Androgens and progesterone in tissues and the gastrointestinal tract

Mapping of tissue-specific sex steroid levels in mice

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ABSTRACT

Sex steroids such as androgens can exert both positive and negative effects in tissues and are important in physiology and pathophysiological states of men and women. Androgens originate mainly from the gonads. Local androgen levels in tissues are also regulated by intracrine processes, resulting in local levels not always being reflected by circulating levels. Mouse models are commonly used to study sex-steroid related disorders, but a lack of sufficiently sensitive and specific methods has prohibited accurate measurement of the low sex steroid levels in mouse tissues. Here, we developed and validated a gas chromatography – tandem mass spectrometry method capable of determining a broad panel of sex steroids in tissues and used it to map local sex steroid levels in tissues of mice in different conditions/treatments. We found that supplementing castrated male mice with dehydroepiandrosterone (DHEA) caused high androgen levels in the prostate and liver, raising concerns about the unsolicited use of DHEA by the public. Also, the gut microbiota was involved in the deconjugation of intestinal androgens, suggesting a possible mechanism for the relationship between androgen-related conditions and the gut microbiota proposed by experimental and epidemiological studies. Finally, progesterone was the most abundant sex steroid in castrated male mice, and the progesterone levels were surprisingly unaffected by both adrenalectomy and castration. We found that dietary progesterone was absorbed into tissues of male mice; therefore, we suggest food as a possible source of progesterone in men, perhaps of relevance for men with prostate cancer.

In conclusion, measurement of local sex steroid levels in tissues is a novel method that could bring new understanding of pathways and mechanisms in androgen-related disorders and contribute to future development of more selective treatments for sex steroid-related diseases.

Keywords: androgens, progesterone, intracrinology, gastrointestinal tract, gut microbiota

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SAMMANFATTNING PÅ SVENSKA

Könshormoner styr flera viktiga processer i kroppen och kan ha både positiva och negativa effekter. Många vävnader kan både bilda och bryta ner könshormoner, därför reflekterar inte alltid nivåerna av hormon i blodet nivåerna som finns lokalt i vävnader. Att mäta könshormoner i vävnader är svårt, eftersom de innehåller så många andra substanser. Dessutom är organ hos försöksdjur, som möss, ofta ganska små så man har inte så mycket material att arbeta med. I dessa studier har vi tagit fram och validerat en ny effektiv metod som kan mäta låga nivåer av könshormoner i svåra material, och använt den för att skapa en karta över könshormonnivåer i vävnader hos möss, och mätt hur de påverkas av olika typer av behandlingar.

Ett intressant fynd som vi gjort är att androgener, de så kallade manliga könshormonerna, utsöndras från kroppen via gallan till tarmen. I tarmen kan tarmbakterier återaktivera hormonet genom att ta bort den molekyl som kopplats på för att möjliggöra utsöndring. Det här kan vara ett av de sätt som tarmbakterier och könshormoner interagerar. Just nu forskas det mycket om just interaktionen mellan tarmbakterier och könshomoner.

För att utreda hur mycket av hormonerna i hanmöss som kommer från testiklarna respektive binjurarna, två viktiga hormonproducerande organ, har vi undersökt lokala hormonnivåer i ett flertal vävnader efter att ha opererat bort testiklar (kastration) och/eller binjurar. Det visade sig att progesteron, som är ett hormon som är viktigt i den kvinnliga reproduktionen men som även finns hos hanar, inte påverkades nämnvärt av någon av operationerna. Överraskande nog fanns det höga progesteronnivåer i maten som mössen åt. Dessutom finns progesteron i mänsklig föda (främst mat som kommer från vuxna hondjur som mejeriprodukter och ägg). Eftersom vi visade att progesteron kan tas upp i tarmen kan man fråga sig om progesteron i mat kan ha någon påverkan för människor. Detta skulle kunna vara relevant för patienter med prostatacancer, då det finns andra studier som visat att progesteron kan ha betydelse för den sjukdomen. När man har undersökt hur kosten påverkar prostatacancer i stora studier på människor har resultaten varit blandade, men man har aldrig direkt funderat på progesteronets roll.

Sammanfattningsvis har studierna i denna avhandling bidragit med ny kunskap om hur könshormoner fördelas i kroppen, liksom uppslag på nya forskningsfrågor för att bättre förstå androgenrelaterade sjukdomar som exempelvis prostatacancer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Colldén H*, Nilsson ME*, Norlén AK, Landin A, Windahl SH, Wu J, Gustafsson KL, Poutanen M, Ryberg H, Vandenput L*, Ohlsson C*.
Comprehensive sex steroid profiling in multiple tissues reveals novel insights in sex steroid distribution in male mice.
Endocrinology 2022; 163(3):bqac001;
doi: 10.1210/endocr/bqac001

II. Colldén H, Nilsson ME, Norlén AK, Landin A, Windahl SH, Wu J, Horkeby K, Lagerquist MK, Ryberg H, Poutanen M, Vandenput L*, Ohlsson C*.
Dehydroepiandrosterone supplementation results in varying tissue-specific levels of dihydrotestosterone in male mice.
Endocrinology 2022; 163(12):bqac163;
doi: 10.1210/endocr/bqac163

III. Colldén H, Landin A, Wallenius V, Elebring E, Fändriks L, Nilsson ME, Ryberg H, Poutanen M, Sjögren K, Vandenput L, Ohlsson C.
The gut microbiota is a major regulator of androgen metabolism in intestinal contents.
Am J Physiol Endocrinol Metab. 2019; 317(6):E1182-E1192; doi: 10.1152/ajpendo.00338.2019.

IV. Colldén H, Hagberg Thulin M, Landin A, Horkeby K, Lagerquist MK, Wu J, Nilsson KH, Grahnemo L, Poutanen M, Ryberg H, Vandenput L*, Ohlsson C*.
Dietary progesterone contributes to intratissue levels of progesterone in male mice.
Endocrinology 2023; 164(8):bqad103;
doi: 10.1210/endocr/bqad103

* Contributed equally

CONTENTS

ABBREVIATIONS

ADT	androgen deprivation therapy
ADX	adrenalectomy/adrenalectomized
CONV-R	conventionally raised (control in gut microbiota-related studies)
CYP17A1	cytochrome P450 17-alpha-hydroxylase/17,20-lyase
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
FSH	follicle-stimulating hormone
GC-MS/MS	gas chromatography – tandem mass spectrometry
GF	germ-free
GnRH	gonadotropin-releasing hormone
HSD	hydroxysteroid dehydrogenase
HPG	hypothalamic-pituitary-gonadal
LH	luteinizing hormone
LLOQ	lower limit of quantification
LOD	limit of detection
ND	not detected
ORX	orchiectomy/orchiectomized
PCOS	polycystic ovary syndrome
SHBG	sex hormone-binding globulin
SRD5A1/2	5α-reductase type 1/2
WAT	white adipose tissue

INTRODUCTION

Androgens such as testosterone have been known for more than eight decades [1]. Their levels in men have been manipulated a lot longer, in ancient times with surgical castration of for example sex offenders and harem employees [2], and nowadays both clinically (castration therapy in prostate cancer) and illicitly (androgen doping i.e. anabolic steroids) [3]. Androgens regulate fetal development of male genitals, and later during puberty development of secondary sex characteristics such as body shape, voice, and reproductive development. They are vital for male reproductive functions and involved in many bodily processes and disease states of both men and women [4]. Importantly, the effects of androgens can lead to both positive and negative end results in different tissues, such that hormonal treatment could treat one illness in one tissue but at the same time infer negative side effects or risks in another tissue.

One might assume that the sex steroid levels of the most common laboratory animal, the mouse, were already well known and characterized allowing research on important androgen-related diseases like prostate cancer. However, even though mice have long been used in androgen research, there has been a lack of sufficiently sensitive and specific analysis methods to characterize local levels in tissues until the last decade. In this thesis, I will start by giving a brief overview of androgens and progesterone, their mechanisms, effects, and clinical relevance, then dive into the different areas in which we have started mapping androgen and progesterone levels. Then I will move on to aspects regarding methods we have used, the results of each included paper and finally discuss their possible meanings and implications.

ANDROGENS

ANDROGEN PHYSIOLOGY

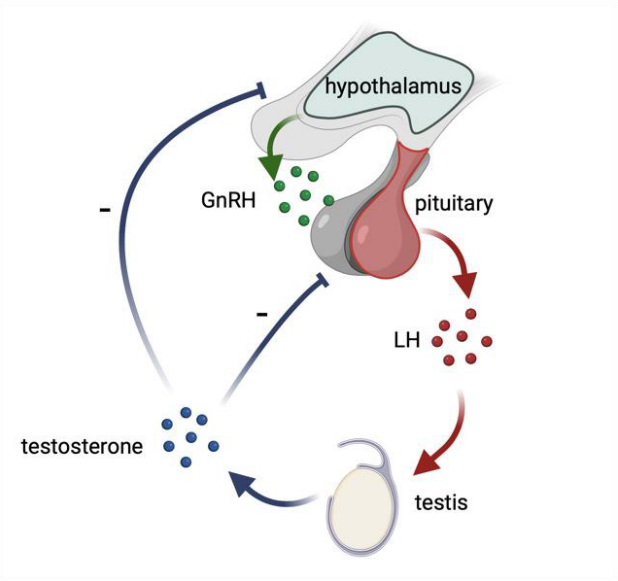

Figure 1 Hypothalamic-pituitary-gonadal (HPG) axis regulation of testicular testosterone production. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which causes the anterior pituitary to release luteinizing hormone (LH) that stimulates testicular testosterone production. Circulating testosterone exerts a negative feedback on both the hypothalamus and the pituitary.

Androgens are the so-called male sex hormones but they are present and have important roles in both sexes. The most abundant androgen is testosterone. In males, testosterone is mainly produced by Leydig cells in the testicles, regulated by luteinizing hormone (LH). The gonadotropins, LH and follicle-stimulating hormone (FSH), are released from the pituitary gland in response to hypothalamic gonadotropin-releasing hormone (GnRH) in the hypothalamic-pituitary-gonadal axis (HPG axis) [5] (Figure 1). The testicles contain extremely high local levels of testosterone to enable spermatogenesis, which is supported by FSH [5].

In the blood, testosterone circulates largely bound to sex hormone-binding globulin (SHBG), prolonging testosterone's half-life and buffering rapid changes in hormone levels [7]. Circulating testosterone exerts a negative feedback on the hypothalamic GnRH and pituitary LH secretion, with an increased serum level of testosterone leading to decreased stimulation of

production [8] (Figure 1). In peripheral tissues such as prostate, skin, liver, bone, and hair follicles testosterone can be metabolized by reduction into the more potent androgen dihydrotestosterone (DHT), that binds more strongly than testosterone to the androgen receptor [9]. The levels of DHT in target tissues such as the prostate are tightly regulated by expression of 5α-reductase enzymes [10]. Another possible metabolic route from testosterone is aromatization into estradiol that binds to estrogen receptors [11] (Figure 2).

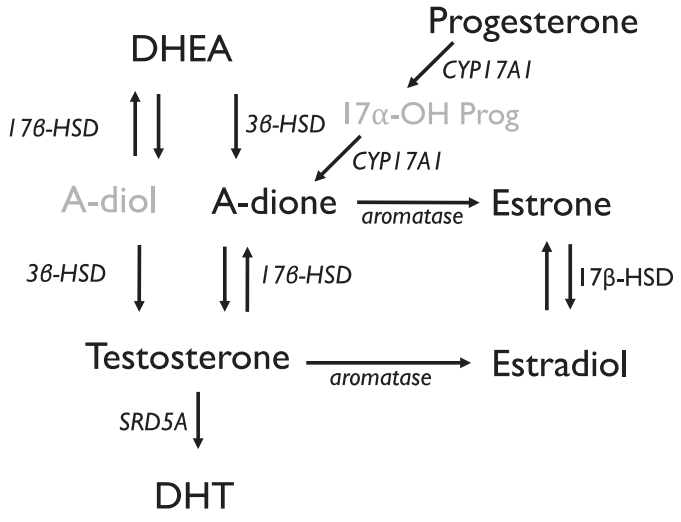

Figure 2 Simplified schematic of sex steroid metabolic routes. Sex steroids in black are measured with the gas chromatography-mass spectrometry assay used in this thesis. DHEA = dehydroepiandrosterone; A-diol = androstenediol; A-dione = androstenedione; CYP17A1 = cytochrome P450 17α-hydroxylase/17,20-lyase; DHT = dihydrotestosterone; HSD = hydroxysteroid dehydrogenase; 17-OH-Prog = 17-hydroxyprogesterone; SRD5A = 5α-steroid dehydrogenase.
Adapted from Schiffer et al 2018 [6].

In addition to the production in testes, testosterone can also be produced in target tissues from adrenal precursors such as dehydroepiandrosterone (DHEA) and androstenedione (Figure 2). Adrenal DHEA production is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, similar to the HPG axis controlling testicular testosterone, although DHEA exerts no negative feedback on the hypothalamus or pituitary [12, 13] (Figure 3). Synthesis from adrenal precursors is the predominant route of androgen production in castrate males [14] and, together with the ovary, in females [15].

Both testosterone and DHT exert their effects by binding to the androgen receptor, an intracellular ligand-activated transcription factor, that is widespread in the body and expressed for example in reproductive organs, intestine, muscles, and brain [16]. The expression of the androgen receptor is subject to tissue-specific regulation, both by the androgen levels themselves and by a variety of transcription factors, in both men and women [9].

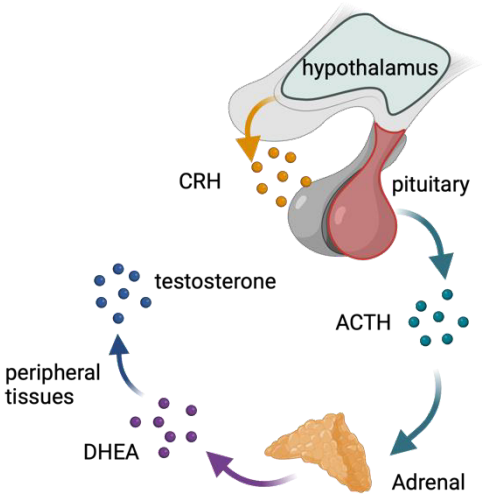

Figure 3 Hypothalamic-pituitary-adrenal axis regulation of adrenal androgen production. The hypothalamus produces corticotropin-releasing hormone (CRH), which causes the anterior pituitary to release adrenocorticotropic hormone (ACTH) that stimulates adrenal hormone production, including dehydroepiandrosterone (DHEA). DHEA can be converted to testosterone in peripheral target tissues.

Clinically, androgens have obvious positive effects [17], ensuring normal physiology and reproduction; but also negative effects, illustrated by side effects of illicit androgen use, including liver toxicity, cardiovascular disease, psychological issues, and acne [18]. The last decades, prescription of testosterone to older men has increased rapidly, despite there being no new approved indications for testosterone supplementation to men without pathological reproductive disorders. The alleged purpose is rejuvenation, with hope for increases in mood, libido, and energy levels. The possible risks with this kind of supplementation on for example prostate malignancy is still not fully known [19]. There has long been discussion of risks connected to cardiovascular disease [20]. Recently, a large randomized trial showed no increase of cardiovascular events in men with hypogonadism treated with transdermal testosterone, compared to placebo [21]. The study did not include

individuals without a diagnosis of hypogonadism. In general, possible negative effects of androgen treatment may be tissue-specific, meaning that increased androgen levels might be beneficial in one tissue but confer a risk of disease in another.

INTRACRINOLOGY

Hormones, including the sex steroids, are by definition chemical messengers. In traditional endocrinology, hormones are produced by designated glands, secreted into the circulation, and exert effects on cells in target tissues by binding to their receptor [22]. Hormones can have endocrine, paracrine, or autocrine effects, corresponding to effects on receptors in the whole body, on nearby cells, or on the cell itself. The last 30 years, however, there has been increasing attention given to the fact that local processes in target cells also regulate local sex steroid levels by transforming precursors or hormones into (more) active hormones that can bind to their receptor in the same cell and thus exert a biologic effect. Local regulation of sex steroid hormones was first described using the term intracrinology by Labrie [23, 24]. Thus, as the required enzymes for androgen production are expressed in several peripheral tissues, measurement of the circulating levels does not always reflect local levels [6]. This was recently demonstrated in a study comparing local sex steroid levels in the periprostatic vein with circulating sex steroid levels in patients undergoing radical prostectomy for prostate cancer [25].

The prostate is the largest producer of DHT from circulating testosterone by expression of the enzyme 5α-reductase (SRD5A), and this local production has been suggested to influence circulating DHT levels [26]. Prostate hyperplasia, a benign condition which can have large effects on patients' quality of life, can be treated with 5α-reductase inhibitors such as finasteride that block local synthesis of DHT and thereby cause a decrease in prostate size and a relief of symptoms [27]. Another example of a clinical application of intracrinological mechanisms is the use of aromatase inhibitors in the treatment of breast cancer, inhibiting the local production of estradiol from testosterone and other precursors [28, 29]. The schematic in figure 2 is a simplified representation as the intracellular DHT production in target tissues can use other pathways by bypassing T and instead use 17-hydroxyprogesterone and androsterone as intermediates, referred to as the backdoor pathway to DHT [6, 30-33]. The backdoor pathway was first discovered in the tammar wallaby (a marsupial, where undeveloped young can be easily accessed in the pouch of the mother) but is also important in mammals including humans [32, 34].

Androgens can be conjugated in target tissues and mainly in the liver to increase water solubility and facilitate excretion (Figure 4). Glucuronidation is the most important conjugation for androgens and the group of enzymes that catalyze this reaction – the uridine diphosphate-glucuronosyltransferases, is expressed in several peripheral androgen target tissues [35]. In addition, DHT, androsterone, and DHEA can be sulfonated by sulfotransferases that are also widely expressed in target tissues [6, 36].

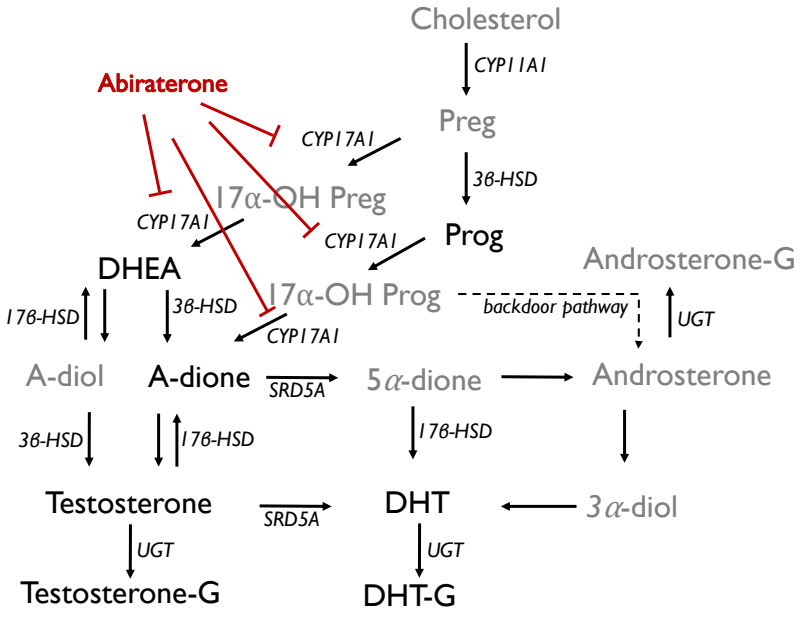

Figure 4 Simplified, expanded, schematic of androgen metabolic routes. Abiraterone is used in prostate cancer to block CYP17A1, inhibiting adrenal androgen production. Sex steroids in black are measured with the gas chromatography mass spectrometry assay used in this thesis. DHEA = dehydroepiandrosterone; 3α-diol = 3α androstanediol, A-diol = androstenediol; CYP = Cytochrome P450; A-dione = androstenedione; Preg = pregnanolone, Prog = progesterone; CYP17A1 = 17α-hydroxylase/17,20-lyase; DHT = dihydrotestosterone; HSD = hydroxysteroid dehydrogenase; 17-OH-Prog = 17-hydroxyprogesterone; -G = glucuronide; UGT = uridine diphosphate-glucuronosyltransferase; SRD5A = 5α-steroid dehydrogenase.
Adapted from Schiffer et al 2018 [6].

PROSTATE CANCER

Intracrinology is of major clinical importance in prostate cancer, the most common non-skin cancer in men. Prostate cancer has a very variable course, from slow-growing tumors that do not alter life expectancy to rapidly growing and metastatic tumors. Nowadays, most cases are detected early, when the disease is largely treatable by surgical removal or radiologic treatment of the tumor. However, in cases when local advanced disease or metastases are discovered, anti-hormonal treatments are used to slow the growth of tumors [12, 37].

Most prostate tumors are, at least at first, dependent on androgens, as shown already in the 1940s when Huggins and Hodges proved that advanced prostate cancer was dependent on androgens, by surgically castrating patients or by suppressing testosterone production by estradiol administration, thereby causing decreased cancer activity [38, 39]. Medical castration, also referred to as androgen deprivation therapy (ADT), is nowadays most often achieved by GnRH analogs (agonists or antagonists) that suppress the testicular production of testosterone and in the early stages of treatment cause a reduction of tumor size and decreased disease burden [40]. However, eventually most cases of metastatic prostate cancer develop into castration-resistant prostate cancer [41]. Additional drugs are added to ADT to further decrease hormone levels or actions. Antiandrogens have been used to counter the effects of castration-resistant prostate cancer for the last 20 years, the currently most used substance is enzalutamide [42]. Additionally, during the last decade, cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) inhibitors such as abiraterone are used to inhibit the adrenal production of androgen precursors in patients with castration-resistant prostate cancer [43] (Figure 4). Resistance to the treatment eventually occurs, but abiraterone treatment can increase overall survival of prostate cancer patients by 3-17 months [43, 44].

The effectiveness of the above treatments is based on the fact that most cases of castration-resistant prostate cancer are still dependent on androgens, as evidenced by local levels of active androgens in tumor biopsies, and upregulation of steroidogenic enzymes and androgen receptors in tumor tissue [44, 45]. The tumor itself can locally produce the potent androgen DHT from circulating precursors and thus can continue to grow despite ADT [12, 33]. Current research efforts aim to identify new inhibitors that target other parts

of the steroid synthesis pathways, to further eliminate androgen precursors from the tumor environment [12, 44, 46].

DHEA AND ITS USE AS A SUPPLEMENT

One of the main sources for local androgen production in patients on ADT for prostate cancer is adrenal-derived DHEA. DHEA is synthesized from cholesterol in the zona reticularis of the adrenal glands and is, together with its sulfate (DHEA-S), the most abundant circulating sex steroid precursor in humans. Circulating levels of DHEA peak around the age of 30, and then decrease gradually with age to levels of only 1/10th of peak levels at 80 years of age. Because of this, DHEA has by some been suggested as a potential "youth hormone" [47-49] and DHEA supplementation has been thought of as a feasible approach to restore physiological levels of androgens and estrogens in target tissues [50-52]. On the one hand, DHEA is classified as a nutritional supplement in the US and there sold freely and it is widely available via uncontrolled online outlets in other parts of the world including Sweden [53, 54]. On the other hand, DHEA is a restricted substance according to the international doping agency due to its androgenic properties [55, 56].

When individuals ingest oral DHEA, serum testosterone levels do indeed increase [57, 58]. However, large-scale supplementation studies in men have failed to show any major beneficial effects of DHEA treatment on metabolic parameters, sexual function, or quality of life [58, 59]. DHEA treatment was previously reported to have positive effects on hip bone mineral density but in later studies those results stayed true for females only [51, 60]. Yet, non-prescribed use of DHEA as a supplement continues with hopes of rejuvenation and possible virilization, despite little knowledge on the potential risks of long-term administration.

PROGESTERONE IN MEN

Another androgen precursor, sometimes overlooked in men, is progesterone. Progesterone in women and its roles in pregnancy and the menstrual cycle are well known, but progesterone in men is less well studied. However, circulating progesterone levels in men are similar to those in postmenopausal women [61-63]. Progesterone exerts its effects via the classical intracellular progesterone receptor that exists in two isoforms, A and B, but progesterone also has membrane-dependent actions. These have been suggested to play a

role in male sperm development [64], LH receptor expression in Leydig cells, and effects in the adipose tissue and kidney [62].

In early studies, testicular extracts from rats, mice, and humans produced androstenedione and testosterone when incubated with radiolabeled progesterone [65-67]. The clinical significance of progesterone's role as a precursor to androgens has however not been extensively studied.

Progesterone is starting to receive attention in the field of prostate cancer. A recent study on castration-resistant prostate cancer found that higher circulating progesterone levels after three months of abiraterone treatment were predictive of poorer treatment effect [68]. The same study investigated the oncogenic effects of progesterone in prostate cancer cell lines and concluded that the effects were both androgen receptor-dependent and -independent (but progesterone receptor-independent), with progesterone inducing the transcription factor MYC in prostate tumor cell lines [68]. Notably, MYC is a driver of the development of prostate cancer tumorigenesis [69]. Other studies have shown that high expression of the progesterone receptor associates with worse progression of prostate cancer tumors [70, 71], although it is unclear if progesterone as a ligand is important for this association.

Only in the last decade, methods have become available that are sensitive enough to measure circulating progesterone levels in males, enabling research on its clinical relevance and effects. Progesterone levels in men did not differ between those who had unprovoked deep vein thrombosis and controls [72] but lower progesterone levels were found in men that had an aeortic aneurysm, compared to controls [61]. Progesterone in men is generally described as being produced in the testicles and adrenals [62]. However, the sources of circulating progesterone in males have to the best of my knowledge not previously been thoroughly investigated.

Another important role of progesterone in both men and women, not further addressed or covered in this thesis or the included papers, is its role as a neurosteroid [73, 74].

ANDROGENS IN WOMEN

As mentioned earlier, androgens such as testosterone and DHT play important roles also in female physiology, and have well-described effects on body hair growth, acne, and sex drive. In women, testosterone is produced in

target tissues from adrenal DHEA (with decreasing levels by age) and, before menopause, in the ovaries [52]. Unlike in males, a female hyperandrogenic disease state exists. Polycystic ovary syndrome (PCOS) is a common condition (incidence between 8% and 13%) and its diagnosis, according to the widely accepted Rotterdam criteria, includes at least two of the following features: hyperandrogenism (either as elevated measured serum levels or distinct symptoms), oligo- or anovulation, and/or polycystic ovaries detected by ultrasound [75]. The exact pathogenesis is still largely unknown, but it is multifactorial and includes genetic, environmental, and transgenerational factors [76].

PCOS is associated with difficulties becoming pregnant, more risks if pregnancy does occur and a significant negative impact on quality of life. In addition, PCOS patients have a high incidence of metabolic disturbances, obesity, and type 2 diabetes. Hormonal treatment of PCOS is mainly estrogenic and progestogenic combined contraceptives and sometimes antiandrogenic drugs; the latter are associated with risks especially if the patient becomes pregnant. The main treatments of PCOS are symptomatic treatment of hirsutism and acne and general treatment of the psychologic, reproductive, and metabolic comorbidities [77].

SEX STEROIDS AND THE GUT MICROBIOTA

The gastrointestinal tract of every living being is the home for trillions of bacteria, fungi, and viruses, together referred to as the gut microbiota. The gut microbiota has several functions in nutrient uptake, production of vitamins, and it contributes to the development of the gastrointestinal immune system [78]. Studies in germ-free (GF) mice that completely lack gut microbiota have established that the gut microbiota is involved in energy uptake from food. Transfer of gut microbiota between mice can, in fact, transfer a metabolic phenotype such as obesity [79, 80]. In addition to these more well-known roles, the gut microbiota is also described as a virtual endocrine organ producing substances that are excreted into the bloodstream of the host, thereby affecting host physiology [81]. Examples of these substances include short chain fatty acids [82, 83], neurotransmitters, precursors to neuroactive compounds such as tryptophan, cortisol, as well as gastrointestinal hormones [81] (Figure 5).

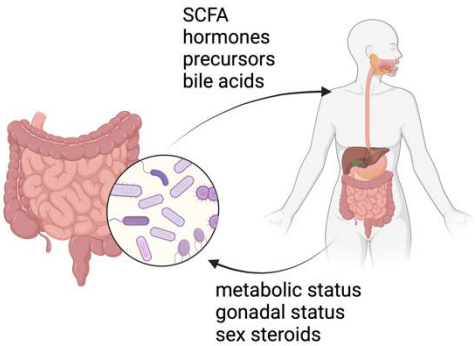

Figure 5 The gut microbiota produces substances that can affect the host, and host factors affect the composition of the gut microbiota. SCFA = short chain fatty acids.

In 2013, Markle and coworkers showed that the gut microbiota (via fecal transplant) could alter a sex steroid-dependent phenotype [84]. In non-obese diabetic (NOD) mice, the incidence of diabetes differs between the sexes with lower prevalence in males. If female pups were treated with intestinal contents from adult male mice, their incidence of diabetes decreased to that of males. This effect was mediated through an increase in serum testosterone (as measured with an immunoassay method) and prevented by treatment with the androgen receptor blocker flutamide. This study sparked an interest in the possibility of altering androgen levels by changing the gut microbiota.

There are several indications supporting the notion that androgen levels can be affected by the gut: CYP17A1, an enzyme vital for the production of androgens and precursors, is expressed and functional in the rat gastrointestinal tract [85]. Also, some strains of bacteria have the ability to metabolize androgens, for example convert testosterone into DHT [86] or catabolically break down testosterone [87]. The opposite relationship has also been shown, that the gut microbiota is affected by sex steroids. Gender-specific differences in gut microbiota composition have been described in some, but not other, studies [88]; but puberty, menopausal status, and castration have all been shown to change the gut microbiota [89-91]. Circulating levels of testosterone and progesterone have been shown to correlate with microbial composition in humans, and fecal transplant from male and female human donors affected the gut microbiota and sex steroid levels of male mice [89]. In patients with PCOS, gut microbial composition correlates with

hyperandrogenism [92-94], but the direction of the causality (i.e. are the androgens, the gut microbiota, or the concurrent metabolic syndrome drivers of the differences) has not been conclusively determined [95].

SEX STEROIDS IN MICE

For obvious and ethical reasons, detailed analyses of local sex steroid levels in certain tissues cannot be performed in humans, and laboratory model species such as mice are indispensable in medical and physiological research. Rodents mainly produce the same sex steroids as humans [96], with the important exception of DHEA which is not found in as high levels [97]. Although DHEA is not abundantly produced, mice adrenals do produce other androgen precursors, as evidenced by the fact that prostate tumor xenografts respond with decreased growth after adrenalectomy [98, 99]. Also in contrast to humans, rodents do not express SHBG which leads to several-fold lower circulating levels of sex steroids than in humans [100, 101].

MEASUREMENT OF SEX STEROIDS

Mice have low levels of sex steroids and sensitive methods are needed to accurately measure them, as well as the low sex steroid levels in children and postmenopausal women. Up until quite recently, even in high-impact journals direct immunoassay-based methods developed for clinical use have been used in preclinical rodent studies. Direct immunoassays without pre-extraction are fast and cheap but can cross-react with other substances present in the sample. This can lead to falsely high levels of sex steroids due to interference, and correlations between direct immunoassay and mass spectrometry (MS) -measurements are poor for estradiol in male serum [102] and for testosterone in mouse serum, testis, and ovary extracts [103]. The last decade, the need for accurate reporting of validated, sensitive, and specific methods for steroid hormone measurements has received increasing attention [104-106]. MS-based methods are considered the gold standard in sex steroid measurements. At the start of the projects included in this thesis, our group had just developed a highly sensitive and specific gas chromatography tandem MS (GC-MS/MS) method and validated it for serum [107]. This is one of the most sensitive sex steroid assays in the world regarding estradiol and DHT [106]. In the works included in this thesis, we set out to explore local sex steroid levels in a way that had not been done previously.

RESEARCH QUESTIONS

To conclude, androgens and progesterone are relevant for various diseases such as prostate cancer in men and PCOS in women, and they have local effects in different tissues. Understanding the local levels of sex steroids in tissues could contribute to finding new possible, more specific, drug targets with the anticipated effect in the target tissue while avoiding side-effects in other tissues. Despite this, only a few studies have been able to accurately determine the sex steroid levels locally in tissues. As a result, we lack understanding of what a normal distribution of sex steroids in the body looks like, and how different conditions affect the local levels of sex steroids. Thus, this thesis aims to start bridging this knowledge gap by providing comprehensive data on local sex steroid levels in mice in different states with its main focus on androgens and progesterone in major body compartments and the gastrointestinal contents.

AIMS

OVERALL AIM

Determine local sex steroid levels in tissues and the gastrointestinal tract of mice and evaluate their dependence on gonads, adrenals, and the gut microbiota (Figure 6).

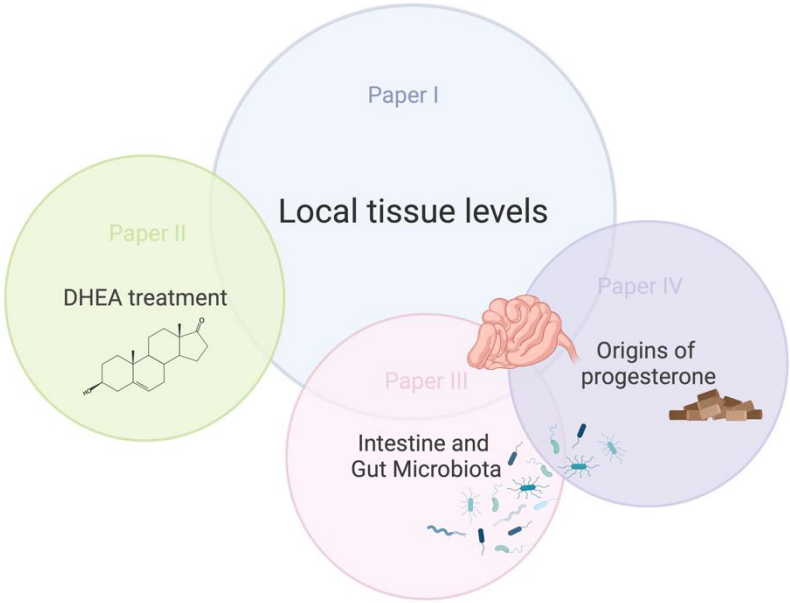

Figure 6 Overview of aims/themes in the different papers and how they are related. DHEA = dehydroepiandrosterone.

SPECIFIC AIMS

- Determine local sex steroid levels in different tissues of intact and orchiectomized male mice (paper I)
- Determine local androgen levels after DHEA treatment in orchiectomized male mice (paper II)
- Determine local sex steroid levels in the gastrointestinal tract (paper III)
- Evaluate the role of the gut microbiota in the regulation of local and systemic sex steroid levels (paper III)
- Evaluate the adrenal and gonadal contribution to androgen and progesterone levels in tissues and the gastrointestinal tract of male mice (paper IV)
- Investigate the origins of progesterone in male mice (paper IV)

METHODS

SEX STEROID ANALYSES

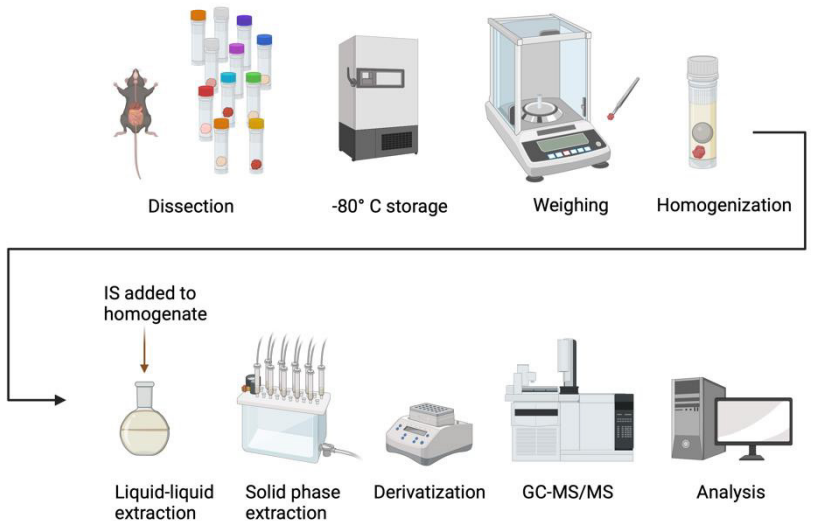

Figure 7 Overview of the workflow for sex steroid analyses in tissues. IS = internal standard, GC-MS/MS = gas chromatography - tandem mass spectrometry.

The GC-MS/MS method used is described in detail in our publications (**paper I, II, III**) [107]. The method consists of the following steps: homogenization, extraction, cleaning, derivatization, and analysis (Figure 7). Weighed samples are homogenized and liquid samples measured volumetrically and diluted. The sex steroids are extracted using liquid-liquid extraction, where an organic solvent is mixed with the water-based homogenate, whereby the fat-soluble steroids end up in the organic phase. In conjunction with the extraction, the homogenates are spiked with stable isotope-labeled steroids as internal standards. Isotope-labeled steroids have the same biochemical properties as the native hormone so these will go through the same losses in the preparation procedure as the native hormone, but they are separated in the mass spectrometric method as they have different masses. In nature, most carbons contain 12 neutrons (^{12}C) but a small proportion of all carbons have 13 neutrons (^{13}C). In the labeled steroids, a known set of carbons are replaced by carbon 13, enabling them to be separated from the endogenous steroids in the MS assay.

After the steroids are extracted into the organic phase, cleaning is necessary to remove interfering compounds. Samples are cleaned using solid phase extraction through silica-based cartridges that bind the steroids, and the cartridges are washed with semi-strong solvents to remove impurities. After the last wash, the strong solvent isooctane is flushed through the cartridges to dissolve the steroids and release them from the silica. Finally, to improve detection of the steroids the samples undergo derivatization, where structures are added to specific sites on the steroids [108]. Derivatization is performed in two steps: oximation and esterification.

In the analysis, seven sex steroids are separated on the GC column and detected simultaneously with electron capture negative chemical ionization by an Agilent 7000 triple quadrupole mass spectrometer. Peaks are integrated using the MassHunter quantitative analysis workstation software from Agilent. The measured concentrations are corrected for input material (wet mass of intestinal contents and tissues or volume of serum and bile).

Table 1 Sensitivity of the GC-MS/MS method in different matrices.

	E2	E1	T	DHT	Prog	A-dione
LLOQ (pg/g)						
Muscle	2.0	2.0	20	8.0	75	7.5
Liver	2.8	4.0	40	4.0	75	7.5
Adipose tissue	2.0	2.0	40	4.0	75	7.5
Bone	2.0	2.0	20	8.0	75	7.5
Intestinal contents			40	20		7.5
Serum (pg/ml)	0.5	0.5	8	2.5	74	12

GC - MS/MS = gas chromatography-tandem mass spectrometry; LLOQ = lower level of quantification; E2 = estradiol, E1 = estrone; T = testosterone; Prog = progesterone; A-dione = androstenedione. Parts previously published in papers I-IV and Nilsson et al. 2015 [107].

VALIDATION

The assay has been validated by spiking samples of serum, tissues, and intestinal content with different concentrations of isotope-labeled or unlabeled sex steroids. All details on the tissue validations are presented in **paper I** and in the supplemental materials in **paper II**, intestinal contents were validated in **paper III**. The lower limit of quantification (LLOQ) is defined as the lowest concentration with a coefficient of variation (CV)

below 20% and an accuracy of 80-120%. Several different levels in the low concentration range have been examined to identify the LLOQ.

DEGLUCURONIDATION

In bile and intestinal contents, a proportion of the sex steroids is expected to be glucuronidated. To assess the levels of glucuronidated steroids we measured levels of each hormone both with and without enzymatic deglucuronidation (**paper III** and **IV**). Each sample was divided into two aliquots before homogenization. After homogenization one of the aliquots was deglucuronidated by adding 50 µl of ß-glucuronidase (from *Escherichia coli*) and incubated. The deglucuronidated sample was denoted "total" and the sample without treatment was denoted "free". The difference between total and free levels was calculated and denoted glucuronidated ("gluc"). During method development we found that using recombinant enzyme from *E. coli* gave more accurate results than using extracted enzymes from the Roman snail, *Helix pomatia*, which is commonly used in the clinic. *H. pomatia* glucuronidase contained measurable levels of several sex steroids (unpublished data). Other researchers have also reported on artifacts using *H. Pomatia* glucuronidase for measurement of sex steroids in urine [109], and rat liver and plasma [110].

STUDY SUBJECTS

MICE

In all studies wildtype mice from the inbred C57BL/6 strain were used. This is the most common inbred mouse strain developed already in 1921 by cross-breeding several generations of siblings and is widely used as a background for gene knock out models and in preclinical studies with various purposes [111]. The two major sub strains J (Jackson laboratory) and N (National Institutes of Health) are genetically similar, but have certain distinct features regarding for example glucose metabolism [112, 113]. In the studies of this thesis both the J and N sub strains have been used due to practical reasons such as availability. Importantly, within each experiment all mice (controls and treated) were from the same sub strain [112].

GERM-FREE MICE

Figure 8 Germ free mice are totally free of bacteria and viruses and are kept sterile by handling inside isolators. Photo from 1966.

GF mice are born to mothers completely free from bacteria, virus, and fungi, living in sterile environments in isolators and they are thus never colonized by commensal microbes [114] (Figure 8). Despite this, GF mice lead fairly normal lives and grow up healthy, with even longer life spans than conventionally raised (CONV-R) mice, probably due to a lack of pathogenic infections [115]. This model has been used to evaluate the role of the gut microbiota for several conditions such as obesity, osteoporosis, and neuroendocrine disorders [115, 116].

GF mice have been used in research for a long time and their use has been widespread since the 1960s. During the last decades, new interest in this model has been sparked with the development of novel analysis methods to investigate microbiota and its metabolites [114]. The phenotype of GF mice is rather well described. Compared to CONV-R mice, GF mice have immature immune systems, enlarged cecums, prolonged intestinal transit time, and smaller hearts, lungs, and livers [117, 118], as well as other differences for example in their brain architecture [119]. GF mice reproduce naturally in their germ-free environment (in the same way as other mice), but their reproduction is somewhat impaired. GF females have longer diestrus periods, so they have longer cycles than CONV-R females and also display smaller

litter sizes and lower implantation rates. GF males have been reported to have lower sperm motility than CONV-R males. All of these reproductive traits have been shown to normalize following accidental colonization with certain bacterial strains [120]. Local sex steroid levels in GF mice had prior to **paper III** not been investigated. In **papers III and IV** we used GF mice of the C57BL/6NTac strain from Taconic, an external company, that housed the mice and performed the tissue dissections according to our instructions.

HUMAN SUBJECTS

For **paper III**, we acquired, through a collaboration, human fecal and serum samples from the baseline measurement of a randomized diet intervention study [121]. Serum and fecal samples from eight healthy men aged 23–31 years were thus analyzed to evaluate if the novel finding of high androgen levels in intestinal contents of mice could be confirmed in men. This diet study also included healthy women, but the female samples had to be excluded as the study was not originally designed to measure sex steroids. Several of the female subjects were taking oral contraceptives and for those who did not, the day of the menstrual cycle was not recorded. Unfortunately, this made sensible interpretation of the female results impossible.

METHODOLOGICAL CONSIDERATIONS

ORCHIECTOMY AND ADRENALECTOMY

The testes produce the majority of the androgens in the male, so removal of testes through surgery, orchiectomy (ORX), induces a hypogonadal state with greatly reduced androgen levels. The decrease in androgens leads to decreased sizes of androgen-responsive organs, among them the prostate, the levator ani muscle, and the seminal vesicles [122-124]. Gonadectomy also typically leads to an increase in the size of the thymus [125]. Finally, the decreased androgens also influence how animals respond to provocative stimuli, most commonly a decrease in aggression [126].

The adrenals produce androgen precursors but also vital glucocorticoids, mineralocorticoids, epinephrine and norepinephrine, as well as aldosterone which affects fluid balance. After adrenalectomy (ADX), surgical removal of the adrenals, mice are supplied with extra saline (in **paper IV** 0.9% NaCl instead of tap water) to maintain sodium balance.

SUPPLEMENTATION STUDIES

To replace hormones after ORX or ADX, supplemental hormones can be given by injection or by implantable pumps or slow-release pellets. In **paper II** we treated ORX mice with DHEA by intraperitoneal injection, five days per week. The dose was based on a previous study by our group and was chosen to reflect a physiological replacement dose [127].

ORAL GAVAGE

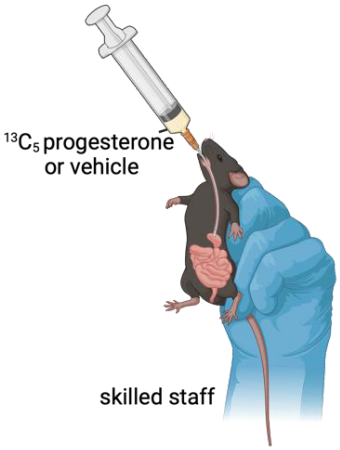

Figure 9 Oral gavage of mice, used in paper IV.

A common method to orally administer substances to mice is oral gavage. A plastic syringe fitted with a blunt gavage needle is used, with the length of the needle adapted so that the tip is located in the stomach when inserted through the mouth and esophagus [128] (Figure 9). This is done in conscious mice and can be a somewhat stressful procedure for the mice but stress and risk are minimized when the procedure is done by skilled staff, and it ensures that each mouse receives the same dose. In **paper IV** we used oral gavage to dose the mice with labeled progesterone. Other options for oral administration such as mixing the hormone solution into a sweet nut spread (Nutella) as described for estradiol [129] were explored. This alternative method did not prove feasible within our time limits, as the mice need to be trained to accept the voluntary feeding. Also, there may be uncertainties in the dosing if some mice do not finish their serving.

WHOLE-BODY SEX STEROID MODEL

In **paper I**, a simplified whole-body model was used to describe and visualize the distribution and total amount of sex steroids in the mouse body. The measured concentration of a steroid in every organ was multiplied by the mass of each organ or tissue type/compartment. For tissues that were weighed at sacrifice the actual mass was used for each individual mouse. For other tissues and compartments the weights were approximated either as fixed weights based on previous experiments (adrenals, testicles) or as percentages of the body weights, based on previous experience with DXA measurements and literature reports. All compartments added up to 100%, with the lean mass as the largest compartment. Then, the calculated amounts for all tissues and compartments were summed to give the total pool.

GONADOTROPIN MEASUREMENTS

Serum LH and FSH were measured by time-resolved immunofluorometric assays by our collaborators in Finland as previously described [130, 131] (**paper III**).

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

To assess relative gene expression in different tissues in **paper III and IV** we used real time quantitative polymerase chain reaction, qPCR. In this common and sensitive method, RNA is extracted from tissues and then reverse transcribed into complementary DNA (cDNA). The cDNA is mixed with primers, oligonucleotides and fluorescence-labeled probes and put through heat cycles where the genes that match the primers are replicated once per cycle, thus, the number of copies doubles once per cycle and grow exponentially. During the cycling, fluorescence is measured and when the number of copies reaches a threshold fluorescence can be detected. The relative expression can be calculated based on in which cycle fluorescence was first detected in each sample. In our studies, we also added primers labeled with a separate fluorescent marker for the ribosomal housekeeping gene 18S as reference gene. We then calculated relative expression of the gene of interest, normalized for the expression of the reference gene, compared to either a control tissue (**paper III**) or a control group (**paper IV**) using the $\Delta\Delta$CT method [132].

STATISTICS

COMPARISONS AND CORRELATIONS

In all studies, comparisons between two or more groups were performed using statistical testing. In the datasets from **papers I-III**, we used non-parametrical tests as hormone level data was not normally distributed due to several samples below the LLOQ with imputed values (either LLOQ or LLOQ/√2). We used Mann-Whitney rank U-test to compare two groups and Kruskal-Wallis with Dunn's post hoc-test to compare more groups.

In **paper IV**, data was log-normally distributed, and the parametric tests Student's t-test and two-way ANOVA were used after log-transformation of hormone levels. Also in this study there were some tissues and hormones with levels below the detection limit, but for tissues and hormones within the main interest of the study all or almost all levels were measurable and parametrical testing was used in order to maintain statistical power.

To evaluate the correlations between serum and tissue sex steroid levels in **paper I**, nonparametric Spearman rank-order correlations were used. To evaluate the correlation between animal fat percentage and progesterone levels in diets in **paper IV**, Pearson correlation was used.

HANDLING OF UNDETECTABLE VALUES

Any analytical method has a detection limit below which the signal cannot be reliably separated from the background (limit of detection, LOD) or accurately quantified (LLOQ). When levels of hormones are lower than this limit, the undetectable values need to be handled so that the full dataset can be used. Commonly used options are removal of undetectable results, or imputation of an estimate such as zero, LLOQ, LLOQ/2, or LLOQ/√2. Removing the undetectable results causes an overestimation of the mean and decreases the power of the study; whereas imputing zero causes an underestimation of the mean, as the undetectable values are distributed between zero and the LLOQ [133]. Imputing zero also prohibits further treatment of the results such as log-transformation. In our studies we have used either imputation of LLOQ (**paper I, paper III)** or LLOQ/√2 (**paper II, paper IV**). The latter was found to introduce the least bias in a study that compared simple substitution methods in a large dataset of sex steroid levels in humans [133]. We also defined a minimum number of detectable values in a group (i.e. half of the samples) to allow reporting of steroid levels. This was

done to avoid false positive findings as we are investigating steroid levels in tissues where few previous reliable measurements are available.

RESULTS

Here, the results from each paper will be briefly presented and discussed (Figure 10). For additional details, see also the appended papers.

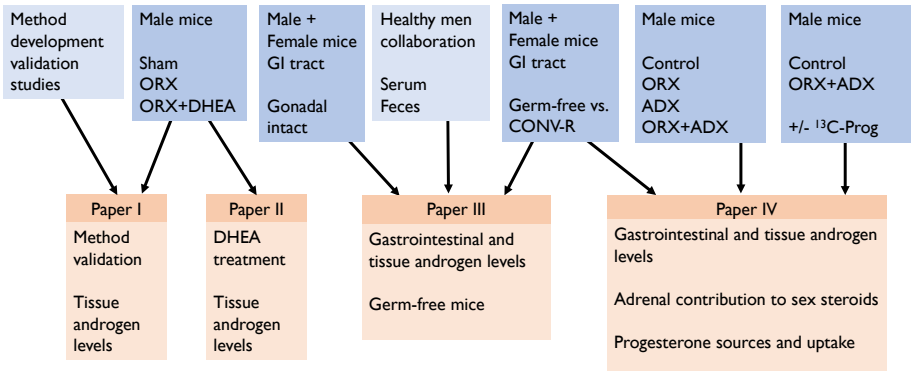

Figure 10 Overview of the different papers and the corresponding studies. Light blue studies were performed mainly by other researchers.
DHEA = dehydroepiandrosterone; GI = gastrointestinal; CONV-R = conventionally raised; ADX = adrenalectomized; ORX = orchiectomized; ^{13}C-Prog = stable isotope-labeled progesterone

PAPER I

COMPREHENSIVE SEX STEROID PROFILING IN MULTIPLE TISSUES REVEALS NOVEL INSIGHTS IN SEX STEROID DISTRIBUTION OF MALE MICE

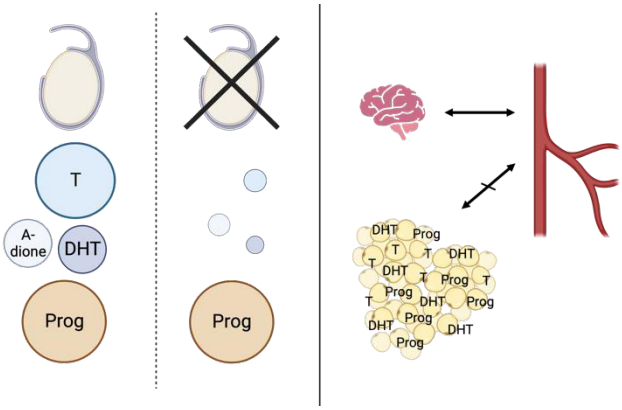

Figure 11 Orchiectomy causes large decreases of the total androgen pools whereas progesterone pools remain after orchiectomy. Brain sex steroid levels correlate with corresponding serum levels, whereas adipose tissue contains high concentrations of sex steroids that do not correlate with their serum levels. A-dione = androstenedione; DHT = dihydrotestosterone; Prog = progesterone; T = testosterone.

In paper I, we published a detailed assay validation for a broad panel of sex steroids with the sensitivity, precision, and accuracy presented for muscle, liver, white adipose tissue (WAT), and bone, representing four very different types of matrices. We then characterized sex steroid levels in serum and ten different tissues of gonadal intact and ORX male mice. The measured levels were presented in two different ways. First, we calculated the amount of hormone in each tissue or compartment of the body and then calculated a total body pool of each sex steroid. Next, we evaluated the tissue/serum correlations in each tissue.

As expected, androgens decreased in most tissues after ORX. In intact mice, testosterone and progesterone both had the largest full-body pools. In ORX mice, progesterone had the, by far, largest pool. There were strong correlations between serum and brain levels of testosterone, DHT, and progesterone but only modest correlations between these hormones in serum and WAT, testis, or adrenals (Figure 11). This first study investigated the levels of several sex steroids in a broad range of tissues, creating an atlas and enabling comparisons of local levels across tissues.

PAPER II

DEHYDROEPIANDROSTERONE SUPPLEMENTATION RESULTS IN VARYING TISSUE-SPECIFIC LEVELS OF DIHYDROTESTOSTERONE IN MALE MICE

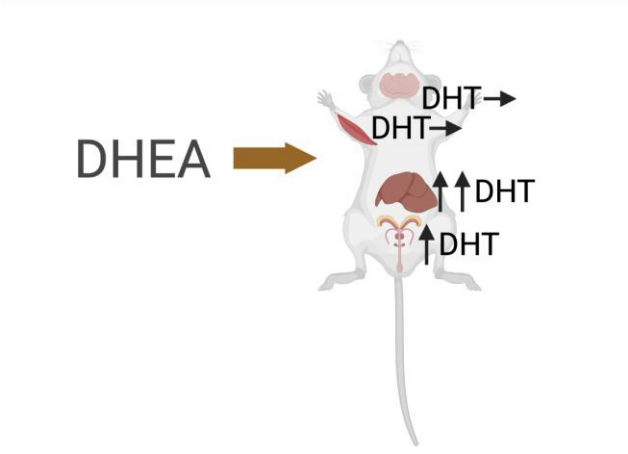

Figure 12 Treatment of orchiectomized mice with dehydroepiandrosterone (DHEA) causes large increases of androgens (testosterone and DHT) in the liver and reproductive tissues but not in brain or muscle. DHT = dihydrotestosterone.

In paper II, we investigated the effect of supplementing castrated male mice with DHEA for three weeks and compared them to the groups already published in paper I.

Downstream metabolites of DHEA, namely testosterone, DHT, and androstenedione, increased in serum and most tissues of DHEA-treated ORX mice compared to ORX mice. The relative recovery of androgen levels (compared to intact mice) markedly differed between tissues, especially for DHT. The DHT levels in the liver were supraphysiological after DHEA treatment while in reproductive organs and serum they were restored to pre-castration levels. On the other hand, in muscle and brain cortex the DHT levels were not significantly increased compared to ORX mice (Figure 12).

The most noticeable finding of this study might be the high androgen levels in the liver after DHEA treatment. This probably reflects the metabolic capacity of the liver to convert endogenous and exogenous compounds and may have implications for the use of DHEA as androgens have been implicated in liver disease.

PAPER III

THE GUT MICROBIOTA IS A MAJOR REGULATOR OF ANDROGEN METABOLISM IN INTESTINAL CONTENTS

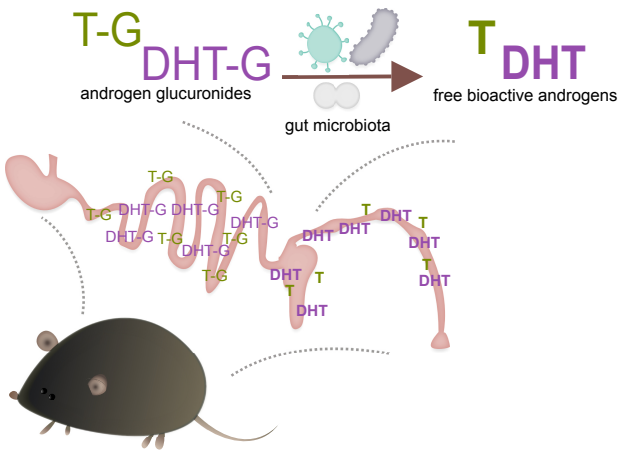

Figure 13 The small intestine contains high levels of glucuronidated androgens (dihydrotestosterone, DHT and testosterone, T) that are deconjugated by the gut microbiota and present in high levels as free sex steroids in the distal intestine.

In paper III, we continued with the mapping of tissue androgen levels and also included the gastrointestinal tract. Unexpectedly, we discovered that the intestinal contents and feces contain high levels of DHT in both male and female mice (Figure 13). These intestinal DHT levels were dependent on deglucuronidation by gut bacteria. In GF mice, the gastrointestinal tract did not contain high levels of free androgens but instead contained very high levels of glucuronidated testosterone and DHT. The GF male mice also had increased DHT levels in seminal vesicles and the liver, and increased FSH levels. In healthy men, we also found high levels of DHT in feces compared to serum levels. The physiological importance of intraintestinal androgens deserves further research.

The findings from this study indicate that the intestine is a route of elimination for androgens, and that deglucuronidation by gut bacteria results in high levels of bioactive androgens available for reuptake or local effects in the intestine. This novel finding suggests a possible mechanism for the interaction between the gut microbiota and androgens.

PAPER IV

DIETARY PROGESTERONE CONTRIBUTES TO INTRA-TISSUE LEVELS OF PROGESTERONE IN MALE MICE

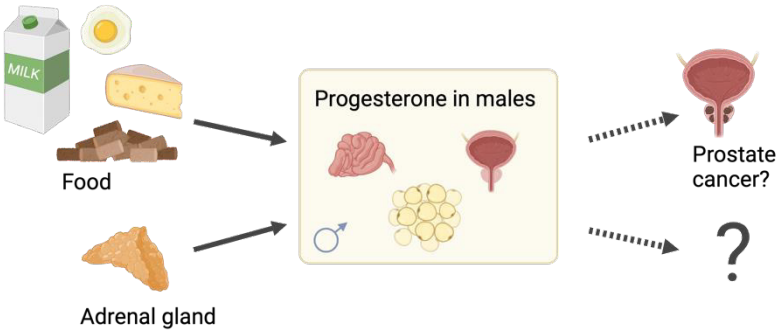

Figure 14 Progesterone levels in male mice can originate from dietary uptake in addition to being produced in the adrenals. The clinical relevance of this is still unknown but may have implications for patients with prostate cancer.

In paper IV, we investigated the effect of ORX and ADX on sex steroid levels in serum, tissues, and the gastrointestinal tract of male mice. Androgen levels decreased substantially with ORX but were not majorly affected by ADX. Surprisingly, the progesterone levels remained largely unchanged after both ORX and ADX in most sites. We then investigated the diet as a possible source of progesterone. We found that the mouse chow contained higher progesterone levels than any tissue in male mice. High and variable levels of progesterone were detected in more than 20 investigated mouse diets and in certain human foods, for example dairy products and eggs (Figure 14). The possibility of intestinal uptake of dietary progesterone into WAT and prostate was confirmed using isotope-labeled progesterone administered via oral gavage to ORX+ADX and sham-operated male mice.

Recently, progesterone has been suggested to play a role in the setting of castration resistant prostate cancer. If dietary progesterone is taken up into tissues, this might be a substrate for intratumoral steroidogenesis and thereby affect prostate cancer. The uptake of dietary progesterone may mediate the effect of dairy product consumption on prostate cancer seen in some epidemiological studies. Also, the high and varying levels of progesterone in rodent diets should be considered by researchers in models with sex steroid-dependent phenotypes.

DISCUSSION

The overall aim of the studies included in this thesis was to determine local sex steroid levels in tissues and the gastrointestinal tract of mice and evaluate their origins and factors affecting them. In **paper I** we presented the validation of the analysis method and investigated the effect of castration on tissue sex steroid levels in male mice. In **paper II** we evaluated the effect of treating ORX mice with DHEA and the possibility of local production of androgens with DHEA as a substrate. In **paper III** we focused on the gastrointestinal tract and the effect of the gut microbiota, this time including both male and female mice and also male human subjects. Finally, in **paper IV**, we followed up our findings of high progesterone levels in ORX mice from **paper I** and high androgen levels in the gastrointestinal tract from **paper III** by investigating the adrenal contribution to local progesterone and androgen levels in both tissues and the gastrointestinal tract. After unexpected findings of progesterone levels still present after ORX and ADX and high progesterone levels in the mouse chow, we also investigated the diet as a potential source of progesterone in **paper IV**.

MEASUREMENT OF ANDROGEN LEVELS IN TISSUES

Serum levels (and, as we have shown, tissue levels) of androgens in male mice show a large interindividual variability within a group, and also vary longitudinally in the same animal [134]. This is partly related to the social dominance pattern, where alpha males typically have higher androgen levels. Different types of social dominance patterns can exist in different cages of the same study [135]. According to the serum testosterone levels no obvious alpha males were present in **paper I**, whereas typical alpha males with tenfold higher androgen levels than their cage mates were present in the gonadal-intact control groups of **papers III and IV**. This contributes to a smaller variability in androgen levels in **paper I** than would be expected in some other studies. For some applications, it might be reasonable to look at tissue/serum concentration ratios rather than the raw concentration. Importantly, if different gonadal intact groups are compared in a study, it would be important to consider if the number of alpha males per group can influence the androgen level results.

The GC-MS/MS method for which the validation was published in **paper I** is currently being used in other studies examining local androgens in heart (unpublished data), prostate [98], and other tissues. The strength of this method is, as mentioned, its high sensitivity, enabling quantification of sex steroids in small samples (as small as 5 mg), and its high specificity, eliminating the risks of misclassification inherent to immunological methods. MS methods are considered the gold standard for sex steroid measurements in serum and tissues [136]. The work-up with several steps of cleaning and removing impurities enables measurement even in complex matrices such as metabolically active organs as the liver, and lipid-rich tissues such as adipose tissue, which contains substances that are difficult to separate from the steroids in a chromatographic assay. The drawback is the requirement of qualified personnel to perform the labor-intensive work-up required for each sample (around 80-120 samples per working week), restricting its use in large-scale studies. For this purpose, our group has also developed a more high-throughput liquid chromatography – MS method that is better suited to measure serum sex steroid levels in large cohort studies [61]. This method includes no pre-extraction or derivatization step but has not been validated for tissue measurements.

A few previous studies have presented methods for the measurement of local sex steroid levels in several rodent tissues. McNamara and coworkers published an LC-MS/MS method in 2010 to measure testosterone, DHT, estrone, estradiol, 3α-androstanediol and 3β-androstanediol and it was validated in mouse ovaries and testis, in addition to serum [137]. Also in 2010, Weng and co-workers published an LC-MS/MS method with derivatization for detection of T and DHT in serum and various reproductive tissues [138]. More recently, a metod for determining several steroids in rat serum and liver samples was published [110]. All of these methods have LLOQs for DHT and estrogens significantly higher than those defined by the method used in this thesis. To the best of my knowledge, no previous studies on sex steroids have included both endocrine organs, androgen-responsive organs, major body compartments, as well as the gastrointestinal tract and used a sensitive, specific, validated method for a broad panel of sex steroids.

TISSUE WEIGHTS AS INDICATORS OF ANDROGEN EFFECTS

As sex steroid measurements in rodents have long been a challenge, monitoring the weights of androgen-responsive tissues is common practice to evaluate androgenic effects. The Hershberger Bioassay suggested by the Organization for Economic Cooperation and Development (OECD) recommends measurement of five tissue weights (ventral prostate, m. levator ani, seminal vesicles, Cowper's glands, and glans penis) of castrated male rats to detect androgenic and antiandrogenic effects. A potent androgen such as testosterone is generally used as a positive control [139]. In **paper II** we assessed not only the tissue weights but also the local androgen levels in three of these tissues after DHEA treatment of ORX mice.

In most studies to date, for several reasons, only sex steroid levels in serum and not tissue levels have been used to monitor sex steroid supplementation in rodent models. This could have underestimated or overestimated the effects locally in the tissues. Measuring local tissue levels provides more in-depth knowledge on the relationship between local and circulating sex steroid levels. This knowledge may contribute to a deeper understanding of disease processes that are dependent on local sex steroids, such as prostate cancer, breast cancer, endometriosis, and PCOS and possible risks or side-effects of sex steroid-affecting treatments.

ANDROGENS AND THE GUT MICROBIOTA

A link between sex steroids and the gut microbiota has in the last decade been suggested in the context of a variety of diseases or physiological states [89, 91, 140]. Specifically, a relationship between the gut microbiota and androgen-related disorders has been suggested in prostate cancer [141-145], PCOS [95, 146-150], and male infertility [151]. The exact mechanisms and dependencies in these correlations still remain to be elucidated [152].

In **paper III** we found that high levels of conjugated androgens are present in the small intestine, and they are deconjugated by the gut microbiota yielding high levels of available androgens in the colonic contents (Figure 15). The pattern of high DHT in colonic contents and high conjugated androgens in the small intestine was found in both male and female mice, although, unsurprisingly, the levels were generally lower in females. Since females do

not have tissues with high local levels of DHT (equivalent to the prostate and seminal vesicles in males), the intestinal and liver contents of DHT were the highest in the female mouse body.

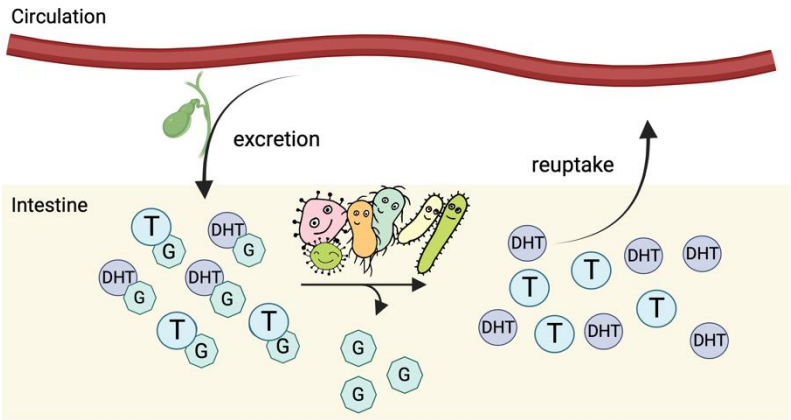

Figure 15 Testosterone (T) and dihydrotestosterone (DHT) are excreted into the intestine from the bile as glucuronidated (G) conjugates, where they are deconjugated by the gut bacteria and available for reuptake or local effects.

These high local androgen levels in intestinal contents may have effects on visceral muscle contractility [153] or on small intestine endothelial function [154]. Androgens have also been implicated to play a role in colorectal carcinoma although the exact mechanisms are still unknown [155]. In addition, testosterone and DHT, as lipophilic compounds, can pass the intestinal wall to the circulation or the lymphatic system and thereby exert systemic effects [156]. Orally administered testosterone is absorbed, even though a part of it is lost to local metabolism in the intestine and first-pass metabolism in the liver [157].

Bacterial deconjugation of excreted conjugated androgens followed by possible reuptake of the hormone is very similar to the proposed effects of the so-called estrobolome (mainly described in females) that involves the gut microbiota deconjugating conjugated estrogens secreted by the liver [158]. The estrobolome has been suggested to be relevant for breast cancer and other estrogen-related conditions [159, 160]. The corresponding concept, the androbolome, has not yet been established but the concept is mentioned in a recent review on the gut microbiota and breast cancer [161]. Our finding of an androbolome in **paper III** that can deconjugate glucuronidated androgens sheds light on one mechanism by which the gut microbiota can affect androgen levels. Another mechanism, that could also be included in the

androbolome concept, is gut microbial metabolism of androgens or precursors which has been demonstrated for several strains of bacteria [86, 87, 162, 163]. In **paper IV** we found that the uptake of dietary progesterone may be influenced by the gut microbiota, as higher progesterone levels were found in the adipose tissue of GF than CONV-R male mice. The mechanism for this interaction is still unknown but must be different from deglucuronidation as progesterone is not glucuronidated.

The notion that changing the gut microbiota can cause relevant changes in androgen-related phenotypes has been demonstrated in several recent preclinical studies (Figure 16). In a mouse prostate cancer model, Pernigioni and coworkers found that castration influences the gut microbiota, and antibiotic treatment, removing most of the gut microbiota, could delay the emergence of castration resistance. Also, prostate cancer phenotypes (hormone-sensitive versus castration-resistant) could be transferred via fecal microbiota transfer [162]. Another study found that male androgen receptor knock-out mice increased their bodyweight more than wildtype littermate controls when exposed to a high-fat diet. Interestingly, antibiotic treatment of the mice abolished this difference, suggesting that the gut microbiota plays a role in the development of hypogonadal obesity in this androgen receptor-deficient mouse model [164]. In other studies, fecal microbiota transfer between mice has been shown to improve sperm quality when it has been diminished by high fat diet or the cancer drug busulfan [165, 166]. In female rats, prenatal treatment with androgens in a PCOS model gave long-term changes in the gut microbiota [167]. Furthermore, when PCOS mice were co-housed with healthy mice their PCOS symptoms improved and a change in the gut microbiota was detected [168].

Another way to affect the gut microbiota is to use probiotics. Probiotic treatment (*Lactobacillus reuteri*) has been shown to affect androgen-dependent phenotypes in aged male mice [169, 170]. However, when we followed up **paper III** by treating castrated male mice with probiotics (*L.mix: Lacticaseibacillus paracasei* DSM13434, *Lactiplantibacillus plantarum* DSM15312 and DSM15313) or vehicle, we could not detect an effect on androgen dependent phenotypes or circulating or gastrointestinal androgens [171]. In the future, it would be interesting to accurately measure local androgen levels in old male mice with phenotypic improvements from probiotic treatment such as those in the studies by Poutahidis and Lee and coworkers [169, 170], to find out if the phenotypic changes are correlated with increased local androgen levels.

Altogether, the prospect of treating or preventing androgen-related disorders by modulating the gut microbiota is still some way off, but this is a highly active field that attracts a lot of attention at the moment.

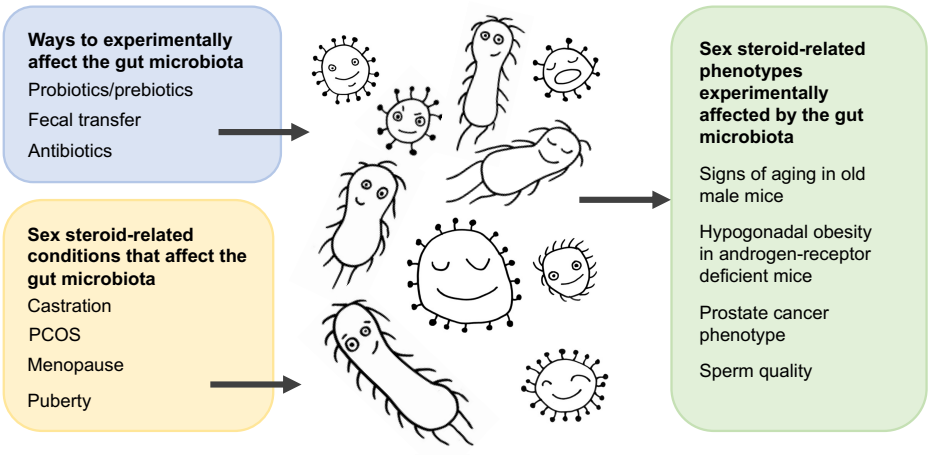

Figure 16 Overview of connections between sex steroids and the gut microbiota. PCOS = polycystic ovary syndrome.

LOCAL ANDROGENS AND CANCER

Prostate cancer is one obvious field for local sex steroid measurements. Steroid metabolizing enzymes are expressed in tumor tissue. In castration resistant prostate cancer, non-androgenic precursors are locally transformed into androgens in tumor tissue thus driving cancer progression [40, 44, 172].

In humans, local levels of androgens in the prostate correlate poorly with circulating androgens [173]. Circulating testosterone levels or treatment with testosterone have not been consistently associated with the development of new prostate cancer in epidemiological studies [174], but a Mendelian randomization study has shown a causal link between genetically determined testosterone levels and an increased risk of prostate cancer [175]. Thus, the fact that the common nutritional supplement DHEA increases androgen levels preferentially in the prostate (and liver) may be a cause of concern (our finding in **paper II).** This increase is however not entirely surprising as these tissues are known to express 5α-reductase enzymes, capable of producing DHT from testosterone [176]. In **paper II** we also found that supplementation with DHEA leads to increased local androgen levels in the liver. Higher serum levels of androgens have been linked to an increased risk of

developing liver cancer, and interestingly, an inverse correlation was seen for DHEA, associating higher DHEA levels with less liver cancer. The authors speculate that the capacity of local production of androgens from DHEA is important for liver cancer risk [177].

PROGESTERONE IN MALES

In **study I** we concluded that progesterone was as abundant as testosterone in intact male mice, and the most abundant sex steroid in castrated male mice. In **study IV** we found that a source other than the adrenals must exist as progesterone levels remained largely unchanged after ORX and ADX. The clinical relevance of progesterone in men is still mostly unknown [62, 63], but levels in men are similar to those in postmenopausal women, and lower progesterone levels have been associated with an increased risk of aortic aneurysm [61].

Progesterone affects prostate cancer cells *in vitro* and it has been described as an oncometabolite [68]. Progesterone levels increase in prostate cancer patients treated with the CYP17A1-inhibitor abiraterone during the first months of treatment as the downstream metabolism of progesterone is blocked by abiraterone [178]. In addition, the progesterone receptor is expressed in prostate tissue and higher expression of progesterone receptor B is associated with worse prognosis for prostate cancer patients, but it is unclear if progesterone is important as a ligand for this mechanism [70]. The role of progesterone in prostate cancer is still far from fully understood.

The finding in **paper IV** that dietary progesterone can be taken up and detected in prostate and adipose tissue of male mice is novel, and in line with previous research reporting high progesterone levels in dairy products and other food items [179-182]. Dietary sex steroids from milk can in fact affect urine or serum sex steroid levels of women and children [183, 184]. The possible clinical relevance of dietary progesterone in men remains to be elucidated.

CONSIDERATIONS OF SEX/GENDER

In this thesis and its accompanying papers, I have used the terms sex and men/women/males/females for individuals based on their sex chromosomes, but I acknowledge that not everyone identifies according to the gender as defined by their sex chromosomes; thus, this a simplified nomenclature. All

studies on physiology should consider differences according to sex – this is especially relevant in research regarding sex hormones [185]. Historically, female rodents have been understudied due to the belief that estrus cycling may induce too much variability, despite that also males show significant, and sometimes greater, variability in several traits [186, 187].

In this thesis, the major focus is on sex hormone measurements in males, whereas androgens are relevant also for female physiology and diseases. In **paper III**, results from females were published alongside those from males. The basic distribution and supplementation effects of sex steroids in tissues published in **papers I and II** have also been performed in females and will be published separately. Further studies in females should be performed to understand local levels of androgens and their relevance for female-specific androgen-related diseases such as PCOS. The connection between the gut microbiota and PCOS is an active area of research, even though it is still unclear if the proposed relationships between the gut microbiota and PCOS are more related to the androgenic or the metabolic component of the disease [95].

Measuring local tissue sex steroids in female mouse tissues is definitely possible with our developed and validated method. Accurate study planning taking into consideration the cycling of intact female mice could benefit analysis. This could be achieved for example by monitoring estrus cycling through vaginal smears during the days before sacrifice, or timing the sacrifices to have all mice in the same cycle phase, depending on the research question [107, 188]. In our experience, slightly larger study groups can be used to compensate for the cycle-dependent variability between female rodents when examining sex steroid levels. Local tissue sex steroid measurements in both female mice and human samples can be important in studies on estrogen-driven diseases such as endometriosis [189] and breast cancer [190].

CONCLUSION

In the papers included in this thesis, we have published an atlas of local sex steroid levels in tissues and the gastrointestinal tract of mice in different conditions (castrated, castrated + DHEA-treated, castrated ± adrenalectomized, germ-free) and specifically mapped local sex steroid levels in intestinal contents of both males and females, as well as the sex steroid levels in mouse and human food.

As these studies have had rather open-ended research questions, we have had a few thought-provoking and unexpected findings, namely the effect of DHEA treatment on androgen levels in the liver, the high DHT levels in the intestinal contents, the effect of the gut microbiota on intra-intestinal androgens, and the possible role of dietary progesterone for male progesterone levels (Figure 17).

Measuring local sex steroid levels in tissues is unlikely to become widespread in clinical practice, as using a GC-MS/MS method such as ours still requires a lot of time and money, and tissue sampling is not always feasible in a clinical setting. Instead, understanding the details of local mechanisms by looking closely at local sex steroid levels together with detailed knowledge of enzyme expression and transcription can result in the identification of novel drug targets or better understanding of the tissue-specific effects and risks of sex hormone-affecting treatments.

One of our findings that is already being actively investigated, and for which we provided a small piece of the puzzle regarding potential mechanisms, is the connection between the gut microbiota and androgen levels and disorders. Since its publication in 2019, **paper III** has been cited 115 times according to Google Scholar (September 2023). This comparatively large number reflects the numerous recent publications discussing the interaction between androgens and the gut microbiota, although in my opinion, review articles are overrepresented and conclusive studies are still lacking. The possibility of targeting the gut microbiota to treat or prevent androgen-dependent disorders requires more high-quality, well-powered studies.

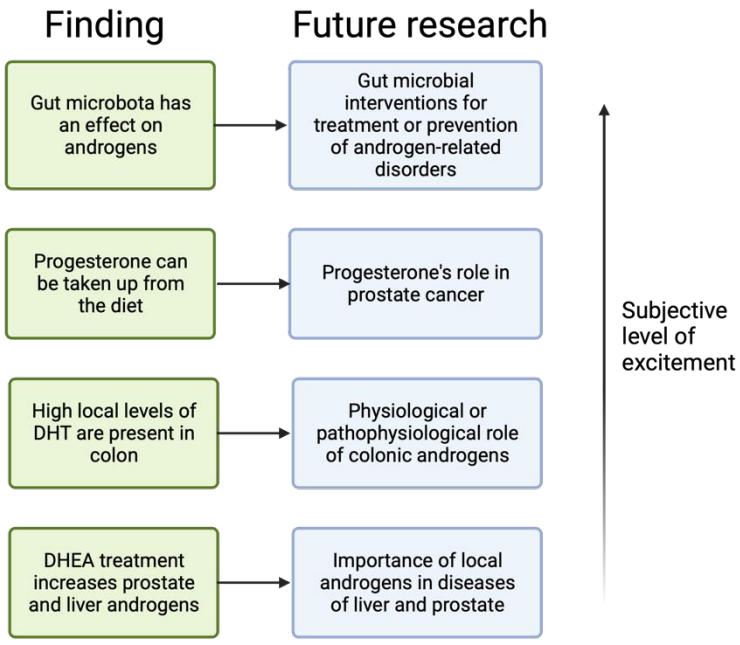

Figure 17 Four main findings and their related future research fields, arranged in order of my personal perceived level of excitement.

FUTURE PERSPECTIVES

The field in which local tissue sex steroid levels are used in research has grown rapidly since the planning of the first studies of this thesis. Our findings will lay a foundation for future research on local sex steroid levels and have produced several interesting research questions.

To understand the relationship between sex steroid levels and the gut microbiota in humans in more detail a more epidemiological approach could be to use data on sex steroid levels and gut microbiome sequencing from large cohort studies, to elucidate the interactions between androgens and the gut microbiota. Our finding of high local androgen levels in feces of young and healthy men should be expanded into other groups such as women and men of other ages, and patient groups with androgen-related or intestinal disorders.

The role of progesterone in prostate cancer is currently under investigation, and our novel finding of uptake of dietary progesterone into prostate tissue requires further studies. An obvious next study would be to investigate the contribution of dietary progesterone to circulating or tissue levels of progesterone by adding progesterone-rich foods to the diets of mice. This could also be done in a prostate cancer mouse model. Also, it would be interesting to plan a clinical diet study for men with prostate cancer on ADT where diets are designed to contain different levels of progesterone, and then follow up the circulating progesterone levels.

Possible long-term effects of DHEA treatment on the liver deserves further investigation, especially if prescribed use of DHEA increase in men or women. At least, careful consideration of the long-term liver effects is warranted in any clinical studies investigating DHEA treatment.

Finally, understanding more about local sex steroid levels in tissues may bring on new ideas and research questions in several different fields, and ultimately lead to new treatment options for sex steroid-dependent diseases or more knowledge about modifiable risk factors for endocrine cancers.

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REFERENCES

1. Brinkmann AO. Molecular mechanisms of androgen action--a historical perspective. *Methods Mol Biol*. 2011;776:3-24. doi:10.1007/978-1-61779-243-4_1

2. Wilson JD, Roehrborn C. Long-term consequences of castration in men: lessons from the Skoptzy and the eunuchs of the Chinese and Ottoman courts. *J Clin Endocrinol Metab*. Dec 1999;84(12):4324-31. doi:10.1210/jcem.84.12.6206

3. Handelsman DJ. Androgen Misuse and Abuse. *Endocrine Reviews*. 2021;42(4):457-501. doi:10.1210/endrev/bnab001

4. Hammes SR, Levin ER. Impact of estrogens in males and androgens in females. *J Clin Invest*. May 1 2019;129(5):1818-1826. doi:10.1172/jci125755

5. Huhtaniemi I. Mechanisms in Endocrinology: Hormonal regulation of spermatogenesis: mutant mice challenging old paradigms. *Eur J Endocrinol*. Sep 2018;179(3):R143-R150. doi:10.1530/EJE-18-0396

6. Schiffer L, Arlt W, Storbeck KH. Intracrine androgen biosynthesis, metabolism and action revisited. *Mol Cell Endocrinol*. Apr 15 2018;465:4-26. doi:10.1016/j.mce.2017.08.016

7. Goldman AL, Bhasin S, Wu FCW, Krishna M, Matsumoto AM, Jasuja R. A Reappraisal of Testosterone's Binding in Circulation: Physiological and Clinical Implications. *Endocrine Reviews*. 2017;38(4):302-324. doi:10.1210/er.2017-00025

8. Naamneh Elzenaty R, du Toit T, Flück CE. Basics of androgen synthesis and action. *Best Pract Res Clin Endocrinol Metab*. Jul 2022;36(4):101665. doi:10.1016/j.beem.2022.101665

9. Hunter I, Hay CW, Esswein B, Watt K, McEwan IJ. Tissue control of androgen action: The ups and downs of androgen receptor expression. *Mol Cell Endocrinol*. Apr 15 2018;465:27-35. doi:10.1016/j.mce.2017.08.002

10. Swerdloff RS, Dudley RE, Page ST, Wang C, Salameh WA. Dihydrotestosterone: Biochemistry, Physiology, and Clinical Implications of Elevated Blood Levels. *Endocr Rev*. Jun 01 2017;38(3):220-254. doi:10.1210/er.2016-1067

11. Nelson LR, Bulun SE. Estrogen production and action. *J Am Acad Dermatol*. Sep 2001;45(3 Suppl):S116-24. doi:10.1067/mjd.2001.117432

12. Desai K, McManus JM, Sharifi N. Hormonal Therapy for Prostate Cancer. *Endocr Rev*. May 25 2021;42(3):354-373. doi:10.1210/endrev/bnab002

13. Barret E. The Adrenal Gland. *Medical Physiology*. ClinicalKey; 2016:1018-1034.e1:chap 50.

14. Labrie F. Blockade of testicular and adrenal androgens in prostate cancer treatment. *Nat Rev Urol*. Feb 2011;8(2):73-85. doi:10.1038/nrurol.2010.231

15. Burger HG. Androgen production in women. *Fertil Steril*. Apr 2002;77 Suppl 4:S3-5. doi:10.1016/s0015-0282(02)02985-0

16. Dart DA, Waxman J, Aboagye EO, Bevan CL. Visualising androgen receptor activity in male and female mice. *PLoS One*. 2013;8(8):e71694. doi:10.1371/journal.pone.0071694

17. Snyder PJ, Bhasin S, Cunningham GR, et al. Lessons From the Testosterone Trials. *Endocr Rev*. Jun 1 2018;39(3):369-386. doi:10.1210/er.2017-00234

18. Linhares BL, Miranda EP, Cintra AR, Reges R, Torres LO. Use, Misuse and Abuse of Testosterone and Other Androgens. *Sex Med Rev*. Dec 6 2021;doi:10.1016/j.sxmr.2021.10.002

19. Cappola AR, Auchus RJ, El-Hajj Fuleihan G, et al. Hormones and Aging: An Endocrine Society Scientific Statement. *J Clin Endocrinol Metab*. Jun 16 2023;doi:10.1210/clinem/dgad225

20. Gagliano-Jucá T, Basaria S. Testosterone replacement therapy and cardiovascular risk. *Nat Rev Cardiol*. Sep 2019;16(9):555-574. doi:10.1038/s41569-019-0211-4

21. Lincoff AM, Bhasin S, Flevaris P, et al. Cardiovascular Safety of Testosterone-Replacement Therapy. *N Engl J Med*. Jul 13 2023;389(2):107-117. doi:10.1056/NEJMoa2215025

22. Stárka L, Dušková M. What is a hormone? *Physiol Res*. Sep 30 2020;69(Suppl 2):S183-s185. doi:10.33549/physiolres.934509

23. Labrie F. Intracrinology. *Mol Cell Endocrinol*. Jul 1991;78(3):C113-8. doi:10.1016/0303-7207(91)90116-a

24. Labrie C, Belanger A, Labrie F. Androgenic activity of dehydroepiandrosterone and androstenedione in the rat ventral prostate. *Endocrinology*. Sep 1988;123(3):1412-7. doi:10.1210/endo-123-3-1412

25. Alyamani M, Michael P, Hettel D, et al. Elevated periprostatic venous testosterone correlates with prostate cancer progression after radical prostatectomy. *J Clin Invest*. Sep 1 2023;133(17):e171117. doi:10.1172/JCI171117

26. Olsson M, Ekström L, Guillemette C, Belanger A, Rane A, Gustafsson O. Correlation between circulatory, local prostatic, and intra-prostatic androgen levels. *The Prostate*. 2011;71(9):909-914. doi:10.1002/pros.21307

27. Kim EH, Larson JA, Andriole GL. Management of Benign Prostatic Hyperplasia. *Annu Rev Med*. 2016;67:137-51. doi:10.1146/annurev-med-063014-123902

28. Sood A, Lang DK, Kaur R, Saini B, Arora S. Relevance of Aromatase Inhibitors in Breast Cancer Treatment. *Curr Top Med Chem*. 2021;21(15):1319-1336. doi:10.2174/1568026621666210701143445

29. Waks AG, Winer EP. Breast Cancer Treatment: A Review. *Jama*. Jan 22 2019;321(3):288-300. doi:10.1001/jama.2018.19323

30. Luu-The V. Assessment of steroidogenesis and steroidogenic enzyme functions. *J Steroid Biochem Mol Biol*. Sep 2013;137:176-82. doi:10.1016/j.jsbmb.2013.05.017

31. Miller WL, Auchus RJ. The "backdoor pathway" of androgen synthesis in human male sexual development. *PLoS Biol*. Apr 2019;17(4):e3000198. doi:10.1371/journal.pbio.3000198

32. O'Shaughnessy PJ, Antignac JP, Le Bizec B, et al. Alternative (backdoor) androgen production and masculinization in the human fetus. *PLoS Biol*. Feb 2019;17(2):e3000002. doi:10.1371/journal.pbio.3000002

33. Wu Y, Tang L, Azabdaftari G, Pop E, Smith GJ. Adrenal androgens rescue prostatic dihydrotestosterone production and growth of prostate cancer cells after castration. *Molecular and Cellular Endocrinology*. 2019/04/15/ 2019;486:79-88. doi:10.1016/j.mce.2019.02.018

34. Renfree MB, Shaw G. The alternate pathway of androgen metabolism and window of sensitivity. *J Endocrinol*. Sep 1 2023;258(3):e220296. doi:10.1530/joe-22-0296

35. Bélanger A, Pelletier G, Labrie F, Barbier O, Chouinard S. Inactivation of androgens by UDP-glucuronosyltransferase enzymes in humans. *Trends in Endocrinology & Metabolism*. 2003;14(10):473-479. doi:10.1016/j.tem.2003.10.005

36. Mueller JW, Gilligan LC, Idkowiak J, Arlt W, Foster PA. The Regulation of Steroid Action by Sulfation and Desulfation. *Endocr Rev*. Oct 2015;36(5):526-63. doi:10.1210/er.2015-1036

37. Teo MY, Rathkopf DE, Kantoff P. Treatment of Advanced Prostate Cancer. *Annu Rev Med*. Jan 27 2019;70:479-499. doi:10.1146/annurev-med-051517-011947

38. Huggins C, Hodges CV. Studies on Prostatic Cancer. I. The Effect of Castration, of Estrogen and of Androgen Injection on Serum Phosphatases in Metastatic Carcinoma of the Prostate*. *Cancer Research*. 1941;1(4):293-297. doi:10.3322/canjclin.22.4.232

39. Huggins C, Steven RE, Hodges CV. Studies on prostatic cancer: II. The effects of castration on advanced carcinoma of the prostate gland. *Archives of Surgery*. 1941;43(2):209-223. doi:10.1001/archsurg.1941.01210140043004

40. Michael P, Roversi G, Brown K, Sharifi N. Adrenal Steroids and Resistance to Hormonal Blockade of Prostate and Breast Cancer. *Endocrinology*. Jan 9 2023;164(3):1-8. doi:10.1210/endocr/bqac218

41. Chang K-H, Li R, Kuri B, et al. A gain of function mutation in DHT synthesis in castration-resistant prostate cancer. *Cell*. 2013;154(5):1074-1084. doi:10.1016/j.cell.2013.07.029

42. Hussain M, Fizazi K, Saad F, et al. Enzalutamide in Men with Nonmetastatic, Castration-Resistant Prostate Cancer. *N Engl J Med*. Jun 28 2018;378(26):2465-2474. doi:10.1056/NEJMoa1800536

43. de Bono JS, Logothetis CJ, Molina A, et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med*. May 26 2011;364(21):1995-2005. doi:10.1056/NEJMoa1014618

44. Penning TM, Detlefsen AJ. Intracrinology-revisited and prostate cancer. *The Journal of Steroid Biochemistry and Molecular Biology*. Feb 2020;196:105499. doi:10.1016/j.jsbmb.2019.105499

45. Stanbrough M, Bubley GJ, Ross K, et al. Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. *Cancer Res*. Mar 1 2006;66(5):2815-25. doi:10.1158/0008-5472.Can-05-4000

46. Poutanen M, Hagberg Thulin M, Härkönen P. Targeting sex steroid biosynthesis for breast and prostate cancer therapy. *Nature Reviews Cancer*. 2023/09/08 2023;doi:10.1038/s41568-023-00609-y

47. Klinge CM, Clark BJ, Prough RA. Dehydroepiandrosterone Research: Past, Current, and Future. *Vitam Horm*. 2018;108:1-28. doi:10.1016/bs.vh.2018.02.002

48. Sahu P, Gidwani B, Dhongade HJ. Pharmacological activities of dehydroepiandrosterone: A review. *Steroids*. Jan 2020;153:108507. doi:10.1016/j.steroids.2019.108507

49. Baulieu EE. Dehydroepiandrosterone (DHEA): a fountain of youth? *J Clin Endocrinol Metab*. Sep 1996;81(9):3147-51. doi:10.1210/jcem.81.9.8784058

50. Labrie F. All sex steroids are made intracellularly in peripheral tissues by the mechanisms of intracrinology after menopause. *J Steroid Biochem Mol Biol*. Jan 2015;145:133-8. doi:10.1016/j.jsbmb.2014.06.001

51. Jankowski CM, Gozansky WS, Schwartz RS, et al. Effects of dehydroepiandrosterone replacement therapy on bone mineral density in older adults: a randomized, controlled trial. *J Clin Endocrinol Metab*. Aug 2006;91(8):2986-93. doi:10.1210/jc.2005-2484

52. Labrie F, Luu-The V, Labrie C, et al. Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr Rev*. Apr 2003;24(2):152-82. doi:10.1210/er.2001-0031

53. Kovac JR, Pan M, Arent S, Lipshultz LI. Dietary Adjuncts for Improving Testosterone Levels in Hypogonadal Males. *Am J Mens Health*. Nov 2016;10(6):Np109-np117. doi:10.1177/1557988315598554

54. Nyce J. Alert to US physicians: DHEA, widely used as an OTC androgen supplement, may exacerbate COVID-19. *Endocrine-Related Cancer*. 01 Feb. 2021 2021;28(2):R47-R53. doi:10.1530/erc-20-0439

55. USADA. What Should Athletes know about DHEA? Updated 2021-05-14. Accessed 2023-09-12, https://www.usada.org/spirit-of-sport/athletes-know-about-dhea/

56. WADA. Exogenous DHEA administration and performance: Possible mechanisms of action and metabolic signature. Accessed 2023-09-12, https://www.wada-ama.org/en/resources/exogenous-dhea-administration-and-performance-possible-mechanisms-action-and-metabolic

57. Zhu Y, Qiu L, Jiang F, et al. The effect of dehydroepiandrosterone (DHEA) supplementation on estradiol levels in women: A dose-response and meta-analysis of randomized clinical trials. *Steroids*. Sep 2021;173:108889. doi:10.1016/j.steroids.2021.108889

58. Li Y, Ren J, Li N, et al. A dose-response and meta-analysis of dehydroepiandrosterone (DHEA) supplementation on testosterone levels: perinatal prediction of randomized clinical trials. *Exp Gerontol*. Nov 2020;141:111110. doi:10.1016/j.exger.2020.111110

59. Corona G, Rastrelli G, Giagulli VA, et al. Dehydroepiandrosterone Supplementation in Elderly Men: A Meta-Analysis Study of Placebo-Controlled Trials. *The Journal of Clinical Endocrinology & Metabolism*. 2013;98(9):3615-3626. doi:10.1210/jc.2013-1358

60. Jankowski CM, Wolfe P, Schmiege SJ, et al. Sex-specific effects of dehydroepiandrosterone (DHEA) on bone mineral density and body composition: A pooled analysis of four clinical trials. *Clin Endocrinol (Oxf)*. Feb 2019;90(2):293-300. doi:10.1111/cen.13901

61. Ohlsson C, Langenskiold M, Smidfelt K, et al. Low Progesterone and Low Estradiol Levels Associate With Abdominal Aortic Aneurysms in Men. *J Clin Endocrinol Metab*. Mar 24 2022;107(4):e1413-e1425. doi:10.1210/clinem/dgab867

62. Oettel M, Mukhopadhyay AK. Progesterone: the forgotten hormone in men? *Aging Male*. Sep 2004;7(3):236-57. doi:10.1080/13685530400004199

63. Liu PY. The Hypothalamo-Pituitary Unit, Testis, and Male Accessory Organs. *Yen & Jaffe's Reproductive Endocrinology*. 285-299.e10.

64. Miller MR, Mannowetz N, Iavarone AT, et al. Unconventional endocannabinoid signaling governs sperm activation via the sex hormone progesterone. *Science*. Apr 29 2016;352(6285):555-9. doi:10.1126/science.aad6887

65. Slaunwhite WR, Jr., Samuels LT. Progesterone as a precursor of testicular androgens. *J Biol Chem*. May 1956;220(1):341-52. doi:10.1016/S0021-9258(18)65359-1

66. Matsumoto K, Mahajan DK, Samuels LT. The influence of progesterone on the conversion of 17-hydroxyprogesterone to testosterone in the mouse testis. *Endocrinology*. Mar 1974;94(3):808-14. doi:10.1210/endo-94-3-808

67. Baggett B, Dorfman RI, Engel LL, Savard K. Biosynthesis of androgens from progesterone by human testicular tissue in vitro. *J Clin Endocrinol Metab*. Dec 1956;16(12):1629-31. doi:10.1210/jcem-16-12-1629

68. Hou Z, Huang S, Mei Z, et al. Inhibiting 3βHSD1 to eliminate the oncogenic effects of progesterone in prostate cancer. *Cell Rep Med*. Mar 15 2022;3(3):100561. doi:10.1016/j.xcrm.2022.100561

69. Qiu X, Boufaied N, Hallal T, et al. MYC drives aggressive prostate cancer by disrupting transcriptional pause release at androgen receptor targets. *Nature Communications*. 2022/05/13 2022;13(1):2559. doi:10.1038/s41467-022-30257-z

70. Grindstad T, Richardsen E, Andersen S, et al. Progesterone Receptors in Prostate Cancer: Progesterone receptor B is the isoform associated with disease progression. *Sci Rep*. Jul 27 2018;8(1):11358. doi:10.1038/s41598-018-29520-5

71. Grindstad T, Andersen S, Al-Saad S, et al. High progesterone receptor expression in prostate cancer is associated with clinical failure. *PLoS One*. 2015;10(2):e0116691. doi:10.1371/journal.pone.0116691

72. Mumoli N, Permunian ET, Vitale J, et al. Progesterone Levels in Men with Unprovoked Deep Vein Thrombosis. *J Am Geriatr Soc*. Jan 2016;64(1):213-5. doi:10.1111/jgs.13917

73. Theis V, Theiss C. Progesterone Effects in the Nervous System. *Anat Rec (Hoboken)*. Aug 2019;302(8):1276-1286. doi:10.1002/ar.24121

74. Gaignard P, Savouroux S, Liere P, et al. Effect of Sex Differences on Brain Mitochondrial Function and Its Suppression by Ovariectomy and in Aged Mice. *Endocrinology*. Aug 2015;156(8):2893-904. doi:10.1210/en.2014-1913

75. Hoeger KM, Dokras A, Piltonen T. Update on PCOS: Consequences, Challenges, and Guiding Treatment. *J Clin Endocrinol Metab*. Mar 8 2021;106(3):e1071-e1083. doi:10.1210/clinem/dgaa839

76. Risal S, Pei Y, Lu H, et al. Prenatal androgen exposure and transgenerational susceptibility to polycystic ovary syndrome. *Nat Med*. Dec 2019;25(12):1894-1904. doi:10.1038/s41591-019-0666-1

77. Joham AE, Norman RJ, Stener-Victorin E, et al. Polycystic ovary syndrome. *Lancet Diabetes Endocrinol*. Sep 2022;10(9):668-680. doi:10.1016/s2213-8587(22)00163-2

78. Bouguen G, Dubuquoy L, Desreumaux P, Brunner T, Bertin B. Intestinal steroidogenesis. *Steroids*. Nov 2015;103:64-71. doi:10.1016/j.steroids.2014.12.022

79. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. *Nature*. Sep 13 2012;489(7415):242-9. doi:10.1038/nature11552

80. Schoeler M, Caesar R. Dietary lipids, gut microbiota and lipid metabolism. *Rev Endocr Metab Disord*. Dec 2019;20(4):461-472. doi:10.1007/s11154-019-09512-0

81. Clarke G, Stilling RM, Kennedy PJ, Stanton C, Cryan JF, Dinan TG. Minireview: Gut Microbiota: The Neglected Endocrine Organ. *Molecular Endocrinology*. 2014;28(8):1221-1238. doi:10.1210/me.2014-1108

82. Arpaia N, Campbell C, Fan X, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*. Dec 19 2013;504(7480):451-5. doi:10.1038/nature12726

83. Martin-Gallausiaux C, Marinelli L, Blottière HM, Larraufie P, Lapaque N. SCFA: mechanisms and functional importance in the gut. *Proc Nutr Soc*. Feb 2021;80(1):37-49. doi:10.1017/s0029665120006916

84. Markle JG, Frank DN, Mortin-Toth S, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science*. Mar 1 2013;339(6123):1084-8. doi:10.1126/science.1233521

85. Dalla Valle L, Couët J, Labrie Y, et al. Occurrence of cytochrome P450c17 mRNA and dehydroepiandrosterone biosynthesis in the rat gastrointestinal tract. *Mol Cell Endocrinol*. Apr 28 1995;111(1):83-92. doi:10.1016/0303-7207(95)03553-j

86. Soory M. Bacterial steroidogenesis by periodontal pathogens and the effect of bacterial enzymes on steroid conversions by human gingival fibroblasts in culture. *Journal of periodontal research*. Mar 1995;30(2):124-31. doi:10.1111/j.1600-0765.1995.tb01261.x

87. Hsiao TH, Chou CH, Chen YL, et al. Circulating androgen regulation by androgen-catabolizing gut bacteria in male mouse gut. *Gut Microbes*. Jan-Dec 2023;15(1):2183685. doi:10.1080/19490976.2023.2183685

88. Kim YS, Unno T, Kim B-Y, Park M-S. Sex Differences in Gut Microbiota. *World J Mens Health*. 1/ 2020;38(1):48-60. doi:10.5534/wjmh.190009

89. Mayneris-Perxachs J, Arnoriaga-Rodríguez M, Luque-Córdoba D, et al. Gut microbiota steroid sexual dimorphism and its impact on gonadal steroids: influences of obesity and menopausal status. *Microbiome*. Sep 20 2020;8(1):136. doi:10.1186/s40168-020-00913-x

90. Yoon K, Kim N. Roles of Sex Hormones and Gender in the Gut Microbiota. *J Neurogastroenterol Motil*. Jul 30 2021;27(3):314-325. doi:10.5056/jnm20208

91. Sisk-Hackworth L, Kelley ST, Thackray VG. Sex, puberty, and the gut microbiome. *Reproduction*. Feb 1 2023;165(2):R61-r74. doi:10.1530/rep-22-0303

92. Insenser M, Murri M, Del Campo R, Martinez-Garcia MA, Fernandez-Duran E, Escobar-Morreale HF. Gut Microbiota and the Polycystic Ovary Syndrome: Influence of Sex, Sex Hormones, and Obesity. *J Clin Endocrinol Metab*. Jul 1 2018;103(7):2552-2562. doi:10.1210/jc.2017-02799

93. Liu R, Zhang C, Shi Y, et al. Dysbiosis of Gut Microbiota Associated with Clinical Parameters in Polycystic Ovary Syndrome. Original Research. *Front Microbiol*. 2017-February-28 2017;8(324):324. doi:10.3389/fmicb.2017.00324

94. Torres PJ, Siakowska M, Banaszewska B, et al. Gut Microbial Diversity in Women With Polycystic Ovary Syndrome Correlates With Hyperandrogenism. *J Clin Endocrinol Metab*. Apr 1 2018;103(4):1502-1511. doi:10.1210/jc.2017-02153

95. Giampaolino P, Foreste V, Di Filippo C, et al. Microbiome and PCOS: State-of-Art and Future Aspects. *Int J Mol Sci*. Feb 19 2021;22(4):2048. doi:10.3390/ijms22042048

96. Couse JF, Korach KS. Exploring the role of sex steroids through studies of receptor deficient mice. *Journal of Molecular Medicine*. 1998/05/01 1998;76(7):497-511. doi:10.1007/s001090050244

97. van Weerden WM, Bierings HG, van Steenbrugge GJ, de Jong FH, Schröder FH. Adrenal glands of mouse and rat do not synthesize androgens. *Life Sci*. 1992;50(12):857-61. doi:10.1016/0024-3205(92)90204-3

98. Huhtaniemi R, Oksala R, Knuuttila M, et al. Adrenals Contribute to Growth of Castration-Resistant VCaP Prostate Cancer Xenografts. *Am J Pathol*. Dec 2018;188(12):2890-2901. doi:10.1016/j.ajpath.2018.07.029

99. Mostaghel EA, Zhang A, Hernandez S, et al. Contribution of Adrenal Glands to Intratumor Androgens and Growth of Castration-Resistant Prostate Cancer. *Clin Cancer Res*. Jan 1 2019;25(1):426-439. doi:10.1158/1078-0432.CCR-18-1431

100. Laurent MR, Hammond GL, Blokland M, et al. Sex hormone-binding globulin regulation of androgen bioactivity in vivo: validation of the free hormone hypothesis. *Sci Rep*. Oct 17 2016;6:35539. doi:10.1038/srep35539

101. Jänne M, Deol HK, Power SG, Yee SP, Hammond GL. Human sex hormone-binding globulin gene expression in transgenic mice. *Mol Endocrinol*. Jan 1998;12(1):123-36. doi:10.1210/mend.12.1.0050

102. Ohlsson C, Nilsson ME, Tivesten A, et al. Comparisons of immunoassay and mass spectrometry measurements of serum estradiol levels and their influence on clinical association studies in men. *J Clin Endocrinol Metab*. Jun 2013;98(6):E1097-102. doi:10.1210/jc.2012-3861

103. Handelsman DJ, Jimenez M, Singh GK, Spaliviero J, Desai R, Walters KA. Measurement of testosterone by immunoassays and mass spectrometry in mouse serum, testicular, and ovarian extracts. *Endocrinology*. Jan 2015;156(1):400-5. doi:10.1210/en.2014-1664

104. Handelsman DJ, Wartofsky L. Requirement for Mass Spectrometry Sex Steroid Assays in theJournal of Clinical Endocrinology and Metabolism. *The Journal of Clinical Endocrinology & Metabolism*. 2013;98(10):3971-3973. doi:10.1210/jc.2013-3375

105. Wierman ME, Auchus RJ, Haisenleder DJ, et al. Editorial: the new instructions to authors for the reporting of steroid hormone measurements. *Endocrinology*. Dec 2014;155(12):4603. doi:10.1210/en.2014-1735

106. Knuuttila M, Hämäläinen E, Poutanen M. Applying mass spectrometric methods to study androgen biosynthesis and metabolism in prostate cancer. *Journal of molecular endocrinology*. May 1 2019;62(4):R255-r267. doi:10.1530/jme-18-0150

107. Nilsson ME, Vandenput L, Tivesten A, et al. Measurement of a Comprehensive Sex Steroid Profile in Rodent Serum by High-Sensitive Gas Chromatography-Tandem Mass Spectrometry. *Endocrinology*. Jul 2015;156(7):2492-502. doi:10.1210/en.2014-1890

108. Higashi T, Shimada K. Derivatization of neutral steroids to enhance their detection characteristics in liquid chromatography-mass spectrometry. *Anal Bioanal Chem*. Feb 2004;378(4):875-82. doi:10.1007/s00216-003-2252-z

109. Hauser B, Schulz D, Boesch C, Deschner T. Measuring urinary testosterone levels of the great apes--problems with enzymatic hydrolysis using Helix pomatia juice. *Gen Comp Endocrinol*. Aug 2008;158(1):77-86. doi:10.1016/j.ygcen.2008.05.006

110. Evangelista S, Vazakidou P, Koekkoek J, et al. High throughput LC-MS/MS method for steroid hormone analysis in rat liver and plasma - unraveling methodological challenges. *Talanta*. Jul 24 2023;266(1):124981. doi:10.1016/j.talanta.2023.124981

111. The Jackson Laboratory. C57BL/6J. Accessed 2023-05-02, https://www.jax.org/strain/000664

112. Fontaine DA, Davis DB. Attention to Background Strain Is Essential for Metabolic Research: C57BL/6 and the International Knockout Mouse Consortium. *Diabetes*. Jan 2016;65(1):25-33. doi:10.2337/db15-0982

113. Mekada K, Yoshiki A. Substrains matter in phenotyping of C57BL/6 mice. *Experimental animals*. May 13 2021;70(2):145-160. doi:10.1538/expanim.20-0158

114. Bhattarai Y, Kashyap PC. Germ-Free Mice Model for Studying Host-Microbial Interactions. *Methods Mol Biol*. 2016;1438:123-35. doi:10.1007/978-1-4939-3661-8_8

115. Luczynski P, McVey Neufeld KA, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. *Int J Neuropsychopharmacol*. Aug 2016;19(8):pyw020. doi:10.1093/ijnp/pyw020

116. Sjogren K, Engdahl C, Henning P, et al. The gut microbiota regulates bone mass in mice. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. Jun 2012;27(6):1357-67. doi:10.1002/jbmr.1588

117. Fox JG, Barthold S, Davisson M, Newcomer CE, Quimby FW, Smith A. *The Mouse in Biomedical Research: Normative Biology, Husbandry, and Models*. 2 ed. Academic Press; 2006.

118. Tung YT, Chen YJ, Chuang HL, et al. Characterization of the serum and liver proteomes in gut-microbiota-lacking mice. *Int J Med Sci*. 2017;14(3):257-267. doi:10.7150/ijms.17792

119. Luczynski P, Whelan SO, O'Sullivan C, et al. Adult microbiota-deficient mice have distinct dendritic morphological changes: differential effects in the amygdala and hippocampus. *Eur J Neurosci*. Nov 2016;44(9):2654-2666. doi:10.1111/ejn.13291

120. Shimizu K, Muranaka Y, Fujimura R, Ishida H, Tazume S, Shimamura T. Normalization of reproductive function in germfree mice following bacterial contamination. *Experimental animals*. Jul 1998;47(3):151-8. doi:10.1538/expanim.47.151

121. Wallenius V, Elebring E, Casselbrant A, et al. Glycemic Control and Metabolic Adaptation in Response to High-Fat versus High-Carbohydrate Diets-Data from a Randomized Cross-Over Study in Healthy Subjects. *Nutrients*. Sep 23 2021;13(10):3322. doi:10.3390/nu13103322

122. Boissonneault G. Evidence of apoptosis in the castration-induced atrophy of the rat levator ani muscle. *Endocr Res*. Aug 2001;27(3):317-28. doi:10.1081/erc-100106009

123. Tanji N, Satoh H, Takagi-Morishita Y, et al. Induction of apoptosis by castration in epithelium of the mouse seminal vesicles. *Arch Androl*. Nov-Dec 2003;49(6):409-15. doi:10.1080/01485010390236369

124. Furuya R, Hisasue S, Furuya S, et al. Fate of seminal vesicles and prostate after medical castration: how long is the optimal duration of neoadjuvant treatment for prostate cancer before radiation? *Urology*. Aug 2008;72(2):417-21. doi:10.1016/j.urology.2007.11.025

125. Oner H, Ozan E. Effects of gonadal hormones on thymus gland after bilateral ovariectomy and orchidectomy in rats. *Arch Androl*. Mar-Apr 2002;48(2):115-26. doi:10.1080/014850102317267427

126. David CD, Wyrosdic BN, Park JH. Strain differences in post-castration sexual and aggressive behavior in male mice. *Behav Brain Res*. Mar 26 2022;422:113747. doi:10.1016/j.bbr.2022.113747

127. Engdahl C, Lagerquist MK, Stubelius A, et al. Role of androgen and estrogen receptors for the action of dehydroepiandrosterone (DHEA). *Endocrinology*. Mar 2014;155(3):889-96. doi:10.1210/en.2013-1561

128. Turner PV, Pekow C, Vasbinder MA, Brabb T. Administration of substances to laboratory animals: equipment considerations, vehicle

selection, and solute preparation. *J Am Assoc Lab Anim Sci*. Sep 2011;50(5):614-27.

129. Ingberg E, Theodorsson A, Theodorsson E, Strom JO. Methods for long-term 17beta-estradiol administration to mice. *Gen Comp Endocrinol*. Jan 1 2012;175(1):188-93. doi:10.1016/j.ygcen.2011.11.014

130. Haavisto AM, Pettersson K, Bergendahl M, Perheentupa A, Roser JF, Huhtaniemi I. A supersensitive immunofluorometric assay for rat luteinizing hormone. *Endocrinology*. Apr 1993;132(4):1687-91. doi:10.1210/endo.132.4.8462469

131. van Casteren JI, Schoonen WG, Kloosterboer HJ. Development of time-resolved immunofluorometric assays for rat follicle-stimulating hormone and luteinizing hormone and application on sera of cycling rats. *Biology of reproduction*. Apr 2000;62(4):886-94. doi:10.1095/biolreprod62.4.886

132. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. Dec 2001;25(4):402-8. doi:10.1006/meth.2001.1262

133. Handelsman DJ, Ly LP. An Accurate Substitution Method To Minimize Left Censoring Bias in Serum Steroid Measurements. *Endocrinology*. 2019;160(10):2395-2400. doi:10.1210/en.2019-00340

134. Michiel Sedelaar JP, Dalrymple SS, Isaacs JT. Of mice and men - warning: intact versus castrated adult male mice as xenograft hosts are equivalent to hypogonadal versus abiraterone treated aging human males, respectively. *The Prostate*. Sep 2013;73(12):1316-25. doi:10.1002/pros.22677

135. Varholick JA, Pontiggia A, Murphy E, et al. Social dominance hierarchy type and rank contribute to phenotypic variation within cages of laboratory mice. *Scientific Reports*. 2019/09/20 2019;9(1):13650. doi:10.1038/s41598-019-49612-0

136. Conklin SE, Knezevic CE. Advancements in the gold standard: Measuring steroid sex hormones by mass spectrometry. *Clinical Biochemistry*. 2020/08/01/ 2020;82:21-32. doi:10.1016/j.clinbiochem.2020.03.008

137. McNamara KM, Harwood DT, Simanainen U, Walters KA, Jimenez M, Handelsman DJ. Measurement of sex steroids in murine blood and reproductive tissues by liquid chromatography-tandem mass spectrometry. *J Steroid Biochem Mol Biol*. Aug 2010;121(3-5):611-8. doi:10.1016/j.jsbmb.2010.02.001

138. Weng Y, Xie F, Xu L, Zagorevski D, Spink DC, Ding X. Analysis of testosterone and dihydrotestosterone in mouse tissues by liquid

chromatography–electrospray ionization–tandem mass spectrometry. *Analytical Biochemistry*. 2010/07/15/ 2010;402(2):121-128. doi:10.1016/j.ab.2010.03.034

139. OECD. *Test No. 441: Hershberger Bioassay in Rats*. 2009.

140. Ma ZS, Li W. How and Why Men and Women Differ in Their Microbiomes: Medical Ecology and Network Analyses of the Microgenderome. *Adv Sci (Weinh)*. Dec 2019;6(23):1902054. doi:10.1002/advs.201902054

141. Kustrimovic N, Bombelli R, Baci D, Mortara L. Microbiome and Prostate Cancer: A Novel Target for Prevention and Treatment. *Int J Mol Sci*. Jan 12 2023;24(2):1511. doi:10.3390/ijms24021511

142. Fujita K, Matsushita M, De Velasco MA, et al. The Gut-Prostate Axis: A New Perspective of Prostate Cancer Biology through the Gut Microbiome. *Cancers (Basel)*. Feb 21 2023;15(5):1375. doi:10.3390/cancers15051375

143. Matsushita M, Fujita K, Hatano K, et al. Emerging Relationship between the Gut Microbiome and Prostate Cancer. *World J Mens Health*. Feb 22 2023;doi:10.5534/wjmh.220202

144. Porter CM, Shrestha E, Peiffer LB, Sfanos KS. The microbiome in prostate inflammation and prostate cancer. *Prostate Cancer Prostatic Dis*. Sep 2018;21(3):345-354. doi:10.1038/s41391-018-0041-1

145. Amirian ES, Petrosino JF, Ajami NJ, Liu Y, Mims MP, Scheurer ME. Potential role of gastrointestinal microbiota composition in prostate cancer risk. *Infectious agents and cancer*. Nov 4 2013;8(1):42. doi:10.1186/1750-9378-8-42

146. Sun Y, Gao S, Ye C, Zhao W. Gut microbiota dysbiosis in polycystic ovary syndrome: Mechanisms of progression and clinical applications. *Front Cell Infect Microbiol*. 2023;13:1142041. doi:10.3389/fcimb.2023.1142041

147. Singh S, Pal N, Shubham S, et al. Polycystic Ovary Syndrome: Etiology, Current Management, and Future Therapeutics. *J Clin Med*. Feb 11 2023;12(4):1454. doi:10.3390/jcm12041454

148. Liu J, Liu Y, Li X. Effects of intestinal flora on polycystic ovary syndrome. *Front Endocrinol (Lausanne)*. 2023;14:1151723. doi:10.3389/fendo.2023.1151723

149. Yurtdaş G, Akdevelioğlu Y. A New Approach to Polycystic Ovary Syndrome: The Gut Microbiota. *J Am Coll Nutr*. May-Jun 2020;39(4):371-382. doi:10.1080/07315724.2019.1657515

150. Guo YJ, Qi Y, Yang XF, et al. Association between Polycystic Ovary Syndrome and Gut Microbiota. *Plos One*. Apr 2016;11(4)e0153196. doi:10.1371/journal.pone.0153196

151. Yan X, Feng Y, Hao Y, et al. Gut-Testis Axis: Microbiota Prime Metabolome To Increase Sperm Quality in Young Type 2 Diabetes. *Microbiol Spectr*. Oct 26 2022;10(5):e0142322. doi:10.1128/spectrum.01423-22

152. Cotton S, Clayton CA, Tropini C. Microbial endocrinology: the mechanisms by which the microbiota influences host sex steroids. *Trends Microbiol*. Apr 24 2023:online ahead of print. doi:10.1016/j.tim.2023.03.010

153. Gonzalez-Montelongo MC, Marin R, Gomez T, Diaz M. Androgens are powerful non-genomic inducers of calcium sensitization in visceral smooth muscle. *Steroids*. Aug-Sep 2010;75(8-9):533-8. doi:10.1016/j.steroids.2009.09.012

154. Ba ZF, Yokoyama Y, Toth B, Rue LW, 3rd, Bland KI, Chaudry IH. Gender differences in small intestinal endothelial function: inhibitory role of androgens. *American journal of physiology Gastrointestinal and liver physiology*. Mar 2004;286(3):G452-7. doi:10.1152/ajpgi.00357.2003

155. Roshan MH, Tambo A, Pace NP. The role of testosterone in colorectal carcinoma: pathomechanisms and open questions. *Epma j*. 2016;7(1):22. doi:10.1186/s13167-016-0071-5

156. Shinohara Y, Baba S, Kasuya Y. Absorption, metabolism, and excretion of oral testosterone in humans by mass fragmentography. *J Clin Endocrinol Metab*. Dec 1980;51(6):1459-62. doi:10.1210/jcem-51-6-1459

157. Newell-Price J, Huatan H, Quirke J, et al. An oral lipidic native testosterone formulation that is absorbed independent of food. *Eur J Endocrinol*. Oct 5 2021;185(5):607-615. doi:10.1530/eje-21-0606

158. Sui Y, Wu J, Chen J. The Role of Gut Microbial beta-Glucuronidase in Estrogen Reactivation and Breast Cancer. *Front Cell Dev Biol*. 2021;9:631552. doi:10.3389/fcell.2021.631552

159. Ervin SM, Li H, Lim L, et al. Gut microbial beta-glucuronidases reactivate estrogens as components of the estrobolome that reactivate estrogens. *J Biol Chem*. Dec 6 2019;294(49):18586-18599. doi:10.1074/jbc.RA119.010950

160. Baker JM, Al-Nakkash L, Herbst-Kralovetz MM. Estrogen-gut microbiome axis: Physiological and clinical implications. *Maturitas*. Sep 2017;103:45-53. doi:10.1016/j.maturitas.2017.06.025

161. Alvarez-Mercado AI, Del Valle Cano A, Fernandez MF, Fontana L. Gut Microbiota and Breast Cancer: The Dual Role of Microbes. *Cancers (Basel)*. Jan 10 2023;15(2):443. doi:10.3390/cancers15020443

162. Pernigoni N, Zagato E, Calcinotto A, et al. Commensal bacteria promote endocrine resistance in prostate cancer through androgen biosynthesis. *Science*. 2021;374(6564):216-224. doi:10.1126/science.abf8403

163. Ridlon JM, Ikegawa S, Alves JMP, et al. Clostridium scindens: a human gut microbe with a high potential to convert glucocorticoids into androgens. *The Journal of Lipid Research*. 2013;54(9):2437-2449. doi:10.1194/jlr.M038869

164. Harada N, Hanada K, Minami Y, et al. Role of gut microbiota in sex- and diet-dependent metabolic disorders that lead to early mortality of androgen receptor-deficient male mice. *Am J Physiol Endocrinol Metab*. Apr 1 2020;318(4):E525-E537. doi:10.1152/ajpendo.00461.2019

165. Zhang P, Feng Y, Li L, et al. Improvement in sperm quality and spermatogenesis following faecal microbiota transplantation from alginate oligosaccharide dosed mice. *Gut*. Jan 2021;70(1):222-225. doi:10.1136/gutjnl-2020-320992

166. Hao Y, Feng Y, Yan X, et al. Gut Microbiota-Testis Axis: FMT Mitigates High-Fat Diet-Diminished Male Fertility via Improving Systemic and Testicular Metabolome. *Microbiol Spectr*. Jun 29 2022;10(3):e0002822. doi:10.1128/spectrum.00028-22

167. Sherman SB, Sarsour N, Salehi M, et al. Prenatal androgen exposure causes hypertension and gut microbiota dysbiosis. *Gut Microbes*. 2018;9(5):400-421. doi:10.1080/19490976.2018.1441664

168. Torres PJ, Ho BS, Arroyo P, et al. Exposure to a Healthy Gut Microbiome Protects Against Reproductive and Metabolic Dysregulation in a PCOS Mouse Model. *Endocrinology*. May 1 2019;160(5):1193-1204. doi:10.1210/en.2019-00050

169. Poutahidis T, Springer A, Levkovich T, et al. Probiotic microbes sustain youthful serum testosterone levels and testicular size in aging mice. *PLoS One*. 2014;9(1):e84877. doi:10.1371/journal.pone.0084877

170. Lee J, Yang W, Hostetler A, et al. Characterization of the anti-inflammatory Lactobacillus reuteri BM36301 and its probiotic benefits on aged mice. *BMC microbiology*. Apr 19 2016;16:69. doi:10.1186/s12866-016-0686-7

171. Lawenius L, Colldén H, Horkeby K, et al. A probiotic mix partially protects against castration-induced bone loss in male mice. *J Endocrinol*. Aug 1 2022;254(2):91-101. doi:10.1530/joe-21-0408

172. Auchus RJ, Sharifi N. Sex Hormones and Prostate Cancer. *Annu Rev Med*. Jan 27 2020;71:33-45. doi:10.1146/annurev-med-051418-060357

173. Cook MB, Stanczyk FZ, Wood SN, et al. Relationships between Circulating and Intraprostatic Sex Steroid Hormone Concentrations. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 2017;26(11):1660-1666. doi:10.1158/1055-9965.EPI-17-0215

174. Yassin A, AlRumaihi K, Alzubaidi R, Alkadhi S, Al Ansari A. Testosterone, testosterone therapy and prostate cancer. *Aging Male*. Dec 2019;22(4):219-227. doi:10.1080/13685538.2018.1524456

175. Ruth KS, Day FR, Tyrrell J, et al. Using human genetics to understand the disease impacts of testosterone in men and women. *Nature Medicine*. 2020/02/01 2020;26(2):252-258. doi:10.1038/s41591-020-0751-5

176. Mahendroo MS, Russell DW. Male and female isoenzymes of steroid 5alpha-reductase. *Rev Reprod*. Sep 1999;4(3):179-83. doi:10.1530/ror.0.0040179

177. Wu Z, Petrick JL, Florio AA, et al. Endogenous sex steroid hormones and risk of liver cancer among US men: Results from the Liver Cancer Pooling Project. *JHEP Rep*. Jul 2023;5(7):100742. doi:10.1016/j.jhepr.2023.100742

178. Moll JM, Kumagai J, van Royen ME, et al. A bypass mechanism of abiraterone-resistant prostate cancer: Accumulating CYP17A1 substrates activate androgen receptor signaling. *The Prostate*. Jun 2019;79(9):937-948. doi:10.1002/pros.23799

179. Okamoto Y, Nunome M, Kondo M, Kitayama I, Suzuki Y, Akiyama H. Quantification of progesterone in beef with marbling using liquid chromatography-tandem mass spectrometry with stable isotope-labelled standards. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. Jan 16 2021:1-9. doi:10.1080/19440049.2020.1869326

180. Goyon A, Cai JZ, Kraehenbuehl K, Hartmann C, Shao B, Mottier P. Determination of steroid hormones in bovine milk by LC-MS/MS and their levels in Swiss Holstein cow milk. *Food Additives & Contaminants: Part A*. 2016/05/03 2016;33(5):804-816. doi:10.1080/19440049.2016.1175186

181. Regal P, Cepeda A, Fente C. Development of an LC-MS/MS method to quantify sex hormones in bovine milk and influence of pregnancy in their levels. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2012;29(5):770-9. doi:10.1080/19440049.2011.653989

182. Tarkowska D. Plants are Capable of Synthesizing Animal Steroid Hormones. *Molecules*. Jul 16 2019;24(14):2585. doi:10.3390/molecules24142585

183. Michels KB, Binder N, Courant F, Franke AA, Osterhues A. Urinary excretion of sex steroid hormone metabolites after consumption of cow milk: a randomized crossover intervention trial. *Am J Clin Nutr*. Feb 1 2019;109(2):402-410. doi:10.1093/ajcn/nqy279

184. Wu J, Shi X, Zhang M, et al. Short-term serum and urinary changes in sex hormones of healthy pre-pubertal children after the consumption of commercially available whole milk powder: a randomized, two-level, controlled-intervention trial in China. *Food Funct*. Oct 17 2022;13(20):10823-10833. doi:10.1039/d2fo02321k

185. Bhargava A, Arnold AP, Bangasser DA, et al. Considering Sex as a Biological Variable in Basic and Clinical Studies: An Endocrine Society Scientific Statement. *Endocrine Reviews*. 2021;42(3):219-258. doi:10.1210/endrev/bnaa034

186. Zucker I. The mixed legacy of the rat estrous cycle. *Biol Sex Differ*. Sep 4 2023;14(1):55. doi:10.1186/s13293-023-00542-7

187. Smarr B, Kriegsfeld LJ. Female mice exhibit less overall variance, with a higher proportion of structured variance, than males at multiple timescales of continuous body temperature and locomotive activity records. *Biology of Sex Differences*. 2022/07/23 2022;13(1):41. doi:10.1186/s13293-022-00451-1

188. Wall EG, Desai R, Khant Aung Z, et al. Unexpected Plasma Gonadal Steroid and Prolactin Levels Across the Mouse Estrous Cycle. *Endocrinology*. Apr 17 2023;164(6):bqad070. doi:10.1210/endocr/bqad070

189. Heinosalo T, Saarinen N, Poutanen M. Role of hydroxysteroid (17beta) dehydrogenase type 1 in reproductive tissues and hormone-dependent diseases. *Molecular and Cellular Endocrinology*. 2019/06/01/ 2019;489:9-31. doi:10.1016/j.mce.2018.08.004

190. Moon JY, McNamara KM, Lee JJ, Chung BC, Sasano H, Choi MH. Improved detectability of sex steroids from frozen sections of breast cancer tissue using GC-triple quadrupole-MS. *J Steroid Biochem Mol Biol*. Apr 2018;178:185-192. doi:10.1016/j.jsbmb.2017.12.012

APPENDIX

I. Colldén H*, Nilsson ME*, Norlén AK, Landin A, Windahl SH, Wu J, Gustafsson KL, Poutanen M, Ryberg H, Vandenput L*, Ohlsson C*.
Comprehensive sex steroid profiling in multiple tissues reveals novel insights in sex steroid distribution in male mice.
Endocrinology 2022; 163(3):bqac001;
doi: 10.1210/endocr/bqac001

II. Colldén H, Nilsson ME, Norlén AK, Landin A, Windahl SH, Wu J, Horkeby K, Lagerquist MK, Ryberg H, Poutanen M, Vandenput L*, Ohlsson C*.
Dehydroepiandrosterone supplementation results in varying tissue-specific levels of dihydrotestosterone in male mice.
Endocrinology 2022; 163(12):bqac163;
doi: 10.1210/endocr/bqac163

III. Colldén H, Landin A, Wallenius V, Elebring E, Fändriks L, Nilsson ME, Ryberg H, Poutanen M, Sjögren K, Vandenput L, Ohlsson C.
The gut microbiota is a major regulator of androgen metabolism in intestinal contents.
Am J Physiol Endocrinol Metab. 2019; 317(6):E1182-E1192; doi: 10.1152/ajpendo.00338.2019.

IV. Colldén H, Hagberg Thulin M, Landin A, Horkeby K, Lagerquist MK, Wu J, Nilsson KH, Grahnemo L, Poutanen M, Ryberg H, Vandenput L*, Ohlsson C*.
Dietary progesterone contributes to intratissue levels of progesterone in male mice.
Endocrinology 2023; 164(8):bqad103;
doi: 10.1210/endocr/bqad103

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