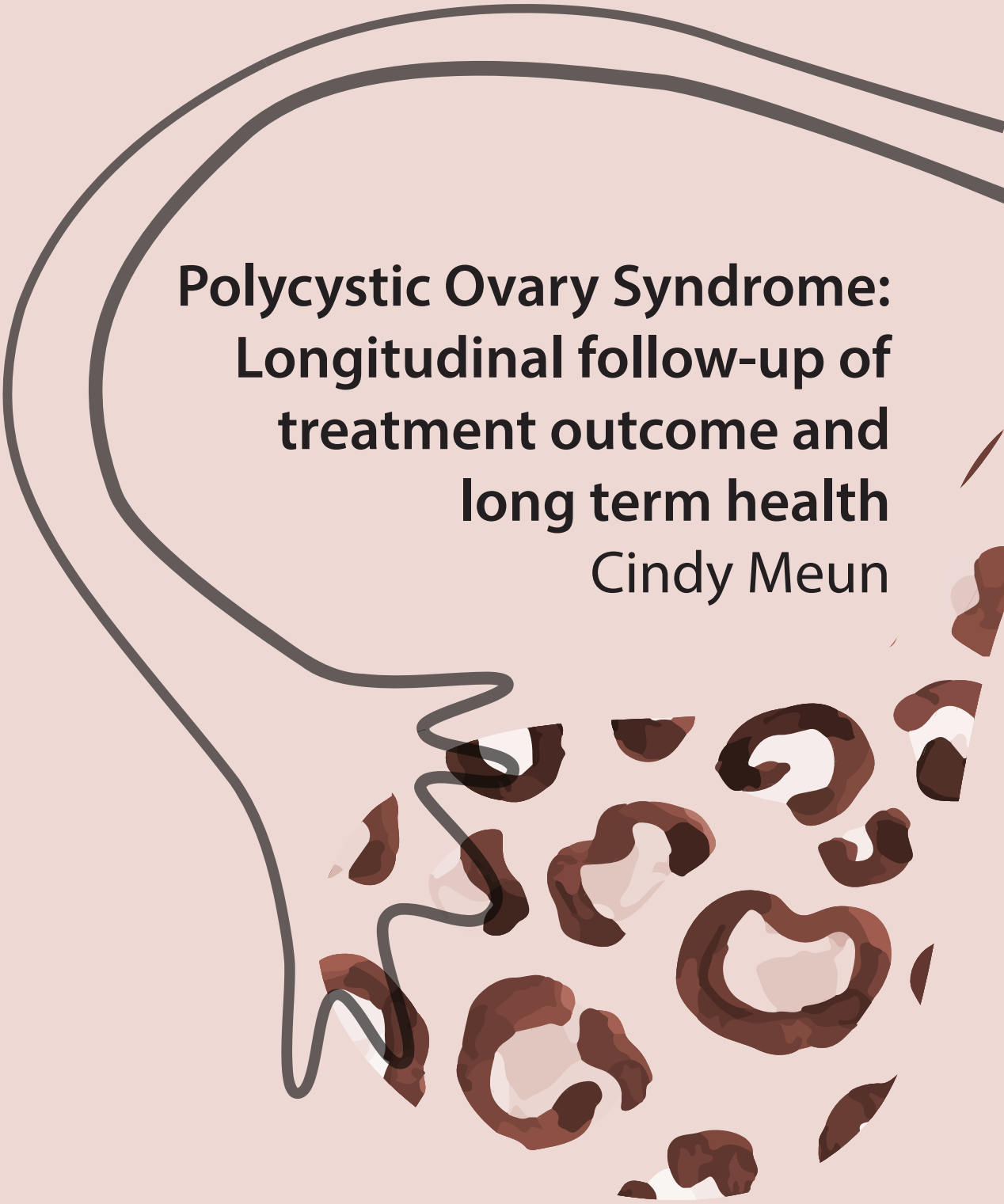
Polycystic Ovary Syndrome: Longitudinal follow-up of treatment outcome and long term health

Cindy Meun



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CHAPTER 1

General Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age and diagnosed in up to 15% of women [1]. Over the last years, PCOS has gained much attention and the importance of diagnosing and treating the syndrome has been much more recognized. PCOS affects women throughout various stages and aspects of life [2]. It is a major cause of anovulatory infertility, often has an impact on self-esteem, bodily image, sexual function, and emotional well-being, and is associated with cardiometabolic disturbances which might increase the risk for cardiovascular disease later in life [3-5]. The costs of evaluating and treating PCOS during the reproductive years, have been estimated to exceed 4 billion dollars in the United States. This estimation does not take comorbidities emerging in the postmenopausal years into account, emphasizing the importance of early and proper identification and treatment [6]. Recently, the international evidence based guideline proposed strategies for the best assessment and management of polycystic ovary syndrome, and hereby aims to improve health outcomes in women with PCOS based on current knowledge of the syndrome. However, the syndrome is still far from fully understood and many knowledge gaps remain which require more research.

The definition of PCOS

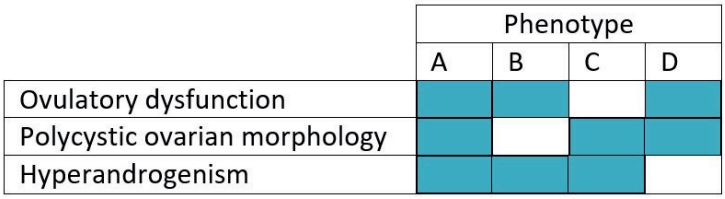
Polycystic ovary syndrome was first described in 1935 by Doctors Stein en Leventhal. They described a syndrome with the simultaneous occurrence of anovulation and bilateral polycystic ovaries in obese women [7]. It was not until 1990 that the first formal criteria for PCOS were proposed, the so called National Institute of Health (NIH) consensus which was actually not reached by a consensus conference [8]. The NIH criteria defined PCOS as a syndrome characterized by hyperandrogenism and ovulatory dysfunction. Later on in 2003, a group of experts established the Rotterdam criteria. In this definition of PCOS, polycystic ovarian morphology (PCOM) was incorporated, resulting in three key features. PCOS could be diagnosed in case a minimum of two out of the three key features were present, resulting in four phenotypes [9]. Other etiologies mimicking features of PCOS (Cushing syndrome, congenital adrenal hyperplasia, androgen-producing tumors and hyperprolactinemia), should be excluded beforehand [10]. Over the years there has been much discussion about the exact diagnostic criteria and the resulting phenotypes of PCOS and their severity. The Androgen Excess-PCOS (AE-PCOS) society issued a statement proposing to establish hyperandrogenism as the key feature of PCOS. The use of these different classifications of the syndrome however, created confusion and delayed progress in the understanding of the syndrome, and hampered the ability to address and manage health issues associated with PCOS because it created a lot of diversity in how to diagnose PCOS [11]. Finally, in 2012 the NIH organized the Evidence-Based methodology workshop on PCOS. All three proposed definitions for PCOS were re-evaluated and it was recommended that the

broader Rotterdam criteria should be maintained and used in research studies and clinical care [11].

The phenotype of PCOS during the reproductive years

Nowadays, the Rotterdam criteria are still the most acknowledged PCOS criteria, recognizing PCOS as a syndrome characterized by ovulatory dysfunction, hyperandrogenism and PCOM. An overview of the possible phenotypes of PCOS are presented in **Figure 1**. Ovulatory dysfunction is the presence of oligomenorrhea (menstrual cycle interval <21 or > 35 days) or amenorrhea (menstrual cycle interval >199 days) [12]. Hyperandrogenism is the excessive circulation of male hormones in females, and can manifest itself clinically in the form of hirsutism, acne or alopecia, or biochemically in the form of an elevated free testosterone or free androgen index (Testosterone x 100/Sex Hormone Globulin (SHBG) serum levels). Biochemical hyperandrogenism should be assessed via high quality assays such as liquid chromatography – tandem mass spectrometry (LC-MS/MS) or extraction / chromatography immunoassays, [13]. PCOM has been defined as the presence of twelve or more follicles in at least one of the ovaries, in the absence of a dominant follicle in case one uses an ultrasound probe of < 8MHz. More recently, due to improvements in US machinery and their resolution, this cut off was increased to ≥20 follicles [14,15]. There is great variation in the expression of the phenotype of PCOS and its' associated comorbidities [2]. While PCOS is generally diagnosed during the early reproductive years, the syndrome probably spans the total life span of women [16]. During adolescence the first clinical symptoms of PCOS may arise in the form of cycle irregularities, acne and hirsutism [2,17]. The majority of women diagnosed with PCOS will suffer from problems with fertility, with a reported prevalence around 70% [18]. Moreover, when they do become pregnant, women with PCOS are at increased risk for pregnancy complications such as gestational diabetes, preterm delivery, and pre-eclampsia, and may have an increased risk for miscarriage [19-21]. Thus making it necessary for women with PCOS to be under the care of a gynecologist during their pregnancy.

Figure 1. PCOS phenotypes according to the Rotterdam criteria



The phenotype of PCOS and risk for cardiovascular disease later on in life

PCOS has been associated with an unfavorable cardiometabolic profile and increased prevalence of risk factors for cardiovascular disease from an early age onwards [3,4,22,23]. Women with PCOS are more often overweight or obese, and the prevalence of central obesity is increased [24]. In addition, the prevalence of hyperinsulinemia and insulin resistance is increased, and women with PCOS exhibit higher serum triglyceride, low-density lipoprotein (LDL) and total cholesterol levels, as well as lower high-density lipoprotein (HDL) levels [4,25,26]. Furthermore. they often develop hypertension and type II diabetes [4,25]. These metabolic disturbances are more often found in all women with PCOS, however the presence of hyperandrogenism and obesity seems to increase the prevalence and exacerbate the severity of the metabolic disturbances associated with the syndrome [3,4].

Central obesity, elevated blood pressure, increased glucose and triglyceride levels and decreased HDL-cholesterol levels are all components of the metabolic syndrome. The metabolic syndrome is a major risk factor for cardiovascular disease [27]. A more than threefold increase in the prevalence of metabolic syndrome has been reported in women with PCOS, already at an early age [28]. The highest prevalence of the metabolic syndrome has been observed in obese and hyperandrogenic women with PCOS [4,28]. In the past it was anticipated that, based on this unfavorable cardiometabolic profile, women with PCOS were at increased risk to develop cardiovascular disease later on in life [3,23].

With increasing age, the phenotype of PCOS changes and generally becomes milder [29]. Over the years, the ovarian reserve declines, the number of follicles decreases and the threshold for PCOM is no longer reached. The menstrual cycle gradually shortens and ceases at the onset of menopause. The only key feature of PCOS which might persist far after the onset of menopause is hyperandrogenism [29,30]. Some of the cardiometabolic disturbances associated with PCOS can persist into the menopausal period as well [4]. Increased blood pressure, triglyceride, and glucose levels have been

described in older women with PCOS, however not many studies on the cardiometabolic profile of older women with PCOS have been performed yet [4,23].

Associations between surrogate markers for cardiovascular disease and PCOS have been described. Previous studies have reported an increased intima media thickness (IMT) and higher coronary artery calcium scores and increased pulse wave velocity in women with PCOS [31-34]. Most of these studies were performed in young women with PCOS. Data on subclinical cardiovascular disease in older women with PCOS is lacking. Available evidence in younger women does however suggest there might be an increased risk for cardiovascular disease later on in life.

To what extent these surrogate markers translate into real hard endpoints remains controversial. Early research on the cardiovascular risk in women with PCOS indicated an association with coronary artery disease and predicted the risk for myocardial infarction in women with PCOS to be 7-fold increased [23,35]. Long-term follow up until a mean age of 70 years did however, reveal a similar prevalence of myocardial infarction compared to age-matched controls [36]. This is still the only available long term follow up study on CVD in women with PCOS. More recent evidence on the matter is inconclusive, but does seem to point into the direction that the risk for cardiovascular diseases, such as myocardial infarction, coronary artery disease, and stroke might not be as high as was assumed in the past [6,33,36,37].

The genotype of PCOS

Long term consequences, as well as the pathogenesis of PCOS are still far from fully understood. PCOS is considered to be a complex genetic disorder. Simple genetic diseases, also known as Mendelian disorders, are caused by a mutation in a single gene [38]. These mutations are rare (prevalence <1%) and give rise to a severe phenotype. Examples of Mendelian diseases are cystic fibrosis and Huntington disease. Common complex disorders such as PCOS, are not driven by one genetic factor, but rather by a combination of genetic factors that might interact with environmental factors. These diseases generally have a milder phenotype, and are caused by common genetic variations with subtle effects [39]. The proportion of a disease or syndrome which is genetic, is called the heritability. Twin studies can be used to estimate the heritability of a disorder. Monozygotic twins share their whole genome, while dizygotic twins share 50% of their genome. In case twins grow up in the same household, they grow up in a relative absence of differences in environmental factors. By comparing monozygotic twins to dizygotic twins, genetic factors can be identified. In PCOS, twin studies have estimated the heritability to be around 70% because of higher degree of concordance in monozygotic twins compared to dizygotic twin pairs [40]. Furthermore, a higher prevalence of PCOS has been observed in first-degree relatives of women affected by PCOS [41]. Traits associated with PCOS, such as hyperandrogenism, metabolic syndrome,

hypertension and insulin resistance also seem to cluster in mothers, fathers, brothers, and sister of patients with PCOS [42-44]. While these studies demonstrate a significant genetic component, they do not provide insight into the exact genes and mechanisms underlying PCOS.

Candidate gene studies were performed to elucidate genes responsible for PCOS. These studies aimed to detect a hypothesized relationship between genetic variants in a gene and a disorder [45]. Candidate genes studies in PCOS have focused on regions regulating steroid biosynthesis and action, gonadotropin secretion and action, folliculogenesis, weight and insulin regulation [45]. Results of these studies have however lacked consistency [6,46].

More recent genetic studies have used the genome wide association (GWAS) approach. In GWAS, a hypothesis free approach is used to test hundreds of thousand genetic variants, also known as single nucleotide polymorphisms (SNPs), for an association with a trait or syndrome or disease under study [39]. A SNP is the substitution of a single deoxyribonucleic acid (DNA) base pair at a specific region in the genome. The substitution of such a base, has a high occurrence (prevalence >1%) and a small effect [39]. A GWAS aims to identify SNPs which are associated with a certain trait or disease, e.g. are more prevalent in people with that trait or disease. Generally this is done by comparing affected individuals to a non-affected control population. Hundreds of thousands SNPs are investigated in a GWAS, and for each separate SNP we test whether this DNA variation is significantly more often present in affected individuals than in controls. For an association to be considered significant a p-value of $<5x10^{-8}$ needs to be reached, hereby correcting for the fact that so many variants are tested. GWAS require large study populations for both discovery and replication of these findings [45]

The first large-scale GWAS in Han Chinese women with PCOS identified three SNPs associated with PCOS, two located on chromosome 2 and one on chromosome 9 [47]. A second GWAS, also in Han Chinese women with PCOS confirmed the previously identified loci, and in addition identified 8 new SNPs [48]. GWAS in study populations of European ancestry, have replicated some of the SNPs found in Han Chinese women, and identified more novel loci, bringing the total to 16 genetic variants significantly associated with PCOS **(Table 1)** [49-51]. Furthermore, cross ethnic analyses Chinese PCOS loci in women of Northern European ancestry indicate that there is a common genetic risk profile for PCOS across populations [52].

Table 1. Genetic loci associated with PCOS identified by GWAS

Number	rsID	SNP (chr:position)	Nearest Gene	Population	Associated with
1	rs13405728	2:48978159	THADA	Chinese Han*	Diabetes Mellitus
2	rs13429458	2:43638838	LHCGR	Chinese Han*	Gonadotropin levels
3	rs2268361	2:49201612	FSHR	Chinese Han*	Gonadotropin levels
4	rs4385527	9:97648587	C9orf3	Chinese Han*	PCOS diagnosis/features
5	rs2479106	9:126525212	DENND1A	Chinese Han*	Androgen excess, anovulation
6	rs1894116	11:102070639	*YAP1*	Chinese Han*	Unknown etiology
7	rs705702	12:56390636	RAB5B	Chinese Han*	Diabetes Mellitus
8	rs2272046	12:66224461	HMGA2	Chinese Han*	Diabetes Mellitus
9	rs4784165	16:52347819	*TOX3*	Chinese Han*	Unknown etiology
10	rs2059807	19:7166109	INSR	Chinese Han*	Insulin resistance
11	rs6022786	20:52447303	*SUMO1P1*	Chinese Han*	Unknown etiology
12	rs11031006	11:30204731	KCNA4/FSHB	European**	Gonadotropin levels
13	rs804279	8:11766130	GATA4/NEIL2	European**	Ovulatory dysfunction
14	rs2178575	2:213391766	ERBB4	European***	Ovarian cancer/steroidogenesis
15	rs13164856	5:131813204	IRF1/RAD50	European***	DNA repair
16	rs1795379	12:75941042	KRR1	European***	Ribosoma assembly factor

*Loci associated with PCOS discovered by GWAS studies. *Shi et al, Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome, **Hayes et al, Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations, ***Day et al, Causal mechanisms and balancing selection inferred from genetic associations with polycystic ovary syndrome*

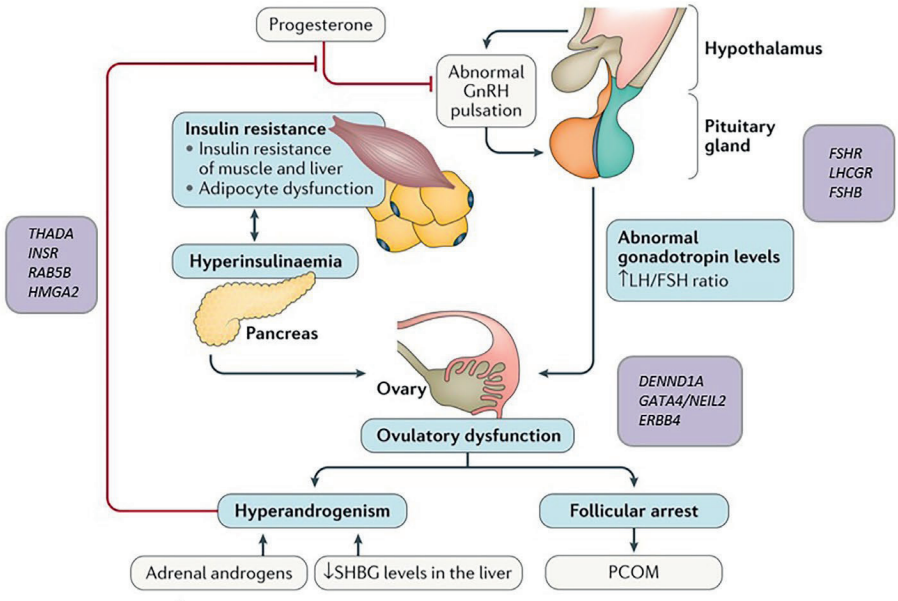
PCOS, from genotype to phenotype

Genome wide association studies have provided us with genetic variants associated with PCOS. GWAS however, only identify SNPs. Identified SNPs are then linked to nearby genes **(Table1)**. These genes may provide us insight into the pathophysiology of the syndrome. Genes of interest for PCOS identified by GWAS are associated with diabetes, insulin resistance, gonadotropin levels, gonadal development, steroid genesis, and DNA repair [45,53]. Studies have been performed to investigate how the identified loci and genes fit into the pathophysiology of PCOS **(Figure 2)**. One of these studies focusses on the DENND1A protein, which is located in the cytoplasm and nuclei of theca cells. Forced overexpression of DENND1A variant 2 in normal theca cells resulted in a PCOS phenotype and androgen biosynthesis, suggesting DENND1A might play a key role in the hyperandrogenemia associated with PCOS [54]. It has been reported that gonadotropin receptor gene LHCGR is overexpressed in non-obese women with PCOS, which could drive excess androgen secretion from the ovary. Furthermore the insulin receptor under expression in metabolic tissues and ovarian overexpression has been linked to insulin resistance and androgen excess [53]. Genetic variations in the FSH receptor could alter the phenotype of PCOS, influence gonadotropin serum levels and might have an influence on the outcome of ovulation induction [55].

Phenome wide association studies (PheWAS) test for an association between genetic variants and a wide range of phenotypes. PheWAS in PCOS has identified shared biology between PCOS and a range of metabolic and endocrine outcomes such as morbid obesity, type 2 diabetes, hypercholesterolemia, disorders of lipid metabolism, hypertension and sleep apnoea [57]. Mendelian randomization analyses indicate a causal role for SNPs associated with PCOS in the etiology for elevations in BMI, insulin resistance and lower sex-hormone binding globulin (SHBG) levels [50]. A higher PCOS risk was associated with genetic susceptibility to later menopause and higher serum anti-Müllerian hormone (AMH) concentrations in girls [50].

AMH plays a crucial role in gonadal function in that it inhibits the unrestricted recruitment of primordial follicles out of the primordial follicle pool generally referred to as ovarian reserve. In women with PCOS, serum AMH levels are often increased. For instance, normal 25 year old women without PCOS have AMH serum levels that are reached at an age of around 45 years in women with PCOS. Recent studies have revealed that AMH induces increased luteinizing hormone secretion by stimulation of the hypothalamic gonadotropin-releasing hormone (Gn-RH) neurons, pinpointing AMH as an important regulator of the Gn-RH system and potential critical role player in the development of PCOS [58]. Elevated AMH plasma levels might contribute to the hormonal alterations which are often observed in PCOS [58]. Finally, PCOS candidate gene studies suggest that disturbances in the fetal ovary are involved in the development of PCOS, pointing to a fetal origin of the syndrome [59]

Figure 2. Genetic areas of interest identified from GWAS



Modified from Azziz, nature reviews [56]

All of these data begin to provide us with more understanding of the etiology underlying PCOS. At this time however, the loci currently known by GWAS still only account for perhaps no more than 10% of the heritability of the disorder [53]. We have gained much more insight into the pathophysiology of PCOS, but we still don't fully understand what causes the syndrome, what the exact long term consequences are, and how we might prevent them. Many knowledge gaps remain, urging the need for further research. This thesis will focus on gaining more insight into the genetics of PCOS, treatment outcomes, and the risk for cardiovascular disease later in life.

Aim of the thesis

1. To identify genetic variants associated with PCOS , by performing a GWAS meta-analysis in a large international consortium
2. To assess the cardiometabolic profile and the risk for cardiovascular disease in middle-aged and older women with (features of) PCOS
3. To study the phenotype and treatment outcome of women with (features of) PCOS

Outline of the thesis

In **chapter 2**, we present the results of a large international genome wide association meta-analysis in women with PCOS of Northern-European descent.

In **chapter 3**, we studied the influence of ethnicity on ovulation induction in women with PCOS. **Chapter 4**, focusses on the cardiometabolic phenotype of young women with WHO2 anovulation, who do not meet the criteria for PCOS.

Chapter 5 focusses on the cardiometabolic disturbances and risk for cardiovascular disease in middle-aged and old women with (features of) PCOS. We assessed the cardiometabolic phenotype (**chapter 5.1**) and presence of coronary and intracranial atherosclerosis (**chapter 5.2 and 5.3**) in middle-aged women with PCOS. Finally, **chapter 5.4**, we studied the risk for atherosclerosis and cardiovascular disease in women with postmenopausal features of PCOS.

In **chapter 6**, we discuss our main findings and provide a general discussion including prospects for future studies.

General design

The research presented in this thesis was based on the following study populations; the Rotterdam Study, the i-PCOS consortium, and the CREW study.

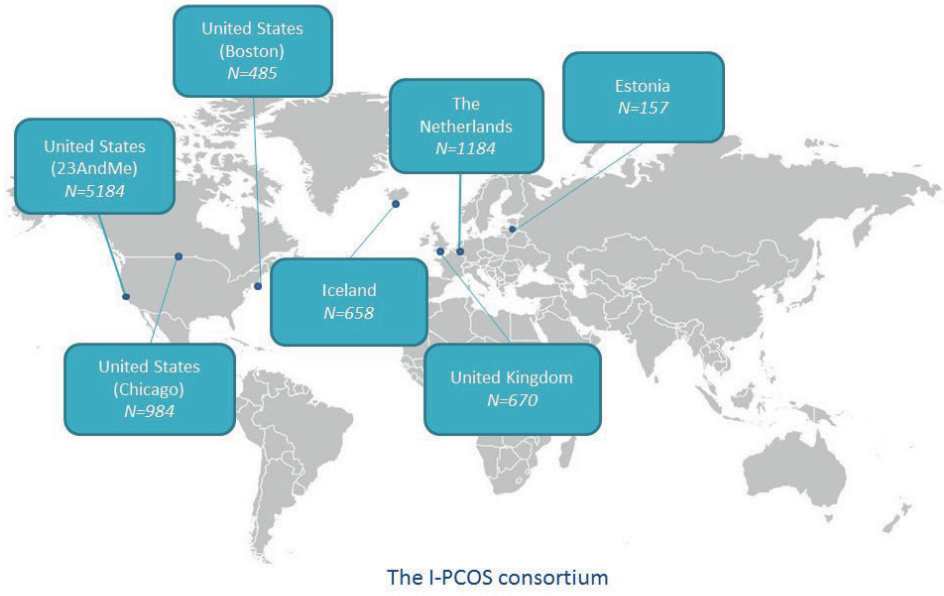
The Rotterdam Study

The Rotterdam study is a prospective population based cohort study among men and women >55 years of age. Recruitment was started in 1990 and comprises inhabitants of Ommoord, Rotterdam. Subjects are examined extensively every 3 to 5 years at the Rotterdam Study research center. Later, the cohort was extended to include inhabitants who had reached the age of 45 or had moved to the study area since the start of the study. The Rotterdam Study investigates risk factors of cardiovascular, neurological, ophthalmological and endocrine diseases in the elderly [60]. Over 16.000 people have been included in the study.

The i-PCOS consortium

The i-PCOS consortium was formed to study the genetics of PCOS. The consortium comprises 7 cohorts originating from Europe and the United states and includes 10.000 cases and 100.00 controls of Northern-European descent **(Figure 3)**. The consortium aims to identify genetic areas of interest and investigate the role of these areas in the pathogenesis of PCOS.

Figure 3. The i-PCOS consortium



The CREW consortium

The CREW consortium was formed to investigate long term cardiovascular health in women who were previously diagnosed with a reproductive disorder. Women diagnosed with PCOS or primary ovarian insufficiency, who had reached the age of 40 underwent an extensive cardiovascular assessment in one of the participating hospitals (Erasmus University Medical Center, University Medical Center Utrecht, and Amsterdam University Medical Center (Location VUmc). Over 300 women were included in this cohort and 200 participants underwent an additional computed tomography to investigate the presence and severity of coronary and intracranial atherosclerosis.

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CHAPTER 2

Large scale genome-wide meta-analysis of polycystic ovary syndrome suggests shared genetic architecture for different diagnosis criteria

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Abstract

Polycystic ovary syndrome (PCOS) is a disorder characterized by hyperandrogenism, ovulatory dysfunction and polycystic ovarian morphology. Affected women frequently have metabolic disturbances including insulin resistance and dysregulation of glucose homeostasis. PCOS is diagnosed with two different sets of diagnostic criteria, resulting in a phenotypic spectrum of PCOS cases. The genetic similarities between cases diagnosed based on the two criteria have been largely unknown. Previous studies in Chinese and European subjects have identified 16 loci associated with risk of PCOS.

We report a fixed-effect, inverse-weighted-variance meta-analysis from 10,074 PCOS cases and 103,164 controls of European ancestry and characterization of PCOS related traits.

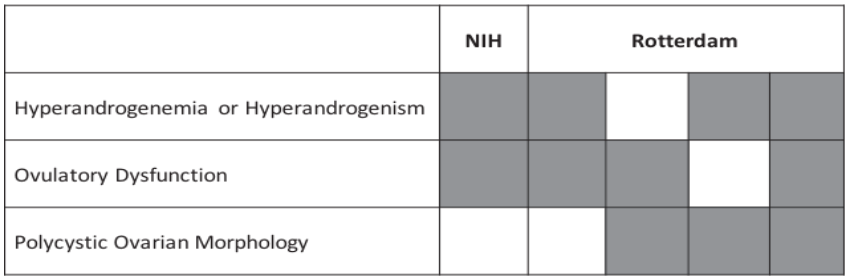
We identified 3 novel loci (near *PLGRKT*, *ZBTB16 and MAPRE1*), and provide replication of 11 previously reported loci. Only one locus differed significantly in its association by diagnostic criteria; otherwise the genetic architecture was similar between PCOS diagnosed by self-report and PCOS diagnosed by NIH or non-NIH Rotterdam criteria across common variants at 13 loci. Identified variants were associated with hyperandrogenism, gonadotropin regulation and testosterone levels in affected women. Linkage disequilibrium score regression analysis revealed genetic correlations with obesity, fasting insulin, type 2 diabetes, lipid levels and coronary artery disease, indicating shared genetic architecture between metabolic traits and PCOS. Mendelian randomization analyses suggested variants associated with body mass index, fasting insulin, menopause timing, depression and male-pattern balding play a causal role in PCOS.

The data thus demonstrate 3 novel loci associated with PCOS and similar genetic architecture for all diagnostic criteria. The data also provide the first genetic evidence for a male phenotype for PCOS and a causal link to depression, a previously hypothesized comorbid disease. Thus, the genetics provide a comprehensive view of PCOS that encompasses multiple diagnostic criteria, gender, reproductive potential and mental health.

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive aged women, with a complex pattern of inheritance [1-5]. Two different diagnostic criteria based on expert opinion have been utilized: The National Institutes of Health (NIH) criteria require hyperandrogenism (HA) and ovulatory dysfunction (OD) [6] while the Rotterdam criteria include the presence of polycystic ovarian morphology (PCOM) and requires at least two of three traits to be present, resulting in four phenotypes (**Fig S1**) [6,7]. PCOS by NIH criteria has a prevalence of ~7% in reproductive age women worldwide [8]; the use of the broader Rotterdam criteria increases this to 15–20% across different populations [9-11].

Supplementary Figure 1. Diagnostic criteria of PCOS



Diagnostic criteria of PCOS. Columns represent the diagnostic phenotypes that result from different diagnostic criteria. Grey squares indicate required traits for diagnosis within each diagnostic phenotype

PCOS is commonly associated with insulin resistance, pancreatic beta cell dysfunction, obesity and type 2 diabetes (T2D). These metabolic abnormalities are most pronounced in women with the NIH phenotype [12]. In addition, the odds for moderate or severe depression and anxiety disorders are higher in women with PCOS [13]. However, the mechanisms behind the association between the reproductive, metabolic and psychiatric features of the syndrome remain largely unknown. Genome-wide association studies (GWAS) in women of Han Chinese and European ancestry have reproducibly identified 16 loci [14-17]. The observed susceptibility loci in PCOS appeared to be shared between NIH criteria and self-reported diagnosis [17], which is particularly intriguing. Genetic analyses of causality (by Mendelian Randomization analysis) among women of European ancestry with self-reported

PCOS suggested that body mass index (BMI), insulin resistance, age at menopause and sex hormone binding globulin contribute to disease pathogenesis [17].

We performed the largest GWAS meta-analysis of PCOS to date, in 10,074 cases and 103,164 controls of European ancestry diagnosed with PCOS according to the NIH (2,540 cases and 15,020 controls) or Rotterdam criteria (2,669 cases and 17,035 controls), or by self-reported diagnosis (5,184 cases and 82,759 controls) (**Tables 1** and **S1**). We investigated whether there were differences in the genetic architecture across the diagnostic criteria, and whether there were distinctive susceptibility loci associated with the cardinal features of PCOS; HA, OD and PCOM. Further, we explored the genetic architecture with a range of phenotypes related to the biology of PCOS, including male-pattern balding [18-21].

Methods

Ethics statement

All research involving human participants has been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participants. The Boston cohort was approved by the Partners IRB (# 2002P001924) and the University of Utah IRB (IRB_00076659). The deCODE cohort was approved by the National Bioethics Committee of Iceland (VSN 03–007), which was conducted in agreement with conditions issued by the Data Protection Authority of Iceland. Personal identities of the participants' data and biological samples were encrypted by a third-party system (Identity Protection System), approved and monitored by the Data Protection Authority. The UK cohort was approved by the Parkside Health Authority (Now—NHS Health Research Authority, NRES Committee—West London & GTAC, UK, London, UK) under EC2359 "The Molecular Genetics of

Table 1. Characteristics of PCOS cases and controls from each cohort included in the meta-analysis.

Cohort	Subject Type	Number	Age (years)	BMI (kg/m2)	PCOS Definition	HA[(1)] n(%)	OD n(%)	PCOM n(%)
Rotterdam	Cases**	1184	28.8 (4.8)	26.1 (6.3)	NIH (41%) & Rotterdam (100%)[(2)]	439 (37.0)	946 (79.8)	661 (55.8)
	Controls	5799	60.5 (7.9)	27.6 (4.7)	Population Based Rotterdam Study	NA	NA	NA
UK (London/ Oxford)	Cases**	670	32.1 (6.8)	28.2 (7.9)	NIH (33%) & Rotterdam (100%)[(2)]	455 (67.9)	537 (80.1)	383 (57.2)
	Controls	1379	45 (0)[$]	26.8 (5.5)	1958 British Birth Cohort	NA	NA	NA
EGCUT	Cases**	157	30.7 (8.2)	26.2 (6.7)	Rotterdam[(2)]	NA	NA	NA
	Controls	2807	31.5 (7.3)	23.1 (5.5)	Population Based	NA	NA	NA
deCODE	Cases**	658	41.3 (8.7)	30.1 (7.8)	NIH (56%) & Rotterdam (100%)[(2)]	644 (97.9)	380 (57.7)	507 (77.1)
	Controls	6774	49.0 (9.9)	25.1 (4.9)	Population Based	NA	NA	NA
Chicago	Cases*	984	28.6 (5.5)	35.9 (8.5)	NIH	984 (100)	984 (100)	NA
	Controls	2963	46.8 (15.2)	27.0 (7.4)	Population Based NUgene	NA	NA	NA
Boston	Cases*	485	28.4 (6.7)	30.8 (8.7)	NIH	485 (100)	485 (100)	441 (90.9)
	Controls	407	27.2 (6.5)	23.8 (4.1)	Screened controls[(3)]	0	0	177 (43.4)
23andMe	Cases***	5,184	45.1 (13.6)	29.2 (8.2)	Self report (defined by questionnaire)	NA	NA	NA
	Controls	82,759	51.1 (15.7)	26.1 (6.1)	No PCOS by self report (defined by questionnaire)	NA	NA	NA

(1) Clinical or Biochemical.

(2) Rotterdam diagnostic criteria include the NIH criteria. All subjects from the indicated cohorts were used in the Rotterdam analysis.

(3) Controls were screened for regular menses and no hyperandrogenism.

* PCOS diagnosis was based on NIH criteria,

** Rotterdam criteria, or

*** self report.

Results are reported as mean (SD) or a number (%).

Abbreviations: BMI: body mass index, NA: not available, HA: hyperandrogenism, OD: ovulatory dysfunction (<10 menses per year), PCOM: polycystic ovarian morphology.

[$]All subjects are from the British Birth Cohort (born in 1958).

Polycystic Ovaries." The Rotterdam PCOS cohort, the COLA study, was approved by institutional review board (Medical Ethics Committee) of the Erasmus Medical Center (04–263). Controls from the Rotterdam Study were approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register and into the WHO International Clinical Trials Registry Platform under shared catalogue number NTR6831. The Chicago PCOS cohort was approved by the Northwestern IRB (#STU00008096). The control subjects from the NUgene study were approved by the Northwestern IRB (# STU00010003). The Estonia cohort was approved by the Research Ethics Committee of the University of Tartu approved the study (198T-18). The Twins UK study was approved by the St Thomas' Hospital Research Ethics Committee (EC04/015). The Nurses' Health Study (NHS I and II) was approved by the Partners Human Research Committee (#1999-P-011114).

Subjects

The meta-analysis included 10,074 cases and 103,164 controls from seven cohorts of European descent. For the analysis of PCOS related traits three additional cohorts, the Northern Finnish Birth Cohort (NFB66) [40], Twins UK [41] and the Nurses' Health Study (NHS) [42] were included. Cases were diagnosed with PCOS based on NIH or Rotterdam Criteria or by self-report. The NIH criteria require the presence of both OD and clinical and/or biochemical HA for a diagnosis of PCOS [6]. The Rotterdam criteria require two out of three features 1) OD defined by oligo- or amenorrhea (chronic menstrual cycle interval >35 days in all cohorts), 2) clinical and/or biochemical hyperandrogenism (HA) and/or 3) PCOM for a diagnosis of PCOS [7]. Non-NIH Rotterdam was defined by OD and PCOM or clinical and/or biochemical hyperandrogenism (HA) and PCOM. Self-reported female cases from research participants in the 23andMe, Inc. (Mountain View, CA, USA) cohort either responded "yes" to the question "Have you ever been diagnosed with polycystic ovary syndrome?" or indicated a diagnosis of PCOS when asked about fertility ("Have you ever been diagnosed with PCOS?" or "What was your diagnosis? Please check all that apply." Answer = PCOS), hair loss in men or women ("Have you been diagnosed with any of the following? Please check all that apply." Answer = PCOS) or research question ("Have you ever been diagnosed with PCOS?") [17]. 23andMe controls were female, only.

Hyperandrogenism was defined as hirsutism and quantified by the Ferriman-Gallwey (FG) score. The FG score assesses terminal hair growth in a male pattern in females, and a score above the upper limit of normal controls (>8) is considered hirsutism [43]. Hyperandrogenemia was defined as testosterone, androstenedione or DHEAS greater than the 95% confidence limits in control subjects in the individual population. OD was defined as cycle interval <21 or >35 days [44]. PCOM was defined as 12 or more follicles of 2–9 mm in at least one ovary or an ovarian volume >10 mL [7]. The quantitative PCOS traits included levels of total testosterone (T), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) and ovarian volume (**Table S1**). An overview of the cohorts, diagnostic criteria and number of subjects included in each subphenotype or trait analysis are summarized in **Tables 1** and **S1**.

Data collection and quality control

Each study provided summary results of genetic per-variant estimates produced in either case-control or trait association analyses. Adjustment for principle components was performed at the study level. The collected files underwent quality control (QC) by two independent analysts using the EasyQC pipeline [45]. Variants were excluded based on minor allele frequency (MAF) < 1%, imputation quality (R^2) < 0.3 or info < 0.4 for MACH and IMPUTE2 respectively [46,47]. Per-cohort QC results from EasyQC are shown (Table S7), and allele frequency spectrum for each cohort, and the combined cohort after meta-analysis is shown (**Figure S4**).

Meta-analysis of PCOS status and PCOS related traits

The per-variant estimates collected from the summary statistics of contributing studies were meta-analyzed using a fixed-effect, inverse-weighted-variance meta-analysis that employed either GWAMA [48] or METAL [49]. In addition to the overall meta-analysis, we performed meta-analyses for studies with available data for the separate PCOS diagnostic criteria: NIH, non-NIH Rotterdam [7] and self-report [17], as well as for the PCOS related traits of HA, OD and PCOM. The meta-analysis of PCOS status was performed using two models; (1) age-adjusted, (2) age and BMI-adjusted, given the high prevalence of obesity in affected women that resulted in cases being significantly heavier than controls in most cohorts (**Table 1**).

We removed any variants that were not present in more than 50% of the effective sample size prior to combining with 23andMe as this was the largest cohort in the meta-analysis, providing approximately 51% of the PCOS cases and 80% of controls. We also removed any variants only present in one study. The meta-analysis of PCOS related traits was performed adjusting for age and BMI. Identified variants were annotated for insight into their biological function using ANNOVAR [50] to assign refGene gene information, SIFT score [51], PolyPhen2 scores [52], CADD scores [53], GERP scores [54] and SiPhy log odds [55].

Comparison of PCOS diagnostic criteria

In order to compare different PCOS diagnostic criteria ((1) NIH, (2) non-NIH Rotterdam and (3) self-reported) included in the PCOS meta-analysis, an additional meta-analysis was performed to test for heterogeneity across these independent PCOS case groups. These three PCOS case groups were combined in an inverse variance weighted fixed meta-analysis and the heterogeneity statistics (Cochran's Q and I^2) were obtained using GWAMA [48]. Any variant with a statistically significant Cochran's Q p-value ($P<0.05/14 = 0.0036$ corrected for multiple testing) and $I^2>70\%$ were considered exhibiting heterogeneity across the PCOS case groups. Further

analysis of the heterogeneity included comparison of the 95% confidence intervals for the direction of effect and overlaps.

Identifying associations between PCOS Loci and PCOS related traits

In order to understand biology relevant to identified PCOS susceptibility, we assessed the association between index SNPs at each genome-wide-significant locus and the PCOS related traits HA, OD, PCOM as well as the quantitative traits testosterone, LH and FSH levels and ovarian volume. The threshold for significance in this analysis was $p<4.5\times10^{-4}$ (Bonferroni correction [0.05/(14 independent loci x 8 traits)].

Identifying shared risk loci between European ancestry and Han Chinese PCOS

In order to identify shared risk loci between the previously reported GWAS in Han Chinese PCOS cases and our European ancestry cohort, 13 independent signals (represented by 15 SNPs) at 11 genome-wide significant loci reported by Chen *et al*. [14] and Shi *et al*. [15] were investigated for association in our meta-analyses of PCOS and PCOS related traits. The adjusted P-value for this analysis was <0.00048 (Bonferroni correction [0.05/(13 independent signals x 8 traits)]).

Biologic function of genes in associated loci

Information on the biological function of the nearest gene (or genes, if variants were equidistant from more than one coding transcript and annotated as such by ANNOVAR [49] to the index SNP of each identified risk locus) was collected by performing a search of the Entrez Gene Database [56], and collecting the co-ordinates of the gene (genome build 37; hg19) as well as the cytogenetic location and the summary of the gene function. In addition to the EntrezGene Database queries, the gene symbol was used as a search term in the PubMed database [57], either alone or combined with the additional search term "PCOS" to identify relevant published literature in order to obtain information on putative biological function and involvement in the pathogenesis of PCOS (summarized in 1.1 Note in Data S1).

Weighted genetic risk score and prediction

One potential use of genetic risk scores is prediction of disease. The ability of genetic risk scores calculated from loci discovered in analysis of the different diagnostic criteria to discriminate cases from alternative criteria was measured. We constructed a weighted genetic risk score based on a meta-analysis excluding the Rotterdam Study subjects. The weighted genetic risk score was divided into quintiles and tested for association with PCOS in the Rotterdam cohort. The middle quintile was used as the reference and the odds for having PCOS based on both Rotterdam and NIH criteria was then calculated.

Additionally, the 23andMe results were used to select independent SNPs with cut-offs of $p<5\times10^{-4}$ to $p<5\times10^{-8}$. The Rotterdam cohort was then used to calculate risk scores and the area-under-the curve (AUC) for both NIH and Rotterdam diagnostic criteria. Analyses were performed using PLINK v1.9 and SPSS v21 (IBM Corp, Armonk, NY) [58].

Linkage disequilibrium (LD) score regression

To assess the level of shared etiology between PCOS and related traits, we performed genetic correlation analysis using LD-score regression [59]. Publicly available genome-wide summary statistics for body mass index (BMI) [60], childhood obesity [61], fasting insulin levels (adjusted for BMI) [62], type 2 diabetes [63], high-density lipoprotein (HDL) levels [64], menarche timing [65], triglyceride levels [64], coronary artery disease [66], depression [36], menopause [17] and male pattern balding [67] were used to estimate the genome-wide genetic correlation with PCOS. The adjusted P-value for this analysis was $p<0.0045$ after a Bonferroni correction (0.05/11 traits).

Mendelian randomization

Phenotypes of interest, both where there was evidence of shared genetic architecture and where there was previous evidence for genetic links, were assessed using Mendelian randomization methods. Mendelian randomization differs from LD score regression in that one phenotype is analysed as a potential causal factor for another. Mendelian randomization was performed using both inverse weighted variance and Egger's regression methods [68], with inverse weighted methods being more powerful, but Egger's methods being resistant to directional pleiotropy (where there are a set of SNPs that appear to have an alternative pathway of effect). We report here the results of the IVW methods as none of the analysis suggested that the MR-EGGERs results were more appropriate given that none of the EGGERs intercepts were significant (**Table 5**). In addition to the phenotypes implicated by the LD-score regression measures, male pattern balding has a strong biological rationale and was

therefore included. The genetic score for childhood obesity substantially overlaps with the score for adult BMI (such that the INSIDE violation—where the effect of SNPs on a confounding factor scales with that on the trait of interest—of Mendelian randomization would likely occur [69], so only a score for BMI was used, with the proviso that this represents BMI across the whole of the life course after very early infancy. The SNPs for depression were drawn from the results of a more recent analysis, for which there was not, at time of analysis, publicly available genome-wide data.

Credible Sets

We defined a locus as mapping within 500kb of the lead SNP. For each locus, we first calculated the posterior probability, π_{cj}, that the jth variant is driving the association, given by:

$$\pi_{cj} = \frac{\Lambda_j}{\Sigma_k \Lambda_k}$$

where the summation is over all retained variants in the locus. In this expression, Λ_j is the approximate Bayes' factor [70] for the jth variant, given by

$$\Lambda_j = \sqrt{\frac{V_j}{V_j + \omega}} exp\left[\frac{\omega \beta^2_{\ j}}{2V_j(V_j + \omega}\right]$$

where β_j and V_j denote the estimated allelic effect (log-OR) and corresponding variance from the meta-analysis. The parameter ω denotes the prior variance in allelic effects, taken here to be 0.04 [70]. The 99% credible set [71] for each signal was then constructed by: (i) ranking all variants according to their Bayes' factor, Λ_j; and (ii) including ranked variants until their cumulative posterior probability of driving the association attained or exceeded 0.99.

Results

We identified 14 genetic susceptibility loci associated with PCOS, adjusting for age, at the genome-wide significance level ($P < 5.0 \times 10^{-8}$) bringing the total number of PCOS associated loci to nineteen (**Table 2** and S2 and **Figure 1**).

Figure 1. Manhattan plot

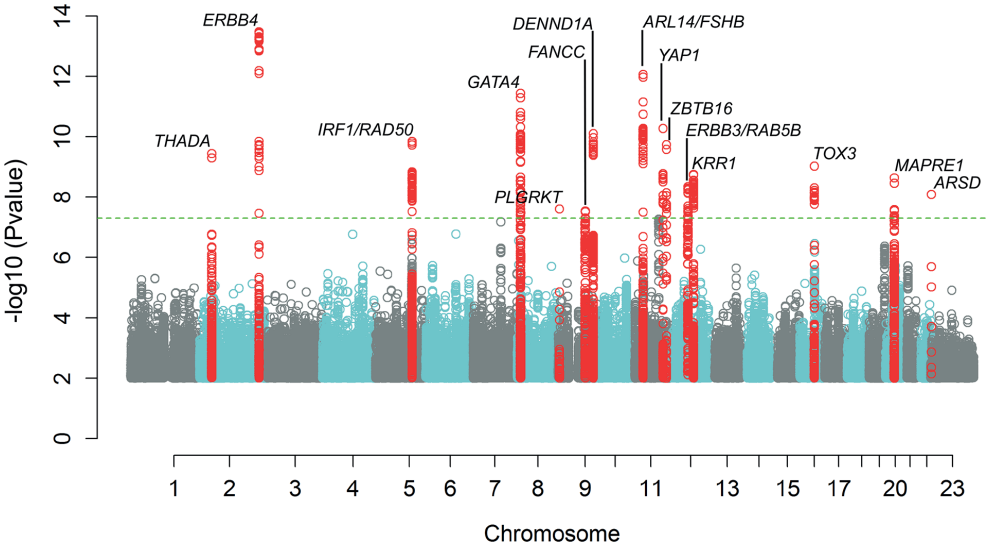


Figure 1. Manhattan plot indicating genome-wide significant variants. The X axis indicates the chromosome depicted by alternating black and grey. The Y axis indicates the inverse log10 of the p-value (-log10(p)). The line designates the minimum p-value for genome-wide significance.

Three of these loci were novel associations (near PLGRKT, ZBTB16 and MAPRE1 respectively; shown bold in table 2.Six of the 11 reported associations were previously observed in Han Chinese PCOS women [14,15]. Eight loci have been reported in European PCOS cohorts [16,17]. Obesity is commonly associated with PCOS and in most of the cohorts, cases were heavier than controls (**Table 1**). However, adjusting for both age and BMI did not identify any novel loci; and the 14 loci remained genome-wide significant. All variants demonstrated the same direction of effect across all phenotypes including NIH, non-NIH Rotterdam, and self-report (**Figure 2** and Table S2). Only one SNP near *GATA4/NEIL2* showed significant evidence of heterogeneity across the different diagnostic groups (rs804279, Het P = 2.6×10^{-5}; **Figure 2** and Table S3). For this SNP, the largest effect was seen in NIH cases and the smallest in self-reported cases. Credible set analysis, which prioritizes variants in a given locus with regards to being potentially causal, was able to reduce the plausible interval for the causal variant(s) at many loci (Table S4). Of note, 95% of the signal at the *THADA* locus came from two SNPs. Examination of previously published genome-wide significant loci from Han Chinese PCOS women [14,15]

demonstrated that index variants from the *THADA*, *FSHR*, *C9orf3*, *YAP1* and RAB5B loci were significantly associated with PCOS after Bonferroni correction for multiple testing in our European ancestry subjects (Table S5).

Table 2. The 14 Genome wide significant variants associated with PCOS in the meta-analysis

Chr:Position[1]	rsID	Alleles[2]	EAF[3]	Beta	Odds Ratio (95% CI)[4]	Std. Error	Nearest Gene	P-value	Effective N[5]	Ref[6]
2:43561780	rs7563201	A/[G]	0.4507	-0.1081	0.90 (0.87–0.93)	0.0172	*THADA*	3.678e-10	17192	
2:213391766	rs2178575	G/[A]	0.1512	0.1663	1.18 (1.13–1.23)	0.0219	*ERBB4*	3.344e-14	17192	17
5:131813204	rs13164856	[T]/C	0.7291	0.1235	1.13 (1.09–1.18)	0.0193	*IRF1/RAD50*	1.453e-10	17192	17
8:11623889	rs804279	A/[T]	0.2616	0.1276	1.14 (1.10–1.18)	0.0184	*GATA4/NEIL2*	3.761e-12	16895	16
9:5440589	**rs10739076**	**C/[A]**	**0.3078**	**0.1097**	**1.12 (1.07–1.16)**	**0.0197**	***PLGRKT***	**2.510e-08**	**17192**	
9:97723266	rs7864171	G/[A]	0.4284	-0.0933	0.91 (0.88–0.94)	0.0168	*FANCC*	2.946e-08	17192	16
9:126619233	rs9696009	G/[A]	0.0679	0.202	1.22 (1.15–1.30)	0.0311	*DENND1A*	7.958e-11	17192	
11:30226356	rs11031005	[T]/C	0.8537	-0.1593	0.85 (0.82–0.89)	0.0223	*ARL14EP/FSHB*	8.664e-13	17192	16,17
11:102043240	rs11225154	G/[A]	0.0941	0.1787	1.20 (1.13–1.26)	0.0272	*YAP1*	5.438e-11	17192	17
11:113949232	**rs1784692**	**[A]/G**	**0.8237**	**0.1438**	**1.15 (1.10–1.14)**	**0.0226**	***ZBTB16***	**1.876e-10**	**17192**	
12:56477694	rs2271194	A/[T]	0.416	0.0971	1.10 (1.07–1.14)	0.0166	*ERBB3/RAB5B*	4.568e-09	17192	17
12:75941042	rs1795379	C/[T]	0.2398	-0.1174	0.89 (0.86–0.92	0.0195	*KRR1*	1.808e-09	17192	17
16:52375777	rs8043701	[A]/T	0.815	-0.1273	0.88 (0.85–0.92)	0.0208	*TOX3*	9.610e-10	17192	
20:31420757	**rs853854**	**A/[T]**	**.4989**	**-.0975**	**0.91 (0.88–0.94)**	**0.0163**	***MAPRE1***	**2.358e-09**	**17192**	

[1]Chr—Chromosome:Position (bp) in hg19;

[2]Alleles are shown as Major/Minor by allele frequency in 1000G EUR cohort, with the effect allele shown within [];

[3]Effect allele frequency;

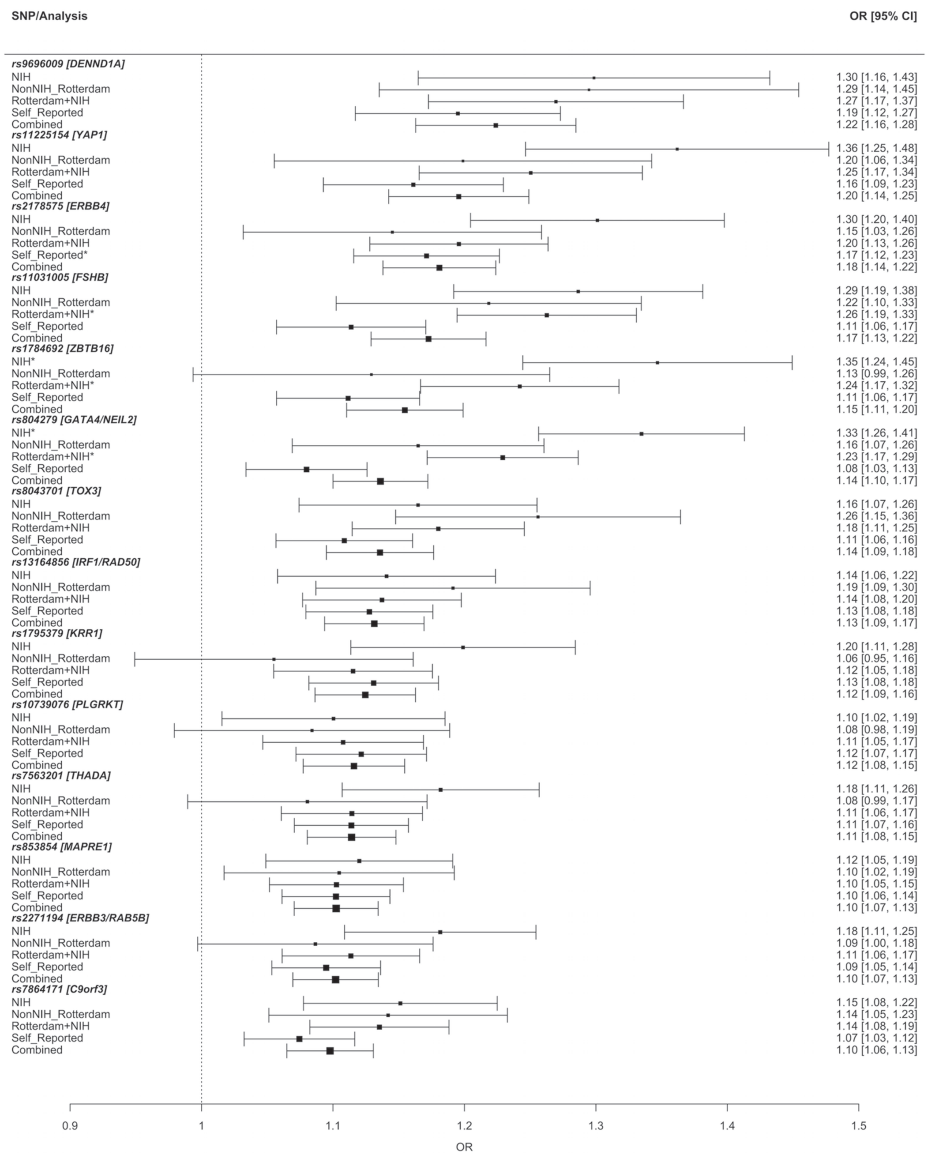
[4]95% Confidence Interval of the Odds Ratio;

[5]Effective N—effective sample size;

[6]Ref = Reference.

Loci previously identified in GWAS studies of European ancestry are referenced. Novel associations with PCOS not previously reported are shown in bold. EAF = Effect Allele Frequency.

Figure 2. Odds of polycystic ovary syndrome as a function of diagnostic criteria applied



*Y-axis: diagnostic criteria; X-axis: OR and 95% CI for PCOS. Data derived as follows: NIH = only NIH diagnostic criteria; NonNIH, Rotterdam = Rotterdam diagnostic criteria excluding the subset fulfilling NIH diagnostic criteria; Rotterdam+NIH = all groups except self-reported; self-reported = 23andMe; and combined = all groups. Specific OR's [95% CI, 5% CI] are indicated on the right. rs804279 in the GATA4/NEIL2 locus demonstrates significant heterogeneity (Het P=2.6x10^{-5}). The * indicates statistically significant association for PCOS and the variant in that specific stratum.*

We assessed the association of the PCOS susceptibility variants identified in the GWAS meta-analysis with the PCOS related traits: HA, OD, PCOM, testosterone,

FSH and LH levels, and ovarian volume in PCOS cases (**Table 3** and S6 and Figure S2). We found four variants associated with HA, eight variants associated with PCOM and nine variants associated with OD. Of the eight loci associated with PCOM, seven were also associated with OD. Three of the four loci associated with HA were also associated with OD and PCOM. Two additional loci were associated with OD alone, one of which was the locus near *FSHB* (Table S6). This locus was also associated with LH and FSH levels. There was a single PCOS locus near *IRF1/RAD50* associated with testosterone levels (Table S6). We repeated this analysis with susceptibility variants reported previously in Han Chinese PCOS cohorts [14,15]. In this analysis, there was one association with HA (near *DENND1A*), three with PCOM and three with OD (Figure S2 and Table S5). A limitation of these analyses is the variable sample size across the phenotypes analysed. Additionally, the known referral bias for the more severely affected NIH phenotype (patients having both OD and HA) may result in more PCOS diagnoses than the other criteria [22], and may have contributed to the number of associations between the identified PCOS risk loci and these phenotypes.

Table 3. Associations of PCOS GWAS meta-analysis susceptibility variants and PCOS related traits

Chr:Position	rsID	Gene	Ref. allele	Other allele	Hyperandrogenism			PCOM		OD	
					EAF	Beta	P-value	Beta	P-value	Beta	P-value
2:213391766	rs2178575	*ERBB4*[*]	G	A	0.83	-0.126	4.3E-03	**-0.24**	**1.4E-05**	**-0.23**	**1.2E-11**
2:43561780	rs7563201	*THADA*[*†]	G	A	0.56	0.061	8.0E-02	**0.16**	**3.7E-04**	0.08	1.5E-03
5:131813204	rs13164856	*IRF1/RAD50*[*]	T	C	0.73	0.092	1.8E-02	0.16	1.4E-03	0.08	5.6E-03
8:11623889	rs804279	*GATA4/NEIL2*[*]	A	T	0.27	0.126	8.7E-04	**0.22**	**1.5E-06**	**0.16**	**9.9E-09**
9:126619233	rs9696009	*DENND1A*[†]	G	A	0.94	**-0.330**	**2.9E-07**	**-0.32**	**4.0E-05**	**-0.36**	**4.4E-15**
9:5440589	rs10739076	*PLGRKT*	A	C	0.30	0.026	5.3E-01	0.10	5.9E-02	0.00	8.9E-01
9:97723266	rs7864171	*C9orf3*[*†]	G	A	0.60	**0.124**	**3.8E-04**	**0.19**	**1.3E-05**	**0.10**	**2.3E-04**
11:30226356	rs11031005	*ARL14EP/FSHB*[*]	T	C	0.85	-0.079	8.2E-02	-0.18	1.3E-03	**-0.13**	**2.8E-04**
11:102043240	rs11225154	*YAP1*[*†]	G	A	0.91	-0.144	1.4E-02	**-0.24**	**3.5E-04**	**-0.23**	**5.7E-08**
11:113949232	rs1784692	*ZBTB16*	T	C	0.85	0.146	4.6E-03	**0.30**	**2.8E-06**	**0.21**	**6.6E-09**
12:75941042	rs1795379	*KRR1*[*]	T	C	0.24	-0.104	8.0E-02	-0.16	1.5E-03	**-0.11**	**1.8E-04**
12:56477694	rs2271194	*ERBB3/RAB5B*[†]	A	T	0.42	**0.126**	**2.7E-04**	**0.17**	**7.9E-05**	**0.13**	**1.4E-06**
16:52375777	rs8043701	*TOX3*[†]	A	T	0.82	**-0.166**	**1.4E-04**	-0.17	1.5E-03	-0.08	9.2E-03
20:31420757	rs853854	*MAPRE1*	T	A	0.50	0.111	9.8E-04	0.10	2.1E-02	0.05	3.8E-02

Significant associations are highlighted in bold. Variant previously reported as a PCOS risk variant in

[*]European or

[†]Han Chinese populations.

In the analyses looking at the weighted genetic risk score in the Rotterdam cohort, we observed an increase in the risk for PCOS (Figure S3). Compared to individuals in the third quintile (reference group), individuals in the top 5th quintile of risk score have an OR of 1.9 (1.4–2.5; 95% CI) for PCOS based on NIH criteria and an OR of 2.1 (1.7–2.5; 95% CI) for Rotterdam criteria based PCOS. Of the associations, only the effect estimate for the Rotterdam criteria was significant, possibly due to the smaller size available with cases diagnosed according to the NIH criteria. When

looking at the area under the ROC curves at SNPs with different P-value thresholds, we found a maximum AUC of 0.54 using SNPs with a P-value < $5x10^{-6}$ for both diagnostic criteria. While this is significantly better than chance, it is unlikely that a risk score generated from the variants discovered to date would represent a clinically relevant tool.

LD score regression analysis revealed genetic correlations with childhood obesity, fasting insulin, T2D, HDL, menarche timing, triglyceride levels, cardiovascular diseases and depression (**Table 4**) suggesting that there is shared genetic architecture and biology between these phenotypes and PCOS. There were no genetic correlations with menopause timing or male pattern balding. Mendelian randomization suggested that there was a causal role for BMI, fasting insulin and depression pathways (**Table 5**). Interestingly, while there was no genetic correlation detected for male pattern balding or menopause timing with PCOS, the Mendelian randomization analyses were significant. The difference in the genetic correlation compared to the Mendelian randomization result suggests that there may be a small number of key biological process that are common between the phenotypes, and that the common genetic causal variants are limited only to the variants shared by the subset of key biological processes. The importance of BMI pathways on reproductive phenotypes was further demonstrated by the attenuation of significance of Mendelian randomization analysis for age-at-menarche when BMI-associated variants were excluded from the analysis.

Table 4 LD Score regression results using the LDSC method

Phenotype	Genetic Correlation	SE	Z	P-value
Body mass index	0.34	0.039	8.60	8.21×10^{-18}
Childhood obesity	0.34	0.066	5.17	2.40×10^{-7}
Fasting insulin levels	0.44	0.087	5.01	5.33×10^{-7}
Type 2 diabetes	0.31	0.068	4.47	7.84×10^{-6}
High-density lipoprotein levels	-0.23	0.059	-3.96	7.40×10^{-5}
Menarche	-0.16	0.042	-3.76	1.71×10^{-4}
Triglyceride levels	0.19	0.052	3.61	3.05×10^{-4}
Coronary artery disease	0.23	0.069	3.32	8.86×10^{-4}
Depression	0.205	0.0582	3.5203	0.0004
Menopause	-0.014	0.0183	-0.762	0.4461
Male pattern balding	0.0149	0.0168	0.8861	0.3756

Table 5. Mendelian randomization using an inverse weighted variant method

Potential Risk factor	IVW method[1]			MR-EGGER intercept p-value[2]
	Beta	SE	P-value	
Body mass index	0.72	0.072	1.56×10^{-23}	0.13
Fasting insulin levels*	0.03	0.007	1.73×10^{-5}	0.06
Male pattern balding	0.05	0.017	0.0034	0.93
Menopause	0.1	0.022	1.31×10^{-5}	0.39
Depression	0.77	0.213	0.00029	0.64

*Loci used were initially reported in an analysis of fasting insulin adjusted for BMI.

[1]IVW = inverse weighted variant,

[2]Mendelian Randomization (MR)-Egger intercept p values were not significant. Therefore, MR-Egger results are not presented.

Discussion

We found 14 independent loci significantly associated with the risk for PCOS, including three novel loci. The 11 previously reported loci implicated neuroendocrine and metabolic pathways that may contribute to PCOS (1.1 Note in S1 Data). Two of the novel loci contain potential endocrine related candidate genes. The locus harbouring rs10739076 contains several interesting candidate genes; *PLGRKT*, a plasminogen receptor and several genes in the insulin superfamily; *INSL6*, *INSL4* and *RLN1*, *RLN2* which are endocrine hormones secreted by the ovary and testis and are suspected to impact follicle growth and ovulation [23]. *ZBTB16* (also known as *PLZF*) has been marked as an androgen-responsive gene with anti-proliferative activity in prostate cancer cells [24]. *PLZF* activates *GATA4* gene transcription and mediates cardiac hypertrophic signalling from the angiotensin II receptor 2 [25]. Furthermore, *PLZF* is upregulated during adipocyte differentiation *in vitro* [26] and is involved in control of early stages of spermatogenesis [27] and endometrial stromal cell decidualization [28]. The third novel locus harbours a metabolic candidate gene; *MAPRE1* (interacts with the low-density lipoprotein receptor related protein 1 (LRP1), which controls adipogenesis [29] and may additionally mediate ovarian angiogenesis and follicle development [30] (1.2 Note in S1 data). Thus, all the new loci contain genes plausibly linked to both the metabolic and reproductive features of PCOS.

We found that there was no significant difference in the association with case status for the majority of the PCOS-susceptibility loci by diagnostic criteria. All susceptibility variants demonstrated the same direction of effect for the NIH phenotype, non-NIH Rotterdam phenotype and self-report, with only one variant demonstrating significant heterogeneity among the groups. It is of considerable interest that the cohort of research participants from the personal genetics company 23andMe, Inc., identified by self-report, had similar risks to the other cohorts where the diagnosis was clinically confirmed. Our findings suggest that the genetic architecture of these PCOS definitions does not differ for common susceptibility

variants. Only one locus, *GATA4/NEIL2* (rs804279), was significantly different across diagnostic criteria: most strongly associated in NIH compared to the Rotterdam phenotype and self-reported cases. Deletion of *GATA4* results in abnormal responses to exogenous gonadotropins and impaired fertility in mice [31]. The locus also encompasses the promoter region of *FDFT1*, the first enzyme in the cholesterol biosynthesis pathway [32], which is the substrate for testosterone synthesis, and is associated with non-alcoholic fatty liver disease [33]. The major difference between the NIH phenotype and the additional Rotterdam phenotypes is metabolic risk; the NIH phenotype is associated with more severe insulin resistance [34]. rs804279 does not show association with any of the metabolic phenotypes in the T2D diabetes knowledge portal so it may represent a PCOS-specific susceptibility locus.

The significant association of PCOS GWAS meta-analysis susceptibility variants with the cardinal PCOS related traits OD, HA and PCOM further strengthened the hypothesis that specific variants may confer risk for PCOS through distinct mechanisms. Three variants at the *C9orf3*, *DENND1A*, and *RAB5B* were associated with all PCOS related traits. The findings were consistent with the Han Chinese *DENND1A* variant association with HA, as suggested previously [35]. Thus, these loci, along with *GATA4/NEIL2* (as discussed above) may help identify pathways that link specific PCOS related traits with greater metabolic risk. In contrast, the variants at the *ERBB4*, *YAP1*, and *ZBTB16* loci were strongly associated with OD and PCOM, and therefore, might be more important for links to menstrual cycle regularity and fertility. In addition, the *FSHB* variant was associated with the levels of FSH and LH [16,17], suggesting that it may act by affecting gonadotropin levels. This variant maps 2kb upstream from open chromatin (identified by DNase-Seq) and an enhancer (identified by peaks for both H3K27Ac and H3K4me1) in a lymphoblastoid cell line from ENCODE, indicating a potential role for a regulatory element ~25kb upstream from the *FSHB* promoter. Furthermore, the association between the *IRF1/RAD50* variant and testosterone levels may indicate a regulatory role in testosterone production.

Of note, results of the follow-up analysis show a high level of shared biology between PCOS and a range of metabolic outcomes consistent with the previous findings [17]. In particular, there is genetic evidence for increased BMI as a risk factor for PCOS. There is also genetic evidence that fasting insulin might be an independent risk factor. This study also confirmed a causal association with the pathways that underlie menopause [17], suggesting that PCOS has shared aetiology with both classic metabolic and reproductive phenotypes. Furthermore, there was an apparent effect of depression-associated variants on the likelihood of PCOS, suggesting a role for psychological factors on hormonally related diseases. However, the links between PCOS and depression might be complicated by pathways that are also related to

BMI, as BMI pathways are causal in both PCOS and depression [36]. In addition, male-pattern balding-associated variants showed strong effects on PCOS, suggesting that this might be a male manifestation of PCOS pathways, as has been previously suggested [18,20,21,37]. This observation may reflect the biology of hair follicle sensitivity to androgens, seen in androgenetic alopecia, a well-recognised feature of HA and PCOS [38,39]. The Mendelian randomization results for male-pattern balding and menopause are significant despite non-significant genetic correlation results, suggesting that the shared aetiology may be specific to only a few key pathways.

In conclusion, the genetic underpinnings of PCOS implicate neuroendocrine, metabolic and reproductive pathways in the pathogenesis of disease. Although specific phenotype stratified analyses are needed, genetic findings were consistent across the diagnostic criteria for all but one susceptibility locus, suggesting a common genetic architecture underlying the different phenotypes. There was genetic evidence for shared biologic pathways between PCOS and a number of metabolic disorders, menopause, depression and male-pattern balding, a putative male phenotype. Our findings demonstrate the extensive power of genetic and genomic approaches to elucidate the pathophysiology of PCOS.

1.1. Supplementary Results.

In addition to these 14 significant loci, there was suggestive evidence of a 15th signal, rs151212108, near *ARSD* on the X chromosome. This SNP shows a relatively large effect size (OR:1.72, CI:1.43-2.07, P=$8.35x10^{-9}$). However, the SNP had low sample number (overall minor allele frequency=0.0765) with poor imputation quality and it was present in only three studies; Oxford, deCODE, and Chicago. Further, the signal showed nominally significant heterogeneity (P =0.028) in the direction of effect estimates between Oxford, where the effect allele had a protective effect, and deCODE and Chicago, where the effect allele increased risk of PCOS. Thus, this signal was less robust than our other signals and will require further confirmation. Accordingly, we have not included this locus in downstream analyses. A detailed review of genes within reported loci is included in the Supplementary Notes (Section 1.2).

1.2 Literature Lookup of genes at PCOS risk loci. Summary of published literature on gene function of PCOS susceptibility loci.

1. THADA (Thyroid Adenoma Associated): Located at 2p21 (Chr 2: 43561780-43561780). Encodes a transcript of largely unknown function. THADA encodes thyroid adenoma-associated protein, which is expressed in pancreas, adrenal medulla, thyroid, adrenal cortex, testis, thymus, small intestine, and stomach.[71] This gene has been identified in GWAS for gestational weight gain, inflammatory bowel disease and PCOS and more specifically with the phenotype trait PCOM.[14,71-74] THADA has been associated with endocrine and metabolic disturbances commonly found in PCOS, such as increased LH, testosterone and LDL levels and T2D.[75] Proposed to modify PCOS risk through metabolic mechanisms.[76]

2. ERBB4 (erb-b2 receptor tyrosine kinase 4; also known as HER4): Located at 2q33.3-q34. Fourth member of the EGFR (epidermal growth factor *receptor*) family and the Tyr protein kinase family (USCS, GeneNetwork, RefSeq). Participates in the YAP/Hippo pathway, which regulates cell proliferation, differentiation and apoptosis and has been associated with the size of the primordial follicle pool in mice, female reproductive capacity in Drosophila, and it is hypothesized that disruption of Hippo signalling can promote follicle growth.[77-81] Tyrosine-protein kinase plays an essential role as cell surface receptor for neuregulins and EGF family members and regulates development of the heart, the central nervous system and the mammary gland.[82] HER4 is characterized by anti-proliferative and pro-apoptotic activity, is co-expressed in 90% of ER positive breast tumors. Proposed to modify PCOS risk through metabolic mechanisms.[76] Suggested to have a pathogenic role in cystogenesis in polycystic kidney disease.[83]

3. IRF1 (interferon regulatory factor 1): Located at 5q31.1 (Chr5: 131813204-131813204). Belongs to the interferon regulatory transcription factor (IRF) family.

Activates the transcription of interferons alpha and beta, and genes induced by interferons alpha, beta and gamma (USCS, GeneNetwork, NCBI gene database). IRF1 displays a functional diversity in the regulation of cellular responses including host response to viral and bacterial infections, inflammation, and cell proliferation and differentiation, regulation of the cell cycle and induction of growth arrest and programmed cell death following DNA damage (UniProtKB). Acts as a tumor suppressor and plays a role not only in antagonism of tumor cell growth but also in stimulating an immune response against tumor cells.[84] It has been shown in fathead minnow that IRF1 may function as early molecular switch to control phenotypic changes in ovary tissue architecture and function in response to androgen or antiandrogen exposure.[85]

4. RAD50 (RAD50 homolog): Located at 5q31 (Chr5: 131892616-131980313). Rad50, a protein involved in DNA double-strand break repair.[82] This protein forms a complex with MRE11 and NBS1. The protein complex binds to DNA and displays numerous enzymatic activities that are required for non-homologous joining of DNA ends, and is important for DNA double-strand break repair,[86] telomere maintenance, and meiotic recombination.[87] Knockout studies of the mouse homolog suggest this gene is essential for cell growth and viability.[88]

5. GATA4 (GATA binding protein 4): Located at 8p23.1-p22 (Chr 8: 11623889-11623889). GATA4 encodes a zinc-finger transcription factor that recognizes the GATA motif in the promoters of various genes (RefSeq). GATA4 is implicated in regulating granulosa cell differentiation, proliferation and function and is expressed in follicles, embryoid bodies and chorion of women with PCOS.[89] Knockdown of GATA4 and GATA6 impairs folliculogenesis and induces infertility.[30,90] The loss of GATA4 within the ovary results in impaired granulosa cell proliferation and theca cell recruitment.[91] Knockdown of both genes affects expression of FSH receptor, LH receptor, inhibin α and β.[30,89] In rats with reproductive and metabolic abnormalities similar to PCOS, GATA4 has been associated with the biosynthesis and metabolism of steroids.[92] It is also proposed to modify PCOS risk through metabolic and inflammatory mechanisms.[76]

6. PLGRKT (Plasminogen receptor, C-terminal lysine transmembrane protein): Located at 9p24.1 (Chr 9; 5440339-5440839). PLGRKT encodes a plasminogen receptor involved in regulating macrophage migration and regulates catecholamine release.[93] The region also includes genes for several members of the insulin superfamily (INSL6, INSL4, RLN1, RLN2), which have roles in spermatogenesis, follicle growth and ovulation.[22,94]

7. FANCC (Fanconi anemia, complementation group C): Located at: 9q22.3 (Chr 9: 97723266-97723266). Member of the Fanconi anemia complementation group which, amongst others, includes FANCD1 (BRCA2). Members of the Fanconi anemia complementation group are related by their assembly into a common nuclear

protein complex (RefSeq). This gene encodes the protein for complementation group C. Fanconi anemia is a recessive repair deficiency disorder, characterized by cytogenetic instability, hypersensitivity to DNA crosslinking agents, chromosomal breakage and defective DNA repair (UCSC, GeneNetwork). FANCC is a DNA repair protein that may operate in a post replication repair or a cell cycle checkpoint function.[82] May be implicated in interstrand DNA cross-link repair and in the maintenance of normal chromosome stability. Was recently shown to have a mitophagy function as well, and is required for clearance of damaged mitochondria.[95]

8. C9orf3. Located at 9q22.32. Has been previously associated with PCOS.[15] However, the region also includes genes for two hormones that regulate gluconeogenesis (FBP1, FBP2), and for PTCH1, which is a receptor for hedgehog proteins. In mice, the hedgehog signalling has been shown to be important for ovarian follicle development and is also implicated in the proliferation and steroidogenesis of theca cells.[96] This is supported by the association between rs4385527 in C9orf3 and anovulation, HA and polycystic ovarian morphology (PCOM).[73]

9. DENND1A (DENN/MADD domain-containing protein 1A): Located at 9q33.3 (Chr 9: 126619233-126619233). Member of the connecdenn family and functions as a guanine nucleotide exchange factor involved for the early endosomal small GTPase RAB35 (UCSC, RefSeq). Regulates clathrin-mediated endocytosis (a major mechanism for internalization of proteins and lipids) through RAB35 activation (USCS, RefSeq). DENND1A variant 2 (DENND1A.V2) protein and mRNA levels are increased in PCOS theca cells and play a key role in the hyperandrogenemia associated with PCOS.[97] The *DENND1A* locus has also been associated with PCOM and elevated serum insulin levels in PCOS women.[73,75] Some SNP's in DENND1A have even been associated with endometrioid carcinoma. It has been suggested that *DENND1A, LHCGR, INSR*, and *RAB5B* form a hierarchical signalling network that can influence androgen synthesis.[98]

10. ARL14EP (ADP-ribosylation factor-like 14 effector protein): Located at 11p14.1. Encodes an effector protein, which interacts with ADP-ribosylation factor-like 14 [ARL14], beta-actin and actin-based motor protein myosin 1E. ARL14 controls the export of major histocompatibility class II molecules by connecting to the actin network via this effector protein (RefSeq).

11. FSHB (Follicle stimulating hormone, beta polypeptide): Located at 11p14.1 (Chr 11; 30226356-30226356). FSHB is a member of the pituitary glycoprotein hormone family and encodes the β-subunit of the follicle-stimulating hormone (FSH) (RefSeq). FSH regulates folliculogenesis. FSHB polymorphisms influence early follicular phase FSH concentrations and IVF treatment outcome.[99] SNPs in the FSHB region are known to be associated with circulating FSH, LH and AMH levels

but also with PCOS.[99-105] Overexpression of FSHB could cause polycystic ovary syndrome in women, whereas inactivating mutations of the FSHB gene, encoding for the hormone's unique β-subunit, cause infertility by primary amenorrhea.[106,107]

12. YAP1 (Yes-associated protein 1): Located at 11q13 (Chr 11; 102043240-102043240). YAP1 is an effector protein in the Hippo pathway involved in development, growth, repair, and homeostasis (RefSeq). This pathway also plays a pivotal role in organ size control and tumor suppression by restricting proliferation and promoting apoptosis.[82] Has been associated with the size of the primordial follicle pool in mice, female reproductive capacity in *Drosophila*, and it is hypothesized that disruption of Hippo signaling can promote follicle growth.[79] The candidacy of YAP1 as a susceptibility gene for PCOS has been highlighted in several studies.[15,108,109]

13. ZBTB16 (Zinc finger and BTB domain containing 16, also known as PLZF): Located at 11q23.1 (Chr 11; 113949232-113949232). Member of the Krueppel C2H2-type zinc-finger protein family and encodes a zinc finger transcription factor that contains nine Kruppel-type zinc finger domains at the carboxyl terminus. This protein is located in the nucleus and is involved in cell cycle progression. The zinc finger protein has a pro-apoptotic and anti-proliferative activity and has been marked as an androgen-responsive gene with anti-proliferative activity in prostate cancer cells.[23] PLZF binds to the GATA4 gene regulatory region and activates GATA4 transcription and mediates cardiac hypertrophic signaling from angiotensin II receptor 2.[24] The loss of PLZF has been related to increased proliferation, invasiveness and motility, and resistance to apoptosis in different cancer cell types.[110] PLZF is considered a tumor suppressor gene in various cell types and tissues. Up-regulated during adipocyte differentiation *in vitro*.[25] Involved in control of early stages of spermatogenesis,[26] and critical for endometrial stromal cell decidualization.[27]

14. ERBB3 (erb-b2 receptor tyrosine kinase 3; also known as HER3): Located at 12q13. A member of the EGFR family of receptor tyrosine kinases (RefSeq). The ERBB3 gene is a potential susceptibility locus for T1D and has also been associated with PCOS.[15,111] ERBB4 together with ERBB3-binding protein 1 may modulate the protein cascade that leads to differentiation of ovarian somatic cells. ERBB3 interacts with the YAP protein in the Hippo pathway and is implicated in ovarian cell tumors.[112] The same region also includes RAB5B and a SNP in this region has been associated with response to glycose stimulation.[113]

15. RAB5B (Member of the RAS oncogene family): Located at 12q13. Member of the RAS oncogene family. RAB5B is an isoform of RAB5, a member of the small G protein family. Rab5 regulates fusion and motility of early endosomes, and is a marker of the early endosome compartment.[114] Endogenous Rab5B may work in conjunction or in sequence with Rab5A to facilitate the trafficking of EGFR.[115]

RAB5b has previously been identified in PCOS in women of Han Chinese and European descent.[109] A variant near this gene has been associated with insulin and glucose levels.[113] It has been suggested that *DENND1A, LHCGR, INSR*, and *RAB5B* form a hierarchical signaling network that can influence androgen synthesis.[98] Proposed to modify PCOS risk through metabolic mechanisms.[76] RAB5B shows lower expression levels in adipose tissue from PCOS women compared to healthy controls.[116]

16. KRR1 (KRR1, small subunit (SSU) processome component, homolog (yeast)): Located at 12q21.2 (Chr 12; 75941042-75941042). Required for 40S ribosome biogenesis. Involved in nucleolar processing of pre-18S ribosomal RNA and ribosome assembly (inferred function based on sequence similarity).[82] The region also includes the testosterone- and estrogen-sensitive GLIPR1, GLIPR1L1 and GLIPR1L2 genes, which encode proteins involved in male germ cell maturation and sperm-oocyte binding.[117-119] Proposed to modify PCOS risk through metabolic mechanisms.[76]

17. TOX3 (TOX high mobility group box family member 3): Located at 16q12.1. This gene regulates Ca2+-dependent neuronal transcription through interaction with the cAMP-response-element-binding protein (CREB).[120] The protein encoded by this gene contains an HMG-box, indicating that it may be involved in bending and unwinding of DNA and alteration of chromatin structure (RefSeq). The C-terminus of the encoded protein is glutamine-rich due to CAG repeats in the coding sequence. A minor allele of this gene has been implicated in an elevated risk of breast cancer. In normal human tissues, TOX3 is largely expressed in the central nervous system (CNS), in the ileum, and within the brain in the frontal and occipital lobe. TOX3 overexpression induces transcription involving isolated estrogen-responsive elements and estrogen-responsive promoters, and protects neuronal cells from cell death caused by endoplasmic reticulum stress or BAX overexpression.[120] *TOX3* has been highlighted as a potential PCOS susceptibility locus before and there is evidence it may modify the hyperandrogenemic aspects of the syndrome.[15,121] Proposed to modify PCOS risk through inflammatory mechanisms.[76]

18. MAPRE1 (Microtubule-associated protein, RP/EB family, member 1, also known as EB1): Located at 20q11.1-q11.23 (Chr 20; 31420757-31420757). EB1 interacts with the low-density lipoprotein receptor related protein 1 (LRP1), which controls adipogenesis[28] and may additionally mediate ovarian angiogenesis and follicle development.[29] EB1 binds to the plus end of microtubules and regulates the dynamics of the microtubule cytoskeleton.[82] It is thought that this protein is involved in suppression of microtubule dynamic instability, regulation of microtubule polymerization and spindle function, and chromosome stability (RefSeq).

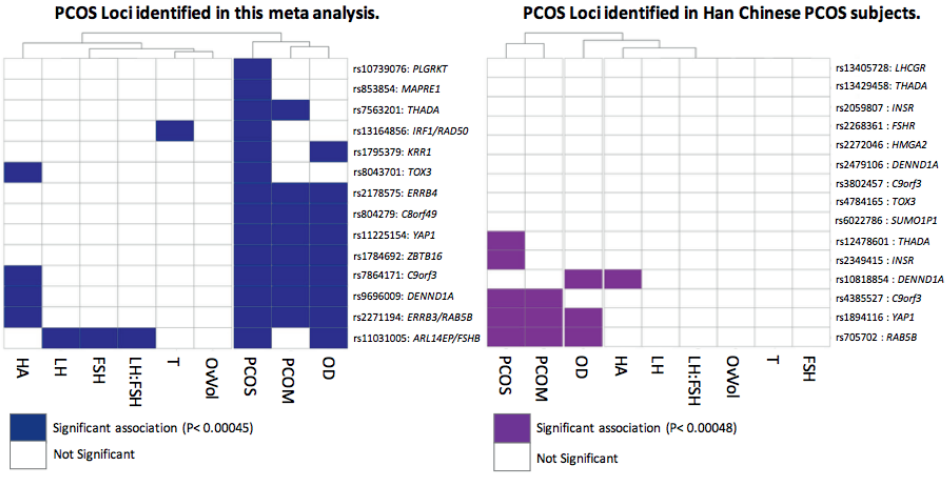
19. ARSD (arylsulfatase D): Located at Xp22.3 (X-chromosome; 2846021-2846021). ARSD is a member of the sulfatase family and located within a cluster of

similar arylsulfatase genes on chromosome X. The encoded proteins are essential for the correct composition of bone and cartilage matrix (RefSeq, GeneNetwork, USCS). This gene has been marked as a prognostic marker in chronic lymphocytic leukemia and has been suggested as a biological mechanism in chroinic lymphocytic leukemia – CLL.[122] The Xp22.3 region also includes the gene for glycogenin 2 (GYG2), which is involved in glycogen biosynthesis and blood glucose homeostasis. It has been shown that glycogen biosynthesis pathways are impaired in PCOS.[123,124]

Supplementary note on gene enrichment analysis.
We used MAGENTA (Meta-analysis Gene-set Enrichment of Variant Associations; version 2.4;[125] and DEPICT (Data-driven Expression-Prioritized Integration for Complex Traits; release 142 for 1000 Genomes imputed data;[126] [127] methods to specifically prioritize genes, pathways and tissues enriched in the genome-wide results of the PCOS meta-analysis. In brief, MAGENTA assesses the over-representation of genes with low P-values in their locus across manually curated databases. For the MAGENTA analysis, curated gene-sets and Gene Ontology (GO) gene-sets were obtained from the Molecular Signatures Database (MSigDB release v4.0;[128]). DEPICT prioritizes genes in the associated loci, detects enriched pathways and tissues based on derived data sets based on patterns of co-expression and the expression levels of the genes at associated loci. Further, we performed functional annotation enrichment analysis using GoShifter (Genomic Annotation Shifter;[129]). Functional annotations used in these analyses were transcription factor binding sites (172 transcription factors)[130] and chromatin states in different tissues (n=196).[131] We investigated whether the 14 PCOS-associated susceptibility variants detected in this study and the variants in LD with them (r^2>0.6) co-localized with specific functional annotations. The results from gene set analysis did not show results that we found particularly trustworthy, and the methods of MAGENTA have been criticized elsewhere so these are only reported in the supplement. No individual pathway appeared to be significant. GoShifter analyses for identification of enriched functional annotations did not reveal any statistically significant finding (all p-value>0.05). DEPICT tissue identification approach reinforced the importance of ovarian morphology, with ovarian follicle, ovum, oocytes, ovary, granulosa cells, fallopian tubes and cumulus cells all showing nominally significant p-values (p-value<0.05). This is alongside the more general enrichment at endocrine cells and adipocytes.

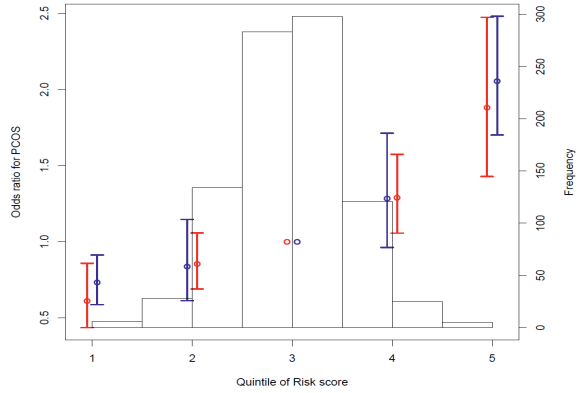
The nominally significant findings for ovaries in the tissue identification analysis suggested the importance of ovarian morphology in PCOS pathogenesis. However, no individual pathway appeared to be significant in gene-set enrichment and gene prioritization analyses. A potential explanation for the lack of significant findings is that these methods are limited by the functional data available for the tissues relevant to PCOS and its related traits, e.g. ovary. In addition, DEPICT and GoShifter analyses were based on the 14 PCOS GWAS meta-analysis susceptibility variants, which may limit the power of these approaches to detect significant enrichments.[129]

Figure S2. Cluster plots showing relationships between PCOS loci and related traits



Loci significantly associated with PCOS in our meta-analysis (shown on left, blue) or in the previously reported meta analyses of Chinese PCOS subjects in the analysis of related traits in our own meta-analysis (shown on right, purple). Clustering by column (phenotype/trait) demonstrates the large proportion of PCOS loci that are also significantly associated with ovulatory dysfunction (OD) and polycystic ovarian morphology (PCOM).

Figure S3. Weighted genetic risk score



The odds for having PCOS by NIH (red) or Rotterdam (blue) diagnostic criteria based on genetic risk score from across the identified genome-wide significant loci. The group with the average number of risk alleles was used as the reference group.

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CHAPTER 3

The influence of ethnicity on outcomes of ovulation induction with clomiphene citrate in women with PCOS

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Abstract

Research question
To study the influence of ethnicity on the outcome of ovulation induction with clomiphene citrate (CC) in women with polycystic ovary syndrome (PCOS).

Design
A retrospective cohort study. In total, 420 women diagnosed with PCOS from Northern European, Mediterranean, African, South-East Asian and South-American descent, who started ovulation induction treatment with CC, were included. All women were treated with CC according to standardized treatment regime. Minimal effective dose of CC, and the prevalence of clomiphene resistance (CRA) were assessed, and we predicted the chance to become ovulatory.

Results
We observed differences in BMI ($p=0.002$), waist circumference ($p=0.036$), LH, insulin, and androgen serum levels (all $p<0.001$) in PCOS women of different ethnicity. Compared to women of Northern European descent, the minimal effective dose of CC in women of other ethnic groups was not different ($p>0.2$). The prevalence of CRA ($p=0.574$) was similar in all ethnic groups. We predicted a similar chance of ovulation ($p=0.5$) in the different ethnic groups.

Conclusions
This is the first study aiming to link ethnicity to ovulation induction outcome in PCOS. Although PCOS women of different ethnicity exhibit variation in phenotype expression, it does not appear that there are differences in the prevalence of clomiphene resistant anovulation as well as the minimal effective dose of CC. Furthermore, a prediction model revealed no significant differences in the predicted chance to ovulate. A larger cohort is needed to validate these findings.

Introduction

Normogonadotropic anovulation is the most common cause of subfertility in women of reproductive age. The majority of women with normogonadotropic anovulation are diagnosed with polycystic ovary syndrome (PCOS) [1]. PCOS is most often diagnosed according the Rotterdam criteria, when either two or three of the key features (cycle irregularities, polycystic ovarian morphology, and elevated androgen levels) are present. This results in four different PCOS phenotypes [2]. Using this definition, PCOS has a reported prevalence up to 13% [3]. Multiple strategies are available for the treatment of anovulation, ranging from lifestyle modification to assisted reproductive techniques and laparoscopic ovarian drilling [4,5].. The recently published international guideline on PCOS suggests letrozole as a first line pharmacological treatment for anovulation [6]. In many countries, such as India, Egypt, parts of the USA, and the Netherlands, Letrozole is not registered as an ovulation induction (OI) drug. Therefore, if used for OI, it is used off label which implies that many doctors will use clomiphene citrate (CC) [6,7]. The chance to regain ovulatory cycles with CC has been estimated around 60-80%, and 30-50% will become pregnant [6,8-10]. Currently, there are no ethnicity based treatment algorithms for the use of clomid in ovulation induction in women with PCOS.

Response to drugs is difficult to predict and variability in the response might be caused by the pathophysiology and severity of the disease [11]. Genetic variants involved in the pathogenesis of PCOS have been associated with a higher chance of clomiphene resistant anovulation (CRA), and a lower chance to achieve an ongoing pregnancy [12,13]. Besides the genetic make-up of the patient, age, as well as PCOS phenotype characteristics influence the response to OI treatment with CC.

Phenotype expression and the severity of PCOS are influenced by ethnicity. South-Asian women with PCOS tend to experience oligomenorrhea at a much younger age, and BMI and androgen levels are generally low in these women [14-16]. At the same time the prevalence of insulin resistance and metabolic syndrome is high in these women [16,17]. Women with PCOS originating from sub-Saharan Africa as well as Mediterranean areas tend to have a higher BMI, together a higher prevalence of obesity and elevated androgen levels [16,18]. In contrast, Hindustani women with PCOS generally present with the highest free androgen index (FAI) [17,18].

Studies have shown that a high body mass index (BMI), as well as high androgen and serum insulin levels, the type of ovulatory disorder (i.e. oligomenorrhea versus amenorrhea) , and a larger ovarian volume are associated with poor response to ovulation induction treatment with CC [10,19-21]. In addition, high AMH levels have been associated with a reduced response to clomiphene citrate [10,22].

As stated ethnicity is related to differences in PCOS phenotype, which could also influence the response to CC. However, response to ovulation induction therapy has

rarely been explored in relation to ethnicity. Further, available algorithms to predict to response to CC do not take ethnicity into consideration.. The first aim of the current study, was to assess the influence of ethnicity on OI with CC, by assessing the minimal effective dose, and the chance to become ovulatory. The second aim of this study was to predict the chance to ovulate after OI treatment in women with PCOS of different ethnicity.

To study these aims we have used an algorithm designed in our university hospital, which can be used to predict the chance to ovulate following CC therapy ([19]. This model includes the free androgen index (FAI), BMI and the type of ovulatory dysfunction and was developed in a cohort of Northern-European women.

Materials and Methods

Study population

Patients diagnosed with normogonadotropic anovulation and PCOS, who visited the outpatient clinic of a university in Rotterdam, the Netherlands, between January 1st 2005, and January 1st 2016, with a wish to conceive, and did not undergo previous fertility treatment, were eligible for inclusion. PCOS was diagnosed according to the Rotterdam criteria, when two or three of the key features; ovulatory dysfunction, hyperandrogenism and polycystic ovarian morphology, were present [23]. A standardized screening was performed by trained physicians. The screening protocol has been described in detail elsewhere [24]. Briefly, information on general, medical and obstetric history and previous fertility treatment was obtained through a questionnaire. Blood pressure, waist and hip circumference, height and weight were measured. An ultrasound probe of <8 MHz, was used to assess the presence of polycystic ovarian morphology, which was defined as ovarian volume >10ml or >12 follicles in one or both ovaries in the absence of a dominant follicle or cyst. Ovulatory dysfunction was defined as menstrual cycle length of <21 days or >35 days (oligomenorrhea) or the absence of a menstrual cycle ≥6 months (amenorrhea). Women with other reasons for infertility, including thyroid disease, were excluded. From 2009 onwards, in the Netherlands, the first line of treatment of anovulation in overweight (BMI >25) or obese (BMI >30) women with PCOS has become lifestyle modification. These women are enrolled in a year lifestyle modification program. Women enrolled in this program were not included in the current study. We excluded patients who did not start ovulation induction treatment with CC. Reasons for not starting OI treatment with CC were having received previous ovulation induction treatment elsewhere, the occurrence of a spontaneous pregnancy, switch to another form of infertility treatment than CC (intra-uterine insemination, IVF of ICSI), getting a second opinion and treatment elsewhere. Finally, some couples choose to not start with infertility treatment after all. Since this study neither implied that patients would receive a particular treatment, nor imposed on their behavior as described in the Medical Research Involving Human Subjects Act (WMO), the local

institutional review board (IRB) of Erasmus Medical Center Rotterdam has officially stated that the WMO does not apply (MEC-2020-0534).

Ethnicity
Self-reported ethnicity and country of birth of the patient and both parents were registered via questionnaires. Based on self-reported ethnicity, eligible patients were classified as either Northern European, Mediterranean, African, South-East Asian or South-American. Due to the small sample size, patients with a mixed ethnicity were excluded from the analyses.

Endocrine assessment
Blood was drawn on a random cycle day after an overnight fast. Luteinizing hormone (LH) and follicle stimulating hormone (FSH), sex-hormone binding globulin (SHBG) and insulin levels were measured with immunoluminometric assay (Immulite® platform, Siemens DPC Los Angeles, CA, USA.). Testosterone, androstenedione (Adione) and dehydroepiandrosterone (DHEA) were measured with (LC-MS/MS). Glucose levels were measured with Cobas 8000 (Roche Diagnostics Almere, Netherlands). Estradiol was measured with Ria assay (Siemens DPC Los Angeles, CA, USA.). Gen II Beckman Coulter, (Beckman Coulter, Inc., Webster, TX) was used to determine serum anti-Mullerian hormone (AMH) serum levels. The free androgen index (FAI) was calculated as 100 x Testosterone/SHBG.

Treatment protocol
According to the standard treatment regimen, patients received an initial CC dose of 50 mg/d on cycle day 3-7 after spontaneous or induced withdrawal bleeding. In case of the absence of ovulation, the dosage was increased to 100mg/d or 150 mg/d. The minimal effective dose is the dosage of CC, after which a regular menstrual cycle was restored. Clomiphene resistant anovulation (CRA) was defined as the failure to ovulate during treatment with CC. The failure to conceive after ovulation has been restored for a minimum of 6 cycles is referred to as clomiphene citrate failure (CCF).

Statistical analyses
Statistical analyses were performed using IBM SPSS version 24 (IBM corp., Armonk, NY, USA). Baseline characteristics were displayed as medians and interquartile ranges (IQR) or numbers and percentages. Mann-Whitney-U was used to compare continuous variables between groups. Proportions were tested with the Chi-square test. An ordinal regression was performed with the polytomous universal model (PLUM) procedure, to assess the minimal effective dose of CC across different ethnicities. A p-value of <0.05 denoted a statistical significant difference. The chance to ovulate after the admission of CC was calculated with a previously designed prediction model [8]. This model predicts the chance to ovulate following CC therapy, based on the height of the FAI, BMI and the presence of oligomenorrhea or amenorrhea. First the linear predictor (LP) is calculated with the formula LP = -4.1768+0.0626*BMI+0.1397*FAI+ 1.2047*(1 (in case of amenorrhea)). Next the

probability for CRA is calculated with; Probability (PR) CRA = 1/(1+exp(-LP)). Finally, this results in the chance to ovulate (1-Pr(CRA)) [8]. We calculated the chance of ovulation for each individual and then compared the group medians.

Results

In total, 1259 diagnosed with women were eligible for inclusion in the current study. We excluded 762 women (60.5%) who did not start with ovulation induction. Reasons for not starting treatment were the occurrence of a spontaneous pregnancy (N=130 (16.9%)), being overweight or obese (N=252 (32.8%)), previous treatment (N=63 (8.2%)) or because they had started with ovulation induction with recombinant FSH or IVF treatment as a first line treatment (N=185 (24.0%). Women who decided not to start their ART treatment (N=132 (17.2%) were also excluded. This resulted in 497 women who began with treatment with CC. Due to the small sample size (N=8) women with a mixed ethnicity were also excluded. Finally, N = 69 (14.1%) women who started treatment with CC were lost to follow up and therefore excluded, resulting in 420 women used in the analyses.

We stratified women according to self-reported descent. We assessed N=278 Northern-European, N=60 Mediterranean, N=20 African, N=44 South-East Asian, and N=18 South-American women with PCOS. Women classified as South-American mainly originate from the Netherlands Antilles (Aruba and Curacao). The baseline characteristics of women with PCOS of different ethnicity are presented in **table 1**.

We observed differences in age ($p < 0.001$), BMI ($p=0.002$), and waist circumference ($p=0.036$), with the highest BMI and waist circumference found in women of Mediterranean (median BMI 25.6 kg/m^2, waist 84.0cm) and South-American descent (BMI median BMI 25.1 kg/m^2, waist 86.5). Lowest median BMI was seen in women of Northern-European descent (23.2 kg/m$^{2)}$ Highest median LH levels were found in women of Mediterranean (12 IU/L) and African (12.2 IU/L) descent, and lowest in Northern-European women (8.2IU/L), $p=0.002$. Furthermore, we observed differences in androgen levels ($p<0.001$), with highest testosterone (2.8 nmol/L) and FAI (median 6.6) in African women with PCOS, and the lowest in women of Northern-European . Insulin levels differed between women of different ethnicity ($P<0.001$), with the highest levels measured in South-East Asian women (82.0 pmol/L), and lowest in Northern-European women). Systolic and diastolic blood pressure, as well as the prevalence of oligomenorrhea and amenorrhea, PCOM, and FSH serum levels were similar in women with PCOS across all ethnic groups. Finally, we observed no significant differences in AMH serum levels in women of Northern-European, Mediterranean, African, South-East Asian and South-American descent.

The prevalence of CRA in women of self-reported Northern-European, Mediterranean, African, South-East Asian, or South-American descent was not significantly different (p=0.574). We performed an ordinal regression analysis to assess the minimal effective dose of CC in women with PCOS of different ethnicity. For this analysis, we used the largest group, women of self-reported Northern-European descent as the reference group, and tested whether women of other ethnicities needed a higher dose of CC to become ovulatory (Figure 1). We found no significant differences in the minimal effective dose of CC in Mediterranean (0.35 (-0.12:0.82), β (95% CI), African (0.33 (-0.43:1.09)), South-East Asian (0.19 (-0.35:0.73)), or South-American (0.08 (-0.91:0.74)) women.

Finally, we used a previously designed algorithm predict the chance to ovulate after treatment with CC [19]. We observed no significant differences in the predicted chance of ovulation in the different ethnic groups (p=0.504). Median predicted chance to ovulate ranged from 0.84 to 0.88 women with PCOS across all ethnic groups (**Figure 2)**.

We performed a sensitivity analysis to compare women of self-reported Northern-European and South-east Asian ethnicity. We compared the prevalence of CRA, the predicted chance to ovulate after CC and the minimal effective dose of CC. In these analyses we observed no significant differences (data not shown).

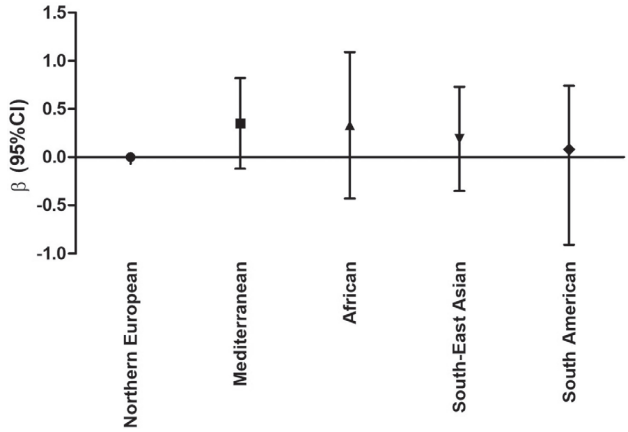
Table 1. Characteristics of the study population stratified by ethnicity

Ethnicity	Northern-European	Mediterranean	African	South-East Asian	South-American	P-value
No. of cases	278 (66.2%)	60 (14.3%)	20 (4.8%)	44 (10.5%)	18 (4.3%)	-
Age	29.6 (27.0-32.3)	26.4 (23.6-28.9)	26.1 (22.8-29.1)	29.4 (26.6-32.4)	29.5 (26.5-33.3)	<0.001
BMI (kg/m^2)	23.2 (21.0-27.6)	25.6 (23.6-29.6)	23.7 (21.1-31.4)	24.2 (21.9-28.3)	25.1 (23.5-34.3)	0.008
Waist (cm)	78.0 (71.0-87.0)	84.0 (74.0-90.0)	75.5 (69.3-87.5)	78.5 (71.8-87.3)	86.5 (75.5-100.00)	0.036
BP systolic (mmHg)	112.0 (110.0-120.0)	110.0 (105.0-120.0)	115.0 (105.0-120.0)	110.0 (110.0-120.0)	115.0 (107.5-125.5)	0.725
BP diastolic (mmHg)	72.0 (70.0-80.0)	70.0 (68.0-80.0)	75.0 (65.0-82.0)	70.0 (70.0-80.0)	80.0 (72.5-83.5)	0.348
LH (IU/L)	8.2 (5.2-12.5)	12.1 (7.5-16.7)	12.2 (8.1-18.1)	9.2 (5.9-14.0)	8.5 (4.6-12.2)	0.002
FSH (IU/L)	5.9 (4.5-7.3)	6.0 (5.0-7.2)	5.9 (3.9-6.8)	6.4 (4.6-7.0)	5.5 (4.4-6.8)	0.728
Testosterone (nmol/L)	1.6 (1.1-2.2)	1.7 (1.2-2.4)	2.8 (1.8-3.6)	1.7 (1.3-2.1)	1.6 (1.3-2.2)	0.002
SHBG (nmol/L)	52.4 (35.9-69.5)	34.1 (21.5-48.9)	34.5 (24.6-52.1)	30.4 (20.8-44.0)	42.0 (28.8-59.3)	<0.001
FAI	3.1 (1.8-5.1)	4.7 (3.1-9.5)	6.6 (4.6-11.4)	5.9 (3.4-8.8)	4.7 (2.6-6.5)	<0.001
FG score	1.0 (0.0-3.0)	4.0 (2.0-7.0)	4.0 (0.0-5.5)	3.0 (1.5-8.5)	3.0 (1.5-4.5)	<0.001
Estradiol (pmol/L)	224.0 (162.5-359.0)	253.0 (154.3-361.8)	292.5 (229.8-395.5)	187.5 (133.3-282.3)	252.0 (196.5-371.0)	0.046
Adione (nmol/L)	9.2 (6.6-12.3)	10.9 (8.1-14.6)	15.1 (10.9-24.2)	10.5 (7.9-13.9)	8.9 (6.3-12.2)	<0.001
DHEA (nmol/L)	31.3 (21.4-49.3)	44.5 (28.2-66.0)	51.4 (38.9-77.4)	33.1 (22.9-50.5)	32.0 (23.9-48.3)	0.002
Glucose (mmol/L)	4.6 (4.3-4.9)	4.7 (4.2-4.9)	4.9 (4.3-5.2)	4.9 (4.3-5.2)	4.7 (4.2-4.8)	0.172
Insulin (pmol/L)	40.0 (23.8-68.3)	54.0 (31.0-83.0)	46.0 (23.0-91.8)	82.0 (27.5-112.7)	53.0 (26.5-99.3)	0.002
AMH ((µg/L)	8.1 (5.1-13.6)	8.4 (6.3-11.9)	11.3 (6.2-16.5)	8.3 (5.1-14.5)	8.7 (5.0-16.4)	0.859

Oligomenorrhea	202 (72.7%)	48 (80.0%)	16 (80.0%)	35 (79.5%)	18 (100.0%)	0.079
Amenorrhea	76 (17.3%)	12 (20.0%)	4 (20.0%)	9 (20.5%)	0 (0%)	0.073
PCOM	265 (95.3%)	55 (91.7%)	20 (100%)	41 (93.2%)	16 (88.9%)	0.596
CRA	43 (15.5%)	10 (16.7%)	5 (25%)	8 (18.2%)	1 (5.6%)	0.574

Values are medians (IQR) or numbers (%). Comparison of continuous variables between groups was done with the Kruskall-Wallis test, a P-value of <0.05 was considered statistically significant. Chi-square test was used to test proportions. Boldface indicates a significant association at P <0.05. Abbreviations: body mass index (BMI), blood pressure (BP), luteinizing hormone (LH), follicle stimulating hormone (FSH), sex-hormone binding globulin (SHBG), free androgen index (FAI), Ferrimann Galwey (FG) score, androstenedione (Adione), dehydroepiandrosterone (DHEA),anti-mullerian hormone (AMH), clomiphene resistant anovulation (CRA)

Figure 1. Minimal effective dose of CC in women with PCOS of different ethnicity.

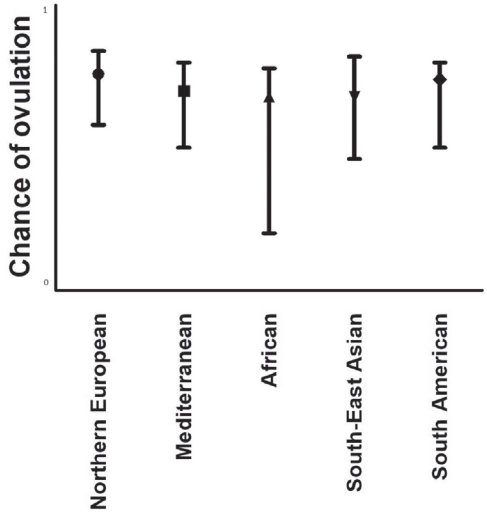


We used ordinal regression to assess differences in the minimal effective dose CC in women with PCOS of different ethnicity. Results are expressed as beta's and corresponding 95% confidence intervals, with Northern-European women used as the reference group.

Discussion

To our knowledge, this is the first study assessing the influence of ethnicity on ovulation induction treatment with clomiphene citrate in women with PCOS. The main finding of the current study is that although we observed differences in the phenotype of women with PCOS of different ethnicity, we found no significant differences in the prevalence of clomiphene resistant anovulation and the minimal effective dose of CC. Furthermore, we used a previously designed algorithm to predict the chance to ovulate after treatment with CC. This model revealed no significant differences in the predicted chance to ovulate, suggesting that the differences in phenotype might balance each other out and result in a similar response to treatment and this model could be used in clinical practice.

Figure 2. The predicted chance to ovulate after the administration of CC in women with PCOS of different ethnicity.



Values are expressed as medians and interquartile ranges. Comparison of groups was done with the Kruskall-Wallis test, a p-value of <0.05 was considered statistically significant.

We assessed the phenotype of women with self-reported Northern-European, Mediterranean, African, South-East Asian, and South-American ethnicity. In line with existing literature, we report significant differences in BMI and waist circumference, and in insulin, LH, and androgen levels in women with PCOS of different ethnicity [16-18]. Similarly, no significant differences were observed in systolic and diastolic blood pressure, and the prevalence of oligomenorrhea or amenorrhea, PCOM, and in FSH serum levels. Despite these observed ethnicity based phenotypic differences in women with PCOS, no significant differences in the prevalence of CRA were observed, nor did we observe differences in the minimal effective dose of CC in women of different ethnicity. One potential explanation for that could be the fact that AMH levels were similar for all ethnic groups. High AMH levels have been associated with a reduced response to treatment [10]. The absence of differences in AMH serum levels in women of different ethnicity one the reasons why we did not observe differences in CC response. Another explanation might be that an increase in one of the predicting factors, could outbalance the impact of other predicting factors resulting in a similar outcome of OI treatment with CC for all ethnicities. Alternatively, the weight of the predictors might be different in women of different ethnicity. It could also be that the current study lacked the power to detect significant differences. We did observe some differences in the prevalence of CRA, but this did not reach statistical significance. This could be due to the relatively small sizes of some of the ethnic groups. We need to be cautious when interpreting these

results, as it is one of the first addressing this topic. Validation of our findings in a larger cohort are necessary. However, based on the current study, we conclude that although ethnic differences exist in the phenotype of women with PCOS, this does not impact the response to CC. Hence, the treatment of women with PCOS of different ethnic descent according to the same standardized protocol seems appropriate.

We used a previously designed algorithm, which uses BMI, the free androgen index and the type of cycle irregularity, to predict the chance to ovulate after treatment with CC in women with PCOS of different ethnicity [8]. We predicted a similar chance to become ovulatory in the different ethnic groups. This is in line with the actual observed prevalence of CRA, which was similar amongst the different ethnic groups. Most algorithms to predict ovulation induction outcome are developed in women of Northern European descent. The weight of the predictors might be different in women of different ethnicity. Possibly the impact of for instance hyperandrogenism on fertility is bigger in some ethnic groups than in others. If is this the case for multiple phenotypic traits, it could be that the combination of traits with a bigger or lesser impact, result in a similar response to OI with CC. However, it is also possible that we did not detect differences either due to the design of the study or other physiologic influences. By not including overweight and obese women who were enrolled in the lifestyle program in our analyses, one could hypothesize that this is why we found no significant difference in the predicted chance to ovulate. However, because the multiplier in BMI is so small, compared to that of the FAI type of ovulatory dysfunction, we expect its influence to be minor.

The current study is one of the largest studies, assessing ethnic differences in women with PCOS and the first study aiming to link ethnicity to ovulation induction treatment outcome. We were able to assess many phenotypical features and had detailed information regarding the follow up of treated women. A limitation of this study is the difference in size of our ethnic groups. We had to categorize our study population into five ethnic groups, to be able to use them for analysis. Some of the ethnic groups, are still relatively small, and we may not have detected small associations. We performed a sensitivity analysis to compare the outcome of OI with CC in women of Northern-European and South-East Asian ethnicity. This yielded no significant differences. When comparing additional ethnicities, it does not appear that there are significant differences. However a larger cohort is needed to validate these findings. Furthermore, we assessed a relatively lean group of women with PCOS, this is because in the Netherlands the first step in the treatment of ovulation is lifestyle modification and weight loss. Before starting pharmacological treatment, women with PCOS who are overweight or obese must attempt to achieve sustained weight loss on their own or by entering a lifestyle program, or sometimes are referred for bariatric surgery. With this, a proportion of these women will regain ovulatory cycles and become pregnant and further treatment might not be necessary. This may have introduced a selection bias. However, we feel that women enrolled in the

lifestyle modification program, and their response to OI treatment after completing this program, need to be analyzed separately.

The definition of ethnicity is complex including non-genetic elements such as diet, language, and other cultural elements as well as elements that are expected to have a genetic component such as biogeographic origin (i.e., genetic ancestry) or the genetic background in general, which may or may not affect the variability of the PCOS phenotype including its characteristics. In medical studies, including those on PCOS, ethnicity is usually assessed by self-reported data. We are aware that self-reported ethnicity can be biased and does not necessarily capture all existing population substructures. We partly address this bias by not only asking women for their ethnicity , but also include country of birth of the patient as well as her parents. We previously showed that the association between self-reported ethnicity and genetic ancestry was moderate ([25]. It would be very interesting to include genetic ancestry data in the prediction of ovulation induction outcome.

To what extent these results are generable to overweight and obese women, needs to be examined. Validation of the current study in a larger multi-ethnic cohort are necessary to validate our findings and to provide more insight on the outcome of treatment with CC.

Conclusions

In conclusion, although women with PCOS of different ethnicity exhibit variation in the phenotypic expression of PCOS, there seem to be no differences in the prevalence of clomiphene resistant anovulation or the minimal effective dose of CC. Furthermore, a prediction model revealed no significant differences in the predicted chance to ovulate. A larger cohort is needed to provide more insight on the outcome of OI treatment with CC in women with PCOS of different ethnicity.

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CHAPTER 4

The cardiometabolic profile of women with WHO2 anovulation differs from that of women with women with polycystic ovary syndrome

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Under review

Abstract

Objective

Study the cardiometabolic phenotype of women diagnosed with WHO2 anovulation, PCOS (phenotype A, B or D), and a normo-ovulatory reference group.

Design, patients, setting

Cohort study from a university hospital. We assessed 277 women with WHO2 anovulation, 1890 women with PCOS and a reference group consisting of 262 normo-ovulatory women.

Interventions

Questionnaires, a physical examination, and fasting blood samples.

Main outcome measures

BMI, blood pressure, lipid levels, as well as well as the prevalence of overweight and obesity, central obesity, hypertension, hyperglycemia, insulin resistance and the metabolic syndrome were assessed.

Results

We observed a lower prevalence of cardiovascular risk factors, such as BMI ($p < 0.001$), insulin resistance ($p < 0.001$), unfavorable LDL ($p = 0.004$) and HDL ($p < 0.001$) cholesterol levels and the metabolic syndrome ($p = 0.007$) in women with WHO2 anovulation, compared to women with PCOS. Furthermore, the cardiometabolic profile of women with WHO2 anovulation seems to be comparable with that of healthy women. Lean (BMI <25) women with PCOS present with an unfavorable cardiometabolic profile compared to women with WHO2 anovulation. Overweight or obese women with WHO2 anovulation, PCOS or from the reference group exhibit a similar cardiometabolic profile.

Conclusion

Women with WHO2 anovulation exhibit a cardiometabolic profile distinctly different from that seen in PCOS, and comparable to that of normo-ovulatory women. Overweight or obese women with either WHO2 anovulation, PCOS or normo-ovulatory women all have a similar cardiometabolic profile which seems to be much more driven by BMI.

Introduction

Normogonadotropic anovulation (WHO2) is the most common type of anovulation found in women of reproductive age. Using the Rotterdam PCOS criteria the majority of women with WHO2 anovulation are diagnosed with polycystic ovary syndrome (PCOS). PCOS is a disorder characterized by ovulatory dysfunction, hyperandrogenism and polycystic ovarian morphology (PCOM) [1]. PCOS is associated with cardiometabolic disturbances and an increased prevalence of risk factors for cardiovascular disease [2,3]. Women with PCOS are more often overweight or obese predominantly due to central obesity [4]. Obesity worsens PCOS features such as hyperandrogenemia and menstrual disturbances and aggravates metabolic disturbances [5,6]. Furthermore, unhealthy dietary patterns in women with PCOS have been associated with a more severe hyperandrogenic PCOS phenotype [7].

Insulin resistance (IR) is detected in approximately 80-95% of women diagnosed with PCOS and has been proposed as a key pathophysiological feature of PCOS, contributing to both reproductive and metabolic disturbances [8,9]. Insulin resistance in women with PCOS often progresses to type II diabetes (10). In addition, IR in PCOS has been associated with dyslipidemia [10]. Obesity, impaired glucose tolerance and dyslipidemia, are all components of the metabolic syndrome (MetS). The MetS is a major risk factor for cardiovascular disease later in life and is more prevalent in women diagnosed with PCOS [11,12]. Recent studies seem to point into the direction that, despite of this unfavorable cardiometabolic profile, long term health in women with PCOS might be better than was previously anticipated [13-15]. Validation of these findings is still ongoing, however, the necessity to assess and follow-up risk factors for cardiovascular disease in women with PCOS has been well recognized [16].

A small proportion of women with normogonadotropic anovulation, do not meet the criteria for PCOS. Evidence on the cardiometabolic risk in women with WHO2 anovulation without PCOS is not yet available. It has been hypothesized that cardiometabolic disturbances are much less prevalent in women with WHO2 anovulation without PCOS, because of the absence of hyperandrogenism [17]. It is not known whether the absence of PCOM is a modifier of the WHO2 phenotype. The first aim of the current study was to assess clinical characteristics and cardiometabolic parameters in women diagnosed with WHO2 anovulation without PCOS, and compare them to both women with PCOS and a normo-ovulatory reference group.

Exposure to certain environmental factors during critical periods of development influence short and long term health outcome in an individual [18]. This concept is commonly known as the Developmental Origins of Health and disease (DOHaD) paradigm, and postulates that adverse exposures during pregnancy, adverse pregnancy outcomes (such as a low birth weight), and an extra-uterine environment reversed of that experienced in utero, increase the risk for coronary heart disease,

and other non-communicable diseases later in life [18,19]. Excessive weight gain during adult life and aging itself further increase this risk [18]. The second aim of this cross sectional study was to assess the influence of BMI on the cardiometabolic profile of adult women with WHO2 anovulation, in comparison to women with PCOS and normo-ovulatory women.

Methods

Patients

Patients were recruited at a large University hospital in Rotterdam, the Netherlands, between January 1st 2005 and January 1st 2020. Women diagnosed with WHO2 anovulation with or without a concurrent PCOS diagnosis were eligible for inclusion. WHO2 anovulation was diagnosed in case of normal LH and FSH (>2 and <10 UL/L), and estradiol serum levels (>100 pmol/L), and absence of hyperandrogenism and polycystic ovarian morphology. PCOS was diagnosed according to the Rotterdam criteria [1]. Patients underwent a standardized examination after an overnight fast. Screening was performed on a random cycle day, or on the last day of the oral contraceptive pill-free interval. [20]. Clinical examination included age, ethnicity, menstrual history, use of oral contraceptives (OC) general medical, gynecological and family history and current use of medication. Height and weight, and waist and hip circumference were measured and blood pressure was measured in sitting position. The presence of hirsutism was objectified with the modified Ferriman Gallwey score. Transvaginal ultrasonography was performed, using a 6 MHz probe, to assess mean ovarian volume and mean follicle count in both ovaries. PCOM was defined as ovarian volume exceeding 10 cm^3 and/ or an antral follicle count ≥ 12 (in the absence of a follicle >10mm). Ovulatory dysfunction was defined as oligomenorrhea (mean interval between 35 days and 199 days), or amenorrhea (absence of menstrual bleeding for more than 199 days). Hyperandrogenism was defined as testosterone >3 nmol/L, or a free androgen index >4,5. The screening protocol has been described in detail elsewhere [10]. We included patients of whom information was available on all components of the metabolic syndrome. Women diagnosed with other endocrine abnormalities affecting menstrual function, such as hyperprolactinemia, thyroid dysfunction and congenital adrenal hyperplasia and other causes of hyperandrogenemia were excluded. Other reasons for exclusion were the use of medication which might influence cardiometabolic parameters; such as antihypertensive or antidiabetic medication and statins. Finally, we excluded women in case data on BMI was missing. All biochemical parameters were assessed in serum after a 12 hour period of fasting. LH, FSH, and SHBG was measured on the Immulite 2000Xpi platform (Siemens). Insulin, estradiol, glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides, were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH). Androstenedione,

testosterone, and dehydroepiandrosterone sulfate (DHEAS) were measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Reference group
A reference group of normo-ovulatory women was derived from the HAVEN study, which is a study designed to identify determinants in the pathogenesis and prevention of coronary heart disease. This study has been described in detail elsewhere [21,22]. In short, the study cohort comprises children with coronary disease and both parents, as well as healthy control-children and their parents, living in the western part of the Netherlands. Mothers of healthy children who visited the hospital for a standardized evaluation around 17 months after pregnancy were eligible for inclusion in the current study. Information on smoking, education, use of oral contraceptives and medication use was gathered via questionnaire. Information on the menstrual was obtained. Height and weight were measured and fasting venous blood samples were taken. Women included in the reference group had information on lipid serum levels available, a regular menstrual cycle, were not pregnant, did not breastfeed and did not have diabetes mellitus. Insulin, glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides, were measured on the COBAS 8000 Modular Analyzer (Roche Diagnostics GmbH).

Cardiometabolic parameters
We defined hypertension as blood pressure (BP) ≥ 140/90 mmHg, increased waist circumference (WC) as a WC ≥ 88 cm. Hyperglycemia was present in case of a fasting glucose ≥ 6.1 mmol/L), insulin resistance when 1/HOMA-IR < 0.47. Dyslipidemia was defined as total cholesterol ≥ 5.0 mmol/L, triglycerides ≥ 1.7 mmol/L, LDL-cholesterol ≥ 3.0 mmol/L, and HDL-cholesterol < 1.29 mmol/L). We assessed the presence of the metabolic syndrome by using the NCEP ATP3 definition (≥ 3 of any of these clinical features; WC ≥ 88cm, fasting glucose ≥ 5.6 mmol/L or on drug treatment for elevated glucose, BP ≥ 130/85 mmHg or on antihypertensive drug treatment, HDL < 1.3 mmol/L or on drug treatment for reduced HDL, TG ≥ 1.7 mmol/L or on drug treatment for elevated triglycerides) [8,23,24].

Calculations
Body Mass Index (BMI) was calculated as weight in kilograms divided by the squared height in meters. The FAI was obtained as the quotient of 100 × total testosterone / SHBG, and defined as biochemical hyperandrogenism if > 4,5 when testosterone was measured with the RAI assay, or > 2.9 when testosterone was measured with the LC-MS/MS assay [25]. Insulin resistance was assessed using the homeostasis model assessment (HOMA-IR): fasting glucose (mmol/L) x fasting insulin (mU/L)/22.5 [26]. LDL-C was calculated with the Friedewald formula: LDL-C = cholesterol − HDL-C − (TG/5) [27].

Statistical analysis
Baseline characteristics were expressed as medians with interquartile ranges or numbers with percentages. The Kruskal-Wallis test was used to compare the cardiometabolic parameters of lean, overweight and obese women with WHO2 anovulation with or without PCOS, and a healthy reference group. Patients were stratified based on BMI as described by the World Health Organization; lean (BMI < 25 kg/m2), overweight (BMI ≥ 25 kg/m2 - < 30 kg/m2) and obese (BMI ≥ 30 kg/m2). The Chi-squared test was used to compare the prevalence of cardiometabolic risk factors. A Bonferroni correction was used, a p-value of < 0.025 was considered to be statistically significant. These analyses were performed with Statistical Package for Social Sciences (SPSS) version 25. (IBM corp. Armonk, NY, USA).

Ethical approval
Since this study neither implied that patients would receive a particular treatment, nor imposed on their behavior as described in the Medical Research Involving Human Subjects Act (WMO), the IRB has officially stated that the WMO does not apply.

Results

In total, 3576 women diagnosed with WHO2 anovulation were eligible for inclusion in this study. We excluded women with missing data on BMI (N = 6, 0.2%), on one or more components of the metabolic syndrome (N = 1349, 37.7%), or women who were over 45 years of age (N = 54, 1.5%). In total, 2167 (60.6%) women were included in our analyses. PCOS was diagnosed in 1890 (87.2%) women and the remaining 277 (12.8%) were diagnosed with WHO2 anovulation only. We compared women who were in and excluded from the analyses. We observed differences in the prevalence of OC use and estradiol and androgen levels, with highest levels reported in women included in the analyses (data not shown). Of the 408 eligible normo-ovulatory women, we excluded women who were pregnant (N = 32, 7.8%), or currently breastfeeding (N = 14, 3.4%), reported having irregular menstrual cycles (N = 52, 12.7%), those who were in a non-fasting state at the day of investigation (N = 14, 3.4%), had diabetes mellitus (N = 1, 0.2%), or were missing data on blood pressure or lipid levels (N = 33, 8.1%). In total, 262 (64.8%) women were included in the reference group. No statistically significant differences were found in the baseline characteristics of the women included and excluded from the reference group (data not shown).

Table 1.Baseline characteristics of women with WHO2 anovulation, PCOS, and healthy controls

	Control (n = 262)	WHO2 (n = 277)	PCOS (n = 1890)	p value
Age	33 (29–35)	32 (29–35.4)	28 (25–32)	<0.001[b]
Ethnicity				<0.001[a,b]
Northern European	224 (85.5)	212 (76.8)	1172 (62.0)	
Mediterranean	21 (8.0)	10 (3.6)	265 (14.0)	
Negroid	11 (4.2)	24 (8.7)	181 (9.6)	
Other/mixed	6 (2.3)	30 (10.9)	272 (14.4)	
Smoking	57 (21.8)	80 (29.0)	612 (32.4)	0.054
OCP	130 (49.6)	244 (88.7)	1619 (86.0)	<0.001[a]
Oligomenorrhea	0	214 (77.3)	1448 (76.6)	0.813
Amenorrhea	0	51 (18.4)	399 (21.1)	0.301
PCOM	0	0	1704 (90.2)	
PCOS phenotype				
OD + AE + PCOM	-	-	1045 (55.3)	
OD + AE	-	-	186 (9.8)	
OD + PCOM	-	-	659 (34.9)	
AE + PCOM	-	-	-	
Endocrine profile				
LH (IU/L)	-	4.3 (3.1 – 7.0)	7.6 (4.7 – 11.9)	<0.001[b]
FSH (IU/L)	-	6.0 (4.5 – 7.5)	5.8 (4.3 – 7.1)	0.032
Estradiol (pmol/L)	-	205 (126–355)	213 (147 – 352)	0.274
Progesterone	-	1.0 (0.6 – 2.6)	1.1 (0.7 – 2.5)	0.102
Testosterone (nmol/L)	-	0.9 (0.7 – 1.2)	1.5 (1.1 – 2.2)	<0.001[b]
SHBG (nmol/L)	-	61 (41 – 80)	41 (27 – 61)	<0.001[b]
FAI	-	1.6 (1.0 – 2.3)	3.9 (2.3 – 6.6)	<0.001[b]
DHEA (nmol/L)	-	22 (14–35)	29 (18 – 46)	<0.001[b]
DHEAS (nmol/L)	-	3.6 (2.4 – 5.1)	4.5 (3.2 – 6.0)	<0.001[b]
Adione (nmol/L)	-	3.4 (2.6 – 4.2)	5.3 (4.1 – 7.1)	<0.001[b]

Values are in medians (interquartile range), or in numbers (percentages). Abbreviations: oral contraceptive pill (OCP), polycystic ovarian morphology (PCOM), ovulatory dysfunction (OD), androgen excess (AE), luteinizing hormone (LH), follicle stimulating horone (FSH), sex-hormone binding globuline (SHBG), free androgen index (FAI), dehydroepiandrosterone (DHEA), . a = WHO2 vs control, b = WHO2 vs PCOS

The baseline characteristics of the total study population are presented in Table 1. All women included in the analysis were in their late twenties to mid-thirties and the majority were of Northern-European descent.

Women with WHO2 anovulation exhibited lower LH, SHBG and androgen serum levels, compared to women with PCOS (p<0.001). No differences were found in the type of ovulatory dysfunction. Compared to normo-ovulatory women from the reference group, women with WHO2 anovulation more often used oral

contraceptives (p<0.001). We observed no other baseline differences between women with WHO2 anovulation and women from the reference group.

Cardiometabolic parameters

The cardiometabolic parameters of women with WHO2 anovulation compared to women with PCOS and normo-ovulatory women are presented in **table 2**.Women with WHO2 anovulation presented with a lower systolic and diastolic blood pressure ($p < 0.05$), a smaller waist circumference ($p < 0.001$), and a lower BMI ($p < 0.001$) compared to women with PCOS. In addition, we observed lower a 1/HOMA-IR ($p < 0.001$), and lower fasting insulin ($p < 0.001$), LDL cholesterol ($p < 0.05$), and HDL cholesterol ($P<0.001$) serum levels in in women with WHO2 anovulation. Compared to women from the reference group, women with WHO2 anovulation presented with a lower BMI, and lower insulin and LDL cholesterol serum levels ($p < 0.05$). No other differences were found between women with WHO2 anovulation and normo-ovulatory women.

Table 2. Cardiometabolic parameters in women with WHO2 anovulation, PCOS, and healthy controls

	Controls (N=262)	**WHO2 (N=277)**	**PCOS (N=1890)**	**p value**
SBP	115 (106 – 120)	110 (105 – 120)	115 (110 – 120)	0.005[b]
DBP	75 (70 – 80)	72 (68– 80)	75 (70 – 80)	0.002[b]
Waist	-	76 (70 – 86)	82 (73 – 96)	<0.001[b]
BMI	24.4 (22.0–27.1)	23 (20.227.8)	25.5 (21.8–30.8)	<0.001[ab]
1/HOMA-IR	0.64 (0.43–1.06)	0.77 (0.45–1.4)	0.58 (0.34–1.04)	<0.001[b]
Fasting glucose	4.8 (4.4 – 4.9)	4.8 (4.5 – 5.1)	4.8 (4.5 – 5.1)	0.254
Fasting insulin	50 (33–75)	44 (26–69)	57 (33 – 92)	<0.001[ab]
Triglyceride	0.92 (0.69–1.24)	0.94 (0.73–1.3)	0.95 (0.71–1.36)	0.510
LDL-C	2.9 (2.6–3.6)	2.8 (2.4–3.4)	2.9 (2.4 – 3.7)	0.003[a,b]
HDL-C	1.4 (1.2 – 1.7)	1.5 (1.2 – 1.7)	1.3 (1.0 – 1.7)	<0.001[b]
Total -C	4.6 (4.2 – 5.4)	4.9 (4.2 – 5.5)	4.8 (4.1 – 5.5)	0.435

Values are in medians (interquartile range). Mann-Whitney U test was used for evaluation of between group differences. Abbreviations: systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), homeostatic model assessment for insulin resistance (HOMA-IR),low-density-lipoprotein (LDL),high-density-lipoprotein (HDL), Cholesterol (C), a = WHO2 vs control, b = WHO2 vs PCOS

Risk factors for cardiovascular disease

We assessed the prevalence of risk factors for CVD, in women with WHO2 anovulation and compared them to women with PCOS, and to the reference group (**Table 3**). Women with WHO2 anovulation were less often overweight or obese ($p < 0.001$), and suffered from central obesity less often ($p < 0.001$) compared to women

with PCOS. Furthermore, insulin resistance ($p < 0.001$), elevated LDL cholesterol ($p < 0.05$) and decreased HDL cholesterol ($p < 0.001$) were less prevalent in women with WHO2 anovulation. Finally they less often suffered from the metabolic syndrome ($p < 0.05$). We found no significant differences in the prevalence of risk factors for cardiovascular disease in women with WHO2 anovulation and normo-ovulatory women from the reference group.

Influence of BMI on cardiometabolic parameters
We assessed the influence of BMI on cardiometabolic parameters in women with WHO2 anovulation, compared to women with PCOS and women from the reference group. We stratified women according to BMI, and assessed the profile of lean, overweight and obese women (Table 3). Lean women with WHO2 anovulation exhibited a significantly lower BMI ($p < 0.001$), lower serum insulin ($p < 0.05$), and a lower 1/HOMA-IR ($p < 0.05$) than women diagnosed with PCOS. Compared to lean women from the reference group, women with WHO2 anovulation presented with a lower BMI ($p < 0.001$), and lower serum insulin ($p < 0.05$), and LDL cholesterol levels ($p < 0.05$).

We observed no significant differences in the cardiometabolic parameters of overweight or obese women with WHO2 anovulation, compared to women diagnosed with PCOS or normo-ovulatory women from the reference group. Finally, we assessed the differences in cardiometabolic parameters in lean, overweight and obese women with WHO2 anovulation, compared to women with PCOS and women from the reference group. This yielded no significant results (Supplementary table 1).

Discussion

We assessed the cardiometabolic profile of women with WHO2 anovulation who do not meet the criteria for PCOS. The main finding of this study is that the cardiometabolic profile of women with WHO2 anovulation seems to be distinctly different from that seen in women with PCOS, and comparable to that of normo-ovulatory women. The second finding of this study is that overweight or obese women with either WHO2 anovulation, PCOS or normo-ovulatory women all seem to have a similar cardiometabolic profile which is apparently much more driven by overweight or obesity.

Women with WHO2 anovulation seem to exhibit a cardiometabolic profile different from that of women with PCOS. In women with WHO2 anovulation, we observed a lower blood pressure and BMI, a smaller waist circumference, and lower insulin levels. In addition they seem to exhibit a favorable lipid profile, with lower LDL-cholesterol and higher HDL-cholesterol serum levels compared to women with

PCOS. An explanation for this finding could be the lower serum androgen levels in women with WHO2 anovulation. Hyperandrogenism, a major driver for cardiometabolic abnormalities, is present in the majority of women with PCOS [10,17,28]. In addition, women with PCOS are more often overweight or obese, and have central obesity [4]. The presence of hyperandrogenism or obesity both exacerbate the severity of metabolic disturbances associated with PCOS [17,29]. The lower BMI and androgen levels found in women with WHO2 anovulation could explain why these women seem to have a cardiometabolic profile which resembles that of the normo-ovulatory reference group.

We assessed the prevalence of risk factors for cardiovascular disease in women with WHO2 around the age of thirty, and compared them to women PCOS and normo-ovulatory women from the reference group. We observed a lower prevalence of central obesity, insulin resistance and dyslipidemia in women with WHO2 anovulation, compared to women with PCOS. No differences were found in the prevalence of cardiovascular risk factors, compared to women from the normo-ovulatory reference group. This points into the direction that the risk for cardiovascular disease in women with WHO2 anovulation might be comparable to that of normo-ovulatory women.

Risk factors for cardiovascular disease cluster in women with PCOS from an early age onwards [10,17,29]. The metabolic syndrome is a major risk factor for cardiovascular disease and has been reported to be up to five times as prevalent in women in PCOS [29,30]. This is in line with the current study, in which we observed an high prevalence of the metabolic syndrome in young women with PCOS. The prevalence of the metabolic syndrome in women with WHO2 anovulation was significantly lower and resembled that of normo-ovulatory women of the reference group. Our findings indicate that the cardiometabolic risk profile of women with WHO2 anovulation is distinctly different from that of women suffering from PCOS.

Table 3. Cardiovascular risk factors in patients with WHO2 anovulation, PCOS, and healthy controls

	Control N=262	**WHO2 N=277**	**PCOS N=1890**	**p value**
Obese	36 (13.7)	49 (17.7)	546 (28.9)	<0.001[b]
Overweight and obese	111 (42.4)	102 (36.8)	1009 (53.4)	<0.001[b]
Central obesity	-	65 (23.5)	755 (39.9)	<0.001[b]
Hypertension	37 (14.2)	54 (19.5)	438 (23.2)	0.100
Hyperglycemia	0 (0.0)	11 (4.0)	65 (3.4)	0.129
Insulin resistance	16 (29.1)	72 (26.0)	725 (38.4)	<0.001[b]
Lipid profile				
Total cholesterol ≥ 5.0 mmol/L	101 (38.5)	115 (41.8)	805 (42.8)	0.440
Triglyceride > 1.7 mmol/L	24 (9.2)	30 (10.9)	286 (15.2)	0.059
LDL cholesterol ≥ 3.0 mmol/L	120 (45.8)	110 (40.0)	924 (49.2)	0.004[b]
HDL cholesterol < 1.29 mmol/L	87 (33.2)	92 (33.5)	879 (46.8)	<0.001[b]

Values are in numbers (percentages). Mann-Whitney U test was used for evaluation of between group differences. Obese ((BMI ≥ 30kg/m^2), overweight and obese (BMI ≥ 25 kg/m^2), Central obesity (WC ≥ 88 cm), Hypertension (BP ≥ 130/85 mmHg), Hyperglycemia (glucose > 6.0 mmol/L), Insulin resistance (1/HOMA-IR < 0.47), *Abbreviations: body mass index (BMI), waist circumference (WC), blood pressure (BP), homeostatic model assessment for insulin resistance (HOMA-IR), low-density-lipoprotein (LDL), high-density-lipoprotein (HDL). a = WHO2 vs control, b = WHO2 vs PCOS*

We assessed the influence of BMI on cardiometabolic parameters of women with WHO2 anovulation, compared to women with PCOS and normo-ovulatory women. In lean women with WHO2 anovulation, we observed a cardiometabolic profile which is favorable to that of women with PCOS, confirming the negative impact of PCOS itself on the cardiometabolic profile. In overweight or obese women however, we observed no significant differences in cardiometabolic parameters of women with WHO2 anovulation, compared to women with PCOS or women from the reference group. With increasing BMI there seems to be a similar increase of cardiometabolic parameters. Our findings are in contrast with earlier studies, in which it was suggested that the prevalence of impaired glucose tolerance, type 2 diabetes and metabolic syndrome was higher in women with PCOS, when compared to a BMI matched control population [11,31,32]. The difference in outcome might be due to the differences in methodology, and could also be influenced by different ethnicities used in the various study populations. However, based on the current study, we conclude that the impact of BMI on the cardiometabolic profile is bigger than the impact of a fertility disorder itself. Of note, in the current study we found higher insulin and LDL cholesterol levels in women from the reference group, compared to women with WHO2 anovulation. This could be due to chance, or explained by the relatively high BMI in the women included in the reference group, stressing the importance of a healthy lifestyle. Maintaining a healthy lifestyle and bodyweight have a positive influence on the risk for cardiovascular disease and non-communicable diseases later in life [18,19,33]. In addition, a healthy lifestyle and BMI not only improve health outcomes in these women themselves, but could also positively influence short and long term health in their future offspring [19,33,34].

To our knowledge this is one of the first studies assessing the cardiometabolic profile in women with WHO2 anovulation, who do not meet the criteria for PCOS. We were able to assess a well phenotyped cohort of women who received extensive standardized screening at the outpatient clinic of a university hospital. Due to the extensive period of screening we were faced with various changes in endocrine and metabolic laboratory assays over time. Fortunately we were able to apply well established conversion factors necessary to correct for potential variations due to these assays alterations. Potentially, the 1/HOMA-IR threshold value used may have underestimated the true prevalence of IR. The gold standard for establishing IR is the euglycemic hyper insulinemic clamp. However, this rather elaborate procedure is not suitable for large-scale clinical use [24].

Conclusions

The cardiometabolic profile of women diagnosed with WHO2 anovulation only, seems to be distinctly different from that of women with PCOS, and to resemble that of normo-ovulatory women. We did not find evidence of an unfavorable cardiometabolic profile or an increased cardiovascular risk in women with WHO2 anovulation. Data regarding the long-term cardiometabolic outcome in these patients is not yet available. However, based on our findings, standardized cardiometabolic screening in women with WHO2 anovulation does not seem to be indicated.

Lean women with WHO2 anovulation exhibit a cardiometabolic profile, which is favorable to that of women with PCOS and resembles that of normo-ovulatory women. In overweight or obese women with WHO2 anovulation, PCOS or normo-ovulatory women, we observed a very similar cardiometabolic profile. in women with WHO2 anovulation, women with PCOS and healthy controls. This indicates that the impact of BMI on the cardiometabolic profile is bigger than the impact of a fertility disorder itself. that the impact of BMI on these cardiometabolic parameters is stronger than the impact of PCOS itself. We recommend a healthy lifestyle and achieving a health body weight before pregnancy, as this does not only has a positive influence on the risk for cardiovascular disease and non-communicable diseases later in life, but could also positively influence short and long term health in their future offspring.

Supplementary table 1. Cardiometabolic parameters in lean, overweight and obese women with WHO2 anovulation, or PCOS and healthy controls

	Lean			Overweight			Obese		
	Control (N=151)	**WHO2 (N=176)**	**PCOS (N=881)**	**Control (N=75)**	**WHO2 (N=52)**	**PCOS (N=463)**	**Control (N=36)**	**WHO2 (N=49)**	**PCOS (N=546)**
Systolic BP	110 (105–120)	110 (105-120)	110 (105-120)	120(110–122)	115(105 – 120)	115 (110 – 120)	120 (110 – 125)	120(113 – 130)	120 (115 – 130)
Diastolic BP	70 (66–80)	70 (65–75)	70 (65-80)	75 (70 – 80)	75 (70 – 80)	75 (70 – 80)	80 (72 – 85)	80 (75 – 90)	80 (75 – 85)
Waist	-	72 (68–76)	73 (68-78)	-	84 (80 – 92)	86 (80 – 92)	-	102 (95 – 116)	102 (95 – 111)
1/HOMA-IR	0.89 (0.53-1.38)	0.99 (0.64-2.03)[b]	0.91 (0.58–1.49)	0.57 (0.39 – 0.73)	0.57 (0.35 – 0.85)	0.53 (0.35 – 0.89)	0.35 (0.26 – 0.61)	0.35 (0.22 – 0.53)	0.31 (0.19 – 0.47)
Glucose	4.50 (4.20-4.80)	4.70 (4.40-5.00)	4.70 (4.40–5.00)	4.85 (4.65 – 5.18)	4.90 (4.35 – 5.20)	4.90 (4.50 – 5.20)	4.90 (4.70 – 5.20)	4.90 (4.60 – 5.50)	5.00 (4.60 – 5.40)
Insulin	41.8 (26.8-58.8)	34.0 (17.3-51.8)[a,b]	38.0 (24.0-57.0)	54.8 (40.9 – 77.9)	59.0(40.0 – 92.0)	61.0 (39.0 – 90.0)	99.8 (62.3 – 121.8)	92.0 (62.0 – 142.0)	103.0 (69.0 – 157.3)
Triglyceride	0.89 (0.67-1.18)	0.89 (0.67-1.15)	0.81 (0.64-1.09)	0.99 (0.73 – 1.34)	1.05 (0.78 – 1.40)	0.99 (0.76 – 1.44)	0.97 (0.73 – 1.61)	1.25 (0.89 – 1.80)	1.22 (0.88 – 1.80)
LDL-C	2.94 (2.48-3.48)	2.78 (2.31-3.22)[a]	2.89 (2.32-3.52)	3.10 (2.74 – 3.80)	2.97 (2.50 – 4.00)	3.05 (2.39 – 3.83)	2.81 (2.59 – 3.46)	2.87 (2.50 – 3.60)	3.10 (2.40 – 3.78)
HDL-C	1.51 (1.26-1.74)	1.55 (1.31-1.78)	1.53 (1.25-1.87)	1.42 (1.16 – 1.67)	1.35 (1.10 – 1.68)	1.25 (0.99 – 1.54)	1.27 (1.09 – 1.40)	1.09 (0.95 – 1.40)	1.05 (0.86 – 1.34)
Total-C	4.60 (4.10-5.40)	4.85 (4.19-5.41)	4.70 (4.10-5.40)	4.80 (4.30 – 5.60)	4.97 (4.40 – 5.72)	4.80 (4.16 – 5.60)	4.45 (3.93 – 5.48)	4.70 (4.07 – 5.61)	4.90 (4.20 – 5.70)

Values are in medians (interquartile range). Mann-Whitney U test was used for evaluation of between group differences. Abbreviations: blood pressure (BP), body mass index (BMI),homeostatic model assessment for insulin resistance (HOMA-IR), low-density-lipoprotein cholesterol (LDL-C), high-density-lipoprotein cholesterol (HDL-C). a = WHO2 vs control, b = WHO2 vs PCOS

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CHAPTER 5

PCOS and the risk for cardiovascular disease

CHAPTER 5.1

The cardiovascular risk profile of middle-aged women with polycystic ovary syndrome

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Abstract

Objectives

Contradictory results have been reported regarding the association between polycystic ovary syndrome (PCOS) and cardiovascular disease (CVD). We assessed the cardiometabolic phenotype and prevalence of CVD in middle-aged women with PCOS, compared with age-matched controls from the general population, and to estimate 10-year CVD risk and cardiovascular health score.

Design

A cross sectional study of 200 women with PCOS aged >45, and 200 age-matched controls.

Measurements

Anthropometrics, insulin, lipid levels, prevalence of metabolic syndrome, and type-II-diabetes. Ten year Framingham risk score and the cardiovascular health score were calculated, and carotid intima media thickness (cIMT) was measured

Results

Mean age was 50.5 years (SD 5.5) in women with PCOS and 51.0 years (SD 5.2) in controls. Increased waist circumference, body mass index and hypertension were more often observed in women with PCOS ($P<0.001$). In women with PCOS the prevalence of type II diabetes and metabolic syndrome was not significantly increased and lipid levels were not different from controls. cIMT was lower in women with PCOS ($P<0.001$). Calculated cardiovascular health and 10-year CVD risk were similar in women with PCOS and controls.

Conclusions

Middle-aged women with PCOS exhibit only a moderately unfavorable cardiometabolic profile compared to age-matched controls, even though they present with an increased BMI and waist circumference. Furthermore, we found no evidence for increased (10-year) CVD risk or more severe atherosclerosis compared to controls from the general population. Long term follow-up of women with PCOS is necessary to provide a definitive answer concerning long term risk for CVD.

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in women worldwide[1]. Risk factors for CVD are more prevalent and tend to cluster in women with polycystic ovary syndrome (PCOS) [2,3]. This syndrome represents the most common endocrine disorder in women of reproductive age, with a prevalence of up to 15 %[4]. PCOS has been associated with cardiometabolic abnormalities such as obesity, dyslipidemia, type II diabetes, hypertension, and the metabolic syndrome, which increase the risk for CVD[5,6]. PCOS is a syndrome characterized by ovulatory dysfunction, hyperandrogenism and polycystic ovarian morphology[7]. The phenotype of PCOS is modified by body mass index (BMI) and ethnicity, generally becomes milder with increasing age, and disappears largely after menopause[8-10]. The cardiometabolic abnormalities associated with PCOS can however, persist beyond the onset of menopause[5,8,11].

In the past it was assumed that women with PCOS would be more prone to develop CVD later in life[12]. The only available long-term follow up study in women with PCOS did however, not reveal an increased risk for CVD[6]. More recent studies in postmenopausal women with features of PCOS, seem to reinforce these findings[11,13]. At the same time, others suggest an increased incidence of CVD in women with PCOS already at an early age [14-16]. Whether or not women with PCOS are at increased risk to develop CVD still remains uncertain. Long before the onset of cardiovascular events, atherosclerosis can be detected. Carotid intima-media thickness (cIMT) is a marker of subclinical atherosclerosis and a can be used to predict future cardiovascular events[17]. In women with PCOS, an increased cIMT has been described, which suggests an increased risk for accelerated atherosclerosis compared to the general population [18]. It remains to be determined to what extent these surrogate markers translate into real cardiovascular events later in life[19].

In addition to markers used to detect (early) signs of CVD, models have been developed to estimate cardiovascular health and cardiovascular disease risk[20,21]. The Framingham study has provided an algorithm to predict the risk for future CVD, based on factors such as smoking, BMI and cholesterol levels[21]. At the same time, the American Heart Association has identified factors and behaviors, which improve cardiovascular health and reduce death from CVD. The simultaneous presence of ideal health factors and behaviors in an individual is associated with longevity and healthy aging[20,21]. Not much is known about the performance of women with PCOS in these models.

The aim of the current study was to assess the cardiometabolic phenotype and prevalence of CVD in middle-aged women previously diagnosed with PCOS, compared to age-matched controls from the general population. We compared the

cardiovascular profile and assessed the presence of subclinical atherosclerosis, by measuring cIMT. In addition, we assessed differences in the estimated cardiovascular health score and 10-year CVD risk between the aforementioned populations.

Methods

Patients

Women aged ≥40 years who were previously diagnosed with PCOS in one of the three participating university hospitals, were eligible for inclusion. All patients had in the past underwent a standardized examination, involving a questionnaire, anthropometric measurements, hormonal evaluation and a transvaginal ultrasonography to assess ovarian volume and follicle count. This protocol has been described in detail elsewhere [22] Diagnosis of PCOS was based on the Rotterdam criteria and established during the reproductive years [10]. According to these criteria, PCOS is diagnosed when either two or three of the key features are present: ovulatory dysfunction, polycystic ovarian morphology and clinical and/or biochemical hyperandrogenism[7]. In total around 850 women had previously been diagnosed and phenotyped by this standardized screening and by now reached the age of 40. Women with a poor ability of speaking or understanding of Dutch or English language or who were currently pregnant were excluded. All other women with PCOS aged >40 were invited to participate in the current study. Women visiting the outpatient clinic underwent an extensive endocrine and cardiovascular assessment which included general medical, obstetric and family history, education level, smoking status, and anthropometric measurements. Visualization of both carotid arteries was done using ultrasound. This study was approved by the institutional review board of the University Medical Center Utrecht, University of Utrecht and registered at www.clinicaltrials.gov, registration number NCT02616510. Written informed consent was obtained from all participants.

Controls

The control group was derived from the Rotterdam Study, a prospective population-based cohort study focusing on health and diseases in the elderly. We selected 200 women included in the third cohort of the Rotterdam Study. The third cohort includes inhabitants of the municipality of Ommoord, Rotterdam aged > 45 years, and was recruited between 2006 and 2008. Participants are examined extensively at the research center every 3 to 5 years. The rationale and design of this study have been described in detail elsewhere[23]. All participants provided written informed consent to participate in the study and to obtain information from their treating physicians. The Rotterdam Study has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of

Health, Welfare and Sports of the Netherlands. National trial registry number NTR6831.

Endocrine and cardiovascular assessment of women with PCOS and controls

On the day of the assessment, fasting blood samples were collected and assessed [2,24]. We measured cholesterols, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride serum levels. Furthermore, insulin and glucose levels, aspartate transaminasel (ASAT) and alanine transaminase (ALAT), androgens, gonadotropins, sex-hormone binding globulin (SHBG) and estradiol (E_2) levels were determined. Brain natriuretic peptide (NT-proBNP), a marker for heart failure, was also measured. The free androgen index was calculated as (Testosterone /SHBG)*100. Waist to hip ratio was calculated as waist circumference/hip circumference. Blood pressure was measured once in cases and twice in controls, in sitting position after at least five minutes of rest with a random-zero sphygmomanometer. Insulin resistance was assessed with the homeostasis model assessment (HOMA-IR). Insulin was converted to mU/L and next the HOMA-IR was calculated as: fasting serum insulin (mU/L) x fasting plasma glucose (mmol/L) /22.5. Diabetes was defined as a fasting glucose level of ≥7.0 mmol/L, use of anti-diabetic medication, or self-reported diagnosis. Hypertension was defined as systolic blood pressure (SBP) >139 mmHg, or diastolic blood pressure (DBP) >89 mmHg or use of anti-hypertensive medication. The National Cholesterol Education Program (NCEP) definition was used to determine the presence of the metabolic syndrome [25]. According to this definition metabolic syndrome is present when ≥3 of the following features are present: waist circumference ≥88 cm, fasting glucose ≥6.1 mmol/L, blood pressure >129/84 mmHg, high density lipoprotein (HDL) <1.3 mmol/L, triglycerides (TG) ≥ 1.7 mmol/L. Vitamin D deficiency was defined as a 25-OH-D serum of <50 nmol/L.

Carotid intima-media thickness for Women with PCOS and controls

We used cIMT to assess subclinical atherosclerosis in middle-aged women with PCOS and age-matched controls from the general population. cIMT was defined as the distance between the lumen intima and the media-adventitia and measured three times at both sides over 1 centimeter length and at least 0.5 centimeters proximal of the bifurcation of the common carotid artery, or at the beginning of the dilatation of the distal common carotid artery across a length of 1 centimeter[26-28]. The mean of the mean of the right and left carotid arteries was used for analysis. Ultrasound measurements were performed by trained professionals at the respective research center. Multiple devices were used for ultrasound measurements. In women with PCOS the Panasonic CardioHealthStation (Yokohoma, Japan), Esaote MyLabTMOne, and the ToshibaAplioArtida Medical System were used. Measurements with the various machines yielded similar results across the different

research centers (supplementary table 1). In controls the ATL UltraMark IV (Advanced Technology Laboratories, Bothell) was used.

Other measurements

Women with PCOS, with FSH serum levels of >40 (U/L) in combination with an amenorrhea were labelled as postmenopausal. In controls, postmenopausal status was self-reported via questionnaire. Information on prevalent CVD (stroke, myocardial infarction and/or coronary heart disease) was self-reported or obtained through general practitioners or hospital discharge reports. Smoking status was labelled as ever or never smoker. Former smokers and current smokers were grouped as "ever smokers" as it was not known how long ago women had stopped smoking. Ethnicity was self-reported.

The 10-year CVD risk was calculated according to the Framingham Risk Score (FRS), and based on age, SBP, HDL and total cholesterol, smoking and the presence of type II diabetes [21]. Low risk was defined as a 10-year CVD risk <10%, 10-20% as intermediate 10-year CVD risk, and >20% was marked as high 10-year CVD risk CVD. Next, we calculated the CHS in women with PCOS and controls[20]. The CHS was introduced by the American Heart Association and encompasses health factors (cholesterol and glucose serum levels, blood pressure and BMI) and behavioral factors (smoking, dietary intake and physical activity). Information on 5 out of the 7 factors (cholesterol, glucose, blood pressure, smoking status and BMI) was available in our study population. We did not have information on dietary intake and physical activity. However, measures of these parameters have been marked to be prone to sampling variability and misclassification[29,30]. Therefore, we calculated a composite CHS based on the 5 available parameters and assessed the mean CHS and performance of cases and controls on each of the health metrics.

Statistical analysis

For each case, a control was age-matched 1:1, from the Rotterdam study cohort, using propensity score matching (PSM) greedy approach. PSM was based on a logistic regression model that include PCOS vs no-PCOS as a dichotomous outcome and age as the only covariate under study. Hosmer-Lemeshow test was used to evaluate goodness-or-fit of the models. Transformation of age was used based on lowess graph in order to choose the best fitting model. Standardized differences and plots of propensity scores distribution between PCOS and control group, before and after matching procedure, were made to evaluate the balance achieved.

All statistical analysis were performed with IBM SPSS statistics version 24 (IBM Corp., Armonk, NY) and STATA version 14.2 (Station College, Texas, USA). A two-sided P <0.05 denoted statistical significance. Baseline characteristics were

presented as mean (standard deviation (SD)) or median (interquartile range (IQR)) for continuous variables and as proportions (%) for dichotomous variables. Continuous variables with a normal distribution were compared with the student t-test and with Mann-Whitney-U for non-normally distributed variables. Chi-square test or Fisher's exact test were used for categorical variables. Linear regression was used to assess cIMT. cIMT was log transformed to obtain a normal distribution. Results were expressed as regression coefficients (β) and corresponding 95% confidence intervals (95%CI). To eliminate the effect of possible confounders, we adjusted for BMI, smoking status, education level, research center, and menopausal status.

Finally, using the propensity score matching (greedy approach), we also matched cases and controls on both age and BMI to and repeated all the analyses to examine the effect of BMI on the cardiometabolic profile of women with PCOS.

Results

General and cardiometabolic characteristics

In this cross-sectional study, we compared 200 women diagnosed with PCOS with 200 age-matched controls from the general population. The baseline characteristics of the total study population are presented in **Table 1**. The mean age was similar in women with PCOS (50.5 years, SD 5.5) and controls (51.0 years, SD 5.2). Women with PCOS had experienced menarche at a later age (13.7, SD 2.6 vs 12.8, SD 1.6 (P<0.001)) and had more often experienced cycle irregularities in the past (69.8% vs 12.5% (P<0.001)). Compared to women with PCOS, a much larger proportion of the control population was already postmenopausal (40.5% vs 12.6% (P<0.001)). Women with PCOS were less often smokers (41.5% vs 64.8% (P<0.001)) and had more often attended higher general education or university (P<0.001). The free androgen index was significantly higher in women with PCOS (1.9 IQR 1.2-2.9 vs 1.2, IQR 0.8-1.7 (P<0.001)), as well as serum levels of E_2 (P=0.028), whereas SHBG levels were significantly lower (P<0.001).

We observed a higher BMI (28.4, IQR 23.8-32.9 vs 26.3, IQR 23.7-29.8 (P=0.015)), higher SBP (130.0, IQR 120.0-140.0 vs 122.0, IQR 112.0-136.0 (P=0.003)) and increased waist circumference (93.0, IQR 84.5-107.0 vs 85.9, IQR 79.5-94.6 (P<0.001)) in women with PCOS. Moreover, the prevalence of hypertension (48.2% vs 26.5% (P<0.001)) was increased and we observed higher glucose levels (5.3, IQR 5.0-5.7 vs 5.1, IQR 4.8-5.5 (P=0.019)). No differences were found in serum lipid levels, NT-pro-BNP, and vitamin D levels, prevalence of cardiovascular disease or type II diabetes. The prevalence of the metabolic syndrome was higher in women with PCOS, but this result did not reach statistical significance. The HOMA assessment of insulin resistance yielded similar results (P=0.647). When we repeated

the analyses in an age and BMI matched control population of 171 women, the analyses yielded similar results (data not shown).

Table 1. Characteristics of the total study population.

	PCOS (N=200)	Control (N=200)	P-value
General / Obstetric parameters			
Age (years)	50.5 (5.5)	51.0 (5.2)	0.35
BMI (kg/m^2)	28.4 (23.8-32.9)	26.3 (23.7-29.8)	0.02
Ethnicity (Northern-European)	170 (85.4%)	175 (87.5%)	0.50
Ever smoker	78 (41.5%)	129 (64.8%)	<0.001
Age at menarche (years)	13.7 (2.6)	12.8 (1.6)	<0.001
Postmenopausal	25 (16.0%)	81 (40.5%)	<0.001
OCP use (ever)	166 (83.4%)	182 (91%)	0.02
Amenorrhea (at age 25)	14 (6.9%)	3 (1.5%)	
Oligomenorrhea (at age 25)	127 (62.9%)	22 (11.0%)	<0.001
Regular cycle (at age 25)	35 (17.3%)	131 (65.0%)	
Education			
Primary	2 (1.1%)	18 (9.0%)	
Lower/intermediate/lower vocational	34 (18.0%)	77 (38.7%)	
Intermediate voc./higher general	70 (37.0%)	54 (27.1%)	<0.001*
Higher voc. or university	79 (31.8%)	50 (25.1%)	
Anthropometrics			
Waist (cm)	93 (85-107)	86 (80-94)	<0.001
Hip (cm)	107 (100-114)	106 (101-11)	0.68
Waist/Hip ratio	0.88 (0.83-0.93)	0.81 (0.77-0.86)	<0.001
Cardiometabolic parameters			
Systolic BP (mmHg)	130 (120-14)	122 (112-136)	<0.01
Diastolic BP (mmHg)	82.7 (11.3)	81.2 (11.3)	0.19
Hypertension	96 (48.2%)	53 (26.5%)	<0.001
Prevalent CVD	3 (1.5%)	3 (1.5%)	1.00
Lipid lowering medication	13 (6.5%)	31 (15.6%)	<0.01
Total cholesterol (mmol/L)	5.3 (4.5-6.0)	5.3 (4.8-6.1)	0.44
	PCOS (N=200)	**Control (N=200)**	**P-value**
HDL cholesterol (mmol/L)	1.5 (1.2-1.8)	1.5 (1.2-1.8)	0.68
LDL cholesterol (mmol/L)	3.3 (2.7-4.0)	3.1 (2.6-3.9)	0.42
Triglycerides (mmol/L)	1.0 (0.8-1.6)	1.1 (0.8-1.5)	0.35
ASAT (U/L)	22.0 (19.0-25.0)	21.0 (18.0-23.0)	0.09
ALAT (U/L)	21.0 (16.0-30.0)	20.0 (17.0-25.0)	0.38
Gamma GT (U/L)	20.0 (15.0-29.0)	17.0 (13.0-27.0)	0.02
NT-pro-BNP (pmol/L)	7.0 (4.0-13.0)	6.2 (4.1-11.1)	0.40
Elevated NT-pro-BNP (> 15 pmol/L)	27 (14.2%)	27 (18.8%)	0.26
Insulin (pmol/L)	74 (47-117)	72 (55-105)	0.83
Glucose (mmol/L)	5.3 (5.0-5.7)	5.1 (4.8-5.5)	0.02
HOMA-IR	2.68 (1.54-4.33)	2.43 (1.71-3.69)	0.65
Diabetes	22 (11.1%)	13 (6.5%)	0.11

Cardiometabolic parameters			
Metabolic syndrome (NCEP)	45 (25.0%)	34 (17%)	0.06
Mean carotid cIMT (um)	612.8 (93.6)	721.7 (118.4)	<0.001
Endocrine parameters			
FAI	1.9 (1.2-2.9)	1.2 (0.8-1.7)	<0.001
Testosterone (nmol/L)	0.9 (0.6-1.2)	0.8 (0.6-1.1)	0.041
SHBG (nmol/L)	48.5 (34.3-70.7)	69.6 (46.7-100.4)	<0.001
Androstenedione (nmol/L)	2.6 (1.9-3.7)	3.0 (2.1-4.0)	0.02
DHEA (nmol/L)	9.8 (6.1-14.3)	13.9 (9.5-20.9)	<0.001
E2 (pmol/L)	151 (41-383)	79 (18-346)	0.03
Vitamin D deficiency	55 (36.9%)	78 (41.7%)	0.48

*Values are displayed as Means (standard deviation) or medians (interquartile range), or as numbers (percentage). Differences were tested with Student's T-test for variables with a normal distribution, Mann-Whitney-U test was used for variables with a skewed distribution. Chi-square test or Fisher's exact test were used for categorical variables Abbreviations: Body mass index (BMI), oral contraceptive pill (OCP), blood pressure (BP), cardiovascular disease (CVD), high-density lipoprotein (HDL), low-density protein (LDL), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT). *use of Fisher's exact test.*

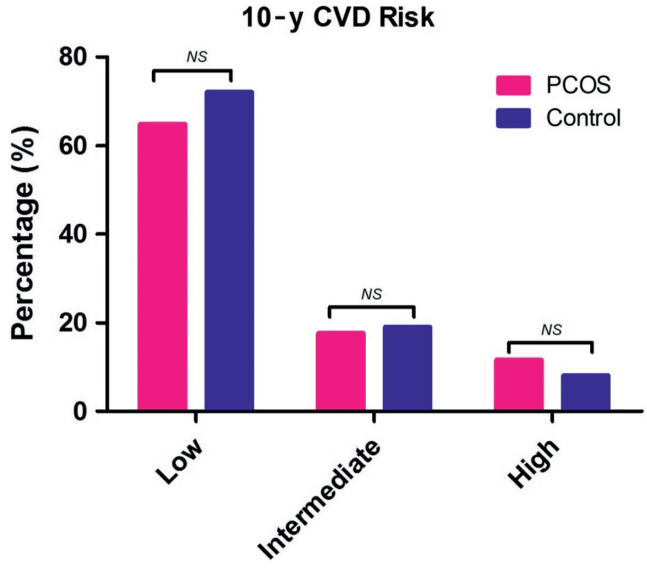
Carotid-intima media thickness

We observed a lower mean cIMT (um) in women diagnosed with PCOS compared to age-matched controls (612.8, SD 93.6 vs 721.7, SD 118.4 (P<0.001)). The latter was consistent across all participating university hospitals (supplementary table 1). In a linear regression model after adjusting for BMI, smoking, SBP, education, measurement center and menopausal status, we found that PCOS was associated with a lower cIMT β (95%CI) -0.212 (-0.283: - 0.142, P <0.001).

Ten-year cardiovascular disease risk and the cardiovascular health score

The median 10-year CVD risk was 5.79% in women with PCOS and 7.38% in controls (P=0.214). Next, we categorized women into low (<10%), intermediate (10-20%) and high (>20%) risk for a cardiovascular event in the subsequent 10 years. We observed no significant differences in the proportion of women with PCOS and controls in each risk category (P=0.388, **Figure 1**).

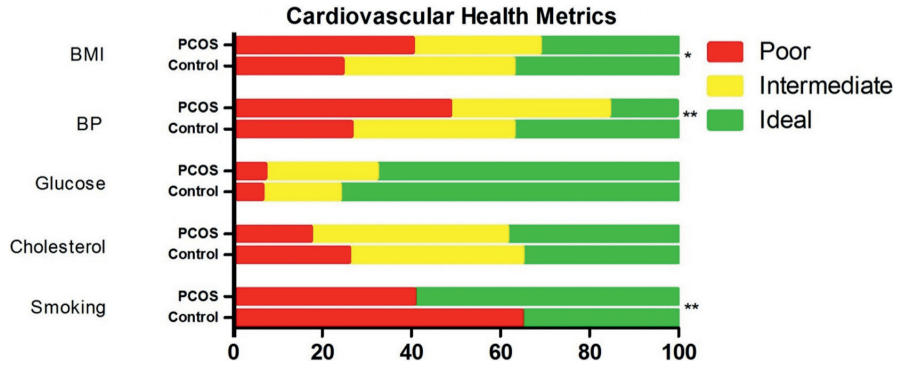
Figure 1. Ten year risk for CVD in women with PCOS and controls.



The 10 year risk for CVD in women with PCOS and age-matched controls. A risk of <10% was marked as low risk, 10-20% as intermediate and >20% as high risk. Abbreviations: polycystic ovary syndrome (PCOS), not significant (NS).

The composite cardiovascular health score was calculated in all patients and controls with available information on all health metrics. We used information on BMI, blood pressure, glucose and cholesterol serum level and smoking status. We were able to calculate the CHS in 158 cases and 199 controls. The mean (SD) CHS in PCOS women was 5.69 (2.18) and 5.71 (1.99) in controls (P=0.915). The performance of women with PCOS and controls on each of the separate cardiovascular health metrics is presented in **Figure 2**.

Figure 2. Performance of women with PCOS and controls on cardiovascular health metrics



*Prevalence (%) of poor, intermediate and ideal cardiovascular heath metrics in women diagnosed with PCOS and controls. A * denotes statistical significance of <0.01, ** denotes a statistical significance of <0.001. abbreviations: BMI: body mass index, BP: blood pressure, PCOS: polycystic ovary syndrome.*

Discussion

In this large cross-sectional study in women with PCOS around the age of 50, we observed that women with PCOS exhibit only a moderately unfavorable cardiometabolic profile compared to age-matched controls, despite a higher BMI and larger waist circumference. The prevalence of major risk factors for CVD or CVD itself was not increased, and we found no evidence for more severe atherosclerosis in women suffering from PCOS. Finally, the aggregated measure of 10-year CVD risk and overall performance on cardiovascular health metrics in women with PCOS were similar to age-matched controls from the general population.

PCOS is associated with cardiometabolic disturbances which can persist throughout life[5,11]. Indeed, we observed that compared to age-matched controls, waist circumference, BMI, SBP and androgen levels were all significantly higher and the prevalence of hypertension was nearly 50%. At the same time, we observed no differences in lipid levels of women with PCOS and age-matched controls. In addition, neither HOMA assessed insulin resistance, nor the prevalence of type II diabetes, metabolic syndrome or CVD were significantly increased at the age of 50, despite of a higher BMI and blood pressure in women with PCOS. Atherosclerosis can be detected with cIMT and used as a predictor for future CVD. In the current study, we measured a lower cIMT in women with PCOS. Variability in measuring techniques and devices may have influenced our results, baring reason for caution.

However, this finding indicates that atherosclerosis is not more advanced in women with PCOS[17,31]. In line with this, both the estimated Framingham 10-year CVD risk and the cardiovascular health score, used to predict longevity and healthy aging, were similar in women with PCOS and age-mathed controls. Of note, repeating all analyses with an age-BMI matched control population yielded similar results, suggesting the outcomes of our study were not driven by the higher BMI in women with PCOS.

Previous studies report an increase of CVD risk factors in women with PCOS, such as dyslipidemia, insulin resistance, type II diabetes and metabolic syndrome. This risk is increased by the presence of PCOS per se, but also strongly correlated with BMI as overweight and obese women with PCOS are most at risk[6,16,32,33]. The relatively low BMI in our PCOS cohort could explain the seemingly contradictory results in the prevalence of metabolic syndrome and type II diabetes[32,33]. However, it also seems as if some metabolic disturbances associated with PCOS are detectable at any age, whilst others seem to disappear over time[2,5,6,34]. Evidence suggests that of the lipid disturbances associated with PCOS, increased triglyceride levels are the only lipid abnormality still detectable at older age[5,6,11,13]. The same pattern seems to apply to the metabolic syndrome, which has been described to be five times as prevalent in young women with PCOS, but to remain only two times as prevalent after the age of 39[5,13]. Another possible explanation for this could also be that these women were already diagnosed early on during their reproductive years and were also informed about their long term health risks which they might have adjusted accordingly in the years following initial diagnosis.

Conflicting results have been reported on cIMT and CVD in women with PCOS[3,18,35]. Data on cIMT in middle-aged and older women with (features of) PCOS is scarce, but most evidence suggests a higher cIMT[3,11,36]. The lower cIMT in women with PCOS compared to age-matched controls in our study could be explained by the small proportion of women with PCOS who were postmenopausal at the age of 50. The menopausal transition is associated with an increase in cIMT[31]. The fact that on average, women with PCOS enter menopause at a later age could have a protective effect on the development of atherosclerosis and risk for future CVD[37,38]. Indeed, most evidence points into the direction that the risk for CVD in women with PCOS is not increased. A recent large Danish study in women with PCOS however, demonstrated higher incidence rates of CVD already an early age [14]. In this study hypertension and dyslipidemia were considered cardiovascular diseases, and comprised the majority of CVD diagnosis. The event rate for stroke was not increased and the event rate for ischemic heart disease in women with PCOS was slightly increased but included milder forms of ischemic heart disease (angina) in the definition[14]. Similarly, in the current study we detected a much higher prevalence of hypertension in women with PCOS, but the prevalence of cardiovascular events was similar to the general population and NT-proBNP as a marker for heart failure

was not increased. We believe most evidence still points into the direction that long term risk for CVD events (stroke, myocardial infarction and/or coronary heart disease) in women with PCOS might not be increased[6,11,13].

How is it possible that so many known risk factors for CVD cluster in women with PCOS already at an early age, yet this does this not seem to translate into an increased risk for cardiovascular disease later in life? It might be that there is an early worsening in risk factors for CVD in women with PCOS, which doesn't seem to progress much over the years[5,6]. Again the latter might be due to the fact that women are aware of these risk factors and do anticipate accordingly to them. This in contrast to controls who seem to develop metabolic abnormalities gradually over time and apparently end at a similar level as women with PCOS. Furthermore, genetic studies have provided us with clues suggesting women with PCOS might be able to compensate the damage caused by this unfavorable accumulation of risk factors. Genetic variants associated with late menopause and associated with better DNA–repair and maintenance are more prevalent in women with PCOS. These variants are correlated with long-term health and longevity, suggesting a potential evolutionary advantage for women with PCOS[39,40]. Indeed in our study the majority of middle-aged women with PCOS were not yet postmenopausal at the age of 50. Finally, hyperandrogenism is present in the majority of women with PCOS and associated with cardiometabolic abnormalities at a younger age. In the past, hyperandrogenism was suggested as a main driver for the CVD risk in PCOS[2,34]. The effects of hyperandrogenism after menopause are still heavily debated. While some consider hyperandrogenism to be a risk factor for CVD, other studies have shown that hyperandrogenism is not associated with a higher risk for CVD, and could even be protective against CVD[9,11,13,41].

All of these proposed mechanisms could protect women with PCOS from developing CVD. Despite of their unfavorable profile at a younger age, long-term cardiovascular health in women with PCOS seems to be similar to that of the general population. Based on the selective enrichment with better DNA repair and maintenance genes one could hypothesize that these women should actually be healthier compared to the general population provided that they had received proper preventive treatment in combination with a healthy lifestyle from an early age on.

This is one of the largest clinical studies assessing the cardiometabolic profile and prevalence of CVD in women diagnosed with PCOS around the age of 50. Besides availability of several subclinical measures of atherosclerosis, detailed information on study subjects made it possible to comprehensively address the cardiometabolic profile of these women and to estimate their risk for future CVD. The limitations of our study also merit consideration. Our dataset is quite precise and complete, unfortunately we were still sometimes faced with missing data. Although this was

only the case for a small proportion of the data, this may have led to an underestimation of the true prevalence of for instance the metabolic syndrome, as we were not able to assess all parameters in all patients. In addition, although our study population comprised a large population of meticulously phenotyped women with PCOS, we might still have lacked sufficient power to detect small associations. Our findings therefore still need to be validated in a large cohort of women with PCOS followed up until old age.

Studies following women with PCOS until very old age will eventually provide definitive answers on the risk for CVD and the involved mechanisms. Therefore, cardiovascular assessment and follow up of women with PCOS is still necessary. At this time however, we conclude that although some metabolic disturbances were present in our large cohort of middle-aged women with PCOS, we found no evidence for premature atherosclerosis or an increased risk for future CVD. Only, time will tell whether this will indeed translate into a better cardiovascular health in women with PCOS than was previously anticipated.

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CHAPTER 5.2

Cardiovascular risk profile - imaging and gender-specific disorders (CREw-IMAGO): rationale and design of a multicenter cohort study

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Abstract

Background

Reproductive disorders, such as polycystic ovary syndrome (PCOS), primary ovarian insufficiency (POI) and hypertensive pregnancy disorders (HPD) like pre-eclampsia (PE), are associated with an increased risk of cardiovascular disease (CVD). Detection of early signs of cardiovascular disease (CVD), as well as identification of risk factors among women of reproductive age which improve cardiovascular risk prediction, is a challenge and current models might underestimate long-term health risks. The aim of this study is to assess cardiovascular disease in patients with a history of a reproductive disorder by low-dose computed tomography (CT).

Methods

Women of 45 - 55 years, who experienced a reproductive disorder (PCOS, POI, HPD), are invited to participate in this multicenter, prospective, cohort study. Women will be recruited after regular cardiovascular screening, including assessment of classical cardiovascular risk factors. CT of the coronary arteries (both coronary artery calcium scoring (CACS), and contrast-enhanced coronary CT angiography (CCTA)) and carotid siphon calcium scoring (CSC) is planned in 300 women with HPD and 300 women with PCOS or POI. In addition, arterial stiffness (non-invasive pulse wave velocity (PWV)) measurement and cell-based biomarkers (inflammatory circulating cells) will be obtained.

Discussion

Initial inclusion is focused on women of 45 - 55 years. However, the age range (40-45 years and/or ≥ 55 years) and group composition may be adjusted based on the findings of the interim analysis. Participants can potentially benefit from information obtained in this study concerning their current cardiovascular health and expected future risk of cardiovascular events. The results of this study will provide insights in the development of CVD in women with a history of reproductive disorders. Ultimately, this study may lead to improved cardiovascular prediction models and will provide an opportunity for timely adjustment of preventive strategies. Limitations of this study include the possibility of over diagnosis and the average radiation dose of 3.5 mSv during coronary and carotid siphon CT, although the increased lifetime malignancy risk is negligible.

Background

Reproductive disorders, including polycystic ovary syndrome (PCOS), primary ovarian insufficiency (POI) and hypertensive pregnancy disorders (HPD) such as pre-eclampsia (PE), are associated with an increased risk of cardiovascular diseases (CVD).

Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) has a prevalence of around 8 to 10% in Caucasian women and is the most common endocrine disorder in women of reproductive age [1]. According to the Rotterdam consensus criteria, PCOS is diagnosed when at least two of the following criteria are present: oligo−/anovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovaries on ultrasonography [2]. Insulin resistance, dyslipidemia and type 2 diabetes mellitus (T2DM) have been associated with PCOS [3-7].

Increasingly PCOS has been associated with cardiovascular risk factors, such as impaired glucose tolerance, obesity, metabolic syndrome (MetS) and hypertension. Several studies have ascertained premature signs of subclinical arterial disease in women with PCOS, such as abnormal carotid intima media thickness on ultrasound or coronary artery calcification score (CACS) on computed tomography (CT) [8-10]. Nevertheless, evidence on the potential association between PCOS and CVD endpoints is still limited [11-13].

Primary ovarian insufficiency

Primary ovarian insufficiency (POI), formerly known as premature ovarian failure, is characterized by secondary amenorrhea for at least 4 months accompanied by elevated FSH levels above 40 IU/L, before 40 years of age [14]. The incidence of POI is reported to be 1-2% [15,16]. POI is associated with elevated gonadotropins, hypoestrogenemia and hypoandrogenemia.

Early age at menopause, including POI, is associated with an increased incidence of coronary heart disease and CVD mortality [17-19]. Epidemiological data showed that the relative risk (RR) on CVD was 1.03 (95% confidence interval (CI) 1.01 – 1.05) for each 1- year decrease in age at menopause [19]. Hypoandrogenemia in women has been associated with an increased risk of atherosclerosis, as measured by CIMT or catheter angiography [20-23] and CVD events [24]. A recent systematic review and meta-analyses identified POI as an independent, modest risk factor for developing or dying from IHD (ischemic heart disease) (hazard ratio (HR) 1.69, 95% CI 1.29-2.21, $p = 0.0001$) and total CVD (HR 1.61, 95% CI 1.22-2.12, $p = 0.0007$) [25]. No relationship was found for POI and stroke (HR 1.03, 0.88 - 1.19, $p = 0.74$). These findings may implicate a decreased cardiovascular health in women with POI. However, like PCOS, it remains unclear to which extent POI is independently associated with CVD due to the paucity of data.

Hypertensive pregnancy disorders

HPD include pregnancy-induced hypertension (PIH), PE and the hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome. Together, this group of disorders complicates 5-12% of all pregnancies worldwide, [26] while PE alone is seen in 3-5% of all pregnancies [27,28]. Several studies showed that both classical CVD risk factors and novel serum biomarkers for CVD were increased in former hypertensive pregnancies (PIH, late-onset PE and especially early-onset PE) compared to normotensive pregnancies in both premenopausal and postmenopausal women [29,30]. Major CVD risk factors (e.g. hypercholesterolemia, hypertension, diabetes and MetS) were 3-4 fold more prevalent in formerly pre-eclamptic patients when compared with healthy controls of the same age at one to three years after index pregnancy [31-33]. However, mainly due to the relative young age (mean 30.5 years), the 10-year absolute risk of a CVD event as estimated by the Framingham Risk Score (FRS) was still low (mean estimated 10-year cardiovascular disease risk 1.08%) [31].

Women who were diagnosed with pre-eclampsia have a twofold future CVD risk [34-38]. The relative risk of developing hypertension later in life in women with a history of pre-eclampsia is 3.74 [39]. Moreover, the risk of developing diabetes later in life is also 2-3 times increased in women with a history of pre-eclampsia compared to women without such a history [40-42]. These findings have led to the hypothesis that pregnancy acts as a stress-test for CVD later in life [43].

The sub-analyses of a longitudinal follow-up study of the HYPITAT trial – the HyRAS study – showed neither significant differences in hypertension and biochemical cardiovascular risk factors postpartum, nor a difference in the estimated 10- and 30 year Framingham cardiovascular event risk, between women with a history of late-onset pre-eclampsia compared to women with PIH [32]. On the other hand, the increased CVD risk does appear to be more pronounced in the subgroup of early-onset of pre-eclampsia (generally defined as pre-eclampsia occurring before 34 weeks of gestation), with a RR of 7 to 8 on IHD and death due to IHD [34,35].

Despite recent advances in long-term follow-up after reproductive disorders, identifying women at increased risk for premature CVD remains a challenge. The use of the FRS and other risk models for IHD, like the Systematic Coronary Risk Evaluation (SCORE), Reynolds risk score and the Pooled Cohort Equations, are limited by their underestimation of lifetime CVD risk in young women. The recently published Dutch guideline "Cardiovascular Risk Management after Reproductive Disorders" recommends all women with a history of a reproductive disorder to optimize lifestyle factors [44]. Patients with a history of pre-eclampsia are advised to generate a risk profile at 50 years of age, as their risk of hypertension and diabetes mellitus is increased and the onset of these cardiovascular risk factors is up to 7 years earlier compared to women without pregnancy complications [39,45]. However, longitudinal follow-up data on biomarkers, signs and symptoms of premature subclinical atherosclerosis are needed to better identify the potential adverse effects of female-specific risk factors and life-events on CVD risk.

Serum biomarkers

Circulating endothelial cells, extracellular vesicles and circulatory inflammatory cells might lead to discovery of new biomarkers for women with reproductive disorders which are at risk for CVD development.

Both HPD and CVD later in life share a pathophysiologic pathway of vascular (endothelium) damage. Pre-eclampsia is associated with an increased number of circulating endothelial cells due to a high degree of endothelial cell activation or injury. Extracellular vesicles (e.g. microvesicles, exosomes) reflect the disease state of pre-eclampsia patients compared to healthy pregnant women [46,47]. In addition, extracellular vesicles -associated polygenic immunoglobin receptor, cystatin C, and complement factor C5a are markedly increased in patients suspected of acute coronary syndrome [48]. As EVs might be involved in both reproductive disorders and CVD, they could possibly serve as a biomarker.

The inflammatory profile of circulating cells is proven to be very different in women suffering from CVD [47]. For example, carotid plaques show sex-dependent inflammatory cell content, including neutrophils [49].

Imaging

CACS acquired with CCT has been shown to have superior predictive value for CVD events to traditional risk factors, risk factor scores and serum biomarkers in asymptomatic persons [50]. Contrast-enhanced CCTA may have additional value over CACS as it can also identify non-calcified plaques, and thereby the total atherosclerotic burden, and assess the presence of coronary luminal narrowing [51-54]. As calcification of plaque occurs at a relatively late stage in atherosclerosis, significant coronary atherosclerosis may be visualized earlier by visualizing the non-calcified coronary plaque with CCTA. Data of CCT as a diagnostic tool, i.e. CACS or CCTA, is scarce and inconclusive for women with reproductive disorders.

Several studies have assessed the presence of coronary calcium in PCOS. Although some of these studies showed an increased CACS in women with PCOS, a recent large, cross-sectional study could not find an association with PCOS and CACS [55-58].

In a retrospective cohort study published in 2007, the relation between HPD and coronary calcification later in life was assessed in 491 women (mean age 66.8 years, standard deviation 5.4 years) [59]. Coronary calcifications (Agatston score ≥ 1) were found in 305 women (62.9%).This study showed that a self-reported history of hypertension during pregnancy is related to higher CACS in the 7th decade of life (Odds ratio (OR) 1.57, 95% CI 1.04 - 2.37). In an adjusted model correcting for age, BMI, waist:hip ratio, systolic blood pressure and diastolic blood pressure the relation did not reach statistical significance anymore (OR 1.52, 95% CI 0.96 - 2.39) [59]. Other retrospective studies assessing coronary artery disease (CAD) by catheter coronary angiography in women suspected for CAD and with a history of HPD showed

contrasting results [60,61]. A recent prospective cohort study conducted among 40 former pre-eclamptic women and 40 age- and parity-matched healthy controls showed increased CACS in former pre-eclamptic women at a mean age of 59.5 ± 4.6 years. The unadjusted OR for having higher CACS due to preeclampsia was 3.54 (95% CI 1.39 - 9.02), although the adjusted model correcting for BMI and hypertension did not reach statistical significance anymore [62].

There is mounting evidence that intracranial carotid artery calcifications are associated with increased large artery and intracranial artery stiffness, as well as ischemic stroke, white matter abnormalities and cognitive impairment [63,64]. Recent studies found a relationship between carotid siphon calcium (CSC) and white matter hyperintensities and lacunar infarcts [65-67]. Other studies, however, could not confirm these findings [68-70].

Collectively, previous studies indicate an association between women with reproductive disorders and CVD later in life. Current risk profiles are inadequate to establish future CVD risk in still relative young premenopausal women. The aim of this study is to assess the diagnostic value of cell-based biomarkers, CCT imaging (both non-contrast CACS and contrast enhanced CCTA) and non-contrast CSC in patients with a reproductive disorder to detect CVD.

Methods

Study design and study setting

In this multicenter, prospective, cross-sectional study of patients with a reproductive disorder (PCOS, POI or HPD) we aim to assess the diagnostic value of cell-based biomarkers and CT imaging of the coronary arteries and carotid siphon in the detection of CVD. Patients will be invited to participate at their regular cardiovascular screening, which is performed at two large University Medical Centers in Utrecht (UMC Utrecht) and Rotterdam (Erasmus MC) in the Netherlands.

Participant characteristics

All patients with a reproductive disorder undergo regular cardiovascular screening at a specialized vascular outpatient clinic in one of the participating hospitals as part of standard care for cardiovascular diseases. The study population consists of women aged 45-55 years within three different groups:

1. Women with PCOS defined by Rotterdam consensus criteria, requiring the presence of at least two of the following criteria: oligo−/anovulation, clinical and/or biochemical hyperandrogenism, and/or polycystic ovaries on ultrasonography.

2. Women with POI defined as women with secondary amenorrhea for at least 4 months accompanied by elevated FSH levels above 40 IU/L, occurring prior to 40 years of age.

3. Women with a history of HPD (PIH, early-onset PE (i.e. delivery before 34 weeks of gestation) and late-onset PE (i.e. delivery after 34 weeks of gestation)) according to the ISSHP criteria, verified in clinical records.

Patients with any serious illness that can compromise study participation, patients with high risk for contrast nephropathy (renal dysfunction with an estimated glomerular filtration rate < 60 ml/min/1.73 m2) or patients with a history of myocardial infarction are excluded from the study.

After written informed consent is obtained, patients will undergo cardiovascular imaging assessment by CCT imaging, biomarkers and a non-invasive vascular measurement.

Coronary CT imaging

CCT is performed using a multislice CT scanner (256 slice Philips CT, Philips Healthcare, Best, the Netherlands or dual source Somaton Force or Drive Siemens CT, Siemens, Forchheim, Germany) with prospective ECG-triggering. A non-contrast coronary CT is acquired first to calculate the CACS (scan parameters 120 kV, 50 mAs or reference mAs of 80 mAs). Participants with a heart rate > 65 beats/min may receive an oral (25-50 mg) and/or intravenous (5-20 mg) beta-blocker (metoprolol, Selokeen AstraZeneca, Zoetermeer, the Netherlands) before the scan. All participants will receive sublingual nitroglycerine just before the CCTA. CCTA scan parameters will be as follows depending on the participant's weight:

1. For the Philips scanner a prospective ECG-triggered acquisition is performed at a mid-diastolic phase (78%) with 80-120 kV; 195-210 mAs; and 90–115 ml non-ionic contrast material (Iopromide, 300 mg I/ml; Ultravist, Bayer Healthcare, Berlin, Germany) followed by 30–40 ml saline, both injected at a speed of 6–6.7 ml/s. A bolus-tracking technique is used to time the arrival of contrast in the coronary arteries. The CCTA scan is initiated once a threshold of 130 HU is reached in the descending aorta followed by a 7-s post-threshold delay. CCTA's are reconstructed with 0.9 mm slice thickness and iDose iterative reconstruction level 4 and 6.

2. For the Siemens scanners a sequential prospective ECG–triggered acquisition is performed with a pulsing window width depending on heart rate or a high-pitch acquisition timed to image the heart in diastole in case of low regular heart rate. KV and mAs are selected using automatic KV selection based on the topogram (range 70-120 kV) and a reference mAs setting of 230 mAs at 120 kV. At lower kVs reference mAs is automatically adapted accordingly. Either a bolus-tracking technique or test bolus injection with 10 ml contrast is used to time the arrival of contrast in the coronary

arteries at the discretion of the technician. Non-ionic contrast material (Iopromide, 370 mg I/ml; Ultravist, Bayer Healthcare, Berlin, Germany) is used followed by 30-40 ml saline, both injected at a speed of 5.4 ml/s. Total contrast volume is calculated as scan time + 8 s multiplied by contrast flow rate. Mostly around 70 ml of contrast is injected for the CCTA. CCTA's are reconstructed with 0.6 mm slice thickness and ADMIRE iterative reconstruction level 3.

The total CCT radiation dose to which participants will be exposed is expected to be within 3.0 mSv.

CT scans are post processed on a workstation (IntelliSpace Portal, Philips Healthcare, QAngio CT software, Medis Medical Imaging or SyngoVia,Siemens) by experienced personnel. CACS is measured on the non-contrast CT with the Agatston scoring method [71]. Coronary artery calcium is defined as a density of >130 Hounsfield units (HU) in a coronary artery. Total CACS is calculated by the sum of all lesions in all four coronary arteries and their side branches. The total Agatston score will be categorized as no calcification (CACS = 0), mild (CACS > 0 and < 100), moderate (CACS ≥ 100 and < 400) and severe (CACS ≥ 400) calcification; and compared with the MESA database [72]. Semi-automated vessel analysis is used to make multiple curved multiplanar reconstructions (MPR) of all coronary arteries on the CCTA data.

All cardiac CT scans will be assessed by an experienced cardiovascular radiologist in both academic hospitals. Image quality, plaque characteristics and coronary lumen stenosis will be analyzed on a 18-segment basis according to the modified American Heart Association classification [73,74]. Plaque composition will be evaluated in a qualitative manner as calcified, mixed (both calcified and non-calcified components) and non-calcified (plaques without calcium). Total atherosclerotic plaque burden will be measured with both the segmental involvement score (SIS) and the segment stenosis score (SSS) based on the 18-segment coronary artery model [74]. Luminal stenosis will be graded as absent, minimal (1 – 24%), mild (25 – 49%), moderate (50 – 69%), and severe (≥ 70%) narrowing on the basis of diameter measurements comparing the diameters of the maximal stenosis to a reference diameter proximal and distal to the stenotic area [75]. If severe calcifications are present and quantification of stenosis is difficult, the radiologist will refrain from stenosis quantification and score the segments involved as 'calcified, stenosis unclear'.

Carotid siphon calcification imaging

A non-contrast CT with 20-40 mm coverage is planned around the sella turcica to include the intracranial carotid siphon and anterior clinoid process. The head is tilted with the chin towards the breast to avoid scanning the eye lens. The CSC radiation dose to which participants will be exposed is expected to be less than 0.5 mSv on average. Scan parameters will be as follows:

1. For the Philips scanner: 20 mm coverage from petrous apex to ICA top with 120 kV, 100 mAs, brain filter B and C, iDose iterative reconstruction level 3, 1 mm and 2 mm reconstruction thickness without overlap.

2. For the Siemens scanner (Somatom Force or Drive): 40 mm coverage from horizontal part of the petrous ICA to ICA top with 120 kV, 100 mAs reference, H31s and H45s head filter, 075 mm reconstruction thickness and 0.4 mm increment.

The axial scans are visually assessed by a radiologist for presence or absence of carotid siphon calcification. The severity of CSC will be visually assessed and categorized according to Woodcock as 0 = absent, 1 = mild (thin, discontinuous), 2 = moderate (thin, continuous or thick, discontinuous) or 3 = severe (thick, continuous) [76,77]. In addition, the CSC will be semi-automatically quantified as described in detail previously [65].

Single center side study: Serum biomarkers and non-invasive vascular measurements

Classical cardiovascular biochemical risk factor assessment (glucose, insulin, triglycerides, total cholesterol, low-density lipoprotein cholesterol and c-reactive protein) and a general hematological profile (total red and white blood cells and differential counts of nucleated cells) will both be determined.

As part of a single center side study in the UMC Utrecht, the neutrophil, monocyte, and lymphocyte cell numbers and subtype distribution will be examined based on established cell surface marker expression by flow cytometry analysis [78,79]. Peripheral blood mononuclear cells and circulating endothelial cells will be isolated and sorted directly after drawing blood. To assess histone modification in inflammatory genes, monocytes are cultured in the presence of atherosclerosis associated antigens (oxLDL, HSP). Plasma will be used to isolate and detect (endothelial) microparticles. Proteomics on isolated microparticles will be performed on selected samples and analyzed. Lupus anticoagulants are phospholipid-dependent coagulation inhibitors that are detected with a functional assay based on a clotting test. Platelet reactivity can be assessed with the platelet activation test (PACT), a whole blood functional test of the total platelet response capacity.

Arterial stiffness measured by pulse wave velocity (PWV) is a predictor of cardiovascular events. The Arteriograph (TensioMed, Budapest, Hungar. EC Directive 93/42/EEC, ISO 13485:2003, ISO 13485:2004) is an oscillometric arterial stiffness measurement, which will be performed directly before the CCT in the UMC Utrecht.

Outcome

The primary outcome is the presence of relevant CAD that will be defined as one or more of the following on CT: a CACS ≥100 AU or luminal stenosis ≥ 50%. All measurements will be discussed with participants individually under supervision of a vascular specialist. For management of cardiovascular risk factors, the current guideline on European Guidelines on cardiovascular disease prevention will be used [80]. All patients with relevant CAD are discussed with a cardiologist. Management and treatment decisions are left to the discretion of the cardiologist. As a general rule, participants with a CACS of 100-400 AU without obstructive CAD will receive lifestyle advice and may be recommended to initiate treatment with statins [81,82]. Participants with a CACS ≥ 400 AU or coronary artery stenosis ≥ 50% will be offered a consultation with a cardiologist to discuss management options. Other relevant cardiac findings will be discussed with the cardiologist. If incidental extra-cardiac findings, such as lung or liver lesions, are considered to be of clinical importance, recommendations of further testing, follow-up or referral to another specialist will be made.

Sample size calculation

No prospective studies have performed CCT (including both CACS and CCTA) in women with reproductive disorders as part of long-term cardiovascular follow up. The estimation of the expected necessary sample size is based on $\alpha = 0.05$ and desired power = 0.90. In addition, the background prevalence of coronary plaque (both non-calcified and calcified) in asymptomatic, healthy females ≥ 45 years old based on the CCTA study by Kim et al. (2013) is estimated 6.7% [83]. We presume a relative risk of around 2 for the development of CVD is women with reproductive disorders [44]. This leads to an (conservative) estimated prevalence of coronary artery disease (plaque) of 13.4% (95% CI 10.0 - 17.6) and a minimal sample size of at least 261 CCT's in both the HPD and the PCOS/POI group.

Based on these results and given the radiation-induced health risks, initial inclusion will be confined to women who are 45-55 years old and we will perform an interim analysis after 300 CCT's (100 in patients with HPD, 100 in patients with PCOS and 100 in patients with POI). If the prevalence of plaque as seen on CCTA is ≥ 10%, which is the lower bound of the 95% CI in our estimated prevalence of coronary artery disease, we expect to find significant differences compared to controls. In that case, we will perform the remaining 300 CCT's (200 in patients with HPD and 100 in patients with PCOS/POI), leading to a total of 300 CCT's in patients with HPD and 300 CCT's in patients with PCOS/POI. The age range (40-45 years and/or ≥ 55 years) and group composition for later inclusion may be adapted based on the findings of the interim analysis. If the prevalence of plaque as seen on CCT is ≤ 10%, we do not expect to find any significant differences compared to controls and therefore we will withdraw the remaining CCTA's and focus on CACS only.

Statistical analysis

Data analysis will be performed using SPSS. A probability (p) less than 0.05 will be considered significant. The characteristics of participants will be described using means +/− standard deviations for continuous variables using a two tailed independent t-test for comparison between groups (e.g. plaque burden). Categorical variables will be expressed as numbers (percentages) or medians (with inter quartile ranges) and compared with a chi-square test (e.g. CACS percentiles). Identical tests will be performed for circulating biomarkers. If several variables are identified to be statistically associated with cardiovascular risk factors, multiple linear regression or multiple logistic regression will be performed to identify the most important associations, appropriate to the endpoint chosen.

Ethical considerations

This study has been approved by the Medical Research Ethics Committee of the University Medical Center Utrecht (MEC number 15-508). This trial is registered in the Dutch Trial Register, NTR 5531, date of registration: October 21st, 2015.

Discussion

In this multicenter, prospective, cross-sectional study in patients with a reproductive disorder (PCOS, POI or HPD) we aim to assess CVD by CCT imaging (both CACS and CCTA) and CSC. Given the increased risk on CVD later in life, we believe the low radiation exposure is justified. Moreover, a sub study of the association of cell-based biomarkers and CCT will be performed.

The results of this study will provide insights in the added value CT imaging in the detection of cardiovascular disease. Ultimately, these insights can lead to improved cardiovascular prediction models in these women and may provide an opportunity for adjusted preventive strategies.

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CHAPTER 5.3

Prevalence and severity of coronary and intracranial carotid calcification on computed tomography in middle-aged women with PCOS

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Under review

Abstract

Background

Women with polycystic ovary syndrome (PCOS) develop risk factors for atherosclerosis and cardiovascular disease. An increased prevalence of coronary artery calcification (CAC) has been described in younger women with PCOS. Data on subclinical atherosclerosis in older women with PCOS is scarce. We determined the prevalence and severity of subclinical coronary and intracranial calcification (ICAC) in asymptomatic women with PCOS aged >45.

Methods

A prospective multicenter study. We measured coronary and intracranial calcifications on computed tomography in 100 asymptomatic women with PCOS and compared them to 200 asymptomatic age- and ethnicity matched controls. Simultaneously, coronary computed tomography angiography was performed to detect calcified and non-calcified plaque and assess the severity of luminal stenosis.

Results

Hypertension (46% vs 30%, $P < 0.001$) and diabetes (8% vs 1.5%, $P < 0.001$) were more prevalent in women with PCOS. At the same time, they were less often post-menopausal (18% vs 41.5%, $P<0.001$). Lipid levels and the prevalence of metabolic syndrome were not increased. The prevalence of CAC > 0-AU was 20% of women with PCOS and 23% of the controls, (OR (95%CI) 1.14 (0.63-2.08). Coronary plaque was detected in 20% of women with PCOS. The prevalence of coronary artery disease was 2%. ICAC was detected in 34.7%.

Conclusions

Despite an increased occurrence of cardiovascular risk factors, such as hypertension and diabetes, the prevalence and severity of observed subclinical coronary artery sclerosis in women with PCOS seems to be comparable to age and ethnicity matched controls. Long term follow-up is required to validate these findings.

Introduction

Despite advances in medical and interventional therapy, cardiovascular disease (CVD) is still the number one cause of death in women[1]. Subclinical signs of atherosclerosis are detectable long before the clinical onset of CVD. Coronary artery calcification (CAC) is a validated strong predictor of future ischemic heart disease[2]. Similarly, intracranial carotid artery calcification (ICAC) was demonstrated to be one of the strongest risk factors for stroke[3].

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women of reproductive age and is diagnosed in up to one in every six women[4]. It is a syndrome characterized by ovulatory dysfunction, polycystic ovarian morphology and hyperandrogenism[5]. Women diagnosed with PCOS are predisposed to develop risk factors for atherosclerosis, such as dyslipidemia, type II diabetes and hypertension[6,7]. These various risk factors along with PCOS itself increase chances for developing CAC, even at an early age[8-10]. Evidence concerning the prevalence and severity of subclinical atherosclerosis in older women with PCOS is scarce[8,9]. Whether or not the risk for CVD in women with PCOS is increased remains unclear[8]. Some studies suggest an increased incidence of CVD in women with PCOS at a young age, while others could not demonstrate increased CVD morbidity and mortality rates in women with PCOS[11-14].

The aim of the current study was to assess the prevalence and severity of subclinical atherosclerosis in women with PCOS aged 45 years and over. We assessed the CAC score in women with PCOS and compared them to age and ethnicity matched controls. Furthermore we assessed intracranial carotid artery calcification (ICAC) on non-contrast CT, and the presence of coronary artery plaque and severity of stenosis on coronary CT angiography (CCTA), in women with PCOS and compared these results to the existing literature.

Methods

Setting

In this prospective, multicenter, cross-sectional study, we assessed women aged 45 years and above who were previously diagnosed with PCOS. The rationale and study design have been described in detail elsewhere[15]. In short, CT imaging took place at the Erasmus University Medical Center in Rotterdam, or the University Medical Center of Utrecht between June 2016 and December 2017. Women diagnosed with PCOS according to the Rotterdam criteria were eligible for inclusion. The Rotterdam criteria recognizes PCOS as a syndrome characterized by ovulatory dysfunction, polycystic ovarian morphology and clinical and/or biochemical hyperandrogenism[16]. When either two or three of the key features are present, PCOS is diagnosed. We excluded women with serious comorbidities compromising study participation (e.g.

medication dependent asthmatic disease, contrast nephropathy, estimated glomerular filtration rate < 60 ml/min/1.73 m^2 or history of myocardial infarction). Patients included in this study had previously participated in a general cardiovascular assessment, approximately 13 months prior to imaging [17]. At that moment, we assessed general medical, obstetric and family history, education level, smoking status, anthropomorphic measurements, and blood pressure. On the day of screening fasting blood samples were collected to assess lipid levels, fasting glucose and insulin levels. This study adheres to the principles of the declaration of Helsinki and has been approved by the local ethics review board of the University Medical Center Utrecht (Netherlands Trial Register, TR5531, NCT00005487). Written informed consent was obtained from all study participants.

Controls

Female controls for CAC score were derived from the Multi-Ethnic Study of Atherosclerosis (MESA), comprising more than 6000 women and men from 45-84 years of age from the United States. The MESA study is a prospective cohort study, designed to investigate characteristics of subclinical cardiovascular disease and risk factors which predict progression to clinically overt cardiovascular disease[18].

Assessment of covariables

Hypertension was defined as systolic blood pressure (SBP) > 139, or diastolic blood pressure (DBP) > 89, or use of anti-hypertensive medication. Diabetes was defined as a fasting glucose level of ≥ 7.0 mmol/L or self-reported diagnosis via questionnaire. The National Cholesterol Education Program definition was used to assess the presence of the metabolic syndrome [19].

Assessment of coronary and intracranial carotid calcification

CT imaging was performed using state-of-the-art scanners (iCT, Philips Healthcare, Best, the Netherlands or Somatom Flash, Siemens Healthcare, Erlangen, Germany). In case of a resting heart rate > 65 beats per minute, either 10mg oral metoprolol was administered one hour prior to the CT scan or up to 20mg intravenous metoprolol was administered on the CT table prior to scanning.

First, a non-contrast scan of the carotid siphon region was performed, with 20mm coverage, 120kV, 100mAs, and 1mm reconstruction slice thickness. Next, an ECG-triggered non-contrast scan of the heart was performed with settings (120 kV with 3 mm thick reconstructed slices) adapted from the MESA protocol[18] to determine the CAC score following the Agatston scoring method [20]. Finally, a prospectively ECG-triggered contrast enhanced scan of the coronary arteries was performed after administrating sublingual nitroglycerine and intravenous non-ionic contrast (Iopromide, Ultravist, Bayer Healthcare, Berlin, Germany). Evaluation of the images was done in a standardized fashion by experienced radiologists.

Intracranial carotid artery calcification (ICAC) was measured bilaterally and used as a proxy for intracranial atherosclerosis. The presence and volume of carotid siphon calcifications (ICAC) were assessed with a semi-automated scoring method. In short, areas of interest were drawn around calcifications and the calcification volumes were calculated by multiplying the number of pixels in excess of 130 Hounsfield units by the pixel size and the increment[21]. The CAC score was calculated using commercially available software (SyngoVia, Siemensand Intellispace portal, Philips Healthcare). The calcification scores were categorized as follows: no calcifications (CAC = 0); mild calcifications (CAC >0 and <100); moderate calcifications (CAC ≥ 100 and < 400); or severe calcifications (CACS ≥ 400) [22]. Calcium scores were converted into age, sex, and race dependent percentiles based on the MESA data.

Assessment of non-calcified coronary atherosclerosis and stenosis
The CCTA images were scored for the presence and type of coronary atherosclerotic plaques (non-calcified, mixed or calcified), and the percentage of luminal stenosis. Luminal stenosis of ≥ 50% was considered to represent a significant stenosis. A CAC score ≥ 100 AU on coronary CT or ≥ 50% luminal stenosis on CCTA was considered relevant coronary artery disease [22]. Data on coronary plaque, stenosis and ICAC was not available in the MESA control population.

Statistical analyses
The prevalence and severity of coronary calcium are heavily influenced by age, gender, and ethnicity[18,23]. Using optimal matching, we selected 200 (ratio 1:2) age-ethnicity matched controls (R studio). We used single imputations following the mean predictive matching procedure for body mass index (BMI) and smoking. Baseline characteristics were presented as means and standard deviations (SD) for data with a normal distribution, and as medians and interquartile ranges (IQR). Chi-square test was performed to test categorical variables. The absence (CAC score = 0) or presence (CAC score > 0) of CAC was assessed, as well as the proportion of patients with a CAC score ≥ 95th MESA percentile. In addition, we assessed the proportion of patients with an increased cardiovascular risk, defined as a CACS ≥ 75th MESA percentile or CACS > 300 AU (according to the European guidelines on cardiovascular disease prevention [24]). Mixed effects logistic regression analyses were performed and adjusted for smoking and BMI. These analyses were performed with Statistical Package for Social Sciences (SPSS version 25 (IBM corp. Armonk, NY, USA).

Results

Baseline characteristics
In total, 100 asymptomatic women with PCOS aged > 45 years were included and compared to 200 age and ethnicity matched controls from the MESA. The baseline

characteristics are depicted in **Table 1**. The mean age in patients and controls was 51 years. We included women of Northern European (89%), black (5%) and Asian (6%) descent. At the mean age of 51, 18% of PCOS women were postmenopausal, against 42% of controls (P<0.001).

Table 1. Characteristics of the study population

	PCOS (N= 100)	Controls (N=200)	P-value
General parameters			
Age	51 (SD 5)	51 (SD 6)	0.24
Postmenopausal	18 (18%)	83 (41.5%)	**<0.001**
BMI (kg/m2)	27.1 (23.5-32.0)	30.0 (23.1-30.7)	0.25
Overweight	64 (64%)	118 (59%)	0.25
Obesity	35 (35%)	55 (28%)	0.14
Waist circumference (cm)	91.0 (25.5-104.0)	89.9 (77.8-102.7)	0.09
Ethnicity			
Northern European	89 (89%)	190 (95%)	
Black	5 (5%)	0 (0%)	**0.01**
Asian	6 (6%)	10 (5%)	
Cardiometabolic parameters			
Systolic BP (mmHg)	130 (SD 18)	113 (SD 18)	**< 0.001**
Diastolic BP (mmHg)	84 (SD 15)	67 (SD 11)	**< 0.001**
Hypertension	46 (46%)	60 (30%)	**< 0.001**
Total Cholesterol (mmol/L)	5.0 (SD 1.3)	5.0 SD (1.0)	0.97
Triglycerides (mmol/L)	1.1 (SD 0.8)	1.4 (SD 1.0)	**0.03**
HDL-C (mmol/L)	1.6 (SD 0.6)	1.5 (SD 0.4)	0.06
LDL-Cholesterol (mmol/L)	3.2 (SD 0.6)	2.9 (SD 0.8)	0.05
Glucose (mmol/L)	4.3 (SD 1.0)	4.6 (SD 1.1)	**0.001**
Metabolic Syndrome	15 (19%)	40 (20%)	0.87
Diabetes	8 (8%)	3 (1.5%)	**< 0.001**
Smoking			
Current	7 (7%)	29 (15%)	
Ever	38 (38%)	72 (36%)	0.06
Never	55(55%)	99 (50%)	

Values are displayed as means (SD) or medians (IQR), or as numbers (%). Differences were tested with Student's T-test or Mann-Whitney-U for continuous variables and chi-square of Fisher's exact test for categorical variables. ***Bold*** *indicates statistical significance. Abbreviations: polycystic ovary syndrome (PCOS), body mass index (BMI), high density lipoprotein (HDL), low density lipoprotein (LDL).*

Women with PCOS presented with higher systolic and diastolic blood pressure (P<0.001), and the prevalence of hypertension (46% vs 30%, P<0.001) and diabetes (8% vs 1.5%, P<0.001) were increased (P<0.001) in women with PCOS. The BMI and lipid profile of women with PCOS was comparable to that of the controls and the prevalence of metabolic syndrome was not significantly increased.

Coronary artery calcification in women with PCOS and controls

We observed a similar prevalence of CAC (> 0 AU) in women with PCOS (20%) and controls (23%), and the risk for a CAC score > 0 was not increased in women with PCOS (OR (95%CI); 1.14 (0.63-2.08)). This result remained similar after adjustment for potential confounders (OR (95%CI); 1.04 (0.56-1.95)). The risk for a CAC score above the 95th MESA percentile was not increased in women with PCOS (OR (95%CI); 0.95 (0.35-2.58)). Finally, based on observed CAC scores, women with PCOS had a similar risk for CVD compared to controls (OR (95%)CI); 0.97 (0.74-1.27) Data on CAC in women with PCOS and controls in presented in **Table 2**.

Table 2. Coronary artery calcium score in women with PCOS and controls

CAC score	PCOS (N=100)	Controls (N=200)	OR (95%CI)	OR (95%CI)*
Any CAC (> 0 AU)	20 (20%)	45 (23%)		
Minimal (0.1–9AU)	9 (9%)	19 (10%)		
Mild (10-99AU)	6 (6%)	17 (9%)	1.1 (0.6-2.1)	1.0 (0.6-2.0)
Moderate (100-400 AU)	5 (5%)	9 (5%)		
CAC ≥ P95	7 (7%)	13 (7%)	0.9 (0.4-2.4)	0.98 (0.4-2.6)
Increased CVD risk †	18 (18%)	37 (19%)	1.0 (0.8-1.3)	0.97 (0.7-1.3)

*Values are displayed as numbers (*precentages*). We performed mixed effects logistic regression analyses (*adjusted* for *BMI and smoking), † (> 300 AU / ≥ P75) abbreviations: coronary artery calcium (CAC), cardiovascular disease (CVD), polycystic ovary syndrome (PCOS), odds ratio (OR), Agatston Units (AU)*

Coronary plaque and stenosis severity in women with PCOS

Coronary plaque was present in 20% of women with PCOS on CCTA. We found non calcified plaque in 7 (7%) women, calcified plaque in 14 (14%) women, and mixed plaque in 5 (%) women. The prevalence of any coronary lumen stenosis was 14%. Significant luminal stenosis was present in 2% of women with PC (**Table 3**).

ICAC in women with PCOS

Information on intracranial carotid artery calcification was available in 92 women (92.9%) with PCOS and presented in Table 3. At the mean age of 51 years, we observed intracranial carotid artery calcifications in 40 women (43.5%) with a median (IQR) volume of 5.8 (2.0-45.4). The prevalence of ICAC was 36.4% in women aged 45-49, 50% in women aged 50-59 and 66.7% in women aged 60-69 years.

Table 3. Coronary plaque and stenosis and ICAC in women with PCOS

	PCOS, N=99*
Coronary computed tomography angiography	
Coronary plaque	20 (20%)
Non calcified plaque	7 (7 %)
Calcified plaque	14 (14%)
Mixed plaque	5 (5%)
Significant stenosis *	2 (2%)
No stenosis	86 (86%)
Minimal, < 25%	6 (6%)
Mild, 25-49%	5 (5%)
Moderate, 50-69 %	2 (2%)
Severe 70-99%	0 (0%)
Coronary artery disease †	2 (2%)
Intracranial computed tomography	N=92
Prevalent ICAC	40 (43.5%)
ICAC volume	5.8 (2.0-45.4)
Prevalent ICAC 45-49 years (N=52)	20 (36.4%)
Prevalent ICAC 50-59 years (N=18)	14 (50.0%)
Prevalent ICAC 60-69 years (N=5)	6 (66.7%)

*Values are displayed as numbers (percentages). * Significant stenosis: stenosis>50%. *in one patient the contrast enhanced CT-scan could not be performed, † (CACS > 100 AU and/or stenosis > 50%). Abbreviations: polycystic ovary syndrome (PCOS), intracranial carotid artery calcification (ICAC)*

Discussion

The current study demonstrates that although cardiovascular risk factors such as hypertension and diabetes, are more prevalent in middle-aged women with PCOS, the prevalence and severity of coronary artery calcification is comparable to age and ethnicity matched controls. In addition, we assessed the prevalence of coronary plaque, coronary stenosis and intracranial atherosclerosis in women with PCOS. We detected coronary plaque in one out of every five women with PCOS on CCTA. The prevalence of coronary plaques with a significant stenosis was 2%. Intracranial atherosclerosis was present in more than a third of middle-aged women with PCOS.

The presence of PCOS in women is expected to increase the risk for coronary atherosclerosis[9,10]. In addition, PCOS has been associated with an increased prevalence of cardiovascular risk factors, such as obesity, dyslipidemia, hypertension and diabetes mellitus [6,17]. Previous studies reported an increased prevalence of CAC and higher CAC scores in women with PCOS, compared to controls of a similar age [8,25,26]. Most of these studies were performed in young women with PCOS. Evidence regarding the prevalence of atherosclerosis in older women

with PCOS is scarce and generated conflicting results [9,27]. In the current study, two major risk factors for atherosclerosis (diabetes and hypertension), were observed more often in middle-aged women with PCOS compared to age and ethnicity matched controls. The prevalence of CAC observed in women with PCOS in the current study is in concordance with earlier studies however, not different from our age and ethnicity matched controls [9,28,29]. In addition, the risk of having any CAC or high CAC scores were not increased in women with PCOS. Even after adjustment for confounders (smoking and BMI) we observed a similar outcome in women with PCOS compared to controls. Despite the fact that major risk factors for CAC are more prevalent in women with PCOS, this does not translate into signs of more severe subclinical atherosclerosis. This could be due to the fact that only 18% of women with PCOS were postmenopausal against 41% of the controls. The menopausal transition increases cardiovascular risk and the progression of CAC [9]. The higher age of menopause and consequently the longer exposure to estrogens in PCOS might protect these women against the development and progression of atherosclerosis. In addition, risk factors for atherosclerosis, such as dyslipidemia, the prevalence of overweight, obesity and the metabolic syndrome were not increased in our study population. This relative lack of unfavorable cardiometabolic characteristics in middle-aged women with PCOS, is in line with other studies, indicating that with increasing age, some of the differences in the cardiometabolic profile of women with PCOS and controls seem to diminish [7,11,30] Also the data from a recent prospective follow-up study in women previously diagnosed with PCOS show similar results. This study indicates that risk factors for CVD do not deteriorate in women with PCOS whereas they do in controls [11,31].

CCTA has additional value over CACs because of the ability to identify non-calcified plaque, and thus the total atherosclerotic burden, and assess coronary luminal narrowing [32,33]. Hereby we might be able to better identify those women with PCOS at risk for future CVD. No data are present on coronary artery plaque formation and stenosis in asymptomatic women with PCOS. We were able to assess these outcomes in our study population, although the size and power of these analyses are relatively small. We observed coronary plaque in 1 out of every 5 women with PCOS, the majority of which was calcified or mixed plaque. The prevalence of coronary artery disease and significant stenosis was 2%. Reference data of a low risk population-based cohort is lacking. One available study in asymptomatic South Korean women around the age of 50, yielded a 6.7% prevalence of coronary plaque and 0.5% prevalence of significant stenosis, mostly non-calcified plaque [34]. We were not able to test these numbers statistically. The prevalence of significant stenosis was low in both study populations, but there seems to be a substantial difference in the prevalence of plaque to the disadvantage of women with PCOS. However, because of genetic and lifestyle differences in European and Asian women, it is very difficult to put these numbers into perspective.

In addition to coronary artery plaque and stenosis, we assessed the prevalence and volume of intracranial calcifications. Intracranial calcifications have been associated with an increased risk for stroke. Diabetes and hypertension have been identified, risk factors for the development of ICAC[21]. Based on the observed increased prevalence of diabetes and hypertension in this cohort of middle-aged women with PCOS, one could hypothesize to find an increased prevalence and volume of intracranial calcifications. There is currently no evidence available on ICAC in women with PCOS, but some reference ranges for the general population have been proposed[21,35]. For women aged 50-59, ICAC is said to be as prevalent as 75.9 % and the median volume 21 mm^3. At a mean age of 51 years, the observed prevalence and volume of ICAC in women with PCOS were lower than the reference values proposed for the normal population [21]. We assessed the prevalence of ICAC of women with PCOS in different age groups; aged 45-49, 50-59 and 60-69. In all groups the prevalence of ICAC was lower than those described in the general population[21]. Although the sample size is small and we need to be careful when interpreting these results, it seems that ICAC is not more prevalent or more severe in women with PCOS, pointing into the direction that their risk for stroke is similar to that of the general population. A previous study of the Swedish group of Dahlgren reported similar stroke events in women with PCOS compared to controls [11]. More studies in women with PCOS and a well phenotyped reference cohort are necessary to be able to provide more insight into the burden of coronary plaque, stenosis and intracranial calcifications in women with PCOS.

We assessed a well phenotyped cohort of women with PCOS, diagnosed in the respective study centers nearly 20 years ago. We were able to compare them to a properly matched control population and controlled for possible confounders. No control groups of European women were available; therefore we compared our group of middle-aged women with PCOS from Europe with a control group originating from the United States. This may have influenced our results.

To our knowledge, this is the first study to report on coronary plaque, stenosis and intracranial calcification in women with PCOS. Unfortunately, at this time no data on these outcomes was available in controls. Another limitation of this study is the limited sample size which could have shifted our results towards the null.

At this time, we conclude that neither evidence for an increased prevalence nor for an increased severity of atherosclerosis in middle aged women with PCOS was found. Based on our findings, women with PCOS do not seem to be more at risk for future cardiovascular events. Follow up of women with PCOS until old age is necessary to provide definitive answers on the burden of atherosclerosis and cardiovascular disease in these women.

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CHAPTER 5.4

High androgens in postmenopausal women and the risk for atherosclerosis and cardiovascular disease: the Rotterdam Study

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Abstract

Context: Polycystic ovary syndrome (PCOS) is closely linked to hyperandrogenism (HA). In PCOS, hyperandrogenism has been associated with metabolic disturbances which increase the risk for cardiovascular disease (CVD).

Objective: To assess the association of high serum androgen levels, as a postmenopausal remnant of PCOS, with the prevalence of atherosclerosis and incidence of CVD in postmenopausal women.

Design The Rotterdam Study, a prospective population-based cohort study. Median follow up was 11.36 years.

Setting General community

Participants 2578 women aged >55 years. Exclusion criteria were missing informed consent or follow-up data, perimenopausal status, menopause by surgical intervention or at an unnatural age (age <40 or >62).

Intervention: None

Main outcomes and measures: Linear, logistic, and cox regression models were used to assess the association of top quartiles (P_{75}) of serum testosterone, free androgen index (FAI), dehydroepiandrosterone, and androstenedione and SHBG with coronary artery calcium, carotid intima media thickness (IMT), pulse wave velocity, peripheral artery disease and incidence of coronary heart disease, stroke, and CVD.

Results: Mean age (standard deviation) was 70.19 (8.71) years and average time since menopause 19.85 (9.94) years. Highest quartile FAI was associated with higher pulse wave velocity [β (95%CI): 0.009 (0.000;0.018)]. Highest quartile dehydroepiandrosterone [β (95%CI): -0.008 (-0.015;-0.001)] and androstenedione [β (95%CI): -0.010 (-0.017;-0.003)] levels were associated with a lower IMT. We found no association between high androgen levels and incident stroke, coronary heart disease, or cardiovascular disease.

Conclusion: Postmenopausal high androgen levels were not associated with an increased risk for CVD. Cardiovascular health in women with PCOS might be better than was anticipated.

Introduction

Androgens in women are present throughout life and produced by the adrenal glands as well as by the ovaries. After menarche, i.e. during early puberty and adolescence, testosterone levels generally rise and reach peak values around the age of 20.[1] After adolescence, serum androgen concentrations gradually decline. Although after menopause the ovarian contribution is minimalized, detectable androgen concentrations will still be present.[2]

The most common cause of elevated androgen levels, i.e. hyperandrogenism (HA), in women of reproductive age is the polycystic ovary syndrome (PCOS). Depending on the population under study and diagnostic criteria used, PCOS is as prevalent as 10-15%.[3,4] HA in PCOS is defined as the presence of hirsutism and/or an increase in serum testosterone or the free androgen index (FAI).[5] In addition, androgen precursors such as dehydroepiandrosterone (DHEA) and androstenedione (Adione) are often elevated among women with PCOS.[6,7]

An increased prevalence of metabolic abnormalities has been observed in women with PCOS, especially in those exhibiting HA.[8,9] In addition, signs of early atherosclerotic disease have been observed in women with PCOS. [10,11] Presumably, this unfavorable cardiometabolic profile persists into the post-menopausal period, suggesting a higher risk for cardiovascular disease (CVD) morbidity and mortality later in life.[12-14] Nevertheless, due to scarcity of large-scale studies with sufficient follow-up time, results regarding long-term CVD incidence among women with PCOS is yet inconclusive.[15,16]

Free testosterone, Adione, and FAI have been suggested as the best markers to predict the presence of PCOS at all ages, especially after the onset of menopause.[7] In the absence of large-scale PCOS patient cohorts with long term follow-up, we aimed to assess the association between the possible postmenopausal remnant of PCOS; e.g. elevated androgen levels, with both prevalent atherosclerosis and incident CVD in a large prospective population-based cohort study of post-menopausal women. Additionally, we defined a PCOS phenotype consisting of testosterone or FAI levels in the top quartile together with reported cycle irregularities at 25 years of age. Prevalence of atherosclerosis and incident CVD events were further examined among women fulfilling the PCOS phenotype criteria.

Materials and Methods

Study Setting

This study was embedded within the Rotterdam Study (RS), a prospective population-based cohort study among subjects ≥55 years in the municipality of Rotterdam, the Netherlands. The rationale and design of the RS has been described in detail elsewhere.[17] The baseline examination was completed between 1990 and 1993 (RS-I). In 2000, the cohort was extended to include inhabitants who had become 55 years of age or moved into the study district after the start of the original cohort (RS-II). Participants were all interviewed at home and then had an extensive set of physical examinations at the research center every 3-5 years. The current study used data from the third examination of the original cohort (RS-I-3,1997-1999) and the first examination of the extended cohort (RS-II-1, 2000-2001). There were no eligibility criteria to enter the Rotterdam Study cohorts except the minimum age and residential area based on ZIP codes. For this study, we excluded women who were not post-menopausal and those who had entered menopause at an unnatural age (<40 or >62) or by a surgical intervention. RS has been approved by the medical ethics committee according to the Population Screening Act: Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. All participants in the present analysis provided written informed consent to participate and to obtain information from their treating physicians.[17]

Sex steroid measurements

Blood for sex steroid assessment was drawn during the postmenopausal period and at the first visit to the study center. Serum levels of testosterone, DHEA and Adione were measured in the fasting blood samples with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The free androgen index (FAI) was calculated as (Testosterone (nmol/l) * 100) / SHBG (nmol/l)). Estradiol (E_2) levels were measured with a radioimmunoassay. E_2 levels below 18.35 pmol/L were under the detection limit of the immunoassay used.

SHBG, insulin and lipid measurements

SHBG and insulin levels were determined with the Immulite platform (Diagnostics Products Corporation Breda, the Netherlands). Total and high-density lipoprotein (HDL) cholesterol, triglyceride and fasting glucose were measured on the COBAS 8000 Modular Analyser (Roche Diagnostics, Almere, the Netherlands).

Other measurements

Type II Diabetes mellitus (DM) was diagnosed on the basis of a fasting plasma glucose level of 7.0 mmol/L or higher (≤126.1 mg/dL), or use of antidiabetic medication. Body mass index (kg/m^2) was calculated as (weight/height^2). Waist circumference was measured in standing position, without heavy outer garments, midway in between the lower rib and iliac crest. Blood pressure was measured twice at the right brachial artery in sitting position with a random-zero sphygmomanometer and the average of the two measurements were used for the analysis. Information on reproductive history including menstrual cycle irregularities at 25 years of age, medication, education, and smoking was obtained during the home interview by a computerized questionnaire.

Subclinical measures of atherosclerosis

Coronary artery calcium (CAC) was measured in the epicardial coronary arteries with either a C-150 electron beam computed tomography scanner (Imatron, South San Francisco, California) or a 16- or 64-slice multidetector computed tomography scanner (Somatom Sensation 16 or 64; Siemens, Forchheim, Germany) and quantified by using the Agatston method.[18] The ATL UltraMark IV (Advanced Technology Laboratories, Bethel, Washington, USA) was used for carotid intima-media thickness (IMT) measurements. Ultrasonography of the left and right carotid arteries was performed, the mean of the maximal measurements from the near and far walls on both the left and right sides was used for the analysis.[18] Pulse wave velocity (PWV) was measured with an automatic device (Complior; Artech Medical, Pantin, France) which measures the time delay between the rapid upstroke of simultaneously recorded pulse waves in the carotid and the femoral arteries in meters per second.[18] Peripheral arterial disease (PAD) was defined as ankle brachial index values of 0.9 or lower.

Incident cardiovascular disease

The clinical events included incident stroke, coronary heart disease (CHD), and a composite CVD outcome (the occurrence of either CHD or stroke). Follow-up data was collected through general practitioners in the study area and subsequent collection of information from letters of medical specialists and discharge reports in case of hospitalization. Follow up was completed until January 2012. Incident CHD was defined as fatal or non-fatal myocardial infarction or death from CHD as described previously. [19] Strokes were diagnosed when a patient had typical neurological symptoms and a computed tomography or magnetic resonance imaging, made within 4 weeks after the occurrence of stroke, confirmed the diagnosis.[20]

Statistical analysis

Analyses were performed using IBM SPSS statistics version 21 (IBM corp., Armonk, NY, USA). A 2-sided P value of less than 0.05 denoted statistical significance. We used the single imputation by the Expectation Maximization method in SPSS to impute the missing values for the co-variables. Participant characteristics were described as mean (standard deviation) for continuous variables and proportions for dichotomous variables. Hormone levels were categorized into quartiles. Androgen levels in the highest quartile were considered indicative for hyperandrogenism. Hormone levels in the middle of the range; P_{25}- P_{50}, were used as the reference category. The relation between hormones and prevalent atherosclerosis was examined cross-sectionally, using linear regression for the association with CAC, IMT, and PWV and logistic regression for the association with PAD. IMT and PWV were log transformed to obtain a normal distribution. CAC was analyzed as natural logarithm of (CAC+1). Results were expressed in regression coefficient (β) or Odds Ratio (OR) and their corresponding 95% confidence intervals (95% CI).The association between hormone levels and incident cardiovascular events; CHD, stroke, and composite CVD, was examined using the Cox proportional hazards models. Results were expressed as hazard ratios (HR) and their corresponding 95% CI.

Adjustments were made to eliminate the effect of possible confounders. The first model was adjusted for age, years since menopause, and cohort. The second model was further adjusted for total and HDL cholesterol, lipid-lowering medication, smoking, systolic blood pressure, treatment for hypertension, DM, waist/hip ratio, and the use of hormones.

We further aimed to identify women with (premenopausal) features of PCOS and assess their risk for atherosclerosis and incident CVD. PCOS was defined as either testosterone or FAI levels in the highest quartile together with a reported history of cycle irregularities at age 25. Prevalence of atherosclerosis and incident CVD events among these women were compared to those of women with no history of cycle irregularities and testosterone and FAI in the reference range (P_{25}-P_{50}). The Kaplan-Meier method was used to generate a cumulative survival plot for CHD, stroke and the composite CVD outcome, using Graphpad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA).

We performed different sets of sensitivity analyses. First, we repeated the cross-sectional analyses regarding the association of hormones with atherosclerosis after excluding women who had prevalent CVD at baseline. Second, we assessed the association between extreme hormone levels (>P90) and atherosclerosis and incident CVD. Last, we repeated the cross sectional and longitudinal analyses after excluding

all women who reported using hormones. We further considered a more conservative Bonferroni adjusted P value of 0.001, corresponding to 42 sets of analyses.

Supplemental table 1. Baseline characteristics of the total study population.

	Women, N = 2578
Age (years)	70·19 (8·71)
Age at menopause (years)	50·33 (3·94)
Years since menopause (years)	19·85 (9·94)
Waist / hip ratio	0·88 (0·09)
BMI (kg/m2)	27·20 (4·37)
Current smoking, n (%)	432 (16.8%)
Systolic Blood Pressure (mmHg)	143·22 (21·32)
Diastolic Blood Pressure (mmHg)	75·60 (10·75)
Cycle irregularities at age 25, n (%)	272 (10·6%)
Hypertension, n (%)	1626 (63·1%)
Cholesterol (nmol/l)	6·01 (0·95)
HDL Cholesterol (mmol/l)	1·50 (0·39)
Triglyceride (mmol/l)	1·52 (0·74)
Lipid lowering medication, n (%)	322 (12·5%)
Fasting blood glucose (mmol/l)	5·89 (1·51)
Diabetes Mellitus, n (%)	316 (12·3%)
Prevalent CVD, n (%)	232 (9·0%)
Hormones	
SHBG (nmol/l)	60·86 (43·70 - 84·78)
Estradiol (pmol/l)	35·35 (18·35 - 60·06)
Androstenedione (nmol/l)	2·27 (1·64 - 3·07)
DHEA (nmol/l)	8·39 (5·36 - 12·68)
Testosterone (nmol/l)	0·84 (0·62 - 1·19)
Free androgen index	1·41 (0·93 - 2·11)

Values are reported in means and standard deviations (SD) or medians (27^{th} – 75^{th} quartile) for continuous variables or number (percentage) for categorical variables. Abbreviations: BMI: body mass index, HDL: High density lipoprotein, CVD (cardiovascular disease), SHBG: sex hormone binding globulin, DHEA: dehydroepiandrosterone.

Results

In total, 3,452 women had data available data on all hormones and were therefore eligible for this study. We excluded women for whom informed consent and follow-up data was not available (N=36) Furthermore, those who were not post-menopausal (N=108), entered menopause by a surgical intervention (N=670) or at an unnatural age (age of menopause <40 or >62, N=60) were excluded. Thus leaving 2,578 women for our analysis. Mean (standard deviation, SD) age was 70·19 (8·71) and the mean time (SD) since menopause was 19·85 (9·94) years. Cycle irregularities at

the age of 25 were reported by 272 (10·6%) women. 316 (12·3%) women had prevalent type II DM and 232 (9·0%) had prevalent CVD at baseline. The baseline characteristics are depicted in supplemental table 1.

Participants were divided into quartiles based on their serum hormone levels. The reported results are for the hormonal levels in the highest quartiles (P_{75}) compared to the middle of the range (P_{25}- P_{50}) as the reference category. Testosterone or FAI levels in the highest quartile were considered indicative for hyperandrogenism. The cut off for the highest quartile of testosterone serum concentrations (>P_{75} i.e. >1.19 nmol/L) in our study to define hyperandrogenism was similar to those suggested by others (1.4 nmol/L (41.0 ng/dl) and 1.1 nmol/L (30.9 ng/dl).[7,16]

Sex steroids and prevalent atherosclerosis

Results regarding the linear and logistic regression models for the associations of the highest quartile of testosterone, DHEA, Adione, the FAI, and SHBG levels with CAC scores, carotid IMT, PWV, and presence of PAD are presented in **Table 1**. Carotid IMT, PWV, and PAD data were available in 2339, 2174, and 2295 women and CAC measurements were available in a smaller sample set (N=1483). After adjustment for cardiovascular risk factors, we found no association between high testosterone levels and any of the surrogate markers of atherosclerosis. FAI in the highest quartile was associated with a higher PWV. High Adione and DHEA levels were associated with lower IMT. Finally, SHBG values in the highest quartile were associated wiath lower PWV. None of the associations were significant at a more conservative Bonferroni corrected P value of 0.001. We found no association between hormone levels in the highest quartile with CAC or with PAD.

Table 1. Association between high levels of sex steroids and SHBG with surrogate markers of atherosclerosis

	CAC (β [95% CI])	IMT (β [95% CI])	PWV (β [95% CI])	PAD (OR [95% CI])
Testosterone				
Model 1	-0·140 (-0·469;0·189)	-0·006 (-0·014;0·001)	0·001 (-0·009;0·010)	0·95 (0·69;1·31)
Model 2	-0·100 (-0·414;0·213)	-0·004 (-0·011;0·003)	0·003 (-0·006;0·012)	0·99 (0·72; 1·38)
FAI				
Model 1	0·303 (-0·014;0·620)	0·002 (-0·005;0·010)	**0·021 (0·012-0·031)**	0·99 (0·72;1·35)
Model 2	-0·093 (-0·407;0·220)	-0·005 (-0·012;0·002)	**0·009 (0·000;0·018)**	0·87 (0·62;1·21)
Adione				
Model 1	-0·161 (-0·476;0·155)	**-0·008 (-0·016;-0·001)**	**0·010 (0·001;0·020)**	1·01 (0·73;1·38)
Model 2	-0·263 (-0·563;0·037)	**-0·010 (-0·017;-0·003)**	0·008 (0.000;0·017)	0·93 (0·67;1·29)
DHEA				
Model 1	-0·149 (-0·463;0·165)	**-0·011 (-0·018;-0·003)**	-0·002 (-0·011;0·008)	**0·70 (0·50;0·98)**
Model 2	-0·152 (-0·450;0·147)	**-0·008 (-0·015;-0·001)**	0·003 (-0·006;0·012)	0·72 (0·51;1·01)
SHBG				
Model 1	-0·252 (-0·580;0·076)	-0·007 (-0·015;0·001)	**-0·022 (-0·032; -0·012)**	1·06 (0·78;1·45)
Model 2	0·054 (-0·271;0·379)	0·002 (-0·005;0·009)	**-0·010 (-0·019; -0·001)**	1·24 (0·89;1·72)

We used linear regression for CAC, IMT, and PWV and logistic regression for PAD. Results are shown in beta (β) estimates or odds ratios (OR) and 95% confidence interval (CI) for hormonal levels at the top quartile (P_{75}) using levels between P_{25}-P_{50} as the reference category. ***Bold*** *indicates a significant association at p<0·05. Model 1: adjusted for age, years since menopause and cohort. Model 2: adjusted for age, years since menopause, cohort, waist/hip ratio, total and high-density lipoprotein cholesterol, lipid lowering medication, smoking, systolic blood pressure, treatment for hypertension, Diabetes Mellitus, and use of hormones. Carotid IMT, PWV, and PAD data were available in 2339, 2174, and 2295 women respectively. Data on CAC was available for 1483 women. Abbreviations: SHBG: steroid hormone binding globulin, DHEA: dehydroepiandrosterone, IMT: Intima-media thickness CAC: Coronary Artery Calcium, PWV: pulse wave velocity, PAD: peripheral artery disease.*

Table 2. Association between high levels of sex steroids and SHBG with incident cardiovascular disease

	CHD	Stroke	CVD
Testosterone			
Model 1	0·81 (0·52;1·24)	1·31 (0·90;1·91)	1·06 (0·79;1·43)
Model 2	0·85 (0·55;1·31)	1·31 (0·80;1·91)	1·10 (0·82;1·47)
FAI			
Model 1	1·04 (0·67;1·60)	0·82 (0·57;1·17)	0·91 (0·68;1·22)
Model 2	0·84 (0·54;1·32)	0·75 (0·52;1·10)	0·80 (0·59;1·09)
Adione			
Model 1	1·10 (0·71;1·72)	1·29 (0·90;1·86)	1·17 (0·87;1·57)
Model 2	1·04 (0·67;1·63)	1·25 (0·87;1·79)	1·13 (0·83;1·52)
DHEA			
Model 1	0·83 (0·51;1·35)	0·89 (0·61;1·29)	0·89 (0·65;1·21)
Model 2	0·87 (0·54;1·42)	0·90 (0·62;1·31)	0·92 (0·67;1·26)
SHBG			
Model 1	1·05 (0·66;1·66)	0·95 (0·66;1·36)	0·97 (0·72;1·30)
Model 2	1·36 (0·84;2·18)	0·98 (0·68;1·42)	1·10 (0·81;1·50)

Results are shown in hazard ratio (HR) and 95% confidence interval (CI) for hormonal levels at the top quartile (P_{75}) using levels between P_{25}-P_{50} as the reference category. Bold indicates a significant association at p<0·05. Model 1 is adjusted for age, years since menopause, cohort. Model 2 is adjusted for age, years since menopause, cohort, waist/hip ratio, total and high-density lipoprotein cholesterol, lipid lowering medication, smoking, systolic blood pressure, treatment for hypertension, Diabetes Mellitus, and use of hormones. Abbreviations: CHD: coronary heart disease, CVD: cardiovascular disease, SHBG: steroid hormone binding globulin, DHEA: dehydroepiandrosterone.

Sex steroids and incident cardiovascular disease

In the analysis concerning incident CVD, only women without prevalent CVD at baseline (N=2346) were included. Compared to women with CVD at baseline, women without prevalent CVD were younger, entered menopause slightly later and had a lower waist to hip ratio. Moreover, a lower diastolic blood pressure, lower fasting glucose, and lower prevalence of type II DM was observed among these women. There were no significant differences in mean serum hormone levels in women with or without prevalent CVD (Data not shown).

The median follow up time was 11.36 years during which 165 women (7.0%) developed CHD and 215 women (9.2%) experienced a stroke. Overall 359 (15.3%) developed CVD (stroke and/or CHD) during follow up. When adjusted for cardiovascular risk factors, we found no significant associations between high serum levels of any of the sex-steroids and SHBG and the incidence of any of the studied clinical CVD outcomes (**Table 2**).

Table 3. Baseline characteristics of women with PCOS and controls.

	PCOS, N = 106	Controls, N = 171	P-value
Age	69·57 (8·72)	69·20 (8·60)	0·73
Age at menopause	51·00 (3·85)	50·19 (3·92)	0·09
Years since menopause	18·56 (9·19)	19·00 (10·15)	0·71
Waist / hip ratio	0·89 (0·08)	0·86 (0·08)	**0·01**
BMI (kg/m2)	27·92 (4·53)	26·84 (3·83)	**0·03**
Current smoking, n(%)	17 (16·0%)	28 (16·4%	0·94
Systolic BP (mmHg)	142·30 (21·74)	143·61 (19·22)	0·60
Diastolic BP (mmHg)	74·55 (10.39)	77·03 (9·92)	**0·05**
Hypertension, n(%)	70 (66.0%)	107 (62.6%)	0.56
Cholesterol (nmol/l)	5·98 (0·85)	6·09 (1·01)	0·34
HDL-C (mmol/l)	1·40 (0·35)	1·57 (0·39)	**<0·01**
Triglyceride (mmol/l)	1·62 (0·86)	1·39 (0·62)	**0·02**
Lipid lowering med n(%)	8 (7·5%)	24 (14·0%)	0·10
Fasting glucose (mmol/l)	6·25 (1·83)	5·79 (1·41)	**0·03**
Diabetes Mellitus	20 (18·9%)	12 (7·0%)	**<0·01**
Prevalent CVD	7 (6·6%)	11 (6·4%)	0·96
Hormones			
SHBG (nmol/l)	43·6 (33·4 - 78·3)	63·0 (57·7- 69·7)	**<0.01**
Estradiol (pmol/l)	38·8 (21·2-69·4)	29·4 (18·4- 58·6)	0.06
Androstenedione (nmol/l)	2·63 (2·00 - 3·31)	2·17 (1·67 - 2·88)	**0.01**
DHEA (nmol/l)	9·12 (6·13 - 14·14)	8·68 (6·20 - 12·24)	**0.68**
Testosterone (nmol/l)	1·30 (1·04 - 1·71)	0·74 (0·68 - 0·80)	**<0·01**
FAI	2·69 (2·13 - 3·49)	1·16 (1·05 - 1·29)	**<0·01**

PCOS was defined as Testosterone and/or FAI serum hormone levels >P_{75} and a history of cycle irregularities. Controls were defined as FAI and T levels P_{25}-P_{50} and no history of cycle irregularities. Values are reported in means and standard deviations (SD) or medians (27^{th} – 75^{th} quartile) for continuous variables or as a number (percentage) for categorical variables. ***Bold*** *values indicate that the association is significant at p<0·05. Abbreviations: BMI: body mass index, HDL: High density lipoprotein, WC: waist circumference, SPB: systolic blood pressure, DBP: diastolic blood pressure, SHBG: sex hormone binding globulin, DHEA: dehydroepiandrosterone, FAI: free androgen index.*

PCOS and the risk for atherosclerosis and CVD

Out of the 272 women with a reported history of cycle irregularities, 106 also had testosterone or FAI levels in the highest quartile and were therefore marked as PCOS. A control group was composed of 171 women with no history of cycle irregularities and hormone levels in the reference range (P_{25}-P_{50}). Women with PCOS had a larger waist/hip ratio, a higher BMI and more often had Type II DM (20% versus 7%). In addition, lower HDL and higher triglyceride levels as well as lower SHBG levels were observed among these women (**Table 3**). After adjusting for cardiovascular risk factors, we found no association with prevalent atherosclerosis, nor did we observe an increased risk for incident CVD in women marked PCOS (**Table 4**). No difference was observed in event free survival for CHD,

stroke or the composite CVD outcome between women fulfilling the criteria for PCOS and the control group (**Figure 1**).

Table 4. Association between PCOS phenotype and surrogate markers of atherosclerosis and with incident cardiovascular disease

	PCOS, N = 106	
	Model 1	**Model 2**
Surrogate markers (β/OR 95% CI)		
IMT	0·009 (-0·008 ; 0·025)	0·010 (-0·007 ; 0·026)
PWV	-0·011 (-0.031 ; 0·010)	-0·016 (-0.036 ; 0·004)
PAD	0·77 (0.37 ; 1·60)	0·66 (0·284 ; 1·51)
Incident CVD (HR 95% CI)		
CHD	1·10 (0·46 ; 2·66)	0·73 (0·27 ; 1·99)
Stroke	1·14 (0·56 ; 2·23)	1·16 (0·55 ; 2·42)
CVD	1·10 (0·61 ; 1·97)	0·94 (0·51 ; 1·76)

Results are shown in beta (β) estimates or odds ratios (OR) or hazard ratios (HR) and 95% confidence interval (CI). PCOS was defined as Testosterone and/or FAI serum hormone levels >P_{75} and a history of cycle irregularities. Controls were defined as FAI and T levels P_{25}-P_{50} and no history of cycle irregularities. ***Bold*** *values indicate that the association is significant at p<0·05. Model 1 is adjusted for age, years since menopause, cohort. Model 2 is adjusted for age, years since menopause, cohort, waist/hip ratio, total and high-density lipoprotein cholesterol, smoking, systolic blood pressure, treatment for hypertension, Diabetes Mellitus, and use of hormones. Abbreviations: PCOS: polycystic ovary syndrome, CHD: coronary heart disease, CVD: cardiovascular disease, PAD: peripheral artery disease,* ***IMT: Intima-media thickness, PWV: pulse wave velocity****. (CAC was excluded due to the low number of available measurements for this analysis).*

Sensitivity analyses

Three sensitivity analyses were performed. First, the cross-sectional analyses regarding the association of sex steroids and SHBG with atherosclerosis were repeated after excluding all women who had prevalent CVD at baseline. Results regarding association of hormones with surrogate markers of atherosclerosis remained the same (**Supplemental Table 2**). Second, we assessed the association between extreme levels of sex steroids and SHBG (>P_{90}) and prevalent atherosclerosis and incident CVD. Results of this set of analyses were in concordance with the analyses regarding hormone levels in the top quartile (>P_{75}) with prevalent atherosclerosis and incident CVD. Extreme FAI levels (>P_{90}) were associated with a higher PWV. Extreme Adione and DHEA levels were associated with a lower IMT. Extreme SHBG values were associated with lower PWV. We found no association between extreme hormone levels with CAC or PAD. In addition, we did not find any association between extreme sex steroid levels and incident CVD. (**Supplemental Table 3 and Supplemental Table 4**). Finally, we

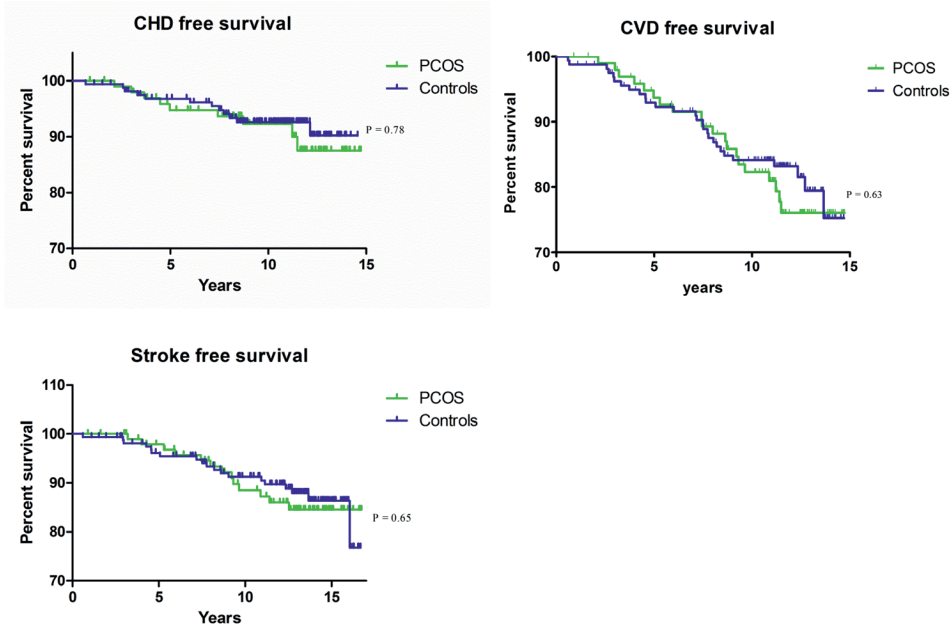
repeated the analyses after excluding women who reported using hormones. The results for this set of analysis were not different from our main analysis (**Supplemental Table 5 and Supplemental Table 6**).

Discussion

In this large population-based study of postmenopausal women we did not find a robust association between high androgen levels and surrogate markers of atherosclerosis or incident cardiovascular disease. Although PCOS women exhibited an unfavorable metabolic profile, this did not translate into a larger prevalence of atherosclerosis or an increased risk for incident CVD among these women.

Surrogate markers of atherosclerotic cardiovascular disease can be prevalent among clinically asymptomatic women and the menopausal transition marks an upward transition of cardiovascular risk.[21] High circulating androgen levels have been associated with an unfavorable cardiovascular risk profile and increased prevalence of subclinical atherosclerosis in postmenopausal women.[21,22] Our findings suggest there might indeed some association between high androgen levels and surrogate markers for cardiovascular disease. However, the association between high androgen levels with surrogate markers for atherosclerosis in our study was not robust and consistent. These findings are in line with several previous reports indicating inconsistent relationships between androgen levels and measures of subclinical atherosclerosis among post-menopausal women. Moreover, to what extent these surrogate markers translate into real hard endpoints remains controversial.[23]

Figure 1. CVD event free survival in women fulfilling the criteria for the PCOS phenotype and controls.



CVD event free survival during follow up in women fulfilling the criteria for the PCOS phenotype and controls. PCOS was defined as Testosterone and/or FAI serum hormone levels >P_{75} and a history of cycle irregularities. Controls were defined as FAI and T levels between P_{25}-P_{50} and no history of cycle irregularities. Abbreviations: PCOS: polycystic ovary syndrome, CHD: coronary heart disease, CVD: cardiovascular disease.

The few available studies on androgens and CVD did not demonstrate a clear relation, indicating that atherosclerosis burden among post-menopausal women might largely be due to adverse cardiovascular risk factors and hyperandrogenism per se might not be a risk factor for CVD.[24,25] In line with these findings, we also didn't find an association between high androgens per se and incident cardiovascular disease. Even when assessing the extreme levels (serum hormone levels > P90), amply exceeding cut offs suggested by previous studies,[7,16] we observed no increased risk for cardiovascular disease.

Hyperandrogenism in PCOS has been associated with an unfavorable cardiometabolic profile during the reproductive years. It has been suggested that these women might be especially at risk for CVD morbidity and mortality later in

life.[26] In comparing PCOS women with non PCOS women we were not able to detect any significant associations with cardiovascular disease endpoints. This could be due to the small number of women included in the PCOS phenotype analyses. However, to our knowledge this is the largest study to assess cardiovascular disease in women exhibiting postmenopausal features of PCOS. In addition, the few available studies on CVD in women with PCOS also did not demonstrate an association with CVD (mortality) and point towards the same direction as the current study.[15,16]

The only available long term follow up study in women with PCOS showed persistence of CVD surrogate markers into post-menopausal life without an increased incidence of stroke and / or CVD.[15] These results are in line with the current findings. Women with PCOS in our study however, were at risk to develop type II DM, which has been reported previously as well.[12]

One could argue we might have studied a relatively healthy sample. As the mean duration between menopause and our study entry was 19 years, women suffering from CVD at early age may have already died before being eligible to be included in the RS. Hence, the possible selection bias could have shifted the associations in the current study towards the null. However, androgen levels in women with or without prevalent CVD at baseline were not significantly different. The pathological mechanism behind early CVD might be different and does not seem to be driven by hyperandrogenism per se.

Several previous reports have suggested a greater burden of atherosclerosis among younger and middle-age women with PCOS, especially in those with both cycle irregularities and hyperandrogenism. [10,11,21,27] Little is known about the real incidence of atherosclerosis at older ages in women who previously suffered from PCOS. Our findings indicate that PCOS is not associated with more severe atherosclerosis at older age.

Recent studies have implicated a common biological mechanism behind both PCOS susceptibility and a later age at menopause which ameliorates ovarian aging and thus, expands the reproductive lifespan.[28] In line with this, women with PCOS in our study had entered menopause on average one year later compared to their control group, albeit non-significant. Reproductive performance seems to constitute a good marker for a women's general health later in life and an extended reproductive lifespan is correlated with long term health and longevity probably due to longer estrogen exposure.[29,30]

Therefore, despite the fact that PCOS is associated with risk factors for CVD such as higher BMI and insulin levels, which are strongly correlated with HA and the known unfavorable cardiometabolic profile, evidence now seems to point in the

direction that long term health in these women might be better than was previously anticipated.

Conclusions/Implications

In conclusion, persistent high androgen levels in women after menopause were associated with an increase in surrogate markers of CVD but did not show a robust association with cardiovascular disease. Long term health in these women might therefore be better than previously thought. Long term follow up in a large group of women diagnosed with PCOS during their premenopausal years is necessary to confirm these findings and to evaluate the necessity for cardiovascular screening.

Supplemental Table 2. Association between high levels of sex steroids and SHBG with surrogate markers of atherosclerosis among individuals free of cardiovascular disease at baseline.

	Women, N=2346			
	CAC (β [95% CI])	**IMT (β [95% CI])**	**PWV (β [95% CI])**	**PAD (OR [95% CI])**
Testosterone				
Model 1	-0·120 (-0·459 ; 0·220)	-0·005 (-0·013 ; 0·002)	-0·003 (-0.013 ; 0·007)	1·04 (0·73 ; 1·47)
Model 2	-0·166 (-0·494 ; 0·162)	-0·003 (-0·010 ; 0·004)	0·001 (-0008 ; 0·010)	1·08 (0·76 ; 1·55)
FAI				
Model 1	0·385 (0·061 ; 0·709)	0·002 (-0·005 ; 0·010)	0·020 (0.010 ; 0·030)	1·06 (0·76 ; 1·49)
Model 2	-0·060 (-0·386 ; 0·266)	-0·005 (-0·012 ; 0·002)	0·008 (-0·001 ; 0·017)	0·94 (0·66 ; 1·34)
Adione				
Model 1	-0·133 (-0·456 ; 0·190)	-0·008 (-0·015 ; -0·001)	0·010 (0·000 ; 0·020)	0·88 (0·63 ; 1·24)
Model 2	-0·248 (-0·559 ; 0·063)	-0·009 (-0·016 ; -0·002)	0·009 (0·000 ; 0·018)	0·82 (0·58 ; 1·16)
DHEA				
Model 1	-0·131 (-0·452 ; 0·190)	-0·010 (-0·018 ; -0·002)	-0·001 (-0·011 ; 0·009)	0·69 (0·48 ; 0·99)
Model 2	-0·151 (-0·461 ; 0·159)	-0·008 (-0·015 ; -0·001)	0·004 (-0·005 ; 0·013)	0·72 (0·50 ; 1·04)
SHBG				
Model 1	-0·211 (-0·548 ; 0·125)	-0·006 (-0·014 ; 0·001)	-0·022 (-0·032;-0·012)	1·10 (0·78 ; 1·53)
Model 2	0·096 (-0·240 ; 0·432)	0·002 (-0·006 ; 0·009)	-0·009 (-0·019; -0.001)	1·28 (0·90 ; 1·82)

We used linear regression for CAC, IMT, and PWV and logistic regression for PAD. Results are shown in beta (β) estimates or odds ratios (OR) and 95% CI for hormonal levels at the top quartile, using the levels between P_{25}-P_{50} as the reference category. Bold indicates a significant association at p<0·05. Model 1 is adjusted for age, years since menopause and cohort. Model 2 is adjusted for age, years since menopause, cohort, waist to hip ratio, total and high-density lipoprotein cholesterol lipid lowering medication, smoking, systolic blood pressure, treatment for hypertension, Diabetes Mellitus, and use of hormones. Abbreviations: SHBG: steroid hormone binding globulin, DHEA: dehydroepiandrosterone, IMT: Intima-media thickness CAC: Coronary Artery Calcium, PWV: pulse wave velocity, PAD: peripheral artery disease. Data on CAC was available for 1483 women.

Supplemental Table 3. Association between extreme levels of sex steroids and SHBG with surrogate markers of atherosclerosis among individuals free of cardiovascular disease at baseline.

	CAC	IMT	PWV	PAD
Testosterone				
Model 1	0·002 (-0·440 ; 0·445)	-0·006 (-0·016 ; 0·004)	0·007 (-0·006 ; 0·019)	1·11 (0·75 ; 1·65)
Model 2	0·059 (-0·480 ; 0·363)	-0·006 (-0·016 ; 0·001)	0·005 (-0·007 ; 0·016)	1·11 (0·74 ; 1·67)
FAI				
Model 1	0·421 (0·012 ; 0·831)	0·004 (-0·006 ; 0·014)	0·029 (0·016 ; 0·014)	0·97 (0·64 ; 1·48)
Model 2	-0·131 (-0·537 ; 0·276)	-0·006 (-0·015 ; 0·004)	0·014 (0·003 ; 0·026)	0·84 (0·54 ; 1·30)
Adione				
Model 1	0·081 (-0·336 ; 0·499)	-0·015 (-0·025 ; 0·005)	0·010 (-0·003 ; 0·022)	0·87 (0·57 ; 1·35)
Model 2	-0·101 (-0·499 ; 0·297)	-0·017 (-0·026 ; -0·008)	0·009 (-0·003 ; 0·020)	0·82 (0·52 ; 1·27)
DHEA				
Model 1	-0·118 (-0·539 ; 0·303)	-0·011 (-0·020 ; 0·002)	0·009 (-0·003 ; 0·021)	0·78 (0·50 ; 1·22)
Model 2	-0·167 (-0·568 ; 0·233)	-0·011 (-0·019 ; -0·002)	0·011 (0·000 ; 0·022)	0·76 (0·48 ; 1·21)
SHBG				
Model 1	-0.399 (-0.842 ; 0·044)	-0·006 (-0·016 ; 0·001)	-0·025 (-0·038 ; -0·013)	1·06 (0·71 ; 1·57)
Model 2	-0·058 (-0·496 ; 0·380)	0·004 (-0·006 ; 0·014)	-0·013 (-0·025 ; -0·001)	1·21 (0·79 ; 1·86)

We used linear regression for CAC, IMT, and PWV and logistic regression for PAD. Results are shown in beta (β) estimates or odds ratios (OR) and 95% CI. for extreme hormonal levels (P_{90}) , using the levels between P_{25}-P_{50} as the reference category. Bold indicates a significant association at $p<0·05$. Model 1 was adjusted for age, years since menopause and cohort. Model 2 was adjusted for age, years since menopause, cohort, waist to hip ratio, total and high-density lipoprotein cholesterol, lipid lowering medication, smoking, systolic blood pressure, treatment for hypertension Diabetes Mellitus, and use of hormones. Abbreviations: SHBG: steroid hormone binding globulin, DHEA: dehydroepiandrosterone, IMT: Intima-media thickness, CAC: Coronary Artery Calcium, PWV: pulse wave velocity, PAD: peripheral artery disease.

Supplemental Table 4. Association between extreme levels of sex steroids and SHBG with incident cardiovascular disease.

	CHD	Stroke	CVD
Testosterone			
Model 1	0·85 (0·49 ; 1·46)	1·46 (0·93 ; 2·30)	1·16 (0·81 ; 1·67)
Model 2	0·87 (0·51 ; 1·50)	1·39 (0·89 ; 2·19)	1·16 (0·81 ; 1·66)
FAI			
Model 1	1·09 (0·62 ; 1·89)	1·02 (0·65 ; 1·59)	1·03 (0·71 ; 1·49)
Model 2	0·85 (0·48 ; 1·50)	0·95 (0·60 ; 1·50)	0·89 (0·61 ; 1·30)
Adione			
Model 1	1·28 (0·74 ; 2·23)	1·18 (0·69 ; 1·82)	1·13 (0·76 ; 1·68)
Model 2	1·27 (0·73 ; 2·23)	1·07 (0·66 ; 1·75)	1·11 (0·75 ; 1·64)
DHEA			
Model 1	1·04 (0·55 ; 1·94)	1·37 (0·87 ; 2·16)	1·23 (0·83 ; 1·83)
Model 2	1·08 (0·57 ; 2·04)	1·37 (0·87 ; 2·16)	1·27 (0·85 ; 1·89)
SHBG			
Model 1	1·41 (0·83 ; 2·41)	0·81 (0·50 ; 1·32)	1·00 (0·69 ; 1·45)
Model 2	2·03 (1·16 ; 3·56)	0·82 (0·49 ; 1·36)	1·16 (0·79 ; 1·71)

Results are shown in hazard ratio (HR) and 95% confidence interval (CI) for extreme hormonal levels (P_{90}), using the levels between P_{25}-P_{50} as the reference category. Bold values indicate that the association is significant at p<0·05. Model 1 was adjusted for age, years since menopause and cohort. Model 2 was adjusted for age, years since menopause, cohort, waist/hip ratio, total and high-density lipoprotein cholesterol, lipid lowering medication, smoking, systolic blood pressure, treatment for hypertension, Diabetes Mellitus, and use of hormones. Abbreviations: CHD: coronary heart disease, CVD: cardiovascular disease, SHBG: steroid hormone binding globulin, DHEA: dehydroepiandrosterone.

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CHAPTER 6

General Discussion

Polycystic ovary syndrome (PCOS) is a very heterogeneous disorder with a high prevalence. Phenotype expression varies amongst women and between the different stages of life [1,2]. Some women will experience severe hirsutism, depression and anovulatory subfertility, and will develop hypertension and diabetes, while others might even go throughout life undiagnosed without any cumbersome complaints. The exact pathophysiology and long term consequences of PCOS are still far from fully understood, urging the need for further research. A better understanding of the pathophysiology will give us the opportunity to provide the best care and management for women suffering from PCOS, and might improve long-term health outcome in these women as well. The focus of this thesis was to gain more insight into the genotype and phenotype of PCOS. We aimed to investigate the risk for cardiovascular disease in women diagnosed with PCOS during their reproductive years. Furthermore, we aimed to identify more genetic variants associated with PCOS and its comorbidities. The current chapter places these results in a broader context and suggests directions for future research.

Cardiovascular disease in women with PCOS: past concept

In the past it was believed that women with PCOS were at increased risk to develop cardiovascular disease (CVD) later in life. This assumption was based on the fact that risk factors for CVD tend to cluster in women with PCOS [3-5]. Women with PCOS are often overweight or obese and tend to have central obesity. Moreover, cardiometabolic disturbances such as hyperinsulinemia, insulin resistance and dyslipidemia are more prevalent in women suffering from PCOS [5-7]. Over time, hyperinsulinemia and insulin resistance can progress into type II diabetes, and in addition many women with PCOS develop hypertension [5,7]. The combination of these metabolic disturbances leads to an increased prevalence of the metabolic syndrome, a major risk factor for the development of cardiovascular disease [8]. From an early age onwards, women with PCOS exhibit such an unfavorable cardiometabolic risk profile [5]. Hyperandrogenism, one of the key features of PCOS and detected in a large proportion of women, exacerbates metabolic disturbances, as does obesity [3-5]. In addition to this, associations between surrogate markers for cardiovascular disease, such as an increased intima media thickness (IMT), higher coronary artery calcium (CAC) scores and an increased pulse wave velocity have been described as well [9-12]. All of this pointed towards an increased cardiovascular risk in women with PCOS.

Cardiovascular disease in women with PCOS: current concept

Initial studies predicted a 7.1 times higher risk to develop myocardial infarction, and reported a higher prevalence of cerebrovascular accidents [13,14]. The only long-term follow up study on CVD in PCOS however, demonstrated no increased cardiovascular morbidity and mortality [15]. Over the years, contradicting results have been published and whether or not women with PCOS are at increased risk for cardiovascular disease remains an ongoing debate [16,17]. At this time, most of the available evidence seems to indicate that the risk for cardiovascular disease in women with PCOS might not be increased after all [11,14,18]. Evidence presented in this thesis, supports this hypothesis. We performed the largest study extensively assessing the cardiovascular status in middle-aged women who were diagnosed with PCOS according to the Rotterdam criteria nearly 20 years ago (chapter 5.1 and chapter 5.3). Compared to age and ethnicity matched controls, women with PCOS did have some unfavorable metabolic characteristics, such as a higher body mass index (BMI), increased waist circumference and increased prevalence of hypertension. Other CV risk factors such as type II diabetes, metabolic syndrome, and dyslipidemia were however not more prevalent in women with PCOS. Furthermore, intima media thickness and CAC scores, both surrogate markers for CVD, were not increased and based on available risk models, we predict cardiovascular health and 10-year cardiovascular risk in these women to be similar to that of the general population. In addition, we assessed postmenopausal women with features of PCOS (hyperandrogenism and a history of irregular menses, chapter 5.4). Here again we did find an association with cardiovascular risk factors such BMI, waist circumference, dyslipidemia and diabetes, but very little association with surrogate markers for CVD, and no evidence for an increased risk for stroke, coronary heart disease or CVD overall. Hence, in our studies, we were not able to demonstrate an increased risk for cardiovascular events in middle-aged or older women with (features of) PCOS. Moreover the observed cardiovascular risk profile in both studies was rather mild and not that different from controls. It seemed as if though some of the metabolic disturbances associated with PCOS can persist throughout life, with increasing age the differences between women with PCOS and controls diminished. This observation has been confirmed by others, who reported that the only remnant of dyslipidemia at older age were elevated triglyceride levels [5,15]. Similarly, the metabolic syndrome has been reported to be five times as prevalent in young women, and only two times higher in women aged over 39 years [5]. Somehow women with PCOS seem to be able to correct for their unfavorable

cardiometabolic baseline profile and the impact this might exert over the years on their CVD status. Indeed, genetic studies in women with PCOS have revealed an association with genes involved in DNA maintenance and repair and the latter were associated with a later age of menopause, which might enable these women to compensate or correct for the damage caused by the unfavorable accumulation of risk factors more efficient (chapter 2) [19,20]. Furthermore, late menopause implies a longer premenopausal estrogen exposure, which is known to be important for a better cardiovascular health and could have a protective effect on the development of atherosclerosis and risk for future CVD as well [21,22]. Indeed, women with PCOS enter menopause some years later, prolonging their reproductive lifespan and thereby increasing longevity and the latter might be completely driven by their genetic enrichment in menopause postponing genetic variants [23,24]. Another factor might be that women with PCOS have become more aware of these risk factors and do anticipate accordingly to them. Indeed, lifestyle interventions have been shown to improve body composition, hyperandrogenism and insulin resistance [25]. Taken together, this might explain that despite the increased presence of cardiovascular risk factors, there is no progression over the years into overt cardiovascular disease.

Cardiovascular disease in women with PCOS: future concept?

The fact that older women with PCOS exhibit a cardiometabolic profile similar to that of the general population, gives rise to another hypothesis. If they are genetically equipped with the more favorable DNA repair and maintenance genes, maybe they should be able to correct for their unfavorable baseline profile. Hence, their cardiovascular health should be better compared to most control populations not selectively enriched with the better DNA repair and maintenance genes.

Many women with PCOS suffer from anovulatory subfertility. However, after being diagnosed and having received treatment, fertility rates are restored and fecundity is not lower compared to women without PCOS [26]. With advancing age their menstrual cycles normalize and it has been suggested that this leads to a catch-up of fecundity and a subsequent increase in the number of a spontaneous pregnancies [27,28]. In addition, their ovarian reserve seems to be better than in normal women, indicated by the presence of higher AMH levels at all ages [27]. Moreover, their reproductive performance in terms of implantation rates and the number of euploid embryos are also better indicating the pivotal role of the DNA repair and maintenance systems [29].

Reproductive performance as well as ovarian reserve are strongly correlated with general health and longevity [20]. Based on this, general as well as cardiovascular

health in women with PCOS should maybe actually be better than that of most reference populations. Although our studies do not reveal any differences between PCOS women and matched control populations, developing proper preventative treatment strategies might lead to a further improvement of their cardiovascular health ultimately leading to a better health status compared to controls. Hence due to selection bias and thereby inclusion of different phenotypes, with a different genetic make-up, between several large population based studies might have led to the seemingly contradictory findings between different populations studies.

Perspective for research in PCOS

Polycystic ovary syndrome remains an interesting topic for research and many challenges lay ahead. Genetic studies in PCOS should focus on the discovery of more genetic variants associated with the syndrome. Even more important they should focus on how identified genes and variants fit in the pathophysiology of the syndrome and its associated comorbidities. Finally, research should establish whether or not some of these genetic factors are protective for the so called non-communicable diseases such as diabetes and cardiovascular disease. The department of reproductive medicine of Erasmus Medical Center has, with the help of many collaborators, initiated a worldwide consortium, the i-PCOS consortium, which focusses on genetic PCOS research. The original consortium consisted of 7 cohorts which included 10.000 cases and 100.000 controls of Northern-European descent. By now, many more research groups have joined, including research groups with cohorts of women with PCOS of other ethnicities. This larger consortium will improve the power to detect genetic variants with a small effect and to define their role in the etiology of the syndrome. It will also allow us to look further into the ethnic differences and similarities.

With regard to ethnic differences in women with PCOS, it will be of interest to explore the influence of ethnicity and other phenotypical characteristics on the outcome of fertility treatment. Not much evidence is available on this subject at this time, and might help to predict outcome of treatment more appropriately in order to circumvent unnecessary treatments.

Studies on cardiovascular health in women with PCOS should focus on follow up in women properly diagnosed during the reproductive lifespan and followed up until old age. By doing so we might be able to once and for all answer the question if their risk for cardiovascular disease is increased or not. Genetic studies on the association of PCOS with genetic variants associated with cardiovascular could provide us with

a strong indication whether they have a better cardiovascular health compared to women without PCOS or not. Subsequently, research in this area should aim at improving the cardiovascular health in women with PCOS at a younger age to prevent deterioration. And last but not least we should starting to promote a healthy lifestyle in combination with the treatment of early metabolic disturbances, by which we might be able to improve long-term health and longevity in these women and simultaneously shift the paradigm on health in women with PCOS.

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CHAPTER 7

Summary
Samenvatting

Summary

The polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age. PCOS is a major cause of anovulatory infertility, often has an impact on emotional well-being, and is associated with cardiometabolic disturbances which might increase the risk for cardiovascular disease later in life. The exact pathogenesis of PCOS is still far from fully understood. PCOS is considered to be caused by a combination of genetic factors that interact with environmental factors. Currently, genetic variants associated with PCOS still only account for perhaps no more than 10% of the heritability of the disorder. This thesis aimed to identify genetic variants associated with PCOS by performing a genome wide association study meta-analysis. Moreover, we assessed the cardiometabolic profile and the risk for cardiovascular disease in women with PCOS later in life. **Chapter 1** provides a general introduction into the diagnostic criteria, and into the genotype and phenotype of PCOS.

Different sets of diagnostic criteria are available to diagnose PCOS, resulting in a phenotypic spectrum of PCOS cases. The genetic similarities between cases diagnosed based on the two criteria have been largely unknown. Previous studies in Chinese and European women have identified 16 loci associated with the syndrome. In **chapter 2**, we conducted a fixed-effect, inverse weighted-variance meta-analysis from 10.074 PCOS cases and 103.164 controls of European ancestry, and characterization of PCOS related traits. In this study, we identified three novel loci, and provided replication for 11 previously reported loci. The genetic architecture of PCOS diagnosed by self-report via questionnaire, or PCOS diagnosed by the NIH or Rotterdam criteria was similar. Identified variants were associated with hyperandrogenism, gonadotropin regulation and testosterone levels in women with PCOS. Linkage disequilibrium score regression analysis revealed genetic correlation with obesity, fasting insulin, type II diabetes, lipid levels and coronary artery disease, indicating shared genetic architecture between metabolic traits and PCOS. Mendelian randomization analyses suggested variants associated with body mass index, fasting insulin, menopause timing, depression and male-pattern balding play a causal role in PCOS. Thus, our study revealed 3 novel loci associated with PCOS and a similar genetic architecture for all diagnostic criteria. The data also provide the first genetic evidence for a male phenotype of PCOS and a causal link to depression.

In the **chapter 3**, we reported on the outcome of ovulation induction with clomiphene citrate in women with PCOS of different ethnicity. We assessed 420 women of Northern European, Mediterranean, African, South-East Asian and South-American descent. We observed differences in body mass index, waist circumference, luteinizing hormone, insulin, and androgen serum levels in women with PCOS of different ethnicity. Despite its phenotypic differences, the minimal

effective dose of clomiphene citrate and the prevalence of clomiphene resistant anovulation were similar. Furthermore, a prediction model designed predict the chance to ovulate after treatment, revealed no significant differences in the predicted chance to ovulate among the ethnic groups, suggesting that the differences in phenotype might balance each other out and result in a similar response to treatment. This is the first study focusing on the influence of ethnicity on ovulation induction treatment with clomiphene citrate in women with PCOS. Our findings suggest that the treatment of women with PCOS of different ethnicity according to the same standardized protocol seems to be appropriate.

Normogonadotropic anovulation (WHO2) is the most common type of anovulation found in women of reproductive age. Using the Rotterdam criteria, the majority of women with WHO2 anovulation are diagnosed with PCOS. PCOS has been associated with an increased prevalence of risk factors for cardiovascular disease. A small proportion of women with normogonadotropic anovulation however, do not meet the criteria for PCOS. Evidence on the cardiometabolic risk in women with WHO2 anovulation without a concurrent PCOS diagnosis is scarce, although it has been suggested that cardiometabolic disturbances are much less prevalent in women with WHO2 anovulation due to the absence of hyperandrogenism. In **chapter 4**, We assessed the cardiometabolic profile of women with WHO2 anovulation, and compared them to women with PCOS and a normo-ovulatory reference population. In addition we assessed the influence of BMI on the cardiometabolic profile of women with WHO2 anovulation, and again compared them to women with PCOS and normo-ovulatory women. Our data suggest a lower prevalence of cardiovascular risk factors, such as a high BMI, insulin resistance, and lipid abnormalities in women with WHO2 anovulation, compared to women with PCOS. Furthermore, the cardiometabolic profile of women with WHO2 anovulation seems to be comparable to that of normo-ovulatory women. In addition, overweight or obese women with either WHO2 anovulation, PCOS, or normo-ovulatory women all have a similar cardiometabolic profile which seems to be much more driven by BMI rather than by the fertility disorder itself.

Contradictory results have been reported regarding the association between PCOS and cardiovascular disease. PCOS has been associated with cardiometabolic abnormalities such as, obesity, dyslipidemia, type II diabetes, hypertension and the metabolic syndrome, which increase the risk for cardiovascular disease. In the past, it was assumed that women with PCOS would be more prone to develop cardiovascular disease later in life. The only available long-term follow up study in women with PCOS did however, not reveal an increased risk for cardiovascular disease. At this time, evidence regarding this matter is still contradicting and inconclusive. In **Chapter 5.1** we studied the cardiometabolic phenotype and prevalence of cardiovascular disease in 200 middle-aged women previously diagnosed with PCOS, and compared them to 200 age-matched controls from the

Rotterdam study. Around the age of 50, women with PCOS were much less often postmenopausal than age matched controls. We observed an increased waist circumference and body mass index, and a higher prevalence of hypertension in women with PCOS. At the same time, the prevalence of type II diabetes and the metabolic syndrome were not significantly increased and lipid levels were not different from age matched controls. Furthermore, intima media thickness was lower in women with PCOS, the cardiovascular health score not different, and we predicted a 10-year cardiovascular disease risk similar to that of age matched controls. Thus middle-aged women with PCOS exhibit only a moderately unfavorable cardiometabolic profile compared to age matched controls, even though they present with an increased body mass index and waist circumference. Furthermore, we found no evidence for increased cardiovascular disease risk or more severe atherosclerosis compared to controls from the general population. In **chapter 5.2** and **chapter 5.3** we describe the methods and present the results of the CREW IMAGO study, in which we assessed the prevalence and severity of subclinical coronary and intracranial calcification in 100 asymptomatic women with PCOS, over 45 years of age. We compared them to 200 age and ethnicity matched controls from the MESA study. We found that although some metabolic disturbances, such as hypertension and diabetes were present, lipid levels and the prevalence of metabolic syndrome were not increased. We reported a prevalence and severity of subclinical coronary artery calcification comparable to age and ethnicity matched controls.

Finally, in **chapter 5.4**, we studied the association of high serum androgen levels, as a postmenopausal remnant of PCOS, with the prevalence of atherosclerosis and incidence of cardiovascular disease in postmenopausal women. We studies 2578 women with a mean age of 70 years and average time since menopause of 20 years. We found that the highest quartile of the free androgen index was associated with higher pulse wave velocity. Furthermore highest quartile dehydroepiandrosterone and androstenedione levels were associated with a lower carotid intima media thickness. We found no association between high androgen levels and the incidence of stroke, coronary heart disease, or with cardiovascular disease. We conclude that postmenopausal high androgen levels were not associated with an elevated risk for cardiovascular disease and that cardiovascular health in women with PCOS might be better than was previously anticipated.

Chapter 6 places the results described in this thesis in broader context and discusses our view on long term cardiovascular health in women with PCOS.

Samenvatting

Het polycysteus ovarium syndroom (PCOS) is de meest voorkomende hormonale aandoening onder vrouwen in de vruchtbare levensfase. PCOS veroorzaakt vaak anovulatie (het uitblijven van de eisprong), al dan niet in combinatie met verhoogde spiegels van het mannelijk geslachtshormoon en een toegenomen aantal eiblaasjes in de eierstokken. Daarnaast heeft PCOS invloed op het geestelijk welbevinden en wordt het syndroom geassocieerd met een ongunstig stofwisseling (metabool) profiel dat het risico op hart- en vaatziekten verhoogt. Hoe PCOS precies ontstaat is nog niet bekend. Wel weten we dat PCOS een complexe genetische aandoening is, wat wil zeggen dat het wordt veroorzaakt door een combinatie van genetische factoren en de invloed hierop van omgevingsfactoren. Genetische variaties die in verband zijn gebracht met PCOS verklaren op dit moment slechts ongeveer 10% van de erfelijkheid van het syndroom. Het eerste doel van dit proefschrift was om meer genetische variaties te identificeren die een rol spelen in het ontstaan van PCOS. Dit hebben we gedaan door middel van een zogenaamde volledige genoom associatiestudie (GWAS) meta-analyse: dit is een studie waarbij een genoom brede reeks DNA variaties wordt getest bij een grote groep individuen die PCOS hebben en wordt vergeleken met een groep vrouwen die geen PCOS hebben. Op deze manier wordt bekeken of een genetische variant in verband kan worden gebracht met een bepaalde eigenschap of aandoening in dit geval dus PCOS. Daarnaast hebben we in dit proefschrift gekeken naar het cardiovasculaire profiel en het risico op hart- en vaatziekten van vrouwen met PCOS op latere leeftijd.

Hoofdstuk 1 beschrijft hoe PCOS wordt vastgesteld en hoe de diagnostische criteria door de jaren heen zijn gevormd. Daarnaast wordt uitleg gegeven over wat er bekend is over de oorzaak van PCOS, het genotype en het fenotype en waarom het syndroom in verband wordt gebracht met hart- en vaatziekten.

PCOS is een syndroom dat bij verschillende individuen wisselend tot uiting komt en een breed scala aan klachten kan geven. Daarnaast worden in de praktijk meerdere diagnostische definities van PCOS gebruikt, met verschillende diagnostische criteria. Dit maakt dat PCOS een zeer heterogene aandoening is. Eerdere GWAS bij vrouwen van Europese en Han-Chinese afkomst hebben 16 genetische varianten geïdentificeerd die in verband worden gebracht met het syndroom. In **hoofdstuk 2** presenteren we de resultaten van een GWAS meta-analyse. In deze studie hebben we 10.074 vrouwen met PCOS van Europese afkomst vergeleken met 103.164 controles (vrouwen zonder PCOS). Het doel van deze studie was om genetische varianten te identificeren en in kaart te brengen hoe deze passen in de ontstaanswijze van en kenmerken geassocieerd met PCOS. In deze studie werden drie nieuwe genetische varianten geïdentificeerd en tevens 11 genetische varianten gerepliceerd uit eerdere GWAS in vrouwen met PCOS. Het gebruik van verschillende

diagnostische criteria leverde dezelfde genetische uitkomsten op. De geïdentificeerde genetische varianten werden in verband gebracht met hyperandrogenisme (teveel aan mannelijk geslachtshormoon), de regulatie van de gonadotrofinen (de hormonen die de eierstokken aanzetten tot het uitrijpen van eicellen) en de testosteron spiegel in het bloed. Met behulp van linkage desequilibrium score regressie analyse werd een genetische relatie (correlatie) gevonden tussen PCOS en vetzucht (obesitas), insuline resistentie en cholesterolspiegels, type II diabetes (ook wel ouderdomsdiabetes genoemd) en ziekten van de kransslagaderen. Mendeliaanse randomisatie, waarbij men de genetische varianten probeert te combineren met bekende ziekten die je vaker ziet bij PCOS, toonde aan dat de varianten een relatie leken te hebben met gewicht (BMI), insuline resistentie en dus diabetes, de leeftijd waarop de menopauze optrad, met depressieve klachten en een mannelijk kaalheidspatroon.

Ovulatie inductie is een behandeling waarbij met medicatie wordt getracht de eisprong op te wekken bij vrouwen met een onregelmatige of afwezige menstruatie cyclus. Wat de invloed van etniciteit op de uitkomst van deze behandeling is, is niet bekend. **Hoofdstuk 3** beschrijft de uitkomsten van een studie naar de invloed van etniciteit op de uitkomst van ovulatie inductie met een medicijn genaamd clomifeencitraat (Clomid®) bij vrouwen met PCOS. Wij vergeleken 420 vrouwen van Europese, Mediterraanse, Afrikaanse, zuidoost Aziatische, of Zuid-Amerikaanse afkomst. Er werden verschillen gevonden in de BMI, navelomtrek, LH, insuline en androgeen spiegels tussen vrouwen met PCOS met verschillende etnische achtergronden. Deze verschillen kwamen overeen met wat eerder in de literatuur beschreven is. Ondanks deze gevonden verschillen trad even vaak een eisprong op en ook was de dosering Clomid® die nodig was om een eisprong op te wekken niet anders. Daarnaast hebben wij een reeds bestaand predictiemodel getest, wat de kans voorspelt om een eisprong te krijgen na het toedienen van Clomid®. Ondanks verschillen in de factoren die worden meegenomen in dit model (BMI, vrije androgenen spiegel en de soort menstruatiestoornis), vonden wij dat de vrouwen van verschillende etniciteit een gelijke kans hadden op het krijgen van een eisprong na behandeling met Clomid®. Het lijkt erop dat de verschillen tussen de etnische groepen elkaar uitbalanceren en uiteindelijk resulteren in dezelfde respons op Clomid®.

Bij het overgrote deel van de vrouwen met een menstruatiestoornis (verminderd voorkomen of uitblijven van de eisprong), worden bij bloedonderzoek geen afwijkingen in de hormoonspiegels gevonden. Daarom worden deze menstruatie stoornissen door de Wereld Gezondheidsorganisatie (WHO) geclassificeerd als een WHO2 menstruatiestoornis. De meesten van deze vrouwen voldoen ook aan de diagnose PCOS. PCOS wordt in verband gebracht met het verhoogd voorkomen van

risicofactoren voor hart- en vaatziekten. Een kleine groep vrouwen voldoet echter niet aan de diagnose PCOS en heeft enkel een WHO2 menstruatiestoornis. Over het risico op hart- en vaatziekten van vrouwen met een WHO2 menstruatiestoornis is weinig bekend. Wij brachten in **hoofdstuk 4** het cardiovasculaire risicoprofiel van vrouwen met een WHO2 menstruatiestoornis in kaart en vergeleken hen met vrouwen met PCOS en met vrouwen met een normale regelmatige cyclus. Vrouwen met een WHO2 menstruatiestoornis hadden een lagere BMI, waren minder vaak insulineresistent en hadden minder vaak afwijkende cholesterol spiegels dan vrouwen met PCOS. Het cardiometabole profiel van vrouwen met een WHO2 menstruatiestoornis leek erg op dat van vrouwen met een regelmatige cyclus. Vervolgens hebben we bekeken wat de invloed van BMI is op het cardiometabole profiel van vrouwen met een WHO2 menstruatiestoornis in vergelijking met vrouwen met PCOS of vrouwen met een regelmatige cyclus. We hebben onderscheid gemaakt tussen vrouwen met een normaal gewicht (BMI <25), vrouwen met overgewicht (BMI 25-30) en vrouwen met obesitas (BMI >30). Vrouwen met een WHO2 menstruatiestoornis, PCOS of normale cyclus met overgewicht of obesitas, hadden allemaal een vergelijkbaar cardiometabool profiel. BMI lijkt dus een veel grotere invloed te hebben op het cardiometabole profiel dan het hebben van een vruchtbaarheidsstoornis.

Of vrouwen met PCOS daadwerkelijk een verhoogd risico hebben op het krijgen van hart- en vaatziekten is nog niet bekend. PCOS is in verband gebracht met risicofactoren voor hart- en vaatziekten zoals overgewicht en obesitas, type II diabetes, hypertensie en het metabool syndroom. Vroeger werd hierdoor gedacht dat vrouwen met PCOS een verhoogd risico hadden op het krijgen van hart- en vaatziekten. Echter, de tot op heden verrichtte, enige lange-termijn studie, toonde geen verhoogd risico op hart- en vaatziekten aan. Sinds die tijd zijn er tegenstrijdige resultaten gepubliceerd, al lijkt het merendeel van de beschikbare data erop te wijzen dat het risico op hart- en vaatziekten bij vrouwen met PCOS niet zo hoog is als aanvankelijk werd gedacht. In **hoofdstuk 5.1** presenteren we de resultaten van de CREW studie, waarin we het cardiometabole profiel van 200 vrouwen met PCOS rond de leeftijd van 50 jaar, hebben vergeleken met 200 qua leeftijd overeenkomstige vrouwen van de algemene populatie (de Rotterdam studie). Op deze leeftijd waren significant minder vrouwen met PCOS al in de menopauze waren. Wij vonden een grotere middel omtrek en hogere BMI in vrouwen met PCOS. Tevens hadden ze vaker hypertensie. Echter, hadden ze niet significant vaker type II diabetes of het metabool syndroom dan op leeftijd gematchte controles. Vrouwen met PCOS hadden dus maar een ietwat ongunstiger profiel dan de op leeftijd gematchte controles. De vaatwanddikte van de halsslagader, een maat voor de mate van aderverkalking, was ook niet toegenomen bij vrouwen met PCOS. Tot slot hebben we de cardiovasculaire gezondheidsscore en het 10-jaars risico op hart- en vaatziekten berekend. Wederom vonden we vergelijkbare resultaten voor vrouwen met PCOS en op leeftijd gematchte controles. Ondanks de hogere BMI en middelomtrek, lijken vrouwen met PCOS rond de leeftijd van 50 jaar dus niet

slechter af te zijn dan gezonde controles van dezelfde leeftijd. Bij dezelfde vrouwen hebben wij het voorkomen en de ernst van atherosclerose van de kransslagaderen gemeten. **Hoofdstuk 5.2** en **hoofdstuk 5.3** beschrijven de methodiek en uitkomsten van de CREW-IMAGO studie. In deze studie is de mate van verkalking van de kransslagaderen en de carotis sifon (gelegen in de schedelbasis) gemeten door middel van een CT-scan. Honderd vrouwen met PCOS, zonder voorgeschiedenis van hart- en vaatziekten werden vergeleken met 200 vrouwen afkomstig van de MESA studie. De controles hadden eenzelfde leeftijd en waren van soortgelijke afkomst. Ook in deze studie kwamen enkele metabole stoornissen zoals hypertensie en diabetes, vaker voor bij vrouwen met PCOS, maar was het cholesterolprofiel niet anders dan dat van de controles en kwam het metabool syndroom niet vaker voor. De mate van aderverkalking in vrouwen met PCOS en op leeftijd en etniciteit gematchte controles was vergelijkbaar. Tenslotte beschrijven we in **hoofdstuk 5.4** de resultaten van de studie waarin is gekeken naar de associatie van hoge androgeen spiegels met aderverkalking en hart- en vaatziekten bij postmenopauzale vrouwen. Androgenen komen bij vrouwen met PCOS vaak in verhoogde concentraties voor. Tevens is dit het enige kenmerk van PCOS dat lang na het optreden van de menopauze meetbaar kan blijven. Wij onderzochten 2578 vrouwen met een gemiddelde leeftijd van 70 jaar, die ongeveer 20 jaar geleden in de overgang gekomen waren. Een hoge vrije androgeen index was geassocieerd met een hogere polsgolfsnelheid. De polsgolfsnelheid is de snelheid waarmee een bloeddrukgolf zich door de slagaderen verplaatst en een maat voor de vaatwandstijfheid (een vroeg teken van een verslechterde functie van de vaten). Hoge spiegels van DHEA en DHEAs waren geassocieerd met een lagere vaatwanddikte. Er werd geen verband gevonden tussen hoge androgeenspiegels bij postmenopauzale vrouwen en het voorkomen van een beroerte, aandoeningen van de kransslagaderen en hart- en vaatziekten als geheel. Wij concluderen dat hoge androgeenspiegels bij postmenopauzale vrouwen niet in verband kunnen worden gebracht met een verhoogd risico op hart- en vaatziekten en dat de gezondheid van hart en bloedvaten bij vrouwen met PCOS wel eens veel beter zou kunnen zijn dan aanvankelijk werd gedacht.

In **hoofdstuk 6** plaatsen we de in dit proefschrift gevonden resultaten in bredere context en beschrijven we onze visie op de lange termijn gezondheid van vrouwen met PCOS.

CHAPTER 8

Curriculum Vitae
Bibliography
PhD portfolio
Dankwoord

Curriculum Vitae

Cindy Meun was born on the 12th of February 1986 in Zoetermeer. According to her mother, this was the only time in Cindy's life that she arrived early.

She attended Erasmus College, where she enjoyed participating in the school's theatre and music activities. After finishing high school, she began medical school at Erasmus Medical Center and relocated to Rotterdam. During her studies, she had the opportunity to conduct research at Flinders Medical Centre in Adelaide, Australia, for six months in the genetic lab. Apart from becoming an expert at pipetting, this experience taught her to drive on the left side of the road and that wine should not come from a cardboard box

In 2013, Cindy graduated from medical school and began working in gynecology at Amphia Ziekenhuis in Breda. She later returned to Erasmus MC to pursue a PhD in Reproductive Medicine, but realized her true passion was in oncology. She is now a resident in radiation oncology at Leiden University Medical Center and lives happily in Rotterdam with her partner Mo and their cat Greta, whom they claim is responsible for them moving in together. Last year their family was expanded with the birth of their son Ibrahim.

Cindy is expected to complete her residency in radiation oncology in early 2025 and is excited to see where life takes her after reaching this milestone.

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This Thesis

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3. *High Androgens in Postmenopausal Women and the Risk for Atherosclerosis and Cardiovascular Disease: The Rotterdam Study*
   Meun C, Franco OH, Dhana K, Jaspers L, Muka T, Louwers Y, Ikram MA, Fauser BCJM, Kavousi M, Laven JSE
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5. *Cardiovascular RiskprofilE - IMaging and gender-specific disOrders (CREw-IMAGO): rationale and design of a multicenter cohort study*
   Zoet GA, **Meun C**, Benschop Hendrik, Boersma E, Budde RPJ, Fauser BCJM, de Groot CJM, van der Lugt A, Maas AHEM, Moons KGM, Roeters van Lennep JE, Roos-Hesselink JW, Steegers EAP, van Rijn BB, Laven JSE, Franx A, Velthuis BK
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6. *The cardiometabolic profile of women with WHO2 anovulation differs from that of women with women with polycystic ovary syndrome*
   Meun C, van Zwol-Janssens C, van Dorp W, Steegers RPM, Laven JSE

Under Review

7. *Prevalence and severity of coronary and intracranial carotid calcification on computed tomography in middle-aged women with PCOS*
Meun C, Gunning MN, Benschop Hendrik, Bos D, Peters H, van Rijn BB, Franx A, Boersma H, Budde RPJ, Appelman Y, Maas AHEM, Lambalk CB, Velthuis BK, Kavousi M, Louwers YV, Fauser BCJM, Laven JSE, CREW-consortium
Under Review

Other Publications

8. *A Polygenic and Phenotypic Risk Prediction for Polycystic Ovary Syndrome Evaluated by Phenome-Wide Association Studies*
Joo YY, Actkins K, Pacheco JA, Basile AO, Carroll R, Crosslin DR, Day F, Denny JC, Velez Edwards DR, Hakonarson H, Harley JB, Hebbring SJ, Ho K, Jarvik GP, Jones M, Karaderi T, Mentch FD, **Meun C**, Namjou B, Pendergrass S, Ritchie MD, Stanaway IB, Urbanek M, Walunas TL, Smith M, Chisholm RL, Kho AN, Davis L, Hayes MG; International PCOS Consortium
Journal of Clinical Endocrinoly & Metabolism, 2020, 105(6):1918-36

9. *Causal mechanisms and balancing selection inferred from genetic associations with polycystic ovary syndrome*
Day FR, Hinds DA, Tung JY, Stolk L, Styrkarsdottir U, Saxena R, Bjonnes A, Broer L, Dunger DB, Halldorsson BV, Lawlor DA, Laval G, Mathieson I, McCardle WL, Louwers Y, **Meun C**, Ring S, Scott RA, Sulem P, Uitterlinden AG, Wareham NJ, Thorsteinsdottir U, Welt C, Stefansson K, Laven JSE, Ong KK, Perry JRB
Nature Communications, 2015, 6:8464

10. *Pre-Conception Characteristics Predict Obstetrical and Neonatal Outcomes in Women With Polycystic Ovary Syndrome*
Christ JP, Gunning MN, **Meun C**, Eijkemans MJC, van Rijn BB, Bonsel GJ, Laven JSE, Fauser BCJM
Journal of Clinical Endocrinoly & Metabolism, 2019, 104(3):809-818

11. *Antimüllerian hormone to determine polycystic ovarian morphology*
Dietz de Loos A, Hund M, Buck K, **Meun C**, Sillman J, Laven JSE
Fertility Sterility, 2021, 116(4):1149-1157

12. *Coronary artery calcification in middle-aged women with premature ovarian insufficiency*
Gunning MN, **Meun C**, van Rijn BB, Maas AHEM, Benschop Hendrik, Franx A, Boersma E, Budde RPJ, Appelman Y, Lambalk CB, Eijkemans MJC, Velthuis BK, Laven JSE, Fauser BCJM, CREW-consortium
Clinalal Endocrinology, 2019, 91(2):314-322

13. *Cardiovascular Risk in Women With Premature Ovarian Insufficiency Compared to Premenopausal Women at Middle Age*
Daan NM, Muka T, Koster MP, Roeters van Lennep JE, Lambalk CB, Laven JS, Fauser CG, **Meun C**, de Rijke YB, Boersma E, Franco OH, Kavousi M, Fauser BC.
Journal of Clinical Endocrinoly & Metabolism, 2016, 101(9):3306-15

14. Sex Steroids, Sex Hormone-Binding Globulin and Cardiovascular Health in Men and Postmenopausal Women: The Rotterdam Study
Jaspers L, Dhana K, Muka T, **Meun C**, Kiefte-de Jong JC, Hofman A, Laven JS, Franco OH, Kavousi M
Journal of Clinical Endocrinoly & Metabolism, 2016, 101(7):2844-52

15. *Associations of Steroid Sex Hormones and Sex Hormone-Binding Globulin With the Risk of Type 2 Diabetes in Women: A Population-Based Cohort Study and Meta-analysis*
Muka T, Nano J, Jaspers L, **Meun C**, Bramer WM, Hofman A, Dehghan A, Kavousi M, Laven JS, Franco OH
Diabetes, 2017, 66(3):577-586

16. *The cardiovascular risk profile of middle age women previously diagnosed with premature ovarian insufficiency: A case-control study*
Gunning MN, **Meun C**, van Rijn BB, Daan NMP, Roeters van Lennep JE, Appelman Y, Boersma E, Hofstra L, Fauser CGKM, Rueda-Ochoa OL, Ikram MA, Kavousi M, Lambalk CB, Eijkemans MJC, Laven JSE, Fauser BCJM, CREW consortium
PLoS One, 2020, 15(3):e0229576

17. *Health in middle-aged and elderly women: A conceptual framework for healthy menopause*
Jaspers L, Daan NM, van Dijk GM, Gazibara T, Muka T, Wen KX, **Meun C**, Zillikens MC, Roeters van Lennep JE, Roos-Hesselink JW, Laan E, Rees M, Laven JS, Franco OH, Kavousi M
Maturitas, 2015, 81(1):93-8

18. *Early Onset of Coronary Artery Calcification in Women With Previous Preeclampsia*
Benschop Hendrik, Brouwers L, Zoet GA, **Meun C**, Boersma E, Budde RPJ, Fauser BCJM, de Groot CMJ, van der Schouw YT, Maas AHEM, Velthuis BK, Linstra KM, Kavousi M, Duvekot JJ, Franx A, Steegers E, van Rijn BB, Roeters van Lennep JE, CREW Consortium
Circulation Cardiovascular Imaging, 2020, 13(11):e010340

PhD Portfolio

Name: Cindy Meun Erasmus MC Department: Obstetrics and Gynaecology, division of Reproductive Medicine PhD period: May 2014 – May 2019 Promotors: J.S.E. Laven & B.C.J.M. Fauser Supervisors: Y.V. Louwers & M. Kavousi		
	Year	**ECTS**
Courses		
Biomedical English Writing and Communication	2015	4
Research Integrity	2016	0.3
Statistics	2016	5.7
BROK ('Basiscursus Regelgeving Klinisch Onderzoek'	2016	1.5
ESP43 Principles of Genetic Epidemiology	2014	0.7
ESP57 Genomics in Molecular Medicine	2014	1.4
ESP29 Genome Wide Association Analysis	2014	0.7
ESP40 Case-control studies	2015	0.7
ESP62 Markers and Prediction Research	2015	0.7
Molmed SNP course (2015)	2015	2
R course (2016)	2016	1.4
Photoshop / Indesign	2016	0.3
Illustrator	2016	0.15
Diagnostic data for dummies: the untapped potential for re-use	2017	0.7
Womens' health course	2015	1
Biweekly research meetings	2014-2019	2
Biweekly Reproductive Medicine – Endocrinology meeting	2014-2019	2
Pijlerdag voortplantingsgeneeskunde	2016	0.2
Sophia research day	2015-2017	0.4
AAV wetenschapsmiddag	2016	0.2

	Year	
(Poster)Presentations at International Conferences		
Endocrine society for reproductive medicine (ENDO), San Diego	2015	1
European Society of Reproductive Medicine (ESHRE), Lisbon	2015	1
American Society of Human Genetics (ASHG), Baltimore	2015	1
Rotterdamse Gynaecologen OpleidingsCluster (RGOC), Rotterdam	2015	1
Women's Health Congress, Washington, USA	2015	2
Society for Reproductive investigation (SRI), Orlando, Florida	2017	1
Int. Society of Gynaecological Endocrinology (ISGE), Florence, Italy	2018	1
European Society of Reproductive Medicine (ESHRE), Barcelona	2018	1
Wladimiroff Award Meeting (RCOG), Erasmus MC, Rotterdam	2018	1
Society for Reproductive investigation (SRI), Paris, France	2019	1
PCOS patientendag 2015 (presenter workshop hart- en vaatziekten)	2015	1
Conferenence in attendence (no presentation)		2
Teaching		
Curriculum Bachelor Medicine, Erasmus University	2015, 2016	2
Tutoraat	2014 -2016	3
Supervising Master's theses	2014-2018	4
Other		
Womens' health course (organizing committee)	2015	1
Arts Assistenten Vereniging Erasmus MC (board)	2016 –	2
AAV Wetenschapsmiddag 2018 (organizer)	2018	2
Workshop onderhandelen (academie voor medisch specialisten)	2018	0.2
Peer reviewing of articles for scientific journals	2017-2019	2

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