PhD Thesis

Androgen Patterns in Polycystic Ovary Syndrome (PCOS)

Submitted by

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Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of „Good Scientific Practice“.

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3. Abbreviations

ACN	acetonitrile
ACTH	adrenocorticotropic hormone
ADA	American Diabetes Association
AES	Androgen Excess Society
AMH	anti-Mullerian hormone
ASRM	American Society of Reproductive Medicine
AUC	area under curve
BMI	body mass index
BMP	bone morphogenic protein
CAH	congenital adrenal hyperplasia
Calib	calibrator
CI	confidence intervals
CNV	copy number variation
CV	coefficient of variation
CYP11A1	cytochrome P450 family-11, subfamily-A polypeptide-1
DCM	dichloromethane
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid

E1	estradiol
E2	estrone
ELISA	enzyme linked immunosorbent assay
ESHRE	European Society of Human Reproduction and Embryology
FAI	free androgen index
FAM	6-carboxyfluorescein
FSH	follicle stimulating hormone
GC-MS/MS	gas chromatography tandem mass spectrometry
GDF9	growth and differentiation factor 9
GnRH	gonadotropin-releasing hormone
HbA1c	glycated hemoglobin
HC	hip circumference
HDL	high-density lipoprotein
HE	heterozygote
HO	homozygote
HOMA-IR	homeostatic model assessment insulin resistance
HPLC	high performance liquid chromatography
HSD	hydroxysteroid dehydrogenases
HWE	Hardy-Weinberg equilibrium
IGT	impaired glucose tolerance
INHA	inhibin alpha
IQR	interquartile range
IR	insulin resistance

ISTD	internal standard
IVD	in-vitro diagnostic
kb	kilobases
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL	low-density lipoprotein
LH	luteinizing hormone
LLOQ	lower limit of quantification
MAF	minor allele frequency
ml	millilitres
mRNA	messenger ribonucleic acid
mm HG	millimetres of mercury
MS	mass spectrometry
n	number
n.a.	not applicable
NCEP	(U.S.) National Cholesterol Education Program
NICHD	National Institute of Child Health and Human Disease
NIH	National Institutes of Health
ng	nanogram
nm	nanometer
oGTT	oral glucose tolerance test
OSM	on-line SPE manager
PCR	polymerase chain reaction

PCOS	Polycystic ovary syndrome
POF	premature ovarian failure
QC	quality control
QUICKI	Quantitative Insulin-sensitivity Check Index
r^2	coefficient of determination
RIA	radioimmunoassay
ROC curves	receiver operating characteristics curves
SD	standard deviation
SHBG	sex hormone binding globulin
SNP(s)	single nucleotide polymorphism(s)
SPE	solid phase extraction
STAR	steroidogenic acute regulatory protein
T2DM	type 2 diabetes mellitus
TGFB	transforming growth factor beta
THF	tetrahydrocortisol
TT/DHT ratio	testosterone to dihydrotestosterone ratio
UK	United Kingdom
USA	United States of America
WC	waist circumference
WHR	waist to hip ratio
WT	wildtype
yr	year(s)

5α-THF	5α-tetrahydrocortisol
17OHP	17α-hydroxyprogesterone
µl	microliter

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6. Abstract in German

Einleitung: Das polyzystische Ovarien-Syndrom (PCOS) ist die häufigste endokrine Erkrankung von Frauen. Zahlreiche klinische Phänotypen mit unterschiedlichen Hormonspiegeln und Symptomen können unterschieden werden. Die genauen Ursachen und pathologischen Hintergründe sind bis heute noch nicht vollständig geklärt. Ziel dieser Dissertation war es, eine umfassende genetische und biochemische Analyse in einer großen, gut charakterisierten Kohorte von PCOS-Patientinnen durchzuführen.

Methoden: Für die Analyse von betroffenen Steroidhormonen wurden zwei unterschiedliche Methoden von Liquid-Chromatographie-Massenspektometrie (LC-MS/MS) durchgeführt. Hierfür wurden ein kommerziell verfügbarer Kit (SteroIDQ, Biocrates, Innsbruck, Österreich) und eine in einem universitären Zentrum für Massenspektrometrie (Manchester Academic Health Science Centre, University Hospital South Manchester, Vereinigtes Königreich) entwickelte Methode für Steroid-LC-MS/MS angewendet. Weiters wurde auf Basis der LC-MS/MS Messergebnisse eine umfassende Analyse der Testosteron-zu-Dihydrotestosteron Ratio (TT/DHT Ratio) und deren Einfluss auf den Hormon- und Glukosestoffwechsel bei PCOS-Patientinnen untersucht. Als weitere Untersuchungsebene wurde der Einfluss von genetischen Polymorphismen von Kandidatengenen des PCOS auf verschiedene Stoffwechsel-Parameter bei PCOS-Patientinnen evaluiert.

Ergebnisse: Die Verwendung des kommerziell erhältlichen Kits SteroIDQ zur massenspektrometrischen Quantifizierung mehrerer Steroidhormone erwies sich als unzureichend für unsere Fragestellungen. Die an der Universität Manchester erhobenen Steroid-Messergebnisse zeigten deutliche Unterschiede zwischen PCOS-Patientinnen und gesunden Kontrollen für Testosteron ($p<0.001$), Androstendion ($p<0.001$), und DHEA ($p<0.001$), aber nicht für Dihydrotestosteron ($p=0.072$). Außerdem wiesen PCOS-Patientinnen eine signifikant höhere TT/DHT Ratio ($p<0.001$) im Vergleich zu gesunden Kontrollen auf. Innerhalb der PCOS-Kohorte wurden signifikante Korrelationen der TT/DHT Ratio mit diversen anthropometrischen Parametern und Parametern des

Glukose-, Lipid- und Hormonstoffwechsels gefunden, die die TT/DHT Ratio als neuen Risikoparameter bei PCOS auswiesen.

Eine Genvariante des Inhibins (rs12720062) zeigte bei heterozygoten Genotypen „AG“ eine signifikante Assoziation mit Messwerten für LH (p=0.042), AMH (p=0.033), ACTH (p=0.022), Cortisol (p=0.042), Testosteron (p=0.004), Androstendion (p=0.005) und freiem Testosteron (p=0.007).

Schlussfolgerung: In der vorliegenden Dissertation wurde eine große Kohorte von PCOS-Patientinnen biochemisch und genetisch charakterisiert und neue Forschungsansätze validiert. Die neu eingeführte TT/DHT Ratio, basierend auf MS-Steroidmessungen, kann als neuer Risikoparameter für metabolische Veränderungen bei PCOS angesehen werden.

7. Abstract in English

Introduction: The polycystic ovarian syndrome (PCOS) is the most common endocrine disorder of women. Various clinical phenotypes with differences in hormone levels and symptoms can be distinguished. The exact pathologic background still needs to be elucidated. The aim of this study was to perform a comprehensive genetic and biochemical analysis in a large and well characterised cohort of PCOS patients.

Methods: For the analysis of steroid hormones, two different approaches of liquid chromatography tandem mass spectrometry (LC-MS/MS) were performed. A commercially available kit (SteroIDQ, Biocrates, Innsbruck, Austria) and a specific MS method at a centre for LC-MS/MS (Manchester Academic Health Centre, University Hospital South Manchester, United Kingdom) were applied. Based on the LC-MS/MS results, a comprehensive investigation of the testosterone to dihydrotestosterone ratio (TT/DHT ratio) and its impact on the hormone and glucose metabolism in PCOS patients was performed. In addition, the influence on several genetic polymorphisms in candidate genes of PCOS on various metabolic parameters in PCOS patients was investigated.

Results: The commercially available SteroIDQ kit for mass spectrometrical quantification of several steroid hormones was not suitable for our research questions. Results from LC-MS/MS measurements at the University of Manchester showed significant differences between PCOS patients and healthy controls for testosterone ($p<0.001$), androstenedione ($p<0.001$) and DHEA ($p<0.001$), but not for dihydrotestosterone ($p=0.072$). Moreover, a significantly higher TT/DHT ratio was assessed in PCOS patients compared to healthy controls. Within PCOS patients, significant correlations of the TT/DHT ratio with various anthropometric parameters and parameters of the glucose, lipid and hormone metabolism were found, therefore considered as a new risk parameter for metabolic changes in PCOS.

Genetic polymorphism of inhibin (rs12720062) showed significant associations of the heterozygous genotype “AG” with LH ($p=0.042$), AMH ($p=0.033$), ACTH ($p=0.022$),

cortisol (p=0.042), testosterone (p=0.004), androstenedione (p=0.005) and free testosterone (p=0.007).

Conclusion: In this thesis, a large cohort of PCOS patients was characterized for biochemical and genetic parameters and new biomarkers have been validated. The new TT/DHT ratio based on MS steroid measurements will open us a new risk parameter for metabolic changes in PCOS patients.

8. Introduction

8.1. The Polycystic Ovary Syndrome

The polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age [100]. Depending on which diagnostic criteria are applied, the prevalence varies from 6 to 20% [33, 69]. In 1935, the first systematic characterization of the syndrome was performed by Stein and Leventhal, who described seven women affected by diverse clinical characteristics (i.e. acne, hirsutism, amenorrhea, obesity) in combination with bilateral polycystic ovaries [115].

8.1.1. Definition of PCOS

PCOS is a complex endocrine condition with heterogeneous symptoms and no clear consensus concerning the key criteria of PCOS exists. So far, several diagnostic criteria for PCOS have been proposed. The first official recommendation for diagnosing PCOS was proposed in 1990 by the National Institutes of Health (NIH) and National Institute of Child Health and Human Disease (NICHD) of the United States of America (USA) [156]. According to the NIH criteria, the combination of hyperandrogenism (biochemical and/or clinical), oligo/anovulation and the exclusion of other related syndromes, such as congenital adrenal hyperplasia (CAH), androgen secreting tumors, hyperprolactinemia, primary ovarian insufficiency, acromegaly, etc., are needed for the diagnosis of PCOS. Since polycystic ovaries have not been included in the NIH criteria, a revision was performed at a meeting of the American Society of Reproductive Medicine (ASRM) and the European Society of Human Reproduction and Embryology (ESHRE) in Rotterdam, Netherlands, in 2003. The criteria for the diagnosis of PCOS were expanded to include any two of three key features of PCOS: biochemical and/or clinical hyperandrogenism, ovulatory dysfunction and polycystic ovaries [100]. This definition of PCOS is referred to as the so called “Rotterdam criteria”. Another modification of the diagnostic criteria was proposed in 2006 by the Androgen Excess Society (AES), which

states that clinical and/or biochemical hyperandrogenism and either polycystic ovaries and/or ovarian dysfunction are needed for the diagnosis of PCOS [8]. Again, other diseases with similar clinical characteristics have to be excluded. Based on the criteria selection, different phenotypes of PCOS patients can be distinguished (Table 1).

Table 1. Overview about all possible PCOS phenotypes based on the presence of hyperandrogenism, polycystic ovaries, oligo-/anovulation and hirsutism (adapted from [9]).

Potential Phenotypes	Hyperandogenism	Polycystic Ovaries	Oligo-/ anovulation	Hirsutism	NIH Criteria	Rotterdam Criteria	AES Criteria
A	✓	✓	✓	✓	✓	✓	✓
B	✓	-	✓	✓	✓	✓	✓
C	✓	✓	✓	-	✓	✓	✓
D	✓	-	✓	-	✓	✓	✓
E	-	✓	✓	✓	✓	✓	✓
F	-	-	✓	✓	✓	✓	✓
G	✓	✓	-	✓	-	✓	✓
H	-	✓	-	✓	-	✓	✓
I	✓	✓	-	-	-	✓	✓
J	-	✓	✓	-	-	✓	-
K	✓	-	-	✓	-	-	-
L	-	✓	-	-	-	-	-
M	-	-	✓	-	-	-	-
N	-	-	-	✓	-	-	-
O	✓	-	-	-	-	-	-
P	-	-	-	-	-	-	-

8.1.2. Aetiology

The aetiology of PCOS is still not quite understood. It is estimated, that PCOS is not the consequence of a single gene defect, but rather the result of a polygenic interaction together with environmental factors [26, 135]. Familiar clustering of PCOS cases and high heritability of clinical and biochemical features provide evidence for a genetic background of PCOS [35, 61]. A wide range of possible candidate genes have been proposed, however, genome wide association studies and case-control cohort studies failed in elucidating the genetic background until present [56].

8.1.3. Pathogenesis

Like the aetiology of PCOS, the pathogenesis is also poorly understood. Because of the heterogeneous character of the syndrome, multiple pathophysiological mechanisms are possible (Figure 1).

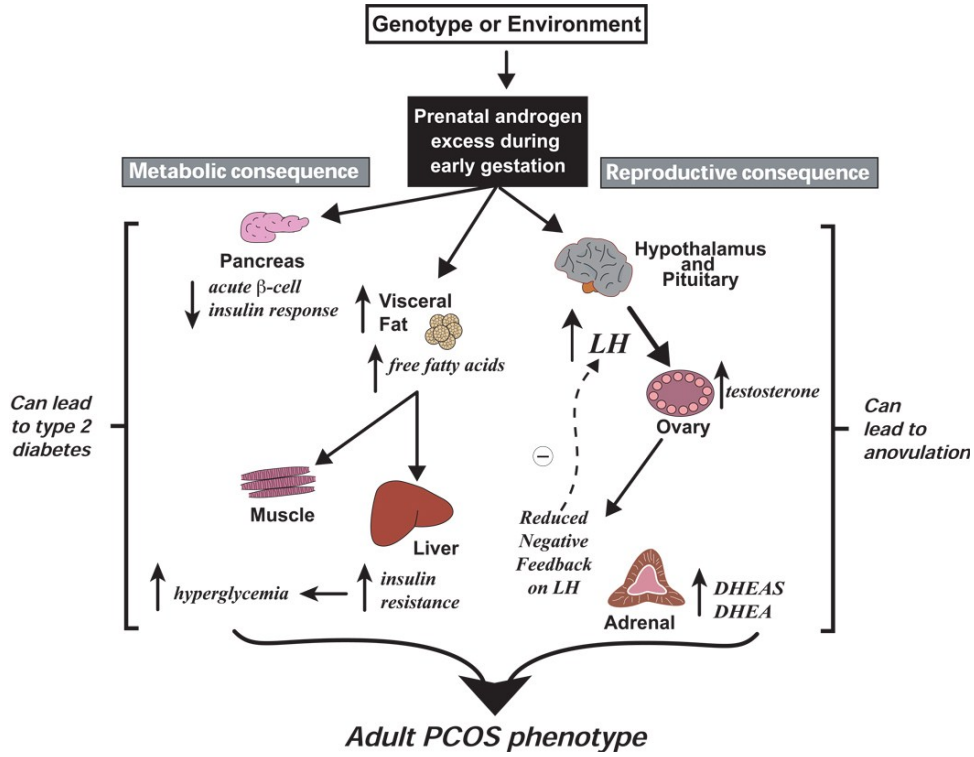

Figure 1. Diagrammatic overview about factors involved in the development of PCOS [27]. Androgen excess leads to a number of metabolic and reproductive consequences and subsequently to the adult phenotype of PCOS.

8.1.4. Gonadotropins

Increased secretion of luteinizing hormone (LH) is a typical characteristic for PCOS. LH is produced by the pituitary gland and released in a pulsatile manner. In PCOS patients, pulse frequency and amplitude are both elevated, resulting in elevated circulating LH levels [15]. Hypersecretion might be a consequence of elevated pulses of hypothalamic gonadotropin-releasing hormone (GnRH), regulating LH secretion. Elevated LH levels lead to an increased ratio of LH to follicle stimulating hormone (FSH) (LH/FSH ratio: >2) [140].

8.1.5. Hyperinsulinemia and Insulin Resistance

Hyperinsulinemia is defined as an excess in circulating insulin levels in the blood compared to glucose levels, whereas in insulin resistance cells lose their ability to respond to insulin. It is characteristic for early onset of type 2 diabetes mellitus (T2DM) and also frequently seen in PCOS patients (60-80%) [62], and can easily be assessed via an oral glucose tolerance test. It is linked to several metabolic conditions, such as obesity, hypertension, glucose intolerance and dyslipidemia [75]. Women suffering from PCOS are at high risk for developing T2DM by the age of 40 [129]. Nevertheless, insulin resistance is also frequent in lean PCOS patients, providing evidence that obesity alone cannot be the only underlying cause for insulin resistance [28]. Furthermore, insulin enhances proliferation and thus androgen secretion of ovarian theca cells by increasing the activity of CYP17A1, a key enzyme in androgen production [80]. It also reduces circulating sex hormone binding globulin (SHBG) levels, which in cause leads to a rise in free circulating testosterone levels [91].

8.1.6. Hyperandrogenism

Increased levels of LH in combination with hyperinsulinemia stimulate androgen production in the ovaries. Furthermore, reduced SHBG levels lead to an elevated amount of free circulating steroids. SHBG levels are even lower in obese PCOS patients compared to lean women with PCOS, thus resulting in higher androgen levels in obese PCOS patients [86].

Increased hair growth is one visible feature of hyperandrogenism, primarily occurring on chin and face in a male pattern hair growth. Other clinical symptoms for hyperandrogenism are oily skin, acne, androgenic alopecia or acanthosis nigricans (thickening and darkening of skin areals, especially in skin folds). The most common used method for assessing hirsutism is the modified Ferriman-Gallway score (Figure 2) [42, 71].

In contrast, scalp hair loss in a male pattern is characteristic for androgenic alopecia in PCOS women and based on an increased sensitivity of hair follicles to

circulating androgens [44]. However, hyperandrogenism does not necessarily lead to androgenic alopecia [51].

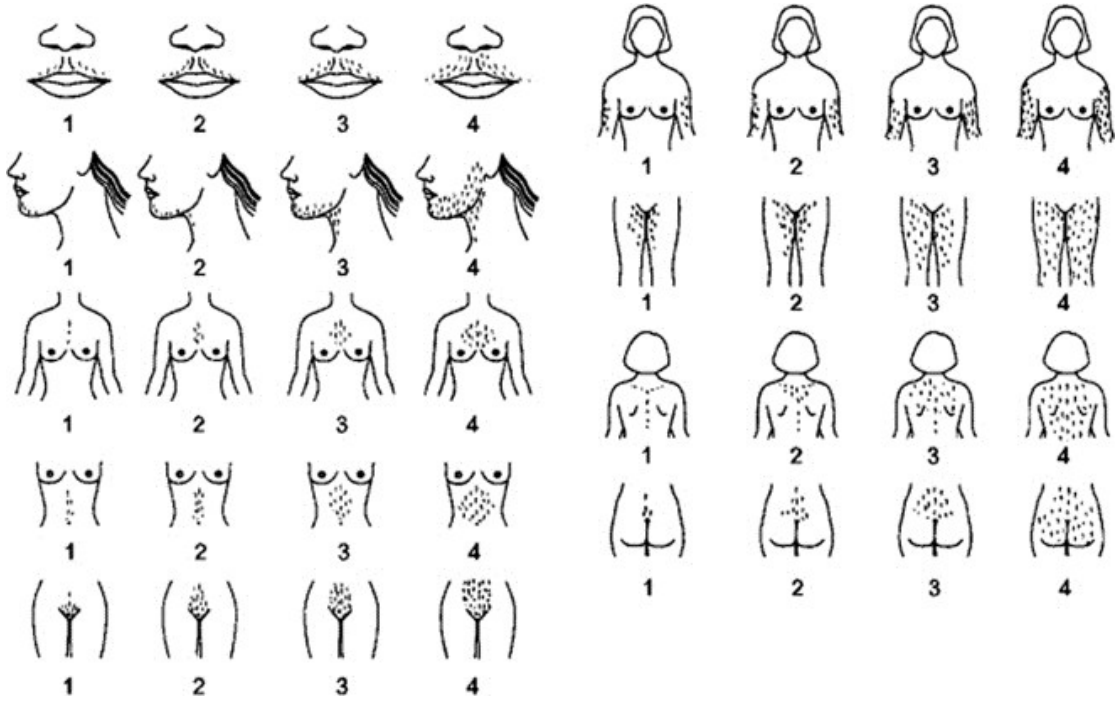

Figure 2. Modified Ferriman-Gallway scoring system for assessing hirsutism [116]. A score of ≥8 is considered as pathologic.

8.1.7. Obesity

Approximately 50% of all PCOS women are overweight or obese [86]. Weight loss can improve insulin sensitivity and glucose tolerance, thus ameliorating metabolic disturbances and improving ovulatory and fertility rates [92]. It can also decrease pulse amplitude of LH production and in turn reducing androgen production [48].

8.1.8. Ovarian Dysfunction and Infertility

Two of the three key symptoms needed for the diagnosis of PCOS according to the Rotterdam criteria are menstrual abnormalities and polycystic ovaries. These cysts can be detected via ultrasound as follicle cysts in the cortex of the ovaries. These cysts represent

follicles that are unable to undergo ovulation. Increased serum androgen levels stimulate the ovary and lead to the development of a polycystic morphology. Only 5-10% of all PCOS patients have regular menses, whereas about 20% demonstrate a complete absence of ovulation, also called amenorrhoea [21]. In overweight and obese PCOS patients, anovulation and menstrual abnormalities are more frequent compared with normal-weight PCOS women [86]. When undergoing assisted reproduction, PCOS women are at high risk for developing an ovarian hyperstimulation syndrome because of the high number of follicles in the cortex of their ovaries [72].

8.1.9. Metabolic Disturbances

According to the Third Report of the U.S. National Cholesterol Education Program (NCEP) Expert Panel, metabolic syndrome is diagnosed when at least three of the following features can be assessed: waist circumference >88cm, elevated systolic and diastolic blood pressure levels (above >130 mm Hg and >85 mm HG, respectively, elevated fasting glucose levels (>110 mg/dl) or prevalent T2DM, blood triglyceride levels above 150 mg/dl and/or HDL cholesterol levels above 50 mg/dl [79].

Many features of the metabolic syndrome, i.e. dyslipidemia, obesity, insulin resistance, hypertension and increased cardiovascular risk, are also frequently present in PCOS. Female and even male first-degree relatives of women diagnosed with PCOS show an increased risk for metabolic complications [11, 101, 132].

8.1.10. Dyslipidemia

Dyslipidemia, abnormal high levels of blood lipids, are common in PCOS patients compared to weight-matched controls [147, 149]. Higher triglycerides, higher high-density lipoprotein (LDL) levels, but lower high-density lipoprotein (HDL) cholesterol levels are found in PCOS women, independent of body mass index (BMI) [148].

8.1.11. Cardiovascular Disease Risk

Several clinical studies provided evidence that women with PCOS show a higher prevalence of classical and non-classical risk factors for cardiovascular diseases [150]. Increased intima-media thickness of the carotid artery and coronary artery calcifications are more frequent in PCOS women, even after adjusting for BMI and age [23, 109, 122].

Based on the increased risk of PCOS women for cardiovascular diseases, patients should be routinely controlled for anthropometric parameters (waist circumference (WC), hip circumference (HC), weight and BMI), blood pressure (systolic and diastolic) and biochemical parameters (insulin and glucose levels, serum lipid levels).

8.1.12. Management of PCOS

So far, no single therapy for PCOS has been found. Treatment remains symptomatic and tailored individually to each patient, depending on symptoms, metabolic characterization and on individual side effects. Most frequent used therapeutic approaches are lifestyle interventions, weight loss, oral contraceptive pills and insulin sensitizers (i.e. metformin). Especially weight loss has shown to improve metabolic and cardiovascular features, menstrual cycle aberrations and to reduce circulating androgen levels [2, 77, 84].

8.2. Steroid Hormones

Steroid hormones are a large group of biochemical compounds that are all synthesized from the same precursor, cholesterol. Hence, they all share a closely related structure. They are secreted from three “steroid” glands: the adrenal cortex, gonads (testes and ovaries) and during pregnancy also from the placenta. Additionally, other tissues like adipose tissue, skin, muscles and liver, are also capable of deriving steroid hormones from precursory.

After secretion, steroid hormones are transported through the bloodstream to target cells and tissues where they bind to specific receptors in order to regulate a variety of physiological and developmental functions [31].

Based on the receptors that bind steroid hormones, five groups can be distinguished: glucocorticoids (i.e. cortisol), mineralocorticoids (aldosterone), androgens (testosterone, dihydrotestosterone, androstenedione), estrogens (estrogen, estrone) and progestins (progesterone) [73]. Glucocorticoids and mineralocorticoids are produced in the liver, whereas androgens, estrogens and progestins are produced in the gonads. The glucocorticoids affect carbohydrate, lipid and protein metabolism and are also included in various vital functions such as inflammatory reactions and stress response. Mineralocorticoids regulate the salt and water balance by the kidneys. Androgens and estrogens regulate the sexual development and function and fertility, and progestins regulate menstrual cycle and pregnancy.

8.2.1. Biosynthesis and Endocrine Regulation

The common precursor of all steroids, cholesterol, is mostly taken up by diet and only a small part of its supply comes from intracellular *de novo* synthesis from acetate, primarily in the endoplasmatic reticulum of the cell [93]. Bound to LDL, lipophilic cholesterol is transported into the mitochondria of the cell via endocytosis. Within the mitochondria, cholesterol is transported by the protein steroidogenic acute regulatory protein (STAR). This transfer from the outer to the inner membrane of the mitochondria is the rate-limiting step in steroid biosynthesis.

In the inner mitochondria membrane, cholesterol can then be further metabolized to prognenolone by the enzyme cytochrome P450 family-11, subfamily-A polypeptide-1 (CYP11A1). Since this conversion is based on a side-chain cleavage, the enzyme CYP11A1 is also referred to as P450scc (scc as acronym for side-chain cleavage). Prognenolone itself can further be enzymatically converted to 17α-hydroxyprogesterone or progesterone, which themselves can also be further metabolized to other steroids.

The cytochrome P450 family comprises a large number of enzymes that regulate the steroid hormone metabolism. The human genome encodes for 57 different CYPs that are located in the mitochondria or endoplasmatic reticulum of the cell. Another family of metabolic enzymes is called hydroxysteroid dehydrogenases (HSDs). The conversion of testosterone to its metabolite dihydrotestosterone is catalysed by the enzyme 5α-reductase, which might be involved in PCOS (see section 8.2.3).

Many enzymes catalyse more than one reaction and are therefore involved in several functions. An overview about the biosynthesis pathway of steroid hormones and the different enzymes involved in this system are presented in Figure 3.

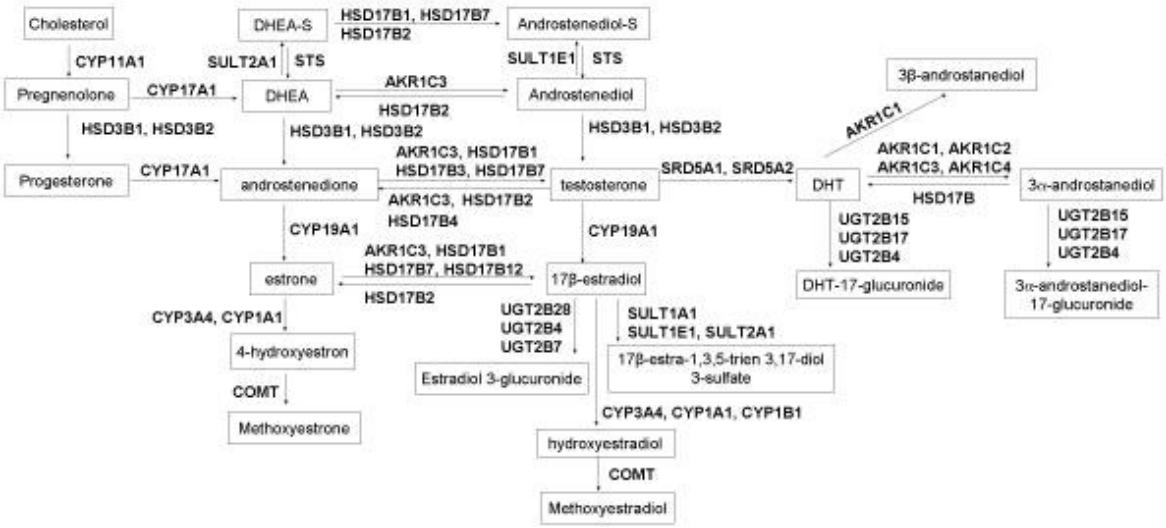

Figure 3. Overview of the steroid hormone metabolism and enzymes involved in biosynthesis [118].

8.2.2. Production and Regulation of Steroid Hormones in the Ovary

In women, circulating androgens are produced by the cortex of the adrenal gland and the ovaries [5]. DHEA is mainly secreted by the adrenal gland, whereas other androgens like androstenedione or testosterone are mainly produced by special cells within the ovary.

The ovary is a major source of steroid hormones in women (Figure 4). It is responsible for the nutrition and growth of follicles. Each ovarian follicle contains one oocyte surrounded by granulosa cells and in later, activated stages of follicular development also two layers of theca cells (an inner and an outer theca cell layer) [67]. In follicles, theca cells are the exclusive producer of androgens.

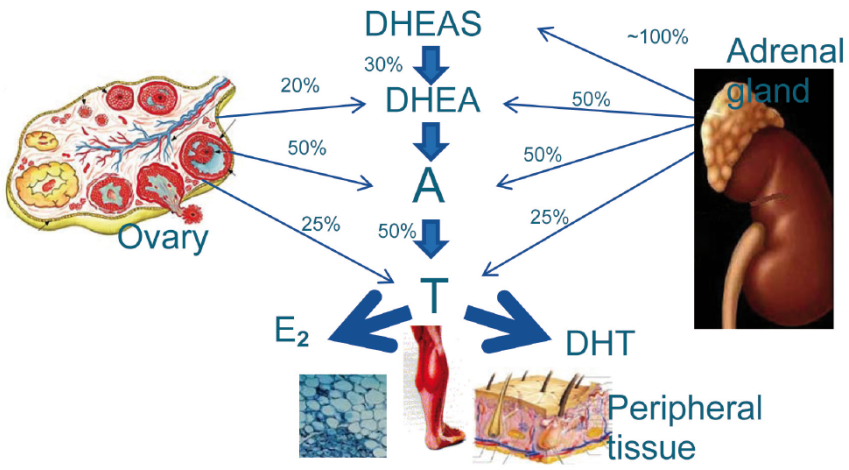

Figure 4. Estimated percentages of steroid hormone production in ovaries, adrenal gland and peripheral tissues in women [34].

As soon as follicles develop from the primordial to antral stage (Figure 5), granulosa cells start to proliferate and theca cells get recruited from the surrounding stromal layer to the follicle. In more developed stages (preantral follicle), granulosa cells start secreting various cytokines such as bone morphogenic proteins (BMPs), activins, inhibins, growth and differentiation factor 9 (GDF9) and transforming growth factor beta (TGFB) in order to control androgen production in theca cells. In response to luteinizing

hormone (LH), theca cells produce and secrete androgens like testosterone or androstenedione [46]. In theca cells, most key enzymes of the steroid hormone biosynthesis pathway are expressed, except the enzyme aromatase, responsible for the production of estrogens. The granulosa cells of the ovary convert androgens into estrogens by aromatization. The enzyme involved in this step is member of the cytochrome P450 family (CYP19A1), but also called aromatase. Its production and activity is regulated by the follicle stimulating hormone (FSH).

One important player in theca cell function and activity is insulin. In several in vitro studies, an increase of steroid hormone production and upregulated activity of key enzymes such as STAR, CYP11A1 or CYP17A1 was demonstrated [18, 78, 112, 151] (Figure 6).

Moreover, members of the TGF-beta superfamily are also important regulators of steroid hormone metabolism, follicle stimulating hormone production and follicular growth and development [155]. This group of proteins includes activins, inhibins, anti-Mullerian hormone (AMH) and BMPs. All of them are produced by the granulosa cells of the ovarian follicles and nearly all have been described as being differently expressed in PCOS in numerous studies [30, 87, 98, 104, 106, 117, 127, 145]. An antagonist of BMPs and activin, called follistatin, is also described as being involved in disturbed steroid hormone metabolism in PCOS patients [81]. Differences in follistatin expression in PCOS patients might be based on genotype variations [17, 49, 85, 130, 133, 134]. Differences in inhibin and activin levels in follicular fluid between PCOS patients and healthy women have also been found [68]

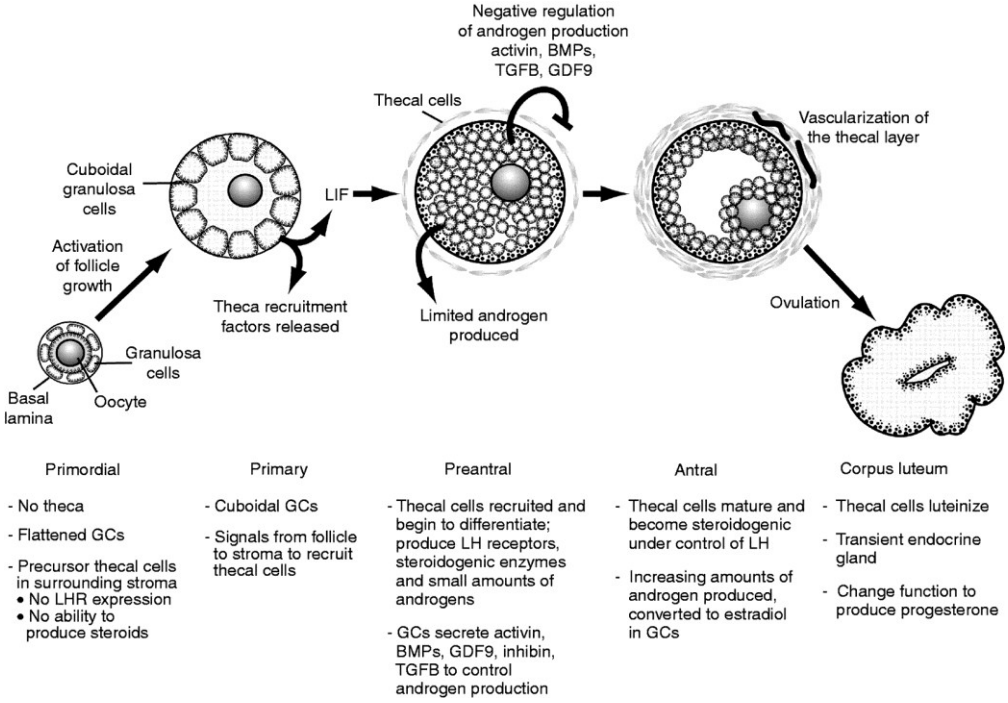

Figure 5. Cellular structure of different stages in follicular development and overview of secreted factors during folliculogenesis [155].

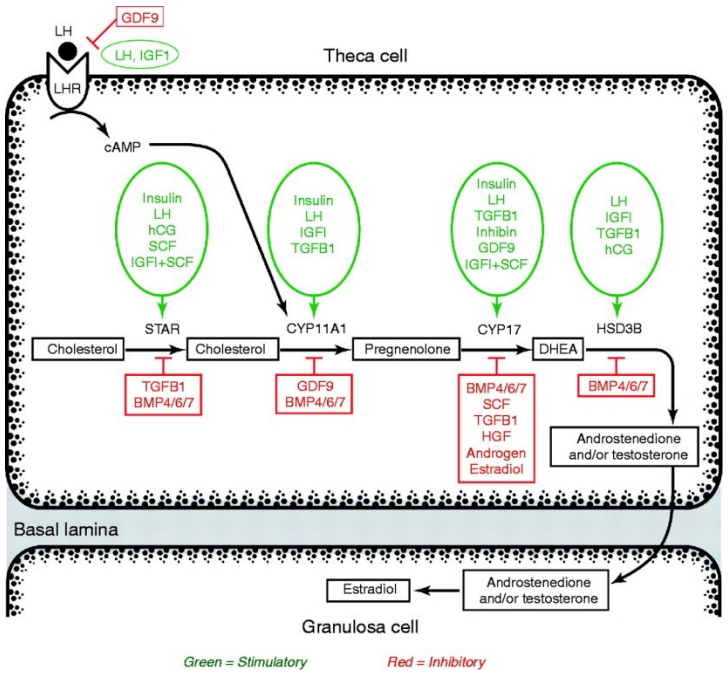

Figure 6. Modulation of key enzymes in steroid hormone biosynthesis. Stimulatory factors are listed in green and inhibitory factors are presented in red [155].

Inhibin

Inhibins are glycoproteins secreted by the granulosa cells of ovarian follicles [14]. They consist of an α-subunit linked to another α-subunit or a β-subunit. Thus, the resulting dimer is either called inhibin A or Inhibin B. When two β-subunits dimerize, the resulting protein is called activin. Both forms can suppress the release of FSH by the pituitary [25]. Inhibin B is produced by pre-antral and small-antral follicles; hence, serum levels are highest in midfollicular phase and decline in late follicular phase [60].

Previous studies about inhibin in PCOS showed conflicting results [4, 24, 59, 89, 127, 144]. In follicular fluids of PCOS women, inhibin A concentrations were found to be lower compared to healthy controls, whereas inhibin B levels seem to be similar [68]. However, anovulatory PCOS women showed significantly higher inhibin B serum levels and the inhibin B response to ovulation induction in clomiphene treatment in artificial reproduction differs to normo-ovulatory women [65, 123].

Follistatin

Follistatin, sometimes also called activin-binding protein, is a glycoprotein expressed in a variety of tissues, including ovaries, adrenal gland and pituitary [131]. Based on alternative splicing and/or posttranslational modification, three isoforms can be distinguished: FS-288, FS-300 and FS-315. It has the ability to inhibit FSH secretion from the pituitary and blocks folliculogenesis and aromatization of steroids [133]. Administration of follistatin to transgenic mice resulted in infertile mice with blocked folliculogenesis and small ovaries [37].

In PCOS patients, higher follistatin levels were found [17, 30, 124]. In previous linkage studies, the follistatin gene has been identified as a candidate gene for the aetiology of PCOS [6, 22, 64, 102, 133, 134].

8.2.3. 5α-Reductase and PCOS

According to the criteria and recommendations for the diagnosis and management of PCOS, testosterone is the most important measurement for identifying androgen excess [7]. However, a recent study by O'Reilly *et al.* provided evidence that biochemical hyperandrogenism might be missed if only serum testosterone is measured [82]. It was demonstrated that also androstenedione is an important steroid hormone in androgen excess, since PCOS patients with normal testosterone but elevated androstenedione are at high risk for metabolic diseases.

Testosterone can be produced directly by the ovaries and the cortex of the adrenal gland, but also by conversion of precursors in peripheral tissues. Like androstenedione, testosterone can also be metabolized to other steroid hormones, involving the activity of distinct genes and enzymes. One key enzyme in testosterone metabolism is 5α-reductase, responsible for the conversion of testosterone to dihydrotestosterone. It is also the catalyser for the metabolism of progesterone to 5α-progesterone and for the conversion of tetrahydrocortisol (THF) to 5α-tetrahydrocortisol (5α-THF) in the kidneys.

5α-reductase, short for 3-oxo-5α-steroid 4-dehydrogenase, is expressed in many tissues in both sexes [10]. Three isoforms can be distinguished: 5α-reductase type 1, type 2 and type 3, encoded by the genes SRD5A1, SRD5A2 and SRD5A3, respectively. However, it is not possible to distinguish between the 5α-reductase isoforms when measuring urinary steroid metabolites [126]. Differentiation between isoforms is only possible via gene expression analysis, since they are all encoded by a different gene.
At the moment, it is not possible to directly measure the systemic effect of the enzyme activity in patients; only measuring gene expression in distinct tissues or via calculating the ratio of serum or urinary hormones to their metabolites, converted by this enzyme, can be performed.

Several studies propose a role of 5α-reductase in the pathogenesis of PCOS [12, 126, 138, 142]. However, there are some discrepancies in the results concerning 5α-reductase activity, which might be based on differences in the used techniques for assessing 5α-reductase activity and/or different sample sizes.

In PCOS patients, 5α-reductase activity was only measured in tissue samples, i.e. subcutaneous adipose tissue [142] or skin tissue [111], or by calculating the ratio of THF to its metabolite 5α-THF in urine samples [128, 138]. Quantifying gene expression levels in tissue samples allows the differentiation between 5α-reductase isoforms; however, gene expression might be variable in different tissues and organs. Therefore, gene expression analysis can only provide information about the gene activity in a distinct tissue and might not be representative for the systemic effect or activity.

8.3. Quantification and Analytics of Steroid Hormones

High-quality assays for the quantification of steroid hormones are crucial for all epidemiological studies. The quality of an assay results from its sensitivity, specificity and the reproducibility of results. Different methods for quantification of steroid hormones are available, but all differ in sensitivity and specificity. Hence, comparison of results from different approaches for quantification is often difficult. Moreover, no gold standard exists to achieve an objective assay validation and maximal quality control [114]. This lack of standardization leads to varying results from epidemiological studies and might lead to false conclusions.

Nowadays, steroid hormone concentrations are mostly measured with immunoassays or chromatography based mass spectrometry.

8.3.1. Immunoassays

The common molecular basis of all immunoassays is an antibody-antigen-interaction used for quantification. Although they have some disadvantages, immunoassays are still the most often used method for steroid hormone quantification in clinics. They are relatively cheap, easy to perform and results can be obtained very quickly.

Radioimmunoassays (RIAs) were the first type of commercial available immunoassay. The first quantified molecule was insulin, followed by LH, FSH and estrogen [1, 83, 153]. In RIAs, radioactive labelled antigens compete with the natural antigens within the test sample for the binding sites of antibodies. High levels of natural antigens in the test sample lower the ratio of bound radioactive antigens. After incubation, unbound antibodies will be washed away and radioactivity gets measured in a gamma counter.

RIAs are highly specific and are available for automated platforms, but need careful handling and authorized laboratories due to the radioactivity.

A nonradioactive variant of immunoassays is enzyme-linked immunosorbent assay (ELISA). Again, antibodies are used for the quantifications of antigens like steroid hormones. Antigens from a test sample get attached to a surface (microtiter plate) and specific enzyme-linked antibodies are allowed to bind to the antigens. Then, a solution containing the substrate for the enzyme will be added, that causes occurrence of a signal, visible as a change of colour. Finally, this signal will be measured and amount of antigen in the test sample calculated.

Nevertheless, immunoassays can be impaired by several problems, such as unspecific antibodies, macroanalytes, high dose effect, cross reactivity with other factors, patient group specific interferences and influencing binding proteins. In samples with low steroid hormone concentrations, such as testosterone levels in female serum samples, these issues can be even more problematic and lead to wrong and overestimating results. Therefore, the Endocrine Society recommends using the more precise and specific mass spectrometry for steroid hormone quantification [38, 99]. It is estimated that the near future, liquid chromatography mass spectrometry (LC-MS/MS) and/or gas chromatography mass spectrometry (GC-MS/MS) will replace immunoassays in clinical research [53]. The Endocrine Society even stated that it will be mandatory to measure steroid hormones with mass spectrometry for submission of research articles by January 2015 [38].

8.3.2. Mass Spectrometry

GC-MS/MS and LC-MS/MS are highly specific and powerful methods for the quantification of molecules such as steroid hormones. First, ionization if the analyte in an ionization source is performed that leading to fragmentation. The resulting ions are separated based on their mass-to-charge ratio in a mass analyser. Separated ions are measured in the detector and results will be visualised on a chart.

Since mass spectrometry (MS) methods are based on the mass and charge of the analyte, high specificity and sensitivity can be achieved. Issues affecting immunoassay results do not disturb mass spectrometry measurements. Furthermore, only small

amounts of test samples are needed and several steroid hormones (multiplex analysis) can be measured within a single assay.

In the last decades, LC-MS/MS has become more often used than GC-MS/MS [19, 95, 108], since it often needs only smaller sample volume and less time for sample preparation, since derivatisation is not always necessary [19, 141]. Therefore, LC-MS/MS can easily be used for large-scale and automated approaches [58]. Nevertheless, chromatographic separation is not as good as it is in GC-MS/MS, which can make it difficult to separate similar analytes from each other [58].

When MS results are compared to immunoassay measurements, quantified steroid hormone levels are generally lower in MS approaches, based on the unspecific character of immunoassays. Thus, new diagnostic cut-off values for mass spectrometric measurements of steroid hormones are needed. However, there are also differences in results from MS methods. Inter-laboratory variations exist, based on differences in the type of internal standard and calibrator, derivative preparation and in the types of instruments [114]. Therefore, quantification protocols used for mass spectrometry have to be developed well and should be evaluated carefully. Moreover, MS is highly complex, needs special equipment and well trained personnel, and is more expensive than immunoassays [53]. Therefore, this technique is usually only available in larger specialised institutes with sufficient resources and expertise.

8.4. Genetic Analyses

Differences among people are mainly based on genomic variability. Genetic differences can range from changes of single nucleotides to large chromosomal abnormalities. Variations can occur as single nucleotide polymorphisms (SNPs), transposable elements, variable numbers of tandem repeats (short DNA sequences of 2 to 6 pairs are repeated several times in the genome; i.e. microsatellites), copy number variations (CNVs; abnormal numbers of copies of one gene or even whole DNA sections) and structural aberrations, such as duplications, insertions (additional base pairs included in the DNA), deletions (parts of the DNA or even of a chromosome are missing) or inversions (rearrangements of chromosome parts, based on double strand breaks of the DNA). Depending on the location of the aberration, its size and nature, transcriptional regulation can be affected and result in benign or pathogenic phenotypes [39].

In this thesis, genomic variation in terms of single nucleotide polymorphism and copy number variations among PCOS patients were investigated.

8.4.1. Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphisms, or SNPs, are differences of one single nucleotide in the genetic code and represent the most common type of genetic variation in humans. SNPs occur approximately every 300 nucleotides in the genome [16]. It is estimated that the human genome contains about 10 million different SNPs. They are the leading cause for diversity among humans.

A variation is called a SNP, if more than 1% of a population carries a different nucleotide at a specific position in the DNA sequence (Figure 7). Polymorphisms with a frequency of less than 1% within a population are called mutations.

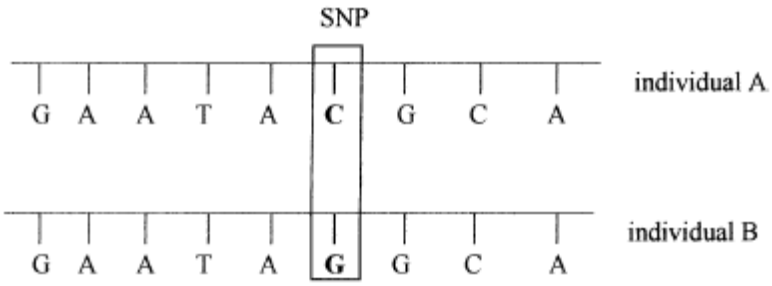

Figure 7. Diagrammatic explanation of differences in DNA code based on single nucleotide polymorphism among two individuals [105]. Individual A and B differ in one single nucleotide at a distinct locus in their genomes.

Although SNPs might not be the causative factor leading to a disorder, many variations have been associated with various diseases and different kinds of cancers. A prominent example for a missense SNP is the *factor V Leiden* mutation, which causes increased blood clotting (hypercoaguability) [13].

Depending on the location of the polymorphism and its effect on protein function, different kinds of SNPs can be distinguished. Synonymous SNPs are variations in the genome that do not alter the encoded amino acid sequence, whereas non-synonymous SNPs change the amino acid sequence [54]. As a consequence SNPs can alter the activity of promotors, conformation of DNA and mRNA and can have a direct effect on the phenotype of the individual carrying the polymorphism [57, 66, 90]. However, most SNPs are located in the non-coding regions of the genome and therefore have no impact on the phenotype of a person, but are of high interest for evolutionary studies [50].

TaqMan Genotyping

TaqMan 5'3' exonuclease assays are a simple but effective method for SNP genotyping, optimal for high-throughput genotyping approaches. Each assay contains two probes that consist of minor groove binder molecules labelled with distinct fluorescent dyes (VIC or FAM), and also two primers in order to detect the SNP of interest (Figure 8). The fluorophores are themselves marked with a quencher molecule at their 5’-ends, which inhibit their fluorescence. During polymerase chain reaction (PCR), the labelled

probe binds to the DNA sequence of the target gene and gets cleaved off by the polymerase. The quenchers get separated from the fluorophores, leading to fluorescence. This fluorescence can be detected. For genotyping approaches, each assay contains two probes, each labelled with a different fluorophore. Therefore, different types of fluorescence measured after the PCR reaction can be used for discriminating between genotypes.

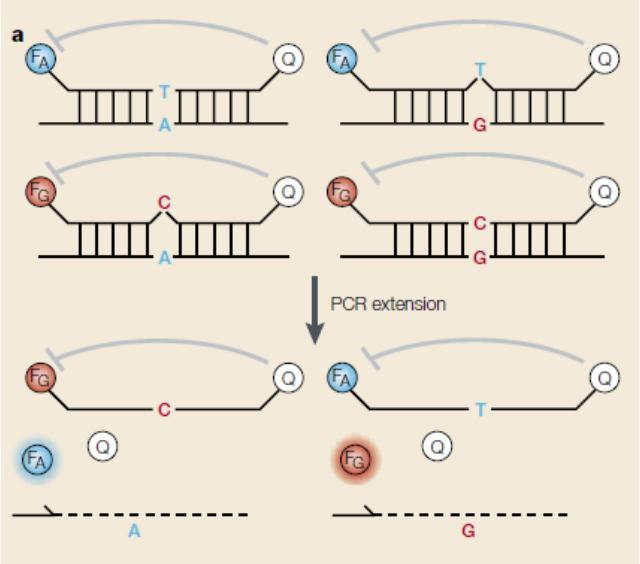

Figure 8. Principle of TaqMan genotyping assays. A quencher (Q) inhibits activity of a fluorophore (F). Each genetic variant (nucleotide) is labelled with a different fluorophore [119]. Cleavage of the hybridized probe during PCR reaction leads to fluorescence.

9. Aims of the Thesis

The aetiology of PCOS still needs to be elucidated. Women suffering from PCOS typically have a variable pattern of different androgens of ovarian or adrenal origin, and vary in their phenotypic appearance. This might be based on a different genetic background and thereby provide insight in the aetiology of PCOS.

We hypothesize that differences in steroid hormone expressions and in ratios of steroid hormone levels lead to different phenotypes and that these phenotypes are based on different genetic variants, e.g. in steroid metabolism.

The aim of the present theses was to perform comprehensive biochemical and genetic analysis in a large cohort of diagnosed and well characterized PCOS patients. Therefore, this thesis is structured into three major parts:

First, performing a comprehensive biochemical steroid hormone profiling in PCOS patients, including several additional steroid hormones that are usually not used for characterizing PCOS patients.

Second, determining the role of the testosterone to dihydrotestosterone (TT/DHT) ratio in PCOS patients and investigating its impact on steroid hormone and glucose metabolism.

Third, analysis of single nucleotide polymorphisms (SNPs) of genes involved in the regulation of steroid hormone metabolism.

10. Material and Methods

10.1. Steroid Hormone Profiling

10.1.1. High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS)

The SteroIDQ Kit (Biocrates, Innsbruck, Austria) is a CE-marked in-vitro diagnostic (IVD) test for steroid hormone quantification based on high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). It is intended for steroid profile screening in serum patient samples, as it allows the simultaneous quantification of 16 steroid hormones within one single assay. Following steroids can be measured: aldosterone, androstenedione, androsterone, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS), estradiol (estradiol-17 beta, E2), estrone (E1), etiocholanolone, 17α-hydroxyprogesterone, progesterone and testosterone. In our approach, dihydrotestosterone (DHT) was additionally quantified. With each Kit a total number of 80 serum samples can be analysed in combination with 16 calibrators and quality controls. Reagents provided within the Kit were stored at -20°C and plates at room temperature until used.

Serum samples of 272 PCOS patients, diagnosed according to the Rotterdam criteria [100], previously recruited at the outpatient clinics of the Department of Endocrinology and Metabolism at the Medical University of Graz and long-time stored at -80°C, were analysed. Procedures were performed according to the manufacturer's recommendations, as described below.

Preparation of Reagents

The internal standard (ISTD) was dissolved with 1200 µl methanol (Sigma-Aldrich Chemie GmbH, Germany), followed by 20 seconds of vortexing. Isotopes as internal standard are presented in Table 2. Dried calibrator matrix was dissolved with 10 ml of distilled water and vigorously shaking. The 7 different calibrators were dissolved with 40 µl, followed by 20 seconds of vortexing. Concentration of target steroid analytes provided in calibrators is presented in Table 3. Afterwards, 1180 µl calibrator matrix was added into each vial of ISTD and calibrators, followed by carefully vortexing. The 3 different types of quality controls (QC1, QC2, QC3) were prepared by dissolving in 600 µl distilled water and vigorously shaking.

Table 2. Internal standards used for assignment of analytes.

Analyte	Internal Standard
Aldosterone	d7-Aldosterone
Androstenedione	d3-Androstenedione
Androsterone	d4-Androsterone
Corticosterone	d8-Corticosterone
Cortisol	d4-Cortisol
Cortisone	d7-Cortisone
11-Deoxycorticosterone	d8-17α-Hydroxyprogesterone
11-Deoxycortisol	d5-11-Deoxycortisol
DHEA	d4-E1
DHEAS	d5-DHEAS
Estradiol (E2)	d3-E2
Estrone (E1)	d4-E1
Etiocholanolone	d4-Androsterone
17α-Hydroxyprogesterone	d8-17α-Hydroxyprogesterone
Progesterone	d9-Progesterone
Testosterone	d5-Testosterone
Dihydrotestosterone	d5-Testosterone

Table 3. Concentrations (ng/ml) of target analytes provided with SteroIDQ calibrators.

Analyte	Calib 1	Calib 2	Calib 3	Calib 4	Calib 5	Calib 6	Calib 7
Aldosterone	0.05	0.1	0.2	0.4	1.5	3	5
Androstenedione	0.032	0064	0.13	0.64	2.4	4.8	8
Androsterone	0.06	0.12	0.24	0.48	1.8	3.6	6
Corticosterone	0.03	0.12	0.6	2.4	9	18	30
Cortisol	1	4	20	80	300	600	1000
Cortisone	0.1	0.4	2	8	30	60	100
11-Deoxycorticosterone	0.03	0.12	0.6	1.2	4.5	9	15
11-Deoxycortisol	0.01	0.04	0.2	0.8	3	6	10
DHEA	0.12	0.24	0.48	2.4	9	18	30
DHEAS	32	64.0	128	640	24	4800	8000
Estradiol	0.02	0.08	0.4	1.6	6	12	20
Estrone	0.03	0.12	0.6	1.2	4.5	9	15
Etiocholanolone	0.06	0.12	0.24	0.480	1.8	3.6	6
17OHP	0.05	0.2	1	4	15	30	50
Progesterone	0.06	0.12	0.24	1.2	4.5	9	15
Testosterone	0.01	0.04	0.2	0.8	3	6	10

Conditioning of the Solid Phase Extraction (SPE) Plate

The solid phase extraction plate (SPE) was placed on a vacuum chamber for 96-well plates. For conditioning of the SPE, 1 ml of methanol was loaded on each well and was allowed to slowly run through the SPE by application of smooth vacuum, followed by 1 ml of distilled water to clean the SPE. Waste solvents were discarded after each step.

Preparation of Samples

Serum samples were stored frozen at -80°C until quantification. 500 µl serum was used for determination of the serum steroid profile. Before samples, calibrators (Calib) and quality controls (QC) can be loaded on the SPE plate, they need to be prepared on the 2 ml 96-well deep well plate. The layout used for preparation is given in Figure 9.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Double Blank	Blank			QC 2							
B	Calib 1	QC 1										
C	Calib 2	QC 2								QC 2		
D	Calib 3	QC 3										
E	Calib 4											
F	Calib 5						QC 2					
G	Calib 6											
H	Calib 7											QC 2

Figure 9. Plate layout used for preparation of serum samples, calibrators, quality controls in 2ml deep well plates.

500 µl of serum samples, calibrators and quality controls were loaded on the deep well plate. First, calibrator matrix was loaded on wells A1 and A2 as double blank and blank. Freshly prepared calibrators (Calib 1 – 7) were pipetted on wells from B1 to H1, and freshly prepared quality controls were loaded on well B2 (QC 1), wells C2, A5, F7, C10, H12 (QC 2) and well D2 (QC 3) to ensure reproducibility of analysis. Serum samples were pipetted into the 80 remaining empty wells. As a next step, 10 µl of the ISTD was added to each well with a multistep pipette, except the one for double blank (well A1). For the dilution of samples, 400 µl of distilled water was added to each well, followed by mixing of the samples by carefully pipetting up and down three times. Finally, the content of the deep well plate was transferred onto the conditioned SPE plate for solid phase extraction.

SPE Procedure

Samples were allowed to run slowly through the SPE plate by gravitation. A smooth vacuum was applied to achieve a flow rate of about 1-2 drops per second. Waste solvents were discarded. 500 µl of distilled water was then used to wash the plate. Again, a smooth vacuum was applied. Waste solvents were discarded, and SPE plate was dried with a nitrogen drying system. Full vacuum and nitrogen flow at 4 bars were applied and the plate was dried for at least 1 hour.

Dichloromethane (DCM) Extraction

Dichloromethane (DCM) (Sigma-Aldrich Chemie GmbH, Germany) was used for the elution of all steroids except DHEAS, as it cannot be extracted with DCM. Therefore, 500 µl of DCM was pipetted into each well of the SPE plate and DCM was allowed to drop through the SPE bed by gravitation into a clean deep well plate. After 15 minutes, a short vacuum was applied to ensure DCM has completely passed the SPE bed. The received DCM extract was dried for about 20 minutes under nitrogen flow at 4 bars and 50°C. Another 500 µl of DCM was added onto each well of the receiving deep well plate, followed by drying of extracts under nitrogen flow as described before. Dried content was afterwards carefully reconstituted with 50 µl of 40% methanol in distilled water, and deep well plate was afterwards sealed with a silicon mate. Plate was centrifuged at 50 x g for about 1 minute before it was placed into the autosampler tray for analysis.

Acetonitrile (ACN) Extraction

Acetonitrile (ACN) (Sigma-Aldrich Chemie GmbH, Germany) was used to elute DHEAS out of the SPE bed. Before extraction, SPE plate was placed on clean paper towels to ensure there would be no contamination with remaining DCM droplets. Afterwards, another clean deep well plate was put under the SPE bed and 600 µl of ACN were pipetted into each well of the SPE plate for elution of DHEAS. A smooth vacuum was applied. Received extracts were diluted with 400 µl distilled water and content of each well was carefully mixed by pipetting three times. The plate was sealed with a silicon mate, centrifuged at 50 x g for about 1 minute and placed into the autosampler tray for analysis.

HPLC-MS/MS Analysis

Chromatographic separation was performed on Ultimate 3000 Rapid Separation LC Systems (Thermo Scientific) and quantification of steroids on an Exactive Benchtop LC/MS Orbitrap Mass Spectrometer (Thermo Scientific) by the experienced staff of the HEALTH – Institut für Biomedizin und Gesundheitswissenschaften of the Joanneum

Resarch Forschungsgesellschaft mbH (Graz, Austria), under the supervision of Mag. Dr. Christoph Magnes. Quantification was performed according to manufacturer's recommendations.

Assessing Stability and Reproducibility

To examine the exactness of the Biocrates kit and whether frequent freeze and thaw cycles can influence the stability of steroid hormone levels in mass-spectrometric analysis, serum samples of eight healthy male and eight healthy female volunteers were analysed. These control serum samples were frozen at -80°C and up to ten freeze/thaw cycles were performed before measuring via mass-spectrometry.

10.1.2. Immunoassays

Beside mass spectrometry, ELISAs were also used for the quantification of androstenedione, 17α-hydroxyprogesterone (DiaMetra, Biovendor, Brno, Czech Republic), and DHEAS (LDN Labor Diagnostika Nord GmbH, Nordhorn, Germany) with intra- and inter-assay variations below 10%. SHBG levels were analysed by luminescence assays (Roche, Basel, Switzerland) with intra- and inter-assay coefficients of variations (CVs) of less than 3%. Testosterone levels were also assessed via luminescence (Siemens, Erlangen, Germany), with inter- and intra-assay CVs of 4.7% and 6.2%, respectively.

10.1.3. Statistics

Descriptive statistics were performed. Results from the HPLC-MS/MS analysis were compared to previous measurements using ELISAs and luminescence immunoassays. Statistical analyses were performed using Graphpad Prism 16 and SPSS 19.0 (SPSS inc., Chicago, IL, USA) and p-values <0.05 were considered being significant. Data are presented as mean ± standard deviation (SD) unless otherwise stated.

10.2. Determining the Role of the Testosterone to Dihydrotestosterone (TT/DHT) Ratio in PCOS Patients

10.2.1. Study Population

In this study, 275 premenopausal, well characterized PCOS patients, previously enrolled in the prospective PCOS cohort study (E. Lerchbaum, B. Obermayer-Pietsch) at the outpatient clinics of the Department of Endocrinology and Metabolism, Medical University of Graz, were included. PCOS was diagnosed according to the Rotterdam ESHRE 2004 criteria [100]. Disorders with a similar clinical presentation, such as Cushing's syndrome, congenital adrenal hyperplasia (CAH), hyperprolactinemia or androgen secreting tumors, were excluded for all patients. Therefore, specific laboratory analysis of distinct hormone parameters [prolactin, cortisol, adrenocorticotropic hormone (ACTH) 17α-hydroxyprogesterone] was applied. Clinical and/or biochemical hyperandrogenism was assessed by the presence of hirsutism (Ferriman-Gallway score ≥8), acne, alopecia and/or elevated serum androgen levels. The modified Ferriman-Gallway score was used for quantifying hirsutism. Polycystic morphology of the ovaries was examined by ultrasound and anovulation and/or oligoovulation were defined by the presence of anovulation and/or oligomenorrhea.

In addition to PCOS patients, 35 BMI-matched, healthy and premenopausal controls were also included in this study. Healthy controls were enrolled at the Medical University of Graz in the NOTHYS study (nitric oxidase in thyroid diseases, B. Obermayer-Pietsch, H. Gruber). Furthermore, a normative cohort of 38 healthy and premenopausal women that were age-matched with the PCOS cohort was enrolled at the University of South Manchester, United Kingdom. This normative cohort was used to compare and validate results from the study cohort of the Medical University with another group of healthy women.

For all controls and PCOS patients, strong exclusion criteria were applied. Exclusion criteria were as follows: systemic illnesses, previous intake of anti-androgen drugs or any drugs known to affect endocrine parameters, carbohydrate metabolism,

serum lipid profile or hepatic P450 enzymes, pregnancy or intake of oral contraceptives with the last three months before study entry.

The study protocols were approved by the Ethics Committee (18-066 ex 06/07) of the Medical University of Graz. Written informed consent was obtained from each patient before entering the studies.

10.2.2. Metabolic Characterization

Metabolic characterization of patients was performed by the investigators and the medical staff at the outpatient clinics of the Department of Endocrinology and Metabolism at the Medical University of Graz.

Standard anthropometric data such as height, weight, hip circumference, waist circumference were obtained. Systolic and diastolic blood pressure was measured. The BMI was calculated by dividing weight in kilograms by height in square meters. Normal weight was defined as BMI between 25 and 29.9 kg/m^2 and obesity as BMI above 30 kg/m^2. All patients had to undergo an overnight fast before blood samples for blood samples for basal hormone levels were obtained.

For evaluation of parameters of glucose metabolism, a fasting 75 g oral glucose tolerance test (oGTT) was performed. Blood samples were collected after 30, 60 and 120 minutes and glucose and insulin levels were determined. Impaired glucose tolerance (IGT) or prediabetes was defined according to the American Diabetes Association (ADA) as glucose levels between 140 mg/dl and 199 mg/dl after 120 minutes [3]. Insulin resistance was identified by levels >2.5 in the homeostatic model assessment-insulin resistance (HOMA-IR). It was assessed by the product of fasting insulin (µIU/ml) and glucose levels (mg/dl), divided by 405 [62]. Quantitative insulin sensitivity check index (QUICKI) was calculated as 1/(log(fasting insulin µlU/ml) - log(fasting glucose mg/dl)) [52].

Metabolic syndrome was diagnosed by the presence of at least three of the following criteria: WC above 88 cm, raised blood pressure, such as systolic values >130 mm Hg and diastolic levels >85 mm Hg, triglyceride levels >150 mg/dl, HDL cholesterol

<50 mg/dl, elevated fasting glucose levels (>110 mg/dl) or an already prevalent T2DM. However, patients with persistent T2DM were excluded from the study.

10.2.3. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

275 PCOS patient serum samples and 35 serum samples of healthy, BMI-matched controls were analysed for testosterone, androstenedione, dihydrotestosterone and DHEA using an established routine method for LC-MS/MS at the Department of Clinical Chemistry at the University Hospital of South Manchester (Manchester, United Kingdom (UK)). Anonymized samples were shipped frozen on dry ice within 24 hours to South Manchester and then analysed by the experienced staff of the Department of Clinical Chemistry.

Extraction of steroids was performed by an automated on-line solid phase extraction by an on-line SPE Manager (OSM) (Waters, Manchester, UK). Cartridges were conditioned with methanol and afterwards washed with distilled water. Samples were allowed to run through cartridges and a 35% methanolic wash was performed. For the elution of compounds the first 0.5 minutes of the mobile phase gradient was used. Chromatography was utilised with a 50 x 2.1 mm Acquity HSS SB column (Waters, Manchester, UK) with a 3 minute gradient of 50% to 70% methanolic mobile phase (o.45 ml/min) containing 0.05% formic acid. The time between injections was 6.5 minutes. Steroid analytes were measured via TQ-S tandem mass spectrometer (Waters, Manchester, UK). Following quantifier and qualifier transitions were used: testosterone 289.3>109.0 (289.3>97.0), DHT 291.3>255.1 (291.3>159.0), androstenedione 287.3>109.0 (287.3>97.0) and DHEA 271.3>213.1 (271.3>253.1). Chromatographic separation between all four steroids was achieved with a run time of 6.2 minutes per sample. The calibration lines were linear (r^2>0.99) to at least 14.42 ng/ml for testosterone, androstenedione and DHEA and 1.45 ng/ml for DHT. Lower limit of quantification (LLOQ) was 0.02 ng/ml for testosterone, DHT and androstenedione, and 0.28 ng/ml for DHEA. The between assay imprecision was low with coefficients of variation (CVs) of <6% for testosterone, <10% for DHT, <10% for DHEA, and <7% for androstenedione at concentrations of 0.17 ng/ml, 0.06 ng/ml, 0.95 ng/ml and 0.48 ng/ml, respectively.

10.2.4. Biochemical Analyses

Standard serum parameters used for the metabolic characterization of PCOS patients were immediately determined at the routine lab of the Department of Endocrinology and Metabolism at the Medical University of Graz.

SHBG was quantified by luminescence immunoassay (Roche, Basel, Switzerland) with intra- and inter-assay CVs of 1.3% and 2.1%. Albumin was assessed via photometry (Roche, Vienna, Austria). Cholesterol, triglycerides, LDL and HDL were quantified by Modular Analytics SWA (Roche, Basel, Switzerland) with intra- and inter-assay CVs below 10%. DHEAS was assessed by ELISAs (LDN Labor Diagnostika Nord GmbH, Nordhorn, Germany). 17α-hydroxyprogesterone was also assessed via ELISAs (DiaMetra, Biovendor, Brno, Czech Republic) with intra- and inter-assay CVs below 10%.

10.2.5. Calculations

Free levels of testosterone and dihydrotestosterone that are not bound to any proteins such as SHBG or Albumin were calculated according to the common used formula described by Vermeulen *et al.* [139]. Furthermore, the free androgen index (FAI) was calculated as follows: total testosterone levels (nmol/l) * 100, divided by SHBG levels (nmol/l).

Moreover, the activity of the enzyme 5α-reductase was assessed by dividing total testosterone levels by total dihydrotestosterone levels determined by LC-MS/MS.

10.2.6. Statistics

Distribution of data was evaluated by Kolmogorov-Smirnov test and descriptive statistics. We assessed differences between PCOS patients and control women by performing Mann-Whitney-U test for non-normally distributed independent samples. Two-tailed Spearman rank correlation test was applied for calculating univariate association. Binary logistic regression analyses were used to determine associations of

TT/DHT ratio with IGT (yes/no), metabolic syndrome (yes/no) and insulin resistance by means of HOMA-IR (yes/no).

To determine the optimal cut-off point of the TT/DHT ratio for the diagnosis of PCOS, IGT, insulin resistance as means of HOMA-IR and metabolic syndrome, receiver operating characteristics (ROC) curves were constructed and the Youden index was calculated [154].

BMI and age were used as covariates in all statistical analysis to exclude potential bias. All data are presented as median with interquartile range (IQR) unless otherwise stated. All statistical analyses were performed using SPSS 19.0 (SPSS inc., Chicago, IL, USA) and p-values <0.05 were considered being significant.

10.3. Single Nucleotide Polymorphism (SNP) Genotyping

10.3.1. Isolation of DNA

For SNP genotyping assays of PCOS patients, deoxyribonucleic acid (DNA) was freshly isolated from peripheral blood by using the NucleoSpin Blood method by Macherey-Nagel (Germany) according to manufacturer's protocol.

First, 200 µl of blood were pipetted into a fresh 1.5 ml microcentrifuge tube, followed by an addition of 25 µl Proteinase K. Samples were diluted by adding 200 µl buffer B3. Tubes were vortexed for 20 seconds and incubated at 70°C and shaking at 700 rpm for 20 minutes. Afterwards, 210 µl ethanol were added and tubes vortexed to adjust DNA binding conditions. For binding of DNA to spin columns, lysate was loaded onto NucleoSpin blood columns placed in collection tubes and centrifuged for 1 minute at 11,000 x g. Flow-through was discarded and columns were washed two times with 500 µl washing buffer. Flow-through was discarded after each washing step and residual ethanol was removed by dry centrifugation of columns placed in new collection tubes. For elution of DNA, 100 µl of preheated elution buffer (70°C) were loaded onto the silica membrane of spin columns placed in fresh 1.5 ml microcentrifuge tubes. After an incubation of 1 minute at room temperature, centrifugation of samples at 11,000 x g for 1 minute was performed and eluted DNA samples were stored frozen at -80°C until further used.

10.3.2. Single Nucleotide Polymorphisms (SNPs)

Study Population

A total number of 233 PCOS patients, previously recruited in the PCOS cohort study at the outpatient clinics of the Department of Endocrinology and Metabolism, Medical University of Graz, were included in this study. PCOS patients were selected from the study database of the Department for Endocrinology and Metabolism. DNA samples were genotyped for different single nucleotide polymorphisms as described below.

Follistatin SNP rs11745088

The follistatin polymorphism rs11745088 leads to a C/G transversion substitution in exon 5 of the follistatin gene with a minor allele frequency (MAF) of C <1%. Context sequence is presented in Table 4.

Inhibin SNP rs12720062

The inhibin alpha 769G>A gene variant is located in an exon of the α-subunit and leads to an amino acid change from alanine to threonine at codon 257 (A257T). This inhibin alpha polymorphism has a minor allele frequency of A<1%. Context sequence is presented in Table 4.

Inhibin SNP rs12720061

The polymorphism rs12720061 leads to an intragenic A/G transition substitution in the α-subunit of the inhibin gene. Context sequence is presented in Table 4.

Table 4. Overview of analysed SNPs, the types of polymorphism and context sequences.

Gene	SNP ID	Location	Polymorphism	Amino Acid Change	Context Sequence [VIC/FAM]
Follistatin	rs11745088	Chr.5: 52779510	Missense Mutation, Transversion Substitution, Intragenic	Q152E	TGCACTCCTAAAGGCAAGATGTAAA**[C/G]**AGCAGCCAGAACTGGAAGTCCAGTA
Inhibin	rs12720062	Chr.2: 220439916	Transition Substitution, Missense Mutation, Intragenic	T257A	GCTGCAGAGGCCTCCGGAGGAACCG**[A/G]**CTGCCCATGCCAACTGCCACAGAGT
Inhibin	rs12720061	Chr.2.: 220439826	Missense Mutation, Transition, Substitution	R227G	CACTCGGACCAGACCACCCAGTGGA**[A/G]**GGGAGAGAGCCCGACGCTCAACTCC

SNP Genotyping: 5´3´Exonuclease Assay

Genotyping was performed using 5´3´exonuclease SNP Genotyping Assays (Life Technologies, Invitrogen, Life Tech Austria, Austria). DNA samples were thawn at room temperature shortly before use. 2 µl of DNA were pipetted into 96-well plates, followed by 2.5 µl TaqMan Gene Expression Master Mix (Life Technologies, Invitrogen, Life Tech Austria, Austria), 0.46 µl SNP Genotyping Assay (Life Technologies, Invitrogen, Life Tech Austria, Austria) and 0.04 µl distilled water. Reactions were overlayed with 5 µl mineral oil (Sigma-Aldrich Chemie GmbH, Germany). Plates were sealed with a PCR foil (Thermo Scientific, UK), centrifuged at 1000 rpm for 1 minute and placed into a PCR cycler (Mastercycler gradient, Eppendorf Austria, Austria). Cycler conditions are presented in Table 5.

Table 5. Cycler conditions used for SNP Genotyping.

Temperature	Length	Number of Cycles
95°C	10 min	1
92°C	15 sec	40
60°C	1 min	
4°C	∞	1

As a quality control, one well containing only water instead of DNA was included in each PCR plate as a negative control. More than 40 DNA samples were genotyped twice to check genotyping quality expressed by the number of discrepancies.

Endpoint fluorescence of PCR reactions were measured in a Fluoroskan Ascent plate reader (Thermo Labsystems, Helsinki, Finland). Genotyping assays contained VIC and FAM labelled dyes that emit light in a distinct part of the visible spectrum and can therefore be used for differentiation between genotypes (wildtype (WT), heterozygote (HE), homozygote (HO)). VIC dye has an absorbance maximum of 538 nanometers (nm) and emits light at 520 nm wavelength, whereas FAM dye absorbs light of 485 nm wavelength and emits light of 520 nm wavelength.

After measuring the fluoresce levels, data are exported into an Excel datasheet and displayed as a scatter plot. The relative fluorescence units of the FAM dyes are presented on the x-axis of the plot, and relative fluorescence units of the VIC dyes on the y-axis. Every single point in the plot represents one analysed patient DNA sample.

In the plot, four different clusters of samples can be depicted. The first cluster is located next to the x-axis and represents the patients with a genotype homozygous for FAM, i.e. homozygote mutated genotypes. The cluster next to the y-axis represents the samples homozygote for VIC, which are wildtype, non-mutated DNA samples. The third cluster in the middle of these two clusters represents the DNA samples from patients with the heterozygous mutated genotype. Samples that cannot clearly be assigned to one of these clusters are manually excluded and not taken for analysis. All remaining samples that didn’t show any or too low fluoresce, including the empty control, were grouped into the forth cluster and were also excluded from analysis. The borders between the different clusters are altered manually by the operating researcher. Examples for raw data of fluorescence measurement and for a scatter plot displaying genotype distribution are given in Table 6 and Figure 10.

Table 6. Raw data from VIC and FAM fluorescence measurement.

Sample	VIC	FAM
Blank	0,42	0,85
Blank	0,45	0,87
A03	1,05	3,69
A04	1,36	1,05
A05	1,49	1,1
A06	1,05	3,75
A07	0,49	5,01
A08	1,43	1,07
A09	1,46	1,04
A10	0,97	3,33
A11	1,47	1,06
A12	1,10	3,90

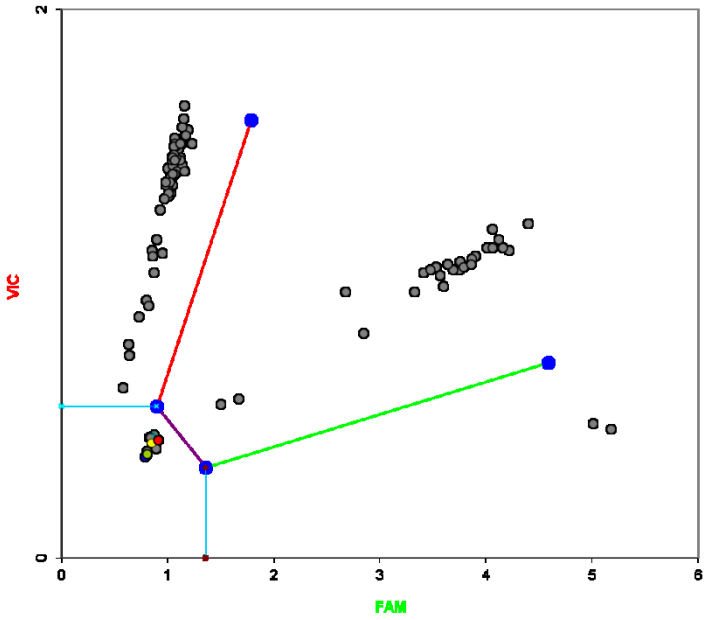

Figure 10. Results from SNP analysis in PCOS patients presented as scatter plot. The cluster next to the y-axis represents the wildtype genotype, the cluster next to the x-axis represents the homozygote mutated genotype and the cluster in the middle represents the heterozygote mutated genotype. The cluster in the left corner contains control samples and excluded samples that could not be assigned to one of the genotype clusters.

The genotype frequency within each analysed PCR plate was compared to the mean genotype frequencies of all analysed samples taken together. Strong deviations from the mean genotype frequencies might be based on mistakes in the PCR setup or analysis of results, thus analysis of genotype distribution in the scatter plot was checked again. If assigning genotypes into the clusters described above was not clear for a PCR plate, the PCR was repeated.

Hardy-Weinberg Equilibrium

As another quality control, the Hardy-Weinberg equilibrium (HWE) was analysed [103]. This theorem declares that genotype and allele frequencies within a population will remain constant from one generation to the next generation, provided there is an absence of influencing factors, such as non-random mating, natural selection, mutations, gene flow or genetic drift. Mutations can disturb the HWE since they change the allele frequency. Without those influencing factors, genotype and allele frequencies would remain constant in equilibrium. Nevertheless, in reality, all these influencing factors usually occur, thus, the Hardy-Weinberg principle can be seen as an idealized principle.

Deviances from the HWE could be the result from a selection bias or indicate an effect of the distinct allele in the population. Therefore, testing for HWE is regarded as an important quality control of SNP analysis and genetic association studies [76].

Within our setup, the HWE was tested with the André Rogatkos method [97].

Statistical Analyses

Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Parametric and nonparametric tests were performed and a p-value <0.05 was considered as being significant.

11. Results

11.1. Steroid Hormone Profiling

The study population consisted of 273 PCOS women fulfilling Rotterdam criteria, with a median age of 26 years and a median BMI of 24.8 kg/m^2. Baseline characteristics of PCOS women are presented in Table 7.

Serum samples were analysed via the HPLC-MS/MS based SteroIDQ kit for the simultaneous quantification of 16 different steroid hormones and dihydrotestosterone, additionally. However, simultaneous quantification of steroid hormones was not effective for all proposed steroids. Strongest problems occurred when determining aldosterone, estradiol and estrone. Since results for these three steroid hormones were in most cases under the lower limit of quantification or not detectable at all, results were not reliable and were not further used. In most samples cortisone levels were higher than the upper limit of quantification; hence, results were not further used. Quantification of 11-deoxycorticosterone resulted in very low serum levels, also often under the lower limit of detection. Additional quantification of dihydrotestosterone was effective for 214 serum samples and failed in the remaining samples due to the very low serum levels for this hormone in female samples.

Table 7. Metabolic characteristics of PCOS study cohort (n=273).

	Median	25th Percentile	75th Percentile
Age (years)	26	22	30
Weight (kg)	69	59	87.9
BMI (kg/m^2)	24.8	21.48	31.6
Fasting glucose (mg/dl)	87	81	93
Fasting insulin (µIU/ml)	7.2	4.2	12.9
SHBG (nmol/l)	39.2	25.02	56.37
LH (IU/l)	7.99	4.48	11.87
FSH (IU/l)	4.93	3.8	6.17

11.1.1. Comparison of Results from HPLC-MS/MS based SteroIDQ Kit and Immunoassays

Differences between results from immunoassays and the SteroIDQ kit were analysed using Spearman correlations, scatter plots and Bland-Altman plots (Figure 11 and Figure 12). Mass spectrometric results were systematically lower compared to immunoassay results (Table 8). Comparison of results showed only weak correlations for androstenedione ($r=0.349$, $p<0.001$), progesterone ($r=0.473$, $p<0.001$) and 17α-hydroxyprogesterone ($r=0.398$, $p<0.001$), moderate correlations for DHEAS ($r=0.696$, $p<0.001$) and testosterone ($r=0.763$, $p<0.001$), but good correlations for cortisol ($r=0.881$, $r<0.001$). Bland-Altman plots show that inter-assay differences increase with rising serum hormone levels of androstenedione, progesterone and 17α-hydroxyprogesterone.

For cortisone, corticosterone, 11-deoxycortisone, 11-deoxycortisol, DHEA, dihydrotestosterone, etiocholanolone and androsterone, no results from immunoassays were available.

A comparison of serum concentrations of quantified hormones using immunoassays and the SteroIDQ kit is presented in Table 8. Statistically significant differences between results from immunoassays and SteroIDQ were assessed via paired sample t-test for testosterone ($p<0.001$), androstenedione ($p<0.001$), progesterone ($p<0.001$), 17α-hydroxyprogesterone ($p<0.001$) and cortisol ($p<0.001$). Only for DHEAS, the difference between results was not statistically significant ($p=0.321$).

Table 8. Differences in quantified steroid hormones in 275 PCOS serum samples using immunoassays and the SteroIDQ kit. A p-value <0.05 was considered being significant.

	Immunoassay			SteroIDQ			
	Median	**25th Percentile**	**75th Percentile**	**Median**	**25th Percentile**	**75th Percentile**	**p-value**
Testosterone (ng/ml)	0.71	0.56	0.85	0.51	0.37	0.69	<0.001
Androstenedione (ng/ml)	3.86	2.84	5.32	2.01	1.56	2.98	<0.001
DHT (ng/ml)	n.a.	n.a.	n.a.	0.16	0.11	0.23	n.a.
DHEA (ng/ml)	n.a.	n.a.	n.a.	8.55	5.44	11.73	n.a.
DHEAS (ng/ml)	2239.5	1560	3015	2172.57	1636.77	2884.16	0.321
Progesterone (ng/ml)	1.62	0.57	4.54	0.12	0.08	0.77	<0.001
17OHP (ng/ml)	1.22	0.80	1.98	0.81	0.51	1.20	<0.001
Cortisol (ng/ml)	154.4	122	190	134.2	106.89	169.96	<0.001

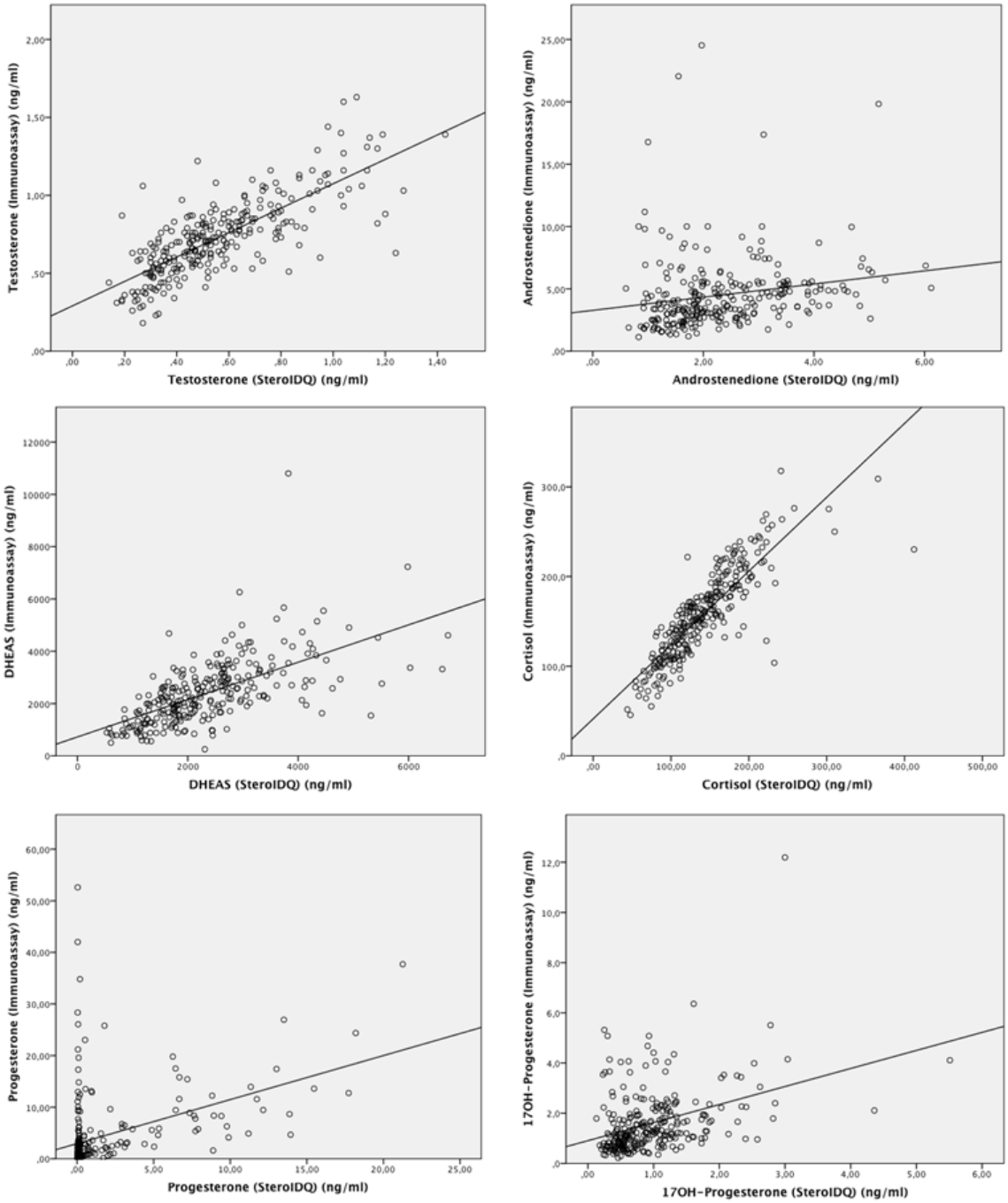

Figure 11. Scatter plots showing inter-assay differences in quantified steroid hormone levels of testosterone, androstenedione, DHEAS, cortisol, progesterone and 17alpha-hydroxyprogesterone.

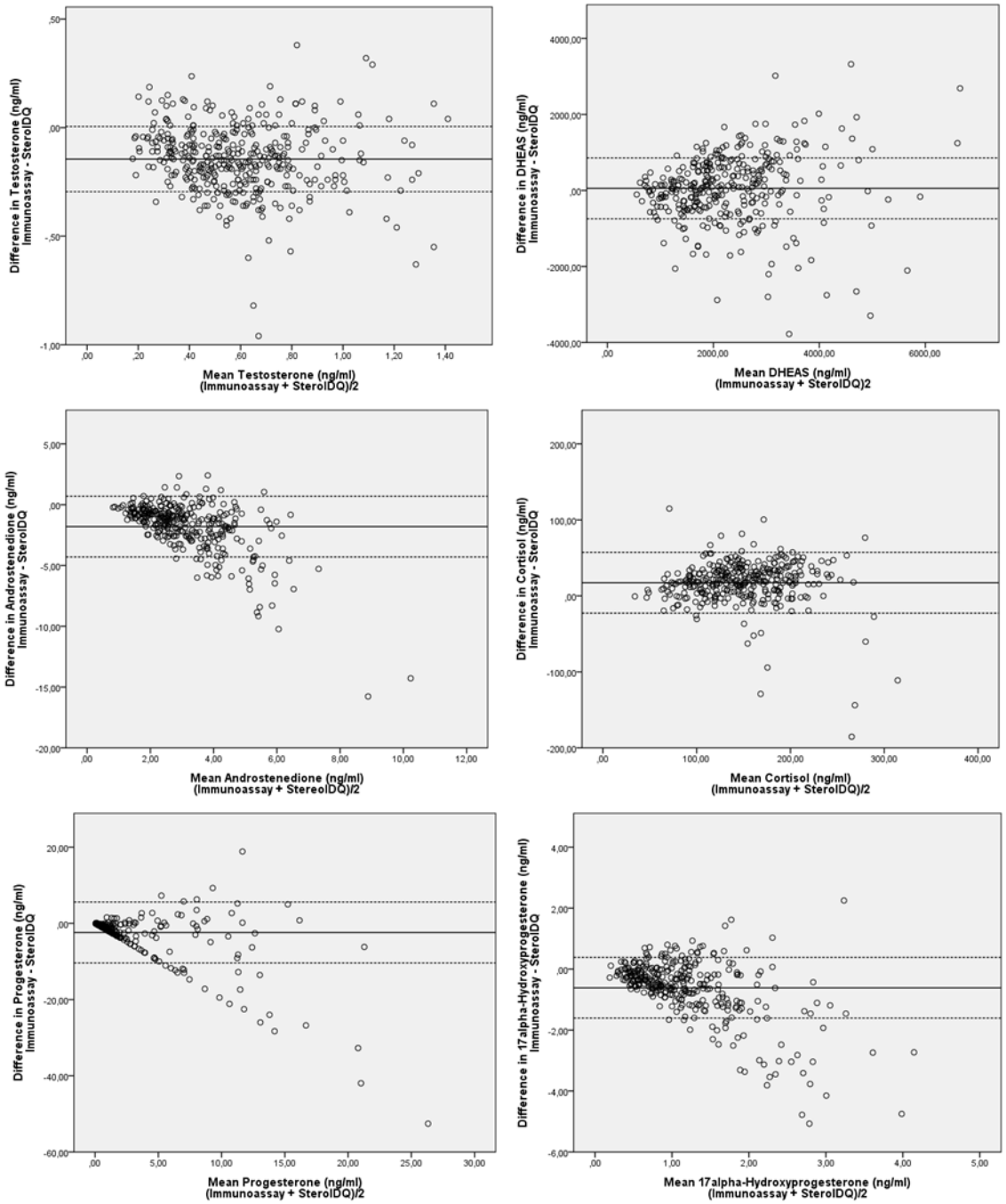

Figure 12. Bland-Altman plots of the differences in serum steroid hormone levels versus the average serum levels measured by the SteroIDQ kit and immunoassay.

11.1.2. Comparison of Results from the HPLC-MS/MS based SteroIDQ Kit and LC-MS/MS

Serum levels of testosterone, androstenedione, dihydrotestosterone and DHEA were analysed using a validated routine method for LC-MS/MS. Spearman correlations of results with previous results from the SteroIDQ kit showed good correlations for dihydrotestosterone ($r=0.726$, $p<0.001$) and DHEA ($r=0.760$, $p<0.001$), and high correlations for androstenedione ($r=0.881$, $p<0.001$) and testosterone ($r=0.867$, $p<0.001$) (Table 9).

Statistically significant differences between results from HPLC-MS/MS based SteroIDQ kit and LC-MS/MS were assessed via paired sample t-test for androstenedione ($p<0.001$), dihydrotestosterone ($p<0.001$) and DHEA ($p<0.001$). Only for testosterone, no significant differences were found ($p=0.887$). Median serum levels of testosterone, androstenedione and DHEA were lower when quantified with the SteroIDQ kit, but dihydrotestosterone levels were slightly higher. A comparison of serum concentrations of quantified hormones using LC-MS/MS and the SteroIDQ kit is presented in Table 9 and Figure 13.

Table 9. Differences in steroid hormone levels of testosterone, androstenedione, dihydrotestosterone and DHEA, quantified using LC-MS/MS and HPLC-MS/MS based SteroIDQ kit. A p-value <0.05 was considered being significant.

	LC-MS/MS			SteroIDQ			
	Median	**25th Percentile**	**75th Percentile**	**Median**	**25th Percentile**	**75th Percentile**	**p-value**
Testosterone (ng/ml)	0.55	0.40	0.71	0.53	0.42	0.73	p=0.887
Androstenedione (ng/ml)	2.70	2	3.56	2.27	1.64	3.06	p<0.001
Dihydrotestosterone (ng/ml)	0.13	0.09	0.17	0.16	0.11	0.23	p<0.001
DHEA (ng/ml)	11.74	7.79	15.75	8.6	5.62	11.66	p<0.001

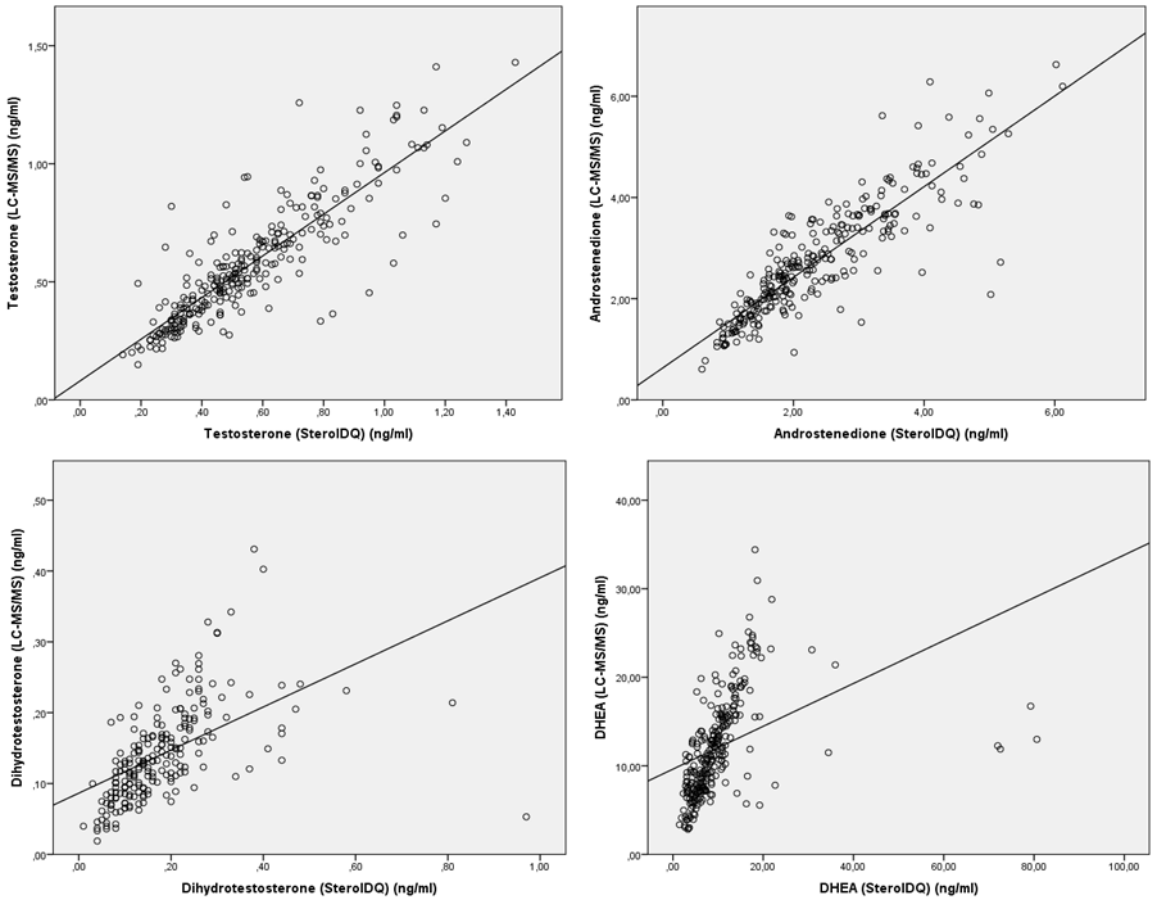

Figure 13. Scatter plots showing differences in quantified steroid hormone levels of testosterone, androstenedione, DHT and DHEA, quantified with LC-MS/MS and HPLC-MS/MS based SteroIDQ kit.

11.1.3. Comparison of Results from LC-MS/MS and Immunoassays

Serum levels of LC-MS/MS measurements were compared to immunoassays. Only for testosterone and androstenedione serum levels determined with LC-MS/MS and immunoassays were available. Spearman correlations showed good correlations for testosterone ($r=0.813$, $p<0.001$), but weak correlations for androstenedione ($r=0.438$, $p<0.001$) (Figure 14).

Differences between results from immunoassays and LC-MS/MS were further analysed using paired sample t-test. Both methods showed significant differences in

quantified hormone levels of testosterone (p<0.001) and androstenedione (p<0.001). A comparison of steroid hormone levels is presented in Table 10.

Table 10. Differences in quantified hormone levels of testosterone and androstenedione, quantified using LC-MS/MS and immunoassays. A p-value <0.05 was considered being significant.

	LC-MS/MS			Immunoassay			
	Median	**25th Percentile**	**75th Percentile**	**Median**	**25th Percentile**	**75th Percentile**	**p-value**
Testosterone (ng/ml)	0.50	0.36	0.67	0.69	0.54	0.83	p<0.001
Androstenedione (ng/ml)	0.69	1.75	3.34	3.58	2.59	5.21	p<0.001

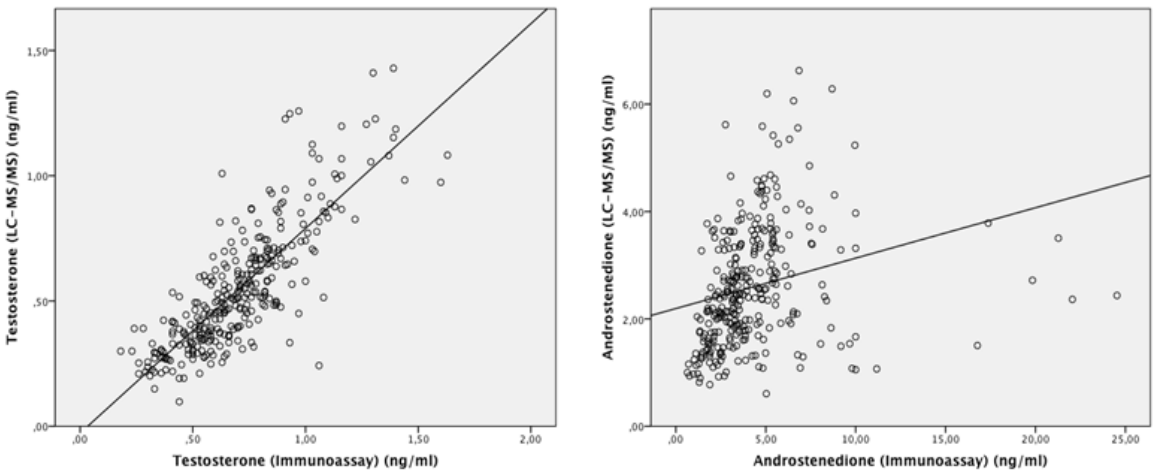

Figure 14. Scatter plots showing inter-assay comparison of LC-MS/MS and immunoassay results for testosterone and androstenedione.

11.1.4. Assessment of Stability and Reproducibility of Results from the SteroIDQ Kit

Serum samples of 8 healthy, volunteering women and 8 healthy, volunteering men were aliquoted and underwent up to 10 freeze/thaw cycles. Quantification was performed using the SteroIDQ kit, but was again not effective for all proposed steroids.

In female control samples (Table 11 and Figure 15), quantification failed for aldosterone, androsterone, corticosterone, cortisone, 11-deoxycorticosterone, 11-deoxycortisone, estrone, estradiol, etiocholanolone and also for DHEAS. Strongest variations were found for 17α-hydroxyprogesterone (up to 50% intra-assay variation) and lowest variations were detected for cortisol (up to 20% intra-assay variation).

In male control samples, quantification failed for aldosterone, corticosterone, cortisone, 11-deoxycorticosterone, 11-deoxycortisone, estrone, estradiol, etiocholanolone and also for DHEAS due to technical problems. Strongest deviations were found when determining concentrations of progesterone (up to 100%) and 17α-hydroxyprogesterone (up to 95%) (

Table 12, Figure 16). Again, cortisol was the most stable steroid (intra-assay variation about 22%).

Table 11. Serum samples of 8 healthy, volunteering women underwent up to 10 freeze/thaw cycles before steroids were quantified using the SteroIDQ kit. Results are given for each single measurement and as mean, standard deviation and difference in percent.

Testosterone (ng/ml)	Median	25th Percentile	75th Percentile	Progesterone (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	0.53	0.41	0.61	**Control 1**	2.33	2.01	2.64
Control 2	0.2	0.19	0.21	**Control 2**	7.11	6.92	7.89
Control 3	0.36	0.29	0.46	**Control 3**	2.72	2.37	3.30
Control 4	0.31	0.25	0.39	**Control 4**	1.85	1.68	2.17
Control 5	0.25	0.22	0.27	**Control 5**	3.65	3.42	4.21
Control 6	0.23	0.22	0.24	**Control 6**	3.03	2.75	3.29
Control 7	0.1	0.09	0.11	**Control 7**	2.74	2.54	2.94
Control 8	0.35	0.33	0.41	**Control 8**	4.1	3.63	4.54

Androstenedione (ng/ml)	Median	25th Percentile	75th Percentile	17OHP (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	0.95	0.89	1.10	**Control 1**	0.26	0.23	0.29
Control 2	0.83	0.82	0.85	**Control 2**	0.11	0.10	0.13
Control 3	0.94	0.91	1.18	**Control 3**	1.96	1.83	2.31
Control 4	0.54	0.48	0.62	**Control 4**	0.08	0.06	0.10
Control 5	0.73	0.69	0.73	**Control 5**	0.18	0.17	0.18
Control 6	0.81	0.79	0.83	**Control 6**	0.63	0.60	0.65
Control 7	0.28	0.27	0.31	**Control 7**	0.08	0.08	0.10
Control 8	1.69	1.62	2.10	**Control 8**	1.15	1.06	1.49

DHEA (ng/ml)	Median	25th Percentile	75th Percentile	Cortisol (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	2.33	2.01	2.64	**Control 1**	282.22	266.39	311.28
Control 2	7.11	6.92	7.89	**Control 2**	221.82	215.97	233.32
Control 3	2.72	2.37	3.30	**Control 3**	113.15	108.34	143.88
Control 4	1.85	1.68	2.17	**Control 4**	193.67	170.81	235.56
Control 5	3.65	3.41	4.21	**Control 5**	116.39	110.49	119.47
Control 6	3.03	2.75	3.29	**Control 6**	65.23	64.58	67.87
Control 7	2.74	2.54	2.94	**Control 7**	52.85	51.87	54.09
Control 8	4.1	3.63	4.54	**Control 8**	84.04	77.22	102.17

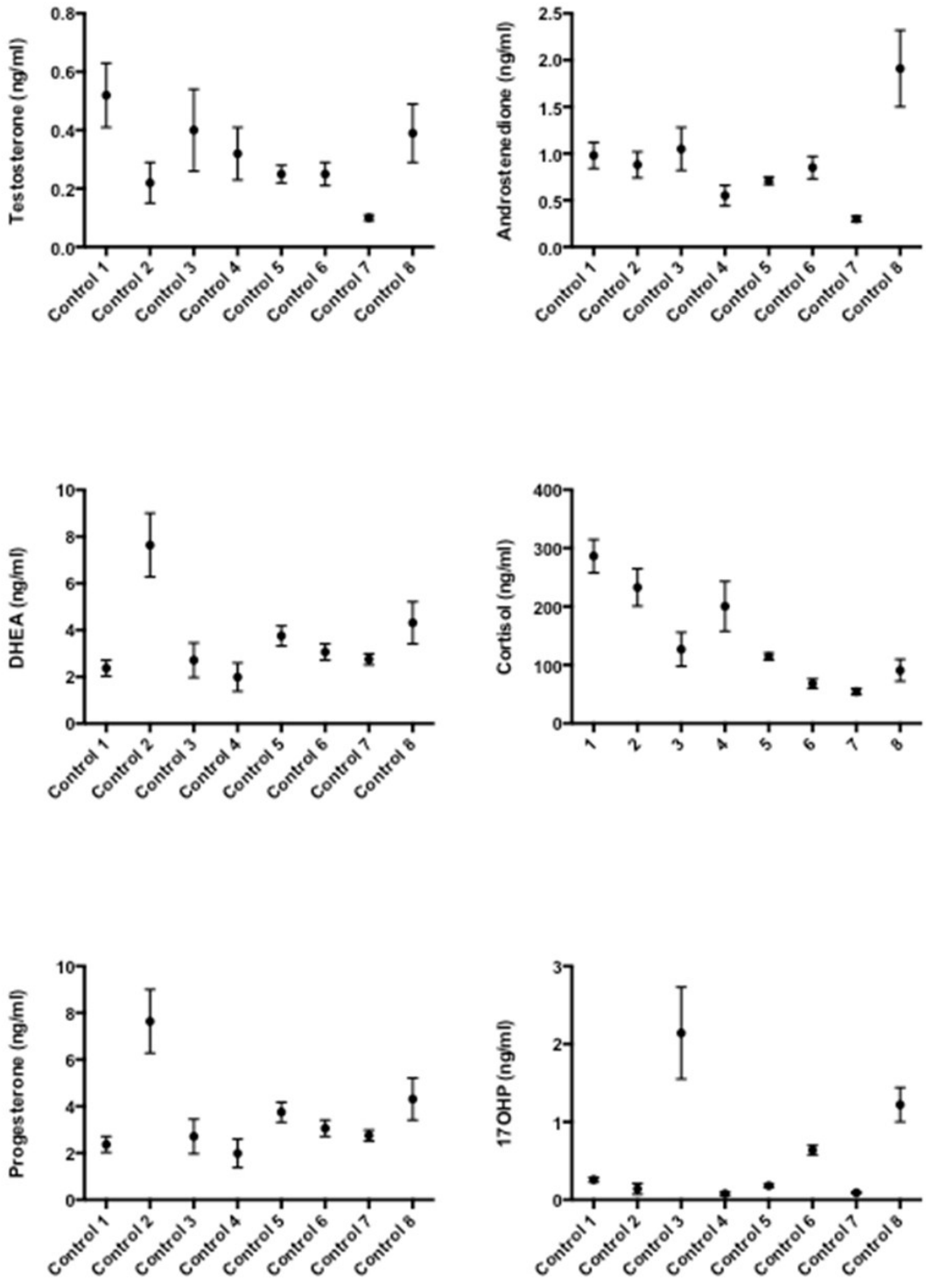

Figure 15. Graphical presentation of quantified steroid hormone concentrations after 10 freeze/thaw cycles of serum from healthy, volunteering women. Data are presented as median and 95% CI.

Table 12. Serum samples of 8 healthy, volunteering men underwent up to 10 freeze/thaw cycles before steroids were quantified using the SteroIDQ kit. Results are given for each single measurement and as mean, standard deviation and difference in percent.

Testosterone (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	4.32	3.99	4.51
Control 2	2.25	2.06	2.39
Control 3	3.28	3.13	3.50
Control 4	5.65	5.50	6.59
Control 5	6.19	5.64	8.01
Control 6	4.52	4.26	4.87
Control 7	3.55	3.48	5.23
Control 8	5.62	5.52	6.47

Progesterone (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	0.07	0.04	0.11
Control 2	0.08	0.04	0.12
Control 3	0.06	0.03	0.08
Control 4	0.06	0.05	0.15
Control 5	0.15	0.05	0.27
Control 6	0.06	0.02	0.11
Control 7	0.10	0.07	0.17
Control 8	0.09	0.03	0.20

Androstenedione (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	0.99	0.65	1.24
Control 2	1.03	0.82	1.18
Control 3	0.71	0.57	0.83
Control 4	0.71	0.59	0.91
Control 5	1.18	1.03	1.32
Control 6	0.78	0.67	1.06
Control 7	0.89	0.71	1.29
Control 8	0.93	0.74	1.62

17OHP (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	0.26	0.04	0.11
Control 2	0.16	0.10	0.28
Control 3	0.16	0.08	0.38
Control 4	0.05	0.04	0.09
Control 5	0.38	0.20	0.48
Control 6	0.23	0.18	0.30
Control 7	0.24	0.16	0.41
Control 8	0.74	0.68	0.85

Androsterone (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	0.38	0.24	0.48
Control 2	0.47	0.40	0.78
Control 3	0.32	0.31	0.38
Control 4	0.40	0.21	0.56
Control 5	0.49	0.43	0.60
Control 6	0.29	0.26	0.43
Control 7	0.39	0.26	0.56
Control 8	0.40	0.26	0.67

Cortisol (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	122.91	121.65	128.33
Control 2	182.91	175.55	190.91
Control 3	62.87	61.78	69.40
Control 4	140.02	137.48	149.66
Control 5	99.03	90.70	122.60
Control 6	58.36	56.63	64.01
Control 7	105.53	102.71	169.86
Control 8	194.03	190.54	221.81

DHEA (ng/ml)	Median	25th Percentile	75th Percentile
Control 1	12.71	10.96	14.09
Control 2	11.70	10.24	14.45
Control 3	5.12	3.48	7.78
Control 4	16.36	14.24	24.96
Control 5	8.15	6.69	12.64
Control 6	17.39	16.89	18.96
Control 7	15.98	13.14	27.70
Control 8	10.55	8.61	11.46

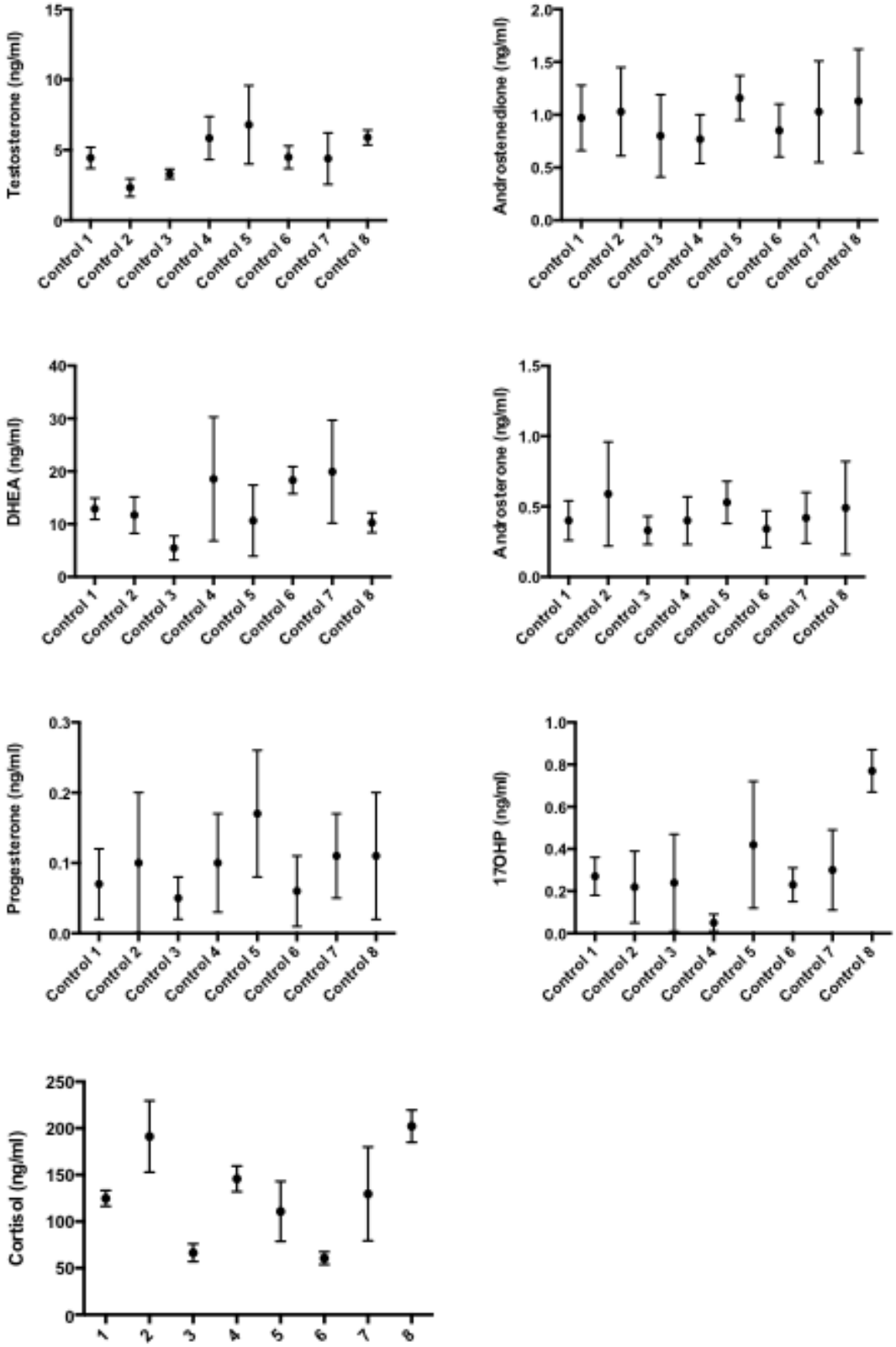

Figure 16. Graphical presentation of quantified steroid hormone concentrations after 10 freeze/thaw cycles of serum from healthy, volunteering men. Data are presented as median and 95% CI.

11.2. Determining the Role of the Testosterone to Dihydrotestosterone (TT/DHT) Ratio in PCOS Patients

11.2.1. Biochemical and Metabolic Characterisation of PCOS Patients and Controls

Investigations concerning the role of the TT/DHT ratio in PCOS patients were performed in a cohort of 275 PCOS patients fulfilling Rotterdam criteria. Patients premenopausal, aged 16 to 48 years, and had a median BMI of 24.8 kg/m^2 (IQR: 24.8-31.6). Additionally, 35 BMI-matched, healthy and premenopausal controls, aged 21 to 50 with a median BMI of 25.2 kg/m^2 (IQR: 20.9-31.1). Clinical and metabolic characterisation of patients and controls is presented in Table 13. Age and BMI were considered as covariate in all statistical analyses in order to exclude potential bias.

Several statistically significant differences between PCOS patients and healthy control patients were found. In PCOS patients, most androgen levels were significantly higher, e.g. testosterone ($p<0.001$), DHEA ($p<0.001$), DHEAS ($p<0.001$) and androstenedione ($p<0.001$). Calculated serum levels of free dihydrotestosterone and free testosterone were significantly higher in PCOS patients ($p<0.001$, $p<0.001$). No difference in steroid hormone level was found for DHT ($p=0.072$).

A normative cohort of 37 healthy, age-matched women was also included in this study, but enrolled at the University of South Manchester. Serum steroid hormone levels were determined with the same method and were within the same range as the control group. No significant differences in the hormonal status between the control group and the normative cohort could be assessed (Table 14).

The calculated TT/DHT ratio was significantly higher in PCOS patients compared to control women ($p<0.001$) or the normative cohort ($p<0.001$) (Figure 17).

Table 13. Clinical and biochemical characterisation of PCOS patients and BMI-matched controls. Differences were assessed via Mann-Whitney U test for non-normally distributed independent samples. Values are presented as median (25th-75th interquartile ranges) and p-values <0.05 are considered significant.

	Controls (n=35)			PCOS (n=275)			
	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile	p-value
Age (yr)	35.8	27.7	42	26	22	30	<0.001
Weight (kg)	70.5	62	89	69	59	88.1	0.716
BMI (kg/m^2)	25.2	20.9	31.1	24.8	21.4	31.6	0.755
Glucose							
Fasting (mg/dl)	89.5	83	92.5	87	81	93	0.304
30 min (mg/dl)	136	116.2	156.5	133.5	115	152	0.865
60 min (mg/dl)	108.5	91.5	128.2	125	97	156	0.091
120 min (mg/dl)	93	78	111	101	85	123	0.078
Fasting insulin (µIU/ml)	4.7	2.3	7.3	7.2	4.2	12.7	0.001
SHBG (nmol/l)	67.7	45.8	75.9	38.7	25.1	56.3	<0.001
DHEAS (µg/ml)	1	0.7	1.6	2.2	1.5	3	<0.001
LH (IU/l)	4.5	3	7.5	7.9	4.4	11.7	0.001
FSH (IU/l)	5.1	3.4	7.1	4.9	3.7	6.1	0.626
Cholesterol (mg/dl)	181.5	157.5	218.5	179.5	156	200	0.301
Triglycerides (mg/dl)	62.5	47.5	81.7	76.5	54	109.2	0.055
Cholesterol/HDL ratio	2.5	1.9	3.1	2.7	2.2	3.5	0.037
HDL (mg/dl)	72	59	86	63	50	78	0.004
LDL (mg/dl)	89	76	129	98	83	117	0.590
HbA1c (%)	5.2	4.9	5.3	5.2	5	5.4	0.295
HOMA-IR	1	0.4	1.6	1.5	0.9	2.8	0.003
Testosterone (ng/ml)	0.3	0.27	0.44	0.5	0.38	0.69	<0.001
Androstenedione (ng/ml)	1.3	1	1.9	2.5	1.9	3.4	<0.001
DHEA (ng/ml)	5	4	9.5	11.2	7.7	15.5	<0.001
DHT (ng/ml)	0.11	0.07	0.14	0.12	0.08	0.17	0.072
Free DHT (pg/ml)	1.4	0.9	1.7	2	1.5	2.6	<0.001
Free testosterone (pg/ml)	13.8	11.1	17.4	28.6	20.7	39.7	<0.001
FAI	1.8	1.4	2.7	4.6	3.2	7.3	<0.001
TT/DHT ratio	3.1	2.7	4.2	4.3	3.1	5.9	<0.001

Table 14. Differences in steroid hormone levels of testosterone, androstenedione, DHEA and DHT quantified with LC-MS/MS and calculated TT/DHT ratio between controls and the normative cohort. A p-value <0.05 was considered being significant.

	Controls (n=35)			Normative Cohort (n=38)			
	Median	**25th Percentile**	**75th Percentile**	**Median**	**25th Percentile**	**75th Percentile**	**p-value**
Testosterone (ng/ml)	0.35	0.27	0.44	0.31	0.22	0.46	0.377
Androstenedione (ng/ml)	1.32	1.08	1.91	1.22	0.99	1.82	0.229
DHEA (ng/ml)	5.08	4.04	9.56	5.33	4.18	8.09	0.667
DHT (ng/ml)	0.11	0.07	0.14	0.10	0.06	0.15	0.774
TT/DHT ratio	3.16	2.77	4.31	3.23	2.21	4.74	0.740

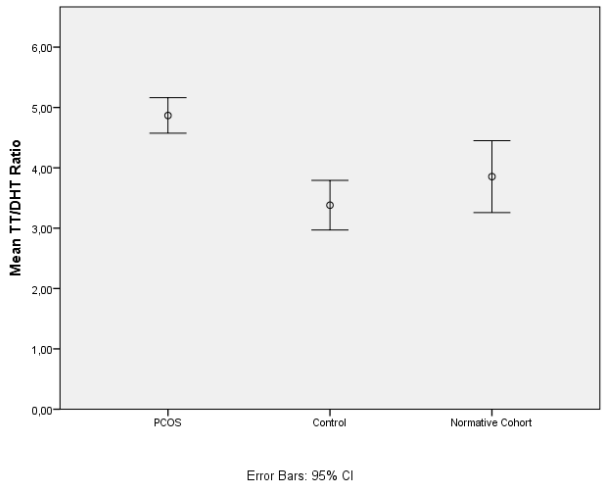

Figure 17. Differences in calculated TT/DHT ratio between PCOS patients, control women and the normative cohort. Results are presented as median and confidence intervals (CI). Data are presented as mean and 95% CI.

11.2.2. TT/DHT Ratio in Metabolic Syndrome and Glucose Metabolism

In PCOS patients alone, 43 (22.32%) women were diagnosed with metabolic syndrome, 81 (44.84%) women with insulin resistance and 64 (31.15%) women with glucose intolerance. PCOS women with metabolic syndrome showed significantly higher serum levels of calculated free testosterone ($p=0.047$) and a significantly higher TT/DHT ratio ($p<0.001$) (Figure 18), but significantly lower levels of free DHT ($p=0.001$) and total DHT ($p<0.001$). No difference between PCOS women with or without metabolic syndrome was found for androstenedione, testosterone, DHEA or DHEAS.

Regarding the glucose metabolism, PCOS patients with an impaired glucose tolerance assessed via oral glucose tolerance test had significantly higher free testosterone levels ($p=0.017$) and also a higher TT/DHT ratio ($p<0.001$), but lower DHT levels ($p<0.001$).

Similar results were found when calculating the homeostatic model assessment for insulin resistance (HOMA-IR). Insulin resistant PCOS patients had significantly higher serum levels of free testosterone ($p<0.001$) and a higher TT/DHT ratio ($p<0.001$), but lower DHT levels ($p<0.001$).

Moreover, hypertensive PCOS patients with systolic blood pressure levels >140 mm HG and diastolic levels >90 mm HG also showed a significantly higher TT/DHT ratio ($p=0.001$).

In order to determine whether the TT/DHT ratio is independent predictors for the presence of metabolic syndrome, IGT or HOMA-IR, binary logistic regression analyses were conducted. TT/DHT ratio, age and BMI were independent predictors for the presence of the metabolic syndrome ($p=0.024$, $p=0.035$ and $p<0.001$, respectively) and IGT ($p=0.011$, $p=0.003$ and $p=0.005$, respectively) in PCOS patients. Using total testosterone instead of TT/DHT ratio in this statistical setup, it turned out that total testosterone is no independent predictor for IGT ($p=0.972$), insulin resistance ($p=0.691$) or metabolic syndrome ($p=0.592$). Using total DHT for a multivariate regression analysis controlled for BMI and age, DHT was an independent predictor for IGT ($p=0.045$), but not for HOMA-IR ($p=0.057$) or metabolic syndrome ($p=0.054$).

11.2.3. TT/DHT Ratio and Obesity

83 (43.47%) PCOS patients were defined as overweight or obese (BMI ≥25kg/m^2). In lean PCOS patients (BMI <25 kg/m^2), significantly higher DHT levels were found ($p<0.001$) compared to obese patients. No difference was found concerning total testosterone levels ($p=0.093$). Concerning the TT/DHT ratio (Figure 18), a significantly higher value was found in the group of overweight and obese patients compared to lean PCOS patients ($p<0.001$).

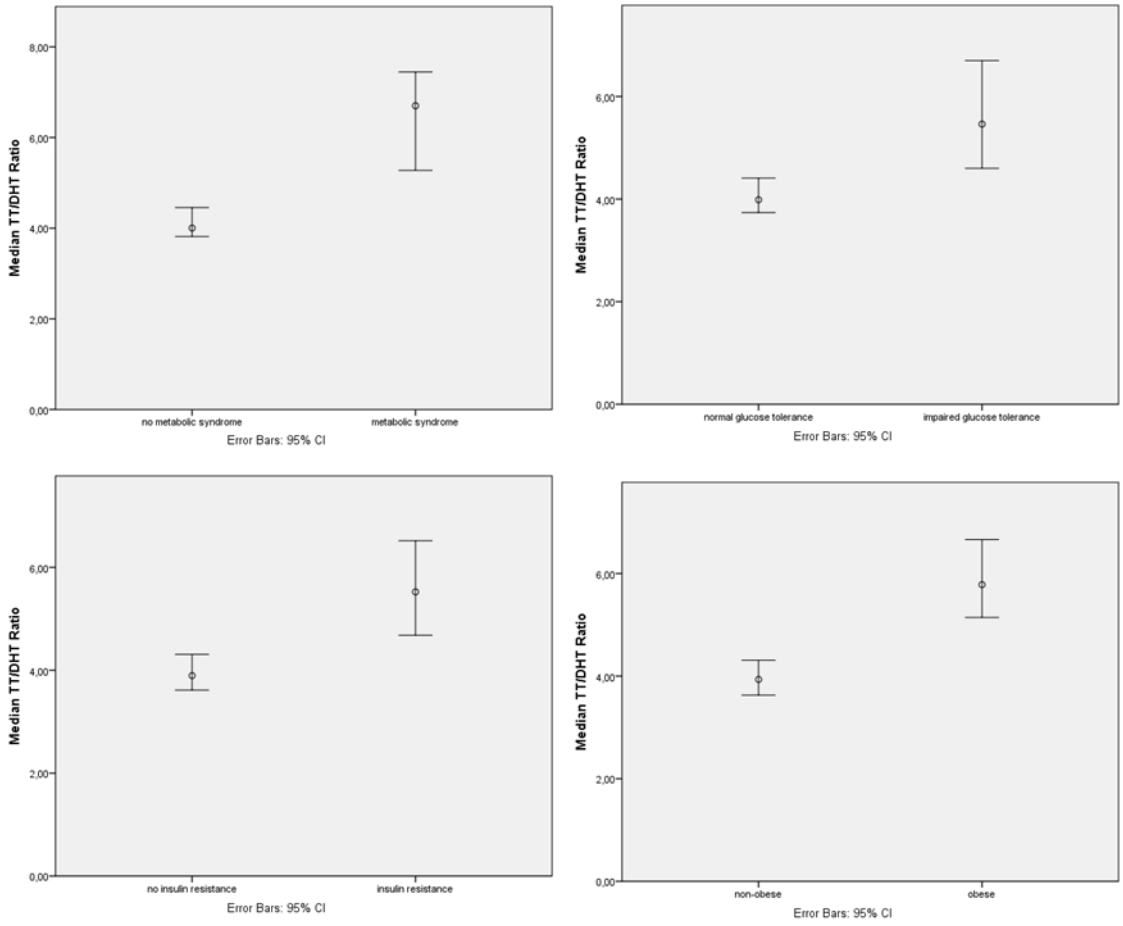

Figure 18. Differences in the TT/DHT ratio in PCOS patients with metabolic syndrome vs. no metabolic syndrome, insulin resistance vs. no insulin resistance, impaired glucose tolerance vs. no impaired glucose tolerance and for obese vs. non-obese/lean PCOS patients. Data are presented as mean and 95% CI.

11.2.4. ROC Curve Analysis

In ROC curve analyses for the determination of the best cut-off value for the TT/DHT ratio for the diagnosis of PCOS, a value of 4.37 was found [sensitivity 50.5%, specificity 82.9%, area under curve (AUC) 0.694]. In PCOS patients alone, the best cut-off for the TT/DHT ratio for the diagnosis of IGT was 3.9 [sensitivity 79.7%, specificity 50.0%, AUC 0.678], for HOMA-IR 4.20 [sensitivity 77.7%, specificity 56.4%, AUC 0.711] and for metabolic syndrome 4.32 [sensitivity 86.0%, specificity 54.9%, AUC 0.768] (Figure 19).

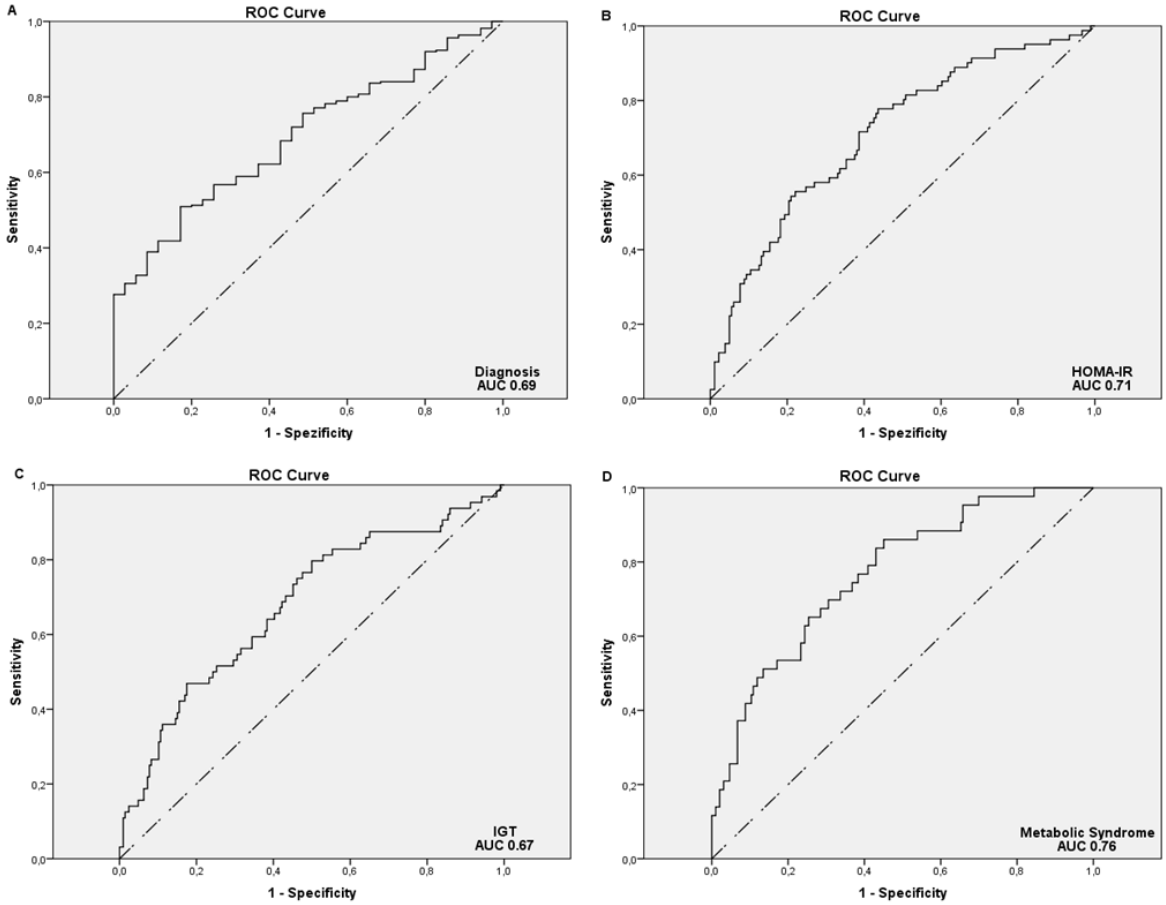

Figure 19. ROC curves analyses were performed to assess the best cutoff for (A) the diagnosis of PCOS, (B) the diagnosis of metabolic syndrome, (C) impaired glucose tolerance and (D) insulin resistance according to the HOMA-IR model.

11.2.5. Correlation of TT/DHT Ratio with Metabolic and Biochemical Parameters

BMI and age adjusted Spearman correlation analyses were performed for the TT/DHT ratio with metabolic and hormonal parameters in PCOS patients (Table 15). Significant associations were found for weight (r=0.198, p=0.001), WC (r=0.165, p=0.009),

adverse parameters of glucose metabolism [fasting glucose (r=0.152, p=0.013) and glucose levels after 30 (r=0.161, p=0.013), 60 (r=0.168, p=0.007) and 120 minutes (r=0.153, p=0.014) in the oGTT, HOMA-IR (r=0.282, p<0.001), insulin and glucose response curve (r=0.174 and p=0.008, r=0.170 and p=0.008, respectively)], adverse liver and lipid parameters [cholesterol (r=0.136, p=0.025), triglycerides (r=130, p=0.032), aspartate transaminase (AST) (r=140, p=0.021) and alanine transaminase (ALT) (r=0.203, p=0.001)], higher FAI (r=0.301, p<0.001) and higher steroid hormone levels [free testosterone (r=0.468, p<0.001) and androstenedione (p=0.321, p<0.001].

Negative correlations of the TT/DHT ratio were found for free dihydrotestosterone (r=-.407, p<0.001), DHEA (r=-.173, p=0.004) and DHEAS (r=-.282, p<0.001). No correlations were found for LH, AMH, progesterone or for the Ferriman-Gallwey score or when testosterone alone was used instead of the TT/DHT ratio in correlation analyses (data not shown).

Table 15. Spearman correlation analysis of the TT/DHT ratio with biochemical and anthropometric parameters in PCOS patients (n=275). A p-value <0.05 was considered being significant.

	TT/DHT ratio			
	r	p-value	r	p-value (age- & BMI-adjusted)
Weight	0.398	<0.001	0.198	0.001
BMI	0.363	<0.001	1	
Waist circumference	0.344	<0.001	0.165	0.009
Hip circumference	0.383	<0.001	0.047	0.464
WHR	0.212	0.001	0.065	0.309
Systolic blood pressure	0.204	0.001	0.029	0.656
Diastolic blood pressure	0.236	<0.001	0.091	0.157
Glucose				
Fasting (mg/dl)	0.272	0.001	0.152	0.013
30 min (mg/dl)	0.200	0.001	0.161	0.009
60 min (mg/dl)	0.229	<0.001	0.168	0.007
120 min (mg/dl)	0.275	<0.001	0.153	0.014
Insulin				
Fasting (mg/dl)	0.327	<0.001	0.245	<0.001
30 min (mg/dl)	0.200	0.001	0.107	0.092
60 min (mg/dl)	0.305	<0.001	0.174	0.005
120 min (mg/dl)	0.235	<0.001	0.099	0.112
HOMA-IR	0.336	<0.001	0.282	<0.001
QUICKI	-.342	<0.001	-.106	0.092
Glucose response curve	0.273	<0.001	0.170	0.008
Insulin response curve	0.300	<0.001	0.174	0.008
HbA1c	0.214	0.001	0.051	0.417
Cholesterol	0.059	0.327	0.136	0.025
Triglycerides	0.330	<0.001	0.130	0.032
HDL	-.213	<0.001	0.076	0.215
Cholesterol/HDL ratio	0.239	<0.001	0.014	0.814
AST	0.173	0.004	0.140	0.021
ALT	0.221	<0.001	0.203	0.001
Androstenedione	0.293	<0.001	0.321	<0.001
Free testosterone	0.590	<0.001	0.468	<0.001
FAI	0.618	<0.001	0.301	<0.001
DHEAS	-.234	<0.001	-.282	<0.001
DHEA	-.113	0.061	-.173	0.004
Free DHT	-.396	<0.001	-.407	<0.001

11.3. Single Nucleotide Polymorphism (SNP) Genotyping

Single nucleotide polymorphisms of two different genes known to be involved in steroid hormone metabolism in women, called follistatin and inhibin, were analysed. Due to the rather small sample size and the small minor allele frequency of the investigated SNPs, only few patients carrying the polymorphisms could be assessed. For the inhibin polymorphism rs12720061, 3 patients showing the heterozygote mutated genotype and 215 PCOS patients with the wildtype genotype could be found. For the tested follistatin polymorphism rs11745088, only 1 PCOS patients with the heterozygote mutated variant and 135 patients with the wildtype genotype were assessed. Due to unclear results after even 2 PCR runs, genotyping for the follistatin SNP failed in many patients. Only for the inhibin polymorphism rs12720062, the number of patients for both genotypes was sufficient to perform statistical analyses.

Inhibin Polymorphism rs12720062

Genotyping for the inhibin polymorphism rs12720062 was effective in 233 patients. For patient DNA samples with no clear genotype after two PCR runs were excluded. 223 patients (95.7%) showed the wildtype genotype “GG” and 10 (4.3%) PCOS patients showed the heterozygote genotype “AG”. No patient with the homozygote genotype “AA” could be found in the study cohort. Genotype distribution did not deviate significantly from the HWE ($p>0.05$).

Clinical and biochemical characterisation in regard of genotype distribution is presented in

Table 16 and Figure 16. Statistically significant differences between the wildtype and the heterozygote genotype could be assessed. PCOS women with the “AG” genotype had a lower AMH ($p=0.033$), lower baseline LH ($p=0.042$) and lower LH levels after 30 and 60 minutes in the LHRH (GnRH) stimulation test ($p=0.013$ and $p=0.009$), lower FSH levels

after 30 minutes in the LHRH stimulation test (p=0.026), a lower LH delta (p=0.010), and also lower levels of ACTH (p=0.022), cortisol (p=0.042), testosterone (p=0.004), androstenedione (p=0.005) and free testosterone (p=0.007). No significant differences in regard of genotype distribution could be found for menstrual cycle disorder (oligo-/anovulation) (data not shown).

Table 16. Metabolic Characterisation according to INHA polymorphism genotype. A p-value <0.05 was considered being significant.

	Genotype AG				Genotype GG				
	n	**Median**	**25th Percentile**	**75th Percentile**	**n**	**Median**	**25th Percentile**	**75th Percentile**	**p-value**
Age (years)	10	26.50	22	29	223	26	22	30	0.821
Weight (kg)	10	63	58	74	222	70	59	89	0.215
BMI (kg/m^2)	10	23.79	20.61	29.2	222	25.18	21.61	31.56	0.337
Fasting glucose (mg/dl)	10	85	80.25	90.25	216	87	82	92.75	0.434
Fasting insulin (µIU/ml)	9	11.6	4.85	14.9	213	7.3	4.15	12.40	0.313
SHBG (nmol/l)	10	35.15	22.17	56.62	223	37.10	24.8	55.7	0.863
AMH (ng/ml)	2	2.95			59	7.2	5	12.3	0.033
LH (IU/l)	10	4.59	2.12	7.83	221	8.16	4.59	11.89	0.042
LH30 (IU/l)	10	18.53	12.8	32.43	213	36.95	19.26	61.88	0.013
LH60 (IU/l)	10	14.8	10.86	28.66	213	33.19	18.13	57.05	0.009
FSH (IU/l)	10	4.36	3.46	5.17	222	4.9	3.74	6.17	0.238
FSH30 (IU/l)	10	4.95	4.36	7.63	212	7.33	5.3	9.37	0.026
FSH60 (IU/l)	10	6.61	4.94	8.96	212	7.87	5.9	10.5	0.152
FSH delta	10	2.22	1.13	4	211	2.98	1.91	4.87	0.128
LH delta	10	11.2	8.78	28.52	210	29.3	15.28	51.48	0.010
ACTH (pg/ml)	10	12.2	9.95	18.35	221	18.1	12.4	24.85	0.022
Cortisol (ng/ml)	10	142.3	111.35	144.72	222	155.45	123.12	190.82	0.042
Testosterone (LC-MS/MS) (ng/ml)	10	0.28	0.25	0.48	223	0.52	0.39	0.68	0.004
Androstenedione (LC-MS/MS) (ng/ml)	10	1.55	1.36	2.18	223	2.56	1.99	3.4	0.005
Free Testosterone (pg/ml)	10	17.44	14.91	26.83	223	28.65	21.82	39.87	0.007

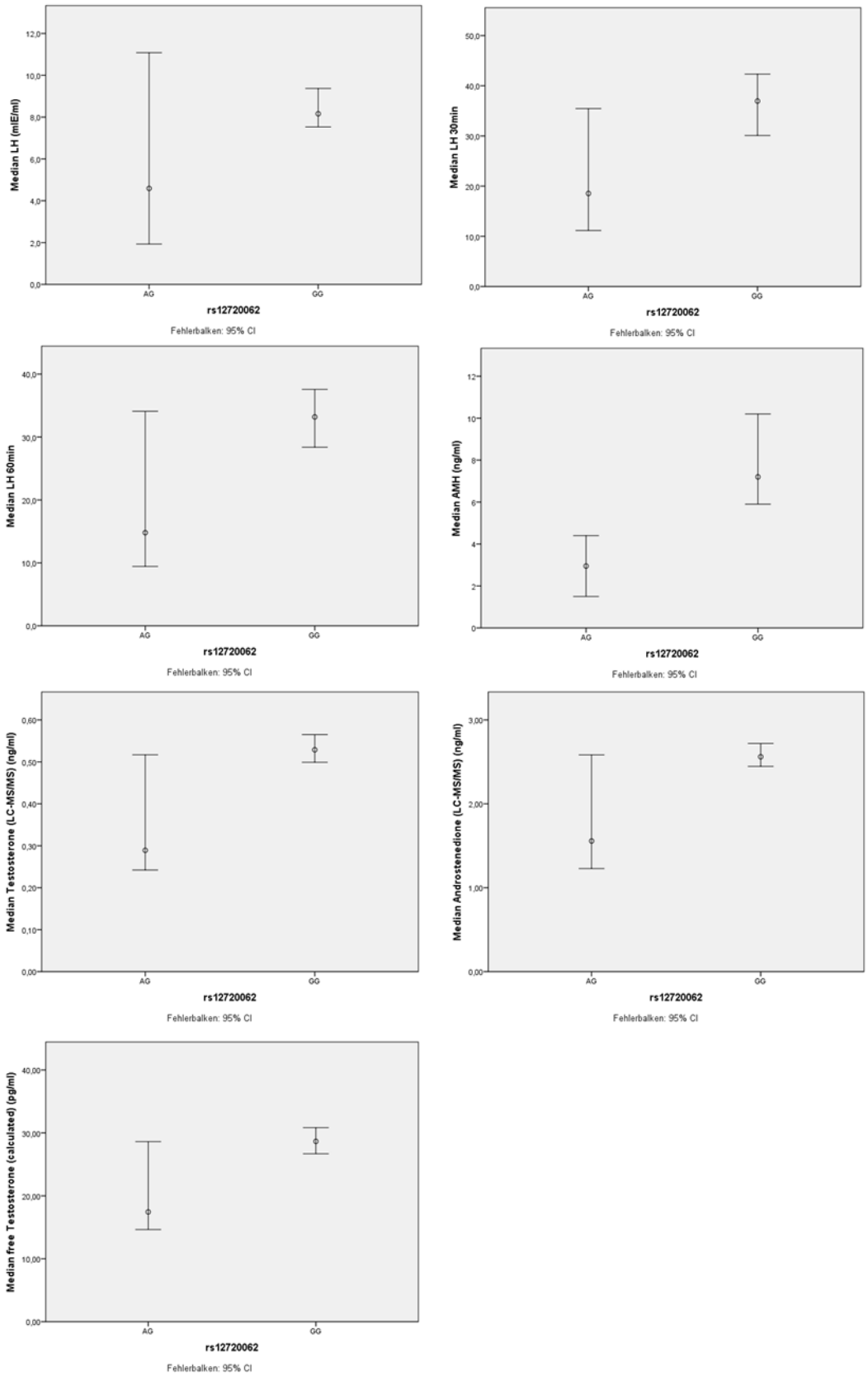

Figure 20. Differences in serum levels of basal LH, LH after 30 and 60 minutes of stimulation, AMH, testosterone, adnrostenedione and free testosterone according to inhibin alpha SNP genotype. Data are presented as median and itnerquartile ranges.

12. Discussion

12.1. Steroid Hormone Profiling

In clinical settings, immunoassays are still the most common technique for the quantification of steroid hormones in diagnostics, though they suffer from several limitations, especially at very low analyte concentrations [146]. In chemical and biochemical research, mass spectrometry methods such as LC-MS/MS and GC-MS/MS are widely used, but need expensive equipment, comprehensive preparation of samples, extraction of analytes and well trained personnel. Based on the much higher sensitivity and selectivity, mass spectrometry has been declared as being the method of choice also for clinical research, especially for steroid hormones.

One big advantage of mass spectrometry is the possibility of measuring several analytes in one single assay in small sample volumes. Especially for endocrine diseases with alterations in the steroid hormone metabolism, such as the polycystic ovary syndrome, Cushing's syndrome or congenital adrenal hyperplasia, quantification of several steroid hormones and creating steroid hormone profiles would be of high interest. Such profiles could provide more information about the metabolic status of one individual than single measurements, and are already used for steroid profiles in 24-hours urine samples. Also for the screening for several diseases in new-borns, mass spectrometry is used for steroid hormone profiling, allowing quantification of several hormones in only a small amount of blood or even blood spots.

Steroid hormones all derive from the same precursor, cholesterol, and show only small structural variations (i.e. presence and localisation of hydrogen bonds). The quality of immunoassays depends on the specificity of the used antibody for epitopes on the antigen. Since steroid hormones share a common ring-structure and show only minor structural differences, cross reactivity of these antibodies with other steroid hormones are possible and can lead to false results. This explains why immunoassays usually give higher results compared to LC-MS/MS or GC-MS/MS. Especially in serum samples of women and children, steroid hormone concentrations can be very low; hence,

quantification with immunoassays can be more challenging and steroid hormone concentrations are often overestimated [74, 120, 152]. A study by Taieb *et al.* demonstrated that in 10 tested immunoassays used for the quantification of testosterone in female serum sample, variation up to 500% occurred [121]. They concluded that none of the tested assays was recommendable for the assessment of hyperandrogenism in women. Several other studies confirmed that at very low serum concentrations (<1 ng/ml), immunoassays are not specific enough and do not give reliable results [19, 74, 113, 141, 152]. Especially in direct immunoassays that do not need any sample preparation or analyte extraction, interfering substances can influence the quantification of hormones and lead to false results [74, 120, 121]. These interferences can be reduced when analyte extraction is performed before the immunoassay [43, 125, 143].

However, also MS-based techniques can give wrong results if they are not well established and validated or performed by untrained staff. Various methods for the simultaneous quantification of steroid hormones are published. These methods are based on in-house methods, since validate, commercially available MS-based kits are still missing. One available HPLC-MS/MS-based kit is the SteroIDQ kit (Biocrates Life Sciences AG, Innsbruck, Austria) [55]. This kit should provide the possibility to measure 16 different steroid hormones in one single assay and in only a small amount of sample volume. However, we had some severe difficulties quantifying all proposed steroids with this approach. Aldosterone, estradiol, estrone, 11-deoxycorticosterone, 11-deoxycortisol and etiocholanolone could not be quantified because analyte levels were often under the lower limit of detection and therefore, results were not reliable. Also for cortisone, strong problems occurred and results could only be regarded as semi-quantitative and were not further used.

Compared to immunoassays, strongest differences to immunoassay results were found for progesterone, 17α-hydroxyprogesterone and androstenedione. Similar results were described by Koal *et al.*, who developed the kit [55], and by Fanelli *et al.*, showing comparisons of LC-MS/MS with immunoassays give poor agreement of both techniques for androstenedione, DHEAS and 17α-hydroxyprogesterone [32]. These limitations could be based on cross-reactivities with components of the matrices or low specificity of the used antibodies in the immunoassays.

Good correlations of the SteroIDQ kit and immunoassays were only found for cortisol and testosterone. Nevertheless, the differences between testosterone and cortisol levels from the SteroIDQ kit and immunoassays were also statistically significant ($p<0.001$). Since higher steroid hormone levels in immunoassays compared to MS-based techniques are frequently found, it is suggested that new reference levels should be established for mass spectrometry [40, 53].

In order to assess stability and reproducibility of the SteroIDQ kit, we used control serum samples for repeated freeze/thaw cycles before analysing of steroid hormone concentrations. Again, strongest deviations in measured hormone levels were found for progesterone and 17α-hydroxyprogesterone (up to 100% variation), whereas highest stability was found for cortisol. These strong variations indicate that the kit might not be effective or well-established for all steroids.

Comparison of quantified steroid hormone concentrations from the SteroIDQ kit and LC-MS/MS measurements, strong correlations and no significant differences were found for testosterone serum levels. Nevertheless, both methods gave statistically significant differences for quantified androstenedione, dihydrotestosterone and DHEA levels. Nevertheless, the protocols used for the LC-MS/MS measurements have been used and validated in several previous studies and high-impact publications [29, 36, 40, 157], whereas no validation and comparison of the SteroIDQ kit to another MS-based steroid hormone quantification has been published so far.

Based on the facts that the SteroIDQ kit was not sufficient for the quantification of all desired steroid hormones, showed severe variations in steroid hormone quantification after freeze/thaw cycles and lack of published validation of the kit with other mass spectrometric techniques, we were not able to establish the kit for a large-scale analysis of all our study cohorts and did not use the results for further investigations.

12.2. Determining the Role of the Testosterone to Dihydrotestosterone (TT/DHT) Ratio in PCOS Patients

This is the first study focussing on the TT/DHT ratio in PCOS patients and its impact on metabolic and hormonal parameters. Our findings are based on comprehensive steroid hormone LC-MS/MS measurements in serum samples of a large cohort of well characterised PCOS patients, carried out by the experienced staff in a centre for steroid hormone analysis at the University of South Manchester, United Kingdom [40, 41, 88].

PCOS is a heterogeneous disease with several metabolic characteristics and clinical phenotypes. Still, no clear consent about the classification and diagnosis of PCOS has been found. It is recommended that androgen levels should be assessed in every woman suspected of having PCOS or any related disease with similar clinical appearance. Because of the high specificity and sensitivity, LC-MS/MS and GC-MS/MS methods have become the gold standard in hormone quantification, especially in research [53]. The Endocrine Society even states that by 2015 that for high impact clinical research journals, assessment of steroid hormones with mass spectrometry will be mandatory [38]. It is estimated that this requirement will also be set for vitamin D measurements in the near future.

So far, testosterone has been considered as the most important androgen in the assessment of biochemical hyperandrogenism in women [7]. It can be produced directly by the ovaries and to a smaller degree by the adrenal gland, but also be generated by the metabolism of its precursor androstenedione in peripheral tissue, such as skin, liver or adipose tissue [94]. Testosterone itself can be enzymatically converted to dihydrotestosterone by 5α-reductase, an enzyme also responsible for the conversion of progesterone to 5α-dihydroprogesterone and of tetrahydrocortisol (THF) to 5α-tetrahydrocortisol (5α-THF).

So far, the role of 5α-reductase in PCOS patients has been investigated in several studies, but with inconsistent results. These variances might be based on differences in the study design, sample sizes and in the techniques used for the assessment of the 5α-reductase activity. In PCOS patients, the activity of the 5α-reductase has been assessed by calculating the ratio of corticosteroid metabolites in urine samples (i.e. THF to 5α-THF) or

by using quantitative real-time PCR in tissue samples (skin, hair follicles, liver) [126, 128, 138, 142]. However, calculating the ratio of urinary corticoid metabolites does not discriminate between 5α-reductase type 1 and type 2 [126]. By using real-time PCR, differentiation between 5α-reductase isoforms is possible, but it only provides information about the enzyme activity in a distinct tissue or organ and will never be representative for the whole organism. Furthermore, real-time PCR approaches can vary by differences in the method for the preparation of tissue and the isolation and stabilisation of RNA and also within sections of the same organ or tissue.

Testosterone is not only precursor for dihydrotestosterone, but also for estradiol. In the theca cells of ovarian follicles, the enzyme aromatase converts testosterone to estradiol. Like most of the other steroid hormones, estradiol and dihydrotestosterone can further be metabolised to other steroids. Various factors can influence the steroid hormone metabolism and lead to higher levels of distinct androgens or estrogens.

Recent studies showed that regarding hyperandrogenism and its consequences in women, not only single hormone levels should be considered, but also the relationship of several hormones to each other [63, 82]. It was also demonstrated, that hyperandrogenism can be missed if only testosterone and not androstenedione levels are evaluated and that PCOS patients with elevated serum androstenedione levels have nearly the same metabolic risk as those patients with high testosterone levels [82]. So far, the relationship of testosterone to its metabolite dihydrotestosterone in PCOS patients has not been deeply investigated.

For our study of the role of the testosterone to dihydrotestosterone ratio in PCOS women, 275 well characterised and premenopausal patients, diagnosed according to the Rotterdam criteria of 2004, have been included. A group of 35 healthy and premenopausal women served as controls. Steroid hormone concentrations were assessed by LC-MS/MS, and the testosterone to dihydrotestosterone ratio was calculated. PCOS patients showed a significant higher TT/DHT ratio compared to healthy controls ($p<0.001$). As a next step, the TT/DHT ratio was investigated regarding anthropometric, hormonal and metabolic parameters. In PCOS patients alone, a significant higher ratio of testosterone to dihydrotestosterone was found in obese patients with a BMI ≤30 kg/m^2.

Moreover, serum levels of dihydrotestosterone were significantly lower in obese PCOS patients. This goes in line with previous studies reporting lower DHT levels in serum of children and teenagers with PCOS [96, 110]. In addition, a higher TT/DHT ratio was assessed in PCOS patients with metabolic syndrome, impaired glucose tolerance and patients with insulin resistance by means of HOMA-IR. These findings were evidenced by several statistically significant associations with parameters of the glucose metabolism (i.e. higher fasting glucose and higher glucose levels at all measured time points in the oGTT, higher HOMA-IR and QUICKI values, higher insulin response curve and glucose response curve). A higher TT/DHT ratio was also associated with several adverse liver and lipid parameters (i.e. ALT, AST, triglycerides, cholesterol) and adverse hormonal parameters (i.e. androstenedione, FAI, free testosterone, free DHT, DHEA and DHEAS).

ROC curve analyses showed high sensitivity and sensitivity of the TT/DHT ratio for the diagnosis of PCOS and for the prediction of metabolic syndrome, impaired glucose tolerance and of insulin resistance.

In contrast, significant lower levels of DHEA and DHEAS were associated with high TT/DHT ratio. DHEA and DHEAS are steroid hormones mostly produced by the cortex of the adrenal gland (~98%) and only to a small degree by the ovary. This finding might support the notion that the ovary is a much stronger contributor to the metabolic deteriorations in PCOS, as suggested in a previous study [63].

A previous study by Stener-Victorin *et al.* investigated steroid hormones and glucoronidated metabolites assessed by LC-MS/MS in PCOS patients, but did not find any differences between PCOS patients and healthy controls concerning the TT/DHT ratio [116]. However, the investigated study cohort was much smaller than our cohort and they did not perform as comprehensive correlations with metabolic parameters as we did. They might have found different results when investigating a larger study cohort.

The use of a large and well characterised study cohort is one clear strength of this study. Similar studies concerning hyperandrogenism in PCOS patients used either a much smaller sample size or did not use a validated mass spectrometric method for steroid hormone quantification. Within our study, measurement of testosterone, dihydrotestosterone, DHEA and androstenedione was performed in an experienced

centre for steroid hormone quantification for diagnostic and research. Our concise group of healthy controls was BMI-matched with the PCOS patients. Enrolment of control women was based on strong exclusion criteria; hence, the control group was rather small. A normative cohort, consisting of 38 healthy, premenopausal and age-matched women, was included as another control group, but was recruited at the University of South Manchester, UK. Comparison of hormonal parameters of women of both control groups showed no significant differences, but significant differences when compared to PCOS patients.

The main focus of this study was put on determining the metabolic consequences of a disturbed TT/DHT ratio, thus we focussed on having BMI-matched controls, based on the strong influence of weight and adipose tissue on the metabolism. Although PCOS patients and controls vary in the mean age, all included women were premenopausal and of reproductive age. Nevertheless, BMI and age were used as covariates in all statistical analyses to exclude a potential bias.

In conclusion, we could provide evidence for a distinct role of the TT/DHT ratio in the metabolic deteriorations in PCOS patients. Our PCOS study cohort showed a significantly higher TT/DHT ratio compared to two different control groups and an associated adverse metabolic phenotype. As suggested by previous studies, it seems important to not only evaluate testosterone levels when determining hyperandrogenism in women, but also to evaluate other steroid hormones and the relationship of hormones to each other. Since our findings of a high TT/DHT ratio and its strong link to an adverse metabolic phenotype was only found in PCOS patients, the TT/DHT ratio might serve as a new biomarker for an adverse metabolic phenotype in the polycystic ovary syndrome. Nevertheless, findings should be evaluated in larger trials and future studies.

12.3. Single Nucleotide Polymorphism (SNP) Genotyping

We are the first to investigate the inhibin polymorphism rs12720062 in PCOS patients, a SNP located in an exon of the inhibin α-subunit. In previous studies, this SNP has already been linked to premature ovarian failure (POF), a disease leading to an early onset of menopause before the age of 40 [70, 107]. However, studies concerning inhibin in PCOS show conflicting results, ranging from lower to higher serum levels of inhibin A and B in PCOS patients compared to healthy women [4, 24, 59, 89, 127, 144].

Inhibin is produced by the follicles of the human ovary and regulates secretion of FSH from the pituitary. There are two forms of inhibin, called inhibin A and B, both consisting of the α-subunit linked to a second α-subunit (in case of inhibin A) or a β-subunit (in case of inhibin B). In the ovary, the α-subunit is expressed by the granulosa cells and the theca cells. Since inhibin blocks FSH secretion, high inhibin levels in PCOS patients could be responsible for a high LH/FSH ratio [60]. Moreover, inhibin directly stimulates androgen production of theca cells in vitro [45, 78].

The “AG” genotype was found in 10 of 233 patients and was significantly associated with lower AMH, LH, ACTH, cortisol, testosterone, free testosterone and androstenedione levels. No statistically association with basal FSH levels could be found. However, AMH and LH are both known to be related to inhibin serum levels; hence, it is likely that the SNP affects bioreactivity of inhibin and thus affects LH and AMH levels. Reduced bioreactivity of inhibin caused by the polymorphism rs12720062 was also found in a previous study in patients with POF [20]. The amino acid change caused by this polymorphism occurs in a highly conserved region which could be crucial for the biological function of the protein. The reduction in bioreactivity could also explain the significant association with lower androstenedione and testosterone serum levels, since inhibin normally stimulates androgen production in theca cells [45, 78]. However, serum inhibin measurements in our study cohort still need to be performed.

So far, an influence of the inhibin polymorphism on ACTH or cortisol levels has not been demonstrated. However, inhibin is expressed by the adrenal cortex and this expression is regulated by ACTH [136]. It was further shown that knockout mice lacking

the inhibin α-subunit develop adrenocortical tumors [47]. Hence, inhibin is regarded as an important factor in adrenal gland physiology and as a possible tumour suppressor [137].

Limitations of our genetic analyses are the relatively small sample size of PCOS patients and a missing genotyping of healthy controls. For further investigations, serum levels of total inhibin as well as inhibin A and B, and genotyping of healthy women for inhibin SNPs will be performed.

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