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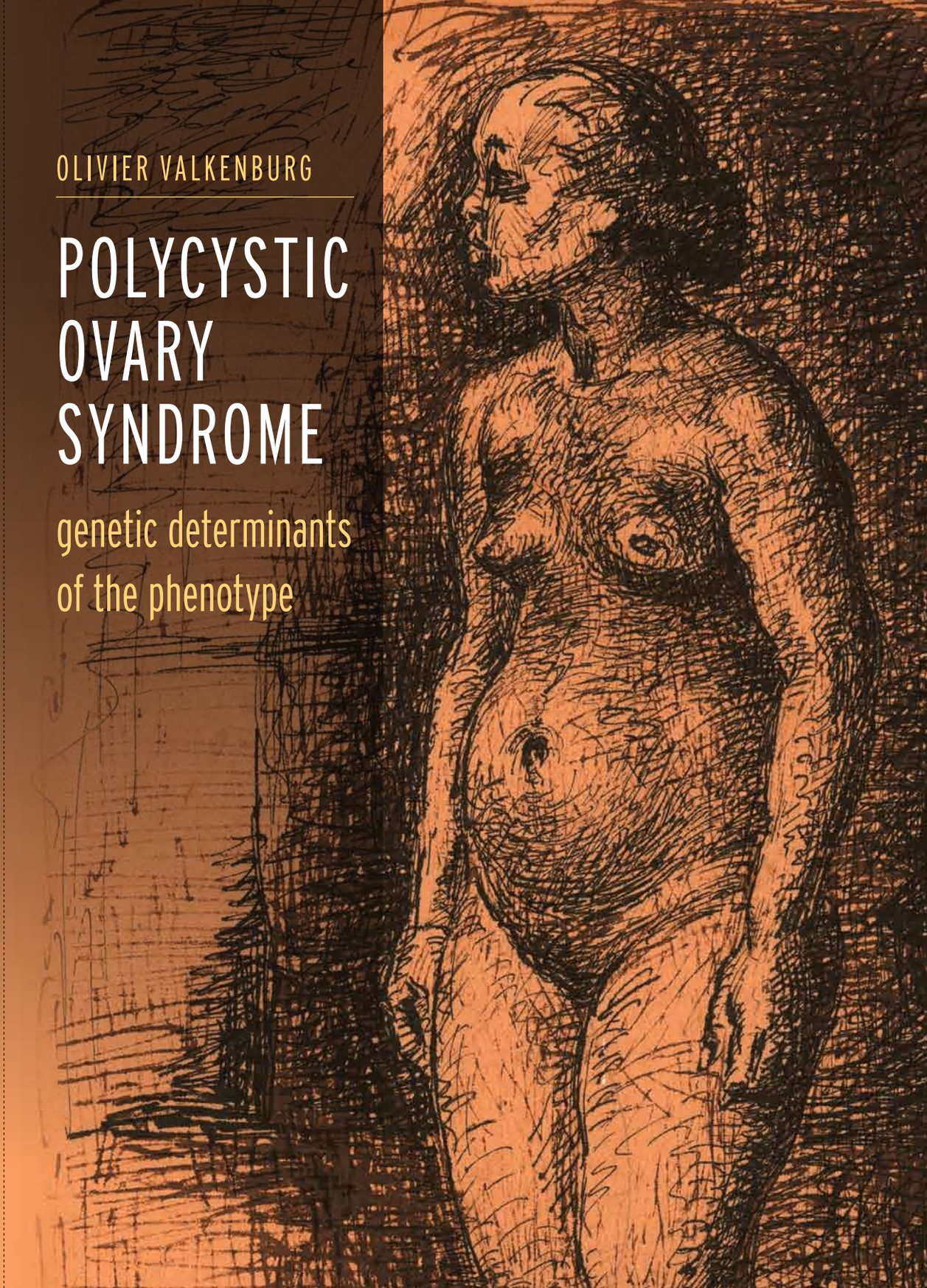

Polycystic Ovary Syndrome

Genetic determinants of the phenotype

Olivier Valkenburg

Polycystic Ovary Syndrome: genetic determinants of the phenotype
Thesis, Erasmus University Rotterdam, The Netherlands

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Polycystic Ovary Syndrome

Genetic determinants of the phenotype

Polycysteus ovarium syndroom

Genetische determinanten van het fenotype

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Olivier Valkenburg
geboren te Riethoven

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"Misschien is niets geheel waar, en zelfs dát niet"

Multatuli (1820-1887)

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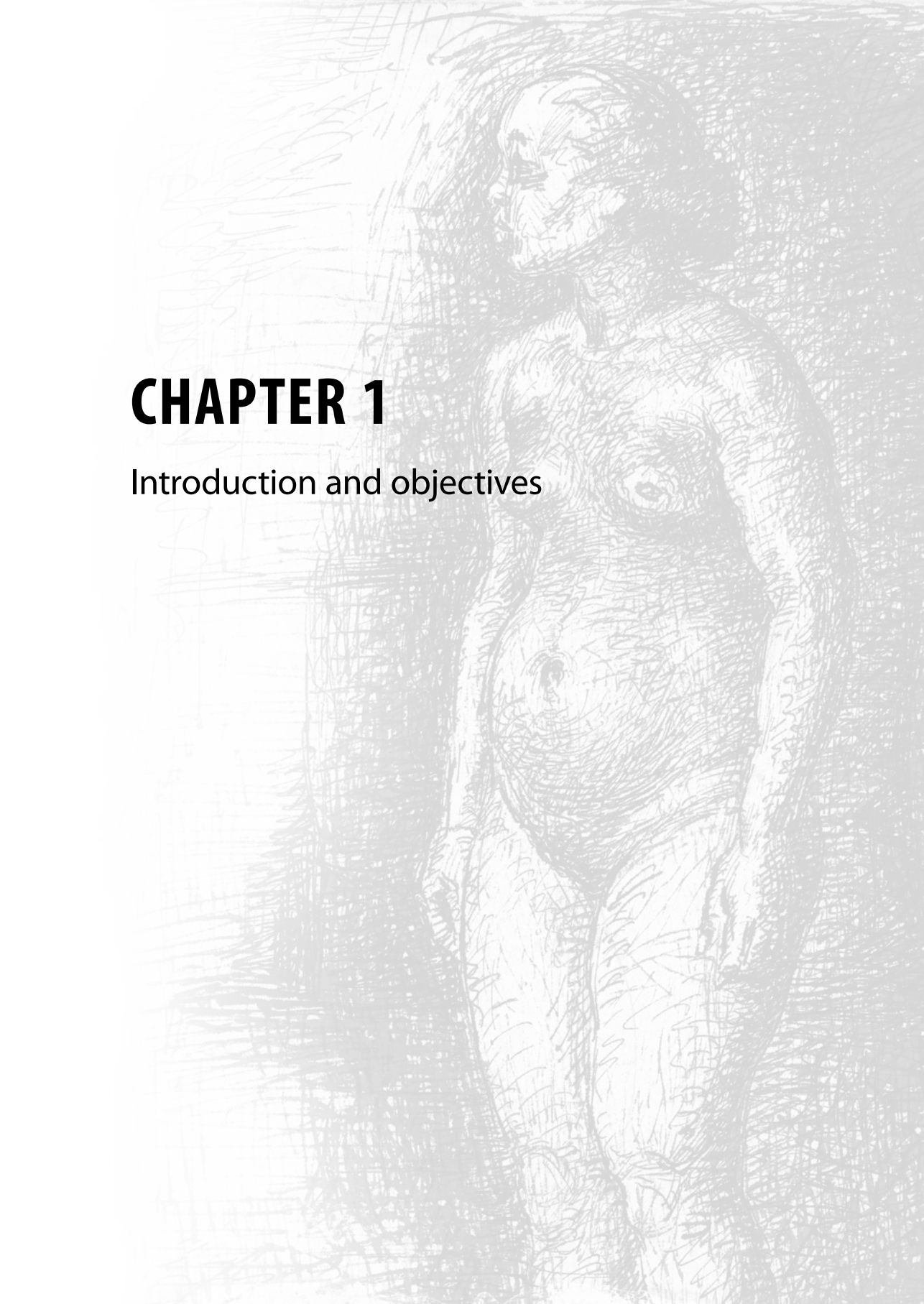

CHAPTER 1

Introduction and objectives

1.1 PCOS PHENOTYPE

The polycystic ovary syndrome (PCOS) was first described in 1935 by Stein and Leventhal as an association of amenorrhoea, obesity and a typical, polycystically enlarged, appearance of the ovaries at laparatomy[1]. Taking into account the absence of advanced imaging techniques and the relatively high risk that was associated with abdominal surgery at that time, it is remarkable that this association was noted, and published, as early as 1935. Perhaps this fact alone serves best to demonstrate the wide impact on population health that is associated with the syndrome that is currently recognised as PCOS. It is the most frequently occurring endocrinopathy among women of reproductive age, with an estimated prevalence of 5-10 % among women of fertile age. The ovulatory disorder that accompanies PCOS is the cause of subfertility and is often the most important reason for affected women to seek medical care. Furthermore, the clinical phenotype of PCOS is characterised by signs of elevated levels of free circulating androgens such as hirsutism, acne and male pattern baldness. However it is important to note that, no matter what diagnostic criteria are used, PCOS constitutes a notoriously heterogeneous phenotype. Due to the absence of a robust aetiologic framework for the pathogenesis of PCOS, physicians are not able to provide a single uniform definition that performs as an accurate diagnostic tool for the diagnosis of PCOS. Therefore, the diagnosis is still based on its description as a clinical phenotype that is characterised by a limited number of typical features (such as oligoovulation, hyperandrogenism and/or polycystic appearance of the ovaries on ultrasound examination).

Definition of PCOS

Science usually begins with a definition. The debate with regard to the exact criteria that should be used for the diagnosis of PCOS is longstanding and shows every promise of continuing well into the 21th century. The first consensus definition of PCOS arose from the proceedings of an expert meeting that was sponsored by the National Institute of Health (NIH, United States of America) in April 1990. This meeting, that gave rise to the so called 1990 NIH criteria[2], was the first to provide a uniform definition of PCOS that found widespread acceptance throughout the field of reproductive medicine. Here, PCOS was defined as the combined presence of 1) hyperandrogenism and/or hyperandrogenemia, 2) oligoovulation and 3) exclusion of related disorders such as Cushing's syndrome, hyperprolactinemia, and congenital adrenal hyperplasia (CAH). While based on consensus rather than empirical evidence, the use of a standardised set of diagnostic criteria still constituted a major step forward. However, the following years were marked by a gradually increasing awareness that the clinical expression of PCOS may be broader than that specified by the 1990 NIH criteria. A more clinically oriented approach towards ovarian dysfunction, whether it be accompanied by oligoovulation or not, marked the

Table 1 Diagnostic criteria for PCOS, after exclusion of other disorders

Clinical phenotype	1990 NIH	2003 Rotterdam	2006 AES
OD + HA + PCO	PCOS	PCOS	PCOS
OD + HA, no PCO	PCOS	PCOS	PCOS
HA + PCO, no OD	X	PCOS	PCOS
OD + PCO, no HA	X	PCOS	X

OD=Ovulatory dysfunction, HA=clinical and/or biochemical evidence of androgen excess, PCO= Polycystic ovary morphology, X= no diagnosis of PCOS

proceedings of a second consensus meeting that was hosted by the Erasmus university in Rotterdam, the Netherlands, in 2003 [3, 4]. Following this meeting, PCOS was recognised as a syndrome of ovarian dysfunction. As such, no single diagnostic criterion (such as hyperandrogenism or PCO) is sufficient for the clinical diagnosis. PCOS is diagnosed by the presence of at least two of the three cardinal features (oligoovulation, hyperandrogenism and polycystic ovary morphology). It is important to note that the 2003 Rotterdam criteria add to, rather than alter, the 1990 NIH criteria. In comparison to the NIH criteria, two additional phenotypes are included in the definition of PCOS that consist of A) women with polycystic ovary morphology (PCO) and clinical and/or biochemical evidence of androgen excess, but without ovulatory dysfunction, and B) women with PCO and ovulatory dysfunction, but no signs of androgen excess. The use of 2003 Rotterdam criteria results in a significantly higher percentage (91% vs 55%) of normogonadotropic anovulatory (World Health Organisatio class 2, WHO2) women who can be diagnosed with PCOS and the inclusion of a phenotype that is characterised by a lower frequency of obesity, hyperglycaemia and insulin resistance compared with the NIH criteria[5]. The 2003 Rotterdam criteria constitute, at present, the most widely acknowledged definition of PCOS and are used in the studies that are described in this thesis.

In 2006, a third expert consensus was put forward as a position statement by the androgen excess society (AES) [6]. Perhaps it should come as no surprise that the taskforce arrived at the conclusion that, based on the risk for metabolic morbidity, PCOS should first and foremost be considered as a disorder of hyperandrogenism. Likewise, the classification criteria for PCOS that are proposed by the AES concede to the inclusion of normoovulatory women with PCO and hyperandrogenism, but no diagnosis of PCOS can be made when hyperandrogenism is not present. Table 1 provides an overview of the various classifications that have been proposed for the diagnosis of PCOS.

Phenotype expression: heterogeneity in the phenotype of PCOS

While the exact definition of PCOS can be debated, there is no question that the clinical signs and symptoms that are present in women with PCOS vary substantially between individual patients. A good example of this is the association with obesity that was al-

ready noted in the original publication by Stein and Leventhal in 1935 [1]. However, while obesity is clearly more prevalent among women with PCOS, it is generally acknowledged that an anovulatory hyperandrogenic phenotype can also exist among lean women. The same holds true for the presence of other signs and symptoms such as hyperandrogenaemia, hirsutism, ovulatory dysfunction and polycystic ovary morphology. All of the above may occur in various combinations in women with PCOS. The search for one overlapping pathofysiologic model that can satisfactorily explain all of the cardinal features of PCOS and that allows for the heterogeneity of the clinical phenotype, so far has not been successful and there is still much debate among experts with regard to the correct set of diagnostic criteria that should be used for the diagnosis of PCOS.

One may speculate that multiple aetiologic pathways can exist that result in a phenotype that can be recognized as PCOS. As such, it is known that disorders of adrenal sex steroïd synthesis that result in high levels of adrenal androgens, may result in a phenotype that cannot be distinguished from PCOS. Other aetiologic pathways may include obesity and/or insulin resistance, the production of gonadotropic hormones or ovulatory dysfunction at the level of the ovaries. At present, phenotype groups have not been clearly specified and the clinician will usually not be able to distinguish between them.

The question remains, from a clinical point of view, whether there exists a real necessity to distinguish between various phenotype groups. From a patient's perspective, the most pressing problem usually is subfertility that is caused by the absence of regular ovulations. The classical treatment paradigm for ovulation induction constitutes first line treatment with clomiphene citrate and, if necessary, second line treatment with follicle stimulating hormone (FSH). Thus far, the treatment protocol is generally not dependent on the specific phenotypic characteristics of the individual patient. However, treatment results may profit from an approach that is better tailored to the individual patient. This has been the subject of multiple studies by Imani and van Santbrink and others, who found that the most important determinants of ovulation induction in WHO2 anovulatory subfertility constitute hyperandrogenism (free androgen index = testosterone/sex hormone-binding globulin ratio), cycle history (oligomenorrhea *vs.* amenorrhea), age and obesity (body mass index) [7-12]: Also, considerable differences may exist with regard to long term sequelae of PCOS such as increased risk of endometrial cancer[13] and the possibility of increased risk for cardiovasular disease[14, 15].

Adding to the heterogeneity of the clinical phenotype of PCOS is the reality of many of today's clinical practices that deal with an ethnically diverse patient population. Lifestyle and health behaviour may vary greatly between ethnic subgroups, adding to overall phenotypic differences; for example the presence of obesity and insulin resistance. It seems plausible that differences in the genetic make-up of ethnic populations may also contribute to disease risk and the phenotype of PCOS. It is conceivable that ethnic

background should be accounted for in order to correctly interpret the phenotype of the individual patient.

1.2 GENETIC RESEARCH IN PCOS PATIENTS

Searching for PCOS genes: the rationale for a genetic predisposition

As early as 1968, Cooper et. al. reported on the hereditary nature of the polycystic ovarian phenotype in women[16]. The following years, numerous studies have convincingly shown evidence for a genetic trait among PCOS probands, although varying definitions of PCOS were used. Genetic research among PCOS patients is greatly hampered by the lack of a well defined phenotype in women and the lack of an undisputed phenotype among men. Moreover, the various studies of inheritance of PCOS within families have not been able to identify a clear Mendelian inheritance pattern for PCOS (Table 2).

It is generally accepted that, as well as most common diseases, PCOS should be regarded as a complex genetic disease that is determined by both genetic and environmental factors such as diet and obesity. In this sense PCOS may be much akin to type 2 Diabetes Mellitus, perhaps even sharing some of the genetic factors that influence diabetes and the metabolic syndrome[17]. The genetic model is illustrated in Figure 1. It is important to realize that the complex genetic model allows for the interaction of multiple small genetic effects that may act together in order to increase disease risk and influence the phenotype of any specific patient. Moreover, disease risk and phenotype will not only be influenced by the genetic predisposition of a certain person but also the amount of

Table 2 Inheritance studies for PCOS

Author	No. Studied	Mode of inheritance
Cooper et al., 1968[16]	18 PCOS women, first-degree relatives and controls	Autosomal dominant
Givens et al., 1988[33]	3 multiply affected families	(?X-linked) dominant
Ferriman et al., 1979[34]	381 PCOS women, relatives and controls	Modified dominant
Lunde et al., 1989[35]	132 PCOS women, 1st and 2nd degree relatives and controls	Unclear
Hague et al., 1988[36]	50 PCOS women and 17 women with CAH and controls	Unclear
Carey et al., 1993[37]	10 kindreds and 62 relatives	Autosomal dominant
Norman et al., 1996[38]	5 multiply affected families	Not stated
Legro et al., 1998[39]	80 PCOS probands, 115 sisters	Autosomal-dominant trait
Govind et al., 1999[40]	29 multiply affected families	Autosomal dominant
Kahsar-miller et al., 2001[41]	90 PCOS probands, 50 sisters, and 78 mothers	Suggesting genetic trait
Vink et. al., 2006[42]	1332 monozygotic twins, 1873 dizygotic twins/ singleton sisters of twins	Suggesting genetic trait

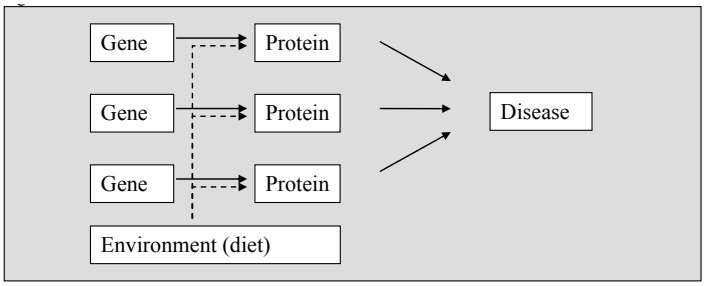

Figure 1

metabolic derangement that may already be present, for example as a result of obesity and insulin resistance. In other words, genetic research that attempts to identify and study risk-alleles for PCOS will be hampered by the fact that effect sizes are expected to be very small, thereby increasing the chance of type II error. There is a definite need for large sample sizes in order to achieve sufficient power to detect risk-alleles.

Candidate gene studies

Genetic research in complex diseases can take either one of two strategies i.e., candidate gene studies *vs.* gene finding. The difference being that candidate gene research is very much hypothesis driven and will relate to genetic variants of genes that are expected to be aetiologically involved in PCOS. This expectation may be the result of the specific function of the gene, for instance in the production of androgens or the occurrence of insulin resistance and type II diabetes, or may stem from the results of other research (gene finding studies). This approach has the advantage that only a limited number of hypotheses are considered, thereby reducing the chance for type I error (multiple testing). Current literature reveals a large amount (>50) of candidate genes that have been associated with PCOS in some way or another. However, the obvious disadvantage of candidate gene studies is that we cannot be certain which, and how many, genes are involved in PCOS. It is expected that different genes will be involved in different pathways and population differences may exist with regard to the specific pathways that are causally related to PCOS. All of the above may explain why most of the candidate genes that have been associated with PCOS could not be reproduced in different populations.

Gene finding

As an alternative to candidate gene studies, it is possible to regard the entire genome as a possible source of disease risk and conduct linkage and association analyses in order to identify a certain locus on the genome that is associated with the disease. Originally, these techniques stem from genetic research in monogenetic diseases that have a well-defined phenotype and a high penetrance. These studies rely heavily on the spatial

relationship between the location of any given marker on the genome and its proximity to the locus of a genetic variant that is causally related to the disease. The closer this marker is positioned to the disease gene, the more likely they will be inherited together in subsequent generations (i.e. no recombination-event takes place). This is the general principle behind linkage disequilibrium (LD) and it allows for research that regards a limited set of highly polymorphic genetic markers, usually restriction fragment length polymorphism (RFLP's), that are spread across the genome. Any marker that is located in the proximity of a disease locus is expected to (at least partially) share similar inheritance with the disease under investigation. The general principle of linkage disequilibrium and genetic association is illustrated in Figure 2. In a population that is marked by relatively limited migration over the course of multiple generations, such a marker would be expected to show a higher frequency among cases compared to healthy controls. In this way, a disease gene can be identified by zooming-in on the specific disease locus with marker sets that are targeted on that specific area of the genome.

One advantage of the procedure is the fact that it does not rely on any preordained hypothesis regarding the aetiology of the disease. The only prerequisite being that a genetic predisposition is undisputedly present. Moreover, genetic techniques are rapidly getting faster and cheaper, allowing for a higher number of genetic variants that can be tested at once. With the advent of *in-silico* genetics, association studies can be performed with a very high spatial resolution of genetic variants across the entire genome. The genome-wide-association (GWAS) studies are limited by the fact that the number of hypotheses that are tested can be so great (exceeding 500 K) that type I error becomes a very real possibility when the usual threshold for significance (P=0.05) is observed. Therefore, it is common practice to adhere to a much stricter threshold for significance

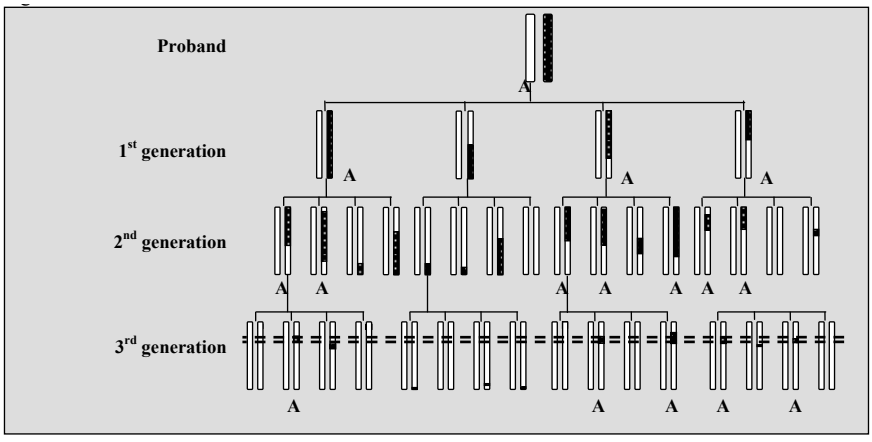

Figure 2

that is usually set at between 10^{-7}-10^{-8}. Also, type 1 error can be overcome by the routine use of replication studies in a different population.

1.3 OBJECTIVES OF THIS THESIS

The studies that are described in this thesis will cross the bridge from a morphometric point of view that serves to identify clinical and endocrine determinants of PCOS to a more genetically oriented approach. However, it is important to realize that the goal of our efforts is similar in that we try to get a grip of those parameters that are directly associated with women's health with respect to anovulation and the PCOS phenotype. These health issues should not be restricted to subfertility that is caused by the ovulatory disorder that is often present in PCOS, but also long-term sequelae such as increased risk for cardiovascular disease and endometrial cancer.

Chapter 2 This chapter will focus on the phenotype of PCOS patients with regard to the possibility of increased risk of long-term cardiovascular disease. Cardiovascular risk factors seem to cluster in women with PCOS compared to the general population[18]. A wide variety of risk factors have been studied in association with PCOS including obesity, insulin resistance, dyslipidemia, endothelial dysfunction, and the presence of the metabolic syndrome[6, 19-21]. Dyslipidemia, that is the result of insulin resistance, is well known and has been studied in type 2 diabetetes[22-25] (Figure 3). The question whether atherogenic changes can be observed in lipid profiles of women with PCOS is the focus

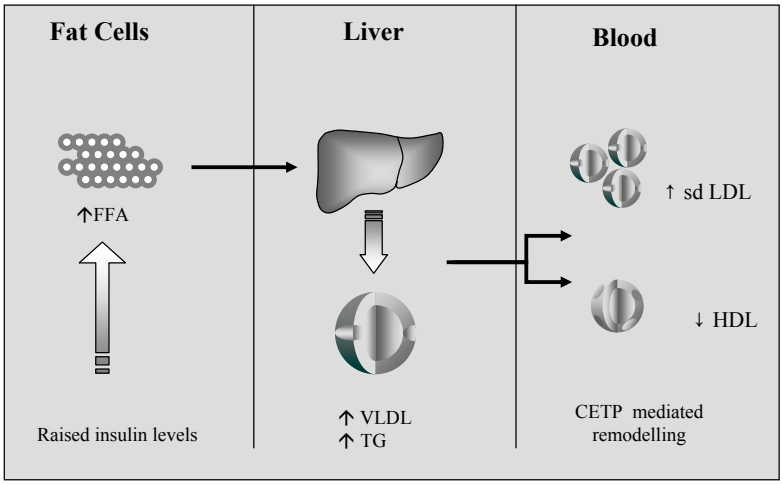

Figure 3

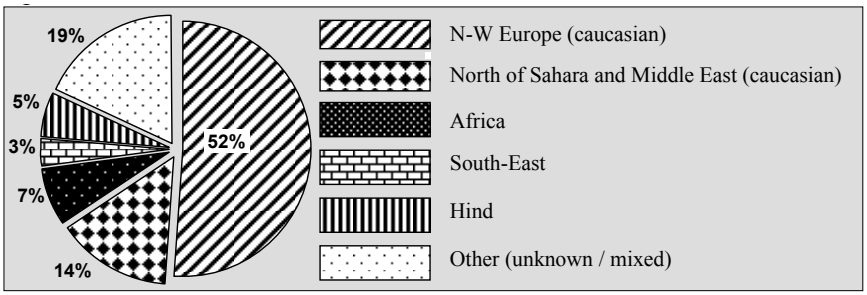

Figure 4

of the first chapter. Furthermore, the influence of hyperandrogenism on lipid profiles of women with PCOS is examined.

The same chapter describes a different study that takes a combined (clinical and genetic) approach to describe ethnic heterogeneity in the phenotype of PCOS. Our academic practice of women's health and fertility medicine is set in an urban population (Rotterdam, the Netherlands) of people with very diverse geo-biographic ancestries (Figure 4). It was questioned whether a specific set of 10 single nucleotide polymorphosms (SNPs)[26] can be used to accurately map phenotypic differences among ethnic subgroups. In addition, we questioned whether their use adds information over self-reported ethnic background.

Chapter 3 This chapter describes two candidate gene studies. The objective of our studies was two-fold; first we aimed to identify risk-alleles for PCOS. In order to achieve this goal, allele frequencies and haplotypes were compared in PCOS cases and healthy controls. Second, in order to understand the possible mechanisms of action, we studied ultrasound and endocrine characteristics of a large cohort of PCOS cases that were uniformly examined at our clinic. Candidate genes were selected for analysis on the basis of their central role in the regulation of ovarian function (gonadotrophin-releasing hormone [GnRH], FSH-receptor, LH-receptor), steroid hormone metabolism (aromatase [CYP19], 11β-hydroxysteroid dehydrogenase type 1 [11βHSD], hexose-6-phosphate dehydrogenase [H6PD]) and signal transduction (estrogen receptor [ESR1 and ESR2], glucocorticoid receptor [GCR]. Genetic variants that were selected for analysis have been identified in prior research as functional genetic variants that are known to have a certain influence on (disease) phenotype. Candidate genes and polymorphisms that are described in this thesis are listed in Table 2.

Table 3 Candidate genes for PCOS (present thesis)

Pathway	Gene	Polymorphism
Estrogen synthesis and signal transduction	Estrogen receptor, ESR1	-397exon2 C/T (rs2234693)
	Estrogen receptor, ESR1	-351exon2 A/G (rs9340799)
	Estrogen receptor, ESR2	+1730 3'UTR G/A (rs4986938)
	Aromatase, CYP19	+1531 A/G (rs10046)
Corticosteroid metabolism and signal transduction	Glucocorticoid receptor	Arg23Lys (rs6190)
	Glucocorticoid receptor	Asn363Ser (rs6195)
	Glucocorticoid receptor	BclI (rs41423247)
	Glucocorticoid receptor	Exon9b A/G
	11BHSD	83557 ins-A
	H6PD	Arg453Gln (rs6688832)
Gonadotropic hormone synthesis and signal transduction	GnRH	Ser16Trp (rs6185
	FSH receptor	Ala307Thr (rs6165)
	FSH receptor	Asn680Ser (rs6166)
	LH receptor	exon 1, insLQ
	LH receptor	Asn291Ser (rs12470652)
	LH receptor	Ser312Asn (rs2293275)

Chapter 4. Following the results of our candidate gene studies, that are very much hypothesis driven, we aimed to search the entire genome for risk loci that are associated with PCOS. In order to overcome the specific issues of genetic heterogeneity and limited LD in the general (outbred) population, we aimed to conduct our research in a genetically isolated population where there is a small number of founders, limited inward migration and rapid expansion of the population in subsequent generations. Such a population was identified in the southwest of the Netherlands (Rucphen). Moreover, detailed genealogical data are available for this population by way of municipal and church records that can be used for linkage analysis.

Chapter 5. This chapter aims to cross the gap between scientific interest and clinical applicability of genotype data. On the basis of our own research and that of others FSH receptor polymorphism comes forward as an important regulator of ovarian function. More interestingly still, it was observed that FSH receptor genotype may influence ovarian response during ovarian hyperstimulation for *in-vitro* fertilisation (IVF). Multiple reports indicate a greater need for exogenous FSH in association with the 680^{Ser} allele of the rs6166 polymorphism[27-29], although some controversy still remains[30-32]. Our research aims to study the influence of this genetic variant on ovulation induction (1^{st} line therapy with Clomiphene Citrate, 2^{nd} line therapy with exogenous FSH preparations) that is the standard therapy for normogonadotropic (WHO2) anovulatory subfertility.

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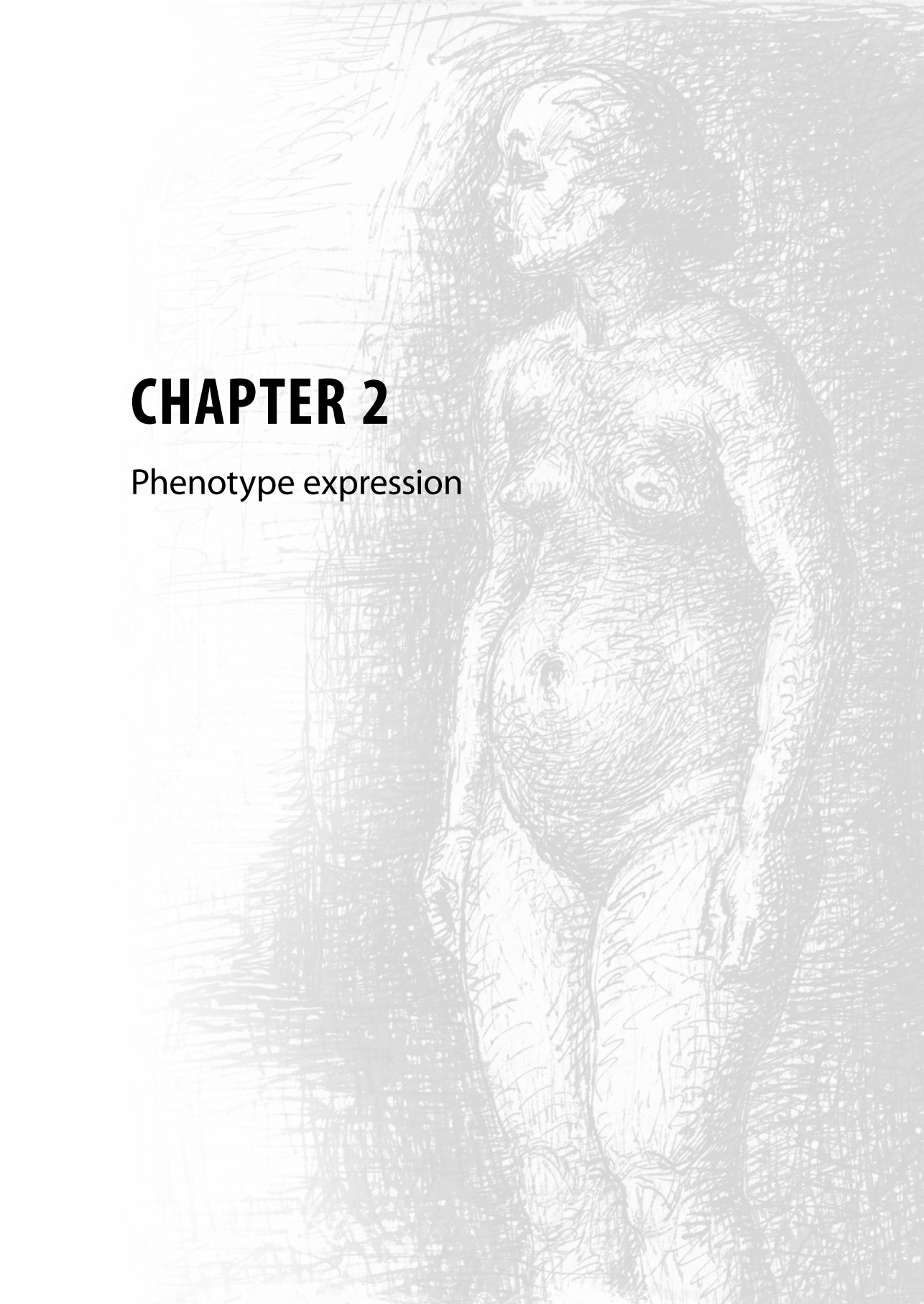

CHAPTER 2

Phenotype expression

CHAPTER 2.1

A more atherogenic serum lipoprotein profile is present in women with polycystic ovary syndrome: a case-control study

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a syndrome of ovarian dysfunction that is characterized by anovulation, hyperandrogenism and/or the presence of polycystic ovary morphology [1]. Obesity and insulin resistance occur frequently in association with this syndrome. Cardiovascular risk factors seem to cluster in women with PCOS compared to the general population [2]. A wide variety of risk factors have been studied in association with PCOS including obesity, insulin resistance, dyslipidemia, endothelial dysfunction, and the presence of the metabolic syndrome [3-6]. In view of the high frequency of cardiovascular risk factors in women with PCOS, it is rather surprising that excess coronary heart disease (CHD) was not found in a retrospective cohort study of women with PCOS [7]. However data on prospective long-term follow-up are not yet available. It has been speculated that women with PCOS may be exposed to a protective factor, such as prolonged exposure to estrogens.

Most studies of dyslipidemia and PCOS have reported on cholesterol (C) levels and triglycerides (TG). The lipid profile that is found in women with PCOS consists of elevated TG levels, together with low levels of HDL-C [8]. These changes are consistent with the lipid profile that is typically found in association with insulin resistance. The effects of insulin resistance on lipid metabolism are well known. Increased secretion of VLDL-particles by the liver results in elevated plasma TG concentrations. Subsequently, triglycerides are exchanged for cholesteryl ester (CE) by the activity of cholesteryl ester transfer protein (CETP). This process results in TG-enriched HDL particles that are catabolized more rapidly, and CE-enriched VLDL particles that are converted into small dense LDL particles [9]. As a consequence, insulin resistance contributes to decreased plasma levels of HDL cholesterol and apolipoprotein A-I (apoA-I), and higher levels of apolipoprotein B (apoB) [10-12].

In addition to insulin resistance, lipid metabolism in women with PCOS may also be affected by ovarian and/or adrenal secretion of sex-steroids. The effects of sex-steroids on lipid metabolism are complex and involve the actions of both androgens and estrogens. Hyperandrogenism has been associated with increased hepatic lipase (HL) activity. This enzyme, that has a role in the catabolism of HDL particles, exhibits strong sexual dimorphism, with exogenous androgens up-regulating, and estrogens down-regulating its activity [13, 14]. A study of 17 female-to-male transsexuals who were exposed to treatment with exogenous testosterone showed a significant increase in HL activity in association with decreased plasma HDL-C levels [15]. Endogenous estrogens may affect LDL metabolism through upregulation of the LDL receptor, resulting in enhanced hepatic clearance of LDL particles from plasma [16, 17].

ApoB and apoA-I are the main structural proteins of atherogenic lipoproteins and HDL particles, respectively. Population based longitudinal follow up studies have indicated

that apoA-I and apoB levels are potent markers for cardiovascular risk [18-21]. ApoB levels reflect the entire spectrum of pro-atherogenic particles whereas apoA-I contributes to the anti-atherogenic properties of HDL particles. At present, only a limited number of studies have investigated apolipoprotein levels in women with PCOS. Typically, these studies are limited by small sample sizes[3, 22-24]. Macut and colleagues reported similar apoB and apoA-I levels in women with PCOS and controls [25]. However the control group was limited to 56 women. Reports with regard to apoA-I levels show either unchanged [3, 22, 25] or lower [23, 24, 26] levels in women with PCOS. Hence, data on plasma apoA-I and apoB levels in women with PCOS remain inconclusive.

It is expected that women with PCOS have a more atherogenic lipid profile than healthy controls. In order to test this hypothesis we performed a case control study in a large cohort of women who were uniformly phenotyped according to the 2003 Rotterdam criteria [1]. Plasma lipids, apoA-I and apoB levels were measured in all PCOS cases and controls. Furthermore, this study aims to assess the relative impact of obesity, insulin resistance and hyperandrogenism on the lipid profile of women with PCOS.

SUBJECTS AND METHODS

Subjects

Patients attended our fertility clinic between 1996 and 2005 with oligomenorrhea (interval between menstrual periods ≥35 days) or amenorrhea (absence of vaginal bleeding for at least 6 months) and serum FSH concentrations within normal limits (1–10 IU/l), i.e. normogonadotropic anovulation (classification according to the World Health Organization, WHOII) [27]. The diagnosis of PCOS was established on the basis of the 2003 ESHRE/ASRM revised Rotterdam criteria [1]. In agreement with the Rotterdam criteria hyperandrogenism was defined as having either biochemical or clinical signs of androgen excess. For the purpose of this study clinical hyperandrogenism was assessed by means of the Ferriman Gallway (FG) score, and was defined as FG score ≥ 8. Biochemical hyperandrogenism was determined by calculation of the free androgen index (FAI) as T (nmol/l) / SHBG (nmol/l) * 100 (to convert into SI-units multiply T(ng/dL) and SHBG (µg/dL) by 0.0347 and 34.72 respectively). A cutoff level of 4.5 was used for the definition of hyperandrogenism [27]. The presence of polycystic ovaries (PCO) was detected by vaginal ultrasound examination. PCO was defined as the presence of 12 follicles or more in one or both ovaries, and/or increased ovarian volume (>10 ml). Exclusion criteria were diabetes mellitus with a fasting glucose level of 126 mg/dl or higher, non-fasting state at the time of investigation and presence of related disorders with similar clinical presentation, such as congenital adrenal hyperplasia and Cushing's syndrome.

Controls were recruited at child health centers in the same geographic area as the study population. The control group consisted of fertile mothers of healthy children who visited the hospital at a standardized study moment of around 17 months after pregnancy. Controls have been described previously [28, 29]. Exclusion criteria for controls were irregular menstrual cycle at the time of investigation, breastfeeding, diabetes mellitus, and non-fasting state. Information on general health and cycle history were gathered by questionnaire. This study was approved by the Central Committee on Research Involving Human Subjects (the Hague, the Netherlands) and the institutional review board at the Erasmus Medical Center. Informed consent was obtained from all participants.

Clinical and endocrine examination

Anovulatory patients underwent a standardized initial examination that was performed after an overnight fast on a random day between 9 a.m. and 11 a.m. Clinical examination included menstrual history and anthropometric measurements (height and weight). Transvaginal ultrasonography was performed to assess ovarian volume and follicle count for both ovaries. Blood samples were obtained by venipuncture and processed within 2 hours. Serum was isolated after centrifugation at 3,000 rpm for 10 minutes at 20ºC and stored at –20 °C until assayed. Endocrine evaluation included serum levels of gonadotropic hormones (LH, FSH) and estradiol (E2), androgens (testosterone (T), androstenedione (AD), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS)), progesterone and 17-hydroxyprogesterone (17-OH-Pg), sex-hormone binding globulin (SHBG), fasting glucose and insulin, thyrotropin (TSH) and prolactin. Hormone assays have been described in detail elsewhere [30]. LH, FSH, TSH, prolactin, and insulin were measured by immunoradiometric assay. T, AD, E2, SHBG, Pg, 17-OH-Pg, DHEA and DHEAS were determined by radioimmunoassays. Intra- and inter-assay coefficients of variation were <5 and <15% for LH, <3 and <8% for FSH, <3 and <5% for testosterone (T), <8 and <11% for androstenedione (AD), <5 and <7% for estradiol (E2), and <4 and <5% for sex hormone-binding globulin (SHBG). Controls underwent a standardized clinical examination that included maternal height and weight. Data on general health, cycle history and the presence of diabetes were collected by questionnaire.

Lipid assays

Venous blood-samples were drawn at the time of examination and stored at –80 °C after centrifugation at 3,000 rpm for 10 minutes at 20ºC. Lipid assays were performed in both cases and controls. Total cholesterol (C), and triglycerides (TG) were analyzed using commercially available assays (Wako Diagnostics, Japan). HDL cholesterol (HDL-C) was analyzed using the direct HDL-assay from Wako diagnostics. Serum apoA-I and apoB were analyzed with a commercially available immunoturbidometric assay (Wako

Diagnostics, Japan). All assays were analyzed on a Cobas Mira autoanalyzer. LDL-C was calculated with the Friedewald formula (LDL-C = C - HDL-C - [TG/2.2]).

Statistical analysis

Distributions of the characteristics in the study groups are presented as the mean and standard deviation (SD) when distributed normally or as the median and interquartile range (P25-P75) when not normally distributed. A nonparametric test (Mann-Whitney U) was used for exploratory comparison of continuous variables between groups. Variables were checked for normal distributions with the one-sample Kolmogorov-Smirnov test and log-transformed when not distributed normally. Analysis of covariance (ANCOVA) was applied to adjust for differences in age and BMI. Between-group differences of categorical variables were evaluated by Pearson's chi-square (X2) test. Statistical significance for all analyses was defined as a two-tailed P value of less than or equal to 0.05. Multiple linear regression analysis was used to identify the best predictors of apolipoprotein levels in women with PCOS. To assess the univariable relation between the initial screening variables and apolipoproteins, Spearman's correlation coefficients were computed. Initial screening variables that were significant in univariate analysis were entered into the multiple regression models in a forward stepwise fashion. Data analysis was performed using SPSS, version 12.0 (SPSS, Inc., Chicago, USA).

RESULTS

Baseline characteristics of women with PCOS and controls

The case group consisted of 638 women who were diagnosed with normogonadotropic anovulation. Eight women were excluded for having diabetes mellitus and seven women had not fasted on the day of venipuncture. Applying the 2003 Rotterdam criteria, 66 women did not meet the criteria for PCOS. All PCOS women reported cycle disorders. The control group consisted of 408 women, of whom 61 individuals were excluded for reasons of being pregnant (N=32), breastfeeding at the time of the study (N=14), not having fasted at the day of investigation (N=14) or the presence of diabetes mellitus (N=1). Of the remaining 347 women, 52 (15%) reported various abnormalities regarding their menstrual cycle. The final group of 295 women all reported regular menstrual cycles.

Oligomenorrhea occurred in 74% (N=412) of women with PCOS. All remaining women had amenorrhea. PCO, as evidenced by vaginal ultrasound, was found in 89% (N=493) of women with PCOS. Hyperandrogenism was present in 64% (N=358) of women with PCOS. There was a small, but significant, difference in age between the case group (median age 28.8 yr) and controls (32.4 yr, P≤0.01). Obesity (BMI≥30 kg/m2) occurred more

frequently in PCOS cases (N=183, 33%) compared to the control group (N=36, 12.2%). The differences in age and BMI between the case group and controls were statistically significant. Therefore, further analyses were adjusted for these two parameters. Analysis of covariance showed that women with PCOS had slightly higher fasting insulin levels than controls (medians adjusted for age and BMI: 9.4 and 7.8 mU/l). Although small, this difference was statistically significant.

Lipid profile

Table 1 summarizes baseline characteristics and lipid profiles in PCOS patients and controls. Women with PCOS had higher levels of C (adjusted medians: 197 vs. 182 mg/dl, P≤0.01), TG (98 vs. 88 mg/dl, P≤0.01) and LDL-C (123 vs. 106 mg/dl, P≤0.01) compared to controls. On the contrary, serum levels of HDL-C (adjusted medians: 46 vs. 55 mg/dl, P≤0.01) and apoA-I (117 vs. 136 mg/dl, P≤0.01) were significantly lower in women with PCOS. Figure 1 illustrates apoA-I levels in PCOS cases and controls. Across the entire range of BMI values apoA-I levels were significantly lower in women with PCOS. No differences in apoB levels were observed among PCOS cases and controls.

Table 1 Baseline characteristics and lipid profiles of women with PCOS and healthy controls

	PCOS (N=557)		***Controls*** (N=295)		***P*** *
	Median	IQR	Median	IQR	
Baseline characteristics					
Age (yr)	28.8	(25.3-32.2)	32.9	(29.2-35.0)	≤0.01
BMI (kg/m^2)	26.6	(22.5-32.0)	24.4	(22.0-27.1)	≤0.01
Fasting insulin (mU/l)	10.1	(6.2-15.5)	6.9	(4.6-9.8)	≤0.01
Testosterone (ng/dL)	58	(43-78)	-		
Androstenedione (ng/dL)	332	(249-434)	-		
SHBG (µg/dL)	1.0	(0.7-1.5)	-		
Free Androgen Index	5.8	(3.3-9.8)	-		
Lipid parameters					
Triglycerides (mg/dl)	95	(71-139)	82	(61-110)	≤0.01
Total Cholesterol (mg/dl)	196	(170-226)	178	(162-209)	≤0.01
HDL cholesterol (mg/dl)	45	(36-56)	56	(46-66)	≤0.01
LDL cholesterol (mg/dl)	125	(102-152)	106	(90-128)	≤0.01
ApoA-I (mg/dl)	118	(106-130)	146	(130-158)	≤0.01
ApoB (mg/dl)	81	(70-95)	79	(69-91)	0.533
ApoB/apoA-I ratio	0.69	(0.56-0.83)	0.55	(0.46-0.63)	≤0.01

Values are unadjusted medians and interquartile range (IQR). * *P* values for age and BMI were calculated with a non-parametric test for significance. Other *P* values were adjusted for differences in age and BMI (ANCOVA). Lipid parameters were log-transformed before analysis.

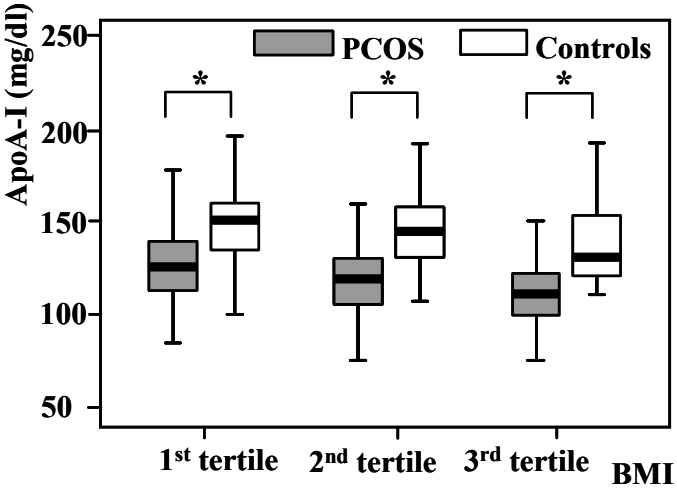

Figure 1 Apolipoprotein A-I levels in women with PCOS and healthy controls
Note: Medians and box (P25-P75) and whisker (p5-p95) plots of apoA-I levels in women with PCOS (grey) and controls (white). Patients and controls were stratified by BMI (tertiles calculated for PCOS cases: P33 = 23.5 kg/m^2 and P66 = 29.8 kg/m^2).

Stratification for BMI

Table 2 shows the result of a comparison of lipid parameters and fasting insulin levels in lean (20≤BMI<25 kg/m2), overweight (25≤BMI<30) and obese (BMI≥30 kg/m2) women with PCOS and controls. First, lean women with PCOS were compared with lean controls. Plasma insulin levels were similar in both groups. Lean PCOS women had significantly higher plasma levels of C, LDL-C and apoB/apoA-I ratio in combination with lower apoA-I levels. Plasma levels of apoB and HDL-C were equal in both groups. Next, we compared plasma lipids and insulin levels in overweight PCOS women and overweight controls. Women with PCOS had higher plasma levels of TG, C and LDL-C, together with lower HDL-C and apoA-I levels and higher apoB/apoA-I ratio. However, overweight PCOS cases had significantly higher BMI and fasting insulin levels than overweight controls (data presented in table 2). Finally, a comparison of obese women with PCOS and obese controls showed higher serum levels of TG, C, LDL-C and apoB in combination with lower levels of HDL-C and apoA-I in obese PCOS cases while we did not observe significant differences in BMI or fasting insulin levels. In order to study the effect of BMI on fasting insulin levels and lipid parameters, P-values for linear trend were calculated in both the case group as well as controls. Highly significant trends were observed for fasting insulin levels, TG, HDL and apoA-I in both PCOS cases and controls (all parameters: P<0.01). Trends for C, LDL-C and apoB were significant in PCOS cases (P≤0.01), but not in controls (P>0.05). However, the obese control group was limited to 36 individuals.

Table 2 Insulin and lipid profiles, stratification according to BMI subgroups

	Lean			***Overweight***			***Obese***		
	PCOS	Control	**P***	PCOS	Control	**P***	PCOS	Control	*P**
N	178	149		134	88		183	36	
BMI (kg/m^2)	22.7	22.6	0.69	27.5	26.8	0.01	34.9	33.2	0.16
Fasting insulin (mU/l)	6.7	6.1	0.07	11.1	7.5	≤0.01	15.2	13.9	0.24
Triglycerides (mg/dl)	81	79	0.58	106	89	≤0.01	133	89	≤0.01
Total Cholesterol (mg/dl)	193	182	0.03	197	186	0.04	205	170	≤0.01
HDL cholesterol (mg/dl)	55	60	0.19	43	55	≤0.01	39	50	≤0.01
LDL cholesterol (mg/dl)	120	106	≤0.01	128	108	≤0.01	135	101	≤0.01
ApoA-I (mg/dl)	124	146	≤0.01	115	147	≤0.01	111	129	≤0.01
Apo-B (mg/dl)	77	78	0.28	82	82	0.28	87	75	≤0.01
ApoB/apoA-I ratio	0.60	0.52	≤0.01	0.74	0.59	≤0.01	0.80	0.57	≤0.01

Values are unadjusted medians. Lean: 20≤BMI<25 kg/m^2, Overweight:25≤BMI<30 kg/m^2, Obese: BMI ≥ 30 kg/m^2.
**P* values for the difference between cases and controls, adjusted for age.

Determinants of dyslipidaemia in women with PCOS

The PCOS group was stratified into four subgroups, based on the presence or absence of hyperandrogenism and BMI≥25 kg/m2. Table 3 shows fasting insulin levels and lipid parameters in the four subgroups and lean controls (20≤BMI<25 kg/m2). First, the subgroup of lean PCOS women who did not have signs of hyperandrogenism (N=102) were compared with lean controls. Notwithstanding the absence of hyperandrogenism and obesity, PCOS cases had significantly higher plasma levels of C, LDL-C and apoB/apoA-I

Table 3 Insulin and lipid profiles in women with PCOS and controls, stratified by the presence of hyperandrogenism (HA) and/or increased body mass (BMI ≥ 25 kg/m**²**)

	Lean controls	*Lean PCOS no HA*	*Lean PCOS HA*[A]	*Overweight PCOS, no HA*	*Overweight PCOS & HA*
N	149	102	76	61	256
BMI (kg/m^2)	22.6	22.3^{NS}	23.2*	29.4**	30.9**
Fasting insulin (mU/l)	6.1	6.0^{NS}	7.9**	10.0**	14.1**
Triglycerides (mg/dl)	79	79^{NS}	82^{NS}	103**	116**
Total Cholesterol (mg/dl)	416	446*	431^{NS}	442*	464**
HDL cholesterol (mg/dl)	136	134^{NS}	116**	105**	89**
LDL cholesterol (mg/dl)	243	272*	283*	284**	310**
ApoA-I (mg/dl)	146	127**	119**	121**	111**
ApoB (mg/dl)	78	76^{NS}	79^{NS}	82^{NS}	86**
ApoB/apoA-I ratio	0.52	0.56**	0.64**	0.70**	0.79**

Values are unadjusted medians. Lean study study subjects had BMI between 20 and 25 kg/m^2. P values were calculated for the differences with lean controls and adjusted for age. [A] P values for this group were adjusted for age and BMI. *P ≤ 0.05. **P ≤ 0.01. [NS]Not statistically significant.

ratio in combination with lower apoA-I levels. Next the influence of hyperandrogenism was examined in lean women with PCOS in a comparison of hyperandrogenic (N=76) and non-hyperandrogenic (N=102) subjects. We observed that hyperandrogenism in lean women with PCOS was associated with higher insulin levels (P=0.01, adjusted for BMI) and apoB/apoA-I

ratio (P=0.03) in combination with lower HDL-C (P≤0.01) and apoA-I (P≤0.01). In a similar fashion the independent influence of body weight on lipid parameters was investigated in non-hyperandrogenic PCOS cases in the absence (N=102) or presence of BMI≥25 kg/m2 (N=61). The latter group showed significantly higher insulin levels, TG,

Table 4 Clinical and endocrine parameters of 557 women with PCOS, forward stepwise multivariate analyses of initial screening parameters for the prediction of Apo-A1 levels

			Univariate[a]		Multivariate[b]							
	Median	**(IQR)**	**Step 0**		**Step 1**		***Step 2***		***Step 3***		***Step 4[c]***	
Endocrine parameters												
Free Androgen Index	5.8	(3.3-9.8)	-0.355	**	In model		In model		In model		In model	
SHBG (µg/dL)	1.0	(0.7-1.5)	0.401	**	0.191	**	0.151	**	In model		In model	
E2 (pg/mL)	62	(46-90)	0.109	*	0.142	*	0.133	*	0.107	*	In model	
Fasting insulin (µIU/mL)	10.1	(6.2-15.5)	-0.338	**	-0.150	**	-0.057		-0.078		-0.072	
HOMA IR	1.8	(1.1-2.9)	-0.327	**	-0.153	**	-0.064		-0.083		-0.080	
17-OH-Pg (ng/dL)	86	(60-129)	0.177	**	0.125	*	0.097	*	0.077		0.025	
Glucose (mg/dL)	72.1	(67-78)	-0.094	*	-0.060		0.018		-0.017	*	-0.028	
Testosterone (ng/dL)	58	(43-78)	-0.071		-		-		-		-	
Androstenedione (ng/dL)	332	(249-434)	-0.063		-		-		-		-	
LH (IU/L)	7.3	(4.5-11.0)	0.043		-		-		-		-	
DHEA (ng/mL)	11.2	(7.6-16.9)	-0.042		-		-		-		-	
FSH (mIU/mL)	5.0	(3.6-6.5)	-0.038		-		-		-		-	
DHEAS (µg/dL)	181	(125-243)	-0.029		-		-		-		-	
Progesterone (ng/mL)	0.6	(0.3-1.0)	0.01		-		-		-		-	
Baseline characteristics and ultrasound parameters												
BMI (kg/m^2)	26.6	(22.5-32.0)	-0.361	**	-0.219	**	In model		In model		In model	
Amenorrhoea (%)	25.8	(%)	-0.089	*	-0.032		-0.041		-0.056		-0.046	
Age (yr)	28.8	(25.3-32.2)	0.058		-		-				-	
Mean follicle count	18.0	(12.5-25.0)	0.036		-		-				-	
Mean ovarian volume (ml)	9.1	(6.8-12.0)	-0.032		-		-				-	

Values are unadjusted medians and interquartile range (IQR). [a]Spearman's correlation-coefficient for the specified variable with apo-A1 levels (step 0 univariate) [b]Partial correlations, corrected for parameters entered into the model (step 1-2, multivariate) [c]Final model, R^2= 0.170 * $p \leq 0.05$ ** $p \leq 0.001$ Variables that did not correlate with apo-A1 in the univariate analysis were not included in the multivariate analysis

apoB and apoB/apoA-I ratio in combination with lower levels of HDL-C and apoA-I (all parameters: P≤0.01). Insulin levels and lipid profiles were most severely affected in the subgroup of PCOS cases who had both hyperandrogenism and BMI≥25 kg/m2.

In order to test the hypothesis that hyperandrogenism and obesity were independent predictors for the presence of a more atherogenic lipid profile in women with PCOS, multiple linear regression analysis was performed. Table 4 shows clinical and endocrine parameters of all women with PCOS. The results of the univariate and multivariate analyses showed that the most important independent determinants for serum apoA-I levels in women with PCOS were FAI, BMI, SHBG and E2. The final model that included all four parameters explained 17 % of the variability in apoA-I levels. Likewise, independent determinants for apoB were calculated as BMI, age and FAI (multiple R^2 0.14, data not shown).

DISCUSSION

The present study confirms the presence of a more atherogenic lipid profile in women with PCOS. We found higher levels of TG, C and LDL-C in combination with decreased HDL-C in women with PCOS. Additional information on cardiovascular risk-factors in women with PCOS was gained by measuring apolipoprotein levels [21]. Interestingly, apoA-I levels were significantly lower in women with PCOS without any difference in apoB levels. Atherogenic changes in lipid profile were most pronounced in a subgroup of obese PCOS women with hyperandrogenism. Furthermore, univariate and multivariate analysis revealed that FAI, BMI, SHBG and E2 were independent determinants of apoA-I levels in women with PCOS. Although these factors accounted for no more than 17% of the variance, this supports the hypothesis that ovarian sex-steroids are involved in the pathogenesis of dyslipidemia in women with PCOS.

The data on TG, C, HDL-C and LDL-C are consistent with prior studies of dyslipidemia in women with PCOS [31]. The strength of the present study lies in the number of patients that were uniformly phenotyped. Moreover, both patients and controls were derived from the same geographic area in the Netherlands. Evidently, studies of dyslipidemia in women with PCOS are hampered by the fact that obesity frequently accompanies this syndrome. It is well known that obesity influences the lipid profile, independently of the presence or absence of PCOS. The stratified analysis shows that PCOS is associated with changes in plasma levels of C, LDL-C and apoA-I and a higher apoB/apoA-I ratio, even when signs of hyperandrogenism or obesity are absent.

Multivariable analysis showed that, after adjustment for age and BMI, women with PCOS had slightly higher fasting insulin levels than controls. The stratified analysis confirms a small difference in insulin levels in the same direction, although this finding

was not statistically significant. In contrast, both cases and controls showed that fasting insulin levels more than doubled in the presence of obesity. Therefore, it is suggested that, although PCOS may contribute to insulin resistance, the influence of BMI is much more pronounced. Likewise, lean PCOS cases did not show higher levels of TG when compared with lean controls.

The results of this study show that there is an additional influence on the lipid profile of women with PCOS that is independent of BMI and seems to be centered on HDL metabolism i.e. apoA-I levels. One possible explanation may be that hyperandrogenism affects lipid metabolism by the induction of hepatic lipase activity. This enzyme, that has a role in the catabolism of HDL particles, was significantly up-regulated by exogenous androgens in a study of female-to-male transsexuals [15] and women using androgenic anabolic steroids [13]. Moreover, increased HL activity was found in a study of 52 PCOS cases and 14 controls who were matched for BMI [32]. The present data support this hypothesis by the fact that the FAI, which is a measure of free circulating androgens, had a strong negative correlation with apoA-I levels that was independent of BMI, serum levels of SHBG and estradiol in the multiple linear regression analysis. .

ApoB levels were not affected by the presence of PCOS, contrary to what was expected. However, this finding is in line with reports that may have lacked sufficient statistical power [22, 23, 25]. Subgroup analysis only showed significantly higher apoB levels in obese hyperandrogenic women with PCOS. Multivariate analysis confirmed that BMI, age and free androgen index were independent determinants of apoB levels in women with PCOS. However the combined model could account for no more than 14% of the total variance.

Taking into account that women with PCOS generally showed a more atherogenic lipid profile than controls, it may be possible to identify subgroups of PCOS patients who have a more adverse cardiovascular risk profile than others. It was observed that both hyperandrogenism and obesity were independently associated with a more atherogenic lipid profile in women with PCOS. Accordingly, insulin levels and lipid parameters were most affected in a subgroup of PCOS patients who presented with BMI≥25 kg/m2 and hyperandrogenism.

It is tempting to speculate that the lack of changes in apoB levels may partly explain the fact that excessive cardiovascular morbidity and mortality were not found in a retrospective follow-up study of women with PCOS [7]. However, it should be taken into account that low apoA-I levels in combination with higher LDL-C and apoB/apoA-I ratio still constitute increased cardiovascular risk. ApoB/apoA-1 ratio is an important risk factor for acute myocardial infarction [33]. In our population increased apoB/apoA-1 ratio resulted primarily from decreased apoA-I levels. While apoA-I is not usually regarded as a risk factor by itself, evidence has mounted for a role of low apoA-I levels in the pathogenesis of CHD [34]. Moreover, apoA-I levels were strong predictors for risk of fatal

myocardial death in a study of 76.831 women who were followed up for a mean of 64,4 months (AMORIS study) [21].

Results from the Framingham offspring study suggest that increased exposure to exogenous estrogens in premenopausal women is associated with decreased levels of apoB and LDL-C [35]. The present study shows that estradiol is an independent determinant of apoA-I, but not apoB. The question whether changes in the exposure to estrogens do indeed exert a protective influence on cardiovascular risk in women with PCOS falls beyond the scope and design of this study. It is possible that other, currently unknown, factors are involved in the regulation of lipid metabolism and cardiovascular risk in women with PCOS.

In summary, a more atherogenic lipid profile, in particular related to HDL metabolism, was found in women with PCOS. We hypothesize that both obesity and hyperandrogenism contribute to these changes. Furthermore, there was evidence for an additional influence of PCOS on lipid metabolism that was independent of obesity. The results of this study may indicate increased risk for cardiovascular disease in women with PCOS. However, this hypothesis still remains to be proven in prospective long-term follow-up studies of women with PCOS.

CHAPTER 2.2

Genetic geo-biographic ancestry affects the phenotype of normo-gonadotropic anovulatory (WHOII) subfertility

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INTRODUCTION

Anovulation is the most important cause of reduced fertility in women of reproductive age. Depending on the set of diagnostic criteria that are used, up to 91 % of women with normogonadotropic (WHO category II) anovulation are diagnosed with Polycystic Ovary Syndrome (PCOS)[36]. PCOS is widely regarded as a syndrome of ovarian dysfunction that is characterized by a multitude of clinical and biochemical manifestations i.e., anovulation, hyperandrogenism, obesity, insulin resistance and is associated with polycystic appearance of the ovaries (PCO). However, from the start it has been recognized that WHOII anovulation and PCOS encompass a notoriously heterogeneous phenotype. Following the 2003 consensus meeting in Rotterdam, the Netherlands, no single diagnostic criterion (anovulation, hyperandrogenism and/or PCO) is mandatory for the clinical diagnosis of PCOS[37]. The treatment algorithm for normogonadotropic anovulatory subfertility remains the same, whether a diagnosis of PCOS can be established or not[38]. Adding to the heterogeneity of the clinical phenotype of WHOII anovulation is the reality of many of today's clinical practices that deal with an ethnically diverse patient population. Lifestyle and health behaviour may vary greatly between ethnic subgroups, adding to overall phenotypic differences; for example the presence of obesity and insulin resistance[39-41]. It seems plausible that differences in the genetic make-up of ethnic populations may also contribute to disease risk and phenotype of WHOII anovulation and PCOS[42].

The present study aims to identify phenotypic differences among WHOII anovulatory patients from different ethnic subgroups. A better understanding of these differences may help to tailor diagnostic criteria to individual ethnic subgroups. Moreover, these results may be used to facilitate the interpretation of genetic studies that aim to elucidate the complex genetic background of PCOS and are frequently hampered by population admixture. A recent paper by Lao and colleagues identified 10 single nucleotide polymorphisms (SNPs) that are highly informative for continental genetic ancestry[43]. Genotyping ethnicity SNPs in these patients carries the benefit that it does not rely on self-reported ethnicity and it allows correction for population admixture at a continental level. We aimed to investigate whether these SNPs can be used to accurately map phenotypic differences among ethnic subgroups. In addition, we questioned whether their use adds predictive power to self-reported ethnic background.

MATERIALS AND METHODS

Subjects and phenotyping

Anovulatory subjects attended our infertility outpatient clinic between 1991 and october 2008. Criteria for inclusion constituted oligomenorrhea (interval between con-

secutive menstrual periods >35 days) or amenorrhea (absence of vaginal bleeding for at least 6 months) and serum FSH concentrations within the normal range (1–10 IU/L)[44, 45]. All patients underwent a standardized initial screening that was performed after an overnight fasting period on a random cycle-day between 9 a.m. and 11 a.m. Clinical examination included a structured interview and physical examination. Transvaginal ultrasonography was performed in order to assess ovarian volume and follicle count for both ovaries and blood samples were taken for endocrine evaluation.

A diagnosis of PCOS was established on the basis of the 2003 ESHRE/ASRM Rotterdam criteria[1]. Following these criteria, hyperandrogenism was defined as the presence of either biochemical or clinical signs of androgen excess. For the purpose of this study clinical hyperandrogenism was assessed by means of the Ferriman Gallway (FG) score, and was defined as FG-score ≥8. Biochemical hyperandrogenism was determined by calculation of the free androgen index (FAI) as: (testosterone [nmol/l] / sex-hormone binding globulin [nmol/l]) * 100. A cutoff level of 4.5 was used for the definition of hyperandrogenism[45]. Polycystic ovaries (PCO) was defined as the presence of ≥12 follicles in one or both ovaries and/or increased ovarian volume (>10 ml). Ethnicity and country of birth were registered by the use of a questionnaire that was checked by the research investigator at the day of investigation. Patients with disorders that show a similar clinical presentation, such as congenital adrenal hyperplasia and Cushing's syndrome, were excluded from analysis.

Genetic controls from parental populations:

Individuals with known bio-geographic ancestry served as controls. Samples were derived from the CEPH–Human Genome Diversity Project Cell Line Panel (CEPH-HGDP)[46]. The control group comprised 804 samples from 43 human populations of global distribution including all continental regions: America (Mexico Brazil and Columbia), Central and East Asia (China, Cambodia, Japan and Siberia), Europe (Italy, France, Russia, Pakistan and the Orkney Islands) and sub-Saharan Africa (Senegal, Namibia, Nigeria, Central African Republic, South Africa and the Democratic Republic of Congo).

Hormones

Blood samples were drawn on the day of clinical examination and processed within 2 hours after withdrawal. Serum was stored at -20° C until assayed. Endocrine evaluation included serum levels of gonadotropic hormones (LH, FSH), oestradiol (E2), androgens (T), androstenedione (AD), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), progesterone (Pg) and 17-hydroxyprogesterone (17-OHP), sex-hormone binding globulin (SHBG), fasting glucose and insulin, thyrotropin (TSH) and prolactin. LH, FSH, TSH, SHBG, Pg, AD, DHEA, prolactin, and insulin were measured by chemiluminescent immunometric assay (Immulite® platform, Diagnostic Products

Corporation, Breda, the Netherlands). T and E2 were determined by radioimmunoassay (Diagnostic Products Corporation) and 17-OHP was determined using an in-house assay. Intra- and inter-assay coefficients of variation were <5 and <15% for LH, <3 and <8% for FSH, <3 and <5% for T, <8 and <11% for AD, <5 and <7% for E2, and <4 and <5% for SHBG. Glucose levels were measured using a Hitachi® 917 analyzer (Roche Diagnostics, Almere, the Netherlands).

Genotyping

The 10 SNPs for continental bio-geographic ancestry were ascertained from a prior study[43]. Genomic DNA was extracted from peripheral venous blood according to standard procedures. SNP genotyping was performed using the Taqman allelic discrimination assay for rs722869, rs1858465, rs1876482, rs1344870, rs1363448, rs952718, rs2352476, rs714857, rs1823718 and rs735612. The PCR reaction mixture included 2 ng (cases) or 3 ng (ethnic controls) of genomic DNA in a 2 µL volume. PCR cycling reactions were performed on an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 15 min at 95 ºC, followed by 40 cycles with denaturation (15 s at 95 ºC) and annealing and extension (60s at 60 ºC).

Statistical analysis

For the cross-sectional analysis of anthropomorphometric data, medians and ranges were computed and compared between the different ethnic subgroups using a non-parametric test for differences between k subgroups (Kruskall Wallis). A nonparametric test (Mann-Whitney

14 U) was used for the exploratory comparison of continuous variables. Variables were checked for

15 normal distributions with the one-sample Kolmogorov-Smirnov test and log-transformed if necessary.

16 Analysis of covariance (ANCOVA) was applied to adjust for age differences. The Chi-square (X2) test was used for the comparison of categorical variables. Statistical analyses were performed using a commercially available software package (SPSS version 12; SPSS Inc, Chicago, USA). In order to infer genetic ancestry in WHOII anovulatory cases, we applied the STRUCTURE algorithm [47] with K=4 clusters. Samples from individuals with known bio-geographic ancestry were used as a parental set. The algorithm provides each individual with a posterior probability of assignment to each of the K populations (the a-priori probability being 1/K). After log-transformation, individual probability scores were entered in a linear regression model in order to examine predictive power with regard to phenotypic characteristerics of anovulatory patients. In a similar fashion, self-reported ethnic background was entered in a linear regression model after binary transformation. A stepwise analysis of self-reported ethnic background (first step) versus

the combination of self-reported and genetically assigned ethnic background (second step) was performed in order to test for a possible advantage for the use of ethnicity SNPs.

RESULTS

Distribution of ethnic subgroups in the study population

Most of the study subjects (62.4 %, N=774) were self-identified from North-Western (NW) European ancestry. In addition, 220 subjects reported a Mediterranean-European background, mostly born in Turkey (N=92), Morocco ((N=55) and the Netherlands (N=41). Other reported self-identified bio-geographic ancestries were sub-Saharan Africa (N=111), Hindustani (N=83) and South-East Asian (N=53) ancestry. Hindustani subjects were mostly born in Surinam (N=53), the Netherlands (N=20), India (n=3) and Pakistan (N=4). In 276 cases information on geographic ancestry was either not reported (118 women of whom 154 were born in Europe and 64 were born outside of Europe) or subjects reported mixed ancestry (58 cases, including Latin-American women).

Phenotypic differences between ethnic subgroups:

All 1241 women who could be assigned to a certain ethnic subgroup were compared for phenotypic differences among the various subgroups.Different ethnic subgroups showed marked differences in phenotypic characteristics (Table 1).

Subjects who reported a Hindustani background were found to have a distinctly more insulin resistant profile compared to all other subjects, showing high fasting insulin levels (median 14.7 mU/L vs. 7.9 mU/L , P≤0.001) and glucose (77 mg/dL vs. 76 mg/dL, P=0.01) in combination with low levels of SHBG (243 µg/dL vs. 342 µg/dL, P≤0.001) which is the cause of high levels of free circulating androgens as represented by the FAI (7.0 vs. 4.5, P≤0.001)

Similar to subjects from Hindustani background, we observed high levels of free circulating androgens in women from Mediterranean-European descent, however these women showed a less insulin resistant profile with insulin levels that were significantly lower (median 7.8 pmol/L vs. 14.7 pmol/L, P≤0.001). The prevalence of hyperandrogenism among both subgroups (Hindustani and Mediterranean-European) was significantly higher compared to other study subjects (75.6% vs. 44.8%, P(X2)≤0.001). Serum levels of androgens (T, AD, DHEA and DHEAS) were uniformly highest in Mediterranean-European women. Also, body mass index was highest in this group compared to other study subjects (27.6 kg/m2 vs. 25.1 kg/m2, P≤0.001).

Women of sub-Sahara African descent were similarly obese (BMI 27.3 kg/m2), however a more pronounced emphasis on waist circumference (89 cm vs. 86 cm in Mediterranean

Table 1 Differences in the phenotype of normogonadotropic (WHOII) anovulation in ethnic subgroups (self-reported ethnic background).

	N-W Europe	Medi-terranean[A]	African	S-E Asian	Hindu	P[B]	Other[D]
N	774	220	111	53	83		276
Baseline parameters							
Age (years)	29,2	25,2	27,5	30,0	27,2		28,6
Obese (BMI≥30) (%)	26,9%	37,6%	39,1%	21,6%	28,9%	≤0.01[C]	32,5%
BMI (kg/m^2)	24,8	27,6	27,3	24,0	25,0	≤0.01[B]	26,0
Waist circumference (cm)	84	86	89	81	84	≤0.01[B]	85
Hyperandrogenism(%)	40,9%	75,9%	63,1%	64,2%	74,7%	≤0.01[C]	54,4%
Mean cycle duration (days)	41	49	43	43	45	ns[B]	45
Amenorrhoea (%)	30,6%	20,9%	26,1%	11,3%	32,5%	≤0.01[C]	25,0%
Nulligravida (%)	75,3%	69,7%	53,2%	65,4%	66,3%	≤0.01[C]	60,4%
Ultrasound							
Mean ovarian volume (ml)	8,1	9,6	9,0	8,4	7,6	≤0.01[B]	8,7
Mean follicle number	16,0	17,8	20,5	17,5	16,0	ns[B]	14,5
PCO (%)	82%	89%	89%	84%	85%	ns[C]	78,2%
Endocrine profile							
LH (U/L)	7,0	8,5	7,9	7,9	7,6	ns[B]	6,2
FSH (U/L)	5,6	5,0	5,0	6,1	5,3	0.03[B]	4,7
Estradiol (pg/ml)	64	60	70	63	56	ns[B]	63
Progesterone (ng/ml)	0.44	0.50	0.41	0.53	0.41	ns[B]	0.72
17 (OH) Progesterone (ng/dl)	83	96	93	83	79	ns[B]	73
Testosterone (ng/dl)	49	58	52	52	58	≤0.01[B]	63
SHBG (µg/dl)	396	252	297	279	243	≤0.01[B]	414
Free Androgen Index	3.8	7.1	5.7	5.7	7.0	≤0.01[B]	5.0
Androstenedione (nmol/L)	289	370	307	307	318	≤0.01[B]	338
DHEA (ng/ml)	11.1	14.0	10.4	11.2	9.3	≤0.01[B]	10.0
DHEAS (µg/dl)	184	203	151	188	166	≤0.01[B]	206
Glucose (mg/dl)	74	77	74	76	77	0.05[B]	74
Insulin (mU/l)	7.1	9.0	10.1	8.0	14.7	≤0.01[B]	10.9
AMH (µg/L)	10,9	11,5	12,4	11,4	10,6	ns[B]	13,0
PCOS							
PCOS Rotterdam criteria (%)	86,4%	95,3%	89,8%	90,6%	90,1%	≤0.01[C]	87,8%
PCOS NIH criteria (%)	40,9%	75,9%	63,1%	64,2%	74,7%	≤0.01[C]	54,4%

Values are medians or percentages where stated. [A] North of Sahara and Middle East. [B]P-value for the difference between subgroups ("other" excluded) using ANCOVA (continuous variables, log transformed if not normally distributed) and corrected for age. [C]P-value for the the difference between subgroups ("other" excluded), chi-square test with 4 degrees of freedom. [D]Ancestry unknown or mixed. ns: P>0.05

women, P=0.046) was observed. Despite somewhat higher levels of fasting insulin (73 vs. 65, P=0.63), African women showed significantly higher SHBG levels (297 µg/dL vs. 252 µg/dL, P=0.01) and consequently lower FAI was observed in this group. (5.7 vs. 7.1, P=0.01). Interestingly, African women seemed to show a more pronounced polycystic appearance of the ovaries. A higher number of antral follicles (mean of left and right ovary) was observed in this group compared to others (20.8 follicles vs. 16.5 follicles, P=0.002). However this difference did not result in a higher frequency of PCO in this subgroup (89% vs. 84%, P(X2)=0.17).

In contrast to all other subgroups, anovulatory women from North-Western European descent showed lower values for BMI compared to other subjects (median 24.7 kg/m2 vs. 26.9 kg/m2). Also, the frequency of hyperandrogenism was strikingly low in this subgroup of patients compared to all others (41% vs. 71%, P(X2)≤0.001). Despite the fact that metabolic profiles were relatively benign, these subjects did show a high frequency of amenorrhoea (31%) compared to other subjects (23%, P(X2)=0.004) that was only surpassed by Hindustani subjects (33%). Moreover, these women showed a relatively high frequency of nulligravidity compared to other ethnic subgroups (75% vs. 65%, P(X2) ≤0.001).

Similar to women from NW-European descent, SE-Asian subjects showed low values for BMI (24.0 kg/m2 vs. 25.6 kg/m2) and waist circumference (81 cm vs. 85 cm). As expected, metabolic profiles with regard to insulin resistance were relatively benign, whereas the frequency of hyperandrogenism was 64%. In contrast to NW-European subjects, these women also showed a low frequency of amenorrhoea (11% vs. 29% in all other subgroups, P≤0.01).

Supervised genetic clustering:

Genotyping was performed in a random set of 637 WHOII anovulatory women who were tested between 1993 and 2004. Call-rates were between 80.1 % (rs1858465) and 95.3% (rs1823718). All but one ethnicity-SNPs (rs735612) were out of Hardy Weinberg equilibrium, showing evidence for population stratification. Complete genotypes were available for 477 subjects. The distribution of self-reported ethnic subgroups was comparable to the overall study-group, i.e., NW-European: n=268 (56%); Mediterranean-European: n=68 (14%); African: n=24 (5%); SE-Asian: n=17 (4%); Hindu: n=26 (6%); Other: n=74 (15.5%). Figure 1 shows the mean of individual probability scores for each (self-reported) ethnic subgroup after the STRUCTURE algorithm was applied. Fisher's Exact Test showed statistically significant correlation between self-reported ethnicity and genetically assigned ancestry (Fisher's Exact Test = 154.047, P<0.5/10.000). However, the current set of ancestry SNP's was not able to distinguish between NW-European, Mediterranean-European and Hindustani ethnic background.

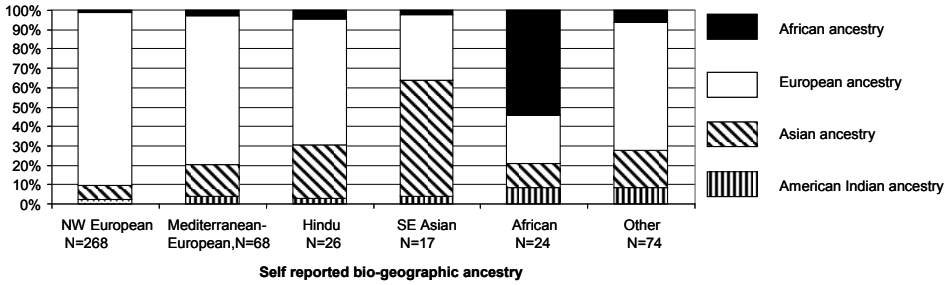

Figure 1 Genetic substructure in 477 WHO2 anovulatory patients (10 ethnicity SNPs)

Univariate analysis of self reported ancestry vs. genetic ancestry SNPs

The log tranformed genetic ancestry probality scores were used to compare to the predictive value of genetic ancestry SNPs versus self-assigned ethnic ancestry. Information from both self-assigned ethnicity and genetic ancestry SNP's were used in a linear regression model. Table 2 shows r-squares and P-values for both models. In addition, the change in F-statistic is shown when the genetic ancestry information is added to the self-assigned ethnicity model. Testosterone levels and FAI showed significant association in both models (self-reported ethnicity and genetic ancestry SNPs). However univariate analysis did not show an additive benefit for the addition of genetic ancestry SNPs to the prediction model. Mean follicle numbers (p=0.018) and 17(OH)Progesterone levels (p=0.010) were significantly associated with genetic ancestry SNPS, but not with self-reported ancestry. In general, r-squares were higher for the self-reported ancestry

Table 2 Linear regression analysis,

	Model fit Self-reported ethnicity		Model fit Genetic ethnicity		Change statistics[a]	
	R^2	P	R^2	P	F change	P change
Age	0.038	0.001	0.019	0.054	1.792	0.148
BMI	0.013	0.148	0.005	0.557	1.044	0.373
Waist circumference	0.005	0.561	0.015	0.114	2.687	0.046
Menarche	0.010	0.287	0.004	0.697	2.298	0.077
Mean ovarian volume	0.028	0.027	0.012	0.284	1.071	0.361
Mean follicle number	0.013	0.196	0.027	0.018	2.119	0.097
LH	0.005	0.586	0.017	0.074	1.957	0.120
FSH	0.020	0.046	0.003	0.713	0.223	0.881
Estradiol	0.021	0.038	0.009	0.323	1.584	0.193
Progesterone	0.000	0.990	0.002	0.830	0.567	0.637

[a] change in predictive value when adding information from ethnicity SNPs to the self-reported ethnicity model.

Table 2 Linear regression analysis, (continued)

	Model fit Self-reported ethnicity		Model fit Genetic ethnicity		Change statistics[a]	
	R^2	P	R^2	P	F change	P change
17 (OH) Progesterone	0.012	0.190	0.028	0.010	2.938	0.033
Testosterone	0.031	0.006	0.025	0.019	1.045	0.373
SHBG	0.060	0.000	0.011	0.215	0.510	0.675
Free Androgen Index	0.039	0.001	0.021	0.038	0.740	0.529
Androstenedione	0.042	0.001	0.016	0.098	1.054	0.369
DHEA	0.045	0.001	0.010	0.311	2.037	0.108
DHEAS	0.023	0.027	0.008	0.359	0.489	0.690
Glucose	0.012	0.212	0.010	0.265	1.802	0.146
Insulin	0.064	0.000	0.015	0.120	0.592	0.621
AMH	0.006	0.530	0.014	0.148	1.226	0.300

[a] change in predictive value when adding information from ethnicity SNPs to the self-reported ethnicity model.

model (between 2.9 x10-4 and 6.4 x10-2) than was the case for the genetic ancestry SNP model (between 2.2 x10-3 and 2.8 x10-2) with the exception of waist circumference, mean follicle number, LH levels, progesterone levels and 17(OH)Progesterone.

DISCUSSION

The present analysis of the influence of ethnic ancestry on the phenotype of women with normogonadtropic (WHOII) anovulation shows that large differences exist with regard to metabolic characteristics of WHOII anovulatory women from different ethnic backgrounds. Genotype information can be used in order to map these differences in individual patiënts. However the present set of 10 ethnicity SNPs that were identified in a prior study by Lao and colleagues[43] was limited by the fact that it was not able to distinguish between subjects from NW-European, Mediterranean-European and Hindustani subjects.

From the time of its first description by Stein and Leventhal in 1935, PCOS has been recognized as a notoriously heterogeneous phenotype. Over the years, diagnostic criteria have been, and still are, hotly debated[48, 49]. The association with a number of metabolic derangements, most importantly hyperandrogenism, insulin resistance and obesity is generally agreed upon. The present study regards all WHOII anovulatory patients, whether a diagnosis of PCOS was assigned or not. The reason for this is two-fold; first, using the revised 2003 Rotterdam criteria[37] a majority of up to 91%[36] of WHOII anovulatory women (between 86 and 95 percent in the present study) can be diagnosed

with PCOS. Second, anovulatory subfertile patients are treated similarly, whether a diagnosis of PCOS is present or not.

The analyses of phenotypic differences among self-identified bio-geographic subgroups in women with PCOS show a number of interesting differences. Two subgroups (Hindustani and Mediterranean-European women) are identified that are more explicitly associated with hyperandrogenism. High levels of free circulating androgens (as represented by the free androgen index) were observed in Hindustani subjects. High FAI levels in this subgroup seem to be the result of low SHBG levels (which is likely the result of increased insulin resistance that is a well documented characteristic of Hindustani subjects[50, 51]). Overall, Mediterranean-European women present with a clinical and endocrine phenotype that seems to indicate increased synthesis of both ovarian (T and AD) and adrenal (DHEA and DHEAS) androgens. Significantly ($P \leq 0.02$) higher levels of T, AD, DHEA and DHEAS remained after correcting for both BMI and fasting insulin levels.

Obesity is a frequent characteristic among women from Mediterranean (37.6%) and African sub-Saharan (39.1%) descent. However, while obesity is accompanied by hyperandrogenism in the majority (75.9%) of Mediterranean-European subjects, this is less often the case among African anovulatory subjects (63.1%, $P=0.01$). It may be that the African sub-Saharan phenotype is more resilient towards the metabolic disturbances that result from the accumulation of abdominal fat. This is also evident from the fact that SHBG levels are higher, instead of lower, in this patient group, compared to all other ethnic subgroups, except NW-European women. Moreover African women showed lowest levels of adrenal androgens (DHEA and DHEAS). In this light, the fact that we observed a tendency towards higher follicle count among African women is unexpected and somewhat contradictory. It may point towards a pathofysiology that is more centered on an ovarian aetiology. On the other hand this observation may also depend on normal morphometric variation among different populations.

With regard to obesity and insulin resistance, NW-European anovulatory wowen show a relatively benign phenotype. Among these women, hyperandrogenism occurs only in a minority of 40.9% of women. This means that, when applying the NIH-criteria, only 41% of anovulatory women are diagnosed with PCOS. While it can be hypothesized that the majority of anovulatory NW-European women indeed do not suffer from PCOS, one should keep in mind that all study subjects show clear signs of ovulatory dysfunction (oligo- or amenorhoea). More so, the frequency of nulligravidity (75.3%) and amenorrhoea (30.6%) are relatively high among these women, giving evidence for ovulatory dysfunction that cannot easily be explained on the basis of an alternative disease process. It is shown that the use of the Rotterdam criteria, that encompass a broader definition, leads to a more even distribution of the frequency of PCOS that was observed to lie between 86% and 95%. This percentage is in line with a prior report concerning the frequency of PCOS among anovulatory patients[36].

The use of genetic ancestry SNPs, as opposed to self reported ancestry, has the advantage that it avoids self-misclassification [52] which can be due to other factors than biological ones [53] and allows to quantify for the presence of population admixture. The present set of 10 genetic ancestry SNPs is effective in distinguishing women from European-Middle East-Central Asian, sub-Saharan African, East Asian and native American bio-geographic genetic ancestry [43]. Due to the limitation in geographic resolution, we could not distinguish between NW-European and Mediterranean European descent or between European and Central Asian ancestry. Additional SNPs would be required in order to enhance the amount of geographic resolution. Because we have shown that significant differences exist in the phenotype of WHOII anovulation, it seems reasonable to state that this is a serious limitation of the use of this set of SNPs in clinical practice. However, as a proof of principle, it is shown that genetic ancestry can be a powerful tool in order to map phenotypic differences between individual subjects with WHOII anovulation. With the arrival of commercially available SNP arrays that allow instant genotyping of up to 500K SNPs and even more, we may be able to use this information to more precisely quantify bio-geographic ancestry. For the individual patient, this approach would allow the physician to quantify how bio-geographic ancestry is related to the specific phenotype (for example with regard to insulin sensitivity) and to correct for this. For medical research purposes, this approach would, at least partly, solve the problem of population-admixture, that is expected to occur with an increasing frequency with the rising number of genome-wide-association studies that are currently performed.

In conclusion, the present report describes metabolic characteristics of normogonadotropic anovulation among different ethnic subgroups that coexist in an urban community in the Netherlands. Knowledge with regard to the presence of these differences may result in diagnostic and therapeutic pathways that are better tailored to the individual patient. The use of a previously validated set of genetic ancestry SNPs among these patients is effective in separating women from Caucasian, African sub-Saharan and SE-Asian biogeographic ancestry. However, at present, this approach is not sufficiently effective to describe all ethnic variation in the phenotype of normogonadotropic anovulation. It is expected that, with the arrival of extended SNP arrays, ancestry detection could be mapped more precisely in the near future.

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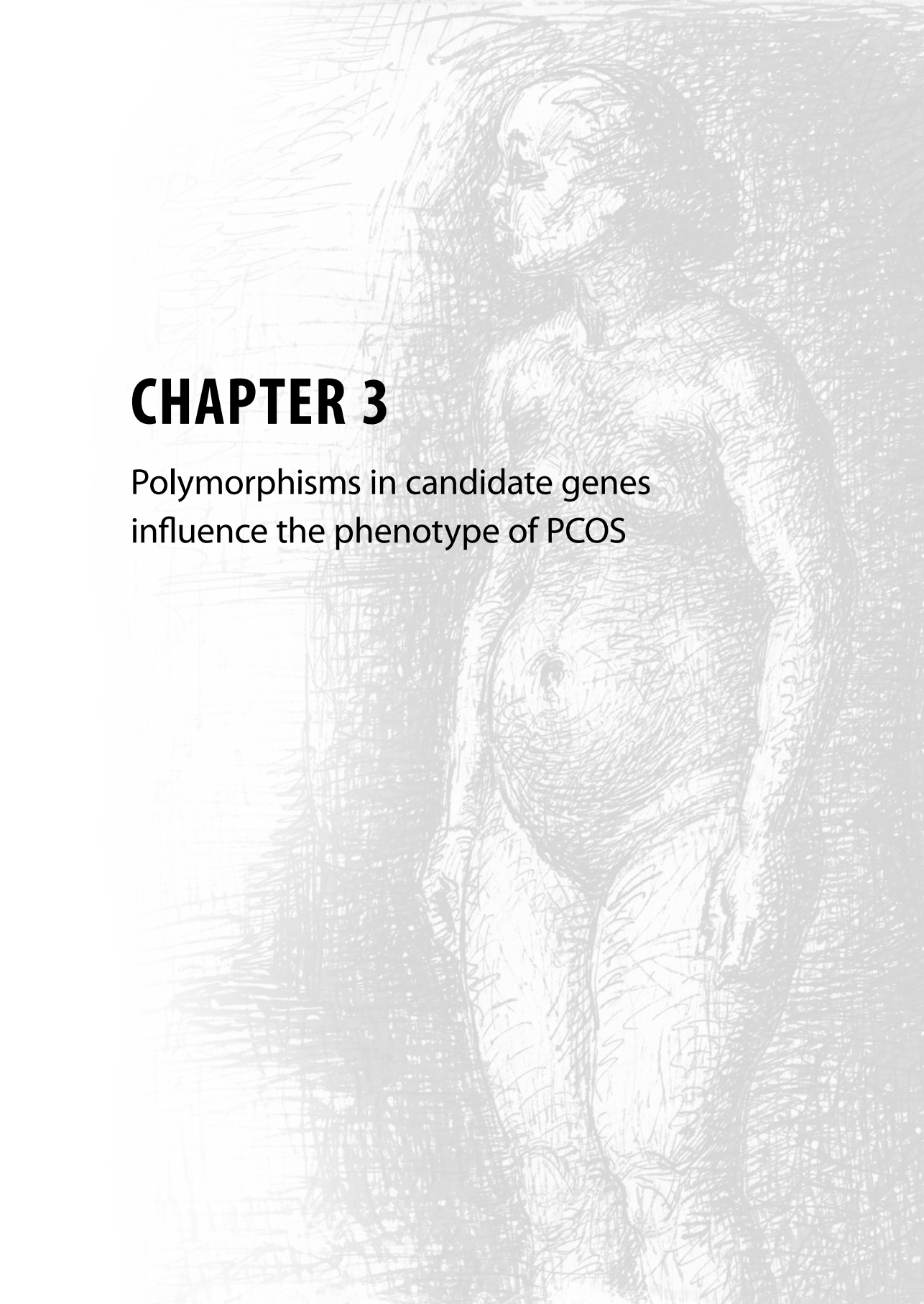

CHAPTER 3

Polymorphisms in candidate genes influence the phenotype of PCOS

CHAPTER 3.1

Genetic polymorphisms of GnRH and gonadotrophic hormone receptors affect the phenotype of Polycystic Ovary Syndrome (PCOS)

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrinopathy that occurs in 5-8 % of women of reproductive age [1]. PCOS constitutes a syndrome of ovarian dysfunction characterized by anovulation, hyperandrogenism and polycystic ovary (PCO) morphology. PCOS is associated with alterations in the function of the hypothalamic-pituitary-gonadal (HPG) axis that may result from increased frequency and amplitude of the hypothalamic gonadotrophin-releasing hormone (GnRH) pulse generator [2]. Changes in the secretion of gonadotrophic hormones consist of elevated luteinizing hormone (LH) levels in combination with normal serum FSH concentrations [3]. However, the extent to which changes in the HPG-axis contribute to the pathogenesis of PCOS is not fully understood. While an intrinsic abnormality of the hypothalamic GnRH pulse generator in women with PCOS has been proposed in the past [4, 5], more recent evidence suggests that this should be regarded as a secondary phenomenon due to disturbed feedback at the level of the hypothalamus [6]. Notwithstanding the uncertainty with regard to the role of the HPG-axis in the pathogenesis of PCOS, this pathway does play a central role in the pathophysiology of the syndrome. Therefore, we hypothesized that common genetic variants of the HPG-axis may affect the phenotype of PCOS and possibly disease susceptibility.

A number of functional SNPs have been described in genes that are involved in the HPG axis. GnRH1 is an important candidate gene for delayed puberty and idiopathic hypogonadotrophic hypogonadism. Thus far, no major defects within GnRH1 have been found in these patients [7, 8]. However, a polymorphism in the first exon of GnRH1 has been described, constituting an amino acid variation at codon 16 (Trp16Ser). This genetic variant has been examined recently in two studies focusing on clinical end-points in relation to altered oestrogen exposure. Rather contradictory, the results of these studies were consistent with both decreased exposure to endogenous oestrogens (decreased bone mineral density [9]), as well as increased oestrogen exposure (shorter disease free survival in breast cancer patients [10]). However, direct assessment of estrogen exposure was not reported in either study.

A well-known combination of two polymorphisms in the FSH receptor gene (FSHR) has been of particular interest with regard to PCOS. Both polymorphisms, that are in near total linkage-disequilibrium, have been identified as coding SNPs at codon-positions 307 and 680 in exon 10 [11]. This exon covers the signal transducing transmembrane domain. It was shown that the presence of the minor allele at position 680 (Ser^{680}) is associated with significantly higher basal FSH levels and altered response to ovarian stimulation using exogenous FSH for *in vitro* fertilisation [12]. A subsequent comparison of allele frequencies in normogonadotrophic anovulatory patients and controls showed a higher frequency of Ser^{680} in anovulatory subjects [13]. However, the presence of this allele did

not negatively affect the chances of success for ovulation induction using recombinant FSH. Apparently, while the Ser^{680} allele is associated with a less responsive FSH receptor, its influence can be overcome relatively easily by the administration of exogenous FSH.

Like FSH, multiple polymorphisms have been described in the LH receptor (LHR) that are associated with altered LHR functionality. LHR is overexpressed in theca cells from PCOS patients [14]. LH promotes the secretion of androgens by ovarian theca cells, which may result in follicular maturation arrest [15]. An insertion of two amino acids (leucine [L] and glutamine [Q]) in the signal peptide of LHR (18insLQ) was shown *in vitro* to result in increased receptor activity [16]. The 18insLQ insertion polymorphism was also associated with shorter disease free survival in breast cancer patients [16, 17]. In addition, exon 10 of LHR contains two coding SNPs that cause a change in amino acids (Asn291Ser and Ser312Asn). Although *in vitro* the Asn291Ser polymorphism was associated with increased receptor sensitivity, this variant was not associated with tumor characteristics or survival of breast cancer patients [10]. A slightly higher frequency of the Asn^{312} allele was noted among breast cancer patients, possibly because of increased action of ovarian steroid hormones [10]. In a different study in men, a lower frequency of the Asn^{312} allele was found in association with impaired spermatogenesis [18], further substantiating the hypothesis of increased receptor activity in association with this polymorphism.

The present analysis of functional polymorphisms of the HPG pathway in patients with PCOS examines the extent to which these subtle genetic variations affect the severity of clinical features of PCOS and disease susceptibility.

SUBJECTS AND METHODS

Subjects and phenotyping

Anovulatory subjects attended our infertility outpatient clinic between 1994 and 2004. Inclusion criteria were oligomenorrhea (interval between consecutive menstrual periods >35 days) or amenorrhea (absence of vaginal bleeding for at least 6 months) and serum FSH concentrations within normal range (1–10 IU/L) [19, 20]. The diagnosis of PCOS was established on the basis of the 2003 ESHRE/ASRM Rotterdam criteria [21]. In agreement with these criteria hyperandrogenism was defined as the presence of either biochemical or clinical signs of androgen excess. For the purpose of this study clinical hyperandrogenism was assessed by means of the Ferriman Gallway (FG) score, and was defined as FG-score ≥8. Biochemical hyperandrogenism was determined by calculation of the free androgen index (FAI) as: (Testosterone [nmol/l] / sex-hormone binding globulin [nmol/l]) * 100. A cutoff level of 4.5 was used for the definition of hyperandrogenism [20]. The presence of polycystic ovaries (PCO) was detected by vaginal ultrasound examination. PCO was defined as the presence of ≥12 follicles in one or both ovaries, and/or increased ovar-

ian volume (>10 ml) [22]. Ethnicity and country of birth were registered. Exclusion criteria were non-Caucasian ethnic origin and/or the presence of related disorders with similar clinical presentation, such as congenital adrenal hyperplasia and Cushing's syndrome.

Controls were derived from the Rotterdam study. The design of this study has been described previously [23, 24]. In short, this is a single-center, prospective, population based study of determinants of chronic disabling diseases in the elderly, aged 55 years and over (n=7012). Participants from the Rotterdam study derive from a specific area near Rotterdam (Ommoord) that constitutes a homogeneous population-based sample of Caucasian elderly men and women. All women with age at onset of menopause > 45 years and available DNA (n=2996) were included in the present analysis. Written informed consent was obtained from all anovulatory patients as well as controls.

Hormones

Anovulatory patients underwent a standardized initial screening that was performed after an overnight fasting period on a random cycle-day between 9 a.m. and 11 a.m. Clinical examination included a structured interview and physical examination. Transvaginal ultrasonography was performed in order to assess ovarian volume and follicle count for both ovaries. Blood samples were drawn on the day of clinical examination and processed within 2 hours after withdrawal. Serum was stored at -20° C until assayed. Endocrine evaluation included serum levels of gonadotrophic hormones (LH, FSH) and oestradiol (E2), androgens (T), androstenedione (AD), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS), progesterone (Pg) and 17-hydroxyprogesterone (17-OHP), sex-hormone binding globulin (SHBG), fasting glucose and insulin, thyrotropin (TSH) and prolactin. Hormone assays have been described in detail elsewhere [25]. LH, FSH, TSH, SHBG, Pg, AD, DHEA, prolactin, and insulin were measured by immunoradiometric assay (ImmuliteP®P platform, Diagnostic Products Corporation, Breda, the Netherlands). T and E2 were determined by radioimmunoassay (RIA, Diagnostic Products Corporation) and 17-OHP was determined using an in-house assay. Intra- and inter-assay coefficients of variation were <5 and <15% for LH, <3 and <8% for FSH, <3 and <5% for T, <8 and <11% for AD, <5 and <7% for E2, and <4 and <5% for SHBG. AMH levels were determined in a subgroup of 354 patients using an in-house double-antibody enzyme-linked immunosorbent assay (ELISA). Intra- and inter-assay coefficients of variance were <10 and <5%. Glucose levels were measured using a HitachiP®P 917 analyzer (Roche Diagnostics, Almere, the Netherlands).

Genotyping

Genomic DNA was extracted from peripheral venous blood according to standard procedures. Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbiosystems.com) was used to set up a Taqman al-

lelic discrimination assay for the FSHR Asn680Ser, GnRH1 Ser16Trp, LHR Asn291Ser and LHR Ser312Asn polymorphisms. Rs numbers, primer and probe sequences are provided in Table 1. The PCR reaction mixture included 2 ng of genomic DNA in a 2 µL volume and the following reagents: probes (200 nM), primers (0.9 µM), 2 x Taqman PCR master mixes (ABgene, Epsom, UK). PCR cycling reactions were performed on an ABI 9700 PCR system (Applied Biosystems Inc., Foster City,P PCA, USA) and consisted of initial denaturation at 95 °C (15 min), and 40 cycles with denaturation (15 s at 95 °C) and annealing and extension (60 s at 60 °C). In addition, all PCOS patients and a sub-set of 2419 controls were genotyped for a 6 base pair insertion polymorphism in exon 1 of LHR (LHR insLQ). Exon 1 was amplified as described by Atger and colleagues [26] using a 5'-hexachlorofluorescein labeled forward primer. Separation and sizing of the PCR fragments and assignment of LHR insLQ genotype was performed on an ABI Prism 3100 automated capillary DNA sequencer using Genescan and Genotyper software packages (Applied Biosystems Inc).

Statistical analysis

Genotype and allele frequencies were determined for all polymorphisms and subsequently tested for Hardy-Weinberg equilibrium. Calculation of linkage disequilibrium (D') and correlation (rP^2P) between multiple SNPs in the same gene was performed using the EMLD software package (https: //epi.mdanderson.org/,qhuang/Software/pub.htm). LHR haplotypes were inferred on the basis of Bayesian linkage disequilibrium analyses [27].

Genotype frequency comparisons were conducted using logistic regression analysis. $P \leq 0.05$ was considered statistically significant. Odds ratios and 95% confidence intervals were calculated to assess risk. For the cross-sectional analysis of anthropometric data, medians and ranges were computed and compared between the different genotypes. Variables were checked for normal distributions with the one-sample Kolmogorov-Smirnov test and log-transformed when necessary. Analysis of variance (ANOVA) was used to test for differences between genotype groups and Bonferroni's correction was used to adjust for the number of SNPs tested. In order to test for allele-dose effects the between group variation was tested for linear association. Statistical analysis was performed using a commercially available software package (SPSS version 12; SPSS Inc, Chicago, USA).

Table 1 Primers and probe sequences.

Gene variant	Rs number	PCR primers		Taqman probes	
GNRH1					
Ser16Trp	rs6185	Fw	AATTCAAAAACTCCTAGCTGGCCTTA	VIC	CACGCACCAAGTCA
		Rv	CATAGGACCAGTGCTGGCT	FAM	ACGCACGAAGTCA
FSHR					
Ala307Thr	rs6165	Fw	GCAACAAATCTATTTTAAGGCAAGAAGTTGA	VIC	TGACCCCTAGTCTGAGTC
		Rv	TGTCTTCTGCCAGAGAGGATCT	FAM	ACCCCTAGCCTGAGTC
Asn680Ser	rs6166	Assay on demand (Applied Biosystems, C_2676874_10)			
LHR					
Asn291Ser	rs12470652	Fw	CTGAAGTCCAAAAGCTCAAATGCT	VIC	CAGACAGAATTTTTC
		Rv	TGTGCTTTCACATTGTTTGGAAAAGT	FAM	CAGACAGAGTTTTTC
Ser312Asn	rs2293275	Fw	TTTTCCAAACAATGTGAAAGCACAGT	VIC	TTACAGTGTTTTGTTATTCACTT
		Rv	GATACGACTTCTGAGTTTCCTTGCA	FAM	CAGTGTTTTGTTACTCACTT

RESULTS

Subjects

From a total of 580 normogonadotrophic anovulatory women, 518 women were diagnosed with PCOS. Hyperandrogenism was present in 51% of all anovulatory women while PCO was present in 81% of subjects. Baseline characteristics, endocrine and ultrasound parameters of the study group are shown in Table 2.

Genotyping

All polymorphisms were in Hardy-Weinberg equilibrium within the PCOS population and controls, except for LHR Asn291Ser, which was out of Hardy-Weinberg equilibrium in the control population. Homozygosity for the presence of the minor allele (SerP^{291}P) at this locus did not occur in PCOS patients and in only 16 controls. As the chi-square test is more prone towards type I errors in case of very low minor allele frequencies, HWE was recalculated with an exact test [28] using the Pedstat software package [29]. Again the LHR Asn291Ser polymorphism was out of Hardy Weinberg equilibrium (P=0.006).

Table 2 Descriptive statistics. Clinical and endocrine parameters of 518 Caucasian women with PCOS

	Median	Interquartile range
Baseline characteristics and ultrasound parameters		
Age (years)	28.7	(25 -31.7)
BMI (kg / m2)	26.2	(22.4 -31.2)
Mean number of folliclesa	18	(13 -25)
Mean ovarian volume (mL)a	8.8	(6.5 -11.6)
Endocrine parameters		
LH (IU/L)	7.6	(4.9 -11.4)
FSH (IU/L)	4.9	(3.6 -6.4)
Oestradiol (pmol/L)	231	(169 -345)
Progesterone (nmol/L)	1.6	(1.0 -2.9)
17 (OH) Progesterone (nmol/L)	2.6	(1.9 -4.0)
Testosterone (nmol/L)	1.9	(1.4 -2.4)
SHBG (nmol/L)	37	(25 -57)
Free Androgen Index (FAI)	5.0	(2.9 -8.2)
Androstenedione (nmol/L)	11.8	(9.2 -15.1
DHEA (nmol/L)	40	(28 -59)
DHEAS (µmol/L)	5.1	(3.6 -7.1)
Glucose (mmol/L)	4.0	(3.7 -4.4)
Fasting insulin (pmol/L)	58	(38 -91)

a mean of left and right ovary

Consequently, this polymorphism was not used for the comparison of allele frequencies and haplotype distributions in PCOS cases and controls.

GnRH

The distribution of the Trp16Ser alleles of GnRH1 was similar in PCOS cases and controls. Results for the comparison of genotype frequencies in PCOS cases and controls are summarized in Table 3.

GnRH1 Trp16Ser was not associated with gonadotrophic hormone levels. The influence of this polymorphism and others on the phenotype of PCOS patients was primarily

Table 3 Distribution of genotypes and minor allele frequencies (MAF) in 518 Caucasian PCOS women and 2996 healthy controls

	PCOS		Control				
Genotypes	N	%	N	%	OR	(95% CI)	PP[b]
GNRH1 16							
Trp / Trp	282	54.9%	1570	53.3%	1	(reference)	
Trp / Ser	187	36.4%	1172	39.8%	0.89	(0.73-1.09)	0.25
Ser / Ser	45	8.8%	202	6.9%	1.24	(0.88-1.78)	0.22
MAF (Ser)		26.9%		26.8%	1.01	(0.87-1.17)	0.91
FSHR 680							
Asn / Asn	123	24.8%	782	26.9%	1	(reference)	
Asn / Ser	248	50.1%	1500	51.5%	1.05	(0.83-1.33)	0.68
Ser / Ser	124	25.1%	630	21.6%	1.25	(0.96-1.64)	0.10
MAF (Ser)		50.1%		47.4%	1.12	(0.97-1.28)	0.12
LHR InsLQP[a]							
non / non	283	55.4%	1220	52.0%	1	(reference)	
non / ins	202	39.5%	934	39.8%	0.93	(0.76-1.14)	0.49
ins / ins	26	5.1%	190	8.1%	0.59	(0.38-0.91)	0.02
MAF (ins)		24.9%		28.0%	0.85	(0.73-0.99)	0.04
LHR 291							
Asn / Asn	466	92.5%	2630	89.8%	1	(reference)	
Asn / Ser	38	7.5%	283	9.7%	0.76	(0.53-1.08)	0.12
Ser / Ser	0	0.0%	16	0.5%			
MAF (Ser)		3.8%		5.4%	0.69	(0.49-0.97)	0.03
LHR 312							
Ser / Ser	184	36.6%	978	33.5%	1	(reference)	
Ser / Asn	240	47.7%	1426	48.9%	0.90	(0.73-1.10)	0.30
Asn/ Asn	79	15.7%	512	17.6%	0.82	(0.62-1.09)	0.17
MAF (Asn)		39.6%		42.0%	0.90	(0.79-1.04)	0.15

P[a]Pgenotyped in all PCOS cases and 2419 controls. P[b]PP-values are not corrected for multiple testing.

Table 4 Clinical and endocrine characteristics of 518 anovulatory women with PCOS, stratifications by genotype.

	GNRH1 Trp16				FSHR 680				LHR 18 InsLQ				LHR 291			LHR 312			
	Trp/Trp	Trp/Ser	Ser/Ser	(P)	Asn/Asn	Asn/Ser	Ser/Ser	(P)	Non/Non	Non/Ins	Ins/Ins	(P)	Asn/Asn	Asn/Ser	(P)	Ser/Ser	Ser/Asn	Asn/GAsn	(P)
Baseline characteristics and ultrasound parameters																			
BMI (kg/m2)	26.8	25.2	26.6	≥ 0.50	25.7	25.5	27.6	≥ 0.50	25.5	26.9	26.8	≥ 0.50	26.2	27.3	≥ 0.50	26.2	26.6	25.1	≥ 0.50
Mean follicle numbera	19	18	16	≥ 0.50	19	18	19	≥ 0.50	19	18	19	≥ 0.50	18.00	17.25	≥ 0.50	18	19	16	≥ 0.50
Mean ovarian volumea	8.8	8.8	7.9	≥ 0.50	8.8	8.8	8.5	≥ 0.50	8.5	9.2	7.0	≥ 0.50	8.57	9.32	≥ 0.50	8.4	9.1	8.2	≥ 0.50
Endocrine parameters																			
LH (IU/L)	7.4	7.6	7.9	≥ 0.50	7.0	7.7	8.7	0.01	7.6	7.6	7.2	≥ 0.50	7.5	8.0	≥ 0.50	7.6	7.4	8.4	≥ 0.50
FSH (IU/L)	4.6	5.1	5.4	0.35	4.2	5.0	5.6	0.00001	4.9	4.8	5.6	≥ 0.50	4.9	4.9	≥ 0.50	4.8	4.9	5.3	0.10
Oestradiol (pmol/L)	232	230	228	≥ 0.50	228	231	230	≥ 0.50	232	241	179	≥ 0.50	229	259	≥ 0.50	227	231	240	≥ 0.50
Testosterone (nmol/L)	1.9	1.7	1.9	0.30	1.7	1.9	2.0	0.08	1.8	2.0	1.9	≥ 0.50	1.8	2.2	≥ 0.50	1.8	1.9	1.8	≥ 0.50
Free androgen index	5.4	4.3	4.8	≥ 0.50	4.7	4.6	5.8	0.22	4.6	5.6	4.7	≥ 0.50	5.0	4.8	≥ 0.50	5.0	5.1	4.2	≥ 0.50
Fasting Insulin (pmol/L)	65	54	56	0.20	55	58	65	0.35	58	58	65	≥ 0.50	58	57	≥ 0.50	63	58	60	≥ 0.50
AMHb (µg/L)	10.4	9.6	10.0	≥ 0.50	10.3	9.6	9.9	≥ 0.50	9.2	11.2	11.1	0.15	10.0	12.4	≥ 0.50	9.6	10.4	9.8	≥ 0.50
Features of PCOS																			
PCO (%)	96.0	94.3	97.6	≥ 0.50	92.1	97.9	93.3	≥ 0.50	94.4	96.9	95.8	≥ 0.50	95.3	97.2	≥ 0.50	94.9	97.0	91.9	≥ 0.50
Hyperandrogenism (%)	60.3	49.7	68.9	≥ 0.50	53.7	52.8	70.2	0.04	53.7	61.4	53.8	≥ 0.50	56.7	68.4	≥ 0.50	58.7	57.5	51.9	≥ 0.50

Values are medians. Hyperandrogenism was defined as the presence of either clinical (Ferriman Gallway score ≥ 8) or biochemical (FAI≥4.5) signs of androgen excess. P values were corrected for the number of SNPs tested (Bonferroni correction). P-values were calculated for allele-dose effects using ANOVA (continuous variables, log transformed if not normally distributed) or Chi-square (PCO and hyperandrogenism). amean of left and right ovary bAMH levels available in a subgroup of 354 PCOS-patients.

tested using a model for allele-dose effects. In this way, no significant associations were observed for this polymorphism. However, a somewhat more benign phenotype was noted in carriers (homozygous or heterozygous) of the minor allele (Ser^{16}) compared to non-carriers, showing 5.3% lower levels of T (1.8 *vs.* 1.9 nmol/L, P≤0.01), lower free androgen index (4.7 *vs*. 5.4, P≤0.01), lower fasting insulin levels (54 *vs.* 65 pmol/L, P≤0.01) and lower follicle count (18 vs. 19, P=0.05). No differences in the frequency of PCO or hyperandrogenism were observed among the various genotype groups (Table 4).

FSHR

In 399 PCOS cases, linkage disequilibrium (D') for the two SNPs in exon 10 of FSHR (Ala307Thr and Asn680Ser) was 0.98 (r^2=0.94), indicating near-complete linkage disequilibrium. All remaining PCOS cases and controls were genotyped for the Asn680Ser polymorphism only. The frequency of the minor allele (Ser^{680}) and the distribution of genotypes at this locus were similar in women with PCOS and controls (Table 3).

There was a strong association of this FSHR polymorphism with phenotypic characteristics of PCOS patients. The Ser^{680} allele was associated with higher levels of gonadotrophic hormones: FSH (0.6 IU/L increase per allele copy, P≤0.01) and LH (1.1 IU/L increase per allele copy, P≤0.01) (Table 4). Likewise, the Ser^{680} allele was associated with a higher frequency of hyperandrogenism (OR [per minor allele copy] 1.41 [95% CI 1.09-1.82], P=0.04) ranging from 53.7% among non-carriers to 70.2% among homozygous carriers of the Ser^{680} allele.

LHR

Minor allele frequencies for the insertion polymorphism at codon 18 of exon 1 (insLQ) in PCOS subjects and controls were 24.9 %, and 28.0% respectively (P=0.04). Genotype frequency comparisons revealed a significant negative association of the minor allele (LHR 18insLQ) with PCOS, showing 15% lower risk for PCOS per minor allele copy (OR 0.85 [95% CI 0.73-0.99], P=0.04). However, the slightly lower frequency of the 18 insLQ insertion polymorphism among PCOS cases compared to controls was not significant after Bonferroni's correction for the total number of SNPs that were tested. Among women with PCOS, homozygous carriers of the minor allele showed 24.1% lower levels of oestradiol (179 vs. 236 pmol/L, P=0.01), in combination with 21.3% lower ovarian volume (7.0 *vs.* 8.9 mL, P=≤0.01) compared to the other genotypes.

The frequency of the minor allele of LHR Ser312Asn (Asn^{312}) was similar in PCOS patients and controls. A significant trend towards higher FSH levels in carriers of the Asn^{312} allele did not persist after correction for the total number of polymorphic variants (P=0.10). As multiple polymorphisms were genotyped within the LH receptor gene, we explored the possibility that specific combinations of allelic variants may have a more pronounced influence on phenotype. To this purpose, the allele frequencies of four pos-

sible LHR haplotypes were calculated in PCOS cases, i.e., H1 (nonLQ^{18}/Ser^{312} 50.4%), H2 (nonLQ^{18}/Asn^{312} 23.6%), H3 (insLQ^{18}/Ser^{312} 9.3%) and H4 (insLQ^{18}/Asn^{312} 16.7%). The distribution of LHR haplotypes was similar in women with PCOS and controls. The presence of H1, which is characterized by the absence of polymorphic variants, was associated with lower levels of FSH. Median FSH levels were 5.1 IU/L (complete absence of H1), 4.5 IU/L (one copy of H1) and 4.4 IU/L (2 copies of H1) (P for allele dose effect = 0.02). Linear regression analysis showed no additional benefit for the use of haplotype 1 over LHR Ser312Asn genotype in predicting FSH levels (P=0.69). No other associations were observed for the presence of haplotypes one to four with the phenotype (including LH-levels) of PCOS patients.

Interaction of FSHR and LHR polymorphisms

Both genetic variants of FSHR (Ser^{680}) and LHR (Asn^{312}) showed (a trend towards) association with higher FSH levels. Therefore, the hypothesis was explored that an interaction between both genetic variants may have a more distinct effect on the phenotype. To this purpose the PCOS population was stratified into five subgroups, i.e., carriers of zero to four polymorphic alleles (FSHR Ser^{680} or LHR Asn^{312}). Median FSH levels rose from 3.9 IU/L in women with no polymorphic variants (n=45) to 6.0 IU/L in women who were homozygous carriers of the variant allele at both polymorphic loci. The total number of variant alleles was significantly associated with increasing FSH levels (P≤0.01) (Figure 1). Carriership of zero to four allelic variants was equally distributed among PCOS women and women from the control population (XP^{2}P test, P=0.34) with an average of two allelic variants per individual (both PCOS women and controls)

DISCUSSION

The present study compares the presence of genetic variants of the HPG axis in Caucasian PCOS patients and unselected controls. All polymorphisms, except LHR insLQ, were equally distributed among cases and controls. A 40% decrease in risk for PCOS resulted from the homozygous presence of a 6-nucleotide insertion polymorphism at codon 18 in exon 1 of the LH receptor gene. Among PCOS cases, homozygous carriers of this genetic variant also showed 24% lower oestradiol levels and 21% lower estimates of ovarian volume. Likewise, multiple associations were identified between genetic variants of the HPG axis and the phenotype of PCOS patients, including gonadotrophic hormone levels as well as the presence of hyperandrogenism.

The primary aim of the current study was to identify risk alleles for PCOS. With the possible exception of the LHR 18insLQ polymorphism, we observed no differences in

the frequency of these polymorphisms in PCOS cases and controls. Contrary to what was expected, we observed a lower frequency of the LHR 18insLQ polymorphism among PCOS patients. However, this finding was not significant after Bonferroni's correction and should therefore be interpreted with caution. No other differences were observed in allele frequencies and/or genotype distributions in PCOS cases and controls. In contrast to a prior report [13], we were unable to confirm a difference in the distribution of the FSHR genotypes in PCOS cases and controls. This discrepancy may originate from the limited number of controls (n=30) that were included in the former study.

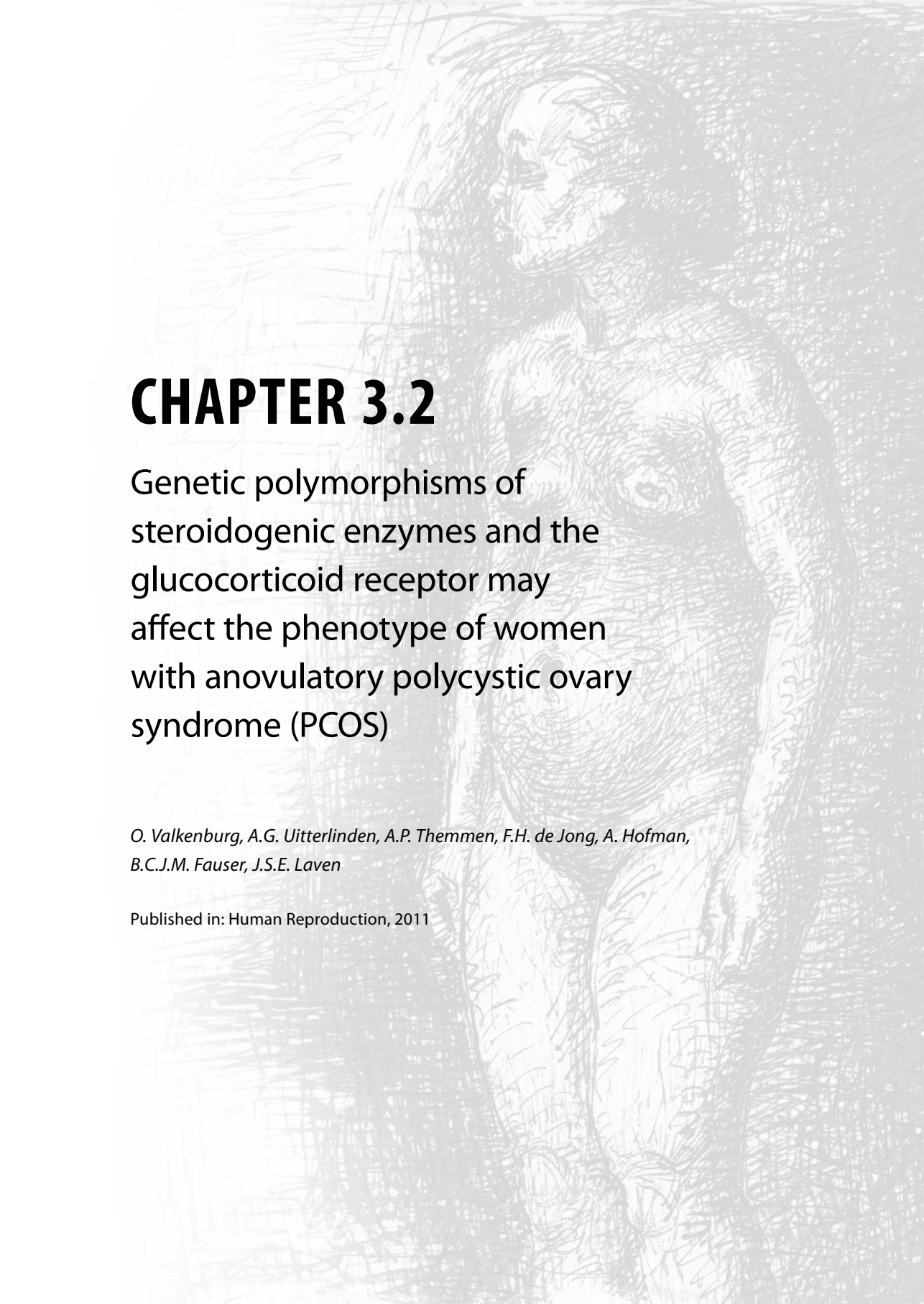
The present study is limited by the absence of phenotype data in controls. Therefore, controls could not be selected for the absence of PCOS. As it is known that 5-8 percent of women in the general population will develop PCOS, we expect that the current results will represent an underestimation of the actual difference between cases and controls. The concomitant loss-of-power will be partially overcome by the relatively large number of patients and controls that were included in the present study. Secondly, there is an apparent age-difference between controls (>55 years) and PCOS cases of reproductive age. However, it is known that the distribution of genotypes will be stable in subsequent generations of a large population. Therefore, provided that the presence of these polymorphisms does not shorten life-span significantly, the higher age of controls is not regarded as a likely confounder. Hence, the lower frequency of the LHR insLQ variant in PCOS cases cannot be easily explained in such a fashion.

The secondary aim of this study was to assess the extent to which these genetic variants influence the severity of clinical features of PCOS. In this regard, the most striking associations were observed for the FSH receptor polymorphisms. The present results are consistent with prior reports concerning the role of FSH receptor variants in normogonadotrophic anovulation [13, 30]. Both studies show higher basal FSH levels in association with the FSHR Ser^{680} allele. In women undergoing ovarian hyperstimulation, this receptor variant results in lower oestradiol levels following FSH stimulation, suggesting lower FSH receptor sensitivity [31]. FSH levels in women with PCOS are within normal limits [15]. Therefore, it does not seem likely that altered FSH sensitivity contributes to the ovulatory dysfunction that is usually present in PCOS. Apparently, the pituitary is capable of a compensatory rise in FSH levels in carriers of the Ser^{680} allele that is able to overcome the increased FSH threshold. Our results further substantiate this hypothesis by the fact that this variant did not constitute a risk allele for PCOS and no association with the number of antral follicles or AMH levels was observed. In complement to the finding of higher FSH levels in carriers of the FSHR Ser^{680} allele, we also report higher LH levels in association with the FSHR Ser^{680}-allele. During folliculogenesis, FSH stimulates the activity and synthesis of aromatase in ovarian granulosa cells [32]. Therefore, decreased FSH receptor sensitivity may disturb normal folliculogenesis causing a decrease in the production of oestradiol and inhibin B that exert an inhibitory feedback action at the level of the

pituitary gland. The resultant increase in FSH and LH levels can also explain the finding of increased androgen levels due to persistent stimulation of ovarian theca cells by LH. Indeed we observed a significant correlation between LH level and the level of ovarian androgens (T [P≤0.01] and AD [P≤0.01]), but not with adrenal androgens (DHEA and DHEAS). While the influence of the FSH receptor variants on gonadotrophic hormone levels and hyperandrogenism are relatively big and consistent with an allele-dose effect, a more subtle influence was observed for the GnRH1 Trp16Ser polymorphism. The presence of either one or two copies of the Ser^{16} allele, which is the minor allele in the European population, is associated with a somewhat more benign phenotype with regard to carbohydrate metabolism and hyperandrogenism (slightly lower testosterone levels and fasting insulin). However, no significant differences in gonadotrophic hormone levels are noted, which would argue against a direct influence of this polymorphism on the function of GnRH as a stimulant at the level of the pituitary. Evidently, this genetic variant does not influence PCOS susceptibility as we found similar genotype distributions in PCOS cases and controls.

We have shown that genetic variations in the HPG axis are capable of altering the phenotype of women with PCOS. More specifically these changes seem to be centered on the levels of gonadotrophic hormones, insulin sensitivity and the presence of hyperandrogenism (either clinical or biochemical). The contribution of these polymorphisms to the phenotype of PCOS is small and may only be relevant in conjunction with other genetic variants that contribute to minor phenotypical variation [33]. Therefore, the combined influence of multiple polymorphisms can be expected to be much more pronounced. This is clearly illustrated by the results for FSH levels, showing nearly two times higher levels in compound homozygous carriers of FSHR-Ser^{680} and LHR-Asn^{312} compared to non-carriers. While there is a clear association with FSH levels, this interaction of genotypes does not seem to influence PCOS susceptibility, as we observed no differences in the distribution of these allelic variants in PCOS cases and controls. These findings show that polymorphic variants of the FSH receptor and LH receptor are important determinants of the physiological setpoint of the HPG axis that might contribute to the pathophysiology of PCOS.

In summary, genetic variants of the HPG axis are associated with a modest but significant effect on the phenotype of PCOS. FSH receptor variants are strongly associated with the severity of clinical features of PCOS, such as levels of gonadotrophic hormones and the presence of hyperandrogenism, but not disease risk.



CHAPTER 3.2

Genetic polymorphisms of steroidogenic enzymes and the glucocorticoid receptor may affect the phenotype of women with anovulatory polycystic ovary syndrome (PCOS)

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women, accounting for nearly 75% of women with anovulatory subfertility [34]. The most important characteristics consist of anovulation, hyperandrogenism, and the appearance of polycystic ovaries (PCO) at ultrasound. While twin studies and studies of familial segregation patterns provide convincing evidence for a genetic aetiology, a clear Mendelian inheritance pattern is lacking [35, 36]. Therefore, PCOS is hypothesized to be the result of an intricate interaction of multiple genetic and environmental factors, i.e., a complex genetic disease.

Besides ovulatory dysfunction, that is often the reason for seeking medical attention, there is a wide variety of metabolic disorders that are frequently found in women with PCOS. These include obesity, insulin resistance and dyslipidemia [37, 38]. Steroid hormones play a central role in the regulation of both ovarian function and body composition. Glucocorticoids (GCs) have numerous effects throughout the human body including the regulation of fat distribution, lipid and glucose metabolism. Increased peripheral cortisol metabolism has been reported in association with PCOS, in a manner that is independent of body mass index (BMI)[39, 40]. It is hypothesized that a compensatory up-regulation of the hypothalamic-pituitary-adrenal (HPA) axis is responsible for the excess of adrenal androgens (dehydroepiandrosterone [DHEA] and dehydroepiandrosterone sulfate [DHEAS]) and their urinary excretion metabolites. Peripheral GC metabolism is dependent on the tissue specific interconversion of cortisol into the non-bioactive steroid cortisone. Two polymorphisms within genes that regulate this enzymatic process (11-beta-hydroxysteroid dehydrogenase type I [HSD11BI] and hexose-6-phosphate dehydrogenase [H6PD]) have been associated with a loss of HSD11BI oxo-reductase activity, resulting in more efficient conversion of cortisol into its non-bioactive metabolites [41].

The influence of genetic variants of the glucocorticoid receptor (GCR) on body-composition and metabolism are wide ranging and include BMI, insulin resistance and cholesterol levels[42-44]. Polymorphic variants of GCR are associated with both higher and lower GC sensitivity, as demonstrated by a variable suppressive response to 0.25 mg of Dexamethasone [44]. Interestingly, a non-synonymous coding SNP in exon 2 of the GCR (rs6190), which results in a relative glucocorticoid resistance, has been associated with multiple clinical endpoints that seem to indicate a more beneficial risk profile with regard to central adiposity, insulin resistance and lipid profiles [42, 43]. The only study to date examining the influence of a single GCR polymorphism (rs6195) in 114 PCOS cases and 92 controls did not find an association with disease phenotype or susceptibility [45].

Notwithstanding the adrenal contribution to the production of androgens, the ovary remains the primary source of sex steroids in women. Androgens that are produced by follicular theca cells, are secreted in follicular fluid where they are converted into

estrogens by the action of the enzyme aromatase. A genetic variant (rs10046) of CYP19, the gene that encodes aromatase, was associated with higher expression of mRNA levels in breast cancer tissue and higher risk for breast cancer (OR 1.5) [46], possibly due to increased estrogen exposure. Furthermore, estrogen signalling may be affected by polymorphic variants of the estrogen receptor. Two forms of estrogen receptors (ESR1 and ESR2) exist that are expressed in tissue-specific patterns during human development. ESR1 is predominantly expressed in the uterus while ESR2 is expressed in the ovary, testis, spleen, brain and skin [47]. Polymorphic variants of ESR1 have been consistently associated with a number of phenotypes that are related to decreased estradiol (E2) signalling, i.e., increased risk of osteoporosis [48], myocardial infarction and stroke [49], later age at onset of menopause [50] and decreased serum E2 levels in postmenopausal women [51]. Furthermore, an association with ovulatory dysfunction [52] and altered ovarian response to controlled hyperstimulation during in vitro fertilisation has been observed [53, 54].

The aim of the present study is to examine to what extent functional genetic variants of the steroid hormone pathway and glucocorticoid receptor can alter disease susceptibility and phenotypic characteristics with regard to PCOS.

MATERIALS AND METHODS

Study population

Anovulatory subjects who attended our infertility outpatient clinic between 1994 and 2004 were eligible for inclusion. Inclusion criteria were oligomenorrhoea (interval between consecutive menstrual periods >35 days) or amenorrhoea (absence of vaginal bleeding for at least 6 months) and serum FSH concentrations within the normal range (1–10 U/l) [19, 20]. Ethnicity and country of birth were registered. The diagnosis of PCOS was established on the basis of the 2003 ESHRE/ASRM Rotterdam criteria [21]. In agreement with these criteria hyperandrogenism was defined as the presence of either biochemical or clinical signs (hirsutism) of androgen excess. For the purpose of this study hirsutism was assessed by means of the Ferriman Gallway (FG) score, and was defined as FG score ≥ 8. Biochemical hyperandrogenism was determined by calculation of the free androgen index (FAI) as follows: FAI= T (nmol/l) * 100 / SHBG (nmol/l) , where T stands for testosterone and SHBG for sex-hormone binding globulin. A cut-off level of 4.5 was used for the definition of hyperandrogenism [20]. The presence of polycystic ovaries (PCO) was detected by vaginal ultrasound examination. PCO was defined as the presence of 12 or more follicles in one or both ovaries, and/or increased ovarian volume (>10 ml). Exclusion criteria were: 1) the presence of related disorders with similar clinical presentation, such as congenital adrenal hyperplasia, hyperprolactinaemia and Cushing's syndrome; 2) non-Caucasian descent.

The control group was derived from The Rotterdam Study, a single-center, prospective, population based study of determinants of chronic disabling diseases in the elderly, aged 55 years and over (n=7012). The design of this study has been described previously [23, 55]. Participants from the Rotterdam study originate from a specific area near Rotterdam (Ommoord) that constitutes a homogeneous population-based sample of Caucasian elderly men and women. Inclusion criteria for the present study were female sex, age at onset of menopause > 45 years and available DNA (n=2996). Written informed consent was obtained from all anovulatory patients as well as controls.

Measurements

Anovulatory patients underwent a standardized initial screening that was performed after an overnight fasting period on a random day between 9 a.m. and 11 a.m. Clinical examination included a structured interview and physical examination. Transvaginal ultrasonography was performed in order to assess ovarian volume and follicle count for both ovaries. Blood samples, taken on the day of clinical examination, were obtained by venipuncture and processed within 2 hours after withdrawal. Serum was stored at -20° C until assayed. Assays used to measure the endocrine and metabolic parameters were as follows. FSH, LH, SHBG, Insulin, cortisol, progesterone, androstenedione and DHEAS were measured in serum by immunoluminescence based assays (Immulite® platform, Siemens DPC, Los Angeles, CA, USA). DHEA, 17-hydroxy-progesterone, E2 and Testosterone concentrations were assayed using coated tube radioimmunoassays) (DPC). Glucose levels were measured using a Hitachi® 917 analyzer (Roche Diagnostics, Almere, the Netherlands). Insulin resistance was estimated by homeostasis model assesment (HOMA). In order to calculate HOMA scores the updated computer model (HOMA2) was used [56].

Genotyping

Genomic DNA was extracted from peripheral venous blood according to standard procedures. Genotypes were determined using Taqman allelic discrimination assays, designed via the Assay-by-Design service (Applied Biosystems). The following polymorphisms were included: four SNPs in GCR (rs6190 [Arg23Lys], rs6195 [Asn363Ser], rs41423247 [C/G] and rs6198 [exon9b A/G]); one SNP in H6PD (rs6688832 [Arg453Gln]) and an insertion polymorphism in 11BHSD (83557ins-A); two SNPs in ESR1 (rs2234693 [-397exon2 C/T] and rs9340799 [-351exon2 A/G]); one SNP in the 3' UTR of ESR2 (rs4986938, [+1730 G/A]) and one SNP in the 3' UTR of CYP19 (rs10046 [+1531 A/G]). Primer and probe sequences are available from the authors upon request. The PCR reaction mixture included 2 ng of genomic DNA in a 2 µL volume and the following reagents: probes (200 nM), primers (0.9 µM), 2 x Taqman PCR master mix (ABgene, Epsom, UK). PCR cycling reactions were performed on an ABI 9700 PCR system (Applied Biosystems Inc., Foster

City, CA, USA) and consisted of initial denaturation at 95°C (15min), and 40 cycles with denaturation (15s at 95°C) and annealing and extension (60s at 60°C). GCR and ESR1 haplotypes were inferred on the basis of Bayesian linkage disequilibrium analysis using the PHASE software package, version 2.1. [27]. Haplotype alleles were coded as numbers (1 to 3 for ESR1 and 1 to 5 for GCR) in order of decreasing population frequency.

Statistical analysis

Genotype and allele frequencies were calculated for all polymorphisms and subsequently tested for Hardy-Weinberg equilibrium with Pearson's chi-square (X2) test. Cross-tables were constructed in order to compare genotypes and allele frequencies between the PCOS-population and controls. Odds ratios and 95% confidence interval were calculated. Medians and interquartile ranges (P25-P75) were calculated in order to describe the distribution of anthropomorphometric data. A nonparametric test (Mann-Whitney U) was used for the exploratory comparison of continuous variables between different genotype groups. Variables were checked for normal distributions with the one-sample Kolmogorov-Smirnov test and log-transformed if necessary. Analysis of covariance (ANCOVA) was applied to adjust for age differences. In order to test for allele-dose effects, the between group variation was tested for linear association. Between-group differences of categorical variables were evaluated by Pearson's chi-square (X2) test. Accounting for the total number of SNPs that were tested (N=10), the threshold for statistical significance was set at a P-value of less than or equal to 0.005. Statistical analysis was performed using SPSS version-12; SPSS-Inc, Chicago, USA). Power calculations were performed for a codominant mode of inheritance with one degree of freedom, using the PGA software package version 2.0 [57]. Assuming minor allele frequency (MAF) of 0.05 and disease prevalence of 5%, we calculated a power of 88% (alpha 0.05) to detect a relative risk of 1.5 at 500 cases and a case:control ratio of 5:1. Lowering MAF to 0.02, the power was calculated as 53% and 97% to detect relative risks of 1.5 and 2.0 respectively.

RESULTS

PCOS phenotype

A total of 580 Caucasian normogonadotropic oligomenorrheic or amenorrheic women were included in the present analysis. A diagnosis of PCOS was confirmed in 518 cases (2003 Rotterdam criteria). PCOS patients were from North-Western European (n=398) and Mediterranean European (North of Sahara and Middle East) (n=120) descent. Median age was 28.9 (range: 14.4-44.7) years. Hyperandrogenism occurred in 51% of cases, whereas polycystic ovaries were present in 85% of cases. Median BMI was 25.8 (range

Table 1 Cycle history, clinical parameters and endocrine profiles in 518 Caucasian PCOS cases

	PCOS (N=518)	
Age and cycle history		
Age (years)	28.7	(25.0 -31.7)
Amenorrhoea (%)	28%	
Mean duration of cycle$P^{a}P$ (days)	43	(38 -63)
Menarche (yrs)	13	(12 -14)
Physical examination and vaginal ultrasound		
Body mass index (kg/m2)	26.2	(25.0 -31.7)
Waist circumference (cm)	87	(77 -98)
Mean number of follicles	18.0	(38 -63)
Mean ovarian volume (ml)	8.8	(12 -14)
Endocrine parameters		
LH (U/L)	7.6	(4.9 -11.4)
FSH (U/L)	4.9	(3.6 -6.4)
Estradiol (pmol/L)	231	(169 -345)
Progesterone (nmol/L)	1.6	(1.0 -2.9)
17-hydroxy-progesterone (nmol/L)	2.6	(1.9 -4.0)
Testosterone (nmol/L)	1.9	(1.4 -2.4)
SHBG (nmol/L)	37	(25 -57)
Free Androgen IndexP^{b}	5.0	(2.9 -8.2)
Androstenedione (nmol/L)	11.8	(9.2 -15.1)
DHEA (nmol/L)	39.8	(27.7 -58.9)
DHEAS (µmol/L)	5.1	(3.6 -7.1)
Cortisol (nmol/L)	357	(271 -439)
Fasting glucose (mmol/L)	4.0	(3.7 -4.4)
Fasting Insulin (pmol/L)	58	(38.0 -90.8)
HOMA IRP^{c}	1.04	(1.61 -0.71)

Values are medians and interquartile ranges (P25-P75)

$P^{a}P$ Amenorrheic subjects excluded
$P^{b}P$ Free androgen index calculated as = T/SHBG * 100
$P^{c}P$ Homeostasis Model Assessment, updated HOMA model (HOMA2).

15.9-59.9) kg/m2 and 30% of cases were obese (BMI>30 kg/m2). Table 1 summarizes clinical and endocrine data of 518 PCOS cases that were included in the present analysis.

Glucocorticoid receptor polymorphisms

All four polymorphisms of the glucocorticoid receptor were in Hardy Weinberg equilibrium in the case group and in controls. GCR genotypes were similarly distributed at all four loci in PCOS cases and controls (Table 2). The phenotype analysis showed 37 % higher LH levels in heterozygous carriers of the minor allele (Lys23) of the rs6190

Table 2 Distribution of genotypes and minor allele frequencies in Caucasian PCOS women and population derived controls (The Rotterdam Study).

Gene	Polymorphism	Allele	PCOS		Control		OR	95% CI
			N	(%)	N	(%)		
GCR	rs6190	Arg / Arg	475	(95.2)	2675	(93.4)	1	reference
		Arg / Lys	24	(4.8)	188	(6.6)	0.72	(0.47-1.11)
		Lys / Lys	0	(0.0)	2	(0.1)		
	MAF	Lys		2.4 %		3.4 %	0.71	(0.46-1.09)
GCR	rs6195	Asn / Asn	463	(92.0)	2672	(92.3)	1	reference
		Asn / Ser	40	(8.0)	222	(7.6)	1.04	(0.73-1.48)
			0	(0.0)	1	(0.0)		
	MAF	Ser		4.0 %		3.9 %	1.03	(0.73-1.45)
GCR	rs41423247	C / C	204	(41.0)	1150	(39.2)	1	reference
		C / G	223	(44.8)	1400	(47.7)	0.90	(0.73-1.10)
		G / G	71	(14.3)	387	(13.2)	1.03	(0.77-1.39)
	MAF	G		36.6 %		37.0 %	0.98	(0.86-1.13)
GCR	rs6198	A / A	300	(66.8)	1956	(67.6)	1	reference
		A / G	128	(28.5)	853	(29.5)	0.98	(0.78-1.22)
		G / G	21	(4.7)	85	(2.9)	1.61	(0.98-2.64)
	MAF	G		18.9 %		17.7 %	1.09	(0.91-1.30)
HSD11B1	83557ins-A	- / -	322	(62.5)	1784	(60.8)	1	reference
		- / ins-A	170	(33.0)	1012	(34.5)	0.93	(0.76-1.14)
		ins-A / ins-A	23	(4.5)	136	(4.6)	0.94	(0.59-1.48)
	MAF	ins-A		21.0 %		21.9 %	0.95	(0.81-1.11)
H6PD	rs6688832	Arg / Arg	303	(59.1)	1672	(59.5)	1	reference
		Arg / Gln	171	(33.3)	972	(34.6)	0.97	(0.79-1.19)
		Gln / Gln	39	(7.6)	167	(5.9)	1.29	(0.89-1.87)
	MAF	Gln		24.3 %		23.2 %	1.06	(0.91-1.24)
CYP19	rs10046	T/T	128	(25.2)	765	(26.0)	1	reference
		T/C	245	(48.2)	1470	(50.0)	1.00	(0.79-1.26)
		C/C	135	(26.6)	705	(24.0)	1.14	(0.88-1.49)
	MAF	C		50.7 %		49.0 %	1.07	(0.94-1.22)
ESR1	rs2234693	T/T	146	(28.7)	892	(30.0)	1	reference
		T/C	252	(49.5)	1449	(48.8)	1.06	(0.85-1.32)
		C/C	111	(21.8)	628	(21.2)	1.08	(0.83-1.41)
	MAF	C		46.6 %		45.6 %	0.96	(0.84-1.10)
ESR1	rs9340799	A/A	213	(41.6)	1293	(43.6)	1	reference
		A/G	229	(44.7)	1322	(44.5)	1.05	(0.86-1.29)
		G/G	70	(13.7)	353	(11.9)	1.20	(0.90-1.62)
	MAF	G		36.0 %		34.2 %	1.09	(0.95-1.25)
ESR2	rs4986938	G/G	198	(38.4)	1100	(37.3)	1	reference
		G/A	243	(47.1)	1437	(48.7)	0.94	(0.77-0.94)
		A/A	75	(14.5)	412	(14.0)	1.01	(0.76-1.01)
	MAF	A		38.1 %		38.3 %	0.99	(0.86-1.13)

MAF: minor allele frequency

Table 3 Clinical and endocrine characteristics of 518 anovulatory women with PCOS, stratifications by GCR genotype

	rs6190			rs6195			rs41423247				rs6198			
	Arg/Arg	Arg/Lys	P[b]	Asn/Asn	Asn/Ser	P[b]	C/C	C/G	G/G	P[b]	A/A	A/G	G/G	P[b]
N	475	24		463	40		204	223	71		300	128	21	
Clinical parameters														
BMI (kg/m2)	26.3	24.5	0.94	26.5	23.6	0.01	26.2	26.1	26.5	0.82	26	26.5	25	0.20
Waist circumference (cm)	87	86	0.51	87	81	0.01	86	87	88	0.93	86	86	86	0.60
Menstrual intervalP[a]P (days)	43	45	0.51	43	46	0.64	46	42	43	0.51	42	45	53	0.09
Amenorrhea (%)	27.4	37.5	0.28	27.6	32.5	0.51	28.9	28.3	23.9	0.70	25	31.3	33.3	0.22
Ultrasound														
Ovarian volume (ml)	8.86	8.03	0.38	8.79	8.99	0.34	9.64	8.05	8.17	0.09	8.54	9.08	9.55	0.36
Mean follicle number	19	17	0.75	18	20	0.85	20	18	17	0.19	18	19	19	0.80
Endocrine parameters														
LH (U/l)	7.6	10.4	0.04	7.6	7.1	0.77	8.1	7.1	6.9	0.13	7.4	8.1	10	0.02
FSH (U/l)	4.9	4.9	0.18	4.9	5.5	0.22	5	4.9	4.4	0.07	4.9	5.2	5	0.15
Estradiol (pmol/L)	230	235	0.90	230	227	0.28	229	231	230	0.11	237	224	219	0.87
Testosterone (nmol/L)	1,9	1,8	0.48	1,9	1,9	0.90	1,9	1,8	1,9	0.08	1,9	1,8	2,2	0.92
Free androgen index	5.1	4.0	0.33	5.1	4.1	0.11	5.0	5.0	5.0	0.60	4.9	4.8	5.9	0.45
DHEA (nmol/L)	40.5	33.6	0.36	40.4	40.5	0.52	43.0	40.0	34.7	0.03	42.3	37.8	47.3	0.82
DHEAS (µmol/L)	5.10	4.69	0.36	5.10	5.65	0.39	5.35	5.00	4.80	0.62	5.02	4.89	6.10	0.45
Fasting glucose (mmol/L)	4,0	3,9	0.53	4,0	4,0	0.78	4,0	4,0	4,0	0.72	4,0	4,0	4,0	0.24
Fasting Insulin (pmol/L)	58	44	0.62	58	51	0.02	61	58	58	0.63	58	58	56	0.85
HOMA IR	1.06	0.78	0.27	1.06	0.94	0.04	1.08	1.03	1.03	0.54	1.04	1.01	1.01	>0.99

Values are medians.

[a] Amenorrheic subjects not included

[b]-values calculated for allele dosage effects, adjusted for age.

Arg23Lys polymorphism (10.4 vs. 7.6 U/L, P=0.04). FSH levels, androgens and other endocrine and ultrasound parameters were not affected by Lys23 (Table 3). Like Lys23, the minor allele of the rs6198 polymorphism (G9β) was associated with a trend towards higher LH levels. LH levels increased with 1.3 U/l per allele copy of G9β (95% confidence interval: 0.1–2.6 U/l, P=0.02). In addition, homozygous carriers of G9β showed longer menstrual intervals compared to other subjects (53 vs. 43 days, P=0.04). Heterozygous carriers of the rs6195 Ser363 allele showed a slightly more benign phenotype with regard to obesity (11% lower BMI, P=0.01) and central adiposity (7% lower waist-circumference, P=0.008). As expected, these changes in body composition were accompanied by lower fasting insulin levels (51 vs. 58 pmol/L, p=0.02) and slightly lower HOMA scores for insulin resistance (0.94 vs. 1.06, p=0.04). Associations of GCR genotypes with the phenotypic characteristics of PCOS did not surpass the more severe threshold for statistical significance (P<0.005) that was applied to correct for multiple testing.

GCR haplotypes

Call-rates for the GCR SNPs varied between 87 % (rs6198) and 97 % (rs6195). A complete data set on all four GCR gene polymorphisms was available for 445 Caucasian PCOS patients and 2755 controls. The degree of correlation between each of the polymorphic variants ranged from 0.001 (r-squared) to 0.13 (Figure 1). Allele-frequencies of the five

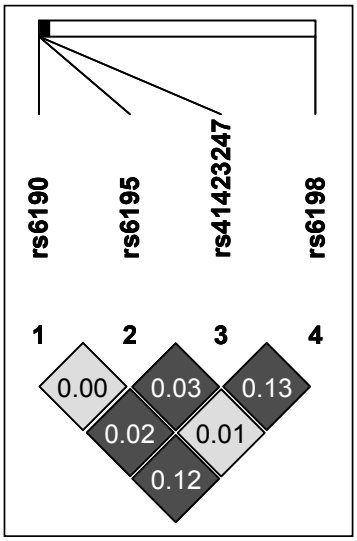
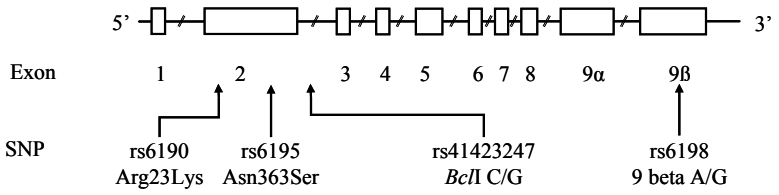


Figure 1 LD-plot of four polymorphisms within the glucocorticoid receptor gene (values for r-squared)



Haplotype		Allele frequency		Odds ratio (95%CI)
		PCOS	Control	
1 (reference)	Arg-Asn-C-A	40.1 %	41.4 %	1
2	Arg-Asn-**G**-A	36.6 %	36.8 %	1.0 (0.8-1.2)
3	Arg-Asn-C-**G**	16.3 %	14.5 %	1.2 (0.9-1.4)
4	Arg-**Ser**-C-A	4.2 %	3.9 %	1.1 (0.8-1.6)
5	**Lys**-Asn-C-**G**	2.7 %	3.3 %	0.8 (0.5-1.3)

Figure 2 Characterization and allele frequencies of GCR haplotypes one to five (allele frequency > 1%) in PCOS cases and controls

most common GCR haplotypes were similar in PCOS cases and controls. No single haplotype was associated with risk for PCOS. Haplotype descriptions and allele-frequencies are presented in Figure 2. Haplotype 1 (H1), which constitutes the absence of minor alleles at all four loci, was the most frequent haplotype among women with PCOS (allele frequency 40.1 %).Homozygous carriers of H1 (n=79) served as a reference group for the comparison of clinical, ultrasound and endocrine characteristics within the entire PCOS group (results in Table 4).

Haplotype 2 (H2) constitutes the presence of the minor allele at the rs41423247 locus. The presence of H2 (0, 1 or 2 alleles) was associated with lower LH levels (0.7 U/l per allele copy, P=0.04) and higher age at the time of study (0.9 yrs increase per allele copy, P≤0.01). Figure 3 shows median LH levels in association with allelic combinations of GCR haplotypes. In contrast, the results for haplotype 3 (H3, presence of the minor allele (G9β) of the rs6198 polymorphism) were the opposite of H2, i.e., carriership of one or two copies of the minor allele was associated with significantly higher LH levels (1.2 U/l increase per allele copy, P=0.05) and lower age (1.2 yrs decrease per allele copy, P≤0.01). Moreover, homozygous carriers of H3 (n=14) showed nearly 1.8 times longer menstrual interval compared to non-carriers (mean increase of 6.7 days per allele copy, P=0.02).

Haplotype 4 (H4), which is characterized by the presence of the minor allele of rs6195 (Ser363), was associated with a more benign metabolic profile. A comparison of H1/H4 carriers with the reference group (H1/H1) showed 10% lower lower waist circumference (P=0.03) (table 4). H4-carriers seemed to be less insulin resistant as indicated by lower insulin levels (45 vs. 65 pmol/L, P=0.03), however fasting glucose levels were higher

Table 4 Clinical and endocrine characteristics of 443 anovulatory women with PCOS, stratified by allelic combinations of haplotypes one to five (four GCR SNPs)

Allelic combination	H1 H1	H2 H2	H3 H3	H1 H2	H1 H3	H1 H4	H1 H5	H2 H3	H2 H4	H2 H5	H3 H4	H3 H5
N	79	63	14	124	52	14	9	52	16	8	6	6
Clinical parameters												
BMI (kg/mP^{2}P)	26.6	26.5	24.8	26.1	26.2	23.5	27.5	29.2	23.1	23.2	22.6	29.3
Waist circumference (cm)	86	88	83	87	85	77P†	95	91	81	84	80	96
Menstrual intervalP^{a}P (days)	48	42	75P†	41	42	50	45	49	45	40	78	45
Amenorrhea (%)	22.8	27	21.4	24.2	25	21.4	33.3	32.7	31.2	37.5	66.7†	50
Ultrasonographic												
Mean ovarian volume	9.7	8.4	10.2	8.1P†	10	8.2	10.8P†	8.3	8.4	6.6	10.3	8
Mean follicle number	21	17	20	18	20	21	22	19	19	16	23	16
Endocrine parameters												
LH (U/l)	8.1	6.9	8.8	7.4	8.4	8.0	10.4	7.3	5.5	6.5	10.3	12.0P†
FSH (U/l)	5.0	4.5	4.8	4.7	5.5	5.7	4.3	5.3	5.6	6.2	5.0	5.6
Estradiol (pmol/L)	246	233	217	230	224	221	261	230	243	196P†	180	331
Testosterone (nmol/L)	1.9	1.9	2.2	1.9	1.8	2.1	2	1.8	1.8	1.7	1.7	2.2
Free androgen index	5.2	5.2	6.0	4.8	4.5	4.0	4.1	6.1	4.1	3.9	4.3	4.3
DHEA (nmol/L)	43.9	34.7	47.3	42.4	38.0	47.3	34.9	39.4	40.5	26.5	19.8P††	51.3
DHEAS (µmol/L)	5.3	4.8	6.2	5.0	5.2	6.3	4.4	4.6	5.7	4.8	3.6	5.6
Fasting glucose (mmol/L)	4.0	4.0	4.0	4.1	4.0	4.3P††	4.2P†	4.0	3.8	3.7	4.0	3.9P†
Fasting Insulin (pmol/L)	65	58	57	55	52	45P†	41	63	43	37	57	48
HOMA IR	0.86	0.98	0.99	1.01	1.11	1.09	1.36	0.92	1.39P†	1.65P†	1.12	1.07

Values are medians.

[a] Amenorrheic subjects not included

[†] P-values calculated for difference with the reference group (H1/H1) and age adjusted, P≤0.05

[††] P-values calculated for difference with the reference group (H1/H1) and age adjusted, P≤0.01

compared to the reference groups (4.3 vs. 4.0 mmol/L, P≤0.01). No significant difference in HOMA score was observed.

11βHSD and H6PD

The allele frequencies of the 11β-HSD and H6PD polymorphisms were 21.0% and 24.3% respectively (Table 2). The 11β-HSD polymorphism was in Hardy Weinberg equilibrium (HWE) in PCOS cases and controls, whereas the H6PD polymorphism (rs6688832) showed a significant deviation from equilibrium in PCOS cases (P = 0.03).

There were no significant differences in the distribution of the 11BHSD genotypes between women with PCOS and the control-population. The presence of the minor alleles of 11β-HSD (ins-A) or H6PD (Gln453) did not influence any of the phenotypic characteristics of PCOS patients, including androgens (T, DHEA and DHEAS), levels of gonadotropic hormones (LH and FSH) and cycle history (mean cycle duration, frequency of amenorrhea). Moreover, no significant differences were observed with regard to the presence of PCO, hyperandrogenism, obesity or insulin resistance among women with PCOS.

Aromatase (CYP19)

CYP19 1531 T/C genotypes were in Hardy Weinberg equilibrium in PCOS cases and controls. No differences were observed for allele frequencies and genotype distributions in PCOS cases and controls (Table 2). Moreover, we observed no differences in the phenotype of women with PCOS (specifically with regard to androgen and estrogen levels) among different CYP19 genotype groups.

Estrogen receptor (ESR1 and ESR2)

All polymorphisms of the estrogen receptor 1 and 2 were in Hardy Weinberg equilibrium in women with PCOS and controls. Polymorphisms of ESR1 (rs2234693 and rs9340799) and ESR2 (rs4986938) were similarly distributed among PCOS patients and controls (Table 2). No associations with the phenotype of PCOS patients were observed for any of the three SNPs in ESR1 or ESR2.

The two polymorphic variants of ESR1 showed a high level of correlation (r-squared = 0.64) Haplotype 1 (ESR1-H1), which was the most common haplotype of ESR1 among women with PCOS and controls, consisted of a combination of the major alleles of rs2234693 and rs9340799 (T-A). The allele frequencies of haplotype 1, haplotype 2 (C-G) and haplotype 3 (C-A) were similar in women with PCOS and controls; 53.4% vs. 54.4% (ESR1 H1); 35.9% vs. 34.3% (ESR1 H2) and 10.7% vs. 11.4% (ESR1 H3). Furthermore the phenotype of women with PCOS was not affected by any of the three ESR1 haplotypes.

DISCUSSION

Steroid hormones play a central role in the regulation of both ovarian function and body composition with regard to (central) obesity and insulin resistance. Therefore, the primary aim of this study was to investigate whether known functional genetic variants of the steroid hormone pathway affect the phenotype of PCOS patients with regard to disease risk, hyperandrogenism and insulin resistance. The present study is the first to assess the role of these functional polymorphisms of the glucocorticoid receptor in PCOS patients and controls. While no differences in genotype distribution were identified in PCOS cases and controls, a number of phenotypic associations were identified that may be related to altered glucocorticoid action.

GCR variants were associated with changes in LH levels that suggest an inhibitory influence of the glucocorticoid receptor on the function of the hypothalamo-pituitary-gonadal axis. We observed higher LH levels in association with the rs6190 and rs6198 polymorphisms. Both variants have been described as negative inhibitors of GCR action in prior research [58, 59]. While these findings are not significant at the 0.005 level (threshold for multiple testing), we did observe significantly higher LH levels in combined carriers of haplotypes three and five, that are associated with one (H3) or both (H5) genetic variants. Our data is consistent with data from the HapMap Project (release 28) in the finding of some correlation between the two polymorphisms ($r^2 = 0.12$, figure 1). Therefore, it is difficult to differentiate between the effects of one or the other polymorphic variant on LH levels. H3, that contains only the rs6198 variant, was associated with higher LH levels in a manner that is consistent with an allele dose effect (1.2 U/L per allele copy) (Figure 3). Conversely, the analysis of haplotypes two and four showed lower LH levels in association with glucocorticoid receptor variants that are known to increase receptor sensitivity (rs6195 and rs41423247) [44]. H2 (the minor allele of rs41423247) was associated with lower serum LH levels (0.7 U/L per allele copy). More so, median LH levels were lowest in patients who carried a combination of H2 and H4 (minor allele of rs6195). Both H2 and H3 were associated with patients' age at the time of study. It is not likely that GCR genotype would have had a direct influence on this parameter, however age could have acted as a confounder of LH-levels. Given the direction of the observed differences, this is not a likely explanation for the associations with LH-levels that were found. Because the observations with regard to the phenotype of women with PCOS were not significant at the 0.005 level, we cannot exclude the possibility that they were the result of multiple testing. However, our findings do show consistency and may indeed reflect the relatively small changes in phenotype that are expected to result from receptor polymorphisms that occur frequently in the general population.

The present study is limited by the absence of phenotype data in the control population. Therefore, controls could not be selected for the absence of PCOS. As it is known

that 5-8 percent of women in the general population will develop PCOS, we expect that the present results will represent an underestimation of the actual difference in cases and controls. The concomitant loss-of-power will be partially overcome by the relatively large number of patients and controls that were included. Secondly, there is an apparent age-difference between controls (>55 years) and PCOS cases of reproductive age. However, it is known that the distribution of genotypes will be stable in subsequent generations of a large population. Therefore, on the condition that the presence of these polymorphisms do not shorten life-span significantly, the higher age of controls is not regarded as a likely confounder.

Animal research has provided convincing evidence for a link between glucocorticoid action and the production of gonadotropic hormones in rats [60], ewes [61] and rhesus monkeys [62], either at the pituitary or hypothalamic level. As was found in animal studies, the results of our study indicate that glucocorticoids may exert an inhibitory influence on gonadotropin (LH) release. However, taking into account that the distribution of genotypes was similar in cases and controls, it does not seem likely that these variants contribute to PCOS susceptibility. The question whether these associations are also present in the general population cannot be answered by the present study as we do not have data on gonadotropic hormone levels in controls. It is tempting to speculate that these results point in the direction of a link between the hypothalamo-pituitary-gonadal axis and the hypothalamo-pituitary-adrenal axis that is certainly well established in animal studies but not in humans.

The most pronounced influence on body composition was noted for the GCR rs6195 polymorphism. The presence of the minor allele (Ser363) was associated with lower waist circumference and lower fasting insulin levels among PCOS patients. While significance levels did not surpass the threshold for multiple testing, similar results were obtained from the haplotype analysis for H4. As expected, changes in waist circumference and fasting insulin levels were highly dependent on the differences in BMI between carriers and non-carriers. In contrast, raised glucose levels in H1/H4-carriers did not depend on differences in BMI and are consistent with a diabetogenic action of glucocorticoids [63]. The influence of this polymorphic variant on body composition has been examined in numerous studies showing contradictory results. Lin et. al found highly significant associations with BMI and obesity in two groups of white Australian subjects [64]. However no apparent influence on body composition and insulin resistance was found in a Swedish study of 284 men [65]. Moreover, a recent meta-analysis found no evidence for associations with either BMI or risk of obesity [66]. The present study seems to be unique in the finding of lower BMI in carriers of the Ser363 allele, which may be a specific finding in PCOS patients. The role of the rs6195 polymorphism in PCOS patients has been evaluated before in a case-control study that did not establish any associations with either adrenal androgen excess or disease risk [45]. However, the authors provide no information on BMI.

Due to the low frequency of this polymorphism in reported PCOS cases (6 of 109 cases), this study may have been severely underpowered. The present study is in agreement with the former, as we do not observe altered disease risk in carriers (or non-carriers) of the minor allele.

Two genetic variants of HSD11BI (83557 insA) and H6PD (rs6688832) have been implicated in the pathophysiology of cortisone reductase deficiency [41]. In vitro, these variants were associated with reduced expression of 11BHSD1 and attenuated enzyme activity. Subsequent analyses in PCOS patients were unable to uniformly establish HSD11BI and H6PD variants as risk alleles for PCOS [67-69]. However, these studies were limited in sample size [68] and lacked the use of consensus diagnostic guidelines for PCOS [69]. While the H6PD variant was associated with disease risk and the presence of increased levels of cortisol and 17-hydroxyprogesterone in one study [68], this finding was not confirmed by the others. The present study describes the same genetic variants of HSD11B and H6PD in a very large cohort of patients and controls and also fails to identify an association with disease risk or phenotype of PCOS patients. Therefore, their role in PCOS seems very limited. In addition, we did not observe associations of the functional SNPs in CYP19, ESR1 and ESR2 with the phenotype of PCOS or disease risk.

In summary, the present study examines the role of functional genetic variants of the steroid hormone pathway in PCOS patients. It is shown that these genetic variants do not constitute risk alleles for PCOS. In addition, we studied associations with the phenotype of PCOS. Results with regard to the glucocorticoid receptor show that polymorphic variants influence LH levels in a way that may be mediated through altered GC signalling. Yet, our results require confirmation in another cohort. It is hypothesized that glucocorticoids can affect the function of the hypothalomo-pituitary-gonadal axis in humans. While this theory is strongly substantiated by the results of animal research, the influence of corticosteroids on gonadotropin production in humans has not been clearly established and needs to be explored further.

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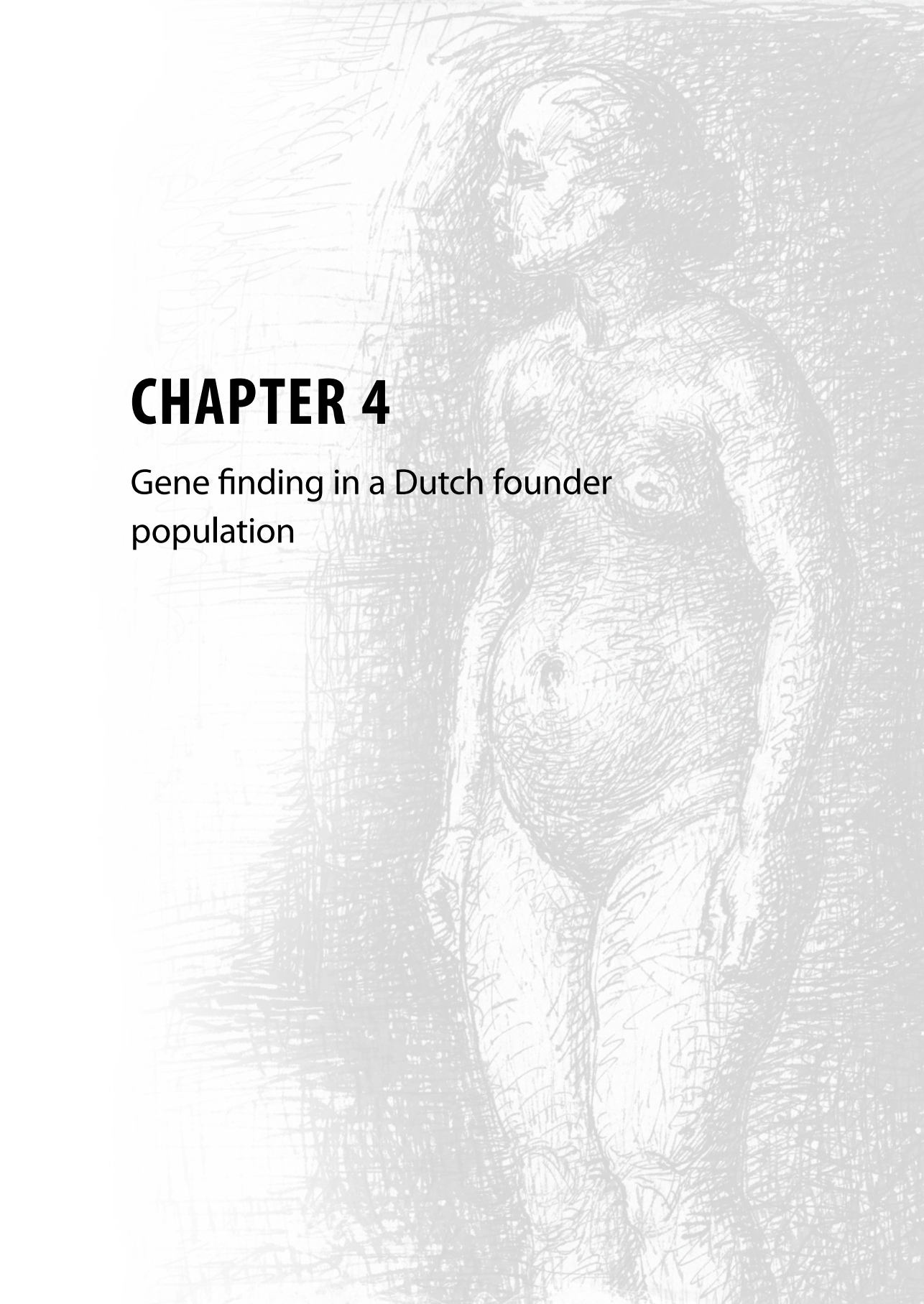
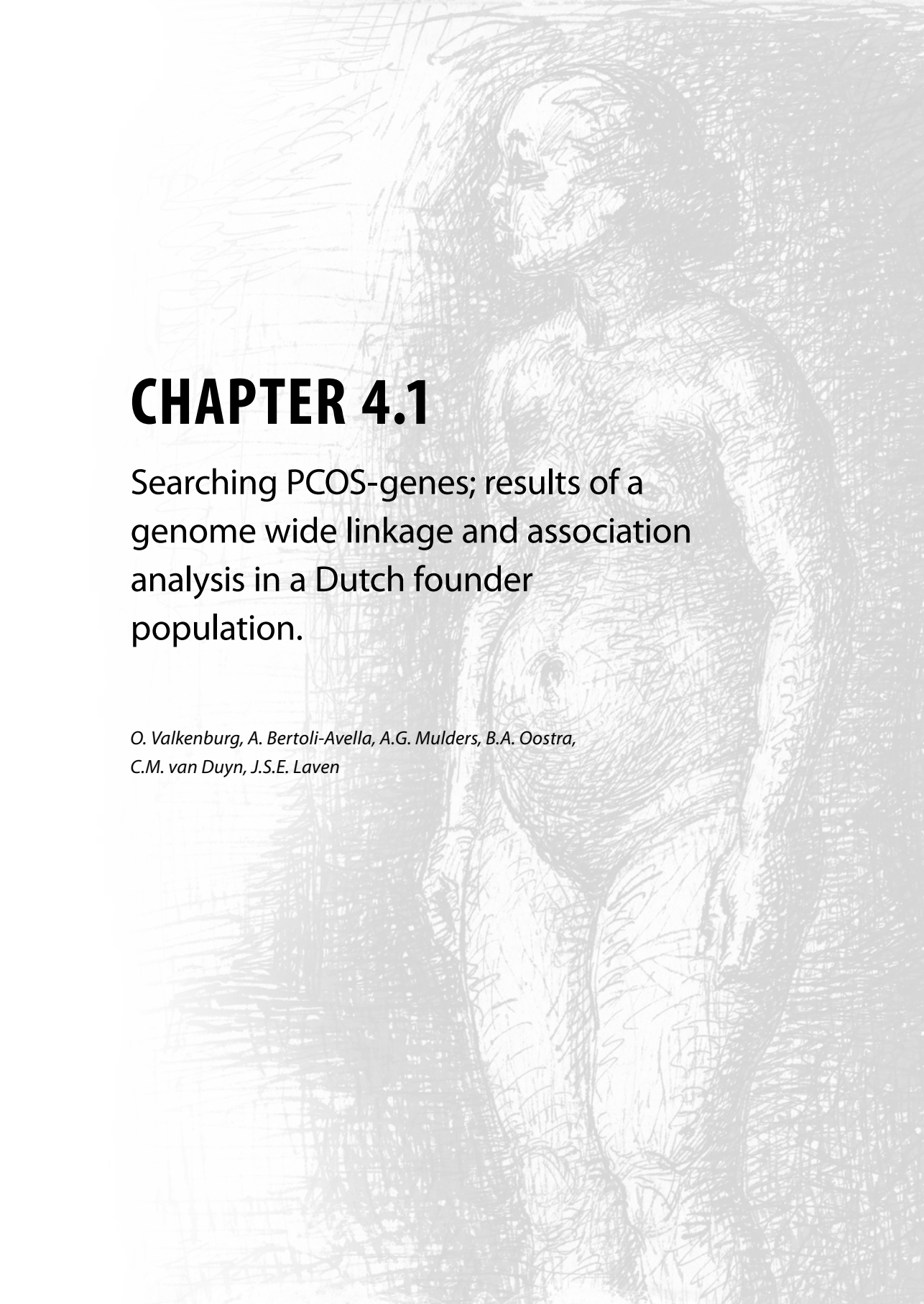


CHAPTER 4

Gene finding in a Dutch founder population



CHAPTER 4.1

Searching PCOS-genes; results of a genome wide linkage and association analysis in a Dutch founder population.

O. Valkenburg, A. Bertoli-Avella, A.G. Mulders, B.A. Oostra, C.M. van Duyn, J.S.E. Laven

INTRODUCTION

Normogonadotropic anovulation is the most common endocrine disorder in women of reproductive age. It is generally understood that this condition is closely, although not exclusively, associated with the polycystic ovary syndrome (PCOS). Depending on the diagnostic criteria that are used 55 % (NIH-criteria) to 91% (2003 Rotterdam criteria) of normogonadotropic (World Health Organisation class 2) anovulatory patients can be diagnosed with PCOS. Subfertility and the association with increased long-term risk for endometrial carcinoma and cardiovascular disease can be counted among the most important sequelae of the condition. Whilst ovarian dysfunction seems to be at the core of the pathophysiology, a broad spectrum of signs and symptoms have been recognised that are closely related to WHO2 anovulation i.e., oligo- or amenorrhoea, hyperandrogenism, polycystic ovary morphology (PCO) [1], obesity, insulin resistance and/or dyslipidaemia. PCOS encompasses a notoriously heterogeneous phenotype. Multiple causal pathways have been proposed that might lead to a similar clinical phenotype. As such, genetic studies that aim to identify genetic variants or loci that are associated with PCOS are hampered by the absence of a homogeneous phenotype. While twin studies [2, 3] and studies of familial segregation [4-6] show evidence for a genetic aetiology, a clear Mendelian inheritance pattern has not been detected. More recent research has indicated that a complex mode of inheritance i.e., an interaction of multiple genetic and environmental factors, might represent a more accurate model of inheritance [7-9].

Genetic association studies rely on the spatial relationship between the location of any given marker on the genome and its proximity to the locus of a genetic variant that is causally related to the disease. The closer a specific marker is located to the disease gene, the more likely they will be inherited together in subsequent generations (i.e. no recombination-event takes place). This is the general principle behind linkage disequilibrium (LD). The pattern of LD in a population results from a complex interplay of genetic factors and the population's demographic history[10]. In large outbred populations, LD will generally only extend over short distances. However in small genetically isolated populations, where there is a small number of founders and limited inward migration throughout subsequent generations, genetic heterogeneity can be much reduced. Due to the influence of the founder effect and genetic drift, LD may be maintained over large regions surrounding disease genes; thereby increasing the power of a genetic association study. Besides the advantage of extended LD, a more homogeneous disease phenotype may be expected to occur in a genetically isolated population. For these reasons it is expected that genes for complex genetic conditions can be more easily identified in isolated populations compared to a general outbred population [11].

We have performed a genome wide association study (GWAS) analysis for normogonadotropic (WHO2) anovulation in a young genetic isolate that was previously identified

in the Netherlands. A high level of LD, extending over large distances (up to 20 cM)[12], and reduced genetic heterogeneity[13] have been described in this population. In order to identify disease loci, WHO2/PCOS cases were ascertained in this population.

MATERIALS AND METHODS

Population and genealogy

This study was performed within the context of the Genetic Research in Isolated populations (GRIP) study program[14]. Population characteristics have been described before [12, 13]. In short: the GRIP population, that resides in a specific area in the southwest of the Netherlands (Rucphen), has been identified as a young genetically isolated population. The community was founded by approximately 150 individuals in the middle of the 18th century. Following years have witnessed a rapid expansion of the original population to, at present, an estimated 20.000 inhabitants that are scattered over eight adjacent communities. Genealogical data of the study population have been collected and computerized through a search of municipal and church records. At present, data are available on more than 90.000 people spanning a total of 23 generations.

Unselected controls were derived from the Erasmus Rucphen Family (ERF) study, which is part of the GRIP study program. The study population essentially consists of one extended family of descendents from 20 related couples who lived in the isolate between 1850 and 1900 and had at least six children baptized in the community church. Detailed information regarding the ERF isolate can be found elsewhere[12, 15, 16]. The medical ethical review board of the Erasmus Medical Center, Rotterdam approved the study and informed consent was obtained from all participants.

Case ascertainment

Patients with normogonadotrophic (WHOII) anovulation were traced in the general Rucphen area through gynaecological records of the adjacent hospitals. The study was approved by the local Medical Ethics Review Boards of the participating hospitals. The individuals were selected by means of their ZIP code and a previous assessment of cycle irregularities, hirsutism and/or anovulation between 1994-2002. Medical charts were handsearched for criteria of WHOII anovulation [17]. Subsequently the clinician involved in the study contacted the patient and written informed consent was obtained. All participants underwent a standardised clinical, sonographic and endocrine evaluation in order to reconfirm the diagnosis of WHO II anovulation. The 2003 Rotterdam consensus criteria were used for the diagnosis of PCOS [18].

Clinical examination and hormone assays

Clinical examination included a structured interview to evaluate cycle history (age at menarche and irregularity of menstrual cycle). Anthropomorphometric measurements included the following parameters: weight, height, waist circumference and waist-to-hip ratio (WHR), extent of hirsutism by means of the Ferriman Gallwey score (FG score)[19]. Ovarian volume and the total number of follicles of both ovaries were assessed by transvaginal ultrasound [20].

Fasting blood samples were obtained by venepuncture and processed within 2 hours after withdrawal. Endocrine evaluation included serum assays for cortisol, prolactin (Prl), thyroid-stimulating hormone (TSH), FSH, LH, estradiol (E_2), testosterone (T), androstenedione (AD), dehydroepiandosterone (DHEA), dehydroepiandosterone-sulphate (DHEAS), SHBG, progesterone (P), 17-hydroxyprogesterone (17-OH-P), inhibin B, anti-Müllerian hormone (AMH), glucose and insulin. The fraction of unbound (biologically active) androgens was estimated by the calculation of the free androgen index (FAI=T [nmol/L]x100 / SHBG [nmol/L]). Serum FSH, LH, Prl, Cortisol, TSH, AD, DHEAS, SHBG, and insulin were assessed by using a chemiluminescent immunoassay (Immulite; Diagnostic Products Corp., Los Angeles, USA). E2 and T were measured by using coated tube radioimmunoassay kits (Diagnostic Products Corp.). Serum DHEA was measured by radioimmunoassay (Diagnostic Laboratories, Webster, USA).

Genotyping

Linkage analysis. Genomic DNA from 90 WHOII anovulatory patients and 169 1st degree family members was extracted from peripheral leucocytes using a standard protocol [21]. Patients and first degree family members underwent a full genome-screen using a set of 382 evenly spaced short tandem repeat polymorphism (STRP) markers from the ABI Prism® Linkage Mapping Set MD-10 (average spacing of approximately 10 cM, Applied Biosystems Inc, Foster City, USA) covering all autosomes. Polymerase chain reactions (PCR) were performed according to the manufacturer's specified conditions. PCR products were separately pooled and loaded on an ABI3100 automated sequencer (Applied Biosystems Inc) and genotypes were analyzed using the Genemapper 2.1 software (Applied Biosystems Inc). Results were read by two independent technicians and discordant results were resolved by a third reader. A total of 366 markers with missing rates less then 5% were included from the initial scan. Information about marker order and distances were obtained from the Marshfield integrated genetic map.

Genome wide association study. WHOII anovulatory cases (N=90) were genotyped using the Illumina 318K platform. Controls were derived from the ERF cohort. Among 1264 controls with available genotypes and phenotype data, 483 subjects were excluded because of male sex. Genotype data were available for this population on four different genotyping platforms which were Illumina 6K, Illumina 318K, Illumina 370K and

Affymetrix 250K (Affymetrix, Inc., Santa Clara, CA, USA), which were merged and ~2.54 × 10^6 SNPs were imputed using MACH 1.0 software (v1.0.16),30, 31 using build 36 HapMap (release 22) CEU population as reference. Within each genotyping batch, only SNPs showing a call rate >98%, MAF>1% and HWE P-value >10−6 were used for imputations.

Data analysis

Genealogy. Genealogic data allowed the calculation of kinship and inbreeding coefficients using the PEDIG software[22]. The amount of kinship and inbreeding was compared with random samples from the isolated population. Random samples were drawn from the genealogic database and matched for age and sex. A total of 1000 sample replicates served to obtain a null distribution for the kinship and inbreeding coefficients. P-values were calculated using a bootstrapping technique.

Linkage analysis. Mega2[23] was used to process the genetic data and perform data validation checks; accuracy of allele segregation within families was checked using PedCheck[24]. Definitive genotyping errors and unlikely genotypes were rechecked using the data from the laboratory. In nuclear families (parents with affected offspring), a transmission disequilibrium test was applied. This statistical test compares the frequency of parent-child transmission of a certain allele with the frequency of non-transmissions. Given the null-hypothesis that no association exists for the allele, the transmission rate is expected to approximate 50%. The test statistic (T) is calculated as follows: $T = (x-y)^2 / (x+y)$, where x represents the number of parent-child transmissions and y represents the number of non-transmissions for a given allele. T is expected to follow a Chi-squared distribution with one degree of freedom. Using Bonferroni correction for multiple testing the threshold for genome wide significance was calculated as 1.35×10^{-5} (1 test per allele, total of 3703 alleles). In order to facilitate the computational challenge of linkage analysis, the complete pedigree was split into multiple subunits. We employed an automated splitting algorithm that allowed a user-defined restriction on bit-size and was validated in the same founder-population (PedCut[25]). The bit-size of a pedigree is calculated as $2n - f$, where n is the number of nonfounders and f is the number of founders [26]. Smaller bit-size allows for more efficient linkage analysis, even when a large number of densely spaced markers are used. The splitting algorithm was set to include the highest number of PCOS patients who share a common ancestor within a maximum of 35 bits. Parametric linkage analysis was performed using the Simwalk2 software that calculates empirical p-values by the use of a Markov-chain Monte Carlo algorithm [27].

GWAS analysis. GWAS analyses were performed for WHOII anovulation and PCOS using the GenABEL R library.[28] Because both cases and controls consisted of related individuals we used genomic control[29] to correct standard errors of the effect estimates for relatedness. The inflation factor lambda was 1.7. GWAS analyses were performed using a mixed model by 'mmscore' option in GenABEL, which combines the Family Based Score Test

for Association (FASTA) method of Abecasis et al.,[30] and kinship matrix estimated from genotyped SNPs[31]. Furthermore, we performed genome wide pedigree based quantitative trait loci (QTL) association analyses using the Genomewide Rapid Asssociation Mixed Model and Regression (GRAMMAR) algorithm[28].

RESULTS

Case ascertainment (Figure 1)

The computer search of women who had undergone a previous endocrine assessment in any of the regional hospitals identified a total of 1602 women who were derived from the general Rucphen area, as defined by zip codes. Medical records were consistent with a diagnosis of normogonadotrophic WHOII anovulation in 252 cases. A total of 118 women agreed to participate in the present study and underwent a clinical reexamination. In 22 cases, the diagnosis of WHOII anovulation was not confirmed (no discernable WHO category, n=12; WHOI anovulation, n=2; WHOIII anovulation, n=4; imminent ovarian failure, n=1 and increased serum prolactin, n=3). In 96 subjects, WHOII anovulation was confirmed at reexamination. A diagnosis of PCOS according to the revised 2003 Rotterdam criteria[18] was valid in 75 of 96 (78%) normogonadotropic anovulatory women. DNA was successfully isolated and available for analysis in 90 women (diagnosis of PCOS, n=72). Six normogonadotropic anovulatory women were dropped from the analysis due to: 1) improbable family genotypes (n=2), 2) no DNA available for analysis (n=2) and two patients withdrew participation after initial consent. Clinical, endocrine and sonographic characteristics of PCOS patients are summarized in Table 1.

Genealogy

We were able to identify 71 of 72 PCOS patients in the genealogical database. From a total of 2485 pair-wise combinations, 1573 patient pairs were related (kinship coefficient>0). Furthermore, 53 probands with PCOS could be connected to one common ancestor within 13 generations (Figure 2) and 33 probands were connected within 10 generations. The probability of finding a similar or higher number of connected subjects among random controls from the same population was high (P=0.95).

The genetic relationship between two individuals can be quantified by the calculation of kinship coefficients. Accordingly, the amount of consanguinity between an individual's parents is represented by the inbreeding coefficient. Kinship and inbreeding coefficients were calculated for all groups: related pairs, inbred subjects and the complete sample. The mean kinship coefficient for PCOS patients was low: 0.0007 (probability of finding lower kinship among random controls P<0.01). In contrast, we observed a relatively high number of inbred patients in the PCOS group (n=41; probability of finding a similar or

Table 1 Descriptive statistics of the study population

	PCOS N=72		PCOS (subgroup founder ≤10 generations) N=33	
	Median	(P5- P95)	Median	(P5- P95)
Age	31.8	(25.1- 43.3)	32.2	(24.0- 44.4)
Menarche	13.0	(10.7- 16.2)	13.0	(11.0- 16.0)
Mean duration of cycle[a]	42	(33- 90)	42	(31- 90)
Gravida	1.0	(0.0- 4.3)	1.0	(0.0- 5.0)
Para	1.0	(0.0- 2.0)	1.0	(0.0- 2.0)
BMI	26.1	(19.9- 45.4)	26.7	(19.7- 50.5)
Waist circumference	91	(73- 128)	92	(69- 131)
Ferriman Gallway score	6.0	(2.0- 18.0)	5.0	(2.0- 13.0)
Mean follicle number	15.5	(4.0- 24.5)	14.5	(3.5- 22.8)
Mean ovarian volume	6.0	(2.2- 15.1)	5.6	(1.7- 14.6)
LH	4.4	(0.8- 16.9)	4.8	(1.6- 23.5)
FSH	4.7	(1.2- 9.8)	4.7	(1.5- 9.2)
Estradiol	203	(69- 654)	193	(78- 1267)
Progesterone	1.2	(0.6- 30.7)	1.2	(0.6- 33.9)
17-OH-Progesterone	1.8	(0.3- 9.9)	2.3	(0.3- 11.7)
Testosterone	1.0	(0.4- 3.0)	1.3	(0.3- 3.6)
SHBG	38	(14- 182)	37	(13- 183)
FAI	2.4	(0.4- 10.7)	2.4	(0.4- 11.0)
Androstenedione	8.5	(3.5- 17.7)	9.1	(3.6- 19.1)
DHEA	41.3	(13.9- 91.3)	40.2	(13.1- 100.0)
DHEAS	3.4	(1.0- 7.1)	3.7	(1.0- 5.9)
Cortisol	370	(204- 777)	401	(199- 655)
Glucose	4.1	(3.4- 6.0)	4.0	(3.3- 5.3)
Insulin	58	(14- 192)	58	(14- 184)
AMH	2.8	(0.3- 8.2)	2.7	(0.4- 9.4)
Inhibin B	71	(0- 279)	81	(2- 422)
Features of PCOS (%)				
Amenorrhoea	13.9 %		12.1 %	
PCO	90.0 %		87.1 %	
Hyperandrogenism	59.0 %		63.6 %	
Obesity (BMI > 27)	44.4 %		45.5 %	
Insulin resistance*	12.5 %		12.1 %	

Values are medians. [A] amenorrhoea excluded

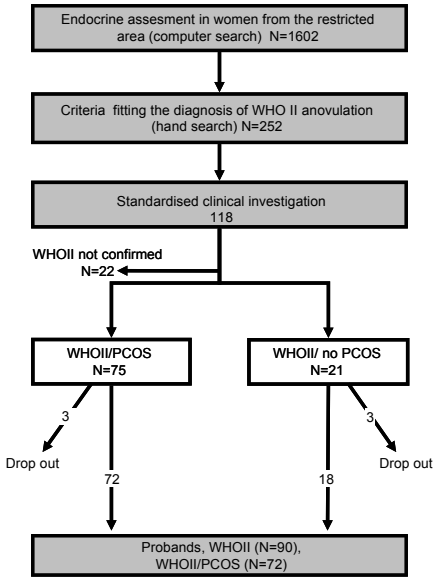


Figure 1 Selection of PCOS patients in Rucphen (the Netherlands) and the general Rucphen area.

higher number of inbred subjects in random controls=0.12); their average inbreeding coefficient was 0.0033. The same finding of an increased number of inbred patients was observed when the analysis was extended to include all WHOII anovulatory patients (89 patients identified in the database, 55 inbred patients, P=0.02). The above indicates

Table 2 Genealogic characterization of 71 PCOS cases

	PCOS	Controls (1000 replications)		P[a]
		P-5	P-95	
Kinship parameters				
Common ancestor (n)	53	52	63	0.95
Related pairs (n)	1573	1511	2067	0.90
Mean kinship coefficient	0.0007	0.0011	0.0022	>0.99
Inbreeding				
Inbred subjects (n)	41	29	43	0.12
Mean inbreeding coefficient	0.0019	0.0011	0.0029	0.49
Close inbreeding (n)	3	0	5	0.44

[a]probability of finding a similar or higher number in random controls (empirical P-value based on 1000 replications)

that, although the amount of inbreeding in the WHOII/PCOS population is quite high, the degree of relationship between PCOS patients was not closer than the rest of the population. Kinship and inbreeding parameters are described in Table 2.

Transmission disequilibrium in nuclear families (Table 3):

In total, we have collected complete genotype data in 50 nuclear families (father / mother / affected offspring). In addition, 14 parent affected-offspring pairs were genotyped where only one parent was genotyped. In 8 cases no parent genotypes were available at all. Therefore, a total of 114 transmissions were included in the transmission disequilibrium test. There was a total of 3703 alleles in 366 markers spread across 22 autosomes (median of 10 alleles per marker [P5–P95: 6–16]). The TDT test-statistic was calculated for each individual allele. In order to avoid type I error a stringent threshold for genome wide significance of $1.35x10^{-5}$ was applied. No significant genome wide association with PCOS was observed for any of 3703 alleles.

Linkage analysis

Using the PedCut splitting algorithm, the extended pedigree was split into 15 subunits containing an average of 3.7 PCOS patients per subunit and an average bit size of 26.3 bits. Parametric linkage analysis was performed by the calculation of empirical p-values using a Markov-chain Monte Carlo algorithm (Simwalk2). None of 15 individual clusters showed clear evidence for linkage with PCOS across their genome (Lod score >3) (Table 4). The strongest results were observed among the members of cluster 2 on chromosome 15 (Lod score 1.98) and in cluster 5 on chromosome 16 (1.80). We observed no evidence for linkage with PCOS when the results of all 11 subunits were pooled.

Table 3 TDT test, association with PCOS (P<0,01)

Marker	Chromosome	Position	Allele	Transmitted/	Untransmitted	Chi^2	P (TDT)
D1S450	1	17.7785	5	16/	4	7.2	0.007
D3S1565	3	197.5809	8	12/	2	7.14	0.008
D4S406	4	127.6907	11	12/	1	9.31	0.002
D6S262	6	136.1863	12	7/	0	7	0.008
D6S308	6	151.6887	2	31/	12	8.4	0.004
D7S530	7	142.6545	3	19/	6	6.76	0.009
D8S264	8	0	3	18/	5	7.35	0.007
D17S784	17	127.2836	3	33/	14	7.68	0.006

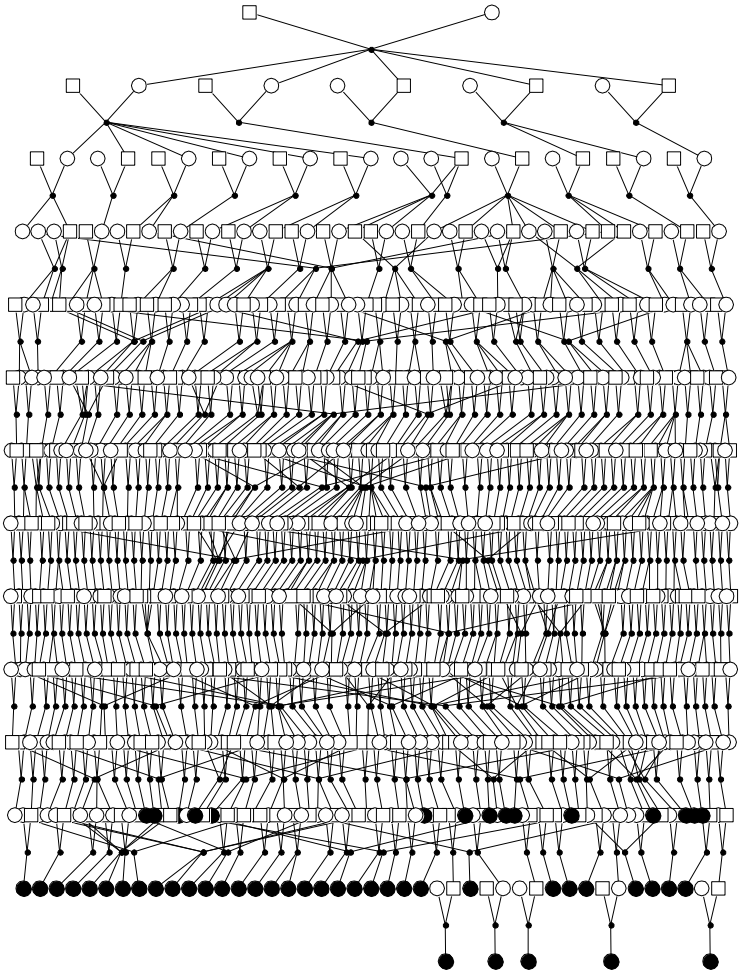


Figure 2 Fifty-three PCOS patients (75%) with PCOS (filled symbols) could be linked to a common ancestor within 13 generations.

Genome-wide association

Using genomic control to correct for relatedness of study subjects, the analysis of the GWAS data identified one SNP that surpassed the threshold for genome wide significance (rs6976312, $P = 3.3 \times 10^{-8}$) and a total of 14 SNPs that showed suggestive association with WHOII anovulation (table 5). The association analysis for quantitative traits showed 39 SNP's that surpassed the threshold for genome wide significance ($P<1.0 \times 10^{-7}$). All of these were associated with fasting insulin levels (adjusted for age and waist circumference) and are located on chromosomes 1, 4, 6, 8, 10, 13 and 17 showed loci with 2 or more SNPs within close proximity of each other.

Table 4 Largest LOD score per chromosome

Chromosome	Position Haldane cM	Cluster	LOD score
1	266.753	10	1.22
2	101.275	5	1.22
3	60.847	2	1.12
4	127.691	12	1.22
5	68.881	9	1.17
6	0.000	10	1.22
7	94.459	8	1.45
8	40.787	6	0.55
9	126.827	1	0.61
10	63.781	12	1.19
11	135.486	5	1.19
12	182.218	12	1.19
13	67.912	8	1.19
14	24.068	10	1.21
15	102.726	2	1.98
16	132.749	5	1.80
17	64.150	9	0.61
18	69.475	1	1.12
19	66.212	3	0.60
20	106.301	6	0.61
21	54.441	6	0.22
22	56.402	3	0.79

Table 5 GWAS, association with WHO2 anovulation in 83 cases and 781 controls

SNP	Chromosome	Position	N	P- value	Corrected* P
rs6976312	7	67977677	1347	<1.00e-10	3.26000e-08
rs1757699	20	61610237	1347	<1.00e-10	1.20100e-07
rs476161	6	161862968	1101	<1.00e-10	1.31500e-07
rs4888517	16	75083929	1347	2.00e-10	1.12480e-06
rs27795	16	47987730	1347	5.00e-10	2.09410e-06
rs1983148	12	62454380	1347	6.00e-10	2.28550e-06
rs7702057	5	115755737	1344	9.00e-10	2.79360e-06
rs228883	17	61874457	1347	1.20e-09	3.28210e-06
rs4234980	4	165972151	1344	1.20e-09	3.40800e-06
rs9990002	3	68248462	1342	2.70e-09	5.44220e-06
rs7914811	10	71921388	1347	3.70e-09	6.53360e-06
rs11626113	14	34700313	226	4.40e-09	7.29100e-06
rs496759	6	161869480	1347	4.90e-09	7.79170e-06
rs10520092	5	130230096	226	7.30e-09	9.86860e-06
rs2560790	6	13614851	1347	7.40e-09	9.93490e-06

* corrected for genomic inflation, ($\lambda = 1.7$)

DISCUSSION

The present study describes the results of a whole genome association and linkage analysis for PCOS that was conducted in a genetically isolated population that resides in the Southwest of the Netherlands. While its geographic location is not especially noteworthy, evidence from prior genetic analyses that were conducted in the same population shows reduced genetic heterogeneity and a high level of linkage disequilibrium. These findings can be attributed to the historical background of this region that is marked by a limited number of founders, rapid expansion of the population and, until recently, limited immigration. In contrast to our expectations the present study did not uncover genetic loci that showed genome-wide significant association with PCOS.

Results of the genealogic investigation showed that PCOS patients were not more closely related to each other compared to random subjects from the same population. In contrast even, the average kinship coefficient among PCOS women was lower than would have been expected from random subjects. This may be caused by the ascertainment procedure. Patients were identified from local hospital charts because they expressed PCOS phenotype and resided within the general Rucphen area, whereas controls were more strictly included on the basis of their descendance from the Rucphen population. Nevertheless, evidence for a genetic predisposition for PCOS was found in a tendency towards a higher frequency of inbreeding among PCOS patients. This tendency became statistically significant when all WHO2 anovulatory patients were included in the analysis, suggesting that the genetic contribution to the phenotype of normogonadotropic anovulatory patients is similar, whether they are diagnosed with PCOS or not. So far, genetic association-studies for PCOS have relied heavily on hyperandrogenism as a required condition in women with PCOS. Whilst effective with regard to hyperandrogenism, this approach excludes a large group of normogonadotropic anovulatory women that are generally recognized as subfertile and treated similarly with ovulation inducing agents and/or *in vitro* fertilisation. The revised 2003 Rotterdam criteria incorporate these women in the definition of PCOS on the condition that polycystic ovaries are present. This means that in the present study the majority of WHOII anovulatory patients (78 %) are diagnosed with PCOS. While this strategy is effective with regard to the selection of women who are treated similarly, it also adds heterogeneity to the phenotype of women that are selected for analysis. As is the case in all complex genetic diseases, it is expected that multiple genetic pathways are involved in various degrees in the pathofysiology of PCOS. As such, it is possible that a wider selection of phenotypes will result in a more heterogeneous genetic background of the study population, resulting in a probable loss-of-power to detect genetic association and linkage.

While the results of the association and linkage analysis do not provide loci that show significant results on a genome wide scale, it is unlikely that these loci do not actually

exist. Evidence for the genetic basis of PCOS stems from studies of familial segregation and a high estimate of heritability (66%) was shown in a Dutch study of monozygotic and dizygotic twins[3]. The fact that these loci remain undetected in the present study can be explained in a number of ways. 1. The number of genes that are aetiologically related to PCOS may be too great, and therefore their individual effect too small to detect in a genome scan, despite the fact that the study was conducted in a genetic isolate. 2. The definition of PCOS that was used in the present study may not succeed in the selection of a homogeneous patient group. Despite their merit as a clinical instrument for the detection and treatment of normogonadotropic anovulatory subjects, the 2003 Rotterdam criteria may not be well suited for the specific purpose of genetic research. 3. The use of a marker set that consists of STRPs with an average spacing of 10 cM may not be adequately sensitive when confronted with a complex genetic disease where the effect of each individual gene can be expected to be limited. 4. The absence of a clear Mendelian inheritance pattern allows only the use of a non-parametric linkage estimate, resulting in a loss of power. 5. the number PCOS patients that we were able to ascertain in the isolated population may have been, certainly in light of the other limitations, inadequate to provide the analysis with sufficient power to detect genome wide association and linkage.

In summary, the present study describes a genome screen in a cohort of PCOS patients who are derived from a genetically isolated population. The lack of consistent results suggests the absence of a consistent genetic background in this select group of PCOS patients. This supports the hypothesis of a complex genetic background for PCOS that allows relatively small contributions of multiple risk-genes to be involved in the pathogenesis of this syndrome. In this founder-population the genetic heterogeneity was not sufficiently reduced to find risk-loci in a genome wide screen using highly polymorphic markers with an average spacing of 10 cM.

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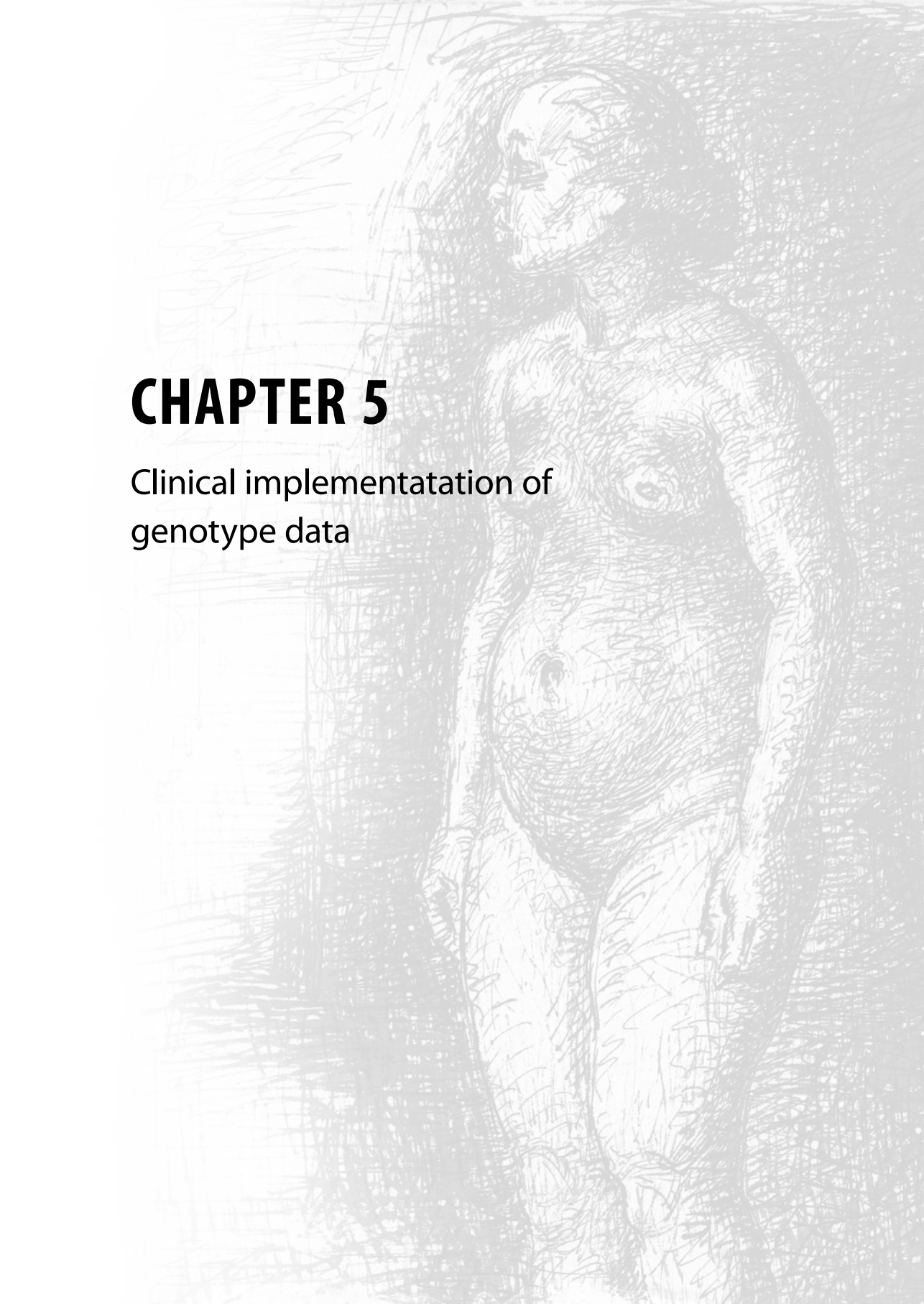
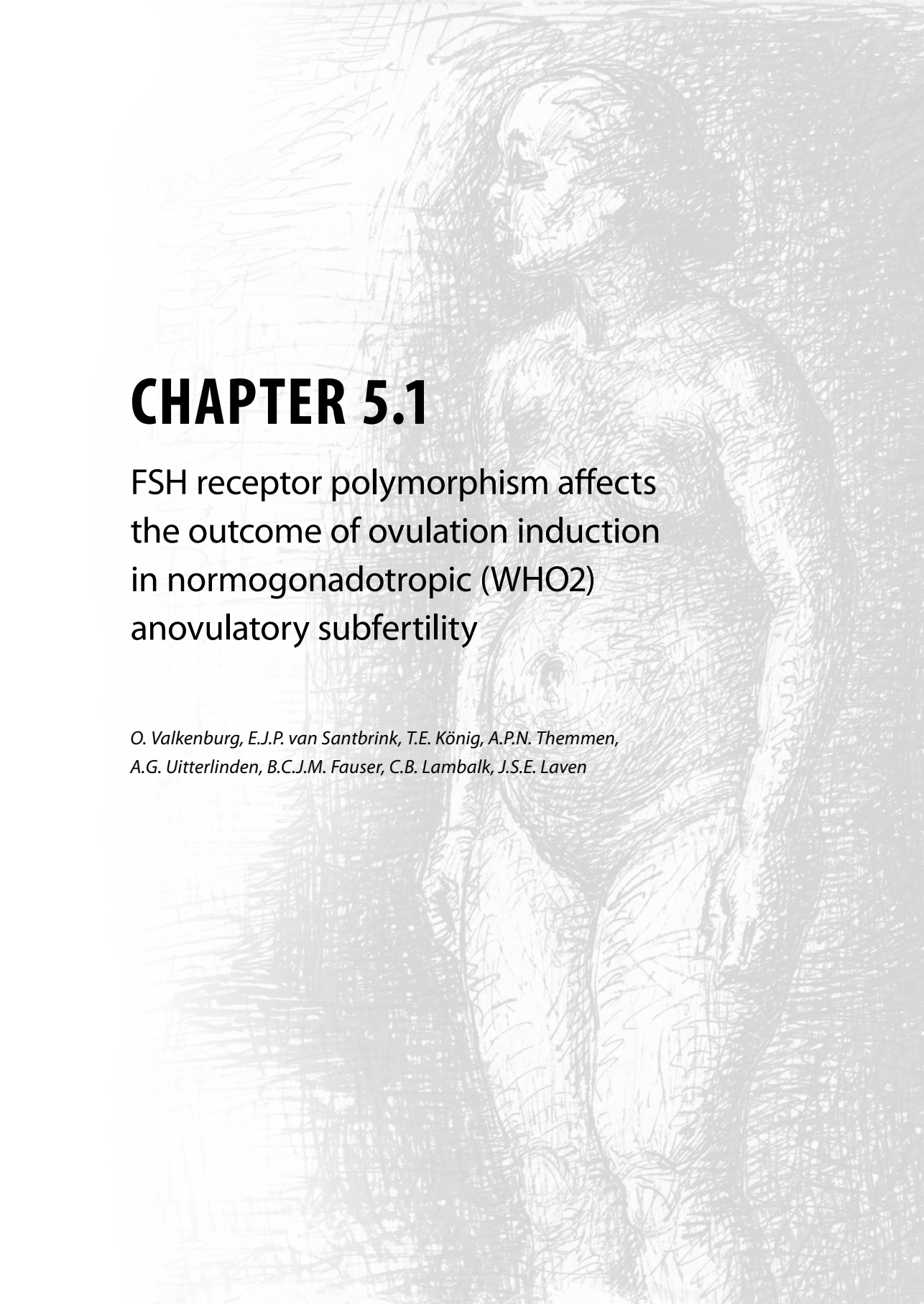


CHAPTER 5

Clinical implementatation of genotype data



CHAPTER 5.1

FSH receptor polymorphism affects the outcome of ovulation induction in normogonadotropic (WHO2) anovulatory subfertility

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INTRODUCTION

Chronic anovulation is a major cause of infertility. Ovulation induction with Clomiphene Citrate (CC) and exogenous FSH preparations are commonly used as first and second line therapy for anovulatory subfertility. Patients presenting with anovulation can be classified according to the World Health Organization (WHO) on the basis of serum levels of gonadotrophic hormones (mainly FSH) and estradiol (E_2).[1] The great majority of anovulatory patients exhibit normal serum concentrations of FSH and E_2 and are classified as WHO2. Patients suffering from polycystic ovary syndrome (PCOS) are considered a subgroup of WHO2 [2]. Applying the 2003 Rotterdam criteria [3], approximately 90% of WHO-II anovulatory patients can be diagnosed with PCOS [4].

Infertility treatment of WHO2 anovulatory subfertility aims to restore normal ovarian physiology i.e., mono-follicular growth and mono-ovulation [ESHRE Capri workshop group][3]. First-line treatment with the anti-estrogen CC has the advantage of low costs and minimal side effects or complications. However, in case of clomiphene resistant anovulation (CRA) or failure to conceive after nine to twelve treatment cycles (CCF), CC may be replaced by exFSH as second-line treatment. High overall effectiveness of the conventional treatment algorithm was observed in a prospective follow-up study of 240 WHO2 patients that reports a cumulative singleton live birth rate of 71% after 24 months of follow-up [5].

Treatment with CC or exFSH is hampered by the limited control of ovarian response due to large inter- and intra-individual variability. The outcome of ovulation induction will depend, in part, on the pharmacological compounds used but also on individual patient characteristics such as age, body mass index, hyperandrogenism and duration of infertility [6, 7]. Recent literature has focused attention on a single nucleotide polymorphism in exon 10 of the FSH receptor gene (FSHR, rs6166). This common polymorphism has been established as a major determinant of ovarian physiology throughout the normal menstrual cycle. The minor allele, that encodes an alternative amino-acid (serine instead of asparagine) at codon 680 (680^{Ser}), has been associated with higher follicular phase FSH levels, a small increase in number of antral follicles and longer menstrual cycle [8, 9]. The relationship of FSHR genotype and ovarian response during ovarian hyperstimulation for *in-vitro* fertilisation (IVF) has been described in multiple reports indicating a greater need for exFSH in association with the 680^{Ser} allele[10-12], although some controversy still remains [13-16].

Among women with WHO2 anovulatory subfertility, 680^{Ser} is associated with higher baseline FSH levels [17]. Moreover, the same polymorphic variant was associated with higher LH and Testosterone levels among women with PCOS [18]. Recent GWAS data have identified the FSHR and LH/choriogonadotropin (LHCGR) locus at 2p16.3 as a PCOS susceptibility locus [19, 20]. 680^{Ser} may constitute a receptor variant that is less responsive to

stimulation with FSH and a higher setpoint for gonadotropic hormones might ensue. For ovulation induction, this would implicate that the success of treatment with CC or exFSH can depend on FSHR genotype. Indeed, 680^{Ser} was associated with a higher frequency of CC resistance in a retrospective analysis of ovulation induction among women with PCOS [21]. The present study aims to investigate the influence of FSHR genotype on the outcome of ovulation induction in a prospective cohort of normogonadotropic anovulatory patients using CC and exFSH.

MATERIALS AND METHODS

Treatment protocol

The treatment protocol and clinical characteristics of the study population have been previously described [5]. In short, 240 anovulatory patients entered the study between November 1992 and May 1999. Inclusion criteria were: a) oligomenorrhoea (bleeding interval ≥35 days) or amenorrhoea (bleeding interval >6 months); b) serum FSH levels within normal limits (1-10 IU/L) and normal serum prolactin and thyroid-stimulating hormone (TSH) levels; c) body mass index (BMI) >18 kg/m^2; d) age between 19 and 40 years; e) no previous treatment with ovulation induction; f) total motile sperm count (TMC) of the partner >1.0x10^6 (TMC=ejaculate volume (ml) x sperm concentration (10^6/ml) x percentage of progressive motile sperm). The study was approved by the institutional review board at the Erasmus Medical Center and written informed consent was obtained from all participants.

Anovulatory patients underwent a standardized initial examination that included a clinical examination and transvaginal ultrasonography to assess ovarian volume and follicle count for both ovaries. Blood samples were obtained by venipuncture and processed within 2 hours. Serum was isolated after centrifugation at 3,000 rpm for 10 minutes at 20ºC and stored at –20 °C until assayed. Serum LH and FSH levels were measured by immunoradiometric assay (Medgenix, Fleurus, Belgium). Serum levels of testosterone (T), androstenedione (AD), sex-hormone binding globulin (SHBG), and dehydroepiandrosterone sulfate (DHEAS) were determined using RIA kits (Diagnostic Products Corp., Los Angeles, CA), as described previously [22, 23].

Participants received an initial CC dose of 50 mg/day (cycle day 3-7). If necessary, the daily CC dosage was increased to 100 mg and 150 mg in subsequent cycles. Ovulation was assessed by sonographic monitoring and/or midluteal progesterone level. Clomiphene resistant anovulation (CRA) was defined as absence of ovulation after 3 months, despite treatment with the maximum daily dose of 150 mg of CC. Clomiphene failure (CCF) was defined as the absence of pregnancy within 6 ovulatory cycles. Treatment with

exogenous recombinant FSH (exFSH) (Puregon®; Organon NV, Oss, The Netherlands) was initiated in case of CRA or CCF. While FSH response dose was determined by the use of a step-up dose regimen during the first treatment cycle, a step-down dose regimen was applied in subsequent cycles as described previously[24].

Replication cohort

Data with regard to ovulation induction with CC in the replication cohort have been previously described [21] The cohort consists of 185 normogonadotropic anovulatory PCOS patients (2003 Rotterdam criteria [3]), who attended the Reproductive Medicine Unit of the Obstetrics and Gynaecology Department of the VU University Medical Center (Amsterdam, the Netherlands) between January 2003 and December 2007. According to local protocol, all patients underwent a standardized evaluation that included clinical data on age, ethnicity, cycle history, BMI and a physical examination for signs of hyperandrogenism, laboratory screening and transvaginal ultrasonography of the ovaries. Hormone assays have been described previously [21]. Ovulation induction consisted of CC in a dose of 50 mg/day for five days from cycle day five to nine, raising the dose in increments of 50 mg/day each cycle until an ovulatory cycle was achieved. CRA was diagnosed after subsequent non-ovulatory cycles with up to 150 mg of CC. Ovulation induction with exFSH was performed using a low-dose step-up dose regimen.

Genotyping

Genomic DNA was extracted from peripheral venous blood according to standard procedures. FSHR genotype (rs6166, Asn680Ser) was determined using the Taqman allelic discrimination assay. The PCR reaction mixture included 2 ng of genomic DNA in a 2 µL volume and the following reagents: FAM and VIC probes (200 nM), primers (0·9 µM), 2xTaqman PCR master mix (ABgene, Epsom, UK). Reagents were dispensed in a 384-well plate using the Deerac Equator NS808 (Deerac Fluidics, Dublin, Ireland). PCR cycling reactions were performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 15 minutes at 95 °C, and 40 cycles with denaturation of 15 seconds at 95 °C and annealing and extension for 60 seconds at 60 °C.

Statistics

Hardy Weinberg equilibrium (HWE) was calculated using a chi-square test. The distributions of clinical characteristics in the study groups are presented as the median and interquartile range (P25-P75). First, we compared clinical characteristics and treatment outcome in patients with and without available DNA. A nonparametric test (Mann-Whitney U) was used for the comparison of continuous variables, whereas categorical

variables were analysed by Pearson's chi-square (X^2) test. Statistical significance was defined as a two-tailed P value of less than or equal to 0·05.

Next, we assessed the influence of polymorphic variants on treatment outcome by univariate Cox regression analysis. Outcomes of ovulation induction with CC and exFSH were analysed separately. The period from the start of CC or exFSH treatment until the first ongoing pregnancy was used as the time variable. Follow-up was censored in case of discontinuation of therapy without an ongoing pregnancy or end of follow-up. The primary outcome parameter was defined as ongoing pregnancy i.e., the presence of a positive fetal heartbeat on ultrasound examination at 12 weeks amenorrhoea. Data analyses were performed with SPSS (version 15).

RESULTS

Baseline characteristics

Stimulation characteristics of the total group of 240 normogonadotropic anovulatory subjects have been described in a prior report [5]. For the present study, genotype data were available in 159 of the original 240 women. Baseline characteristics and outcome of ovulation induction were similar in patients who were genotyped and patients who were not (Table 1). Figure 1 describes the course of treatment of 159 women who initiated ovulation induction. CC was successful in restoring ovulations in 116 patients (73%) of whom 67 became pregnant (63 ongoing pregnancies). There were two ongoing twin pregnancies. The remaining 46 women did not conceive (CCF). In 43 cases (27%), ovulatory cycles could not be restored with CC (CRA). A total of 29 patients did not start treatment with exFSH while treatment with CC had not resulted in pregnancy. Treatment with exFSH was initiated in a total of 68 patients who had shown either CRA (n=37) or CCF (n=31). The use of exFSH added 42 pregnancies of which 3 ended in a miscarriage. There were five multiple pregnancies (three twins, one triplet and one quadruplet). Patients who remained anovulatory with CC showed a significantly higher FSH response-dose than CCF-patients (median 112 *vs.* 75 IU/liter, P≤0.01).

Influence of FSHR genotype on outcome of ovulation induction

Table 2 shows the effect of the FSHR polymorphism on the outcome of ovulation induction using CC and exFSH. FSHR genotypes were in Hardy-Weinberg equilibrium in the study population. Following the administration of CC, it was observed that the presence of the FSHR 680^{Ser} allele was associated with a lower chance of ongoing pregnancy (hazard ratio [HR] per allele copy, 0.76 [0.55-1.05], P=0.10). A comparison of homozygous carriers of the 680^{Ser} allele to both other genotypes, showed significantly decreased chance of ongoing pregnancy in these subjects (HR, 0.51 [0.27-0.98]). Paradoxically, we

Table 1 Baseline characteristics and outcomes in genotyped and ungenotyped patients

	Not genotyped (N=81)		Genotyped (N=159)		
	Median	(IQR[B])	Median	(IQR[B])	P
Baseline characteristics					
Age (years)	28·0	(24·0 -30·4)	27·6	(24·7 -30·9)	0·69
BMI (kg/m^2)	25·3	(22·2 -31·0)	25·8	(22·0 -30·1)	0·81
Mean cycle duration[a] (days)	45	(40 -64)	45	(40 -59)	0·37
Duration of infertility (years)	1·3	(1·0 -2·0)	1·6	(1·0 -2·6)	0·21
Amenorrhoea (%)		22·2 %		22·0 %	0·97
Polycystic ovaries (PCO) (%)		70·0 %		76·7 %	0·26
Hyperandrogenism (%)		35·8 %		39·6 %	0·57
CRA (%)		17·3 %		27·0 %	0·09
Endocrine profile					
FSH (U/liter)	4·7	(3·8 -5·4)	4·5	(3·5 -5·4)	0·19
LH (U/liter)	6·4	(4·4 -10·6)	6·5	(4·5 -10·3)	0·94
17β-estradiol (pmol/liter)	205	(147 -261)	227	(177 -294)	0·06
Testosterone (nmol/liter)	2·2	(1·6 -2·8)	2·4	(1·8 -3·1)	0·07
Androstenedione (nmol/liter)	13·7	(8·9 -16·9)	14·1	1(1·0 -18·9)	0·21
DHEAS (µmol/liter)	6·3	(4·3 -8·3)	6·9	(4·6 -10·1)	0·17
Sex hormone-binding globulin (nmol/liter)	43	(34 -64)	47	(30 -70)	0·58
Insulin (pmol/liter)	11·9	(7·0 -18·7)	10·5	(7·5 -17·7)	0·98
Overall outcome of ovulation induction					
Cum· rate of ong· pregnancies, 1 yr (%)		75 %		76 %	0·81
Cum· rate of singl· live birth, 1 yr (%)		72 %		70 %	0·67

[A]amenorrhoeic patients excluded [B]interquartile range

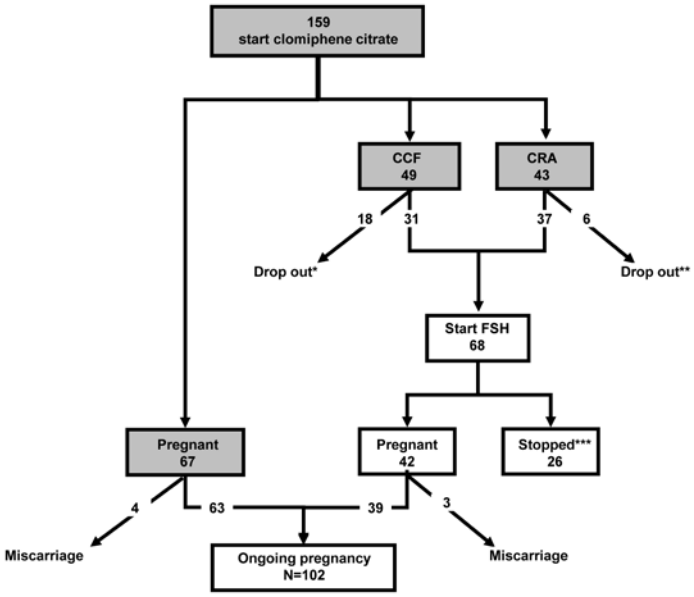

Figure 1 Ovulation induction in 159 normogonadotrophic anovulatory women.
* Drop-out after CC in 18 patients with CCF because of medical problems (N=5), no more child wish (N=6) or start of another therapy (N=7). ** Drop-out after CC in 6 patients with CRA because of medical problems (N=1), no more child wish (N=3), initiation of another therapy (N=1) and because treatment was found too burdensome in one case. *** Discontinuation of FSH in 26 cases because of medical problems (N=2), no more child-wish (N=3), initiation of IVF (N=18). Three couples had not yet planned another treatment at the end of follow-up.

observed that the 680^{Ser} was associated with increased chance of achieving an ongoing pregnancy following the initiation of ovulation induction with exFSH. The presence of 680^{Ser} was associated with 1.8 times higher chance of ongoing pregnancy (HR per allele copy, 1.77 [95% CI 1.11-2.82]) following treatment with exFSH. Regarding the outcome of the combined treatment protocol as a whole (CC, if necessary followed by exFSH), similar chances of achieving an ongoing pregnancy in all three genotype groups were observed. Figure 2 shows the outcome of ovulation induction of the combined treatment protocol and the outcome of treatment with CC and exFSH separately. Secondary ovarian response characteristics (CC-dose that was needed to achieve ovulation and the number of ovulatory cycles that were needed to achieve an ongoing pregnancy) were not influenced by the FSHR genotype. Secondary response characteristics for ovulation induction with exFSH i.e., FSH response dose, frequency of cycle cancellation and number of ovulatory cycles needed to achieve (ongoing) pregnancy, were not affected by the FSHR genotype.

Table 2 Outcome of ovulation induction (ongoing pregnancy) in 159 women, treated for WHO2 anovulatory subfertility, by FSHR genotype

Genotype	Clomiphene Citrate		Exogenous FSH		Combined protocol	
	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)
Asn / Asn	1	reference	1	reference	1	reference
Asn / Ser	1·04	(0·60 -1·82)	2·65	(0·89 -7·90)	1·54	(0·95 -2·48)
Ser / Ser	0·52	(0·25 -1·08)	3·76	(1·24 -11·38)	1·22	(0·71 -2·10)

* Hazard ratio's (univariate Cox regression analysis) calculated for ongoing pregnancy using CC and FSH

Replication cohort (Table 3)

The replication cohort consisted of 185 anovulatory PCOS patients. Genotype frequencies, BMI (median 24.0 kg/m^2) and the frequency of amenorrhoea (23.2 %) and hyperandrogenism (43.4%) were similar in both cohorts (P>0.05). Median age was slightly higher compared to the study cohort (29.6 *vs.* 27.6 years, P<0.01). Similar ongoing pregnancy rates were noted following the initiation of 1st line therapy with CC in the study cohort (63%) and the replication cohort (69%, P=0.69). However, the replication cohort showed

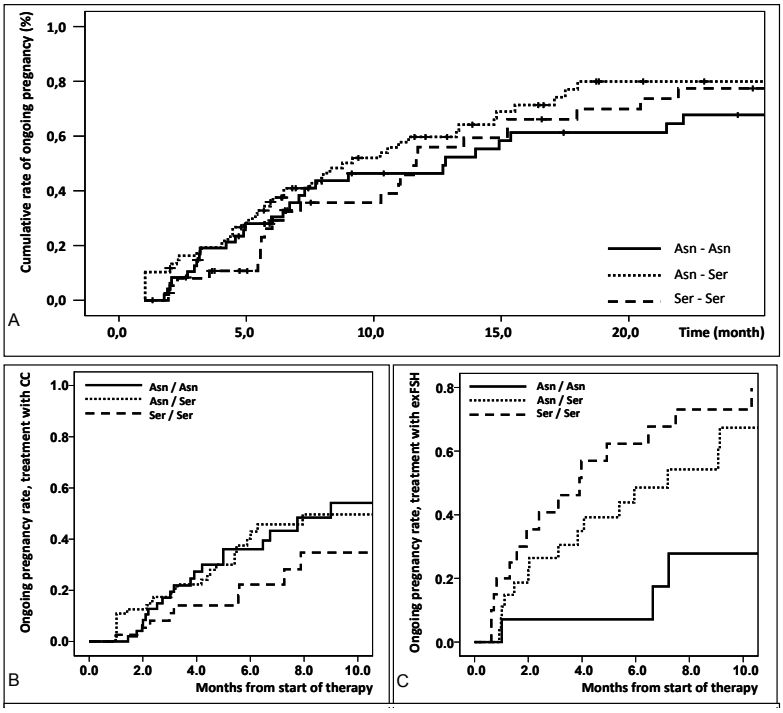

Figure 2 Outcome of ovulation induction. A: combined treatment protocol (1st line treatment CC, 2nd line treatment exFSH. B: ovulation induction with CC C: ovulation induction with exFSH.

lower ongoing pregnancy rate following 2nd line therapy with exFSH compared to the study cohort (24% *vs.* 39%, P<0.01).

Table 4 shows a comparison of outcome variables in both the study cohort and the replication cohort. A higher frequency of CRA was noted among homozygous carriers of the 680^{Ser} allele compared to other genotypes (28.3% *vs.* 15.0%, P=0.05). The pooled analysis of both cohorts showed 1.9 times higher probability of CRA among homozygous carriers of 680^{Ser} (OR 1.9 [95%CI 1.1-3.3], P=0.03) compared to other genotypes. However, ongoing pregnancy rates (following treatment with CC or exFSH) were not affected by FSHR genotype in the replication cohort.

DISCUSSION

The present study investigates whether a genetic polymorphism of the FSH receptor (rs6166) can affect the outcome of ovulation induction in women with normogonadotropic (WHO2) anovulatory subfertility. It was observed that the FSHR 680^{Ser} allele is significantly associated with failure to regain ovulatory menstrual cycles, following the use of ovulation induction with CC. In addition, the current prospective cohort study indicates that carriers of the 680^{Ser} allele may be more likely to achieve an ongoing preg-

Table 3 FSH receptor genotype and ovarian response characteristics in the study cohort and the replication cohort

	Study cohort N=159		Replication cohort (N=185)		P (X^2)
	N	(%)	N	(%)	
Genotype					0·29
Asn - Asn	49	(31·6)	43	(24·0)	
Asn - Ser	68	(43·9)	90	(50·3)	
Ser - Ser	38	(24·5)	46	(25·7)	
Ovarian dysfunction					
Amenorrhoea	35	(22·0)	42	(23·2)	0·79
Hyperandrogenism	63	(39·6)	76	(43·4)	0·48
Polycystic ovaries (PCO)	122	(76·7)	174	(95·1)	<0·01
Ovulation induction					
CRA	43	(27·0)	34	(18·4)	0·06
CCF	49	(30·8)	37	(23·7)	0·16
Ongoing pregnancy (CC)	63	(39·6)	69	(37·5)	0·69
Commence OI with exFSH	68	(42·8)	76	(41·1)	0·75
Ongoing pregnancy (exFSH)	39	(24·5)	24	(13·3)	<0·01
Ongoing pregnancy (total)	102	(64·2)	90	(50·0)	<0·01

Table 4 Ovarian response characteristics in both the study cohort and the replication cohort

	Study cohort		Replication cohort		Pooled analysis	
	OR[A]	(95%CI)	OR[A]	(95%CI)	OR[A]	(95%CI)
Clomiphene citrate	(n=159)		(n=185)		(n=344)	
CRA	1·6	(0·7-3·7)	2·2	(1·0-4·9)*	1·9	(1·1-3·3)*
CCF	1·3	(0·6-2·8)	0·5	(0·17-1·19)	0·8	(0·4-1·5)
Ongoing pregnancy	0·5	(0·2-1·1)	1·3	(0·6-2·5)	0·8	(0·5-1·4)
CC-dose > 50 mg/day	0·7	(0·3-1·6)	1·1	(0·5-2·5)	0·9	(0·5-1·6)
Exogenous FSH	(n=68)		(n=76)		(n=144)	
Ongoing pregnancy	3·1	(1·0-10·1)*	1·0	(0·3-3·2)	1·8	(0·9-3·9)
FSH response dose > 75 IU/day	0·9	(0·2-3·8)	1·9	(0·6-6·2)	1·5	(0·6-3·7)

[A]Odds ratios were calculated for homozygous carriers of the 680^{Ser} allele· * P ≤ 0·05

nancy, when subsequently treated with exFSH. However, the latter observation could not be confirmed in the replication cohort.

This study is the first to describe the rs6166 FSHR polymorphism in a prospective cohort of normogonadotropic anovulatory patients who are treated with ovulation induction therapy. The same polymorphic variant has been studied extensively in ovarian hyperstimulation for IVF where it was shown that the 680^{Ser} allele is associated with reduced ovarian responsiveness and a higher need for exFSH [10-12]. It was hypothesized that minor variations in FSHR function might be more relevant for ovulation induction therapy compared to ovarian hyperstimulation for IVF, because this treatment modality depends on a more subtle increase in FSH levels. In agreement with the data from IVF studies we find that there is a noticeable difference in ovarian responsiveness in association with the 680^{Ser} allele. The pooled analysis shows that homozygous carriers of the 680^{Ser} allele have a 90% higher probability of CRA during ovulation induction with CC. This finding was further substantiated by the fact that the survival analysis showed that these patients were two times less likely to achieve an ongoing pregnancy following treatment with CC in the prospective cohort.

Results with regard to 2nd line therapy with exFSH were more heterogeneous. While it is tempting to speculate on the presence of a more resilient ovarian physiology in homozygous carriers of FSHR 680^{Ser}, one should keep in mind that this represents a common genotype that is present in approximately one quarter of healthy women in the general population. Moreover, the present study shows that these women are as likely (retrospective cohort) or even more likely (prospective cohort) to achieve ongoing pregnancy compared to the other FSHR genotypes. Nor did we observe a higher need for exFSH in association with the 680^{Ser} genotype. We speculate that homozygous

carriers of FSHR 680^{Ser} who remain anovulatory during treatment with CC, represent a subgroup of patients that is selected for anovulation as the leading cause of subfertility.

The success of ovulation induction will also depend on oocyte quality and the receptive capacity of the endometrium. One can hypothesize that differences in oocyte quality may exist in the various FSHR genotype groups. As is evident from animal studies, FSH has an important role in the maturation of oocytes in antral and pre-ovulatory follicles [25, 26]. While the oocyte itself does not express FSH receptors, the influence of FSH can be mediated through the FSH receptors that are present on the granulosa cells that make up the cumulus-oocyte-complex. However as long as adequate serum markers of oocyte quality are lacking, one will not be able to take this factor into account. It was shown that the endometrium expresses FSHR in a cycle dependent manner [27]. Endometrial receptivity may be affected by the polymorphic variant. However these hypotheses remain highly speculative and are not directly supported by our data.

In short, the present study was conducted in order to investigate the influence of FSHR genotype on the outcome of ovulation induction in normogonadotropic anovulatory patients using CC and exFSH. Our data show that the minor allele of rs6166, that is located at exon 10 of FSHR, is associated with failure to regain ovulatory cycles and ongoing pregnancy during treatment with CC. Also, we observed evidence for the hypothesis that these patients are more effectively treated with exogenous gonadotrophins. Therefore, these data may enable us to design a treatment algorithm that is based on individual patient characteristics including FSHR genotype, and that is more efficacious and tailored to the individual patient.

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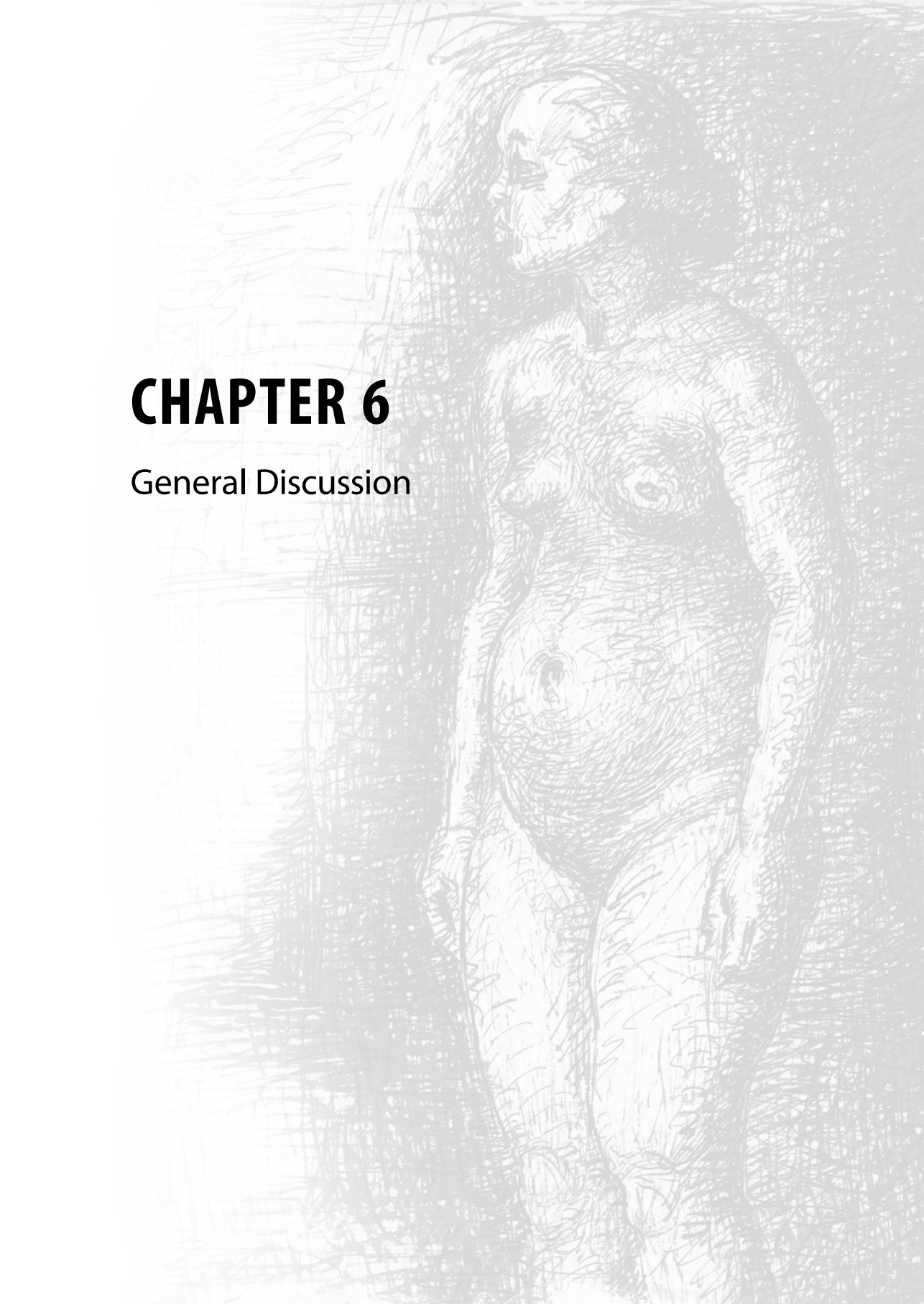

CHAPTER 6

General Discussion

The present thesis aims to study the phenotypic and genotypic characteristics of women with PCOS. In a broader perspective, we study women's health with regard to the ovarian dysfunction that is present in women with PCOS. The main characteristics of PCOS encompass a variety of signs and symptoms that can have a profound influence on women's health, either in the short term or in the long term. For the individual patient, menstrual cycle irregularity and/or (sub)fertility are often the most important reasons for seeking medical attention. However it has become clear that endocrine and metabolic changes that are also present in these women may have severe long-term sequelae. For example, it was observed that women with PCOS are exposed to nearly three times higher life-time risk for developing endometrial carcinoma[1], possibly due to altered exposure to estrogens that is unopposed by progesterone.

Cardiovasular health in women with PCOS

We have studied insulin resistance and dyslipidemia in women with PCOS. Both are well known determinants of cardiovascular health later in life. Insulin resistance occurs frequently in women with PCOS. However its presence cannot be interpreted independent of body mass and (central) adiposity that is also, but not exclusively, associated with PCOS. Analyses of our PCOS cases and unselected controls showed that the presence of PCOS does independently contribute to insulin resistance in these women (**chapter 2.1**). However, in a broader perspective, multivariable analysis suggests that BMI is a much more important determinant of insulin resistance than the presence of PCOS alone, stressing the need for life-style intervention in obese women with PCOS.

The study of lipid profiles in women with PCOS shows two results that are important with regard to cardiovascular health. First, more atherogenic lipid profiles were observed as a consequence of insulin resistance i.e., elevated levels of TG, apoB and apoB/apoA-I ratio. Second, we observe an additional drop in ApoA-I levels in PCOS cases *vs.* controls that is independent of BMI. Multivariate analysis for the prediction of Apo-A1 levels shows that hyperandrogenism (as represented by FAI level) is the most important determinant of disturbed ApoA-I metabolism. It can be hypothesized that the influence of hyperandrogenism on lipid metabolism in women with PCOS can be mediated by increased hepatic lipase activity. This enzyme, that has a role in the catabolism of HDL particles, is known to exhibit strong sexual dimorphism, with exogenous androgens up-regulating, and estrogens down-regulating its activity[2-5]. Indeed, multivariate analysis identified estradiol as an independent positive determinant of apoA-I but not apoB. The possibility that altered exposure to estrogens might exert a protective influence on cardiovascular risk profile in women with PCOS is very interesting indeed, but it falls beyond the scope and design of the studies that are presented in this thesis. It is possible that other, currently unknown, factors are involved in the regulation of lipid metabolism and cardiovascular risk in women with PCOS.

From a clinical point of view, it may prove possible to identify subgroups of PCOS patients who have a more adverse cardiovascular risk profile than others. We have observed that insulin resistance and dyslipidemia were more severe in a subgroup of PCOS patients who presented with a combination of BMI≥25 kg/m^2 and hyperandrogenism. While evidence from retrospective cohort studies is slowly mounting[6-9], it will be of interest to await the results of prospective longitudinal follow-up studies to see whether increased morbidity and mortality can be confirmed in women with PCOS.

For clinical purposes, it is important to increase the awareness of physicians and other care-givers that PCOS constitutes a condition that encompasses far more than just anovulation and subfertility that can be treated with ovulation induction. The present thesis shows clearly that metabolic and endocrine changes exist that may have severe consequences later in life. Obese as well as hyperandrogenic women with PCOS seem to be particularly at risk. As such, screening programs and regimens for lifestyle intervention and for the primary prevention of cardiovascular disease and endometrial carcinoma have to be designed that could be beneficial and cost-effective in this specific population at risk. Periodic screening for type II diabetes mellitus as well as for cardiovascular risk factors such as unfavorable lipid profiles and hypertension should be offered to these women, starting at an early age. At present, it seems that all too often the patient is kept unaware of these health risks.

Geo-biographic ancestry

The reality of today's clinical practice in most Western countries is that our patient population encompasses a broad variety of different ethnic backgrounds. Genetic make-up, lifestyle and health behavior will vary greatly between ethnic subgroups, adding to overall phenotypic heterogeneity; for example the presence of obesity and insulin resistance[10-12]. Our clinical practice is set in a central urban part of the Netherlands that shows high ethnic diversity. Four major subgroups were identified in our study population i.e., North-Western European, Mediterranean European, African sub-Saharan, South East Asian and Hindustani subjects. Some of phenotypic differences were well expected, such as a higher frequency of insulin resistance among Hindustani subjects. However, hyperandrogenism is also more frequent in this subgroup, possibly due to the fact that a diagnosis of hyperandrogenism currently relies heavily on SHBG levels that are typically lowered by insulin resistance. The question remains whether diagnostic criteria, such as the one that is currently in use for biochemical hyperandrogenism, should not be adjusted for ethnic background. The present thesis does show unexpected support for the use of the 2003 Rotterdam criteria for PCOS[13]. Diagnosing PCOS according to this consensus results in a similar frequency of PCOS (between 86% and 91%) among anovulatory women from different ethnic backgrounds. In contrast, the use of the 1990 NIH

criteria results in considerable differences in the frequency of PCOS amongst different ethnic groups (frequencies ranging between 41% and 76%).

We were interested to explore whether a genetic test can be used to adequately map ethnic differences in our patient population. Taking into account the complexity of human migration and the possibility of ancestral information that is not known to the individual patient, it may even be possible that a genetic test is more successful in mapping ethnic differences than self-assigned or self-reported ethnic background. We selected a set of 10 single nucleotide polymorphisms (SNPs) that were shown to be highly informative for continental genetic ancestry in prior research [14]. While promising, the use of these genetic ancestry SNPs in our study population did not provide sufficient geographic resolution (**chapter 2.2**). As a proof of principle, it is shown that genetic ancestry can be a tool to map phenotypic differences in anovulatory PCOS subjects. As such, much is expected from the arrival of commercially available SNP arrays of up to 500K SNPs and more. In short, we substantiate the use of the 2003 Rotterdam consensus criteria that are currently broadly accepted worldwide. In doing so, a more even distribution of PCOS frequencies may be observed among anovulatory women of varying ethnic backgrounds. In the future, genetic fine mapping of a women's ethnic background might provide further discriminative power in order to distinguish different PCOS phenotypes.

Genetic modifiers of PCOS phenotype and disease risk

Shifting attention to the study of genetic determinants of PCOS, we have started our efforts by selecting candidate genes in regulatory pathways for ovarian function, steroid hormone metabolism and signal transduction. It is important to realize that genetic variants were studied that occur regularly in a healthy population. Indeed, small changes in the phenotype of PCOS were observed, most importantly with regard to gonadotrophic hormone levels. These observations seem to substantiate a complex genetic model for ovarian function and PCOS that allows for the interaction of multiple variants that modify disease expression. For example, it was observed that a specific polymorphic variant of the FSH receptor (FSHR $680^{Ser/Ser}$) is associated with higher FSH as well as LH serum levels and slightly higher frequency of hyperandrogenism amongst PCOS patients (**chapter 3.1**). It is such variants that will determine the extent of metabolic derangements that can be observed in the individual patient. A better understanding of these genetic modifiers of PCOS may, in the future, enable the physician to estimate more accurately what kind of risks the individual patient is exposed to and provide guiding for pharmacological and/or life-style intervention. In this respect, one may regard the results of the study that is described in chapter five as a promising step towards a future where treatment protocols may become better tailored to the individual patient on the basis of her genetic make-up. What we have observed is that FSHR $680^{Ser/Ser}$ is significantly associated with failure to regain ovulatory menstrual cycles, following the

use of ovulation induction with CC. In addition, carriers of the 680^{Ser} allele may be more likely to achieve an ongoing pregnancy, when treated with an exogenous FSH preparation (**chapter 5**). To our knowledge, this is the first report that demonstrates such a direct relationship between genetic variants and treatment outcome in anovulatory subfertility. Future research should make it feasible to design a treatment regimen that is more efficacious and cost-effective.

It is interesting to interpret the findings of our study of the glucocorticoid receptor in PCOS patients. GCR variants are associated with changes in LH levels that suggest an inhibitory influence of the glucocorticoid receptor on the function of the hypothalamo-pituitary-gonadal axis (**chapter 3.2**). While a link between the hypothalamo-pituitary-adrenal and gonadal axes is well known from animal research[15-17], surprisingly little information can be found concerning the human species. The present study shows higher LH levels in association with both the rs6190 and rs6198 polymorphisms that have been described as inhibitors of GCR action in prior research. Conversely, haplotype analysis showed lower LH levels in association with glucocorticoid receptor variants that are known to increase receptor sensitivity (rs6195 and rs41423247). Therefore, our data are consistent with a model that allows "cross-talk" between the adrenal and gonadal axes. In other words, the less sensitive the GC receptor the higher the LH serum levels in women with PCOS will be. Because LH levels correlate with an array of other phenotypical characterisics of PCOS it seems worthwhile to investigate and substantiate some of these findings in larger studies.

Risk alleles for PCOS and gene finding in a Dutch genetically isolated population

An important aim of the studies that are described in chapter 3 and 4 was to identify genetic variants that are associated with disease risk. In the case of our candidate gene analyses (**chapter 3.1 and chapter 3.2**) we have compared allele frequencies in PCOS cases and controls. The control group consisted of unselected women from the general population (The Rotterdam Study[18]). In a worst case scenario, up to 10% of these women will also have suffered from some form of PCOS. This may have resulted in a loss of power to detect risk alleles. The present approach did however allow us to include a large number of controls without introducing the risk of other selection bias. Both the study group and controls consisted of Caucasian females, minimizing the risk of population admixture. Our candidate gene analyses did not detect risk alleles for PCOS. Even in the case of the FSHR Asn680Ser polymorphism, that is associated with basal FSH levels in our study and others, no disease risk was observed. Keeping in mind that we deal with an allelic variant that is very common in the general population, it may not come as a surprise that its influence on the (anovulatory) phenotype is subtle. One may hypothesize that its effect, by itself, is so small that it is difficult to detect in the present case-control analysis. However, recently published genome wide association analyses

have implicated 2p16.3, the locus containing both the FSH as well as the LH receptor gene, as a risk locus for PCOS[19-21]. It is expected that the interaction with other genetic variants and/or environmental factors such as diet, will significantly affect disease risk. At present, it seems that the candidate gene approach does not constitute a powerful tool to detect risk alleles in complex genetic diseases such as PCOS.

The genome wide association analysis that was conducted for normogonadotropic anovulatory women has the advantage that it covers the entire genome. In contrast to our candidate gene studies, this approach is not hypothesis driven. The very large number of statistical tests that are performed in the course of these analyses do not only pose a computational challenge but is also problematic in the interpretation of results. The problem of multiple testing can only be overcome by adhering to a very stringent threshold for statistical significance. In the case of complex genetic diseases, effect-size and/or the number of study subjects that can be included may be insufficient to be able to reach this threshold. Also, one would expect that a multitude of SNPs that are located at a certain disease locus would generate a signal. Certainly in the case of a genetically isolated population, where linkage disequilibrium is maintained over longer genetic distances[22, 23], multiple SNPs at, or near, a certain disease loci are expected to be associated with the disease. Such loci were not detected in the present analysis (**chapter 4**). We identified a SNP at chromosome 7 (rs6976312) that just exceeded the threshold for statistical significance. This specific SNP is located at an intergenic spot on the long arm of chromosome 7. No known gene is located within 150K nucleotides of this locus. No other SNP at this locus was associated with PCOS and the locus did not show up in recent GWAS analyses[19, 21]. Nor did the same locus show up in the linkage analysis that was performed in the same population using a different set of polymorphic markers and genealogical data. While we do report the finding as a valid result, it should be interpreted with care and, so far, awaits confirmation from other studies.

Directions for future research

Ours and other studies have provided sufficient evidence to state that cardiovascular risk factors are more prevalent among women with PCOS. While cardiovascular risk factors in women with PCOS are largely determined by obesity, there is sufficient evidence for an independent influence of PCOS itself. On the one hand, our study of dyslipidemia points in the direction of hyperandrogenism and HDL metabolism. On the other hand (V)LDL metabolism seems to be much more affected by insulin resistance. It is hypothesized that up-regulation of hepatic lipase activity may be responsible for the atherogenic changes in the HDL metabolism. However, there is still a knowledge-gap with regard to cardiovascular disease risk in women with PCOS as long as results of prospective long-term follow-up studies are lacking. At present, prospective data are scarce and do not indicate increased CVD or stroke risk in older women previously diagnosed with PCOS[24].

One may also hypothesize that increased life-time exposure to estrogens may partly counterbalance the negative influences of hyperandrogenism and insulin resistance. Therefore although all surrogate markers do predict an increased risk of CVD it might well be that in the end real life events are not increased. Long term follow-up studies with closely defined protocols are urgently needed.

A lot still has to be achieved where it comes to the study of genetic determinants of PCOS. With the results of our studies and others it seems obvious that PCOS should be regarded as a complex genetic disease that is determined by multiple genetic pathways and environmental factors. Each factor by itself is expected to contribute only in a very minute fashion to disease risk. Recently published results from large scale GWAS analyses have begun to identify multiple disease loci[19-21, 25].

It seems that in complex diseases, such as PCOS, recently emerged technologies hold promising possibilities that may allow us to shed some light on the genetic background of the syndrome. The more hypothesis free approaches are the most promising tools available. However, drawbacks such as phenotype differences due to different diagnostic criteria used and the use of different assays to define hyperandrogenism are issues that have to be dealt with. The most important issue researchers have to deal with is how to combine data sets in order to increase the resolution of these genetic tools. Much is expected from international collaborative studies that have already been proven to be very successful in other areas of reproductive research[26, 27].

It seems that there is a lot to be gained by the study of genetic modifiers of PCOS that is of practical use to clinical practioner. Our studies with regard to FSH receptor polymorphism show that the results of ovulation induction therapy is influenced by FSH receptor genotype. In the near future it may even become possible to individualize treatment on the basis of genotypic features. In the same way, one questions whether genetic determinants of hyperandrogenism, insulin resistance and cardiovascular health can be identified in PCOS patients. It is expected that opportunities for primary and secondary prevention can be found in these areas. As such, the study of the anovulatory phenotype and its genetic determinants can be expected to contribute significantly to women's health in the near future.

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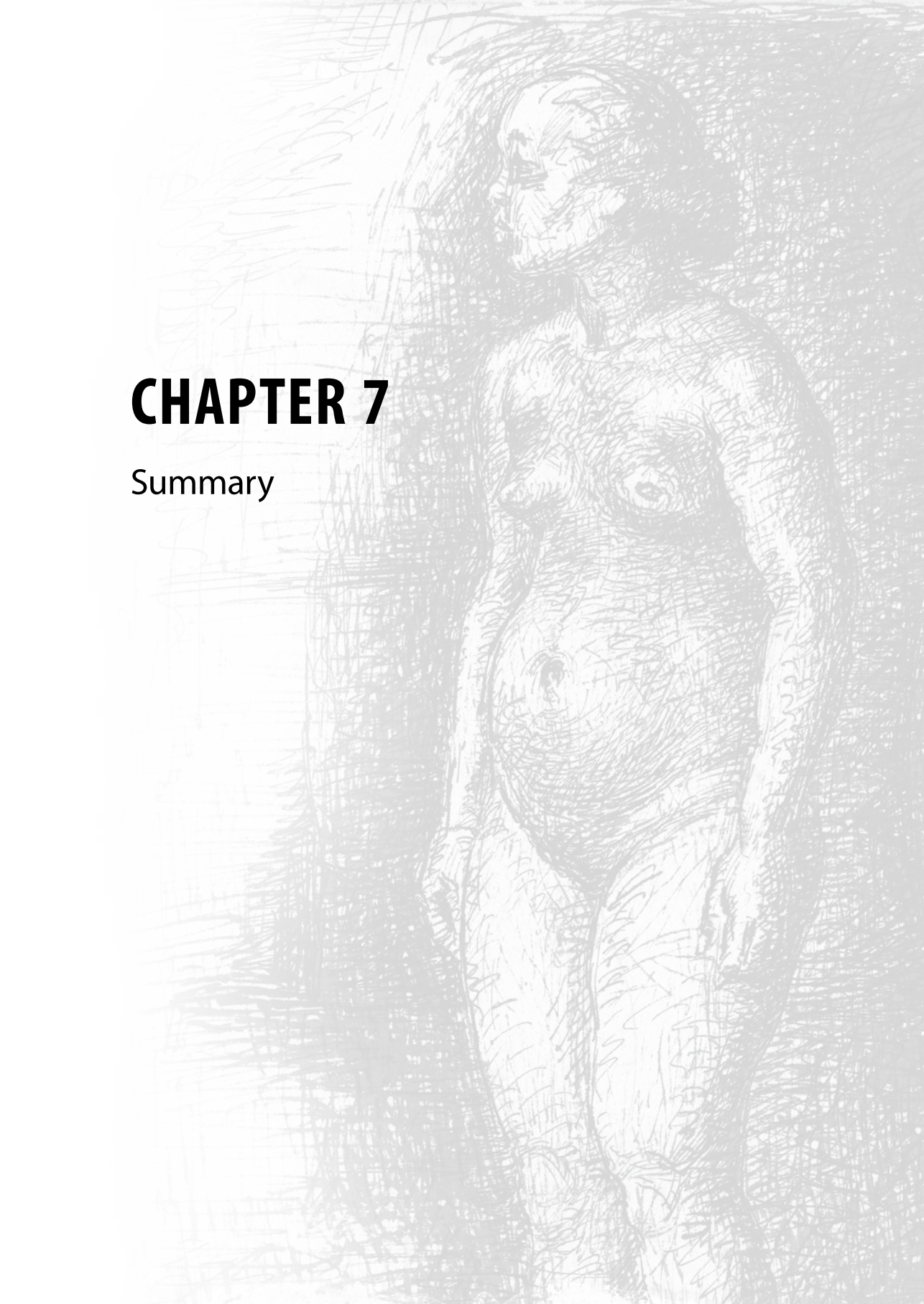


CHAPTER 7

Summary

SUMMARY

Chapter one provides an introduction into the phenotype of PCOS. It describes the main features of PCOS i.e., anovulation, hyperandrogenism and PCO. Moreover it provides a history of the different sets of diagnostic criteria that have been used for the diagnosis of PCOS. The studies that are described in the current thesis all use the 2003 Rotterdam criteria for the diagnosis of PCOS that are currently regarded as the international standard.

Genetic research in complex diseases such as diabetes mellitus and PCOS is hampered by (expected) small effect sizes, heterogeneity in disease phenotype and, in the case of PCOS, lack of a clear pathofysiologic model and uniform diagnostic criteria. Genetic research can either be hypothesis driven (candidate gene studies) or regard the entire genome in order to identify disease loci (gene finding). A number of objectives of this thesis are formulated that serve to cross the bridge from a morphometric to a genetic point of view to describe the polycystic ovary syndrome.

Chapter two describes the phenotype of PCOS patients with regard to the possibility of increased long-term risk for cardiovascular disease. Atherogenic changes in lipid metabolism were compared in 557 PCOS patients and 295 healthy controls. After correction for age and body mass index (BMI) PCOS-women showed higher levels of fasting insulin, increased TG, increased total cholesterol and LDL-cholesterol in combination with lower levels of HDL-cholesterol and apoA-I. We observed that PCOS is associated with risk factors for cardiovascular disease (a more atherogenic lipid profile) that is independent of obesity and may be (partly) determined by hyperandrogenism..

The same chapter describes a different study that takes a combined (clinical and genetic) approach to describe ethnic heterogeneity in the phenotype of PCOS. Ours and most other practices of women's health and fertility is set in an urban population of people with very diverse geo-biographic ancestries. Ethnic subgroups constituted individuals from North-Western European (n=774), Mediterranean European (north of Sahara, Turkey and Morocco, n=220), African (south of Sahara, n=111), South-East Asian (n=53) and Hindustanian (n=83) origin. Marked differences were present in insulin resistance and the presence and severity of anovulation and hyperandrogenism within the study population. Genetic testing was able to differentiate between these subgroups, however we did not observe improved informativeness when these data were used in a prediction model.

Chapter three describes the influence of selected genetic variants in candidate genes on the phenotype of PCOS. Candidate genes were selected in the hypothalamic-pituitary-gonadal axis (FSHR, LHR and GnRH1), steroid hormone synthesis (HSD11B1, H6PD, CYP19) and signal transduction (estrogen receptor [ESR1 and ESR2] and the glucocorticoid receptor [GCR]). Genetic variants constituted single nucleotide polymorphisms (SNPs) that were reported as functional variants in prior research. The primary aim of

both candidate gene studies was to identify risk-alleles for PCOS. In order to achieve that goal, a allele frequencies and haplotype frequencies were compared in PCOS cases and healthy controls. The present studies were unable to identify risk loci for PCOS. However, it was observed that certain polymorphic variants are associated with changes in the phenotype of PCOS patients. The most striking result were observed for the FSHR Asn680Ser polymorphism (rs6166). In complement to the finding of higher FSH levels, we also observed higher LH levels and a higher frequency of hyperandrogenism in association with the FSHR Ser^{680} allele.

The study of functional variants of the steroid hormone pathway showed that GCR variants are associated with changes in LH levels that suggest an inhibitory influence of the glucocorticoid receptor on the function of the hypothalamo-pituitary-gonadal axis. We observed higher LH levels in association with the rs6190 and rs6198 polymorphisms that have been described as negative inhibitors of GCR action in prior research. Conversely, haplotype analysis showed lower LH levels in association with glucocorticoid receptor variants that are known to increase receptor sensitivity (rs6195 and rs41423247). In addition, we did not observe associations of functional SNPs in HSD11B, H6PD, CYP19, ESR1 and ESR2 with the phenotype of PCOS or disease risk.

Chapter four describes the results of a genome wide linkage and association analysis that was performed in a genetically isolated population in the Netherlands (Rucphen, the Netherlands). While its geographic location is not especially noteworthy, evidence from prior genetic analyses that were conducted in the same population shows reduced genetic heterogeneity and a high level of linkage disequilibrium. Linkage analysis was performed, using a set of 382 evenly spaced short tandem repeat polymorphism (STRP) and incorporated genealogical data from municipal records. Risk loci were not detected. Whole genome association was performed for normogonadotropic (WHO2) anovulation. One SNP was detected on chromosome 7 that surpassed the threshold for genome wide significance ($p = 3.3 \times 10^{-8}$).

Chapter five aims to cross the gap between scientific interest and clinical applicability of genotype data. A prospective analysis of FSH receptor genotype was conducted in women with normogonadotrophic anovulatory subfertility who undergo ovulation induction with Clomiphene Citrate (1^{st} line therapy) or exogeneous FSH preparation (2^{nd} line therapy). It is shown that the rs6166 FSHR 680^{Ser} allele is significantly associated with failure to regain ovulatory menstrual cycles, following the use of ovulation induction with CC. In addition, the current prospective cohort study indicates that carriers of the 680^{Ser} allele may be more likely to achieve an ongoing pregnancy, when subsequently treated with exFSH. However, the latter observation was not confirmed in a replication cohort.

SAMENVATTING

Hoofdstuk één beschrijft het fenotype van vrouwen met normogonadotrope anovulatie en PCOS. De typische kenmerken van PCOS worden beschreven, t.w. anovulatie, hyperandrogenisme en PCO. In deze introductie wordt aandacht besteed aan de verschillende diagnostische criteria die worden gebruikt voor het stellen van de diagnose PCOS. Alle studies die worden beschreven in dit proefschrift maken gebruik van de 2003 Rotterdam criteria voor het stellen van deze diagnose. Deze criteria worden op dit moment algemeen beschouwd als de internationale standaard.

Ziekten als Diabetes Mellitus en PCOS zijn voorbeelden van complexe genetische ziekten. Genetisch onderzoek naar complexe genetische ziekten wordt bemoeilijkt door kleine effect grootte, heterogeniciteit van het ziektebeeld (fenotype) en, in het geval van PCOS, het ontbreken van een éénduidig pathofysiologisch model en uniforme diagnostische criteria. Aandacht wordt besteed aan de verschillende vormen van onderzoek die in dit proefschrift worden beschreven. Kandidaat-gen onderzoek wordt gebaseerd op een vooropgestelde hypothese m.b.t de invloed van (kandidaat) genen op het fenotype van de ziekte of risico op het ontstaan ervan. Aan de andere kant wordt in dit proefschrift ook een "gene finding" studie beschreven waarbij getracht wordt ziekteloci te identificeren in het gehele genoom zonder vooropgestelde hypothese m.b.t een beperkt aantal genen.

Aan het einde van dit hoofdstuk worden een aantal doelstellingen geformuleerd voor dit proefschrift met het uitgangspunt te bestuderen hoe het fenotype van PCOS wordt beïnvloed door de variatie in het menselijk genoom die ons allen tot unieke individuen maakt.

Hoofdstuk twee beschrijft een tweetal studies waarin het fenotype van PCOS wordt bestudeerd met betrekking tot cardiovasculair risico-profiel en ethnische heterogeniciteit. In de eerste studie worden veranderingen beschreven in de vetstofwisseling van 557 vrouwen met PCOS in een vergelijkend onderzoek met 295 gezonde controles. Na correctie voor leeftijd en body-mass index (BMI) blijkt dat in het serum van vrouwen met PCOS hogere spiegels worden gevonden voor insuline, triglyceriden, totaal cholesterol en LDL-cholesterol in combinatie met lagere serum-spiegels van HDL-cholesterol en apoA-I. Uit deze studie blijkt dat PCOS gepaard gaat met het verhoogd voorkomen van risicofactoren voor cardiovasculaire ziekte (i.c. atherogene veranderingen van het lipiden-profiel). Deze veranderingen zijn (deels) onafhankelijk van overgewicht. Mogelijk worden zij mede-veroorzaakt door hyperandrogenisme.

In hetzelfde hoofdstuk wordt een tweede studie opgevoerd die het fenotype van PCOS bestudeerd aan de hand van ethnische verschillen. Deze studie beschrijft fenotypische kenmerken van PCOS bij verschillende bevolkingsgroepen. Als zodanig sluit de studie

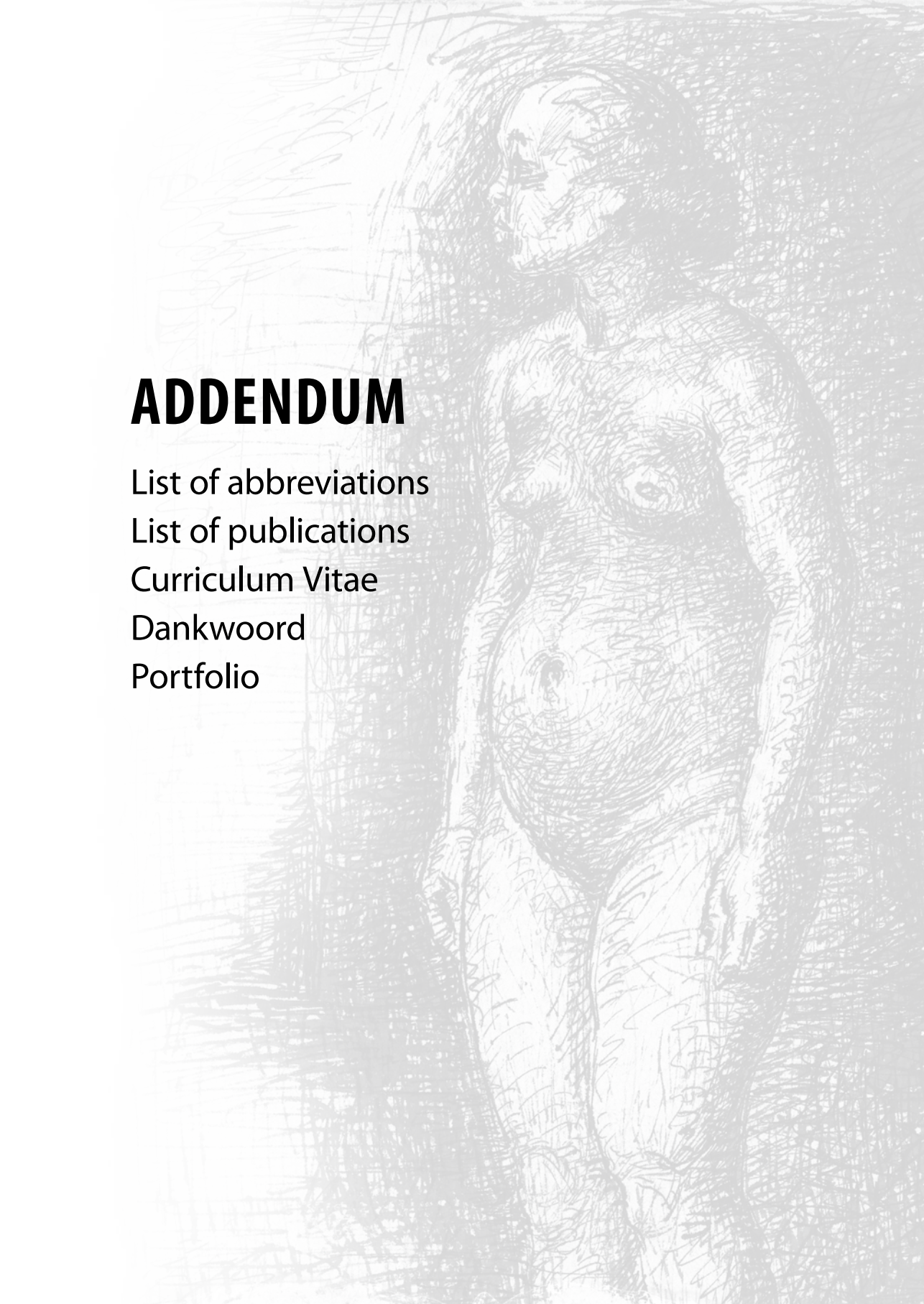
aan bij de realiteit van de meeste praktijken in Nederland en elders die tegenwoordig een zeer heterogene patiëntenpopulatie behandelen. Ethnische subgroepen bestonden uit: Noord-West Europees (n=774), Mediterraans-Europeesn (ten Noorden van de Sahara, Turkije en Marokko, n=220), Afrikaans (ten zuiden van de Sahara, n=111), Zuid-Oost Aziatisch (n=53) en Hindustaans (n=83). Het blijkt dat er grote verschillen bestaan tussen de verschillende bevolkingsgroepen in deze studie m.b.t. insuline resistentie, de aanwezigheid en de mate van anovulatie en hyperandrogenisme. Met behulp van een genetische test voor geografische afkomst konden deze verschillen gedeeltelijk worden geïdentificeerd binnen de studie-populatie; echter, het onderscheidend vermogen van de test die in deze studie werd gebruikt is nog onvoldoende voor klinische toepasbaarheid.

Hoofdstuk drie beschrijft de resultaten van het kandidaat-genen onderzoek dat werd verricht bij vrouwen met PCOS en controles. Kandidaat genen werden geselecteerd in de hypothalamus-hypofyse-gonade (FSHR, LHR en GnRH1), steroïd hormoon synthese (HSD11B1, H6PD, CYP19) en signaal transductie (oestrogeen receptor, [ESR1 en ESR2] en de glucocorticoïd receptor [GCR]. Genetische varianten bestaan in deze hoofdstukken uit z.g. single nucleotide polymorfismen (SNPs) die werden geselecteerd op grond van het feit dat zij reeds werden geïdentificeerd als mogelijke functionele varianten in voorgaand onderzoek. Het doel van de twee kandidaat-gen studies die worden beschreven in dit hoofdstuk is enerzijds de identificatie van genetische varianten die het risico op PCOS vergroten. Anderzijds wordt beschreven hoe deze varianten het fenotype van PCOS beïnvloeden. Hoewel er in dit onderzoek geen aanwijzingen naar voren kwamen dat deze genetische varianten geassocieerd zijn met ziekterisico, worden in de eerste studie wel duidelijke aanwijzingen gevonden dat specifieke kenmerken van het ziektebeeld, zoals hogere basale FSH- en LH-spiegels en de frequentie van hyperandrogenisme, hierdoor worden beïnvloed. Dit was met name het geval voor een specifiek polymorfisme van de FSH receptor (rs6166, FSHR Asn680Ser).

De tweede studie van dit hoofdstuk beschrijft de invloed van functionele genetische varianten van de synthese en signaaltransductie van steroïd hormonen. Het blijkt dat varianten van GCR (rs6190 en rs6198, die eerder reeds werden geassocieerd met inhibitie van GCR, gepaard gaan met hogere LH spiegels. De analyse van GCR haplotypen lijkt ook het omgekeerde aan te wijzen. Er werd geobserveerd dat receptor varianten die eerder in verband werden gebracht met een gevoeligere receptor (rs6195 en rs41423247) geassocieerd zijn met lagere LH-spiegels bij vrouwen met PCOS. Van de overige genen (HSD11B, H6PD, CYP19, ESR1 en ESR2) kon niet worden aangetoond dat genetische varianten het fenotype van PCOS of het ziekterisico significant beïnvloeden.

Hoofdstuk vier beschrijft de resultaten van een genoom wijde linkage en associatie studie voor PCOS. Deze studie werd uitgevoerd binnen een genetisch geïsoleerde populatie in Nederland (Rucphen, Noord-Brabant). Hoewel deze populatie in geografisch opzicht niet opvallend is, hebben een beperkt aantal founders in combinatie met relatieve isolatie en binnenwaardse migratie over opeenvolgende generaties geleid tot een reductie in genetische heterogeniciteit en toename van z.g. linkage disequilibrium. In de huidige populatie werden vrouwen met PCOS geïdentificeerd en onderzocht. Genotypering bestond in eerste instantie uit 382 short tandem repeat polymorfismen (STRPs), verdeeld over het gehele genoom. Door gebruik te maken van genealogische data van de gehele populatie kon een linkage analyse verricht worden waarbij geen locus kon worden geïdentificeerd op het genoom die was geassocieerd met PCOS. Een genoom wijde associatie studie (GWAS) werd daarna verricht voor normogonadotrope anovulatie waarbij één SNP werd geïdentificeerd op chromosoom 7 die de grens voor genoom-wijde significantie ($p=3.3 \times 10^{-8}$) overschrijdt.

Hoofdstuk vijf poogt een brug te slaan naar de klinische toepasbaarheid van de data uit dit proefschrift. In dit hoofdstuk wordt een prospectieve studie gepresenteerd naar de resultaten van de behandeling van normogonadotrope anovulatie bij paren met een actieve kinderwens. De behandeling bestaat uit ovulatie inductie met (eerste lijn) Clomifeencitraat (CC) of (tweede lijn) exogeen FSH. Uit de resultaten blijkt dat de genetische variant van FSHR (rs6166, 680^{Ser}) significant is geassocieerd met het het uitblijven van ovulatoire cycli bij behandeling met Clomifeencitraat (clomifeen resistente anovulatie). Uit dezelfde studie blijkt ook dat draagsters van het 680^{Ser} allel die in tweede instantie worden behandeld met exogeen FSH een hogere kans hebben op doorgaande zwangerschap. Echter deze laatste observatie kon niet worden gereproduceerd in een replicatie cohort.



ADDENDUM

List of abbreviations
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LIST OF ABBREVIATIONS

11ßHSD	11ß-hydroxysteroid dehydrogenase type 1
17-OH-Pg	17-hydroxyprogesterone
95%CI	95% Confidence Interval
AD	Androstenedione
AES	Androgen Excess Society
AMH	anti-Müllerian Hormone
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
apoA-I	Apolipoprotein A-I
apoB	Apolipoprotein B
Asn	Asparagine
ASRM	American Society for Reproductive Medicine
BMI	Body Mass Index
C	Cholesterol
CAH	Congenital Adrenal Hyperplasia
CC	Clomiphene Citrate
CCF	Clomiphene Failure
CE	Cholesteryl Ester
CEPH	Centre d'Etude du Polymorphisme Humain
CEPH-HGDP	CEPH–Human Genome Diversity Project Cell Line Panel
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
cM	centi-Morgan
CRA	Clomiphene Resistant Anovulation
CVD	Cardiovascular Disease
CYP19	Aromatase
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulfate
E2	Estradiol
ELISA	Enzyme-Linked Immunosorbent Assay
ERF study	Erasmus Rucphen Family study
ESHRE	European Society of Human Reproduction and Embryology
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
exFSH	exogenous recombinant FSH
FAI	Free Androgen Index
FG	Ferriman Gallway

FSH	Follicle Stimulating Hormone
FSHR	FSH receptor gene
GCR	Glucocorticoid Receptor
GC	Glucocorticoid
GnRH	Gonadotrophin-Releasing Hormone
GRAMMAR	Genomewide Rapid Asssociation Mixed Model and Regression
GRIP	Genetic Research in Isolated Populations
GWAS	Genome Wide Association Study
H6PD	Hexose-6-Phosphate Dehydrogenase
HA	Hyperandrogenism
HDL	High Density Lipoprotein
HDL-C	HDL cholesterol
HL	Hepatic Lipase
HOMA	Homeostasis Model Assesment
HOMA2	HOMA score, updated computer model
HPA	Hypothalamic-Pituitary-Adrenal
HPG	Hypothalamic-Pituitary-Gonadal
HWE	Hardy Weinberg Equilibrium
IVF	In-vitro Fertilisation
LD	Linkage Disequilibrium
LDL	Low Density Lipoprotein
LDL-C	LDL-Cholesterol
L	Leucine
LH	Luteinizing Hormone
LHR	LH receptor
LHR insLQ	6 base pair insertion polymorphism in exon 1 of LHR
MAF	Minor Allele Frequency
NIH	National Institute of Health
NW-European	North-Western European
OD	Ovulatory Dysfunction
OR	Odds Ratio
PCO	Polycystic Ovary Morphology
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain reaction
Pg	Progesterone
Prl	Prolactin
Q	Glutamine
RFLP	Restriction Fragment Length Polymorphism

RIA	Radio-Immunoassay
SD	Standard Deviation
SE-Asian	South East-Asian
Ser	Serine
SHBG	Sex Hormone Binding Globulin
SNP	Single Nucleotide Polymorphism
STRP	Short Tandem Repeat Polymorphism
T	Testosterone
TDT	Transmission Disequilibrium Test
TG	Triglyceride
TMC	Total Motile Sperm Count
TSH	Thyroid Stimulating Hormone
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
WHR	Waist-to-Hip Ratio
X2	Pearson's chi-square

LIST OF PUBLICATIONS

Brown ZA, Louwers YV, Fong SL, Valkenburg O, Birnie E, de Jong FH, Fauser BC, Laven JS. The phenotype of polycystic ovary syndrome ameliorates with aging. Fertil Steril. 2011 Nov;96(5):1259-65. Epub 2011 Oct 1.

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Geachte professoren B.C.Fauser, A.G Uitterlinden, F.H. de Jong, B. Oostra, A.P. Themmen, A. Hofman, F.J. Broekmans, RP Steegers-Theunissen, E. Steegers, C.M. van Duyn, en C.B. Lambalk.Ik sta slechts op de schouders van giganten ! Stuk voor stuk delen jullie een passie voor de wetenschap. Het plezier om die passie te delen met anderen straalt van jullie af en werkt aanstekelijk. Het was, en is, mij een eer om te delen in jullie kennis, te discussiëren, samen een artikel te produceren. Terugkijkend met de wijsheid van vandaag zou ik sommige zaken wellicht anders hebben aangepakt, een studie anders hebben opgezet, mijn eigen voortgang beter hebben bewaakt ! Maar ook dat is wetenschap. Ik heb nooit een gebrek aan hulp of aandacht ervaren. Mijn grote dank voor de mogelijkheden die jullie als groep voor mij hebben gecreëerd.

Lieve papa. Een klein bericht van een zoon aan zijn vader. Ik stel me voor dat je trots geweest zou zijn. Trots op het feit dat jouw zoon zich handhaaft en ontwikkelt in zijn gekozen vakgebied. Misschien niet eens zozeer op het resultaat. Hoewel ik blij ben dat het boekje er ligt. Jouw eigenaardige mix van mildheid en vrijdenkendheid, anderen zullen het eigenwijsheid noemen, heeft mij altijd verwonderd en werkte aanstekelijk.

Ik herinner mij mijn bewondering bij het lezen van jouw fraaie taalgebruik in formele brieven aan instanties. Veel daarvan is (hopelijk) terug te lezen in dit boekje. Ik voel je aanwezigheid iedere dag in mijzelf. Eigenlijk ben je nooit ver weg !

Lieve Madeleine, lieve Vibeke en Tjaline, lieve Thijs en Sander. Wat ben ik trots op jullie ! Op alle neven, Viggo, Jitse, Tjibbe, Tjalling en nichtje Julot. We hebben samen zo veel doorstaan. Het heeft ons samengebracht en hechter gemaakt. Dat neemt niemand ons af.

En dan een woord aan mijn collega's. Eerst "de onderzoekskamer". Toen ik begon, trof ik daar Mark, Aagje en Manon, Esther H, Esther B en Christien. Jullie maakten mij wegwijs in onderzoeksland. Jazz in Kopenhagen. Hele goede herinneringen ! Wendy en Yvonne: wat een super-collega's zijn jullie. Dank aan jullie voor alle steun en niet in 't minst voor alle gezelligheid. Lieve Sharon, jij bent het meest mijn onderzoeksmaatje geweest. Samen naar "de baas". Voel je je net wat beter ! Ik heb bewondering voor je doorzettingsvermogen. Niet voor niks ben jij eerder gepromoveerd dan ik ! Lieve Lindy, wie goed doet, goed ontmoet ! Annelinde, wat een dappere move naar de dermatologie ! Alle anderen: Sam, Paul, John, Fatima, Dineke, Annelous, Sarah, Melek, Robert, Judith, Geranne, Mariana. Ook mijn dank aan alle andere collega's, IVF artsen, research verpleegkundigen, collega arts-assistenten met wie ik de afgelopen jaren heb gewerkt, lief en leed heb gedeeld. Teveel om volledig te kunnen zijn. Ook jullie waren een onlosmakelijk deel van mijn tijd als promovendus in het Erasmus Medisch Centrum en daarna in het Albert Schweitzer Ziekenhuis. Aleid en Cindy, aan jullie de taak het onderzoek weer een stapje verder te dragen en er een eigen wending aan te geven. Een speciale dank aan mijn paranimfen Cindy Meun en Yvonne Louwers. Drie generaties PCOS-research op rij !

Een speciale dank gaat uit naar mijn opleiders prof. C.W. Burger, dr. M.J. ten Kate-Booy, dr. G.S. Kooi, dr. L.N. Hofman en als laatste dr. E.J.P. van Santbrink. Jullie hebben je gecomitteerd aan de niet-geringe taak in zes jaar tijd een, soms eigenwijze, promovendus op te leiden tot gynaecoloog. Ook bij jullie vond ik begrip voor de vaak lastige combinatie van opleiding, gezin en onderzoek. Ik ben jullie zeer erkentelijk voor de begeleiding op professioneel vlak, maar zeker ook voor de persoonlijke benadering en commitment. Time-management blijft een aandachtspunt !

Als laatste een dankwoord aan mijn vrouw wiens leven ik eindeloos veel complexer maakte dan zij ooit voor zichzelf voorzag. Lieve Liselot, jij was de eerste die mij zag en daagde mij uit mijn ambities waar te maken. Onze levens zijn als twee bomen die in elkaar zijn vergroeit. Samen met de kinderen vormen wij een verbond dat niet meer, nooit meer, kan verdwijnen. Ik hoop dat wij erin slagen onze eigenheid op elkaar af te stemmen. Wat ben ik trots op onze kinderen, Tiemon, Halle en Seppe die de wereld, ieder op hun eigen wijze, ontdekken en vol vertrouwen tegemoet treden. Wat spannend ….!

CURRICULUM VITAE

Olivier Valkenburg was born on June 18th in the village of Riethoven, the Netherlands. He attended secondary school at the Stedelijk Gymnasium in Breda. After graduation he studied Medicine at the Royal University of Leuven. After two years he transferred back to the Netherlands where he completed his doctoral exam in 2000 at the Rijks Universiteit Leiden. He performed a clinical residency at the department of Gynaecology and Obstetrics of the United Bulawayo Hospitals in Bulawayo Zimbabwe under supervision of dr. D.A.A. Verkuyl.

After graduation in 2002 Olivier worked as a resident at the department of Gynaecology and Obstetrics at the Diaconessenhuis Leiden (one year) and the Erasmus University Medical Center (two years) in Rotterdam, the Netherlands. Under the tutelage of prof. J.S.E. Laven he started his PhD research project in 2005 at the division of Fertility Medicine of the Erasmus University Medical Center that was focussed on the genetic basis of polycystic ovary syndrome (PCOS). His work was presented at multiple national and international conferences and was awarded with the 2007 Yuriy Wladimiroff research prize at the Erasmus University Medical Center in 2007.

Olivier started specialist training in gynaecology and obstetrics in 2008 at the Erasmus University Medical Center under supervision of dr. M.J. ten Kate-Booij and, alternately, at the Albert Schweitzer Hospital in Dordrecht under supervision of dr. G.S. Kooi and dr. L.N. Hofman. He is currently employed as a resident in-training at the devision of reproductive medicine of the Erasmus University Medical Center

PORTFOLIO

1. PhD training

	Year	Workload (ECTS)
General courses		
Biomedical English Writing and Communication	007	4
Specific courses		
NIHES summer programme, Erasmus MC, Rotterdam: "Principles of Genetic Epidemiology"	2005	0.7
Seminars and workshops		
Voor- en najaarsbijeenkomst Vereniging voor Fertiliteitsstudie, 2005	2005	0.25
Voor- en najaarsbijeenkomst Vereniging voor Fertiliteitsstudie, 2006	2006	0.25
Voor- en najaarsbijeenkomst Vereniging voor Fertiliteitsstudie, 2007	2007	0.25
Voor- en najaarsbijeenkomst Vereniging voor Fertiliteitsstudie, 2008	2008	0.25
Oral presentations		
FSH receptor polymorphism affects the outcome of ovulation induction in WHO-II anovulatory infertility. O.Valkenburg, CB Lambalk, E.J.P. van Santbrink, A.G. Uitterlinden, B.C.J.M. Fauser, J.S.E. Laven. Annual Meeting American Society for Reproductive Medicine 2011	2011	2
FSH receptor polymorphism affects the outcome of ovulation induction in normogonadotropic anovulatory infertility (WHOII). O. Valkenburg, E.J.P. van Santbrink, A.P.N. Themmen, A.G. Uitterlinden, B.C.J.M. Fauser, J.S.E. Laven. Annual Meeting European Society of Human Reproduction and Embryology 2009	2009	2
Genetic variation in multiple candidate genes for polycystic ovary syndrome affect the phenotype but not risk of PCOS. Valkenburg O, Lie Fong S, Uitterlinden AG, de Jong FH, Themmen AP, LavenJSE. Annual Meeting, Society for Gynaecologic Investigation, 2008, San Diego, California, USA	2008	2
Genetic variation in GnRH and gonadotropic hormone receptors affect the phenotype, but not risk of polycystic ovary syndrome (PCOS).Valkenburg O, Piersma D, Bruggemans S, Uitterlinden AG, de Jong FH, Themmen APN, Laven JSE. Jaarlijkse Onderzoeksdag Rotterdamse Gynaecologen Opleidings Cluster, 2008, Erasmus MC, Rotterdam	2008	0.5
Searching PCOS genes: results of a whole-genome screen for PCOS in a Dutch founder population.Valkenburg O, Mulders AG, Bertoli-Avella A, Oostra BA, Laven JSE. Najaarsbijeenkomst Vereniging voor Fertiliteitsstudie, 2007, Academisch ziekenhuis V.U., Amsterdam	2007	0.5

Insulin resistance and dyslipidaemia in women with Polycystic Ovary Syndrome, a case control study. Valkenburg O, Steegers-Theunissen RPM, Smedts D , Dallinga-Thie GM, Fauser BCJM, Westerveld HE, Laven JSE. Jaarlijkse Onderzoeksdag Rotterdamse Gynaecologen Opleidings Cluster, 2007, Erasmus MC, Rotterdam.	2007	0.5
Apolipoprotein levels in Polycystic Ovary Syndrome. Valkenburg O, Dallinga-Thie GM, Lie Fong S, Eijkemans MJ, Fauser BCJM, Laven JSE, Westerveld HE Dutch Atherosclerosis Society, symposium 2007	2007	2

Poster presentations:

Genetic variation in GnRH and gonadotropic hormone receptors affect the phenotype, but not risk of polycystic ovary syndrome (PCOS). Valkenburg O, Piersma D, Bruggemans S, Uitterlinden AG, de Jong FH, Themmen APN, Laven JSE Annual Meeting European Society of Human Reproduction and Embryology 2008	2008	1
Insulin resistance and dyslipidaemia in women with Polycystic Ovary Syndrome (PCOS), a case control study. Valkenburg O, Steegers-Theunissen RPM, Dallinga-Thie, GM, Fauser BCJM, Westerveld HE, Laven JSE Annual Meeting American Society for Reproductive Medicine 2007	2007	1
Searching PCOS-genes: results of a genome screen for PCOS in a Dutch founder population Valkenburg O, Mulders AG, Bertoli-Avella A, Oostra BA, Laven JSE Annual Meeting Society for Gynaecologic Investigation, 2008, San Diego, California, USA	2008	1
Single nucleotide polymorphisms (SNPs) in candidate genes for normogonadotrophic anovulatory infertility and polycystic ovary syndrome (PCOS).Valkenburg O, Lie Fong S, Nipius RM, Uitterlinden AG, Themmen, APN, de Jong FH, Fauser BCJM, Laven JSE Annual Meeting European Society of Human Reproduction and Embryology 2006	2006	1

International conferences

Annual Meeting European Society of Human Reproduction and Embryology 2005	2005	1
Annual Meeting European Society of Human Reproduction and Embryology 2006	2006	1
Annual Meeting European Society of Human Reproduction and Embryology 2007	2007	1
Annual Meeting American Society for Reproductive Medicine 2007	2007	1
Dutch Atherosclerosis Society, symposium 2007		
Annual Meeting, Society for Gynaecologic Investigation, 2008, San Diego, California, USA	2008	1
Annual Meeting European Society of Human Reproduction and Embryology 2008	2008	1
Annual Meeting European Society of Human Reproduction and Embryology 2009	2009	1
Annual Meeting American Society for Reproductive Medicine 2011	2011	1

2. Teaching

	Year	Workload
Lecturing		
Vaardigheidsonderwijs bij thema 3.1 "Stoornissen in de reproductieve cyclus"	2007	1
Vaardigheidsonderwijs bij thema 3.1 "Stoornissen in de reproductieve cyclus"	2008	1
Supervising practicals and excursions, Tutoring		
Supervisor 3rd year medical student, N.Bakker; keuze-onderwijs.	2007	0.25
Supervisor 3rd year medical student, L. Goedhart; keuze-onderwijs.	2008	0.25
Supervisor 3rd year medical student, Z. Brown; keuze-onderwijs.	2008	0.25
Supervisor 4th year medical student , N. Bakker, wetenschap stage.	2008-2009	0.25
Supervisor 3rd year medical student, R. Rasker; keuze-onderwijs.	2009	0.25
Supervisor 3rd year medical student, J. Burgers; keuze-onderwijs.		0.25