New sensitive and specific methods for sex steroid measurements in rodent serum and tissues

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ABSTRACT

Accurate measurement of sex steroids (androgens, estrogens, and progesterone) is essential in research and clinical settings for common diseases including osteoporosis, prostate cancer, breast cancer, and cardiovascular diseases. Sex steroids are not only produced in the gonads and adrenals but may also be locally synthesized in peripheral target tissues. As many conventional immunoassay-based methods proved to be inaccurate, especially in the lower concentration range, the first aim of this thesis was to develop and validate a highly sensitive and specific gas chromatography-tandem mass spectrometry technique for sex steroid profiling in rodent serum and tissues. In addition, to increase the understanding of the local sex steroid metabolism, we aimed to use this assay to determine the sex steroid levels in multiple rodent tissues and assess the impact of castration and obesity on these levels. We first successfully developed and validated a highly sensitive method for the analysis of a broad panel of sex steroids (testosterone (T), dihydrotestosterone (DHT), estradiol, estrone, dehydroepiandrosterone, androstenedione, and progesterone) in both serum and tissues of rodents. Using this assay, we accurately measured a comprehensive sex steroid profile in serum of female rats and mice according to estrous cycle phase. Next, we developed the first detailed atlas of the levels of a broad panel of sex steroids in multiple tissues in both gonadal intact and castrated mice. This comprehensive atlas can be used by the research community as a reference database to compare sex steroid levels in different tissues. The majority of sex steroids in male mice was found within white adipose tissue, which could possibly act as a buffer for circulating sex steroids. Also, progesterone was the most abundant sex steroid found in castrated male mice. We observed that high-fat diet-induced obesity reduced the DHT/T ratio, reflecting 5α-reductase activity, in muscle and seminal vesicles of male mice but increased DHT levels in the liver. Finally, obesity reduced progesterone levels in adipose tissue. In conclusion, our highly sensitive assay allows mapping of sex steroid levels in serum and peripheral target tissues and can as such contribute to the understanding and potential development of new treatments involved in the regulation of sex steroid action in sex steroid-dependent diseases.

Keywords: sex steroids, mass spectrometry, rodents

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SAMMANFATTNING PÅ SVENSKA

Korrekta mätningar av könshormoner (androgener, östrogener och progesteron) är nödvändiga både för forskning och i klinisk verksamhet när det gäller vanliga sjukdomar så som benskörhet, prostatacancer och bröstcancer. Könshormoner produceras i könskörtlarna (testiklar och äggstockar) och binjurarna men kan även produceras lokalt i perifera vävnader. Många konventionella immunkemiskt baserade metoder har visat sig ge felaktiga resultat, särskilt i de lägre koncentrationsområdena. Därför var vårt första mål med denna avhandling att utveckla och validera en ytterst känslig och specifik gaskromatografi-masspektrometriteknik för analys av könshomonprofiler i serum och vävnader från gnagare. Dessutom var vårt mål att använda analysmetoden för att bestämma könshormonnivåer i olika vävnader och därmed kunna utvärdera förändringar av dessa nivåer efter kastrering och vid fetma hos gnagare. Först utvecklade och validerade vi en mycket känslig och specifik metod för att kunna analysera en bred panel av könshormoner (testosteron (T), dihydrotestosteron (DHT), östradiol, östron, dehydroepiandrosteron, androstenedion och progesteron) i både serum och vävnader från gnagare. Med hjälp av denna analysmetod kunde vi noggrant mäta en omfattande könshormonprofil i serum från honråttor och honmöss enligt deras brunstcykel. Därefter analyserade vi en omfattande könshormonopanel i flera olika vävnader från både gonadintakta och kastrerade möss, vilket resulterade i en detaljerad atlas över könshormonfördelningen hos dessa möss. Denna omfattande atlas kan användas av forskarsamhället som en referensdatabas för relativa nivåer av könshormonnivåer i olika musvävnader. De högsta nivåerna av könshormoner i hanmöss uppmättes i vit fettväv, som möjligtvis kan fungera som en buffert för cirkulerande könshormoner. Dessutom var progesteron det vanligast förekommande könshormonet hos kastrerade hanmöss. Det visade sig att fetma orsakad av högfettdiet reducerade DHT/T kvoten, vilket återspeglar 5α-reduktasaktiviteten, i muskel och sädesblåsor hos hanmöss medan DHT-nivåerna ökade i levern. Slutligen orsakade fetma minskade progesteronnivåer i fettväv. Sammanfattningsvis kan vår mycket känsliga analysmetod kartlägga könshormonnivåer i både serum och vävnader, vilket kan bidra till förståelsen av könshormonaktivitet lokalt i olika vävnader och därmed möjliggöra utveckling av nya behandlingar vid könshormonberoende sjukdomar.

LIST OF PAPERS

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

I. **Nilsson ME***, Vandenput L*, Tivesten Å, Norlén A-K, Lagerquist MK, Windahl SH, Börjesson AE, Farman HH, Poutanen M, Benrick A, Maliqueo M, Stener-Victorin E, Ryberg H*, Ohlsson C*. Measurement of a comprehensive sex steroid profile in rodent serum by high-sensitive gas chromatography-tandem mass spectrometry. Endocrinology, 2015; 156(7):2492-2502; doi: 10.1210/en.2014-1890.

II. Colldén H*, **Nilsson ME***, Norlén A-K, 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.

III. **Nilsson ME**, Colldén H, Norlén A-K, Landin A, Windahl SH, Wu J, Sjögren K, Palsdottir V, Ryberg H, Poutanen M, Vandenput L, Ohlsson C. The effect of high-fat diet-induced obesity on tissue levels of sex steroids in male mice. Manuscript

* Contributed equally

CONTENTS

ABBREVIATIONS

A-dione	androstenedione
CV	coefficient of variation
CYP	cytochrome P450
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
E1	estrone
E2	estradiol
GC-MS/MS	gas chromatography-tandem mass spectrometry
GnRH	gonadotropin-releasing hormone
HFD	high-fat diet
HPG	hypothalamic-pituitary-gonadal
HSD	hydroxysteroid dehydrogenase
LC-MS/MS	liquid chromatography- tandem mass spectrometry
LLOQ	lower limit of quantification
LOD	limit of detection
ND	not detectable
ORX	orchiectomy
OVX	ovariectomy
Prog	progesterone

SHBG	sex hormone-binding globulin
SRD5A	steroid 5alpha-reductase
T	testosterone
WAT	white adipose tissue

1 INTRODUCTION

Sex steroid hormones have long been recognized as being vital for the development and maturation of secondary sexual characteristics. Nonetheless, our understanding of the biology of sex hormones has changed dramatically since then and androgens, estrogens, and progesterone are now thought to play an important role in multiple human physiological and disease processes. Sex hormones are to a large extent produced by the gonads but can also be synthesized locally in target tissues from adrenal sex steroid precursors or *de novo* from cholesterol [1, 2]. This suggests that circulating levels of sex steroids may not always reflect sex steroid metabolism within the target tissues [3].

Even though sex steroids are increasingly important in clinical medicine and research, accurate measurement of androgens, estrogens, and progesterone in human samples remains challenging [4]. The much lower levels of sex steroids present in rodents compared to humans make the measurement of sex steroid levels in animal models even more difficult. The assay would require maximum sensitivity for several steroids while limiting any interfering peaks and matrix effects. In this thesis, we aim to develop and validate a sensitive and specific method to measure a panel of sex steroids in serum and tissues. In addition, to increase the understanding of the local sex steroid metabolism, we aim to use this assay to determine the sex steroid levels in multiple rodent tissues and assess the impact of castration and obesity on these levels.

1.1 SEX STEROID METABOLISM IN HUMANS

Dehydroepiandrosterone (DHEA) and its sulfate (DHEAS), mainly secreted by the adrenals, are the most abundant circulating steroids in humans. DHEA acts as an inactive precursor which is converted into active androgens and/or estrogens in peripheral target tissues [5] (Figure 1). The weak androgen androstenedione (A-dione) is produced from DHEA by 3β-hydroxysteroid dehydrogenase (HSD3B) activity or, alternatively, from progesterone (Prog) in 2 steps by cytochrome P450 17A1 (CYP17A1) exhibiting both 17α-hydroxylase and 17,20-lyase activities. A-dione can then be further reduced by 17β-HSD isoforms (HSD17B) into the major circulating androgen testosterone (T), which can either be activated by 5a-reductase (SRD5A) into the most potent androgen dihydrotestosterone (DHT) or converted into the major

circulating estrogen estradiol (E2), by the aromatase enzyme (CYP19A1). E2 can also be synthesized via aromatization of A-dione into estrone (E1), which is converted by HSD17B enzymes to yield E2 [2, 3, 6-8]. Alternatively, DHT can be synthesized from Prog through alternative pathways that bypass DHEA, A-dione, and T (Figure 1) ([3, 8].

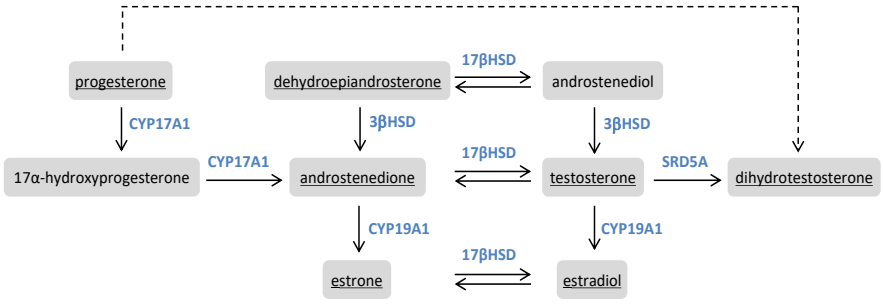

Figure 1 Simplified schematic overview of the biosynthesis of androgens and estrogens. The underlined sex steroids are measured with the gas chromatography-tandem mass spectrometry method developed in this thesis. The dashed line represents an alternative pathway from progesterone to dihydrotestosterone by avoiding testosterone. CYP, cytochrome P450; CYP17A1, 17a-hydroxylase/17,20 lyase; HSD, hydroxysteroid dehydrogenase; SRD5A, steroid 5a-reductase.

In men, the main source for androgen production is the testes with the adrenals also releasing androgen precursors, which can be further metabolized in peripheral tissues [9]. In premenopausal women, the primary source of estrogens is the ovaries, whereas the most abundant precursor for estrogens in postmenopausal women is adrenal-derived DHEAS [5]. Gonadal production of sex steroids is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 2) [10]. Gonadotropin-releasing hormone (GnRH) released by the hypothalamus stimulates the anterior pituitary to secrete luteinizing hormone and follicle-stimulating hormone which in turn stimulate the testes and the ovaries to secrete androgens and estrogens, respectively. Increased circulating levels of T or E2 result in a suppressive effect on hypothalamic and pituitary secretion via a negative feedback mechanism. Adrenal secretion of DHEA and DHEAS is stimulated by adrenocorticotropin hormone [11-13].

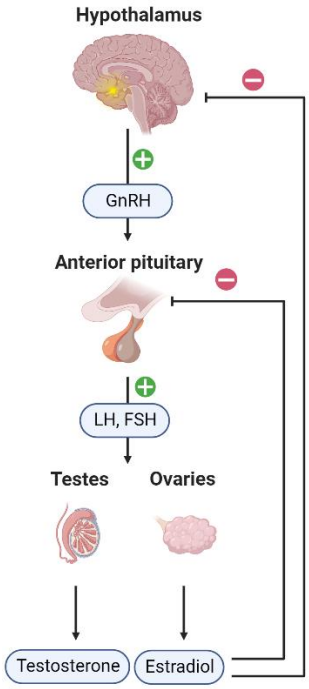

Figure 2 Regulation of gonadal sex steroid production via the hypothalamic-pituitary-gonadal axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) to stimulate pituitary production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The testis and ovaries are in turn stimulated by LH and FSH to secrete androgens and estrogens which are part of the negative feedback loop to the hypothalamus and pituitary with the aim of regulating the blood levels.

In humans, the majority (50-60%) of T and E2 in the circulation is bound with high affinity to sex hormone-binding globulin (SHBG) which regulates the transport, distribution, and metabolism of the active sex steroids. It prolongs the half-life of the active sex steroids and acts as a buffer against rapid changes in steroid levels [14]. A smaller fraction (40-50%) is bound loosely to albumin whereas 1-3% is unbound [15]. Inactivation of sex steroids occurs when active androgens and estrogens are further metabolized, first by reductions and hydroxylations, followed by conjugation of accessible hydroxyl groups with either a glucuronosyl group or a sulfate [16]. Sulfonation of sex steroids by sulfotransferases is reversible whereas glucuronidation by UDP glucuronosyltransferases is irreversible [6, 17].

Both genetic and environmental factors may influence serum sex steroid levels. Twin and family studies indicated high heritability (50-80%) for serum sex

steroid levels [18]. In this context, our research group identified several genetic determinants of serum sex steroid levels in men [19-22]. Health and lifestyle factors including smoking, physical activity, alcohol consumption, and dietary intake were shown to affect serum levels of sex steroids [23-29].

1.2 SEX STEROID METABOLISM IN RODENTS

There are two major differences in sex steroid metabolism between humans and rodents. First, rodents lack SHBG as a carrier protein; this results in low and fluctuating circulating sex steroid levels and explains the pronounced inter-individual variations in mice [30, 31]. Second, rodents do not secrete substantial amounts of the adrenal androgen precursor DHEA [32]. However, recent studies reported reduced growth of prostate cancer xenografts following adrenalectomy in male mice, suggesting that the mouse adrenals do produce certain androgen precursors [33, 34].

These differences influence the total serum concentrations of sex steroids and sex steroid precursors in rodents, which are thus substantially lower compared with humans, making them more difficult to quantify. In addition, sex steroid levels fluctuate substantially from day to day during the female estrous cycle, with extremely low levels observed during parts of the cycle [35]. Furthermore, when male mice are housed together, the alpha-cage mouse will display higher T levels compared to the subordinate mice due to the social dominance hierarchy [36, 37].

1.3 SEX STEROID METABOLISM IN TARGET TISSUES

Steroid hormones are vital for reproductive development as well as the optimal function of many non-reproductive tissues. Since all steroidogenic enzymes and steroid receptors are present in many peripheral target tissues, these tissues can synthesize their own sex steroids. The expression of HSD3B2, HSD17B, CYP19A1, and SRD5A2 enables the conversion of circulating DHEA and DHEAS into more active androgens and estrogens. In addition, expression of steroidogenic acute regulatory protein and CYP11A1 allows for *de novo* steroidogenesis from cholesterol. This distinct mechanism of androgen precursor activation, action, and inactivation in peripheral sex steroid-sensitive

target cells was termed “intracrinology” and first postulated by Labrie in the early 1990s [1]. The local metabolism of androgen precursors into more active androgens and estrogens in target tissues is a means to prevent both overstimulation of the sex steroid receptors and releasing significant amounts of active sex steroids back into the circulation. This ensures that the effects of the androgen precursors are maintained in a certain target tissue and any systemic effects are reduced [1]. The HSD17B enzymes play a central role in these steroid transformations locally in target tissues and are considered gatekeepers controlling the amount of steroids available for the steroid receptors [38, 39]. As a result of this local control, circulating sex steroid levels may not always reflect the tightly regulated sex steroid levels locally in target tissues [3]. This has not been explored in detail previously due to the lack of sufficiently specific and sensitive validated techniques for analyses of sex steroids in tissues.

When translating this concept into the treatment of hormone-dependent disorders such as breast cancer and prostate cancer, the classical sources of sex steroids, the ovaries and testes, can be controlled by GnRH agonists [40, 41]. In addition, sex steroid signaling can be blocked via androgen receptor blockers such as enzalutamide [42] and selective estrogen receptor modulators such as tamoxifen [43]. However, after inhibition of T and E2 synthesis combined with signaling blockade at the receptor level, oncologic progression often continues with resistance to these hormonal therapies due to the adrenal production of androgen precursors and their subsequent local conversion to active sex steroids [44]. Interestingly, the enzymatic processes in peripheral target tissues are recognized as novel therapeutic targets in an attempt to control local sex steroid production in hormone-dependent diseases [8, 45]. This has already been successfully applied to women with estrogen receptor-positive breast cancer who are treated with aromatase inhibitors to prevent the local formation of E2 from T or A-dione [46]. Furthermore, in men with castration-resistant prostate cancer, CYP17A1 inhibitors such as abiraterone are used to inhibit both adrenal and intratumoral steroidogenesis [8, 12].

1.4 SEX STEROIDS AND ADIPOSE TISSUE

The prevalence of obesity has increased significantly during the last decades [47]. Obese individuals are at higher risk of developing cardiometabolic diseases such as insulin resistance, type 2 diabetes, hepatic steatosis, cardiovascular diseases, and several types of cancer. Body fat distribution differs between men and women, with the former showing an android pattern with central fat distribution and the latter a gynoid pattern with peripheral subcutaneous fat distribution. Also, differences in metabolic and endocrine functions of the various fat depots in men and women are related to sex-specific differences in the risks for cardiometabolic diseases [48].

Circulating sex steroid levels are associated with various obesity-related cardiometabolic diseases and metabolic disorders [49-51]. More specifically, low circulating T levels are associated with increased fat mass [24, 52-56] and an increased risk of cardiometabolic diseases in men [57-61]. By using bidirectional Mendelian randomization to infer causality, our research group showed that high BMI reduces serum T levels in men whereas T levels do not influence BMI [62]. It remains uncertain, though, whether treating hypogonadal men with T is safe, especially with respect to the risk of cardiovascular events. Results from randomized controlled trials have been inconsistent [63, 64] but the most recent trial indicated no difference in the incidence of major adverse cardiac events between the treatment group and the placebo group [65, 66].

As described previously, sex steroids are not only produced by the gonads and adrenals but can also be synthesized and metabolized in peripheral tissues that express all necessary enzymes [6, 8]. A recent study showed that human adipose tissue expresses all required enzymes for converting circulating DHEA and DHEAS into more active sex steroids (Figure 1) as well as for *de novo* steroidogenesis [67]. As such, white adipose tissue (WAT) can act as a steroidogenic organ with locally produced sex steroids modulating adipose tissue activity and function [68-70]. In relation to this, it is important to mention once again that circulating sex steroids levels do not always reflect sex steroid metabolism and action in peripheral target tissues, WAT in this case. The effects of obesity on local sex steroid concentrations in metabolically active tissues, such as adipose tissue, remain unknown.

1.5 MEASUREMENT OF SEX STEROIDS

Accurate measurement of sex steroids such as T, DHT, E2, E1 and Prog is necessary for preclinical and epidemiological studies and to aid in clinical diagnosis and patient care. In hospital settings, immunoassay-based methods are routinely used because of their high sample throughput but are neither sufficiently sensitive nor specific because of matrix effects and cross-reactivity with other analytes in the sample [71, 72]. General concerns regarding the use of immunoassays for measuring E2 [73, 74] and T [75, 76] have been raised by academic and clinical communities. Serum T levels are clinically measured in pediatric patients with precocious or delayed puberty [77], adult men with hypogonadism [78], women with suspected hyperandrogenemia such as polycystic ovary syndrome or with irregular periods [79], and in transgender individuals to verify gender-affirming therapy [80, 81]. In addition, low T levels need to be quantified accurately in prostate cancer patients undergoing androgen deprivation therapy to ensure adequate suppression [82]. Clinically, serum E2 levels are measured in breast cancer patients treated with aromatase inhibitors [83], pregnant women [84], women undergoing fertility treatment [85], and postmenopausal women [86]. Also, samples from men with suspected gynecomastia [87] and prepubertal children [88] may be analyzed for E2. The biggest challenge in measuring serum E2 is the large concentration range present in the different patient populations. E2 levels in breast cancer patients treated with aromatase inhibitors are <2 pg/mL while women undergoing *in vitro* fertility treatment may have levels up to 10,000 pg/mL (Table 1) [73, 74].

Table 1. Large variation in average estradiol (E2) levels in different patient populations and animal models.

E2 source	E2 levels (pg/mL)
Prepubertal children	<20
Menstrual cycle	30-800
Pregnancy	Up to 20,000
Postmenopausal women	<20
Men	<40
Patients on aromatase inhibitors	<2
Postmenorpausal homone replacement therapy	20-100
Fertility treatment	Up to 10,000
Female mice	<0.3-50
Male mice	<0.3

Adapted from Stanczyk & Clarke 2014 [74] and own data.

The need for highly sensitive, specific, and accurate assays for T, DHT, E2, E1 and Prog is as critical in epidemiological studies as it is in the clinical setting. Studies often evaluate differences across the normal concentration ranges rather than distinguishing between normal and abnormal levels. The use of inaccurate assays can result in less strong (or absent) associations with a disease or outcome than those actually present [73]. Several studies have reported questionable specificity for serum E2 and T measurements by immunoassay, especially in the lower concentration ranges [89-95]. In addition, in a study of middle-aged and older men, we compared serum E2 levels measured by immunoassay and MS on the same samples and found only poor to moderate correlations. Additionally, we found a potential interference in the immunoassay, possibly by C-reactive protein or a related factor [96]. This is also reflected in the conflicting results presented regarding the associations between serum sex steroid levels, mostly measured by immunoassays, and several clinical outcomes such as mortality and cardiovascular diseases [97-101].

Recognizing these limitations, laboratory-developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays have become the gold standard when low concentrations of these hormones are present in patient samples [102, 103]. Most methods use an extraction step and a chromatographic step to separate the analyte from similar compounds in the sample before quantification by MS. Gas chromatography-tandem MS (GC-MS/MS) assays are equally specific and sensitive but require an extra derivatization step which makes them more labor-intensive with a lower throughput for routine clinical use. In addition, MS-based methods do have the advantage of being able to analyze multiple analytes at once. The greater sensitivity and specificity of MS methods is especially important for measuring the low E2 levels commonly observed in children, postmenopausal women, and breast cancer patients treated with aromatase inhibitors [73, 74].

Serum sex steroids levels in rodent samples are lower compared with human samples for the reasons indicated above. Moreover, the rodent serum sample volume is substantially lower compared with the human serum sample volume. This implies that even more sensitive and specific assays are needed to accurately quantify these hormone levels in rodents [104, 105]. Detectable levels of serum T but not E2 were previously reported in male and female mouse samples using LC-MS/MS [106, 107]. Caldwell et al. reported measurable E2 levels (>2.5 pg/mL) in serum from female mice when collected

at the diestrus stage [108]. However, many studies failed to report accurate levels during the estrous cycle in rodents when using immunoassay-based methods [109-116] with the reported E2 levels often overestimating the true levels because of the non-specificity of the assays used.

Based on the above, our aim is to develop a sensitive and specific in-house MS method to assess a broad panel of sex steroids in human serum as well as in rodent serum and tissues. A special effort was made to reach a remarkably high sensitivity for serum E2 to enable analyses in female rodents. Also, to further progress the knowledge regarding local sex steroid metabolism from adrenal androgen precursors in physiological and pathophysiological conditions, it is necessary to accurately measure tissue-specific sex steroid levels. As this is not ethically feasible in humans, we will infer our findings from animal models to clinical situations in humans. As such, an additional aim is to use our assay to determine the sex steroid levels in multiple rodent tissues and assess the impact of castration and obesity on these levels in order to increase the understanding of the local sex steroid metabolism.

2 AIM

The overall aim of this thesis is to develop and validate sensitive and specific methods for the measurement of a comprehensive panel of sex steroids in both serum and tissues of rodents. In addition, we aim to use these methods to determine the sex steroid levels in multiple rodent tissues and assess the impact of castration and obesity on these levels (Figure 3). In doing so, we will evaluate the concept that local sex steroid metabolism and resulting sex steroid levels within target tissues are critical for local sex steroid action.

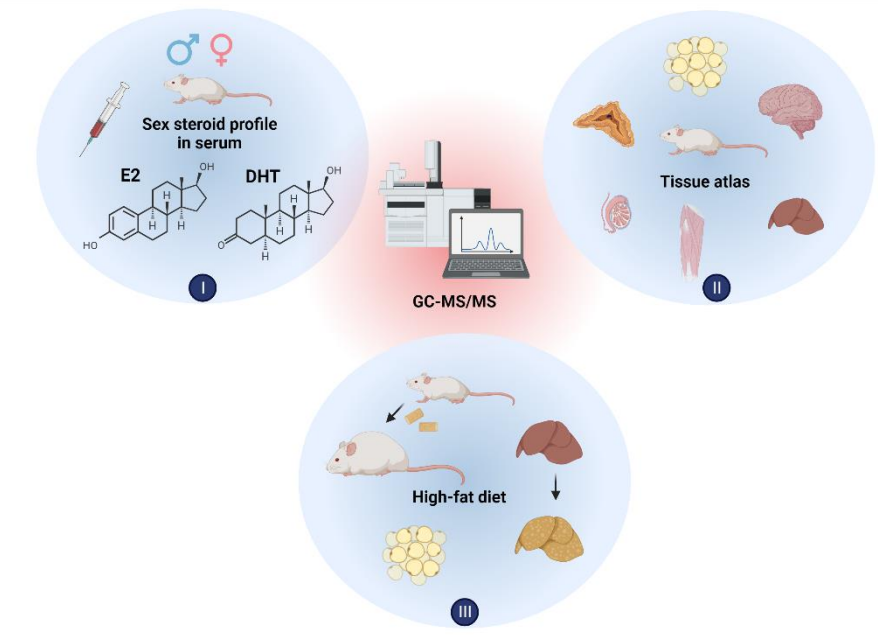

Figure 3 Overview of the different papers included in this thesis. The central aim of this thesis was to develop and validate a sensitive and specific gas chromatography-tandem mass spectrometry (GC-MS/MS) method for the measurement of a comprehensive sex steroid panel. In the first paper, we developed a GC-MS/MS method to evaluate a sex steroid profile in rodent serum with a specific focus on reaching a high sensitivity for estradiol (E2) and dihydrotestosterone (DHT). In paper two, we further developed our method to measure a comprehensive sex steroid profile in multiple tissues in male mice. Lastly, we applied our validated GC-MS/MS assay to determine the effect of high-fat diet-induced obesity on sex steroid levels in multiple tissues in male mice.

The specific aims are

- to develop a sensitive and specific GC-MS/MS method to evaluate a comprehensive sex steroid profile in rodent serum (paper I),

- to develop a sensitive and specific GC-MS/MS method to measure a comprehensive sex steroid profile in multiple tissues in mice (paper II),

- to apply our validated sensitive GC-MS/MS assay to generate a detailed atlas of the sex steroid levels in multiple tissues in male mice (paper II), and

- to determine the effect of castration (paper II) and high-fat diet-induced obesity (paper III) on the sex steroid levels in multiple tissues in male mice.

3 METHODOLOGICAL CONSIDERATIONS

In this section, the methods used are discussed in general. Detailed information is provided in the Materials and Methods section of each individual paper.

3.1 ANIMAL MODELS

To study human biology and disease development, mice are commonly used as a laboratory model. Advantages to be noticed are the similarities between the human and the mouse genome as well as their commonly shared physiology and anatomy. Their small size and short lifespan also contribute to its benefits. Although there are many advantages with mouse models, some comparisons could be questioned. For example, women experience menopause while female mice do not. This condition of sex steroid deficiency can be mimicked in mice by ovariectomy (OVX).

The mouse strain used in our projects is the C57BL/6 with a mean lifespan of about two years and sexual maturation at 6-8 weeks [117]. Genetic manipulations can relatively easily be performed in mouse models and by using these techniques the consequences of a modified genome can be investigated. In paper I, we investigated the serum sex steroid levels of female mice with estrogen receptor-alpha gene inactivation since previous reports had indicated disturbed negative feedback regulation of sex steroids in these mice [118, 119].

In addition to C57BL/6 mice, we also used adult female Wistar rats (Charles River Laboratories) in paper I to investigate serum sex steroid levels according to the estrous cycle phase.

3.1.1 GONADECTOMY AND SEX STEROID REPLACEMENT

In male rodents, most sex steroids are produced in the testes and in female rodents, the main part is produced in the ovaries. Gonadectomy, the surgical removal of either the testes in males (orchiectomy, ORX) or the ovaries in females (OVX), reduces the endogenous sex steroids markedly. The sex steroid levels in these castrated mice can be restored through intraperitoneal

injections, subcutaneous slow-release pellets, or osmotic pumps. The efficacy of the gonadectomy procedure and the replacement dose can be monitored by the wet weights of several sex steroid-responsive organs (e.g., seminal vesicles, prostate, levator ani muscle, and uterus) [120, 121]. In paper I, OVX was performed with subsequent treatment with E2 (830 ng 17β-E2 per mouse per day administered via subcutaneous slow-release pellets), resulting in supraphysiological E2 levels.

3.1.2 ESTROUS CYCLE PHASE

In rodents, the estrous cycle refers to the reproductive cycle which is 4-5 days long and consists of four phases: proestrus, estrus, metestrus, and diestrus. The human reproductive cycle, known as the menstrual cycle, consists of the follicular, ovulatory, and luteal phase and the total cycle lasts for about 28 days. Various methods can be used to determine the estrous phases such as vaginal smear/cytology, visual assessment of female reproductive organs and biochemical measurements in urine. The vaginal cytology is widely used and is a non-invasive, accurate, and relatively inexpensive method, where the different stages of the estrous cycle are determined by the proportion of leukocytes, cornified epithelial cells, and nucleated epithelial cells on the microscope slide [122]. In paper I, we used such vaginal cytology to determine the estrous cycle phase of rodents.

3.1.3 WHOLE-BODY SEX STEROID MODEL

In paper II, we used a simplified whole-body model to approximate the total amount of sex steroids in the different mouse body compartments. The total body pool for an analyte was calculated as the sum of the amounts of analyte in each tissue, calculated as tissue weight x measured concentration. For tissues obtained at sacrifice, the actual wet weights were used; for other tissues and body compartments, the weights were approximated as fixed weights based on previous experiments (adrenals, testes) or as a percentage of the mouse body weights based on body composition measurements or literature reports.

3.1.4 HIGH-FAT DIET-INDUCED OBESITY

To study obesity and its related biomarkers, the laboratory mouse is commonly used since it grows fast and consumes less food than other larger experimental animals. The standard method to generate diet-induced obesity in mice is by administering a high-fat diet (HFD). The metabolic phenotype observed in HFD-fed obese mice compared with control animals includes obesity with both

adipocyte hyperplasia and hypertrophy, insulin resistance and glucose intolerance [123]. A differential fat distribution pattern, similar to that seen in men and women, is observed in male and female mice following a HFD [124]. In paper III, we investigated the influence of HFD-induced obesity on sex steroid tissue distribution in male mice fed either a normal diet (macronutrient composition: 16% protein, 48,5% carbohydrate, 4% fat by energy, 3.30 kcal/g; 2016 Teklad Global, Envigo, NJ, USA) or HFD (macronutrient composition: 20% protein, 20% carbohydrate, 60% fat by energy, 5.24 kcal/g; #D12492, Research Diets, New Brunswick, NJ, USA) for 6 weeks (Figure 4).

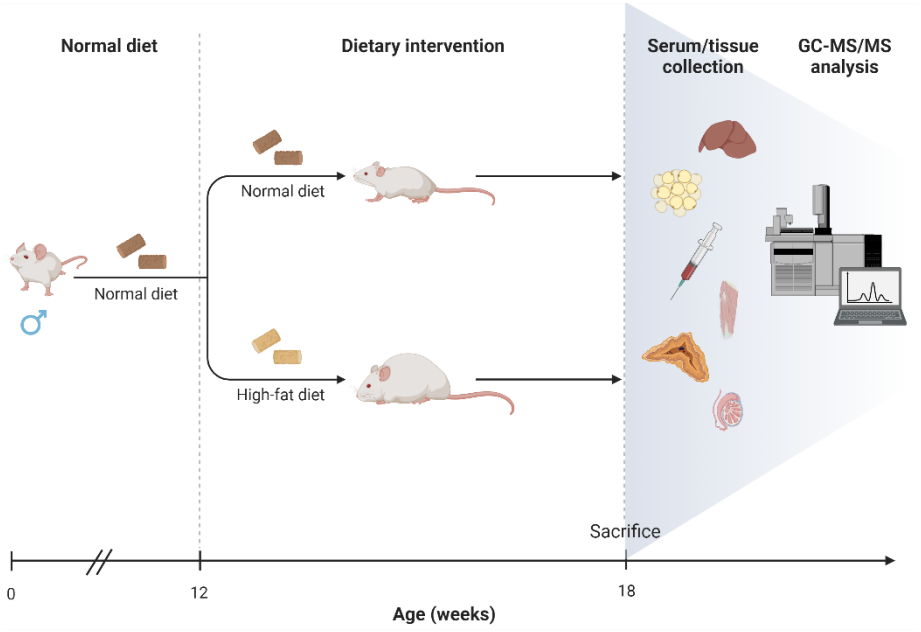

Figure 4 Overview of the high-fat diet-induced obesity project. Twelve-week-old male mice were given ad libitum access to either normal diet (n=10) or high-fat diet (n=10) for 6 weeks. Serum and tissues were collected at sacrifice. Samples were prepared and analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS) to quantify sex steroid concentrations.

3.2 SEX STEROID MEASUREMENTS

During the process of developing our in-house method for the measurement of sex steroid levels in rodent serum and tissues, we chose a GC-MS-based approach instead of an LC-MS method. Specifically, we wanted a method providing a very high sensitivity for serum E2 enabling analyses of female

rodent serum samples. In addition, we also wanted to be able to achieve a sufficiently high sensitivity for serum DHT to allow quantification in rodents. At the time of development, GC was better suited to achieve these goals than LC, as there were no reported LC methods reaching the required sensitivity for serum E2 and/or DHT back then. The more labor-intensive workup required for the GC samples compared to the LC samples is a necessary drawback in order to reach our required high sensitivity.

The development process includes the following steps: sample preparation, sex steroid extraction, derivatization, and GC-tandem MS analysis, followed by the evaluation of the assay performance and method validation. We first developed an assay for the measurement of a broad panel of sex steroids in rodent serum (Figure 5).

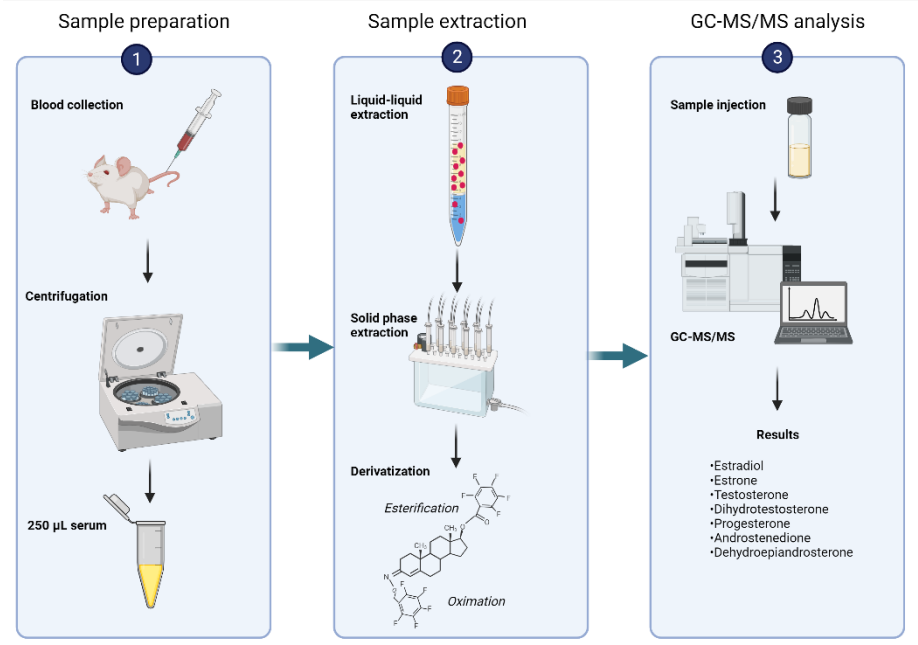

Figure 5 A general flow chart of the preparation and analysis of serum samples. Box 1 shows the blood sample centrifugation followed by pipetting of 250 µL of serum sample. During sample extraction (box 2), the serum samples go through a liquid-liquid extraction. The organic phase is then applied onto the solid phase extraction column. In a next step, the sex steroids are derivatized by oximation of the keto-groups followed by esterification of the hydroxyl groups. Finally, the prepared samples are injected onto the gas chromatography-tandem mass spectrometry (GC-MS/MS) equipment (box 3) and the detected sex steroids are quantified.

This method was then further optimized to allow measurement of the same panel of sex steroids in multiple rodent tissues (Figure 6).

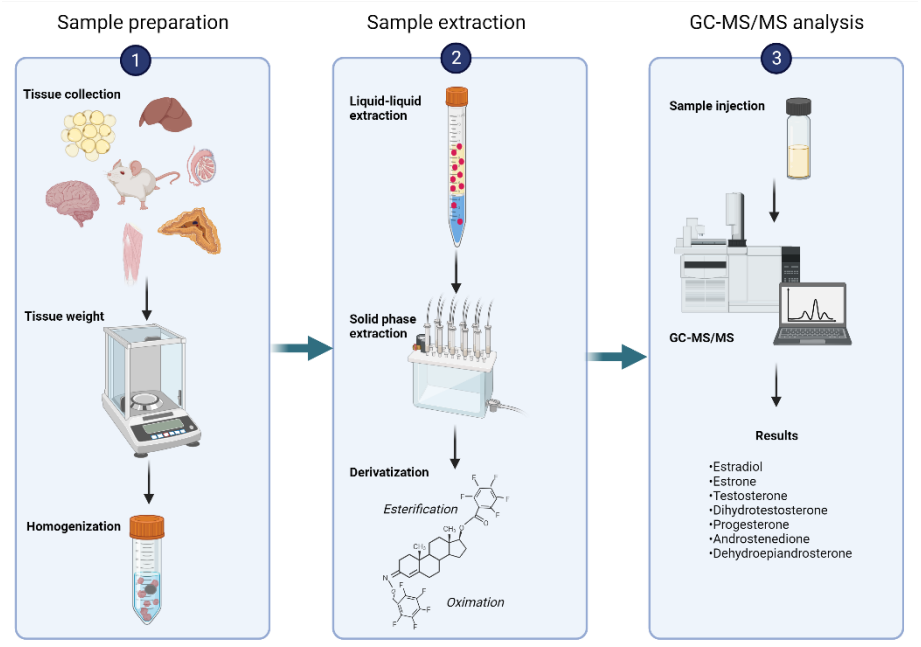

Figure 6 A general flow chart of the preparation and analysis of tissue samples. Box 1 shows the tissue collection followed by weighing of the samples and homogenization. During sample extraction (box 2), the tissue samples go through a liquid-liquid extraction. The organic phase is then applied onto the solid phase extraction column. In a next step, the sex steroids are derivatized by oximation of the keto-groups followed by esterification of the hydroxyl groups. Finally, the prepared samples are injected onto the gas chromatography-tandem mass spectrometry (GC-MS/MS) equipment (box 3) and the detected sex steroids are quantified.

3.2.1 SAMPLE PREPARATION

Serum samples (250 µL) from all three papers were adjusted to a volume of 450 µL with deionized water. The frozen tissues in paper II and III were thawed on ice. Small organs weighing under 125 mg were used in whole for sample preparation while for larger organs/tissues we aimed to use 125 mg for sample preparation. To obtain homogenized samples suitable for injection on the GC-MS/MS, tissues were homogenized in a Tissuelyzer II (Qiagen, Hilden, Germany). Internal standards (isotope-labeled steroids) were added to the samples. The isotope-labeled standards contain modified versions of the molecules we want to measure, in which some carbons or hydrogens are

replaced by stable isotopes and therefore are expected to behave similar to the investigated compounds. Since the chemical properties are the same, it is just a small difference in mass which separates them during the detection process. We used isotope-labeled standards containing ^{13}C or deuterium in paper I-III.

3.2.2 SEX STEROID EXTRACTION

A hydrophobic solvent, chlorobutane was added to initiate the liquid-liquid extraction (Figure 7). The hydrophobic sex steroid molecules accumulate in the organic phase formed by chlorobutane, which makes it possible to separate the sex steroids from other possible interfering substances. The organic extracts were then collected and purified on solid phase extraction columns (Figure 7). The adsorbed fraction includes both impurities and the sex steroids of interest, therefore the properties of the washing solution are important. The optimal washing solution has high affinity for impurities and low affinity for the compounds of interest. The columns were washed twice, except for liver and adipose tissue samples, where three washing steps were needed to reach an optimal performance in the GC-MS/MS assay. The sex steroids of interest were finally eluted, and the organic solvent was evaporated (Figure 7).

3.2.3 DERIVATIZATION

The transformation during which the chemical form of a substance is changed to improve analytical capabilities, such as polarity, thermal stability, volatility, increased detector response, or improved performance of the GC separation, is generally called sample derivatization. A comprehensive sex steroid analysis depends on selective derivatization of hydroxyl groups but also other derivatizable groups. The sex steroid GC-MS assay requires derivatization prior analysis but no standard methods or established protocols are readily available for sex steroid derivatization, making the method more difficult to establish [125]. In our hands, the best performance was achieved when starting with oximation of the keto-groups, followed by esterification of the hydroxyl groups (Figure 7).

Derivatization of sex steroid hormones with pentafluoro-groups produces stable derivatives with favorable ionization properties for subsequent use in negative chemical ionization mode as we used in the GC-MS/MS method. To optimize the derivatization and to be able to expand the numbers of analytes, the reagent solutions, incubation times, and temperatures were thoroughly tested.

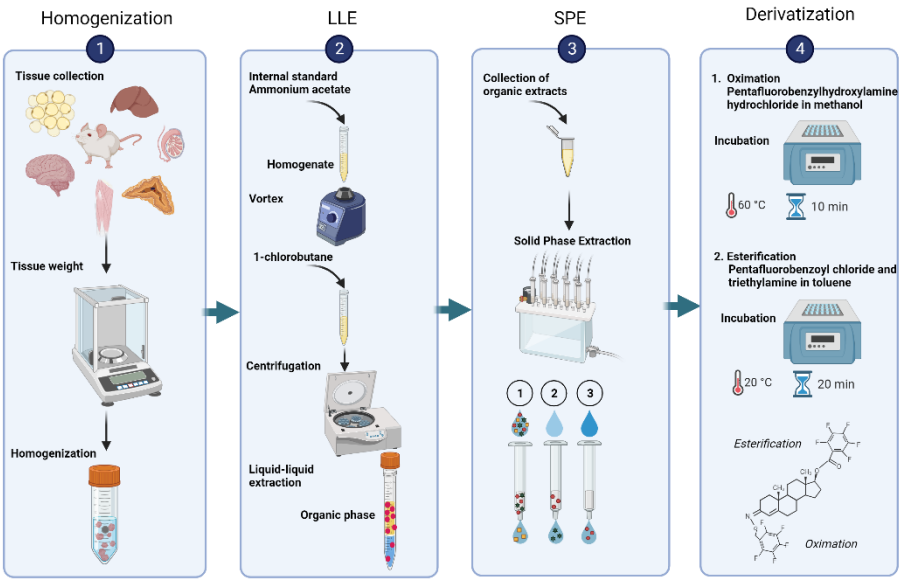

Figure 7 A detailed flow chart of the preparation of tissue samples. Box 1 shows the tissue collection followed by weighing of the samples and homogenization in phosphate-buffered saline with a steel bead. Before extraction, each sample is mixed with isotope-labeled internal standard and ammonium acetate and then vortexed. Thereafter, 1-chlorobutane is added to start the liquid-liquid extraction (LLE, box 2), during which the hydrophobic sex steroids prefer to stay in the organic phase. After centrifugation for 10 min, the samples are frozen overnight to easily decant the organic phase. During the solid phase extraction (SPE, box 3), this organic phase is then applied onto the SPE column where the sex steroids initially stay bound to the solid phase (①) while impurities are washed away (②). In the last step, the sex steroids of interest are eluted in repeated steps with the eluting solvent (③). Before analysis by gas chromatography-tandem mass spectrometry, the sex steroids are derivatized (box 4) by oximation of the keto-groups followed by esterification of the hydroxyl-groups to produce stable derivatives with favorable ionization properties.

3.2.4 GC-TANDEM MS

Solid, gaseous, and liquid samples can be analyzed by GC-MS/MS. The dissolved analytes are injected on the GC and vaporized into a gas phase on an inert liner before passing on to the column (Figure 8). The various components are separated by boiling point on a capillary column placed in an oven, which results in the different retention times of the analytes in the chromatogram. When the sample leaves the GC column, it enters the MS ionization source for ionization. Different degrees of fragmentation of the analyte also occurs depending on the ionization mode and energies used. Either electronic or chemical ionization can be used; in our case we used chemical ionization with ammonium gas, which is a soft ionization method resulting in low degree of

fragmentation and negative charged ions. Through the mass analyzer, often a quadrupole, the ionized molecules separate based on their mass to charge (m/z) ratios, selecting the right "mother ions" that continue over to the collision cell. The "mother ions" are fragmented into "daughter ions" by the collision gas, nitrogen gas in our case. The fragmented molecules were passed on to the second mass filter or quadrupole where the selected "daughter ions", chosen for detection, are separated based on their m/z and sent to the mass detector (for transitions see Table 2) (Figure 8).

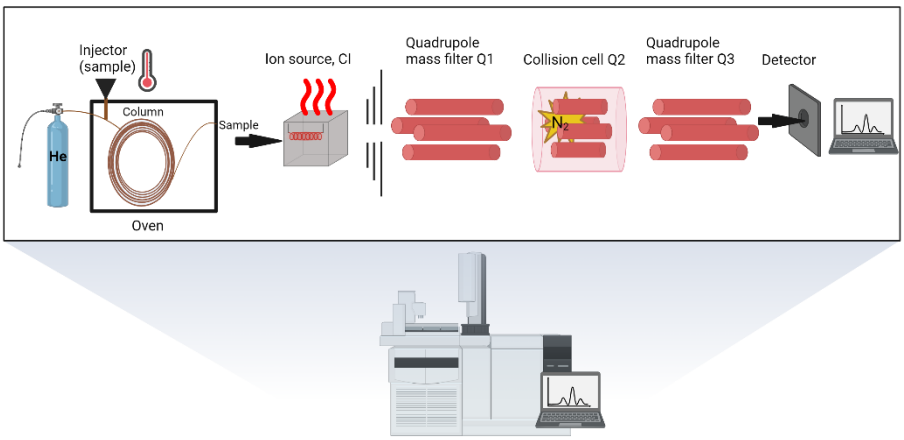

Figure 8 Schematic diagram of the gas chromatography-tandem mass spectrometry (GC-MS/MS) equipment. The sex steroids are dissolved in isooctane and injected on the GC, in which they are vaporized in an inert liner before passing on to the column, with helium as the carrier gas, where the analytes are separated by boiling point. The separated analytes pass through the ion source where chemical ionization (CI) with ammonia gas results in highly negatively charged ions due to the pentafluoro-groups attached to the steroid molecules. The ions are guided further via focusing lenses into the first quadrupole (Q1) where they are separated according to mass/charge (m/z) ratios. Only the selected "mother ion" from each analyte passes on to the collision cell (Q2). The "mother ions" collide with nitrogen gas (N_2) in the collision cell and this generates new fragments, so called "daughter ions". Those predetermined "daughter ions" with certain m/z ratios are selected to pass through the third quadrupole (Q3) and then sent to the detector.

Analytes were detected in multiple reaction monitoring mode covering a wide range of m/z ratios, using an Agilent 7000 triple quadrupole mass spectrometer. The MassHunter quantitative analysis Workstation Software from Agilent was used for automatic integration of all peaks. The Agile integrator was used for E2, T, DHT, Prog, DHEA, and their corresponding internal standards. For E1, A-dione, and their corresponding internal standards,

the General integrator was used. The oximation step could generate two peaks for the ketosteroids, but just a single peak was detected in the chromatogram due to co-elution of all steroids except for DHT. Two baseline-separated peaks were eluted for DHT with seemingly equal intensities. As one of the peaks was disturbed, the automatic peak integration was done with only one peak, thus the second eluted DHT peak was chosen for integration and quantitation.

Table 2. Mass transitions for the analytes measured using our established gas chromatography-tandem mass spectrometry method.

Analyte	Mass transition
Progesterone	489.2 → 459.3
Dehydroepiandrosterone	482.2 → 167.0
Androstenedione	461.2 → 431.2
Testosterone	677.2 → 496.2
Dihydrotestosterone	679.2 → 181.0
Estradiol	660.1 → 596.2
Estrone	464.1 → 400.2

Adapted from Nilsson et al. 2015 [126].

3.2.5 ASSAY PERFORMANCE

The analytical performance of our developed assays has been documented in detail for the measurement of a broad panel of sex steroid levels in serum (paper I) and tissues (paper II).

Specificity

Analytical specificity is one important aspect of assay performance and can be defined as the ability to measure a specific analyte in the sample rather than other closely related substances with similar properties. The MS detector can normally identify the analyte with an extremely high degree of specificity due to its ability to create and measure specific ions of analytes. This is especially true in the MS/MS mode where collected ions are fragmented and a second ion is filtered and measured. Moreover, an MS detector makes it possible to utilize isotope-labeled internal standards. By incorporating stable isotopes, molecules can be created with the same chemical properties as the analytes of interest, except for a small shift in mass that easily can be detected by the MS detector. Comparing the area and retention time of the internal standard peak to those of the analyte peak enables the detection of interferences, which is a powerful tool to evaluate the performance of the assay.

Sensitivity

The sensitivity of an assay is reflected in the detection limit, which specifies the lowest concentration of the analyte that can be distinguished from a blank

matrix with a defined confidence level. We defined the limit of detection (LOD) as the lowest peak having a signal at 3 times the noise level. We defined the lower limit of quantification (LLOQ) as the lowest peak that was reproducible with a precision of less than 20% and an accuracy of 80%-120%. To evaluate each analyte, at least seven different levels in the lower concentration range were used to determine the LLOQ. As a reference, the exceptionally high sensitivity for estradiol with our established method can be compared to trying to detect sugar when two sugar cubes are dissolved in the Sisjön lake in Gothenburg.

Linearity
To determine the linearity of response, a sample spiked at the highest point on the standard curve was serially diluted before the extraction procedure was performed and R^2 was calculated.

Accuracy
The accuracy is defined as percent recovery of known amounts of analyte added to specimens. We used serum aliquots spiked with calibrator standard solutions containing target analytes at two levels. The accuracy was calculated as follows: [(observed value – baseline value)/amount spiked]*100.

Precision
The precision of an assay refers to the closeness of repeated measurements to each other. We analyzed aliquots from two pools of serum (with low and high levels of the analytes) on different occasions and calculated the intra- and interassay coefficients of variation (CVs). The intraassay (or within assay) CVs reflect the variability among replicate measurements of the same sample within the same run and were calculated by analyzing 12 aliquots of both pools of serum on one occasion. The interassay (or between assay) CVs represent the variability among replicate measurements of the same sample over time and were calculated by analyzing three aliquots of both pools of serum on four different occasions. Analyses were performed using $^{13}C_3$-labeled (E2, E1, T, DHT, Prog, A-dione) or d_6-labeled (DHEA) analytes to distinguish from the levels of endogenous analyte present in rat serum.

Recovery
Assay recovery is assessed by comparing the concentration of an analyte measured in the sample with that actually stated to be present (ratio of observed vs. expected). To evaluate the efficiency of our solid phase extraction columns, we used isotope-labeled steroids and calculated the ratio between samples spiked before and after the extraction. The extraction recovery, even if reduced,

does not affect the absolute quantification of the analytes because internal standards for all analyzed steroids are added to every sample before the initiation of the extraction.

Matrix effects

The presence of matrix effects must be investigated as they can affect the analytical performance of an assay. Matrix effects occur when the ionization efficiency of target analytes is altered due to the presence of co-eluting compounds in the same matrix. This may result in either a loss in response (ion suppression) or an increase in response (ion enhancement) of the target analytes. To reduce the risk of matrix effects, use of stable isotope-labeled internal standards are common since ion suppression or enhancement should be in the same range for both the isotope-labeled analogue and the target analyte [127, 128]. Also, the additional third washing step for liver and adipose tissue samples during the solid phase extraction reduces the matrix effects significantly.

For our method in tissues, we performed a detailed validation in mouse WAT, muscle, bone, and liver, having substantially different matrix compositions. Matrices with high fat content, low fat content, and those that are calcified or metabolite-rich were included by the choice of these four different tissues. The LLOQ was defined as the lowest peak reproduceable with a precision of less than 20%, an accuracy of 80% to 120% and having a signal at > 3 times the noise. The intra-assay CV of samples in the same concentration level was used to calculate the precision. The accuracy for tissue measurements was calculated in the same way as for serum. Samples of WAT and muscle were used to evaluate the extraction recovery, which was tested by spiking the samples with isotope-labeled steroids before the liquid-liquid extraction and after the solid-phase extraction, after which the ratio was calculated.

Potential problems

During the method development, several problems occurred. We experienced some contaminations with estradiol. To avoid future contaminations, we put additional procedures in place such as sample processing on a designated desk space only and the use of clean gloves and separately handled pipette tips by the laboratory staff. Another problem can be instrument leaks and therefore skilled personnel is required for proper instrument handling. Initially, we also experienced varying batch-to-batch SPE column quality. This was solved by switching to another supplier whose SPE columns showed stable and high quality over time. When analyzing tissues with high fat content (adipose tissue)

or metabolite-rich samples (liver), an extra washing step is needed on the SPE column to get sufficiently clean samples to avoid matrix effects in the GC-MS/MS.

3.2.6 METHOD STANDARDIZATION

The Centers for Disease Control and Prevention initiated the Hormone Standardization Program in collaboration with clinical, laboratory, and research communities to address standardization of hormone measurement procedures [129]. We participated in the program with our serum T assay, which was validated against their reference measurement procedure using isotope-dilution LC-tandem MS [130]. In paper I, the assay agreement was visually assessed using Passing-Bablok regression analyses and Bland-Altman analyses and was evaluated by calculating the R^2.

3.3 TISSUE ANALYSES

In paper III, triglyceride concentrations in liver homogenate samples were measured by a colorimetric assay (Cayman Chemical, Ann Arbor, MI, USA).

3.4 GENE EXPRESSION ANALYSES

Measurement of mRNA levels by real-time quantitative polymerase chain reaction (RT-qPCR) is a very sensitive method to evaluate the expression levels of a particular gene of interest. The mRNA from the investigated tissue sample was first isolated and then reverse transcribed into cDNA. Next, the cDNA is amplified using sequence-specific primers and fluorescent-labelled probes that are complementary DNA strands for the gene of interest. During cDNA replication, fluorescence is emitted in proportion to the amount of amplified cDNA. In our studies, quantification of expression of the gene of interest is done in relation to the housekeeping gene 18S using the StepOnePlus Real-Time PCR System (Applied Biosystems). In paper III, RNA was isolated from liver, muscle, and seminal vesicle and analyzed for expression of *Srd5a1* and *Srd5a2*.

3.5 STATISTICAL ANALYSES

For statistical analyses either parametric or non-parametric tests may be relevant. If a normal distribution of data is assumed, parametric tests are used. If any other particular distribution of data is assumed, non-parametric tests based on a ranking of individual observations are used.

3.5.1 AGREEMENT OF ASSAYS

When developing a new analytical method, it is important to compare the performance of the new assay to that of a reference method. Passing-Bablok regression is a statistical method for the estimation of assay agreement; to assess if both methods yield similar results and by estimating possible systematic bias between them. It is a non-parametric, robust method with low sensitivity for distribution errors or outliers in the data set [131]. Alternatively, Bland-Altman analysis can be used to estimate the agreement between two quantitative measurements by assessing the mean difference and establishing limits of agreements. The difference of two paired measurements is plotted against the mean of these measurements. According to Bland-Altman recommendation, 95% of the data points should lie within ± 2 standard deviations of the mean difference [132]. In paper I, we evaluated the agreement between our established GC-MS/MS assay and a reference method for serum T using Passing-Bablok regression and Bland-Altman analysis.

Table 3. Statistical models used in this thesis.

Statistical models	Paper
Agreement of assays	
Passing-Bablok regression	I
Blant-Altman plot	I
Comparisons of groups	
Student´s t test	III
Mann-Whitney U test	I, II, III
Wilcoxon rank sum test	II
Kruskal-Wallis test	I
Correlations	
Spearman rank test	II, III

3.5.2 COMPARISONS AND CORRELATIONS

Non-parametric tests were used to analyze sex steroid levels, as the data was not normally distributed. We used the Mann-Whitney U test to compare two

groups (Paper II and III) and the Kruskal-Wallis test (Paper I) to evaluate differences between several groups. For paired samples, groups were compared using the Wilcoxon rank sum test and the Holm-Sidak method was used to adjust for multiple comparisons (Paper II). Correlations between sex steroid levels in different groups was done by the non-parametric Spearman rank test (Paper II and III).

4 RESULTS

4.1 PAPER I

Measurement of a comprehensive sex steroid profile in rodent serum by high-sensitive gas chromatography-tandem mass spectrometry

Immunoassay-based techniques are commonly used to measure serum sex steroid levels in clinical and research settings. Nevertheless, these assays display limited sensitivity, accuracy, and precision, especially in the lower concentration range. As serum sex steroid levels, in particular E2, E1, DHT, and T levels, are low in rodents, postmenopausal women, and children, accurate measurement techniques are essential to investigate sex steroid-related disorders. In this paper, we developed a sensitive and specific GC-MS/MS method to assess a comprehensive panel of sex steroids in rodent and human serum. Our priorities were reaching an exceptionally high sensitivity for serum E2 levels and succeeding in quantifying serum DHT in rodents.

Main results

- We established a GC-MS/MS assay for measuring 7 analytes (E2, E1, T, DHT, Prog, A-dione, and DHEA) in a single run in human and rodent serum.
- Sensitivity, accuracy, and precision were excellent for all analytes and calibration curves were linear.
- Using this assay, we were able to accurately measure a comprehensive sex steroid profile in female rats and mice according to estrous cycle phase.
- The assay also allowed for distinction between the serum sex steroid profiles in gonadal intact and castrated rodents.

Conclusion

We established a highly sensitive and specific GC-MS/MS method to assess a comprehensive sex steroid profile in human and rodent serum. Accordingly, this assay is suitable for the characterization of the sex steroid metabolism in a variety of sex steroid-related rodent models and in human samples with low E2, E1, DHT, and T levels.

4.2 PAPER II

Comprehensive sex steroid profiling in multiple tissues reveals novel insights in sex steroid distribution in male mice

In addition to sex steroid production by the classical endocrine organs, sex steroids can also be synthesized and metabolized by several peripheral tissues that express all necessary enzymes. As a result, circulating levels of sex steroids may not always reflect those within the target tissues. In this paper, we adapted and validated our GC-MS/MS method to quantify a comprehensive sex steroid profile in multiple rodent tissues and assess the correlation between circulating and tissue levels of sex steroids.

Main results

- Our tailored GC-MS/MS assay was optimized and validated in detail with sensitivity, accuracy and precision reported for four different types of matrices (muscle, liver, WAT, and bone).
- Sex steroid profiles were mapped in endocrine, reproductive, and major body compartment tissues, generating the first detailed atlas of tissue sex steroid levels in male mice.
- We used our assay to compare tissue sex steroid profiles in gonadal intact and ORX male mice. T and Prog were the most abundant sex steroids in gonadal intact mice. Most of the active androgens (T and DHT) and Prog were stored in WAT. Prog was the most abundant sex steroid after ORX with the majority stored in WAT.

Conclusion

We adapted and validated our GC-MS/MS method to assess a comprehensive sex steroid profile in multiple rodent tissues, leading to new insights in tissue sex steroid distribution and the development of the first detailed atlas of tissue sex steroid levels. The major pool of sex steroids in male mice was found within WAT, leading us to suggest that WAT might act as a buffer for circulating sex steroid levels in mice that do not express SHBG. Moreover, in ORX mice, we found Prog to be the most abundant sex steroid. Further studies are needed to define the role and origin of Prog in male mice.

4.3 PAPER III

The effect of high-fat diet-induced obesity on tissue levels of sex steroids in male mice

Sex steroid levels in serum associate with metabolic diseases but it is still unknown if local sex steroid concentrations in metabolically active tissues are affected by diet-induced obesity. As suggested in paper II, WAT may act as a reservoir for most of the androgens and Prog in male mice. The aim of this paper was, therefore, to assess the effects of HFD-induced obesity on sex steroid levels in tissues in male mice.

Main results

- We used our validated GC-MS/MS assay to map the effects of HFD-induced obesity on the sex steroid levels in multiple tissues.
- HFD-induced obesity reduced the DHT/T ratio, reflecting 5α-reductase activity, in muscle and seminal vesicles of male mice.
- In HFD-fed obese mice, liver DHT levels were increased, and this was accompanied by significantly increased liver triglycerides.
- Fat depot-specific differences in DHT levels were observed in the control group but not in the HFD group.
- HFD-induced obesity reduced Prog levels in WAT.

Conclusion

Using our validated assay, we provided a comprehensive overview of the effects of HFD-induced obesity on tissue sex steroid profiles in male mice. The DHT/T ratio decreased in muscle and seminal vesicles following HFD-induced obesity while the liver DHT levels increased. We speculate that the increased hepatic DHT levels observed in HFD-fed mice may contribute to obesity-related liver diseases.

5 DISCUSSION

The measurement of sex steroids in both clinical and research settings is challenging and depends heavily on accurate and precise methods. This is especially relevant for patient populations with low sex steroid concentrations and rodent samples in which sex steroid levels are substantially lower compared to human samples. In this thesis, we aimed to develop and validate sensitive and specific methods for the measurement of a comprehensive panel of sex steroids in serum and tissues of rodents. Furthermore, we aimed to use these methods to develop a detailed atlas of the tissue sex steroid levels and assess the impact of castration and obesity on these levels. This allowed us to evaluate the concept that local sex steroid synthesis and metabolism within target tissues are critical for local sex steroid action and are not (always) reflected by circulating sex steroid levels.

5.1 ESTABLISHMENT OF AN IN-HOUSE GC-MS/MS ASSAY FOR SEX STEROID PROFILING IN RODENT SERUM AND TISSUES

Sex steroid profiling in rodent serum

MS is considered the gold standard technique for measurement of sex steroids in clinical practice and translational research [73, 75]. In addition, we demonstrated that, specifically for serum E2, MS is preferred as immunoassay-based measurements showed potential interference in the low concentration range present in men [96]. Following this, we decided to develop an in-house MS method to quantify a panel of sex steroids in human and rodent serum. The objective was to establish an assay that was specific and highly sensitive to allow quantification of serum sex steroids levels across a large concentration range in human samples as well as the low sex steroid levels present in rodent samples. In paper I, we described the development and validation of our highly specific assay for the measurement of 7 analytes (E2, E1, T, DHT, Prog, A-dione, and DHEA) in one single run in serum samples. With this method, we were able to distinguish between the sex steroid levels from gonadal intact and gonadectomized mice as well as measure the serum E2 levels across the estrous cycle in both mice and rats. Since the development of our GC-MS/MS assay in 2015, only Handelsman and colleagues managed to obtain an LLOQ for

serum E2 in the same range as ours [133]. Our method and the method from the Handelsman group currently have the highest sensitivity for both serum E2 and E1. Also, to the best of our knowledge, our assay has by far the highest sensitivity for serum DHT reported and provides a reference method for the measurement of sex steroid levels in multiple tissues. Conventional LC-MS methods have detection limits between 3 and 5 pg/mL for serum E2 that are not sensitive enough to measure serum E2 in all postmenopausal women (Table 1) [73, 134, 135]. Handelsman et al. reported, using their method with an LOD for serum E2 in the same range as that for our assay, that serum E2 was quantifiable in almost all samples from healthy postmenopausal women as well as postmenopausal women with breast cancer treated with an aromatase inhibitor, in which serum E2 levels were on average 85% lower [133]. Moreover, serum E2 was detectable in most female mouse samples but not in any male mouse samples [133]. Recently, using the same method, a detailed steroid profile across the estrous cycle of C57BL/6J mice was presented, with serum sampling at 8-hour intervals [35]. These data confirmed the elevated serum E2 levels in diestrus and proestrus reported in paper I.

One disadvantage of our established method is that we were not able to detect serum estrogen levels in intact male mice, consistent with a recent report [133]. This suggests that even more sensitive assays are required to measure the presumably very low circulating estrogen levels in male rodents. The fact that estrogens are generally recognized to be involved in different aspects of male physiology [56, 136-138] indicates the increased significance of the intracrine production and metabolism of estrogens locally within the tissues in which they subsequently act through local receptors with very limited release into the circulation [6].

The unparalleled sensitivity and precision of our in-house assay allows for comprehensive sex steroid profiling in rodent serum. Accordingly, we are nowadays considered a reference lab for sex steroid measurements in sex steroid-related rodent disease models and our methods paper is highly cited (304 citations, Google Scholar, accessed 12 October 2023). Nevertheless, sex steroid levels measured using immunoassay-based methods, known to be inaccurate especially in the lower concentration range, are still regularly published for various rodent models, e.g., [139-143].

A few methodological aspects of our developed GC-MS/MS assay deserve consideration. The high sensitivity of our assay comes at the cost of limited

sample throughput because of the labor-intensive sample preparations. LC-MS/MS assays with comparable sensitivity have a less complex sample workup as most do not include a derivatization step and are thus more favorable in the clinical setting. As such, our research group has recently developed and validated a high-throughput LC-MS/MS with almost equivalent sensitivity as our original GS-MS/MS for the measurement of sex steroid profiles in epidemiological research [144]. Yet, our initial developed GC-MS/MS method currently still has the highest sensitivity for serum DHT, which is far better than any reported LC-MS/MS assay. Another aspect to consider is the required sample volume. Our method requires 250 µl, which may be challenging when measuring rodent serum samples. Finally, most results presented are from male mice (Paper II and III). The sex steroid levels in peripheral tissues as well as the sex steroid distribution were also evaluated in female mice and these results will be published separately. The fact that sex steroid levels vary extensively in female mice due to the estrous cycle makes the measurements and interpretations more demanding in female mice but, as shown in paper I, our assay can measure the varying concentrations of sex steroids according to estrous cycle phase.

Sex steroid profiling in rodent tissues

In peripheral target tissues, sex steroid action is believed to be largely dependent on the intracrine conversion of androgen precursors into active androgens and estrogens [1, 8]. Yet, the extent to which the circulating levels of sex steroids correlate with their corresponding levels produced locally remains, however, largely unknown due to the lack of sufficiently sensitive methods to quantify sex steroid levels in rodent tissues. In paper II, we adapted and validated our GC-MS/MS assay to allow for sex steroid profiling in multiple rodent tissues and to assess correlations between the circulating and tissue levels of the measured sex steroids. Previous studies using MS methodology reported sex steroid levels in a few selected tissues only [33, 34, 107, 145-150]. We were the first to comprehensively analyze a panel of sex steroids to map sex steroid distribution in endocrine, reproductive, and major body compartment tissues. This detailed atlas can be used by the research community as a reference database for tissue sex steroid levels. Most of the androgens and Prog in intact male mice were found in WAT, which may function as a reservoir for sex steroids in rodents in the absence of an equivalent for human SHBG and avoid extreme fluctuations of sex steroid levels. Prog was the most abundant sex steroid in ORX mice and was mostly found in WAT. To further evaluate the origins of androgens as well as Prog in

male mice, our research group examined sex steroid levels in mice that were both ORX and adrenalectomized to remove all sources of sex steroids and sex steroid precursors [151]. Low DHT levels were still detectable in prostate and several other peripheral tissues, indicating that another source besides the gonads and the adrenals contributes to local DHT levels. Surprisingly, Prog levels in serum and many tissues appeared to be largely independent of the presence of testes and adrenals. This was linked to high amounts of Prog found in the mouse chow, with the dietary Prog contributing to the local levels of Prog, as was functionally shown in that study [151]. The remaining high levels of Prog can in turn serve as precursors for androgens in peripheral tissue such as WAT and prostate [3, 8]. Further studies are warranted to define the role of Prog in men [152].

5.2 EFFECT OF HIGH-FAT DIET ON TISSUE LEVELS OF SEX STEROIDS

Several external environmental factors, such as smoking, alcohol, physical activity, and dietary intake, can affect circulating sex steroid levels [23-29]. In relation to this, it is proposed by the results from both clinical and preclinical studies that sex steroids play an important role in the regulation of fat mass in men [24, 52-56, 153, 154]. In paper II, we showed that the largest reservoir of sex steroids in ORX mice is found in WAT. This then led us to investigate the sex steroid distribution following HFD-induced obesity in male mice in paper III. HFD-induced obesity decreased the DHT/T ratio, reflecting 5α-reductase activity, in muscle and seminal vesicles of male mice but increased liver DHT levels. Given the possible association between androgens and certain liver diseases such as nonalcoholic fatty liver disease (nowadays referred to as metabolic dysfunction-associated steatotic liver disease) and hepatocellular carcinoma [155, 156], we speculated that the increased hepatic DHT levels following HFD-induced obesity could contribute to these disorders. Future studies should investigate the associations between sex steroid levels in metabolically active tissues such as WAT and metabolic disorders with the aim to potentially identify novel therapeutic targets.

5.3 IMPLICATIONS

Several common diseases such as osteoporosis, breast cancer, prostate cancer, and cardiovascular diseases are a global public health concern and account for considerable morbidity and mortality. Epidemiological studies indicated that these diseases are associated with circulating sex steroid levels. Consequently, accurate measurement of serum sex steroid levels using assays with high specificity and sensitivity is crucial for adequate patient care [4, 73, 75]. As discussed previously, the methods used need to be accurate over a large concentration range with different sensitivities needed for specific patient populations (Table 1). Also, as we have shown in this thesis, it is important to consider that circulating levels of sex steroids may not always reflect sex steroid metabolism within the target tissues. This is especially relevant when assessing serum sex steroid levels for diagnostic purposes or verification of treatment responses in patients, as the corresponding tissue sex steroid levels are largely not available for ethical reasons.

In this thesis, we have developed and validated an MS method to enable comprehensive sex steroid profiling in rodent serum and tissues. The ability to map sex steroid synthesis and metabolism at the level of target tissues in animal models of sex steroid-dependent disorders will aid in progressing research on sex steroid physiology and pathophysiology. Sex steroid-dependent diseases are initially driven largely by gonadal sex steroids. Understanding how these active androgens and estrogens are also synthesized from adrenal precursors and metabolized at the target tissue level is, therefore, critical, as these pathways are primary targets for treatment of these diseases [8, 45]. Our multicomponent assay with the ability to measure several analytes in a single run, even closely related sex steroids, provides the opportunity to investigate the local sex steroid conversions in more detail and permits complex interpretations when combining the results from several sex steroid target tissues. In this regard, our detailed atlas of tissue sex steroid levels will surely assist the sex steroid research community in their future endeavors.

6 CONCLUSION

The ability to accurately and reproducibly measure a broad panel of sex steroids is essential for patient care as well as for progressing research within the fields of sex steroid-dependent diseases. With the concept of intracrinology, the formation of active sex steroids from adrenal precursors locally in target tissues, generally recognized, there is a need for sensitive and specific methods to accurately quantify sex steroid levels in serum and tissues of rodent models for sex-steroid dependent diseases.

In this thesis, we have developed and validated a highly sensitive MS method to measure a comprehensive sex steroid profile in both human and rodent serum. In a next step, we optimized our method for the measurement of sex steroid levels in endocrine, reproductive, and major body compartment tissues. We have shown distinct patterns of sex steroid levels between gonadal intact and castrated mice, characterized the sex steroid profile according to estrous cycle phase in rodents, and provided an atlas of sex steroid levels in a variety of mouse tissues. Finally, we used our established assay to map the tissue distribution of sex steroids following diet-induced obesity in male mice.

Altogether, these studies have provided novel insights in sex steroid distribution in male mice with the role of Prog to be further elucidated. Given the importance of local sex steroid synthesis and metabolism for sex steroid action in target tissues, our developed assay may aid in identifying novel therapeutic targets and improved diagnosis and prevention of sex steroid-related disorders.

7 FUTURE PERSPECTIVES

To be able to compare sex steroid levels over time and between different methods, standardization of assays is urgently needed, especially when MS-based methods will become routine clinical practice. External validation against reference methods, currently developed for serum T [130] and E2 [157] by the Centers for Disease Control and Prevention or other external quality schemes, needs to be performed. In addition, uniform reference ranges for MS assays that are age- and sex-specific need to be established across the range of E2 and T concentrations in the various clinical populations to aid in the identification of specific biological events such as the stage of puberty, menstrual cycle, hypogonadal state, or menopause. This will allow accurate assessment of the effects of sex steroid levels on disease risk and patient survival in epidemiological studies. In the long term, this may lead to establishing threshold levels of sex steroids to identify patients at risk for disease, and simultaneously increasing the diagnosis, treatment, and prevention.

MS-based methods for measuring sex steroids are currently predominantly used in research settings as well as in selected clinical laboratories. Switching from immunoassay-based methods to MS is not always feasible for laboratories because of financial issues and the fact that the sex steroid immunoassays are often part of commercial multiplex platforms providing fast results. Nevertheless, accurate and standardized sex steroid assays based on MS methodology and harmonized reference ranges are needed in both research and clinical care of sex steroid-related diseases [158].

An obvious pathway to investigate is whether our assay can be optimized further. One significant improvement could be reducing the sample volume required for sex steroid analysis while still maintaining assay sensitivity. This would especially benefit sex steroid profiling in rodent samples given the limited volume of terminal blood obtainable. Another way to optimize our method is by expanding the panel of analytes that is measured in a single run. This would provide an even more comprehensive picture of the various intracrinology pathways present in peripheral target tissues. This could aid in identifying novel therapeutic targets for sex steroid-dependent diseases: characterizing the pathways and enzyme isoforms used by malignant tissues to synthesize their own hormones will help identify potential enzyme inhibitors and/or explain the occurrence of drug resistance [8, 45]. One important aspect

to keep in mind is that competing pathways may exist for the synthesis of active steroids in malignant tissues and that inhibition of one pathway may result in compensation by another. Measuring the tissue sex steroid profiles in animal models of hormone-dependent diseases may assist in the process of finding the relevant enzyme targets.

Finally, an important next step would be to increase the sensitivity of our assay to enable quantification of estrogens in male mice. The sensitivity can be refined by using the best chromatography instrumentation, improved sample preparation, and new improved and more sensitive MS technology. Also, derivatization of estrogens before quantification by LC-MS/MS is considered to improve sensitivity to achieve the low LLOQ targets as it increases the ionization efficiency [159].

8 RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

1. Ohlsson C, **Nilsson ME**, Tivesten A, Ryberg H, Mellström D, Karlsson MK, Ljunggren Ö, Labrie F, Orwoll ES, Lee DM, Pye SR, O'Neill TW, Finn JD, Adams JE, Ward KA, Boonen S, Bartfai G, Casanueva FF, Forti G, Giwercman A, Han TS, Huhtaniemi IT, Kula K, Lean ME, Pendleton N, Punab M, Vanderschueren D, Wu FC, EMAS Study Group, Vandenput L. Comparisons of immunoassay and mass spectrometry measurements of serum estradiol levels and their influence on clinical association studies in men. J Clin Endocrinol Metab, 2013; 98(6):E1097-102.

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5. Colldén H, **Nilsson ME**, Norlén A-K, 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.

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