies in which sex steroids have been administrated to humans or animals provide evidence of the impact of these hormones on skeletal muscle insulin sensitivity. Administration of testosterone to female-to-male transsexuals thus results in insulin resistance at peripheral sites of insulin action, as shown by hyperinsulinemic-euglycemic

clamp studies.[106] In the same vein, female rats given testosterone develop muscular insulin resistance. This is due to a defective glycogen synthase system and translocation of glucose transporters, as well as to morphological muscle changes such as reduced capillarization resulting in less insulin-sensitive muscles.[107,108]

The dyslipidemia seen in many insulin-resistant conditions often includes high levels of TG and free fatty acids (FFA) and low levels of HDL-C.[109] Excessive accumulation of lipids into non-adipocytes, such as muscle and liver, is thought to induce impaired insulin sensitivity. Originally, it was showed that fatty acids compete with glucose for substrate oxidation, leading to decreased glucose uptake – a process known as the Randle cycle.[110] Later studies suggest that fatty acid derivatives of accumulated intramyocellular lipids cause a disturbance in the insulin signaling pathway, eventually leading to reduced glucose uptake.[111]

Skeletal muscle as an endocrine organ

It has recently become apparent that skeletal muscle produces and secretes a multitude of signaling peptides and cytokines exerting autocrine, paracrine and endocrine effects. Molecules produced, expressed, and released by myocytes are termed myokines.[112] These factors, some of them inflammatory molecules, may play an important role in regulating glucose metabolism and contribute to insulin resistance. The list of identified myokines is constantly growing and includes e.g. monocyte chemotactic protein-1 (MCP-1).[112-114] Interestingly, skeletal muscle from insulin-resistant and type 2 diabetic subjects exhibit increased expression of inflammatory molecules, which correlates inversely with insulin sensitivity.[114]

Adipose tissue

Insulin-stimulated adipose tissue glucose uptake is thought to represent only about 2% of total glucose disposal.[103]

Distribution of adipose tissue

Human adipose tissue can be divided into subcutaneous and visceral depots. The visceral adipose tissue depot can be divided anatomically into omental, mesenteric, and retroperitoneal fat depots. In rats, the visceral adipose tissue also includes gonadal adipose tissue – epididymal or parametrial according to gender. The omental depot does not exist

in rats. The adipose tissues studied in this thesis are the inguinal (subcutaneous), mesenteric and parametrial depots. Their localizations are shown in Fig.3.

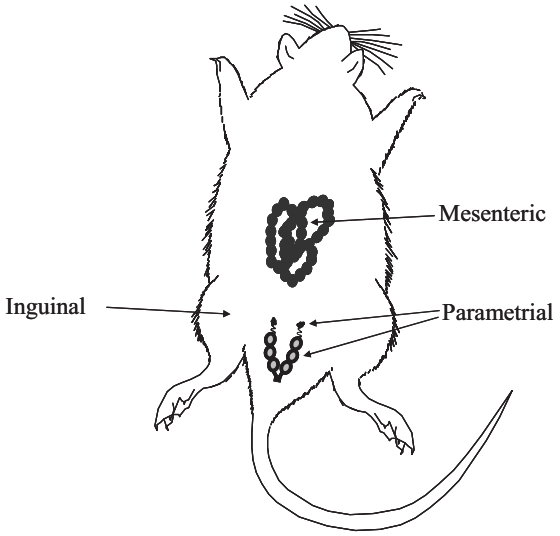

Figure 3. Localizations of studied rat adipose tissues (mesenteric, parametrial and inguinal). Modified with permission from Elisabet Stener-Victorin.

The total amount and distribution of adipose tissue is different in men and women. Women have a higher percentage of body fat than men, with greater accumulation of subcutaneous fat in the gluteofemoral region but with less visceral fat. Some of these differences can partly be explained by the action of sex hormones.[85,86]

Adipose tissue distribution affects metabolic outcome, with increased visceral adipose tissue being strongly associated with insulin resistance and T2DM.[85,115,116] The localization and the high metabolic activity of visceral fat may be the key factors predisposing to the complications of central obesity.[85] For example, the visceral depot is drained by the portal venous system and has therefore a direct connection with the liver. This is believed to contribute to the development of insulin resistance via the release of FFA from visceral adipose tissue. The production and secretion of cytokines and other bioactive molecules may also be involved in the development of complications related to visceral obesity.[109,117] Another factor that may influence the development of metabolic disorders is the size of the adipocytes. Enlarged human subcutaneous adipocyte size is associated with insulin resistance and is an independent risk factor for T2DM.[118] The main role of adipocytes is

to store excess TG. However, when adipocytes become enlarged, reaching their fat storage capacity, lipid excess may instead accumulate ectopically, for example in skeletal muscle and the liver impairing insulin sensitivity.[119] In addition, hypertrophic adipocytes are less sensitive to the antilipolytic effect of insulin [120] promoting increased circulating FFA levels resulting in insulin resistance.[121] Increased FFA delivery to the liver promotes hepatic glucose production.[122] Furthermore, accumulation of FFA in the liver increases TG production, raising the levels of circulating lipids even further.[109]

Adipose tissue as an endocrine organ

White adipose tissue is an active endocrine organ that secretes various adipokines. In this way adipose tissue is able to communicate with the brain and peripheral tissues, including skeletal muscle.[123] Adipokines are involved in a variety of processes, e.g. lipid metabolism, appetite and energy regulation, immunity, insulin sensitivity, inflammation and blood pressure regulation. The expression of some of the adipokines varies with adipose tissue location[124] and changes depending on adipose tissue mass.[125,126] The expression and release of some adipokines seems to be correlated to adipocyte size.[127-130] It has been suggested that a number of the adipokines regulate insulin sensitivity through cross-talk with skeletal muscle.[117,123] Some adipokines protect against insulin resistance, whilst others induce it.[117] Co-culture of human fat and skeletal muscle cells indicates that adipocytes can signal directly to skeletal muscle by the release of adipocyte factors, which leads to impaired muscle insulin signaling and subsequent insulin resistance.[131]

Ovaries

Testosterone, estradiol and progesterone are involved in the maintenance of normal insulin sensitivity, as shown by many human and animal studies.[87] As sex hormones in females are predominantly produced by the ovaries, this organ becomes an important mediator of systemic insulin resistance. Physiological states with increased sex steroid levels, such as puberty,[132] normal pregnancy[133] and the luteal phase of the menstruation cycle, are associated with a physiological insulin resistance.[134,135] A relative insulin resistance is also seen after the menopause, which can partly be explained by low estrogen levels.[136,137] In female rats, insulin sensitivity varies with the estrus cycle,[138] and ovariectomy results in insulin resistance as measured by the euglycemic hyperinsulinemic clamp technique. This is paralleled by decreased insulin-stimulated glucose transport in

muscle. Estrogen treatment restores insulin sensitivity, suggesting that estradiol is an important regulator of glucose uptake in muscle.[139]

It has been suggested that a vicious circle may explain the association between insulin resistance and hyperandrogenism seen in women with PCOS. This explanation involves chronic androgen excess of ovarian and/or adrenal origin, possibly already present in early life, causing abdominal obesity and skeletal muscle insulin resistance, promoting further hyperandrogenism.[140]

AIMS

Overall aim

To elucidate the potential metabolic and ovarian programming effects of postnatal sex steroid exposure in adult female rats and the potential prenatal androgen programming effects on metabolism and anthropometry in adult women.

Specific aims

The following issues were to be addressed:

- **Comparison between early postnatal programming effects of androgens and estradiol in adult female rats** *(paper I)*

Does testosterone exposure affect insulin sensitivity, adipose tissue distribution, and adipocyte size and/or lipid profile?

Are the effects of testosterone mediated via androgen receptor (AR) and/or ERα activation?

- **Early postnatal programming effects of estradiol in adult female rats** *(papers II, III)*

Does postnatal estradiol exposure affect ovarian morphology and genes related to follicular development?

Does postnatal estradiol exposure affect parametrial adipose tissue mass, adipocyte size, lipoprotein lipase (LPL) activity and gene expression related to adipose tissue metabolism? Is it possible to carry out volumetric estimation of this fat depot by using magnetic resonance imaging (MRI)?

Does postnatal estradiol exposure affect metabolic parameters, including insulin sensitivity, adipose tissue distribution and adipocyte size?

Are the effects on metabolism found in postnatal estradiol-exposed rats accompanied by changes in circulating inflammatory markers and/or by the expression of genes involved in inflammation, lipid and glucose metabolism in skeletal muscle and/or adipose tissue?

- **The influence of having a male twin on metabolism and anthropometry in women** *(paper IV)*

Is the metabolic phenotype, i.e. BMI, weight, lipid profile and T2DM, of adult women influenced by having a male twin, and hence- tentatively- having been exposed to increased androgen levels prenatally?

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in the Material and Methods sections of the individual papers, while more general comments are presented below.

Animal studies *(papers I-III)*

Experimental animals *(papers I-III)*

Time-mated female Wistar rats (B&K Universal. Sollentuna, Sweden) were housed individually until parturition. Pups were raised with a lactating mother until 21 days of age. All experimental animals were maintained in a controlled environment (21˚±2˚C; humidity 55-65%; 12:12 light/dark cycle). Standard principles of laboratory animal care were followed. All experimental procedures were approved by the animal ethics committee of the University of Gothenburg.

Animal models *(papers I-III)*

An overview of the study designs of the first and second studies is depicted in Fig.4 A and B.

Postnatal testosterone, estradiol or DHT (paper I)

In the first study, male pups were removed after birth and female pups were assigned to either treatment or control groups. Within 3 hours of birth, the treatment groups were injected subcutaneously with testosterone propionate (1mg, Apoteksbolaget, Stockholm, Sweden), estradiol benzoate (0.5mg, Apoteksbolaget, Stockholm, Sweden) or DHT propionate (1mg, Steraloids, Newport, RI). Controls received vehicle. The testosterone dose was chosen according to previous studies, in an attempt to mimic the endogenous testosterone peak in male pups.[141]

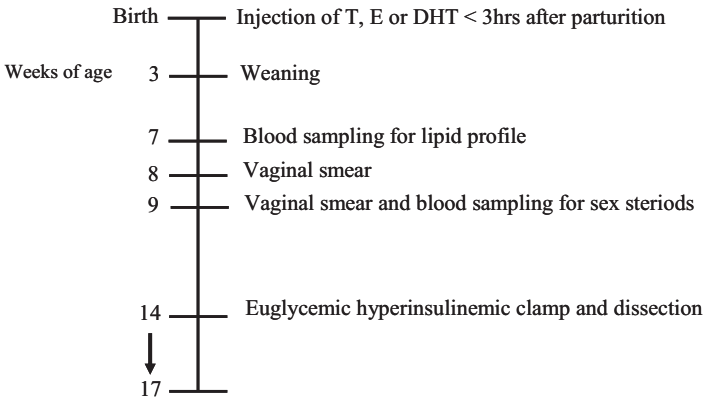

Figure 4A. Time axis for the first study.

Abbreviations: T= testosterone, E=estradiol, DHT= dihydrotestosterone

Postnatal estradiol (papers II-III)

In the second study, male pups were removed after birth, and female pups were assigned either to treatment or control groups. Within 3 hours of birth, pups in the treatment group were injected subcutaneously with estradiol benzoate (0.35mg, Apoteksbolaget, Stockholm, Sweden) and controls received vehicle.

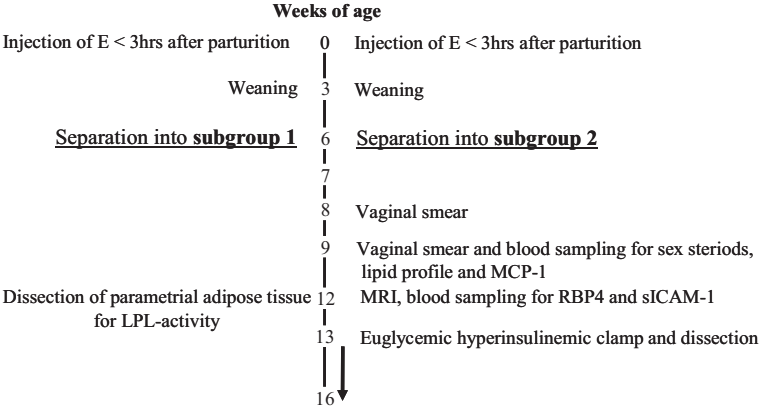

Figure 4B. Time axis for the second study.

Abbreviations: E=estradiol, MCP-1= monocyte chemoattractant protein-1, MRI=magnetic resonance imaging, RBP4=retinol binding protein 4, sICAM-1=soluble intracellular adhesion molecule-1

Vaginal smears *(papers I-III)*

Comments: To determine the estrus status, daily vaginal smears were performed over 10-14 consecutive days. Vaginal samples were collected by immersing a sterile Pasteur pipette in water and then gently inserting the pipette into the vagina. The vaginal contents were then mounted onto a slide and examined under the microscope. Smears were classified according to the relative proportion of leukocytes, nucleated epithelial cells, and cornified cells. The normal estrus cycle of the rat is 4-5 days and consists of four different stages: estrus, diestrus 1, diestrus 2 and proestrus. Estrus is characterized by the presence of cornified cells, diestrus 1 and 2 by the presence of large numbers of leukocytes, some cornified cells and almost no nucleated cells, while proestrus is characterized by the presence of large round nucleated cells (Fig.5).[142] Blood sampling for sex steroid analysis and clamp experiments were performed in the estrus phase of the cycle, because this phase is easy to identify and because estradiol and progesterone levels are relatively stable during this period.

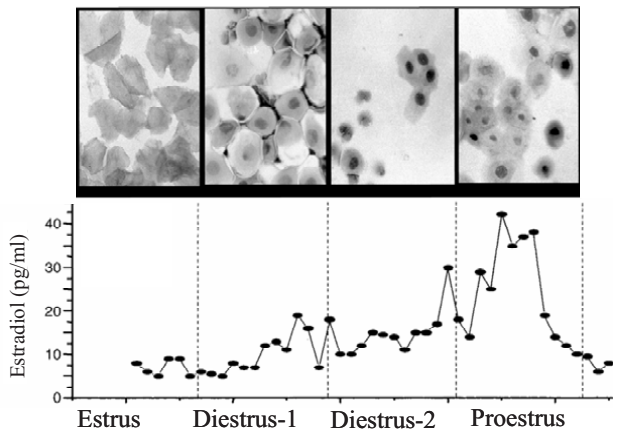

Figure 5. The photographs show cell appearance in vaginal smears during the different phases of the estrus cycle. The diagram shows estradiol pattern in rat throughout the estrus cycle. Photographs of vaginal smears are reprinted with permission of Jovanna Dahlgren.

Assessment of body composition and dissection of tissues *(papers I-III)*

Comments: After clamping the rats were decapitated, and mesenteric, inguinal *(papers I, III)* and parametrial adipose *(paper II)* tissues as well as the ovaries *(paper II)* and soleus, extensor digitorum longus and tibialis anterior muscles *(paper III)* were dissected and snap frozen in liquid nitrogen and stored at -80°C. One ovary from each rat and parametrial

adipose tissue pieces were placed in formaldehyde *(paper II)*. Mesenteric and inguinal adipose tissues were placed in medium for computerized determination of adipocyte size *(papers I, III)*.

To ensure that the removal of tissues was carried out identically in all animals, one or two persons performed the dissections in a similar fashion.

Magnetic resonance imaging *(paper II)*

Comments: MRI was used to measure parametrial adipose tissue volume as described in *paper II*. To distinguish parametrial adipose tissues from the remaining intra-abdominal adipose tissue, we identified an axial level that served as a border between the two depots. After localization of the pelvic girdle, vertebrae L5 (Fig. 6) was set as the skeletal reference for this level. Visceral adipose tissue volume caudal to the axial level served as a proxy measure of parametrial adipose tissue volume.

MRI is a non-invasive method that can be used to measure body composition and body fat content.[143] It provides detailed images of the body in any plane by using a powerful magnetic field, radio waves and a computer to produce detailed pictures of organs, soft tissue, bone and other internal body structures. Other non-invasive imaging methods are dual energy X-ray absorptiometry (DEXA) and computerized tomography (CT). The DEXA body composition technique can provide information on total and regional bone content as well as on body fat and lean body mass. The DEXA scanning technique measures the attenuation difference between two X-ray energies (dual X-ray) and exposes the subject to a very low radiation dose <0.1 uSV. However, this method is limited by the fact that it is not possible to quantify specific tissues.[144] CT provides images of cross-sectional areas of the body related to the density of the different tissues in each area. The X-ray beam passes through the body, and detectors on the opposite side of the body detect the transmitted radiation. The radiation dose is a concern in CT studies.[143] There is generally good agreement between CT- and MRI-derived measures of adipose tissue distribution and both methods are highly reproducible and reliable. However, with MRI there is no harmful ionizing radiation.[143] Studies have also shown the ability of MRI to quantify specific tissues accurately in rats.[145]

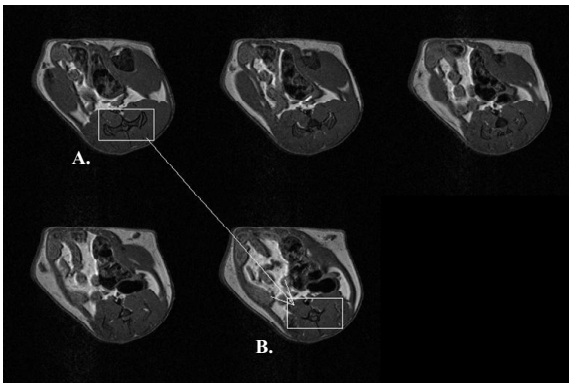

Figure 6. The pelvic girdle, shown in part (A), was chosen as a starting point. Part (B) shows vertebrae L5 which was set as a level that served as a border between parametrial and remaining intra-abdominal adipose tissue.

Euglycemic hyperinsulinemic clamp *(papers I, III)*

Comments: The euglycemic hyperinsulinemic clamp is a standard method of measuring insulin sensitivity[146] and has been adapted for use in rats.[147,148] A steady-state hyperinsulinemic level is achieved by the infusion of a bolus dose of insulin followed by a continuous infusion. To maintain glucose levels at euglycemic levels, glucose is infused at a variable rate. The glucose infusion rate (GIR) required to maintain basal plasma glucose levels serves as index of the response to a defined insulin concentration, i.e. the insulin sensitivity. The euglycemic clamp is reported to be sensitive enough to determine changes in GIR of approximately 10%.[149] In rats, GIR has an intra-assay (i.a.) coefficient of variation (CV) of 8.5%, whereas the inter-assay (it.a.) CV is 15.7% and the CV for maintaining blood glucose to the euglycemic level is 3-7%.[147,150] The euglycemic hyperinsulinemic technique is based on the suppression of basal hepatic glucose production by the infusion of insulin and glucose. It is important to bear this in mind because there is a risk of underestimating glucose utilization, especially in insulin-resistant subjects in whom the capacity to suppress hepatic glucose production is impaired. The hyperinsulinemic euglycemic clamp can be combined with the use of radioactively labeled glucose, making it possible to measure glucose uptake by specific tissues and to distinguish peripheral insulin sensitivity from insulin sensitivity in the liver. Alternative methods used to determine whole-body glucose uptake in rodents include the glucose tolerance test[151] and the minimal model technique.[152]

Computerized determination of adipocyte size – isolated adipocytes *(papers I, III)*

Comments: The technique for computerized analysis is a valuable tool for measuring adipocyte size.[153] It is quick and allows the assessment of a sufficient number of cells to provide reliable data on size distribution; about 10 times more cells than with conventional methods. In addition, images of cell preparations may be stored for future reference. One limitation of the technique is that lipid droplets that are included in the sample must be excluded manually from these images. It may sometimes be difficult to discriminate between small adipocytes and small lipid droplets (20-35 μm).

Computerized determination of adipocyte size – adipose tissue sections *(paper II)*

Comments: This technique is based on manual delineation of fat cell contours and automatic determination of the corresponding fat cell areas. We devised a strict protocol in order to minimize subjective evaluation, i.e. all closed contours in each image were delineated and all images were analyzed by the same operator. This approach is a good alternative when images of isolated adipocytes are not available.

Histological analysis of ovarian morphology *(paper II)*

Comments: Ovarian morphology was analyzed descriptively by scanning each slide with ScanScope (Aperio Technologies, Vista, CA) for measurements and photos and further analyzed with ImageScope virtual microscopy software (Aperio Technologies). All sections were analyzed by two observers in a blinded fashion. Antral follicles, defined as follicles with an antrum and CL, were included in the analysis, while primordial and primary follicles were deliberately excluded because of the risk of double-counting.

Real-time PCR *(papers II, III)*

Comments: Before designing the low density arrays (LDA), five different reference genes (18S rRNA, glyceraldehyde-3-phosphate dehydrogenase, peptidylprolyl isomerase A, and hypoxanthine guanine phosphoribosyl transferase, β-actin) were evaluated in the different tissues of interest using 384-well plates. LDA were then designed in 48-format, including the target genes of interest and four of the most appropriate evaluated reference genes (18S rRNA, glyceraldehyde-3-phosphate dehydrogenase, peptidylprolyl isomerase A, and hypoxanthine guanine phosphoribosyl transferase).

Real-time PCR is a sensitive method for the quantification of specific mRNAs. The method is based on the 5´nuclease assay, which uses the 5´nuclease activity of Taq polymerase to cleave a reporter dye on an oligonucleotide probe during PCR. The cleavage terminates the activity of a quencher dye, and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage and is monitored in real-time. Determination of mRNA levels of reference genes, i.e. genes not affected by the treatment, are crucial to ensure equal quantity and quality in different samples. LDA is a 384-well microfluidic card pre-loaded with probe and primers for the genes of interest. The cards can be designed in formats of 12-96 different genes on the same card. Consequently the experimental part of this approach is very accurate, convenient and quick.

Lipoprotein lipase activity *(paper II)*

Comments: LPL activity was measured in parametrial adipose tissue from rats that had been without food for 3-4 hours in the morning. This protocol was chosen to standardize the nutritional state of the animals. Preliminary experiments showed that at this point LPL activity was reduced and the variation in activity between animals was low. Therefore, food was removed at 07:00 a.m., 3-4 h before decapitation and dissection of parametrial adipose tissue. Dissection was alternated between controls and estradiol rats.

Analytical methods *(papers I-III)*

Comments: Blood samples were taken from the tail and collected in serum, heparinized or EDTA-coated microtubes and centrifuged immediately. Samples for analysis of blood lipids were taken after an overnight fast and determined enzymatically with Konelab autoanalyzer version 2:0. The it.a. CV was below 2.5% for all analyzed lipids. LDL-C was calculated as total cholesterol (TC) – (HDL-C + TG)/2, and the atherogenic index was calculated as (TC – HDL-C)/HDL-C. Insulin, collected during the clamp procedure, was measured with a human insulin ELISA kit (Mercodia, Uppsala, Sweden), with no cross-reactivity with c-peptide or proinsulin. The i.a. CV was 2.8-4.0% and the it.a. CV 2.6-3.6%. Testosterone (i.a. CV 6.7-8.1%, it.a. CV 5.7-10.5%), estradiol (i.a. CV 3.4-3.9%, it.a. CV 4.1-9.9%) and progesterone (i.a. CV 3.3-6.4%, it.a. CV 5.7-10.5%) were determined with radioimmunoassay kits (Diagnostic Systems Laboratories, Webster, TX). Commercial enzyme-linked immunosorbent assays were used for the analysis of plasma

MCP-1 (i.a. CV 4.0-7.8%, it.a. CV 6.7-9.7%) (Invitrogen, Carlsbad, CA), retinol binding protein 4 (RBP4) (i.a. CV 1.2-6.2 %, it.a. CV 2.8-7.0%) (Adipogen, Seoul, Korea) and sICAM-1 (i.a. CV 4.7-4.9%, it.a. CV 6.2-9.4%) (Quantikine, Minneapolis, MN).

Swedish Twin Registry study *(paper IV)*

Comments: The data we have used in our study were collected as part of Screening Across the Lifespan – Twin study (SALT), based on the Swedish Twin Registry (STR). The STR includes more than 172 000 twins, both mono- and dizygotic, and is the largest twin registry in the world.[154-156] The registry offers unique opportunities for the study of the role of genetic and environmental factors in the development of disease. Initially, the STR was established to investigate the influence of smoking and alcohol consumption on the risk for cancer and cardiovascular diseases, whilst taking genetic predisposition into account. By today the subject field has been broadened to include most common complex diseases. In order to enable more detailed study of genetic factors, a biobank of blood and saliva samples is now being established.[154]

Statistical analysis

Animal studies *(papers I-III)*

Results are expressed as mean ± standard error of the mean (SEM). The unpaired *t*-test or Mann Whitney non-parametric U test was used for comparison between treatment and control groups. Multiple comparisons of GIR in *paper I* were made with ANOVA and Fisher's test. Correlation analysis was performed using Simple regression.

Staview statistics software 5.0 for Windows (SAS Institute Inc., Cary, NC) was used for all statistical calculations except for gene expression statistics *(papers II-III)* when SPSS 13.0 (SPSS Inc., Chicago, IL) was used. Adipocyte size distributions *(paper I)* were compared by using two-sample Kolmogorov-Smirnov statistics.[157] An exact *P*-value for the comparison of the two groups was calculated through permutations. For these comparisons, statistical calculations were made using the R language (http://www.R-project.org).

Swedish Twin Registry study *(paper IV)*

Because both twins in same-sex pairs were included in the model, the generalized estimated equation was used for all analyses to control for dependence within pairs. For

comparisons of groups with respect to continuous outcome variables, i.e. BMI, weight and height, a normal distribution and an identity link function were assumed. For comparisons of groups with respect to dichotomous traits, i.e. self-reported dyslipidemia yes/no, self-reported T2DM yes/no, overweight yes/no and underweight yes/no, a binomial distribution with a logit link function was used. For adjusting age differences between the two groups, age at the time of interview was added as a covariate in all analyses, with the exception of the one concerning self-reported weight at birth. All data analyses were carried out using SPSS (Version 15.0) software.

RESULTS

Paper I: The effects of early postnatal testosterone exposure on insulin sensitivity, adipocyte size and lipid profile in adult female rats – comparisons with estradiol and DHT

The postnatally testosterone-exposed rats displayed insulin resistance and an atherogenic lipid profile at adult age, the former being in line with a previous study from our group.[67] In addition, mesenteric adipose tissue weight and adipocyte size were increased. Estradiol-exposed rats exhibited insulin resistance, increased body weight and increased mesenteric adipocyte size, but not an increase in adipose tissue weight. The effects on insulin sensitivity and adipocyte size were even more pronounced in estradiol-exposed rats than in those given testosterone. Furthermore, estradiol-exposed rats had increased TG levels. Rats exposed to DHT, which activates only AR, displayed reduced insulin sensitivity but none of the other changes observed in testosterone- or estradiol-exposed rats. Circulating levels of testosterone were lower in testosterone-exposed rats, and progesterone concentrations were lower in all groups (testosterone-exposed: 18.0 ± 5.1, $P<0.001$; estradiol-exposed: 21.5 ± 5.1, $P<0.05$; DHT-exposed: 37.6 ±5.1 nmol/l, $P<0.05$) compared to controls (58.6 ± 6.2 nmol/l) (unpublished data). Estradiol concentrations did not differ between groups.

In conclusion, by comparing the outcome in rats exposed to testosterone with the outcome in rats given estradiol or DHT, we suggest that activation of ER induces stronger metabolic programming effects than activation of AR.

Paper II: The effects of early postnatal estradiol exposure on parametrial adipose tissue and the ovary in adult female rats

Estradiol exposure resulted in markedly decreased parametrial adipose tissue weight and volume as well as in increased LPL activity. The adipose tissue displayed altered expression of some genes related to adipose tissue metabolism [complement component 3 (*C3*) and *ERα*]. Expression of leptin, *LPL* and hormone-sensitive lipase (*HSL*) was not influenced. The estradiol-exposed rats had decreased ovarian size with altered morphology (Table 2), as well as disrupted cyclicity and a total absence of CL. In addition, estradiol exposure also altered the ovarian expression of genes related to follicle

development [adiponectin, *C3*, *ERa, Glut 3* and *4*, and transforming growth factor β1 (*TGFβ1*)]. We found decreased testosterone and progesterone levels and unaltered estradiol levels in estradiol-exposed rats compared to controls.

A novel approach, using MRI, was employed for the non-invasive volumetric estimation of parametrial adipose tissue. The MRI-estimated volume correlated with the weight of dissected tissue, indicating that the method was reliable.

In conclusion, these findings suggest that early postnatal estradiol exposure of female rats, by means of one single injection, results in long-lasting effects on the ovary and parametrial adipose tissue at adult age.

Paper III: The effects of early postnatal estradiol exposure on insulin sensitivity, skeletal muscle, adipose tissue and circulating inflammatory markers in adult female rats

Postnatal estradiol exposure reduced insulin sensitivity and increased plasma levels of MCP-1 and sICAM-1. In skeletal muscle, estradiol increased the expression of genes encoding *C3*, *MCP-1*, *RBP4*, and *TGFβ1*. The expression of genes encoding *Glut 4,* carnitine-palmitoyl transferase 1b (*CPT1b*)*,* peroxisome proliferator-activated receptor delta (*PPARd*), and uncoupling protein 3 (*UCP3*) was downregulated. Expression of several of the inflammatory genes in skeletal muscle correlated negatively with whole-body insulin sensitivity. In subcutaneous inguinal adipose tissue, expression of *TGFβ1, PPARd,* and *C3* was decreased, while expression of *RBP4* and *CPT1b* was increased. Inguinal adipose tissue weight was increased but adipocyte size was unaltered, suggesting an increased number of adipocytes. In mesenteric adipose tissue, estradiol did not affect depot weight, adipocyte size, or expression of the studied genes. The lipid profile did not differ between the groups (FFA, TG, LDL-C, HDL-C and TC) (data not shown, unpublished data).

In conclusion, early postnatal estrogen exposure may reduce insulin sensitivity at adult age by inducing chronic, low-grade systemic and skeletal muscle inflammation and disturbances of glucose and lipid metabolism in skeletal muscle.

Paper IV: The effects of having a male twin on BMI, lipid profile and T2DM in women

BMI was found to be moderately but significantly higher in women with a twin brother compared to those with a twin sister. Whereas there was no difference in height, women with male twins weighed more than those with same-sex twins. There was also a significantly higher percentage of overweight subjects in women with male twins. Self-reported birth weight did not differ between groups. Self-reported dyslipidemia was significantly more common in women with a male twin compared to those with a same-sex twin. Self-reported T2DM was somewhat more common in women with female twins; this significance however did not survive correction for multiple comparisons. In those younger than 60 years, there was no difference in body weight or BMI. In those aged 60 years and older, the difference between groups was significant for both weight and BMI.

In conclusion, these data indirectly suggest that women with a twin brother may be exposed to slightly higher levels of androgens *in utero* than other women; in this regard, they are in line with previous reports were same-sex and opposite-sex twins have been compared with respect to other traits. By suggesting an influence of early androgen exposure on metabolism and anthropometry, they are also in line with animal studies reporting similar effects (such as those presented in this thesis).

Overview of results *(papers I-IV)*

Table 1. Summary of the results of each study. Results are given as a comparison with the relevant control group. ⇧ = increased, ⇩ = decreased, ⇔= unaltered, — = not studied

	1st STUDY (paper I)			2nd STUDY (papers II, III)	TWIN STUDY (paper IV)
Exposure dose	1mg	0.5mg	1mg	0.35mg	
Substance	**T**	**E**	**DHT**	**E**	
Body composition					
Body weight	⇔	⇧	⇔	⇔	⇧
Adipose tissue depot weight	⇧ Mes	⇔ Mes	⇔ Mes	⇧ Ing ⇩Para	—
Parametrial adipose tissue volume	—	—	—	⇩	—
Adipocyte size	⇧ Mes	⇧ Mes	⇔ Mes, Ing	⇔Mes, Ing, Para	—
Skeletal muscle weight	—	—	—	⇧ Tib, EDL	—
LPL activity	—	—	—	⇧	—
Insulin sensitivity	⇩	⇩	⇩	⇩	—
Lipid profile	⇧	⇧	⇔	⇔ (unpublished)	⇧
Hormones					
Testosterone	⇩	⇔	⇔	⇩	—
Estradiol	⇔	⇔	⇔	⇔	—
Progesterone	⇩ (unpublished)	⇩ (unpublished)	⇩ (unpublished)	⇩	—
Circulating inflammatory markers					
MCP-1	—	—	—	⇧	—
ICAM-1	—	—	—	⇧	—

Abbreviations: T= testosterone, E= estradiol , DHT= dihydrotestosterone, BW= body weight, Mes= mesenteric adipose tissue, Ing= inguinal adipose tissue, Para= parametrial adipose tissue, Tib= tibialis, EDL= extensor digitorum longus, LPL= lipoprotein lipase, MCP-1= monocyte chemoattractant protein-1, sICAM-1= soluble intracellular adhesion molecule-1

Table 2. Alterations in ovarian morphology after postnatal estradiol exposure as compared to control group (*paper II*). ⇧ = increased, ⇩ = decreased, ⇔= unaltered

Parameter	**E** (0.35 mg)
Ovarian area (mm^2)	⇩
Ovarian weight (mg)	⇩
Corpora lutea (n)	⇩
Follicles (n)	⇔
Number of follicles	
Atretic	⇧
Healthy	⇔
Ratio healthy/atretic follicles	⇩
Follicle area (mm^2)	⇧
Theca interna thickness (μm)	
Healthy follicle	⇔
Atretic follicle	⇩

Abbreviations: E= estradiol

GENERAL DISCUSSION

Programming effects on insulin sensitivity and skeletal muscle

In *paper I,* insulin resistance was most pronounced in estradiol-exposed rats, while testosterone- and DHT-exposed rats displayed a reduction in insulin sensitivity of similar magnitude. The result in estradiol-exposed rats was confirmed in *paper III,* showing a reduction in insulin sensitivity to the same extent as that seen in *paper I*. As the euglycemic-hyperinsulinemic clamp technique, which was used in both studies, chiefly measures insulin sensitivity in muscle,[158] these results suggest that there is a time point of vulnerability directly after birth when sex steroids, especially estradiol, influence muscle glucose metabolism. The results suggest that ER activation has a greater effect on insulin sensitivity but that AR activation without simultaneous ER activation is enough to cause insulin resistance.

In *paper IV* opposite-sex twins did not have an increased risk of self-reported T2DM compared to same-sex twins. This is not consistent with our animal experiments which suggest that early androgen exposure impairs insulin sensitivity. However, T2DM is a heterogenous condition caused by a mixture of genetic and environmental factors, and its cause cannot be ascribed to one single pathophysiological mechanism. Insulin resistance is generally believed to be the basic trigger of T2DM,[159] but there are also studies suggesting that the role of β-cell dysfunction relative to that of insulin resistance in the pathogenesis of T2DM has been generally underestimated.[160] However there is no denying that insulin resistance is an important factor since at any given level of β-cell function, the degree of insulin resistance would reduce the threshold for developing impaired glucose tolerance and subsequently T2DM. In other words, the greater the insulin resistance, the lower the threshold, the earlier the onset and the greater the severity of the diabetes disease.[160] Due to the fact that numerous factors, such as parental diabetes, obesity and metabolic syndrome traits are involved in the development of T2DM, it is however very difficult to predict diabetes risk based only on insulin resistance.[161]

We found signs of inflammation in the circulation and also in skeletal muscle in estradiol-exposed rats. Sexual dimorphism exists in immune function for which , in rodents, early androgen exposure during development is thought to be responsible[162].

T2DM is associated with a general activation of the innate immune system, including chronic, cytokine-mediated state of low-grade inflammation, which may contribute to several features of the metabolic syndrome, including insulin resistance.[163] In our postnatal estradiol programming model, further work has to be done to describe the inflammation in terms of location and nature; such studies should also address the possible importance of additional inflammatory markers and their relationship to insulin resistance.

Possible mechanisms of skeletal muscle contribution to insulin resistance

Paper I shows that insulin resistance may be an effect induced by the activation of either ER or AR in the early postnatal phase. Previous mouse knockout studies lend credence to the view that both AR and ERα (but not β) influence insulin sensitivity.[164,165] Our data on insulin resistance are in agreement with previous programming studies showing that insulin resistance at the muscle level follows neonatal androgen treatment in female rats.[67,68] The major novelty of *paper I* is that the effect of postnatal testosterone exposure on insulin resistance is compared to that of early postnatal estradiol and DHT exposure. In addition, this paper adds information on alterations in adipose tissue distribution, adipocyte size and lipid profile at adult age. *Paper III* further investigates the possible contribution of adipose tissue and skeletal muscle to the insulin resistance phenotype in estradiol-exposed female rats. To do this we investigated if insulin resistance was accompanied by changes in circulating inflammatory markers and/or in the expression of genes involved in inflammation and in lipid and glucose metabolism in skeletal muscle and adipose tissue, respectively. In this paper we suggest that changes in skeletal muscle may reduce insulin sensitivity in adulthood by inducing chronic, low-grade systemic and skeletal muscle inflammation and disturbances of glucose and lipid metabolism following early postnatal estrogen exposure. Although *paper III* adds useful information about factors possibly contributing to reduced insulin sensitivity, the underlying mechanism is still unclear, i.e. where the muscle dysfunction is located. Although estradiol-exposed rats display reduced muscle *Glut 4* expression, this does not make clear whether the defect lies here or earlier in the insulin signaling cascade. Further, studies of key genes at the protein level are needed. Only one testosterone programming study has investigated the insulin

signaling cascade.[55] The authors found no difference in muscle insulin signaling and insulin sensitivity as measured by the intraperitoneal insulin tolerance test, which is a less sensitive method than the euglycemic-hyperinsulinemic clamp, although serum insulin levels were increased.

Although not studied in this thesis, it is possible that postnatal sex steroid exposure may affect skeletal muscle directly and alter insulin signaling irreversibly. Studies made on the effects of androgen administration in adult female rats suggest that androgens induce insulin resistance by acting directly on muscle cells, reducing the insulin response of glycogen synthase and translocation of Glut 4.[107,108,166,167] Administration of estradiol to adult female rats suggests that the reduced insulin sensitivity is dose-dependent and that the insulin resistance associated with high doses of estradiol is related to the downregulation of insulin receptor substrate-1 expression.[168] Distinguishing the direct and adult effects on skeletal muscle from insulin sensitivity caused by early postnatal sex steroid exposure should be possible to do by conducting a time-response study on muscle insulin signaling and the expression of genes involved in regulating insulin sensitivity. If these parameters are studied in relation to the occurrence of insulin resistance, it would be possible to find out e.g. whether the increased expression of inflammatory markers precedes the reduction in insulin sensitivity and disturbed insulin signaling, thereby contributing to the phenotype. Considering the increased expression of inflammatory markers in muscle from estradiol-exposed rats, it would also be valuable to examine skeletal muscle morphology in terms of inflammatory cells. In fact, a previous study showed increased CD154 (T-cell marker) levels and infiltration of macrophages in muscle biopsies from T2DM patients, indicating an inflammation process in skeletal muscle that possibly contributes to insulin resistance.[169]

Both neonatal testosterone and estradiol programming studies have shown alterations in circulating sex steroid levels in adulthood.[67,68,170] This could potentially induce insulin resistance at adult age, as sex steroids are known to influence insulin sensitivity.[87] Adipose tissue is also able to influence insulin sensitivity by the secretion of adipokines and FFA, which interfere with skeletal muscle insulin signaling.[117] The possible contributions of adipose tissue, lipid profile, the ovary and endocrine factors to insulin resistance are discussed in the sections below.

In conclusion, skeletal muscle from estradiol-exposed rats shows changes in the expression of inflammatory markers and genes involved in lipid and glucose metabolism, which potentially could contribute to insulin resistance. Establishing the exact mechanism in skeletal muscle responsible for the insulin resistance phenotype requires further investigation.

Programming effects on adipose tissue

In the first study, estradiol increased mesenteric adipocyte size without affecting the weight of the adipose tissue, although there was a tendency towards an increase. Testosterone increased mesenteric adipose tissue absolute weight, relative weight and cell size. DHT had no effect on mesenteric adipose tissue weight or adipocyte size. In this study, inguinal adipose tissue weight and adipocyte size remained unaltered in all groups, although the weight tended to be higher following estradiol exposure. The lack of effect on adipose tissue in DHT-exposed rats suggests that the effect of testosterone is mediated by ER rather than AR. The effect of estradiol on the weight of mesenteric adipose tissue did however not reach statistical significance. This may be due to low statistical power as this group included few rats. It is also possible that activation of ER leads to an increase in adipocyte size with an accompanying reduction in adipocyte number resulting in unchanged adipose tissue weight. Activation of AR might, on the other hand, maintain or even increase adipocyte number. Therefore, in testosterone-exposed rats, the reduction in adipocyte number induced by ER may be counteracted by the parallel activation of AR, leading to increased mesenteric adipose weight. In the second study, estradiol increased inguinal adipose tissue absolute weight and mesenteric adipose tissue relative and absolute weight remained unchanged while parametrial adipose tissue weight was reduced. All three adipose tissues displayed unaltered adipocyte size compared to controls. The second study suggests that early postnatal estradiol exposure increased adipocyte number in the inguinal adipose tissue while parametrial adipocyte number was reduced. In our first study, interestingly, mesenteric adipocyte size increased following estradiol exposure but this was not the case in the second study. As the dose is a major factor that determines the programming outcome, as shown in other programming studies,[3,4] this discrepancy is most likely to be due to dose, as this was the only parameter that differed between the studies. In addition, inguinal adipose tissue

weight did not increase in the first study following early postnatal estradiol exposure, although a tendency was seen. However, in the second study inguinal absolute weight reached significance between the groups probably because the analysis was done on a greater number of animals.

In *paper II*, MRI was used for the first time to estimate parametrial adipose tissue volume. Although the MRI-estimated volume correlated with the weight of dissected tissue, indicating that the method is reliable, the image acquisition can probably be further improved in future studies. For example, reducing breathing-induced motion artifacts is important. Contiguous slices can also be acquired to improve the accuracy of the volumetric assessment. Images with higher adipose tissue contrast might also improve the assessment. In addition, there is also potential for improvement in the choice of reference used to define depots. Since the parametrial depot has never been evaluated with MRI before, the development of this approach will be a useful tool for future studies where a non-invasive estimation of parametrial adipose tissue is warranted.

Taken together our animal studies suggest that the adipose tissue in the various depots respond to early programming with sex steroids with an increase or a decrease in adipocyte number or, in the case of mesenteric adipocytes, with an increase in adipocyte size, as shown in the first study. Different fat depots contain adipocytes with distinct intrinsic characteristics, including receptors, adipokines and transcription factors.[171] The adipogenic response to sex steroids is depot-specific, as shown by both human and animal studies.[172,173] These depot-differences in sensitivity to sex hormone action might be explained by variations in sex steroid receptor expression between the different adipose tissue regions.[174] Therefore, it is not surprising that early postnatal estradiol and testosterone exposures have different outcomes regarding adipose tissue weight, adipocyte size and adipocyte number in the various depots.

Interestingly, it has recently been shown that fat cell number in humans is largely set during childhood and adolescence and that changes in fat mass in adulthood can mainly be attributed to changes in fat cell volume. Although the adipocyte number thus is static in adults, there is a remarkable turnover within this cell population, suggesting that the adipocyte number is tightly controlled.[175] These data strongly support the need for further studies exploring factors, such as sex steroids, which may act during early life at critical

developmental time points and set adipocyte number influencing development of obesity and related adverse effects in adulthood.

Our studies, as well as other animal studies on neonatal and adult rodents, have shown that estradiol has a major impact on adipose tissue, e.g. on adipocyte number. The outcome is dependent on the age and physiological state at the time of exposure as well as on the adipose tissue being studied.[86] A previous testosterone programming study in female rats carried out by Nilsson et al. demonstrated changes in adipose tissue distribution with decreased relative weights of parametrial, retroperitoneal and inguinal adipose tissue while mesenteric adipose tissue tended to increase.[67] The latter is consistent with our results in testosterone-exposed rats. However, we did not observe any alteration in inguinal adipose tissue weight. This might be explained by the use of different strains of rats. In addition, in Nilsson's study, the rats were younger at the time point when inguinal adipose tissue was weighed. Testosterone exposure of 5-day old female rats results in increased visceral adiposity mass,[68] as seen in our *paper I*. However, the specific visceral adipose tissue/s included in the definition of visceral fat was not specified in the study carried out by Perello et al.[68] Prenatal androgen treatment of female rats showed an increase in subcutaneous adipose tissue at adult age.[55] The inconsistency between this study and our results may be due to the use of 5 mg testosterone injected daily on embryonic day 16 to 19. When studying programming effects, careful consideration must be given to the dose and timing of drug administration as well as to the age at which the relevant parameters are measured.

Apart from our first study, no other studies have explored if the adipose tissue effects seen in rats exposed to neonatal androgens are mediated via AR and/or ER. The effects observed in neonatal androgenized female rats in these studies could thus be mediated via AR and/or by aromatization of androgens to estrogens acting via ER. This is certainly a field which requires further investigation.

Adipose tissue – a contributory factor in insulin resistance?

It is generally believed that adipose tissue can contribute to reduced insulin sensitivity by secretion of lipids and adipokines. In addition, adipocyte size has been shown to be an independent risk factor for T2DM[118] and also to affect adipokine secretion.[127,129,130,176] Visceral adipose tissue is of special interest as it seems to correlate with T2DM and

insulin resistance.[85,115,116] In order to shed some light on the role of adipose tissue and its contribution to insulin resistance in estradiol-exposed rats, we studied inguinal and mesenteric adipose tissue weight, adipocyte size and gene expression in *paper III*. Although mesenteric adipocyte size was increased in *paper I* but not in *paper III,* the rats were insulin-resistant to the same extent in both studies. The discrepancy in adipocyte size between the studies is probably a dose-effect, as mentioned above. Furthermore, estradiol exposure had no effect on mesenteric weight or gene expression in *paper III*. This suggests that, in this programming model, mesenteric adipose tissue probably only has a minor influence on the development of insulin resistance.

Inguinal gene expressions of some immune-related adipokines were decreased. This brings into question the contribution of inguinal adipose tissue to the insulin resistance phenotype. As we only analyzed some adipokines we cannot tell whether whole-body insulin sensitivity was affected by changes in the expression of other adipokines. It is also important to emphasize that a change in gene expression is not necessarily followed by a corresponding change in circulating levels.[177] In *paper I*, DHT-exposed rats displayed insulin resistance to the same extent as testosterone-exposed rats but without alterations in adipose tissue weight or adipocyte size, either in the inguinal or mesenteric depots. Consequently there was no apparent relationship between adipose tissue traits and the effect on insulin sensitivity following AR stimulation.

Studies in mice have shown that gender differences in expression of adipokines could be explained by a programmed sexually dimorphic pattern.[178,179] For example, the level of adiponectin is higher in adult females than in adult males. Neonatal castration allows adiponectin levels of adult males to reach female levels while castration in adults does not lead to female levels. Therefore, the set point which determines basal adiponectin levels in males and females is suggested to be established by the neonatal testosterone surge.[178] It is possible that AR stimulation by DHT affects adipokine release without altering adipose tissue morphology. With this knowledge in mind it would have been interesting to study gene expression and circulating levels of adipokines in female rats exposed in early postnatal life to testosterone or DHT.

Parametrial adipose tissue, which we studied in more detail in *paper II*, is implicated in insulin sensitivity. For example, this tissue expresses various adipokines mediating insulin

resistance.[179] However, we did not focus on parametrial adipokine gene expression in our studies and hence cannot draw any conclusions regarding this matter. In female rats estrogenized at adult age, insulin sensitivity is thought to be improved and parametrial adipose tissue mass reduced with altered function.[180] Furthermore, removal of parametrial adipose tissue in both lean and obese female mice improves glucose tolerance, suggesting a role for this tissue in glucose homeostasis.[181] In our second study, female rats exposed postnatally to estradiol had reduced parametrial adipose tissue weight, but despite this they were insulin resistant.

In conclusion, although our studies clearly show that early postnatal estradiol and testosterone exposure has major effects on various adipose tissues, we found no strong direct evidence that adipose tissue contributes to insulin resistance in adult female rats exposed directly after birth to sex steroids.

Programming effects on lipid profile

In *paper I*, testosterone-exposed rats displayed increased LDL-C, TG, TC and atherogenic index, while estradiol-exposed rats only displayed increased TG levels. No similar changes were found in DHT-exposed rats. Lipid profiles were unaltered between controls and estradiol-exposed rats in *paper III*. As discussed previously, the different outcomes in the two studies could possibly be explained by the different doses of estradiol. In *paper IV,* opposite-sex female twins displayed higher lipid values and BMI/weight, tentatively du to enhanced *in utero* exposure to androgens. This suggestion naturally leads to the question if such an effect may be mediated directly by androgen receptors and/or by the aromatization of androgens to estrogens. Although estradiol-exposed rats in *paper I* had less marked dyslipidemia than testosterone-exposed rats they had, like opposite-sex twins, increased TG levels and enhanced body weight. DHT-rats, on the other hand, displayed no alterations in lipid profile or body weight at adult age, as shown in *paper I*.

Differences in BMI, dyslipidemia (unpublished), and weight (unpublished) in opposite-sex female twins compared to same-sex twins were observed in women of 50 years and older, but not in those below 50 years of age. One can therefore speculate that the dyslipidemia and increase in weight and BMI seen in opposite-sex female twins are mediated by androgen receptors and/or estrogen receptors, and that this effect is masked

by protective levels of estradiol until the menopause, when estradiol levels decline. Exploring this further would be of great interest and could be done by analyzing weight, BMI and lipid profile in the pre- and postmenopausal states.

The dyslipidemia in testosterone-exposed rats reported in *paper I* is consistent with transient exposure to androgens during late gestation, which increases TG and cholesterol in the adult female rat offspring. In addition, this study revealed increased hepatic TG content.[55]

The effects of testosterone and estradiol on serum lipids may be explained by the existence of an estrogen programming effect on the hepatic enzymes involved in cholesterol metabolism, as discussed in *paper I*. Another plausible explanation is an effect on adipose tissue lipid metabolism. To our knowledge, the effects of perinatal sex steroid exposure on adipose tissue metabolism have not been studied previously. In adult rats, androgens are able to affect LPL activity and lipolysis[182,183] and estrogen is able to decrease LPL activity[184] and to affect lipolysis either by inducing the lipolytic enzyme hormone-sensitive lipase[185] or by increasing the lipolytic effects of epinephrine.[186] Although the increased LPL activity in parametrial adipose tissue, as displayed in *paper II*, is not consistent with the studies on estrogen administration in adult animals, it is clear that estradiol has profound effects on adipose tissue lipid metabolism, the exact outcome however differing depending on age at exposure. It is not clear if the effect on LPL activity in *paper II* is a direct effect of early postnatal estradiol exposure on adipose tissue, which may have irreversibly altered this parameter, or if it is mediated by changes in adulthood, e.g. by reduced testosterone levels. Unfortunately lipid metabolism was not studied in *paper I,* but it is possible that postnatal estradiol exposure may have affected LPL activity and/or lipolysis in the first study as well. Although LPL activity is increased in parametrial adipose tissue, this does not exclude the possibility that estradiol affects lipid metabolism in other adipose tissues in a different way, thereby contributing to the observed lipid profile in *paper I*. The suggestion that sex steroid effects on lipid metabolism are site-specific is supported by the fact that estradiol affects lipolysis in human subcutaneous fat but not in the intra-abdominal fat depot[187] and, as previously discussed, by the fact that sex hormone receptor density varies between different adipose tissues.[174] Enlarged adipocytes are less responsive to the antilipolytic effect of insulin[120]

which induces increased lipid mobilization.[188] In addition, visceral adipose tissue has high metabolic activity and releases a large amount of FFA,[85] which contributes to the increased production and release of circulating lipids from the liver.[109] Consequently, it is not surprising that the testosterone- and estradiol-exposed animals in *paper I,* which exhibited increased mesenteric weight and/or enlarged mesenteric adipocyte size, also had altered lipid profiles.

Lipid profile – a contributory factor in insulin resistance?

In *paper I*, dyslipidemia was less marked in estradiol-exposed animals than in those given testosterone. Despite this, insulin resistance was even more pronounced. DHT-exposed rats displayed no lipid profile abnormalities but had reduced insulin sensitivity equivalent to that of testosterone-exposed animals. In *paper III,* estradiol-exposed rats displayed insulin resistance to the same extent as in *paper I*, but without alterations in lipid profile. We cannot exclude that the altered lipid profile seen in testosterone-exposed rats may have contributed in part to the insulin resistance seen in this group. However, data from the first and second studies put together suggest that in our sex steroid programming models, circulating lipids are not likely to be a mediator of insulin resistance. In line with this view, the twin study revealed a possible effect of early androgen exposure on serum lipids and BMI that was not accompanied by enhanced risk for T2DM.

Programming effects on the ovary and sex hormone levels

In *paper II,* estradiol exposure resulted in altered ovarian morphology, decreased ovarian size, increases in follicle area and the number of atretic follicles, decreased thickness of the theca interna of atretic follicles, and absence of CL. The rats also became anovulatory. In *paper II*, we found alterations in gene expression that could possibly contribute to the ovarian phenotype. Although not assessed in our study, effects of early estradiol exposure on the nervous system and the hypothalamic-pituitary-gonadal axis might also have contributed to the ovarian phenotype in estradiol-exposed rats, as indicated by other studies.[71,170]

In *paper I,* testosterone levels were reduced in testosterone-exposed rats only. In *paper II* however, testosterone levels were decreased in estradiol-exposed rats. The low number of animals included in the first study, and possibly also the dose difference, may explain the

difference between the studies. Previous studies on the effect of neonatal androgen exposure on testosterone level in adult female rats have yielded conflicting results.[67,68,141,189,190] The low testosterone levels seen in estradiol-exposed rats are consistent with the study made on female rats neonatally exposed to estradiol valerate, which showed reduced levels of the androgen and estrogen precursor, androstenedione, at adult age.[170] Since the ovary seems to be the major source of testosterone in female rats,[190] and neonatal androgen and estrogen administration have been shown to exert a permanent influence on ovarian morphology,[72,73,170] it is reasonable that the ovary is the relevant site with regard to low testosterone levels in testosterone- and estradiol-exposed animals. In *paper II* we discussed ovarian alterations (such as reduced thickness of the theca interna and increased adiponectin gene expression) as possible contributory factors in the low testosterone levels seen in estradiol-exposed rats. However, it is important to emphasize that there are several sites where the effect on testosterone levels could possibly be exerted, e.g. ovarian enzymatic activity,[191] the hypothalamic regulation of follicle stimulating hormone/luteinizing hormone,[192] the adrenals[193] and the hepatic enzymes involved in the breakdown of sex steroids.[194]

In both *papers I and II,* treated rats displayed unchanged estradiol levels. This is consistent with the findings of another study of neonatal estrogenization,[170] which also showed unchanged estradiol levels measured at the estrus phase of adult control rats. The fact that no significant differences were found (in either our first or second study) when hormone-exposed rats were compared to controls, that were all sampled in the estrus phase, is in line with the notion of neonatally androgenized or estrogenized, anovulatory female rats being in a state of constant estrus.[71,72] This does not rule out the possibility that differences between exposed rats and controls in our studies could have been found if the controls had been sampled at other phases of the cycle. This view is further strengthened by the results of another study which showed reduced estradiol levels following neonatal androgenization.[68] Unfortunately, the phase chosen for sampling of the controls was not specified by the authors.

In *paper I*, all three hormone-exposed groups displayed decreased progesterone levels compared to controls. The reduced progesterone levels in estradiol- and testosterone-exposed rats in *papers I and II* are consistent with other testosterone and estradiol

programming studies.[67,170] As vaginal openings were very small or absent in all the treated groups in *paper I*, assessment of estrus cyclicity by means of vaginal smears could not be carried out. In *paper III* however, vaginal smears from estradiol-exposed rats displayed acyclicicty. In the first study, ovarian postmortem morphological examinations from rats given testosterone or estradiol revealed an absence of CL, and DHT-exposed rats displayed reduced amounts of CL compared to controls. It is generally believed that neonatal testosterone and estradiol exposure causes anovulation, and the results from our first and second study indicate that this is also the case in our programming models. The DHT-exposed rats in *paper I* had disrupted cyclicity and were probably oligoanovulatory. As estradiol and testosterone exposure resulted in the most pronounced effects on ovarian morphology and cyclicity compared with DHT exposure, it is tempting to speculate that ER stimulation has stronger effects on these features than AR stimulation, although activation of AR alone seems to be enough to disturb cyclicity.

The ovary and circulating sex steroids – contributory factors in insulin resistance?

Hyperandrogenicity is associated with insulin resistance in both humans and rats. [88,108] It may seem contradictory that testosterone-exposed rats had low testosterone levels and yet displayed metabolic and anthropometric changes normally associated with high levels of testosterone in females. In the second study, estradiol-exposed rats also displayed reduced testosterone levels and insulin resistance. Although testosterone was not increased, there is a possibility that receptor responsiveness to this hormone was altered. The term "hormonal imprinting" refers to a general biological phenomenon which takes place when the developing receptor meets its target hormone for the first time, and this determines the efficiency of receptor- hormone binding for life.[195] It is notable that all hormone-exposed groups in *paper I* displayed insulin resistance, despite considerable differences in their testosterone levels. For example, testosterone and DHT rats were insulin resistant to the same extent although testosterone levels differed between testosterone rats and controls but were not significantly altered in DHT rats. Therefore, there is no obvious relationship between testosterone levels and insulin resistance.

Disrupted sex steroid cyclicity may contribute to insulin resistance, since both estrogen and progesterone regulate insulin sensitivity in rats.[139] In *paper I,* the rats given

testosterone or DHT had reduced insulin sensitivity to a similar degree, but testosterone-exposed rats were probably anovulatory while DHT-exposed rats were probably oligoanovulatory. In addition, estradiol-exposed rats had reduced insulin sensitivity compared to testosterone-exposed rats although both groups were anovulatory. This indicates that the influence of early hormone administration on insulin sensitivity could not be due to the absence of cyclic variation in female sex steroids *alone*.

Possible mechanisms for programming effects

The biological mechanisms underlying perinatal programming are poorly understood and one may only speculate about the primary factors and sites of action involved. Different possible mechanisms for the permanent effects on specific tissues have been suggested by others. During the process of organogenesis, changes in cell position, shape, differentiation, proliferation, cell-cell interactions and cell migration may result in permanent alterations in a developing organ.[196] An area that has come increasingly into focus is the study of heritable changes in gene expression that are not caused by changes in DNA sequence – a phenomenon known as epigenetics.[197] DNA methylation is the most fully described epigenetic mechanism. Epigenetic mechanisms enable the maintenance of cell-type specific gene expression as cells proliferate throughout life, and epigenetic changes may also be the subject of transgenerational inheritance.[198] There is an ongoing debate about DNA methylation as a possible mechanism for the effects of early-life environmental estrogens.[199] For example, mice exposed *in utero* to the phytoestrogen genistein are protected from obesity by hypermethylation occurring during development and this persists into adulthood.[200] DNA-methylation would be an area of interest when examining possible mechanisms behind the effects seen following early postnatal estradiol exposure. Although nutrition and environmental factors may influence DNA methylation and lead to persistent changes, it is clear that metabolism will also feed back to affect DNA methylation and also, perhaps, other epigenetic modifications.[198] In this thesis we have analyzed gene expression in skeletal muscles, adipose tissues and the ovary, and we have identified several changes. To find out if the changes in gene expression are actually causally related to the phenotype or are just consequences of the morphological and/or metabolic effects, it is necessary to do a time-response study on possible epigenetic

modifications, gene expression and protein expression in relation to the appearance of phenotype traits such as insulin resistance.

SUMMARY AND CONCLUSIONS

This thesis can be summarized as follows:

- **Comparison between early postnatal programming effects of androgens and estradiol in adult female rats**

Postnatal testosterone exposure results in adult insulin resistance, altered adipose tissue distribution, including increased mesenteric weight, and enlarged mesenteric adipocyte size as well as altered lipid profile at adult age. Estradiol exposure results in markedly reduced insulin sensitivity and also increased TG levels and mesenteric adipocyte size, while DHT-exposed rats display insulin resistance only.

- **Early postnatal programming effects of estradiol in adult female rats**

Estradiol exposure alters ovarian morphology and gene expression related to follicular development.

Parametrial adipose tissue mass and volume is decreased, LPL activity is increased, and gene expressions involved in adipose tissue metabolism are altered.

Estradiol exposure results in insulin resistance, confirming our previous results. Adipose tissue distribution is altered with increased inguinal adipose tissue mass.

Skeletal muscle displays increased expression of inflammatory markers and alterations in genes associated with lipid and glucose metabolism. Levels of circulating inflammatory markers increase. Adipose tissue gene expression in the mesenteric depot remains unaltered, and the inguinal depots display reduced levels of inflammatory markers and alterations of genes involved in adipose tissue metabolism.

- **The influence of having a male twin on metabolism and anthropometry in women**

Elderly women with a twin brother display increased BMI, weight and dyslipidemia compared to women with a twin sister.

In conclusion, the results contained in this thesis suggest that perinatal exposure to sex steroids affects the developing organism, predisposing it to reproductive and endocrine abnormalities and features of the metabolic syndrome at adult age. Adverse changes in insulin sensitivity, lipid profile, adipose tissue distribution and metabolism, as well as in

ovarian morphology, can thus be programmed perinatally, with possible long-lasting implications for adult health. By comparing the metabolic outcome of early postnatal testosterone exposure with that of exposure to estradiol and DHT, we conclude that activation of ER exerts stronger metabolic programming effects than activation of AR. The primary mechanisms underlying the observed effects are not clear, several mechanisms are probably involved. Although alterations are found in adipose tissue, lipid profile, sex steroid levels and the ovary in adulthood, the respective contributions of each of these changes to insulin resistance are uncertain. However, early postnatal estradiol exposure may cause insulin resistance partly by inducing low-grade systemic and skeletal muscle inflammation and disturbances of glucose and lipid metabolism in skeletal muscle.

By demonstrating that our observations of BMI, weight and dyslipidemia in opposite-sex female twins are at least partly consistent with the results of our animal studies, we have provided support for the notion that the programming effects of early androgen exposure observed in animals may be of relevance also for humans.

FUTURE PERSPECTIVES

This thesis raises a number of questions. Further studies are needed to identify the mechanism behind the observed effects. Establishing causality requires a time course delineating the chronology of the different aberrations, such as insulin resistance, gene expression and lipid profile. In addition, the site of action in skeletal muscle responsible for insulin resistance should be studied by exploring insulin signaling. Issues surrounding skeletal muscle inflammation could be investigated in more detail.

Research into the effects of hormone-like substances found in the environment is highly relevant not only to reproduction, but also, increasingly, to obesity and its complications. At a time when the prevalence of obesity and type 2 diabetes mellitus is rapidly increasing, it is important to understand how complex events, including exposure to sex steroids during development, may contribute to this epidemic. Once specific mechanisms underlying programming phenomena in animals have been identified, the significance of these mechanisms to human development can hopefully be established.

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”Sic itur ad astra”

(*”Så når man stjärnorna”*)

Vergilius

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