GENETICS OF OVARIAN AGEING

Genetic association studies on natural menopause
and Primary Ovarian Insufficiency

Marlies Voorhuis

Cover design and layout: Ruth Visser (VisserVisuals, Amsterdam)
Printed by: Gildeprint Drukkerijen BV, Enschede, the Netherlands

ISBN: 978-94-91082-04-7

This project was funded by the Department of Reproductive Medicine and Gynecology, University Medical Center Utrecht, the Netherlands, and The Netherlands Organisation for Health Research and Development (ZonMW, project 92003575).

The author gratefully acknowledges financial support for printing this thesis by the Gilles Hondius Foundation, Division Woman & Baby University Medical Center Utrecht, Merck Sharp & Dome BV, Origio Benelux BV, Merck Serono, Goodlife, Ferring BV, Biomedic Nederland BV, BMA Mosos BV, Abbott BV, Memidis Pharma BV and Medical Dynamics.

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GENETICA VAN OVARIËLE VEROUDERING

Genetische associatiestudies naar natuurlijke menopauze en primaire ovariële insufficiëntie (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op vrijdag 28 juni 2013 des middags te 4.15 uur

door

Marlies Voorhuis
geboren op 6 februari 1981 te Oldenzaal

Promotoren:	Prof. dr. F.J.M. Broekmans
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Co-promotor:	Dr. N.C. Onland-Moret

Voor mijn ouders

CONTENTS

Chapter

One

General Introduction

GENERAL INTRODUCTION

Age at natural menopause

Menopause occurs when the ovarian follicle pool has become exhausted and is insufficient to maintain menstrual cycles. It is the endpoint of a process referred to as ovarian ageing. The mean age at final cessation of menses and thus menopause is approximately 51 years, but varies widely between 40 to 60 years of age [1-3]. Approximately 1% of all women experience menopause before the age of 40 years, which is referred to as primary ovarian insufficiency (POI) [4]. Timing of menopause has great implications for female fertility and general health. During the last decades, this has resulted in a growing attention for the physiological mechanisms underlying the wide variation of age at natural menopause.

Menopause and fertility

Menopause marks the definite end of a woman's reproductive lifespan. The parallel decay in oocyte quality together with the decrease in follicle numbers contributes to the gradual decline in fertility and the final occurrence of natural infertility, roughly 10 years before menopause occurs [3, 5, 6]. As the rate of ovarian ageing and thus timing of natural menopause varies widely, consequently, the age of natural sterility ranges widely and some 50% of women will become sterile before the age of 41 [3].

In the last decades the number of educated women has increased, resulting in a higher participation of women in the labour market. In combination with the wide availability of reliable contraception methods, women have been postponing childbirth [7]. This tendency of postponed childbearing will lead to an increase in age related infertility, consequently increasing the utilization of assisted reproductive technologies (ART) and unwanted childlessness [6, 8]. An increase in the overall uptake of ART causes a financial burden to society. Currently, in the Netherlands, the estimated annual costs of IVF treatments amount to approximately 50 million euro. In addition to these high costs, ART obviously has an emotional impact on the couples faced with fertility problems [9, 10]. Moreover, it has been recognized that postponing to start a family until women are well in their thirties will frequently lead to permanent loss of a women's own reproductive potential, even with the use of ART treatment [11]. Additionally, studies that have evaluated development of children born after ART showed that these children are at increased risk for several types of health problems [12, 13].

Menopause is the only point in the ovarian ageing process that can be assessed quite clearly [14]. Assuming a fixed ten year interval between infertility and the onset of menopause, the latter event will retrospectively mark the reproductive lifespan (RLS) of an individual woman. Identifying genetic markers for (early) age at menopause may provide us with tools for predicting a woman's reproductive lifespan and could help in the development of individualized preventive

management strategies of RLS and early loss of fertility. By providing these tools, women can be helped to make informed decisions about their current lifestyle and reproductive behavior and potentially to take action to safeguard future fertility where risks exist (i.e. by opting for oocyte vitrification).

Menopause and women's health

Timing of menopause is linked to a variety of health risks [15, 16]. Early menopause is associated with a higher risk of cardiovascular disease [17-19], colorectal cancer [20] and osteoporosis [21]. A late menopausal age on the other hand has been associated with an increased risk of breast-, ovarian- and endometrial cancer [22-25]. Generally, the age-adjusted mortality is reduced by 2% for each increasing year of menopausal age [15].

The identification of genetic determinants of timing of menopause might shed light on the mechanisms involved in the development of these health risks. Eventually, this could benefit measures of early detection and possible medical or lifestyle interventions as part of preventive management of the menopause related diseases and health risks.

Genetics of ovarian ageing

It is yet unclear what exact physiological processes determine the variation in timing of menopause. However, as demonstrated in several mother-daughter, twin and sib-pair studies, genetic factors have proven to play a major role in this respect. Estimations of heritability in menopausal age range from 31% to 87% [26-29]. In addition to genetic factors, several environmental and life-style factors have been suggested to influence menopausal timing. Examples of these associated factors are smoking, body mass index, use of alcohol and parity. However, the effects of these factors are small and have been inconclusive, except for smoking which has consistently been associated with an earlier age at menopause [30-35].

The group women that reach menopause before the age of 40 is separately labelled as POI. POI can be caused by a number of monogenetic mutations, such as premutations of the fragile-X mental retardation 1 (FMR1) gene and mutations in the forkhead transcription factor L2 (FOXL2) gene, auto-immune diseases (most commonly auto-immune thyroid disease), or can be iatrogenic (i.e. after surgery, radiation- or chemotherapy). However, in most cases there is no conclusive factor causing POI (idiopathic POI) [36, 37]. Although it is possible that idiopathic POI cases are part of the variation towards the entire distribution of natural menopause, it remains unclear whether idiopathic POI should be considered as a separate entity.

Throughout the last decade, several genetic studies have been conducted in order to identify genetic factors involved in determining the variation in timing of menopause. To find the genetic variants involved in complex traits (i.e. traits that are influenced by both genetic and environmental factors, like timing of menopause), several genetic approaches can be applied.

These genetic approaches each primarily aim at different specific genetic variants. In timing of menopause, genome-wide linkage analysis, candidate gene association studies and, more recently, genome-wide association studies (GWAS) have been used. These different genetic study designs are explained in chapter two. To date, a number of genetic variants have been discovered that are associated with age at menopause. However, they merely account for only a small part of the heritability of age at natural menopause (chapter 2; [38]).

The identification of genetic variants associated with (early) ovarian ageing and thereby infertility and consequences for health later in life, constitutes the core of this thesis. In order to identify these genetic variants we conducted: (I) candidate gene association studies on SNPs and structural variants (chapters 3, 5, 6 and 7), (II) a genome-wide association study (GWAS, chapter 8) and (III) a gene-gene interaction analysis (chapter 4).

Once genetic variants associated with timing of menopause have been identified, studies can be carried out to unravel the role for these variants in processes that may direct the variation of ovarian ageing. Finally, these studies may yield genetic markers that will be analyzed for their possible role in preventive management of age related infertility and menopause related diseases.

AIMS AND OUTLINE OF THE THESIS

Aims

To identify and validate genetic variants that are involved in ovarian ageing.

Part One:

Identifying genetic variants that are associated with the variation of age at natural menopause in a general population

Part Two:

Identifying genetic variants that are associated with early menopause in a cohort of women with POI and early menopause (EM) compared to the general population

Outline

Part One

- **Chapter 2** gives a systematic review of the genetic studies in timing of natural menopause performed until September 2009;
- **Chapter 3** describes the results of a candidate gene study in five genes involved in primordial follicle recruitment in association with age at natural menopause ;
- In **Chapter 4** pairwise interactions between 23 SNPs in five candidate genes studied in chapter 3 were searched for their role in determining timing of menopausal;
- **Chapter 5** studies the role of 46 SNPs that are associated with coronary artery disease and Type 2 Diabetes as determinants for age at natural menopause;
- In **Chapter 6** the role of normal and intermediate sized FMR1 CGG repeats in timing of natural menopause is investigated.

Part Two

- **Chapter 7** studies the role of normal and intermediated sized FMR1 CGG repeats in Primary Ovarian Insufficiency (POI);
- **Chapter 8** provides the preliminary results of a genome-wide association study in POI and early menopause.

Finally, in **Chapter 9** the results and conclusions of the conducted studies are discussed and future prospects for genetic research on timing of menopause are presented

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Chapter

Two

Human studies on genetics of the age at natural menopause: a systematic review

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Hum Reprod Update. 2010, 16(4):364-377

TABLE OF CONTENTS CHAPTER 2

ABSTRACT

Background

Timing of natural menopause has great implications for fertility and women's health. Age at natural menopause (ANM) is largely influenced by genetic factors. In the past decade, several genetic studies have been conducted to identify genes in ANM, which can help us unravel the biological pathways underlying this trait and the associated infertility and health risks. After providing an overview of the results of the genetic studies performed so far, we give recommendations for future studies in identifying genetic factors involved in determining the variation in timing of natural menopause.

Methods

The electronic databases of Pubmed and Embase were systematically searched until September 2009 for genetic studies on ANM, using relevant keywords on the subject. Additional papers identified through hand search were also included.

Results

Twenty seven papers emerged from our literature search. A number of genetic regions and variants involved in several possible pathways underlying timing of ANM were identified, including two possible interesting regions (9q21.3 and chromosome 8 at 26 cM) in linkage analyses. Recent genome-wide association studies (GWAS) have identified two genomic regions (19q13.42 and 20p12.3), containing two promising candidate genes (BRKS1 and MCM). In the candidate gene association studies on ANM, very few consistent associations were found.

Conclusion

A number of genetic variants have been discovered in association with age at natural menopause, although the overall results have been rather disappointing. We have described possible new strategies for future genetic studies to identify more genetic loci involved in the variation in menopausal age.

INTRODUCTION

Implications of menopausal age on fertility and women's health

In the last decades the interest in the mechanisms behind ovarian ageing and the timing of natural menopause has increased since menopause has great implications for women's fertility and health. The gradual decline in female fertility with age has been long known from several sources [1-3]. As a result of an increase in the number of educated woman, growing participation of women in the labour market together with the wide availability of reliable contraception methods, women have been postponing childbirth in the last decades [4]. This tendency of postponed childbearing will consequently lead to an increase in age related infertility, subsequently increasing the utilisation of assisted reproductive technologies (ART) and unwanted childlessness [1, 5-7]. An increase in the overall uptake of ART causes a financial burden to society as well as a large emotional impact on the couples that are faced with fertility problems. With the ability to predict age at menopause, and thus the end of the reproductive lifespan for an individual woman, family planning and fertility treatment can be greatly influenced [8, 9].

Next to fertility implications, menopause is linked to a variety of health risks [10]. Early menopause is associated with a higher risk of cardiovascular disease [11-15] and osteoporosis [16], whilst a late menopause age has been associated with an increased risk of breast cancer [17].

Menopausal age as a complex genetic trait

Menopause occurs when the follicle pool in the ovaries has become exhausted and insufficient to maintain menstrual cycles. Menopause therefore marks the definitive end of a women's reproductive lifespan [18]. The age at natural menopause (ANM) varies widely amongst women and ranges roughly from 40 to 60 years of age [19, 20]. Although the exact physiological processes underlying the timing of menopause are far from elucidated at present, genetic factors have proven to play a major role in determining this variation in menopausal age as demonstrated in several mother-daughter, twin and sib-pair studies. Estimations of heritability in menopausal age range from 31 to 87% [21-25]. This wide range in heritability estimates of ANM reflects the difficulty of measuring heritability for this trait. Besides this, heritability estimates cannot be used to assess the magnitude of the genetic contribution to a trait [26, 27]. This is because the components of the variance, genes and environment, are in most cases not independent, but interact. Causes of phenotypic variation cannot be separated into genes and environment, and the different genetic and environmental components cannot simply be summed up to 100%. In addition, genetic variance comprises different types (i.e. additive, dominant and epistatic variance) resulting in incomparable heritability estimates from parent-offspring and twin heritability studies. Next to multiple genetic factors, several environmental and life-style factors like smoking, body mass

index, use of alcohol and parity have claimed to influence menopausal timing as well, although the effects seem small and been inconclusive [28-33].

As menopausal age is influenced by both multiple genetic and environmental factors it is considered as a complex genetic trait. Identification of the environmental factors and genes that contribute to the endowment and wastage of follicles in the ovaries and thus timing of menopause will add to the understanding of the physiological mechanism of this trait and the associated infertility and health risks. In the past decade various research groups around the world have carried out genetic studies in ANM cohorts' using a candidate-gene and genome-wide approach, including both a linkage mapping and association design. In this paper we will review the current status of genetic studies conducted on ANM in humans. Before summarising current published literature, the basis of genetic analysis for complex genetic traits will be presented. After providing an overview of the results of the association studies and subsequent replication studies performed so far, we aim to give recommendations for future studies to identify genetic factors involved in determining the variation in timing of natural menopausal age.

GENETIC APPROACHES IN DETERMINING AGE AT NATURAL MENOPAUSE

In order to elucidate the loci or genes involved in complex traits, like the timing of ANM, the three most commonly used genetic approaches are: genome-wide linkage analysis, candidate gene association studies and genome-wide association studies (GWAS). The designs of all these three types of genetic epidemiologic studies have been described extensively in other papers [34-36], and will be briefly summarised below.

(Genome-wide) linkage analysis

In linkage analyses, genetic loci that contribute to a trait can be identified using a set of (genome-wide) genetic markers in related individuals. Linkage occurs when loci are transmitted together from parents to their offspring more often than expected under independent inheritance. If a marker can be identified that is passed down through a family such that it consistently accompanies the disease or trait of interest, this suggests that in the region surrounding the marker (linkage region) a genetic variant is located with a functional effect on a disease or distinct trait (such as height or menopausal age) [35, 37]. Using mathematical models it is possible to estimate the evidence for linkage in a certain genetic region. In these models, the recombination fraction is important and indicates the probability of recombination between two loci, i.e. the separation of the two loci due to crossing over of homologous chromosomes. The further apart two loci are, the

higher the chance that there will be recombination with a maximum of 50% [35]. So a recombination fraction < 0.5 indicates that the chance of recombination between two loci smaller is than 50%. Usually the LOD (logarithm of odds) score is used as an estimate for the evidence of linkage. The LOD score is a function of the recombination fraction. A LOD score > 3 is generally accepted as significant evidence of linkage, whereas a LOD score smaller than -2 is regarded as significant evidence against linkage.

After significant linkage regions are found, extensive candidate gene studies are required to identify the causal genes for a trait within this linkage region (see: candidate gene association studies, below). Genetic linkage approaches have been particularly successful for the identification of genes underlying monogenetic diseases. However, in complex traits where several genes and environmental factors contribute to the trait, there is no clear mode of inheritance. Although methods for model-free linkage analysis exist, these methods have not been very successful in the identification of genes that play a role in complex traits. The most important reason for this is lack of statistical power. Thus, linkage analysis is less suitable for identifying genes in this type of traits [35, 38, 39].

Candidate gene association studies

Analysis of the association between a genetic variant and a trait is in fact the traditional epidemiologic disease-exposure association analysis applied to genotypes or alleles in a population [35]. This method can be applied to candidate genes, or genome-wide. In this part, we will discuss the principles of a candidate gene association study.

Candidate genes are genes that are believed to influence complex traits due to their expected involvement in the biological pathway of that trait, or to their location near a region previously determined in a linkage analysis of the trait. Thus candidate genes are selected based on at least some knowledge about the physiology of the examined trait. Next, genetic variants are selected within the gene that are then associated to the trait. Several types of genetic variation can be studied, the most common one being single nucleotide polymorphisms (SNPs), which are DNA variants that represent variation in a single nucleotide (A, T, C or G). When candidate gene studies were first performed, SNPs were often chosen because of their effect on amino acid change (referred to as non-synonymous SNPs). However, there are also other types of SNPs, such as synonymous SNPs in protein-coding regions (which do not result in amino acid changes) and SNPs in introns or intergenic regions. All types of SNPs may influence complex traits or diseases as they may, for instance, cause variation in gene regulation and expression or differential splicing [40]. With the completion of the first phase of the HapMap project, information on many SNPs in the human genome became available. Because of this large amount (which is still growing) it is impossible to predict which variants will be functional. As a result, the field moved towards indirect association studies. In this type of study the SNP is a proxy for a causal variant [40]. Due

to correlations between SNPs (linkage disequilibrium, LD), it is possible to identify total genetic variation in a gene without genotyping every SNP. In this so called tagging SNP approach, the fact that SNPs are passed on to the next generation in fixed non-recombining blocks of genetic information is used. This means that with a limited number of SNPs the total genetic variation in that block can be described by just a few tagging SNPs [41, 42].

Genome-wide association studies (GWAS)

Since the publication of the International HapMap project in 2005, a public database containing common genetic variants (allele frequency ≥ 1%) is now generally available [43]. Next to this, reduced genotyping costs in the last years have made it feasible to perform GWAS. A GWAS investigates genetic variations underlying a trait or disease that can be explored without prior assumptions on biological pathways and physiology. This is in contrast to the aforementioned association studies using candidate gene studies where prior assumptions on biological pathways are needed. However, it is often stated that GWAS is a hypothesis-free search, which is not the case. In fact GWAS are based on the common disease, common variant hypothesis [44, 45]. Therefore, nowadays people refer to it as an agnostic scan.

In GWAS around 500.000 SNPs are genotyped. These SNPs were chosen to best cover the genetic variation of the entire genome and are mostly tagSNPs. Thus these SNPs were not chosen on the basis of their possible functional effects.

In the last few years, an exploding number of GWAS have been published. Although not all GWAS hits could be replicated, the results illustrate that an agnostic scan of the entire human genome can produce findings that would have been missed with candidate gene approaches. Also, the GWA findings can lead to new hypotheses on the aetiology of complex traits.

METHODS OF THE SYSTEMATIC REVIEW

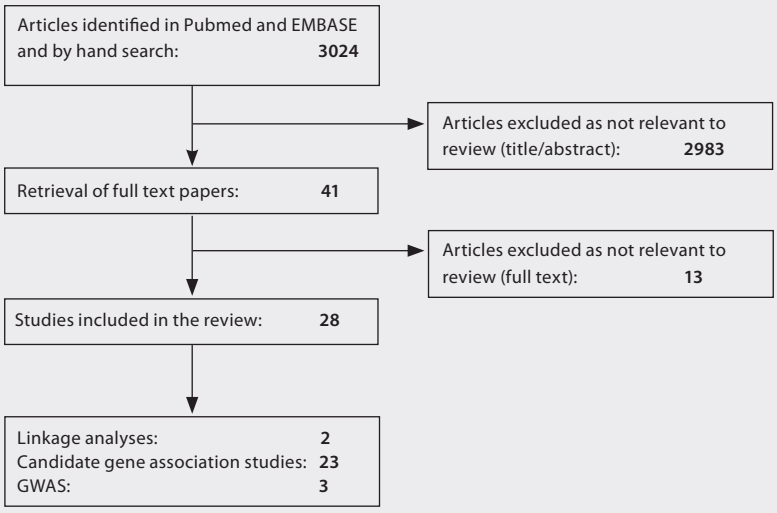
The electronic databases of MEDLINE (Pubmed) and Embase were searched for English-language articles until September 2009, using the keywords: 'menopause' and 'age at menopause' in combination with the keywords: 'genetics', 'polymorphism', 'candidate gene', 'association study' or 'genome wide'. In addition, we hand searched related articles and reference lists of selected studies on the subject for other relevant articles. We included all articles on genome-wide linkage mapping and genome-wide or candidate gene association studies in timing of natural menopausal age as a single endpoint or natural menopausal age as part of a number of reproductive determinants without any selection on quality criteria, as in that case too few studies would have remained.

RESULTS

A total of 28 papers on genetics in ANM emerged from our literature search: two papers on genome-wide linkage analysis, 23 papers on association studies using candidate genes and another three papers on GWAS (figure 1).

All studies used the WHO definition of menopause (≥ 12 consecutive months of amenorrhea not because of surgery or other obvious causes) in determining ANM [46]. Also, all 28 studies determined ANM through self-reportage by either in-person interviews or questionnaires.

Figure 1 Flowchart of selection of genetic studies on age at natural menopause.



Genome-wide linkage analysis

Two genome-wide linkage analyses on ANM have been published to date (Table I). Van Asselt and colleagues conducted the first study in 2004 in a population of 579 extremely discordant and concordant sib-sister pairs from 165 families. The study subjects were genotyped for a total of 417 microsatellite markers. Two regions showed suggestive linkage with ANM: region Xp21.3 (LOD score 3.1) and region 9q21.3 (LOD score 2.6) [24]. The observed linkage region on the X-chromosome has also been described as a linkage region in primary ovarian insufficiency (POI), which is defined as the onset of menopause under the age of forty [47]. After exclusion of women with a menopausal age less than 40 years of age, the linkage peak attenuated, although suggestive linkage of the region remained. This implies that the Xp21.3 region not only influences premature menopause,

but might also affect early menopause and/or menopausal age as a whole. The linkage region on chromosome 9 contains multiple genes, of which one gene that encodes for a protein of the BCL2 family is particularly interesting. This protein is known for its involvement in apoptosis, and the rate of apoptosis in the ovarian follicle pool could well play a role in determining onset of menopause [48]. The *BCL2* encoding gene could therefore be a possible contributing candidate gene for ANM [49]. To date, as far as we are aware, no additional studies on *BCL2* in association with ovarian ageing have been published.

In 2005, a second genome-wide linkage study was carried out in a population-based sample of 861 postmenopausal women from 291 families. Genotyping was performed with a genome-wide scan of 401 microsatellite markers. In unadjusted analysis, two suggestive linkage regions for ANM appeared: chromosome 8 at 26 centimorgan (cM) (LOD score 2.6) and chromosome 16 at 11cM (LOD score 2.4). After adjustment for body mass index (BMI) and cigarette use, another region with suggestive linkage emerged chromosome 11 at 113 cM (LOD score 2.1) [50]. Similar to the linkage study of van Asselt and colleagues, women with early onset of menopause (≤ 40 years) were excluded for an additional linkage analysis. As a result the LOD scores lowered, suggesting a role for these loci in premature and/or early menopause. Near the observed linkage peak on chromosome 8 lies the *gonadotrophin-releasing hormone 1 gene* (*GNRH1*) at locus 8p21-p11.2. The gonadotrophin-releasing hormone (GnRH) is a key molecule in the hypothalamic-pituitary-gonadal axis controlling human reproduction [51]. As the release of follicle-stimulating hormone (FSH) from the pituitary gland is at least in part controlled by pulsatile GnRH and the magnitude of FSH exposure may affect the rate of follicle loss [52], the *GNRH1* gene could be considered as an interesting candidate gene for timing of menopause.

Table I Genome-wide linkage analyses; regions with (suggestive) linkage with ANM

Study	Linkage region	LOD score	N[a]	N markers [b]
Van Asselt '04	9q21.3	2.6	579 [c] (165 families)	417
	Xp21.3	3.1		
Murabito '05	chrom 8 at 26 cM	2.6	861[d] (291 families)	401
	chrom 16 at 11 cM	2.4		
	chrom 11 at 113 cM	2.1[e]		

a Number of women with ANM in study population
b Number of genotyped microsatellite markers
c Extremely discordant and concordant sib-pairs
d General population based sample
e After adjusting for BMI and smoking

Candidate gene association studies

We found a total of 23 papers on association studies using candidate genes, in which 25 genes were examined on association with ANM. In the 25 studied candidate genes on ANM, the majority of the genes can be divided in two major biological pathways: genes involved in steroid pathways and vascular-function related genes.

Candidate genes involved in steroid pathways

Oestrogen-related genes have been of particular interest in the search of genes influencing ANM, as is shown by the large number of candidate gene studies conducted in the pathway of this particular steroid (Table II). Candidate genes for determination of ANM involved in oestrogen pathways have been chosen based upon the general and organ-specific effects of oestrogen on growth, differentiation and function of reproductive tissues. However, a clear foundation for this assumed involvement of oestrogen-related genes on ANM remains absent. No obvious effect upon endowment of wastage of follicles has so far been attributed to gonadal steroid effects, although a recent publication has suggested the presence of functionally active oestrogen receptors in primary follicles in mice [53].

To date, nine genes involved in oestrogen biosynthesis and metabolism have been examined as to their role in timing of menopause. Five genes were at least once reported to be significantly associated with this trait. The first candidate-gene association study on ANM was conducted in 1999 by Weel and colleagues in the *oestrogen receptor gene* (*ESR1*). A significant association was found between the PvuII SNP in the *ESR1* gene and ANM in Caucasian women [54]. However, in three subsequent studies this finding concerning the PvuII SNP could not be replicated. Moreover, no associations were found in other studied SNPs in *ESR1* [55-57].

In the oestrogen-inactivating gene *cytochrome P-450 1B1* (*CYP1B1*), one SNP (rs1800440) was significantly related with ANM in a large population of 1360 Caucasian women [58]. Although that particular SNP was not replicated, three other SNPs (rs100212, rs1056827 and rs1056836), which are not in complete LD with the earlier associated SNP rs1800440, were associated with ANM in a sample of 797 Chinese women [59]. However, Mitchell and colleagues could not replicate these associations with ANM found by Long and colleagues in a small population of 64 Caucasian women [60].

Mitchell an colleagues examined two polymorphisms in the *cytochrome P-450 19 gene* (*CYP19A1*), which is involved in oestrogen synthesis. In this study of 64 postmenopausal white women the TTTA repeat was associated with ANM; women carrying two *CYP19A1* 7r(-3) alleles had a 2.6-year later onset of menopause, compared to those with no 7r(-3) alleles [60]. He and colleagues also examined the role of *CYP19A1*. Although they did not include the TTTA repeat polymorphism in their study, they did find an association with ANM in two (rs1065778 and rs2255192) of a total of 28 examined SNPs in this gene [61]. However, after correction for multiple testing, taking the total number of tests into account, these associations were no longer significant.

Next to *CYP19A1*, the *oestrogen receptor β gene* (*ESR2*) has also been studied in relation to the timing of menopause. Although no significant association with ANM was found in 17 SNPs in the *ESR2* gene, an association was found in two blocks with respectively 8 and 9 SNPs in LD with each other [61]. Of note, no other study tried to replicate this association thus far.

Finally, carriers of AG alleles of SNP rs2830 in the oestrogen-synthesizing gene *hydroxysteroid dehydrogenase* (*HSD17B1*) experienced a later menopause compared to those with AA alleles, in a small study population of 64 white women [60]. Again, to our knowledge, this association has not been examined in subsequent studies.

Four other oestrogen-related genes were examined, but no association with ANM was found: the oestrogen-synthesizing genes *cytochrome P-450 17* (*CYP17A1*) and *17-β-hydroxysteroid dehydrogenase type 1* (*HSD17B1*), and the oestrogen-inactivating genes *catechol-O-methyltransferase* (*COMT*) and *Cytochrome P-450 1A1* (*CYP1A1*) [55, 56, 58, 60, 61].

Other examined candidate genes involved in the steroid pathway are the *anti-Müllarian hormone gene* (*AMH*) and *AMH type 2 receptor gene* (*AMHR2*). Because AMH inhibits primordial follicle recruitment in ovaries of mice, it was hypothesized that AMH also regulates the ovarian follicle pool in women and therefore could influence menopausal timing. Using single tagSNPs for the *AMH* and the *AMHR2* gene, the total genetic variation in these genes could be described [62]. Only the SNP in the *AMHR2* gene was found to be significantly associated with ANM in interaction with parity in a large Caucasian population [63] and was also associated with estradiol synthesis in the dominant follicle in an earlier study [62].

No association with ANM was observed in other studied steroid-related candidate genes like *steroid 5-alpha-reductase type 2 gene* (*SRD5A2*) [64] and *FSH receptor gene* (*FSHR*) [65].

Vascular-function related candidate genes

Based on the hypothesis that vascular ageing and reproductive ageing are connected, a variety of studies have focused on vascular-related candidate genes in association with ANM (Table III). A earlier onset of menopause has been associated with an increased risk of cardiovascular disease [11-13, 15]. Convertibly, cardiovascular risk factors may be involved in determining timing of ANM as well [66]. Moreover, it is hypothesized that ageing of the ovarian vasculature influences individual variation in ovarian ageing and consequently plays a role in the individual variation in timing of menopause, making cardiovascular risk factor genes plausible candidate genes for ANM [67, 68]. Three vascular-related candidate genes have been significantly associated with ANM. Carriers with at least one minor allele of the coagulation *factor V Leiden* (*FV*) experienced an earlier onset of menopause [69, 70], whereas carrying at least one minor allele of the *factor VII gene* (*FVII*) would delay menopause [71]. The third gene that was described to be associated with ANM is the *apolipoprotein E gene* (*APOE*) [70, 72, 73], which is involved in lipoprotein metabolism. However, in subsequent studies the association of *FV* and *APOE* with timing of ANM could not be confirmed [71]. No replication study in *FVII* was

Table II Candidate genes involved in steroid pathways in association with ANM

Candidate gene	Polymorphism	Study	N [a]	Ethnicity	Inheritance model	Effect ANM [b]	P-value
AMH	rs10407022	Kevenaar '07	2381	white	co-dominant	+ 0.1	0.66
		Kevenaar '07	248	white	co-dominant	+ 1.0	0.58
AMHR 2	rs2002555	Kevenaar '07	2381	white	co-dominant	- 2.6	**0.005** [c]
		Kevenaar '07	248	white	co-dominant	-2.8	0.054
COMT	Hsp II 92	Gorai '03	250	Japanese	dominant	0	0.735
	Val158Met	Hefler '05	1348	Caucasian	dominant	- 0.1	0.7
CYP1A1	Msp 1	Gorai '03	250	Japanese	co-dominant	+ 0.9	0.287
		Hefler '05	1346	Caucasian	dominant	+ 0.1	0.7
	Ile462Val	Hefler '05	1349	Caucasian	dominant	- 0.1	0.9
CYP1B1	rs1056836	Hefler '05	1360	Caucasian	dominant	+ 0.4	0.3
		Long '06	797	Chinese	dominant	- 1.0	**0.004**
		Mitchell '08	64	white	co-dominant	- 1.8	0.18
	rs1800440	Hefler '05	1360	Caucasian	dominant	- 0.8	**0.007**
	rs1056827	Long '06	797	Chinese	dominant	+ 1.2	0.04
		Mitchell '08	64	white	co-dominant	- 1.3	0.45
	rs10012	Long '06	797	Chinese	dominant	+ 0.7	0.02
	rs1056837	Long '06	797	Chinese	dominant	- 0.3	0.78
CYP17A1	MspA 1	Gorai '03	250	Japanese	co-dominant	- 0.6	0.496
		Kok '05	341	Caucasian	co-dominant	- 0.1	0.51
	A2 allele T/C	Hefler '05	1348	Caucasian	dominant	+ 0.4	0.07
	7 SNPs [d]	He '07	229	Caucasian	co-dominant	N.A.	> 0.05
	rs743572	Mitchell '08	64	white	co-dominant	- 0.5	0.79
CYP19A1	7r(-3)	Mitchell '08	64	white	co-dominant	+ 2.6	**0.04**
	rs10046		64	white	co-dominant	- 0.9	0.59
	rs1065778	He ' 07	229	Caucasian	co-dominant	not stated	**0.048**
	rs2255192		229	Caucasian	co-dominant	not stated	**0.035**
	rs700519	Hefler '05	152	Caucasian	dominant	+ 0.2	0.7
	C1558T		152	Caucasian	dominant	+ 0.4	0.2
	HSD17 v1V	Hefler '05	984	Caucasian	Dominant	- 0.2	0.8
ESR1	rs2234693	Weel '99	726	Caucasian	co-dominant	- 1.1	**0.03**
	(PvuII)	Gorai '03	315	Japanese	co-dominant	- 0.3	0.863
		Kok '05	311	Caucasian	Dominant	+ 0.5	0.63
		Dvornyk '06	248	Caucasian	co-dominant	- 0.6	0.590
	rs9340799 (XbaI)	Gorai '03	315	Japanese	co-dominant	+ 0.4	0.842
		Kok '05	309	Caucasian	Dominant	+ 0.2	0.96
ESR2	Block 1 [e]	He '07	229	Caucasian	co-dominant	not stated	**0.048**
	Block 2 [f]		229		co-dominant	not stated	**0.035**
FSHR	rs6166	Zerbetto '08	251	Italian	Dominant	1	0.132
HSD17B1	rs2830	Mitchell '08	64	white	co-dominant	+ 1.9	**0.03**
	rs615942		64	white	co-dominant	- 2.0	0.07
	rs592389		64	white	co-dominant	+ 1.8	0.09
SRD5A2	V89L, codon89	Huber '05	323	Caucasian	co-dominant	- 1.1	0.5

P-values < 0.05 are in bold

a Number of women with ANM in study population

b Effect on ANM in years (minor allele, or at least one mutant allele in case of binary variables)

c In nulliparous women

d All 7 SNPs (rs11191416, rs6163, rs3740397, rs10883783, rs4919685, rs4919682 and rs619824) had P-values > 0.05 (range 0.482-0.917)

e Block 1 with size 31 kb, consisting of 8 SNPs in high LD, spanned from promoter to intron 3

f Block 2 with size 36 kb, consisting of 9 SNPs in high LD, spanned from intron 4 to 3`UTR

Table III Vascular-related candidate genes in association with ANM

Candidate gene	Polymorphism	Study	N [a]	Ethnicity	Inheritance model	Effect on ANM [b]	P-value
AGT	Met235Thyr	Temfer '05	354	Caucasian	dominant	- 0.6	0.1
APOE	rs7412	Temfer '05	354	white	dominant	+ 1.5	**0.03**
		v Disseldorp '08	742	white	co-dominant	- 2.4	0.32
		He '09	253	Caucasian	co-dominant	not stated	0.255
	rs769450	He '09	253	Caucasian	dominant	-1.93	**0.007**
	rs429358	Temfer '05	354	white	dominant	0	0.9
		He '09	253	Caucasian	co-dominant	not stated	0.592
F II	G20210A	Temfer '05	354	Caucasian	dominant	+ 0.3	0.8
		v Disseldorp '08	741	white	co-dominant	- 8.03	**0.05**
F VII	Ins/del -323	v Disselorp '08	742	white	co-dominant	+ 0.8	**0.02**
	Arg353Gln				co-dominant	+ 0.5	0.15
F V Leiden	Arg506Gln	v Asselt '03	373	Caucasian	dominant	- 3.1	**0.035**
		v Disseldorp '08	743	white	co-dominant	- 1.96	0.64
	G1691A	Temfer '05	354	white	dominant	- 2.4	**0.03**
MTHFR	Haplotype B [c]	Liu '09	210	Caucasian	dominant	- 2.67	**0.04**
	Haplotype D [d]				dominant	- 2.65	**0.04**
	6 SNPs [e]				dominant	N.A.	>0.05
Nos3	T-786C	Worda '04	87	Caucasian	dominant	+ 0.8	0.49
		Temfer '05	354	Caucasian	dominant	+ 0.1	0.8
	Glu298Asp	Worda '04	87	Caucasian	dominant	0	0.98
		Temfer '05	354	Caucasian	dominant	0	0.9
	Intron 4	Hefler '02	91	Caucasian	dominant	4	0.56
PAI-1	4G/5G	Temfer '05	354	Caucasian	dominant	- 0.9	0.1

P-values < 0.05 are in bold
a Number of women with ANM in study population
b Effect on ANM in years (minor allele, or at least one mutant allele in case of binary variables)
c Haplotype B consisting of 3 SNPs: rs1476413, rs4846048 and rs4846049
d Haplotype D consisting of 4 SNPs: rs1801133, rs1476413, rs4846048 and rs4846049
e All 6 SNPs (rs2066470, rs17037390, rs1801133, rs1476413, rs4846048 and rs4846049) individually all had P-values >0.05 (range: not stated)

performed to date. Finally, in other examined vascular-related genes, like *nitric oxide synthase* (*Nos3*), *clotting factor II* (*FII*) and *angiotensinogen* (*AGT*) no association with ANM was found [70, 71, 74, 75].

Miscellaneous candidate genes

A few studied candidate genes cannot be classified as vascular or steroid-related (Table IV). An example is the *histidine decarboxylase gene* (*HDC*), which is the crucial enzyme for synthesis of histamine in humans. Histamine was found to directly stimulate the secretion of GnRH, which in turn plays an important role in female reproduction [76]. Of the 11 examined SNPs in HDC, one SNP was significantly associated with ANM in a population of 265 Caucasian women. Women carrying the T allele of SNP rs854163 were older at menopause compared to non-carriers [77]. No subsequent studies on *HDC* in relation to ANM were performed.

Furthermore, the *Interleukin-1 receptor antagonist gene* (*IL-1RA*) that encodes for the cytokine Interleukin-1 involved in autoimmune mechanisms was studied as a candidate gene in ANM because of the reported role of autoimmune mechanisms in POI [78]. In a population of 90 postmenopausal Caucasian women with a mean ANM of 50 years, no association with timing of menopause was found [79].

Table IV Miscellaneous candidate genes in association with ANM

Candidate gene	Polymorphism	Study	N [a]	Ethnicity	Inheritance model	Effect on ANM [b]	P-value
DAZL	DAZL260 A/G	Zerbetto '08	251	Italian	dominant	0	0.97
HDC	rs854163	Zhang '07	265	Caucasian	log-additive	+ 1.58	**0.015**
IL-1RA	86-bp repeat	Riener '04	90	Caucasian	co-dominant	+ 1.0	0.4
VDR	rs1544410	Dvornyk '05	260	Caucasian	co-dominant	+ 0.9	0.641
		Grimm '05	507	Caucasian	co-dominant	0	0.7
	3 SNPs [c]	Dvornyk '05	260	Caucasian	co-dominant	N.A.	> 0.05

P-values < 0.05 are in bold
a Number of women with ANM in study population
b Effect on ANM in years (minor allele, or at least one mutant allele in case of binary variables)
c All 3 SNPs (rs2238136, rs2228570 and rs731236) had P-values > 0.05 (range 0.590-0.936)

Genome-wide association studies

Up until September 2009, one rather small and two large GWAS on ANM have been published (Table V). The first GWAS was conducted in 2007 as part of a GWAS on longevity-related traits [80]. A total of 70.987 SNPs on a 100K Affymetrix GeneChip were examined with ANM in a community based sample 438 women from 330 families with natural menopause. A population-based approach (generalized estimating equation, GEE) was used to test association between the 100K SNPs and ANM while taking the correlation among related individuals into account [81]. Top ranked SNP associations in the GEE-model included rs6910534, located on chromosome 6 near the *FOrkhead Box O3a* (*FOXO3a*) gene (number 11 in the top ranked SNP; P=0.00003). *FOXO3* functions at the early stages of follicular growth as a suppressor of follicular activation [82, 83], making it a potential interesting candidate gene in ovarian ageing and thus timing of menopause [84].

Next, in 2009 two large GWAS were published simultaneously [85, 86]. He and colleagues performed a GWAS in two separate cohorts concerning age at menarche and ANM in a total of 17,438 women. In the joint analysis of the two studies, 13 SNPs reached genome-wide significance for ANM. Of these 13 associated SNPs, one SNP is located on chromosome 20p12.3, six SNPs on 19q13.42 , five SNPs on 5q35.2 and one SNP on 6p24.2 [85].

Stolk and colleagues included a total of 5,465 women, using a two-stage design. Stage 1: baseline GWAS in and stage 2: meta-analysis of 4 association studies on 32 top ranked SNPs from 24 loci found in the baseline GWAS. In the combined stage 1 and 2 analyses, six SNPs reached

genome-wide significance for ANM: four SNPs are located on chromosome 19q13.4, one SNP on chromosome 20p12.3 and one SNP on chromosome 13q34 [86].

There are no overlapping results between the two recent large GWAS and the smaller GWAS conducted in 2007 by Lunetta. In the two recent GWAS however, overlapping results were reported for chromosome 19q13.42 and 20p12.3, which are located in or near the *BR serine threonine kinase 1 gene* (*BRSK1*, also known as *SAD1*) and the *minichromosoma maintenance complex component 8 gene* (*MCM8*), respectively. *BRSK1* encodes for AMPK-related kinase (AMPK-RK). The highest expression of *BRSK1* is located in the human brain where it is required for neuronal polarization, but intermediate expression in adult ovaries was also detected. In experiments, mice homozygous for deletion of only one *BRSK1* gene were healthy and fertile, but double-knockout mice died soon after birth [87]. *MCM8* encodes for minichromosome maintenance protein, which is essential for genome replication [88]. A possible role for both *BRSK1* and *MCM8* in reproductive ageing and thus timing in menopause has not yet been investigated and needs further elucidation.

Table V Genome-wide significant SNPs in association with ANM

Chromosome	SNP	Study	Effect on ANM [a]	P-value	Nearby genes
19q13.4	rs1172822	He et al. '09	- 0.49	1.8E-19	BRSK1, THEM224, SUV420H2
		Stolk et al. '09	+ 0.391	6.28E-11	
	rs2384687	He et al. '09	- 0.47	2.4E-18	BRSK1, THEM224 ,SUV420H2
		Stolk et al. '09	- 0.381	1.39E-10	
	rs1551562	He et al. '09	- 0.43	2.6E-12	BRSK1,THEM224 ,SUV420H2
		Stolk et al. '09	- 0.428	1.04E-09	
	rs897798	He et al. '09	- 0.40	1.1E-14	BRSK1, THEM224, SUV420H2
		Stolk et al. '09	- 0.308	3.91E-08	
	rs7246479	He et al. '09	+ 0.36	2.3E-12	BRSK1
	rs12611091	He et al. '09	+ 0.33	6.6E-10	HSPBP1, BRSK1
20p12.3	rs16991615	He et al. '09	+ 1.07	1.2E-21	TRMT6, MCM8
	rs236114	Stolk et al. '09	+ 0.495	9.71E-11	MCM8
5q35.2	rs365132	He et al. '09	+ 0.39	8.4E-14	UIMC1
	rs7718874	He et al. '09	+ 0.39	1.3E-13	UIMC1
	rs402511	He et al. '09	+ 0.39	1.4E-13	UIMC1, ZNF346
	rs691141	He et al. '09	+ 0.36	3.9E-12	HK3, UIMC1
	rs2278493	He et al. '09	- 0.30	7.2E-08	UNC5A, HK3
2	rs10496265	Lunetta et al. '07	not stated	1.1E-08	
	rs10496262	Lunetta et al. '07	not stated	3.3E-07	
13q34	Rs7333181	Stolk et al. '09	+ 0.520	2.50E-08	ARHGEF7
6p24.2	rs2153157	He et al. '09	+ 0.29	5.1E-08	GCM2, SYCP2L

a Effect on ANM in years (minor allele)

DISCUSSION

The results of the different genetic studies on ANM clearly show the difficulties in finding genetic loci for this complex trait. First, we have been able to identify only few, if any, consistent findings among candidate gene association studies. Second, when comparing the different types of studies (i.e. candidate gene studies, linkage analysis and GWAS), there is almost no overlap in the genes that are associated with ANM. Several explanations can be proposed to underlay these inconsistent findings.

Inconsistency among candidate gene association studies

A striking inconsistency among candidate gene association studies on ANM becomes obvious when exploring the data of the studies conducted in the past decade. A number of polymorphisms in various candidate genes have been associated with ANM. However, often no subsequent study was conducted. If a subsequent study had been performed, the initial finding could never be replicated unanimously. Moreover, a few studies even reported conflicting results on the direction of the association between a certain variant with ANM [54, 56, 58-60]. Several possible factors may explain the inconsistent findings in candidate gene association studies. At first, a potential explanation can be found in the heterogeneity of the populations in the different studies. For example, three SNPs within the *CYP1B1* gene were found to be positively associated with higher ANM in a Chinese population [59]. This significant association could not be replicated in a population of Caucasian women [60], whereas this study reported a significant association in another SNP in this population. These different results can occur due to differences in allele frequencies and possible discrepancies in the effect of variants on the trait in separate ethnic populations. Also, when studies do not sufficiently account for ethnic differences within their study population, this can lead to the finding of spurious results, a phenomenon refered to as population stratification [89-92].

Another explanation for inconsistency could be the lack of power caused by limited sample sizes. The sample sizes in our reviewed candidate gene association studies varied widely from 64 to 2,381 women. Because it is expected that common genetic variants will only have a small effect on complex traits, small studies will lack the power to detect these common variants, and can therefore falsely claim that the variant is not associated with the disease or trait (false-negative results) [93-95].

A third explanation may stem from the fact that some studies did not adjust for multiple testing and therefore had a higher chance of finding false-positive results. This could explain why these associations could not be confirmed in subsequent studies. For this reason, replication of associated gene variations should be standard performed in these studies [95].

Finally, age at onset of natural menopause can be divided between women with POI (i.e. onset of menopause under forty years of age), early menopause (EM, i.e. woman with

ANM between forty and forty-five years of age) and women who experienced menopause later than forty-five years of age. Whether POI cases with no obvious cause (idiopathic POI) may be considered as a genetic entity or to be part of the variation towards EM or even the entire distribution of natural menopause remains unclear. However, observations that women with POI and EM are found within the same families, suggests a genetic relationship between POI and EM [96, 97]. So, genes involved in idiopathic POI might also be associated with EM or later menopause [7, 98]. Some reviewed candidate gene association studies excluded women suffering from POI and/or EM from their study population, whereas others made no distinction between the three categories of menopausal age. These differences in study populations might be a reason for finding false-positive or false-negative results and could also be a reason for failure of replication.

For the feasibility of replication in candidate gene association studies, the quality and transparency of the performed studies is essential. In this paper we did not limit the inclusion of studies based on their quality, as for one this would have left us with few studies, and second, not all studies provided enough information to judge their quality. While reviewing the papers concerning a possible association with ANM, we found that basic data regarding methods and results were not always mentioned. Examples of these missing data include ethnical homogeneity of the studied population, in- or exclusion of POI-patients, genotyping call rate, and the direction of the observed effect on ANM. By transparent and complete reporting of genetic studies, it becomes more feasible to compare results and enhance development of further studies [99].

Taking the previous possible reasons for non-association and non-replication into account, inconsistency of associations between separate studies does not necessarily mean that the reported results were false-positive or false-negative. Replication therefore remains the key step in candidate gene association studies.

Lack of overlap in the results of linkage analysis, candidate gene and genome-wide association studies

When comparing the results obtained by linkage studies, candidate gene association studies or GWAS, no overlapping genes or loci were observed. A number of factors can possibly explain this lack of overlap in the genetic regions identified as related to ANM.

Difference in study design between linkage analysis and GWAS

None of the suggestive linkage regions that were found in the two genome-wide linkage analyses, also emerged in the two GWAS on ANM. However, since the two designs are based on different assumptions (see introduction) discrepant findings may not come as a surprise and are even to be expected. As explained above, linkage analysis is most suitable for the detection of genes involved in monogenetic traits. Because of the low heritability of most complex traits, linkage

analyses are less suitable for these types of traits [42]. In addition, the underlying assumption of association studies is the common disease, common variant hypothesis [44, 45]. This means that these studies aim at finding a set of common variants that together are involved in the etiology of the trait. At the same time, rare variants with moderate to strong effect may be better detected by linkage analysis [100].

Lack of overlap between candidate gene and genome-wide association studies

Due to a number of reasons, the associated genes in the candidate gene association studies on ANM were not detected in the two conducted GWAS. First, GWAS often do not have enough power to detect genes with small effect sizes because of strict correction for multiple testing [101]. By controlling the false-positive rate in GWAS, the false-negative rate increases. Among the multiple SNPs that do not reach genome-wide significance there will probably still be many genes which are involved in ANM. Therefore, if no significant associations of genetic variants in certain candidate genes are detected, this does not imply that the association found in the candidate gene study was a false-positive.

Second, genomic coverage of GWAS genotyping arrays have been estimated to capture up to 80% of common SNP variation in the population [102]. Even high-density arrays do not cover all common SNPs and thus may not cover possible causal SNPs for ANM, which could lead to false-negative results [103].

In addition, reasons that might explain non-replication in candidate gene studies, such as population stratification and lack of power in genes with small-effect sizes, may also represent reasons for inconsistency between candidate gene association studies and GWAS [104].

Finally, another reason for the lack of overlap could be due to the fact that candidate gene association studies may have focused on the wrong pathways, genes or polymorphisms. Selecting candidate genes is notoriously inaccurate because of the often poor understanding of underlying mechanisms of complex traits such as ANM [95]. If a candidate gene is selected for a certain trait, susceptible variants possibly influencing the trait need to be identified first. Initial studies have focused on known polymorphisms of a candidate gene preferably with a hypothesized functional effect of the polymorphism [100]. However, more and more common variants in the genome have become known, although it was not yet clear whether these genetic variants had any functional implications. It therefore has become custom to also genotype variants for which this information was not yet available [95, 105]. The publication of the HapMap data in 2005 enabled researchers to describe the total variation in a gene by using a limited amount of tag SNPs (as described in the introduction) [43, 106].

Future prospects for genetic research on timing of menopause

Given the lack of consistency between findings within and between the various studies and

analytic approaches, essential methodological issues will have to be taken into account in future genetic research.

Until now, genetic studies in ANM have been small and therefore have lacked power to detect genes with small effect sizes. In recent years different study populations have been pooled by the formation of large consortia to increase power [107, 108]. Collaboration of study groups has resulted in large studies with dramatically increased power, as seen in the two recent GWAS by He and Stolk on ANM [85, 86]. However, attention must be paid to heterogeneity between the studies when pooling the data of multiple study populations [93].

Instead of focusing mainly on common variants, emphasis can be put on rare variants. Candidate gene studies as well as GWAS are based on the assumption that common variants underlay common diseases and traits [44, 45]. In addition to these common variants, a significant proportion of the inherited susceptibility to complex common diseases and traits may be due to the summation of the effects of a series of rare variants in different genes (minor allele frequency between 0.1 and 2-3%), known as the rare variant hypothesis [101, 107, 109, 110]. GWAS and candidate gene association studies are not designed for or capable of finding these rare variants [109]. In GWAS, indirect mapping is performed using tagSNps, which probably do not cover rare variants due to weak correlations between them. To associate rare variants with a disease or trait, it is necessary to perform direct mapping, and rare variants within a sample must first be identified by sequencing of candidate genes or entire genomes [111]. Detection of rare variants becomes feasible because of the availability of low-cost, high-throughput sequencing technology (Solexa). As a result, there is now an increasing interest in the rare variant hypothesis for all sorts of traits, including ANM.

Both for studies on rare variants and for future candidate gene association studies, selection of correct candidate genes is pivotal. In candidate genes studies that have been performed on ANM so far, it is possible that wrong candidates were chosen. Studies have mainly focused on genes involved in steroid pathways and vascular-function related genes, while convincing evidence of involvement of these pathways in ANM is lacking. More probable candidate genes in underlying ANM might be found in pathways involved in cell apoptosis and development of the follicle pool [112, 113]. Also, genes emerged from GWAS on ANM could give rise to new pathways in this trait, although the results of the two GWAS performed to date have not provided us with clear pathways from which possible candidate genes can be easily harvested.

The understanding of structural genetic variants like copy number variations (CNVs), insertion-deletion variants and inversion variants, has increased greatly in recent years [114, 115]. Although SNPs are the most prevalent genetic variants in the genome, it has been suggested that structural variants account for at least 20% of all genetic variants and are also likely to contribute to common diseases or complex traits [101, 116]. With growing knowledge of structural variants, and more importantly, increasing technological ability to detect structural variants in the genome, it will become feasible to conduct genome-wide association studies involving structural variants [101].

Next, it is likely that complex traits are influenced not through single genetic variants alone, but particularly through gene-gene (also referred to as epistasis) and gene-environment interaction [117]. This suggests the importance of studying joint effects of genes combined with environmental risk factors. Until now, association studies have mainly used single locus analysis, in which one SNP at a time is analyzed, without investigating epistasis and gene-environment interactions. This has resulted in the growing recognition that multi-locus analysis tools, in which gene-gene, gene-environment or even higher order interactions are modeled are needed to help elucidate the etiology of complex traits [118, 119].

Finally, next to genetic studies to identify structural genetic variants, epigenetics may help us to elucidate genetic factors underlying common diseases or traits. Epigenetics focuses on heritability that is not related to DNA sequence and that is involved in regulation of gene expression [120]. An example of epigenetic modification is DNA methylation, which has been studied particularly in cancer genetics [121]. In recent years, interest in epigenetic approaches in addition to genetic approaches for common diseases and traits has increased. Epigenetic variation might help to explain the quantitative nature of complex traits and diseases as well as the role of environment in their development [121].

CONCLUSION

Over the past decaes, huge efforts have been made to identify genetic factors underlying timing of natural menopause by conducting linkage analysis, candidate gene association studies and GWAS on the subject. The ultimate endpoint of this search is to unravel the biological pathway of ANM and allow for prediction of timing of menopausal age in sufficient advance.

So far, linkage analyses have detected a few regions of suggestive linkage with ANM. However, these regions need to be further examined to find the candidate genes underlying these linkage peaks. The candidate gene association studies conducted on ANM in the last decade found very little consistent associations, if any. The recent published results of the two GWAS conducted in ANM have detected potential new genetic loci that may help us in elucidating the physiology of ovarian senescence and thus timing of menopause. Despite all the efforts made in discovering genetics underlying ANM, the overall results have been rather disappointing thus far. In this paper we described possible new strategies that might help us to identify more genetic loci involved in variation in timing of natural menopause.

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Chapter

Three

Genes involved in initial follicle recruitment may be associated with age at natural menopause

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J Clin Endocrinol Metab. 2011, 96(3):E473-9

ABSTRACT

Context

Timing of menopause is largely influenced by genetic factors. Since menopause occurs when the follicle pool in the ovaries has become exhausted, genes involved in primordial follicle recruitment can be considered as candidate genes for timing of menopause.

Objective

To study the association of 23 tagging single nucleotide polymorphisms (tagSNPs) in five genes (*AMH*, *AMHR2*, *BMP15*, *FOXL2* and *GDF9*) involved in recruitment of the primary follicle pool, including the AMHR2 gene, which has recently been associated with age at menopause.

Design

Cross-sectional association study

Setting and Participants

Population-based sample of 3616 Dutch women with natural menopause

Main outcome measure

Age at natural menopause

Results

Both studied *AMHR2* tagSNPs (rs2002555 and rs11170547) in the *AMHR2* gene were associated with age at natural menopause in interaction with parity. Parous rs2002555 G/G carriers had 1 year later menopause compared with A/A carriers (P=0.01). For rs11170547 each minor allele (T) was associated with a 0.41 yr later onset of menopause in parous women (P=0.01). Additionally, rs6521896 in *BMP15* was associated with later menopause (β=0.41, P=0.007). Variants in the *AMH*, *FOXL2* and *GDF9* gene were not associated with timing of menopause.

Conclusions

The present study confirms an earlier finding that variation in the *AMHR2* gene modifies the relation between parity and age at natural menopause. In combination with the association of *BMP15* with menopausal age, we find that there is evidence that genes involved in primary follicle recruitment influence timing of menopause.

INTRODUCTION

The average age at menopause in women is 51 years, but varies widely from 40 to 60 years of age [1, 2]. Genetic factors have proven to play a major role in determining age at onset of menopause. In several twin, sib-pair and mother-daughter studies estimations of heritability ranged from 31-87% [3-5]. Because age at natural menopause is influenced by genetic factors as well as environmental and lifestyle factors, it is considered to be a complex trait.

Throughout the last decade, several genetic studies have been conducted in order to elucidate genetic factors involved in determining the variation in timing of menopause. However, only few genetic variants have been discovered so far, and they mostly account for only a small part of the heritability of age at natural menopause [6]. Moreover, replication studies have not supported a consistency in the relation between genetic variants and age at menopause.

Menopause occurs when the follicle pool in the ovaries has become exhausted and is insufficient to maintain menstrual cycles [7]. During fetal life, the primordial follicle pool is formed and contains a peak of 6 to 7 million oocytes at 20 weeks of gestation [8]. Hereafter, the primordial follicle pool decreases dramatically until approximately 300.000-400.000 remaining oocytes at birth [9,10]. Constant recruitment of primordial follicles into the growing follicle pool takes place throughout life, which is referred to as initial recruitment. During reproductive years, ongoing growth of follicles into antral stage and loss of follicles due to atresia leads to a gradual decrease of the original follicle pool, with menopause as the final result [11]. The exact mechanisms that regulate both the initial endowment of follicles in the developing ovaries and the rate of primordial follicle loss thereafter remain elusive. However, it is generally believed that factors belonging to the transforming growth factor-beta (TGFβ) superfamily are prime candidates for regulating initial follicle recruitment [12-14]. Therefore, genes encoding proteins belonging to the TGFβ superfamily and thus involved in primordial follicle recruitment, can be considered as candidate genes for timing of menopause.

Recently, an association between the rs2002555 polymorphism in the *anti-mullerian hormone type II receptor* (*AMHR2*) gene and age at menopause in interaction with parity was reported [15]. The *AMHR2* gene belongs to the family of type II receptors for TGFβ-related proteins and plays an important role in initial follicle recruitment [16]. Nulliparous women with the rs2002555 G/G genotype had a 2.6 years earlier onset of menopause compared to women with the A/A genotype (P=0.005), whereas in the parous women the G/G genotype tended to have a 1.5 years later age at menopause compared to women carrying the A/A genotype (P=0.072).

Two other genes that belong to the TGFβ superfamily, the *bone morphogenetic protein 15* (*BMP15*) and *growth differentiation factor-9* (*GDF9*) genes, have been studied in ovarian ageing and were found to be associated with primary ovarian insufficiency (POI) [17-20]. Women with POI

experience an onset of menopause before the age of 40 years and might be considered to be part of the variation towards natural menopause [21, 22]. Another gene that has been associated with POI is the *forkhead transcription factor L2* (*FOXL2*) gene [23]. *FOXL2* is required for granulose cell function, which plays an important role in follicle formation and activation [24, 25]. Although the exact molecular mechanism of *FOXL2* action is unclear, it has been speculated that *FOXL2* regulates TGFβ-related signaling pathways involved in ovarian function and could therefore play a role in primordial follicle loss. Moreover, germline inactivation of *FOXL2* affects follicle assembly and completely inhibits recruitment of primordial follicles [26].

In the present study we aim to replicate the finding in the *AMHR2* gene and age at natural menopause in a large cohort of postmenopausal women in the Netherlands and extended our study genetic study with four additional genes involved in initial recruitment of the primary follicle pool: *AMH*, *BMP15*, *GDF9* and *FOXL2*.

MATERIALS AND METHODS

Study population

The Prospect-Epic cohort is one of the two Dutch contributions to the European Prospective Investigation into Cancer and Nutrition (EPIC). The design and rationale of this study has been described previously [27]. In brief, this cohort consists of 17357 white women living in Utrecht and surroundings, aged 49 – 70 years, who were invited to participate in the study through the national breast cancer screening program between 1993 and 1997. All women filled out detailed questionnaires about dietary, reproductive, and medical history and underwent a physical examination at enrollment. In addition, women donated a 30-mL non-fasting blood sample which was fractioned into serum, citrated plasma, buffy coat and erythrocyte aliquots of 0.5 mL each. The samples were stored under liquid nitrogen at -196^{0}C for future research.

Natural menopause was defined according to the Word Health Organization as amenorrhea for at least 12 consecutive months without other obvious reasons. A total of 3497 women were premenopausal or perimenopausal at time of enrolment and therefore excluded. All women who experienced a surgical menopause (i.e. hysterctomy and/or uni- or bilateral ovariectomy) (N=4449), used hormones during the menopausal transition (N=2161) or women with an unknown menopausal status or age (N=1194) were excluded. Next, all women who were younger than 58 years at inclusion in the Prospect-Epic cohort were excluded to avoid bias due to differential inclusion of women with an early menopause N=2248). Finally, 192 women were excluded because of missing buffy coat samples or failed DNA extraction, leaving a total of 3616 women available for final analysis.

SNP selection

Tagging SNPs in the five genes were systematically selected using the general guidelines for SNP selection in candidate genes/regions described by Pettersson et al. [28]. In summary, for all five candidate genes, gene regions were identified using the public available online databases HapMap (http://hapmap.ncbi.nlm.nih.gov/) and UCSC browser (http://genome.ucsc.edu/). Next, the gene regions were expanded by 10kb both at the start and the end of the gene region to capture any upstream regulatory elements. Then, tagSNPs (with a minor allele frequency (MAF) >0.05) were selected for each candidate gene region using Haploview 4.1 (http://www.broadinstitute.org/haploview/haploview). In addition, we used the UCSC browser to identify any functional SNPs with a MAF >0.05 within the candidate gene regions. These functional SNPs were then "forced" in the list of tagSNPs. The following 23 SNPs were selected for genotyping in our study cohort: rs10407022, rs7249235, rs733846, rs886363, rs3746158 and rs4806834 in AMH, rs2002555 and rs11170547 in AMHR2, rs3810682, rs6521896, rs17249566, rs5961233 and rs3897937 in BMP15, rs7641989, rs13064974, rs11924939 and rs10804661 in FOXL2 and rs10491279, rs254286, rs803224, rs4705974, rs30177 and rs11748063 in GDF9.

Genotyping

Genomic DNA was extracted from buffy coat aliquots by KBioscience using their own in-house silica based systems for buffy extractions (http://www.kbioscience.co.uk/ purification/purification_intro.html). Genotyping of the 23 SNPs was performed by KBioscience (http://www.kbioscience.co.uk) using their in-house KASPar chemistry, which is a competitive allele specific PCR SNP genotyping system using FRET quencher cassette oligos (http://www.kbioscience.co.uk/genotyping/genotyping-chemistry.htm). As quality-control tests, blind duplicates, plate-identifying blank wells and Hardy-Weinberg equilibrium tests were used.

We removed 171 of our 3616 samples for overall call rates <95%. The overall genotyping success rate in the remaining 3445 samples was over 95% for all 23 SNPs (99.3% on average).

Data analysis

Characteristics of the study population were described using means and standard deviations for normally distributed continuous variables and frequencies and percentages for categorical variables. Genotypic deviations of Hardy-Weinberg equilibrium were assessed using a chi-square test with 1 degree of freedom. A p-value <0.001 was considered statistically significant. Single SNP associations were tested using linear regression analyses, using an additive genetic model to assess this association, which assumes that there is a linear gradient in risk of each additional risk allele. Association analyses between SNPs and menopausal age were adjusted for age at enrolment into the Prospect-EPIC study by including age in the multivariate linear regression analysis. BMP15 has proven to play a role in primary ovarian insufficiency (POI). To assess whether possible associations

are driven by the women that could have POI in our sample, we did a sensitivity analysis for the BMP15 SNPs significantly associated with age at menopause by excluding women with a menopausal age under 40 years (i.e. POI). In addition, effect modification by parity was tested by adding the interaction term between SNP and parity to the regression model containing also the two individual variables. When the *P*-value for the interaction term was ≤0.15 we presented the stratified association analyses of age at menopause for parity in the two subgroups of parous women and nulliparous women. All models were adjusted for smoking (current smoking yes/no), use of oral contraception (never/ever use of oral contraceptives) and body mass index (BMI, kg/m^2), to test for possible confounding. Statistical analyses were performed using SPSS for Windows (version 15.0, SPSS Inc., Chicago IL) and PLINK software (http://pngu.mgh.harvard.edu/~purcell/plink /download.shtml). A *P*-value ≤0.05 was considered statistically significant.

RESULTS

Characteristics of the women in our study cohort are summarized in Table 1. Mean age at menopause was 50.31 (± 4.22) years in our cohort. The majority of women delivered one or more children (84.6%) and the proportion of ever smokers was considerable (50.7%). The 23 analysed SNPs show no significant deviations from Hardy- Weinberg equilibrium (data not shown).

Table I Population characteristics of the study cohort (N=3445)

Age at inclusion (yrs, mean ± SD)	63.0 ± 3.41
Age at natural menopause (yrs, mean ± SD)	50.31 ± 4.22
Range (yrs)	18 – 64
Parity (mean ± SD)	2.65 ± 1.84
Nulliparous	532 (15.4%)
Parous	2913 (84.6%)
Breastfeeding	2415 (70.1%)
Ever used oral contraceptives (n)	1188 (34.5%)
Ever used HRT [a] (n)	227 (6.6%)
Smoking Never (n)	1748 (50.7%)
Current or past (n)	1697 (50.3%)
Body Mass Index (kg/m^2, mean ± SD)	26 ± 4
Systolic Blood Pressure (mmHg)	138 ± 21
Diastolic Blood Pressure (mmHg)	80 ± 10

a HRT, hormone replacement therapy

Analysis between SNPs in the AMHR2 gene and age at menopause

The two selected SNPs in the *AMHR2* gene both were statistically significantly associated with menopausal age. The G allele of rs2002555 was associated with an on average 0.30 years later age at menopause (*P*=0.02) and carriers of the minor allele (T) of rs11170547 had a 0.31 years delay of menopausal age per allele (*P*=0.05) (table 2). Furthermore, both SNPs in the *AMHR2* gene modified the association between parity (parous vs. nulliparous) and timing of menopause (*P*-values for interaction *P*=0.081 and *P*=0.103 respectively). For both SNPs, parous women carrying a minor

Table II HWE and single SNP association analysis with age at menopause (additive model)

Gene	Chromosome	SNP	Tested allele [a]	MAF	Single SNP association analyses	
					B	P[b]
AMH	19p13.3-p13.2	rs10407022	G	0,176	- 0.04	0,742
		rs7249235	A	0,083	- 0.15	0,423
		rs733846	G	0,155	- 0.08	0,547
		rs886363	A	0,22	- 0.10	0,401
		rs3746158	C	0,356	- 0.12	0,276
		rs4806834	T	0,03	- 0.35	0,243
AMHR2	12q13	**rs2002555**	**G**	0,193	0.30	**0,021**
		rs11170547	**T**	0,115	0,31	**0,049**
BMP15	Xp11.22	rs3810682	G	0,219	0,11	0,37
		rs6521896	**G**	0,131	0,41	**0,007**
		rs17249566	C	0,046	- 0.15	0,545
		rs5961233	A	0,107	0,12	0,454
		rs3897937	G	0,288	0,17	0,138
FOXL2	3q23.3	rs7641989	A	0,055	- 0.07	0,751
		rs13064974	T	0,077	- 0.16	0,399
		rs11924939	T	0,239	- 0.10	0,388
		rs10804661	G	0,085	- 0.17	0,355
GDF9	5q31.1	rs10491279	T	0,176	0,04	0,794
		rs254286	G	0,451	- 0.04	0,689
		rs803224	C	0,094	0,01	0,95
		rs4705974	T	0,17	- 0.15	0,263
		rs30177	C	0,287	- 0.03	0,766
		rs11748063	T	0,355	- 0.08	0,43

SNPs with *P* values <0.05 are in bold
a Tested allele is minor allele
b Linear regression model

allele tended to have a later onset of menopause (rs2002555; additive effect 0.38 years, *P*=0.005 and rs11170547; additive effect 0.41 years, *P*=0.01). These associations with age at menopause were not present in nulliparous women (figures 1 and 2). Adjustment for smoking, oral contraceptive use and BMI did not alter any of the results.

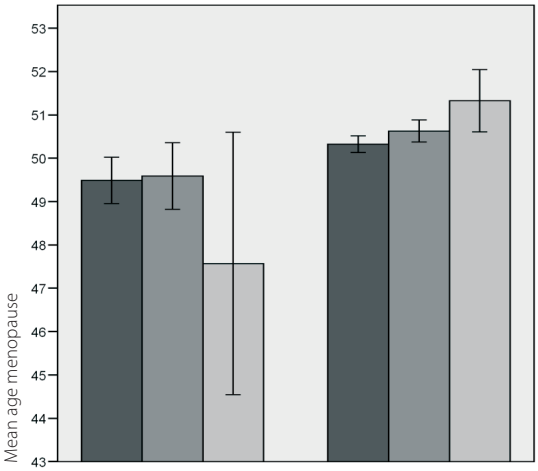
Analysis between SNPs in the BMP15 gene and age at menopause

Women carrying a minor allele (G) of the rs6521896 SNP in *BMP15* experienced menopause 0.41 years later per allele (*P*=0.007) (table 2). After exclusion of women with a menopausal age under 40 years (i.e. POI, N=58) from our study cohort, the association of *BMP15* with age at menopause attenuated (β=0.24, *P*=0.075). Stratified association analysis of menopausal age for parity in the studied SNPs in the *BMP15* gene did not reveal any statistically significant findings (data not shown).

Analysis between SNPs in the AMH, GDF9 and FOXL2 genes, and age at menopause

Single SNPs in the *AMH*, *FOXL2* and *GDF9* genes did not reveal any statistically significant associations with timing of menopause (table 2). Also, stratified analysis by parity did not disclose effect modification by parity of the association between SNPs in the *AMH*, *FOXL2* and *GDF9* genes and age at menopause (data not shown).

Figure 1 Interaction between AMHR2 rs2002555 genotypes and parity

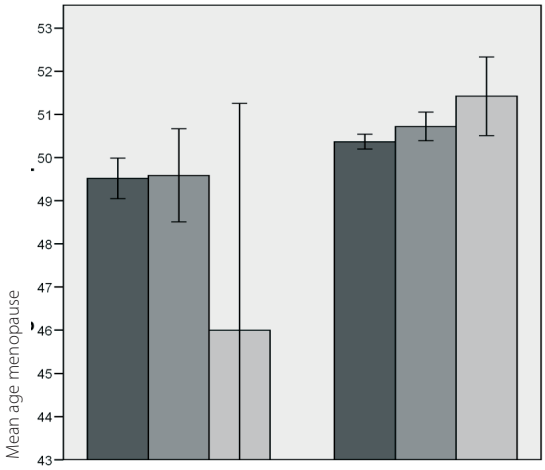


	Nulliparous			Parous		
	A/A	A/G	G/G	A/A	A/G	G/G
N	343	167	14	1879	905	108
ANM	49.49	49.59	47.57	50.32	50.63	51.32

β= -0.22, P=0.58 β= 0.38, P=0.005

ANM, age at menopause

Figure 2 Interaction between AMHR2 rs11170547 genotypes and parity



	Nulliparous			Parous		
	C/C	C/T	T/T	C/C	C/T	T/T
N	422	99	7	2281	562	55
ANM	49.52	49.59	46.00	50.37	50.72	51.42

β= -0.38, P=0.44 β= 0.41, P=0.01

ANM, age at menopause

DISCUSSION

Our study investigated the association between tagSNPs in five genes that are involved in primordial follicle pool recruitment and timing of menopause in a large cohort of Dutch postmenopausal women. In the present study we found interactions between both rs2002555 and rs11170547 in the *AMHR2* gene and parity in determining age at menopause. In addition we observed a statistically significant association between rs6521896 in the *BMP15* gene and age at menopause.

The association between SNPs in the *AMHR2* gene and timing of menopause in interaction with parity confirms an earlier finding that parous women carrying the G/G genotype had a later menopause than parous women carrying the A/A genotype [15]. In our study, parous women carrying the G/G genotype had a 1 year later menopause than parous women carrying the A/A genotype (*P*=0.01). In contrast, in the study by Kevenaar et al. the nulliparous women with the rs2002555 G/G genotype had a 2.6 years earlier menopause compared with nulliparous

women with the A/A genotype [15]. We also confirmed this; nulliparous women with rs2002555 G/G genotype had a 1.9 years earlier menopause compared to nulliparous women with A/A (*P*=0.16). The fact that this latter effect did not reach statistical significance is probably due to lack of power as we had fewer nulliparous women in each genotype category compared to Kevenaar. Together these results suggest that the SNP variation in the *AMHR2* gene modifies the relation between parity and age at menopause.

Next to the association in *AMHR2*, we also observed an association between a SNP in the *BMP15* gene (rs6521896) and menopausal age. Mutations in *BMP15* have been associated with the aetiology of POI in several studies [17, 18, 29], although the exact functional role of *BMP15* in this phenotype remains unclear. To find out to what extent the association of *BMP15* and age at menopause in our cohort was driven by POI cases, we excluded all women with reported menopausal age under 40 years (N=58). After exclusion of POI patients from analyses, the association between *BMP15* and age at menopause attenuated to borderline statistically significant (β=0.24, *P*=0.07*)*, suggesting that *BMP15* is primarily associated with early menopause and has a less important role in determining normal menopausal age variation.

No associations between common genetic variants in *AMH*, *GDF9*, *FOXL2* and timing of menopause were found in our study. Possibly, these genes are truly not involved in timing of natural menopause. It is also possible that the effect sizes of studied SNPs on age at menopause are small and our study lacked power to detect these associations. Finally, it is likely that complex traits, like timing of menopause, are not only influenced through single genetic variants, but that they are particularly influenced through gene-gene (referred to as epistasis) and gene-environment interaction [30]. So, we cannot rule out that *AMH*, *GDF9* and/or *FOXL2* have an effect on menopausal age in interaction with other genetic variants or environmental factors.

Our confirmation of a previous finding [15] that SNPs in the *AMHR2* gene modify the relation between parity and age at menopause, urges to find a biological explanation. Parity is related to timing of menopause, as has been demonstrated in many epidemiological studies [31-33]. Parous women are older when they reach menopause compared to nulliparous women. This effect is also present in our cohort of postmenopausal women, where nulliparous women entered menopause 0.97 years earlier compared with parous women (*P*<0.001). The exact mechanisms behind the relationship between parity and timing of menopause are still unidentified at present. One explanation for this relationship is that prolonged elevation of circulating progesterone during pregnancy may suppress initial follicle recruitment which could result in a delayed onset of menopause [11, 31]. However, it has also been suggested that age at menopause and parity are both a reflection of the pace in the process of ovarian aging, thus lacking a strict causal relationship. In other words, women who reach menopause early generally will have fewer children than women with a normal age at menopause due to underlying reduced fertility [34]. Thereby, the chance of remaining nulliparous will be increased in women who are destined to reach menopause at a relatively early age.

It is unclear, however, how the apparent relationship between parity and age at menopause is influenced by the *AMHR2* gene. It could be hypothesized that changes in hormone levels during pregnancy alter the expression or function of this specific receptor subtype. These altered hormone levels during pregnancy may have a stronger effect on the expression and/or activity of the G-allele than on the A-allele of rs2002555 SNP in the *AMHR2* gene. This would then result in a stronger inhibition of primordial follicle recruitment during pregnancy resulting in a later menopause.

Another explanation for the interaction between parity and *AMHR2* in association with age at menopause could be that *AMHR2* is in fact a fertility-related gene. Recently, an association was found between genetic variants of AMHR2 and unexplained infertility. Common variant allele frequencies of the *AMHR2* gene polymorphisms (amongst which the rs2002555 SNP in the *AMHR2* gene) were statistically lower in infertile patients compared with controls [35]. This is in accordance with the findings in our population, in which women with the G/G genotype were less frequently nulliparous (11.5%) compared to women with the A/G (15.6%) and A/A genotype (15.4%). This suggests that women with the G/G genotype are possibly more fertile and have later menopause compared to women with the other two genotypes.

When interpreting our results, some strengths and limitations should be taken into account. To our knowledge, we conducted the first comprehensive candidate gene association study of genetic variants in five genes involved in the pathway of primordial follicle recruitment. In addition, data from a large cohort of natural menopausal women were available. A limitation that should be considered is that as in most genetic studies conducted in timing of menopause, age at menopause was self-reported and determined retrospectively in the Prospect-EPIC cohort, which can be susceptible to bias [36, 37].

It is nevertheless unlikely that misclassification due to recall bias differs across genotypes. Secondly, we analysed a total of 23 SNPs and therefore had a higher chance of finding false-positive results. However, as this study was hypothesis driven and aimed at confirming earlier reported associations, strict adjustment for multiple testing is not necessary. Moreover, as the 23 tests are not uncorrelated tests strict correction for multiple testing would be over-conservative and it may result in a decrease in power to detect real effects.

In our comprehensive genetic association study of five genes involved in recruitment of the primary follicle pool, we observed an association between variants in the *AMHR2* gene and the *BMP15* gene and timing of natural menopause. These findings suggest a role for this genetic pathway in the process of ovarian ageing in humans and may thereby provide more insight in the biological mechanisms underlying ovarian ageing. Furthermore, we found that the *AMHR2* gene is associated with age at menopause in interaction with parity, which confirms an earlier finding by Kevenaar et al. [15]. This finding may provide more insight into the mechanisms that drive the relationship between menopausal age and parity.

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Chapter

Four

Interactions between genetic variants in AMH and AMHR2 may modify age at natural menopause

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PLoS One . 2013;8(3):e59819

ABSTRACT

The onset of menopause has important implications on women's fertility and health. We previously identified genetic variants in genes involved in initial follicle recruitment as potential modifiers of age at natural menopause. The objective of this study was to extend our previous study, by searching for pairwise interactions between tagging single nucleotide polymorphisms (tSNPs) in the 5 genes previously selected (AMH, AMHR2, BMP15, FOXL2, GDF9).

We performed a cross-sectional study among 3445 women with a natural menopause participating in the Prospect-EPIC study, a population-based prospective cohort study, initiated between 1993 and 1997.

Based on the model-based multifactor dimensionality reduction (MB-MDR) test with a permutation-based maxT correction for multiple testing, we found a statistically significant interaction between rs10407022 in *AMH* and rs11170547 in *AMHR2* (p=0.019) associated with age at natural menopause. Rs10407022 did not have a statistically significant main effect. However, rs10407022 is an eQTL SNP that has been shown to influence mRNA expression levels in lymphoblastoid cell lines.

This study provides additional insights into the genetic background of age at natural menopause and suggests a role of the AMH signaling pathway in the onset of natural menopause. However, these results remain suggestive and replication by independent studies is necessary.

INTRODUCTION

The timing of the end of a women's reproductive life has important health implications. An early onset of menopause is associated with a higher risk of cardiovascular diseases, osteoporosis, and overall mortality, whereas a later menopausal age may increase the risk of breast, ovarian, and endometrial cancer [1-4]. The underlying biological mechanisms for these associations remain poorly understood and much effort has been devoted to explain the observed variation in age at natural menopause (ANM) in an attempt to comprehend the etiology of these complex traits. The age at which menopause occurs varies between 40 to 60 years, with an average of 50-51 years in women of Northern European descent [5, 6]. Numerous studies focused on lifestyle and reproductive factors in association with ANM. Some evidence for an association with ANM has been observed for smoking, parity, and body mass index (BMI), but results have been mainly inconsistent [7]. Furthermore, the individual variation in ANM is thought to be under genetic control. The heritability estimates range from 31 to 78% [1, 5, 8]. So far, genome-wide association studies (GWAS) have identified seventeen menopause loci that function in diverse pathways including hormonal regulation, immune function and DNA repair [9-11]. Despite the large efforts made in unraveling the genetic background of ANM, only a small part can be explained through genetic factors identified so far. Most studies investigated the effect of only one SNP at a time, while it is obvious from biological studies that biological processes are influenced by multiple genes in complex networks [12]. Investigating gene-gene interactions might be a first step towards complex interaction analysis.

In a previous study, we investigated genetic variants in genes involved in initial follicle recruitment in association with ANM among 3445 Dutch women participating in Prospect-EPIC [13]. In that study, we observed an association between ANM and two single nucleotide polymorphisms (SNPs) in *AMHR2* (rs2002555 ($\beta = 0.30$, $p=0.021$) and rs11170547 ($\beta = 0.31$, $p=0.049$)), and one SNP in *BMP15* (rs6521896 ($\beta = 0.41$, $p=0.007$)) [13]. Moreover, we found that the two SNPs in the *AMHR2* gene were associated with age at natural menopause in interaction with parity (i.e., rs2002555: $\beta=0.38$, $p=0.005$ for parous women; $\beta=-0.22$, $p=0.58$ for nulliparous women and rs11170547: $\beta=0.41$, $p=0.010$ for parous women; $\beta=-0.38$, $p=0.44$ for nulliparous women). In the present study, we aim to extend the previous study by exploring gene-gene interactions among genes involved in initial follicle recruitment in association with ANM. In addition, we aim to further explore gene-environment interactions between these genes and parity, smoking and BMI.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Institutional Review Board of the University Medical Center Utrecht. All women signed informed consent.

Study Design

Prospect-EPIC is one of the two Dutch prospective cohort studies participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) which is a multi-center cohort study including 10 European countries [14]. Between 1993 and 1997, a total of 17,357 women aged 50-69 years, living in Utrecht and vicinity were recruited through the national breast cancer screening program. At recruitment, all women completed a questionnaire with detailed questions on reproductive factors, physical activity, smoking, education level, and other life-style related factors. Moreover, all women underwent physical examination and donated a 30-ml nonfasting blood sample. A full description of the study design and cohort has been published elsewhere [15].

Study Population

We describe an extension of a previous study by Voorhuis *et al.* for which participant selection has been described in detail [13]. Briefly, women were excluded if they were pre- or perimenopausal at time of enrollment (n=3,497), if they experienced a surgical menopause (n=4,449), if they used hormone therapy (n=2,161), if they were younger than 58 years at inclusion (n =2248), when menopausal status or age was unknown (n=1,194), or when buffy coat samples were missing or DNA extraction failed (n=192). Eventually, a total of 3,616 postmenopausal women with a known ANM were eligible for the current study.

Outcome Measure

ANM was extracted from the baseline questionnaire. Natural menopause was defined according to the World Health Organization as amenorrhea for at least 12 consecutive months without other obvious reasons [16].

Laboratory Methods

Methods of blood collection, DNA extraction and genotyping have been described in detail [13]. Duplicate samples were included to assess the quality of the genotyping process. Women with a call rate smaller than 95% were excluded (n=171). The average genotyping success rate in the remaining 3445 samples was 99.3%.

Gene and SNP Selection

The selection procedure for genes and SNPs has been described in detail previously [13]. A total of 23 tagging SNPs were selected among 5 genes involved in initial follicle recruitment: rs10407022, rs7249235, rs733846, rs886363, rs3746158, and rs4806834 in *AMH*; rs2002555 and rs11170547 in *AMHR2*; rs3810682, rs6521896, rs17249566, rs5961233, and rs3897937 in *BMP15*; rs7641989, rs13064974, rs11924939, and rs10804661 in *FOXL2*; and rs10491279, rs254286, rs803224, rs4705974, rs30177, and rs11748063 in *GDF9*. We excluded two SNPs with a minor allele frequency smaller than 0.05 (i.e., rs4806834 and rs17249566), leaving a total of 21 SNPs. For the investigation of gene-gene interactions it should be avoided to include SNPs that are in linkage disequilibrium (LD) with each other in order to prevent spurious interactions. Therefore, we used PLINK to generate a pruned subset of SNPs considering a window of 21 SNPs, a shift of 1 SNP forward, and an r^2 of 0.75. The r^2 of 0.75 was advised by the developers of MB-MDR (personal communication). None of the SNPs were removed based on these parameters.

Data analysis

Deviation from HWE was tested in PLINK v1.07 using a X^2 test with 1 degree of freedom. We corrected for age at inclusion by using rank-transformed age-adjusted residuals for ANM (GenABEL v1.6-7). Missing genotypes were imputed using BEAGLE v3.3.2.

All possible pairwise interactions (n=210) were investigated using model-based multifactor dimensionality reduction (MB-MDR) v2.7.5 [17, 18]. MB-MDR is an extension of the multifactor dimensionality reduction (MDR) method, a nonparametric exhaustive data mining method that considers all possible interactions between SNPs and classifies individuals into high and low risk groups [19]. MB-MDR, in contrast to MDR, is capable of analyzing quantitative traits and is able to adjust for main effects. Moreover, it introduces an additional 'no evidence' group. A full description of MB-MDR is available in references 17 and 18. As suggested by the authors, we adjusted for main effects by adjusting for the lower-order effects of the SNPs in the SNP-pair under investigation using a co-dominant coding scheme. This method provides the best balance between type I error and power [17]. Multiple testing was accounted for by adopting a permutation-based maxT correction with 999 replicates.

Gene-environment interactions were evaluated using MB-MDR by including the environmental factor of interest as a categorical variable in the MB-MDR analysis. We investigated interactions between all SNPs and parity (parous (yes/no)), smoking (never, current, former), and BMI (<20, 20-25, ≥25).

RESULTS

Characteristics of the 3445 women in our study cohort have been described previously [13]. Briefly, the mean age at inclusion and at natural menopause were 63 (SD: 3.4) and 50 years (SD: 4.2), respectively. The majority of women delivered one or more children (84.6%). Only 34.5% used oral contraceptives. Half of women were ever smokers (50.7%).

No significant deviations from Hardy-Weinberg equilibrium were observed. Results from the single SNP analysis have been published previously [13].

Interaction results for the top 10 SNP-SNP interaction models are presented in Table 1. The interaction between rs11170547 in *AMHR2* and rs10407022 in *AMH* was statistically significant after correction for multiple testing (p=0.019).

We also tested the interaction between each SNP and parity (parous (yes/no)), smoking (never, current, former), and BMI (<20, 20-25, ≥25). After correction for multiple testing no significant gene-environment interaction was observed. Our MB-MDR analysis did thus not replicate the previously observed interaction between *AMHR2* and parity based on linear regression.

Table I Overview of the Top 10 Pairwise Interactions between tSNPs in Genes Involved in Initial Follicle Recruitment

tSNP 1	Corresponding Gene	tSNP 2	Corresponding Gene	F-test	P-value [a]
rs11170547	AMHR2	rs10407022	AMH	18.162	0,019
rs11170547	AMHR2	rs733846	AMH	13.733	0,199
rs2002555	AMHR2	rs10407022	AMH	10.753	0,622
rs11170547	AMHR2	rs7249235	AMH	10.217	0,727
rs13064974	FOXL2	rs10491279	GDF9	9.470	0,84
rs2002555	AMHR2	rs733846	AMH	9.302	0,86
rs7249235	AMH	rs886363	AMH	8.333	0,958
rs7641989	FOXL2	rs2002555	AMHR2	7.265	0,996
rs10491279	GDF9	rs3746158	AMH	7.049	0,998
rs10491279	GDF9	rs11170547	AMHR2	7.024	0,998

Abbreviations: tSNP, tagging Single Nucleotide Polymorphism
a P-values are reported after adjustment for multiple testing, based on a permutation-based maxT correction with 999 replicates.

DISCUSSION

In this large cross-sectional study we investigated interactions between 21 SNPs in genes involved in initial follicle recruitment in association with ANM. We observed a statistically significant pairwise interaction between rs10407022 in *AMH* and rs11170547 in *AMHR2* after permutation-based maxT correction. No gene-environment interactions were observed between these SNPs and parity, smoking or BMI.

The present study is the first study investigating interactions between these 5 genes involved in initial follicle recruitment in relation to ANM. We previously observed statistically significant associations between the two SNPs that tag the gene encoding the AMH receptor (*AMHR2*; rs2002555 and rs11170547) and ANM [13]. No associations for tSNPs in *AMH* with ANM were found. In the present study we extended this study and searched for pairwise interactions between the SNP in the genes previously selected. We found an interaction between SNPs in the *AMH* gene and its receptor gene *AMHR2*. This might imply that complex interactions between these genes play a role in ovarian aging and thus in onset of menopause. However, this is the first report of an interaction between *AMH* and *AMHR2*, therefore, replication by independent studies is necessary to confirm these findings.

One of the SNPs involved in this interaction, rs10407022 in the *AMH* gene, is an expression quantitative trait locus (eQTL) for *AMH*, which means it influences expression levels of mRNAs [20, 21]. Moreover, this missense mutation is predicted by SIFT to have damaging protein function [22]. We have shown that this SNP by itself does not influence ANM, but that it may modify ANM in interaction with rs11170547 in *AMHR2*. Interestingly, this SNP is the only known eQTL SNP in these genes [20].

Anti-Müllerian hormone (AMH), produced solely by small, growing follicles in the ovary, is a validated biomarker of ovarian aging, as serum levels of this hormone are strongly correlated with the number of growing follicles [23, 24]. AMH levels have also been shown to be a strong predictor of time to menopause [23]. Genetic association studies might provide additional understanding of the biological processes underlying the correlation between AMH and onset of menopause. Motivated by the considerations outlined above, a more thorough investigation of the AMH signaling pathway in onset of menopause seems worthwhile. It may help us to better understand the biological processes that influence variation in ANM, a trait with many health implications.

In this study, we observed no gene-environment interactions between genes involved in initial follicle recruitment and parity, smoking and BMI. This is in contrast with our previous study, in which we found an interaction between parity and tSNPs in *AMHR2* using linear regression models [13]. This interaction was a replication of a finding by Kevenaar *et al.* [25]. The lack of replication in the present study might be attributed to either the different parameterization in linear regression

(additive models) compared to MB-MDR (non-parametric) or to the very stringent correction for multiple testing used with MB-MDR. On the other hand, the previously observed interactions between parity and SNPs in *AMHR2* might be false positive findings. In fact, a clear biological mechanism for these interactions has not been found.

This large study with detailed information on exposures and outcome inevitably has some limitations. We were not able to replicate our findings in an independent population. However, by using a very strict correction for multiple testing, we tried to reduce to chance of false positive findings. Moreover, the statistical power of our study might have been too low to detect real interactions.

In conclusion, we observed a pairwise interaction between 2 SNPs in *AMH* and *AMHR2* in association with ANM. More studies are needed to provide additional evidence for a role of the AMH signaling pathway in the onset of natural menopause.

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Chapter

Five

Are coronary artery disease and type 2 diabetes causally related to age at menopause? A Mendelian randomization study

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Manuscript in preparation

ABSTRACT

Background

Women with early menopause are at an increased risk of cardiovascular disease (CVD) later in life. Besides an association with CVD, early menopause is also associated with an increased risk of type 2 diabetes (T2D), which is one of the major risk factors of CVD.

Methods

To investigate whether coronary artery disease (CAD) and T2D are causally related to age at menopause, we have tested the association of previously reported GWAS hits in CAD and T2D for an association with age at natural menopause in nearly 50,000 women, exploiting the principle of Mendelian randomization. A total of 18 GWAS hits for CAD and 28 for T2D were selected and analyzed to study a possible association with age at natural menopause.

Results

We found no statistically significant associations for any of the SNPs for CAD with age at menopause. For the SNPs associated with T2D, two suggestive associations with menopausal age were identified located near the HNF1A gene and near the NOTCH2 gene.

Conclusion

A previous finding that women with an increased risk of CAD experience an earlier onset of menopause could not be supported by our data. The results in the T2D SNPs imply a role for this metabolic disease in timing of menopause.

INTRODUCTION

Women with early menopause are at an increased risk of cardiovascular disease (CVD) later in life [1-5]. Besides an association with CVD, early menopause is also associated with an increased risk of type 2 diabetes (T2D), which is one of the major risk factors of CVD. In a recently published very large European cohort (EPIC-InterAct) the hazard ratio (HR) for T2D per 1 standard deviation (SD) younger menopausal age was 1.08 (95% CI: 1.02-1.14) [6]. Whether these associations are causal has not been clearly substantiated. The increased T2D and CVD risk in women with early menopause has generally been ascribed to a deprivation of estrogens leading to unfavourable cardiovascular risk profile. Study results in this respect have been quite conflicting [7,8].

It is well known that smoking affects menopausal age; menopause occurs 0.8 to 2 years earlier in women who smoke [9-11]. Smoking has been identified as one of the most important risk factors for coronary artery disease (CAD) [12] and occlusive thrombotic disease [13]. Smoking could thus promote atherosclerosis of the ovarian arteries and subsequently lead to ischemia [11,14]. Following this line of reasoning, other diabetes and cardiovascular risk factors may also accelerate menopause through promotion of ovarian artery atherosclerosis. In support of this theory, we have previously suggested that development of an unfavourable cardiovascular risk profile in premenopausal women (higher total cholesterol levels, increase in blood pressure, and increase in relative weight) indeed accelerates the onset of menopause [15]. Furthermore, a reduced ovarian reserve was recently found in premenopausal women with T2D, based on a significant difference in mean follicle-stimulating hormone (FSH) levels and antral follicle count [16]. However, in a large European study we recently found that menopausal onset was delayed in women developing diabetes after 50 years of age [17].

Genotypes are randomly assigned at meiosis, independent of non-genetic confounders, and are unmodified by disease processes. The Mendelian randomization concept uses these characteristics to test the hypothesis that the association of a risk factor with a disease is causal. Age at natural menopause is a complex trait and believed to be highly heritable, with heritability estimates ranging from 31% to 78% [18-22]. To date, genome-wide association studies have identified 17 loci that are associated with timing of menopause [23-25]. Together these genetic variants account for 2.5-4.1% of the variation in natural age at menopause and they do not provide clear leads to the biological mechanisms underlying the ovarian ageing process menopause [25].

Previously, in candidate gene association studies variants in vascular related genes, such as *APOE*, *F7*, *F5* Leiden and *MTHFR*, were associated with menopausal age, albeit not consistently [26-30]. Recently, a large candidate gene association study in 12,723 naturally postmenopausal women, found no statistically significant associations between 2072 SNPs in 32 genes in the thrombophilia and vascular homeostasis pathway, including *APOE* and *F5*, and timing of natural menopause [31].

Candidate-gene association studies have been rather disappointing in general, which is mainly due to the fact that often results cannot be replicated. Among other reasons, a reason for non-replication may be that candidate-gene studies may have been focussing on the wrong pathways, genes or variants. Genome-wide association studies now have revealed genetic variants that are confirmed to be associated with the risk of CAD and T2D. To investigate whether CAD and T2D are causally related to age at menopause, exploiting the principle of Mendelian randomization, in the current study we test the association of these GWAS hits for an association with age at natural menopause. Here, we present the results of a large meta-analysis in nearly 50,000 women.

METHODS

Study design

To answer our research question we used data from eight studies that genotyped the ITMAT/Broad/CARe (IBC) array (the IBC consortium; Institute of Translational Medicine and Therapeutics (ITMAT)/Broad/Candidate Gene Association Resource (CARe)). The IBC array is a gene-centric genotyping array developed for replication and fine mapping, and incorporates about 50,000 SNPs to efficiently capture genetic variation across more than 2000 genetic regions, related to cardiovascular, inflammatory, and metabolic regions [32].

All GWAS hits for CAD and for T2D available on the IBC array (or proxy with an r^2>0.8) were selected and analyzed for studying a possible association with age at natural menopause. To increase power, we subsequently meta-analyzed the associations with results from ReproGen and EPIC-InterAct. All three consortia are described below.

Study populations

ITMAT/ Broad/CARe (IBC) consortium

Eight cohorts with 9960 naturally postmenopausal women were genotyped using the IBC array. Three studies (Framingham Heart Study (FHS), Cardiovascular Health Study (CHS) and Atherosclerosis Risk in Communities (ARIC)) were later excluded as they also provided data to the ReproGen consortium. Table 1 shows all studies that contributed data to the IBC consortium..

All women with a known age at natural menopause between 40 & 60 years were included. Menopause was defined as the age at the last menstrual period, after at least 12 consecutive months of amenorrhea (as derived from questionnaire data). Women with a hysterectomy and/or a bilateral ovariectomy were excluded, as were women in whom menopause was induced by irradiation or chemotherapy. Furthermore, women were not allowed to have used HRT prior to their menopause. Only Caucasian women were included.

ReproGen

ReproGen is a large consortium that conducted a GWAS study on age at natural menopause in 38,968 women from 22 studies. Three studies overlapped with the IBC consortium; ARIC, CHS and FHS. As in the lookup it was not possible to exclude these studies from ReproGen, we decided to exclude them from the analyses of the IBC consortium. All studies contributing to the ReproGen consortium are shown in table 1.

Table I Studies contributing to the CARe, ReproGen and EPIC-InterAct cohorts

Cohort	Subcohort	N
CARe	Women's Health Initiative (WHI)	3434
	The Multi-Ethnic Study of Atherosclerosis (MESA)	844
	European Prospective Investigation into Cancer and Nutrition (EPIC NL)	1691
	Cooperative Health Research in the Region of Augsburg (KORA)	485
	CARe Whhitehall II	699
ReproGen	AGES-Reykjavik Study	1315
	ARIC	2576
	Cardiovascular Health Study (CHS)	958
	deCODE	5857
	EGCUT	279
	Erasmus Rucphen Family study (ERF)	373
	Framingham Heart Study (FHS)	1452
	Heredity and Phenotype Intervenstion (HAPI) Heart study	240
	InChianti	460
	Nurses' Health Study (NHS)-cgems	1344
	Nurses' Health Study (NHS)-Hu	1772
	Netherlands Twin Register	331
	QIMR	430
	Rotterdam Study (RS) I, II and III	3458
	SardiNIA	828
	Study of Health in Pomerania (SHIP)	4310
	TwinsUK I, II and III	1606
	Women's Genome Health Study (WGHS)	11379
EPIC-InterAct	EPIC-InterAct (Illumina 660W-Quad)	1872
	EPIC Interact (Metabochip)	1843
Total		49836

In ReproGen age at menopause was defined as age at last menstrual period that occurred naturally with at least 12 consecutive months of amenorrhea. Women with natural menopause aged 40–60 were included. Women of self-reported non-European ancestry were excluded, as were women with menopause owing to hysterectomy and/or bilateral ovariectomy, chemotherapy or irradiation, if validated by medical records, and women using HRT before menopause.

EPIC-InterAct

InterAct is a case-cohort study of 12,403 incident type 2 diabetes cases and a random subcohort of 16,835 individuals, nested within the European Prospective Investigation into Cancer and Nutrition Study (EPIC). The participants, methods, study design, and measurements have been described previously [33].

Ascertainment of incident type 2 diabetes involved a review of the existing EPIC datasets at each center using multiple sources of evidence including self-report, linkage to primary-care registers, secondary-care registers, medication use (drug registers), hospital admissions and mortality data. Cases in Denmark and Sweden were not ascertained by self-report, but identified via local and national diabetes and pharmaceutical registers and hence all ascertained cases were considered to be verified. Follow-up was censored at the date of diagnosis, 31 December 2007 or the date of death, whichever occurred first. In total, 12,403 verified incident cases were identified. A subcohort of 16,154 individuals was randomly selected from those with available stored blood and buffy coat, stratified by center.

DNA was not available for Danish (N=4,037) participants, leaving a total maximum sample size of 10,348 incident cases and 14,671 random subcohort participants, including 13,394 non-diabetic InterAct subcohort participants. Of these, a total of 19,651 participants, including 8,582 cases and 11,069 non-diabetic subcohort participants had DNA available for genotyping. DNA was extracted from up to 1 ml buffy coat for each individual from a citrated blood sample. Standard procedures on an automated Autopure LS DNA extraction system (Qiagen, Hilden, Germany) with PUREGENE chemistry (Qiagen) were used and the DNA was hydrated overnight prior to further processing. DNA samples were quantified by Picogreen and normalised to 50 ng/µl. A total of 10,027 participants (4,644 cases) were selected across all except the Danish centres for genome-wide genotyping using the Illumina 660W-Quad Bead Chip (Illumina, San Diego, CA, USA) at the Wellcome Trust Sanger Institute. Of these, a total of 9,431 samples passed QC criteria following genome-wide genotyping (call rate >95%, no conflict between gender and X-heterozygosity, concordant candidate genotyping, not an outlier for autosomal heterozygosity or ethnicity), with 99.9% and 99.5% of samples at call rates of 97% and 99%, respectively. In addition, 9,794 InterAct participants with available DNA and not selected for genome-wide measurement were genotyped using an extended proprietary Illumina metabochip [34]. Genotyping was completed in 9,467 InterAct samples with 99.8% and 98.2% of samples at call rates of 97% and 99%, respectively.

Single nucleotide polymorphism (SNP) selection

The electronic database Huge Navigator version 2.0, GWAS Integrator [35]; http://hugenavigator.net/HuGENavigator/home.do) was searched for GWAS-hits in the traits Coronary Artery Disease and Type 2 Diabetes (until August 2012). Next, GWAS-hits for both traits were checked for presence of the SNPs (or proxy SNP) on the IBC chip (Illumina CARe iSelect) using the Broad Institute SNP Annotation and Proxy Search (SNAP; http://www.broadinstitute.org/mpg/ snap/index.php) [36] with the following search options: population panel: CEU, r2 threshold: 0.8 and distance limit: 500. Of the SNPs present on the IBC chip, we excluded SNPs that were not replicated in a separate study population after initial discovery GWAS. SNPs associated with CAD or T2D in a population other than European, white or Caucasian were also excluded.

For CAD (traits Coronary Heart Disease and Coronary Artery Disease) 68 GWAS-hits were retrieved from the Huge Navigator database of which a total of 18 SNPs in 20 different genes were included in our study (figure 1). For 45 SNPs that were not available on the IBC chip no suitable proxy could be found. Four SNPs were found only in other ethnicities and 1 SNP was never successfully replicated.

For T2D (trait Type 2 Diabetes) 110 GWAS-hits were retrieved from the Huge Navigator database of which 24 SNPs in were included in our study (figure 1). 56 SNPs were found only in non-Caucasian ethnicities. 33 SNPs were not available on the IBC chip and no suitable proxy could be found. An extra 7 SNPs associated with T2D in 4 different GWAS were retrieved from an additional search in PubMed NCBI (figure 2). So, a total of 46 (proxies of) SNPs associated with CAD or T2D were included in our study.

Data analysis

Data analysis was performed in two stages.

Stage 1:

First, we analyzed all selected SNPs in the IBC consortium. Each study contributing to the IBC consortium uploaded study specific summary statistics per SNP for the IBC chip-wide analyses on the server of the Framingham/BU server. All SNPs were analysed using linear regression analysis with age at natural menopause, adjusted for cohort/site and principal components causing population stratification where applicable, assuming an additive genetic model. A look-up of the study specific results was performed for the CAD and T2D SNPs mentioned above. Subsequently, study specific results for all cohorts except FHS, CHS, and ARIC were combined using fixed-effect meta-analysis with inverse variance weighting using METAL [37].

Next, we performed a look-up in ReproGen of all SNPs mentioned above in studies included in the IBC consortium. In ReproGen each study performed a linear regression analysis,

Figure 1 Flowchart of SNP selection in CAD

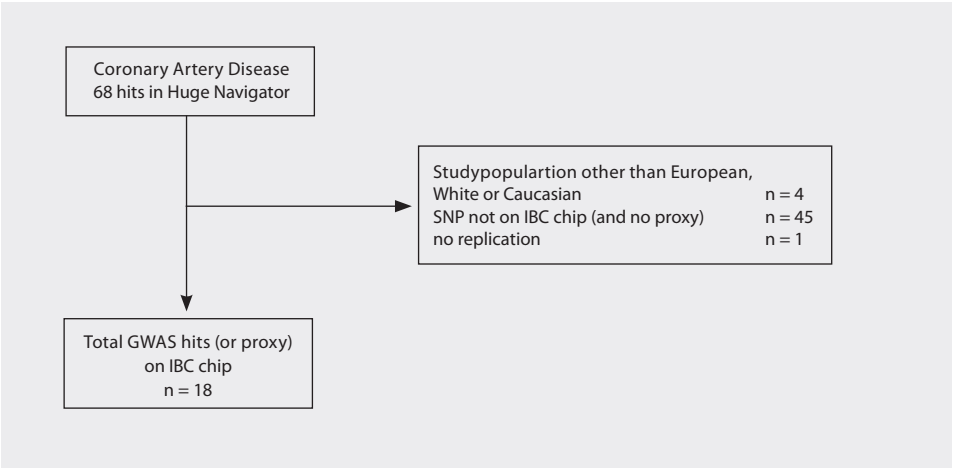
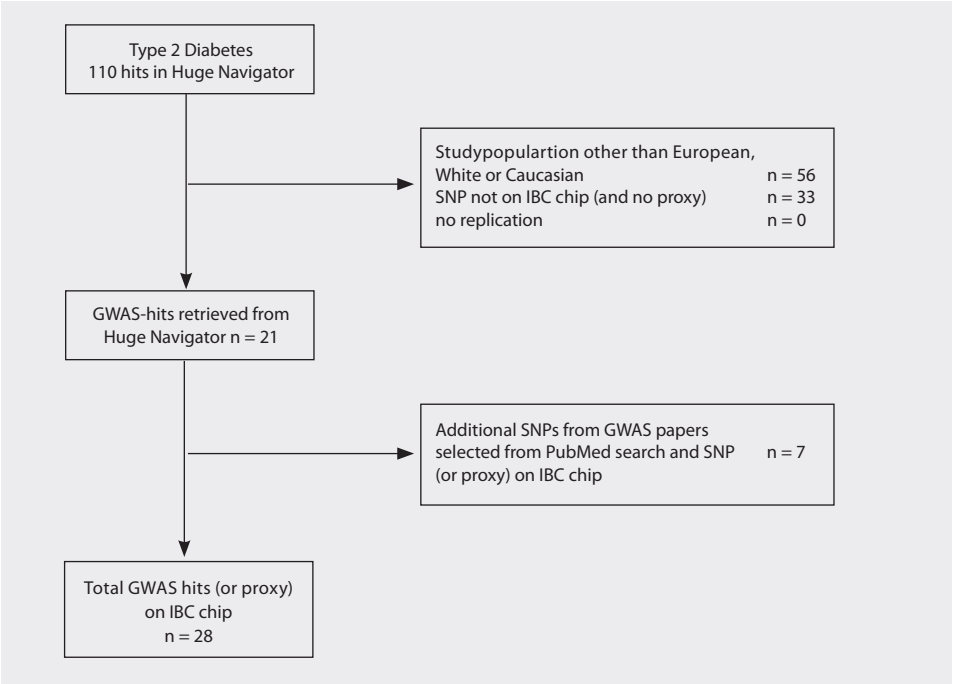


Figure 2 Flowchart of SNP selection in T2D



adjusted for principal components causing population stratification. Inverse variance weighted meta-analysis was performed with METAL using genomic control [37].

Furthermore, in InterAct we used the same criteria as in the IBC consortium to select women that could be included in the analyses. We performed linear regression analysis separately for women genotyped with the Illumina 660W-Quad Bead Chip and with the Metabochip and for women in the random sample and non-random sample type 2 diabetes cases. Subsequently, the analyses within each genotyping platform (i.e. for women in the random sample and non-random sample cases) were meta-analysed using METAL, again using a fixed effect inverse variance weighting [37]. Results for the two platforms (i.e. Illumina 660W-Quad Bead chip array and Metabochip) were reported separately as not all SNPs from the GWAS were available on the Metabochip and chip-specific proxies were used for the latter platform.

Stage 2:

In stage 2 we combined all results, combining proxy SNPs with the original GWAS SNPs where applicable, using a fixed effect, inverse variance weighting meta-analysis using the Metan package in STATA (STATA/SE 11.1 for windows, Stata Corporation, College Station, Texas, USA). We used I^2 to quantify study heterogeneity, where InterAct metabochip and InterAct GWAS were treated as two different studies.

Calculating the appropriate significance threshold is challenging in hypothesis-driven genetic association studies. Per pathway (CAD and T2D) we calculated a significance threshold based on the number of independent tests (r^2 <0.5). For CAD 15, and in TD2 22 independent SNPs were analyzed. This implies that for the pathways the significance threshold is set at a *P*-value of 0.003 for CAD and a *P*-value of 0.002 for T2D. However, as this is a hypothesis-driven study we also refer to all p-values below 0.05 as suggestive associations.

RESULTS

CAD

Our study included 18 SNPs in 14 loci for CAD. We found no statistically significant associations for any of the SNPs for CAD with age at menopause at the predefined significance level of 0.003. Furthermore, no suggestive associations with P-values below 0.05 were found either. In table 2 the results of the individual SNP association analysis per SNP for each of the four consortia (i.e. CARe, ReproGen, InterAct Metabochip, InterAct GWAS) and the joint analysis are shown.

Table 2 Meta-analysis of CAD associated SNPs with timing of natural menopause

					CARe				ReproGen				InterAct Metabochip				InterAct Illumina				Joint analysis	
SNP	Chr	Gene	Allele1	Allele2	Freq allele 1	Beta	95% CI	s.e.	Freq allele 1	Beta	95% CI	s.e.	Freq allele 1	Beta	95% CI	s.e.	Freq allele 1	Beta	95% CI	s.e.	Beta	P-value
rs17465637	1	MIA3	a	c	0,277	-0,093	-0.24, 0.06	0,0768	0,276	-0,028	-0.24, 0.19	0,111	0,281	0,017	-0.25, 0.28	0,1345	0,284	-0,330	-0.70, 0.04	0,191	-0,08	0,153
rs599839	1	SORT1	a	g	0,776	0,035	-0.17, 0.24	0,1057	0,771	-0,005	-0.07. 0.06	0,036	0,772	0,145	-0.14, 0.43	0,146	0,773	-0,092	-0.40, 0.21	0,155	0	0,954
rs646776	1	CELSR2, PSRC1, SORT1	t	c	0,78	0,045	-0.11, 0.20	0,079	0,781	-0,000	-0.07, 0.07	0,035	0,778	0,172	-0.12, 0.46	0,1476	0,777	-0,040	-0.34, 0.26	0,152	0,01	0,685
rs4299376	2	ABCG8	t	g	0,688	-0,005	-0.15, 0.14	0,0717	0,689	0,052	-0.01, 0.11	0,032	0,675	-0,176	-0.44, 0.08	0,1324	0,664	0,123	-0.14, 0.38	0,133	0,04	0,193
rs2706399	5	IL5	a	g	0,509	0,086	-0.04, 0.22	0,0663	0,499	0,035	-0.02, 0.09	0,029	0,531	0,136	-0.10, 0.38	0,1228	0,531	-0,058	-0.31, 0.20	0,130	0,04	0,091
rs10755578	6	LPA	c	g	0,522	0,032	-0.10, 0.17	0,0683	0,529	0,009	-0.05, 0.07	0,030	0,523	-0,294	-0.53, -0.06	0,1215	0,528	0,044	-0.21, 0.30	0,129	0	0,997
rs3127599	6	LPAL2	t	c	0,294	-0,039	-0.18, 0.11	0,0734	0,289	-0,024	-0.09, 0.04	0,033	0,319	0,192	-0.07, 0.45	0,1326	0,305	-0,261	-0.53, 0.01	0,138	-0,03	0,361
rs6922269	6	MTHFD1L	a	g	0,323	-0,044	-0.19, 0.10	0,0752	0,271	0,017	-0.05, 0.08	0,033	0,277	-0,298	-0.57, -0.03	0,1377	0,274	0,107	-0.18, 0.39	0,145	0	0,934
rs11556924	7	ZC3HC1	t	c	0,386	-0,003	-0.14, 0.13	0,0678	0,382	-0,067	-0.13, -0.01	0,031	0,381	0,078	-0.17, 0.33	0,1286	0,388	-0,146	-0.44, 0.14	0,148	-0,05	0,053
rs17321515	8	TRIB1	a	g	0,523	-0,151	-0.28, -0.02	0,0666	0,524	0,038	-0.02, 0.10	0,029	0,519	-0,121	-0.36, 0.12	0,1247	0,532	0,309	0.06, 0.56	0,127	0,01	0,558
rs1333049	9	Intergenic	c	g	0,493	0,010	-0.12, 0.14	0,0665	0,474	0,003	-0.06, 0.06	0,031	0,485	0,119	-0.12, 0.36	0,1215	0,475	-0,023	-0.28, 0.23	0,13	0,01	0,74
rs4977574	9	CDKN2A, CDKN2B	a	g	0,496	0,073	-0.06, 0.20	0,0665	0,523	0,001	-0.06, 0.06	0,029	0,503	0,059	-0.18, 0.30	0,1219	0,478	0,045	-0.20, 0.29	0,127	0,02	0,533
rs579459	9	ABO	t	c	0,787	-0,071	-0.23, 0.09	0,08	0,785	0,011	-0.06, 0.08	0,036	0,775	-0,042	-0.34, 0.25	0,15	0,751	-0,253	-0.54, 0.03	0,144	-0,02	0,589
rs12413409	10	CYP17A1, CNNM2, NT5C2	a	g	0,099	-0,041	-0.26, 0.18	0,1128	0,087	-0,036	-0.14, 0.07	0,052	0,091	0,453	0.03, 0.88	0,2171	0,099	0,062	-0.35, 0.48	0,211	-0,01	0,799
rs2246942	10	LIPA	a	g	0,657	0,016	-0.12, 0.15	0,0694	0,652	0,050	-0.01, 0.11	0,031	0,669	-0,136	-0.39, 0.12	0,1292	0,659	-0,012	-0.28, 0.25	0,136	0,03	0,218
rs501120	10	CXCL12	t	c	0,866	-0,220	-0.42, -0.02	0,1003	0,865	-0,030	-0.12, 0.06	0,044	0,861	-0,012	-0.35, 0.33	0,1747	0,855	-0,045	-0.40, 0.31	0,181	-0,06	0,135
rs4773144	13	COL4A1, COL4A2	a	g	0,566	-0,003	-0.13, 0.13	0,0671	0,554	-0,055	-0.12, 0.01	0,033	0,555	0,076	-0.17, 0.32	0,1245	0,53	-0,140	-0.43, 0.16	0,151	-0,04	0,14
rs46522	17	UBE2Z, GIP, ATP5G1, SNF8	t	c	0,541	0,023	-0.11, 0.16	0,0692	0,531	-0,032	-0.09, 0.02	0,029	0,538	-0,036	-0.28, 0.21	0,1257	0,53	-0,218	-0.47, 0.03	0,128	-0,03	0,21

Table 3 Meta-analysis of T2D associated SNPs with timing of natural menopause

					CARe				ReproGen				InterAct Metabochip				InterAct Illumina				Joint analysis	
SNP	Chr	Gene	Allele1	Allele2	Freq allele 1	Beta	95% CI	s.e.	Freq allele 1	Beta	95% CI	s.e.	Freq allele 1	Beta	95% CI	s.e.	Freq allele 1	Beta	95% CI	s.e.	Beta	P-value
rs10923931	1	NOTCH2, ADAM30	t	g	0,105	-0,009	-0.23, 0.21	0,111	0,106	0,126	0.03, 0.22	0,048	0,093	-0,097	-0.51, 0.32	0,212	0,108	-0,022	-0.43, 0.38	0,207	0,09	0,028
rs7578326	2	IRS1	a	g	0,665	-0,175	-0.31, -0.04	0,070	0,645	0,016	-0.04, 0.08	0,031	0,645	-0,091	-0.35, 0.16	0,130	0,642	0,445	0.19, 0.70	0,130	0,00	0,965
rs7578597	2	THADA	t	c	0,887	0,208	0.00, 0.42	0,108	0,897	0,023	-0.07, 0.12	0,048	0,893	0,175	-0.22, 0.57	0,201	0,902	-0,131	-0.55, 0.29	0,213	0,05	0,218
rs7593730	2	RBMS1, ITGB6	t	c	0,200	-0,077	-0.24, 0.09	0,084	0,212	-0,019	-0.09, 0.05	0,036	0,204	0,131	-0.17, 0.43	0,154	0,219	0,217	-0.09, 0.52	0,157	-0,01	0,710
rs1470579	3	IGF2BP2	a	c	0,685	0,037	-0.10, 0.18	0,071	0,686	-0,034	-0.10, 0.03	0,031	0,690	0,139	-0.12, 0.40	0,133	0,682	0,111	-0.16, 0.39	0,140	-0,01	0,703
rs1801282	3	PPARG	c	g	0,878	0,020	-0.18, 0.22	0,101	0,878	-0,035	-0.12, 0.05	0,045	0,883	-0,090	-0.47, 0.29	0,193	0,887	-0,041	-0.43, 0.35	0,201	-0,03	0,460
rs4402960	3	IGF2BP2	t	g	0,314	-0,038	-0.18, 0.10	0,071	0,312	0,031	-0.03, 0.09	0,032	0,307	-0,137	-0.40, 0.13	0,134	0,315	-0,114	-0.39, 0.16	0,141	0,01	0,783
rs4607103	3	ADAMTS9	t	c	0,258	0,189	0.04, 0.34	0,078	0,252	0,019	-0.05, 0.09	0,035	0,261	0,040	-0.24, 0.32	0,141	0,247	0,135	-0.16, 0.43	0,150	0,05	0,096
rs10946398	6	CDKAL1	a	c	0,686	-0,148	-0.29, 0.00	0,074	0,688	-0,037	-0.10, 0.02	0,031	0,686	0,029	-0.24, 0.29	0,135	0,666	-0,005	-0.27, 0.26	0,136	-0,05	0,078
rs7754840	6	CDKAL1	c	g	0,314	0,148	0.00-0.29	0,074	0,312	0,037	-0.02, 0.10	0,031	0,314	-0,025	-0.29, 0.24	0,135	0,334	0,002	-0.26-0.27	0,136	0,05	0,078
rs7756992	6	CDKAL1	a	g	0,734	-0,173	-0.33, -0.02	0,078	0,734	-0,042	-0.11, 0.02	0,033	0,716	-0,032	-0.30, 0.24	0,137	0,706	0,116	-0.15, 0.39	0,138	-0,05	0,066
rs864745	7	JAZF1	t	c	0,500	0,071	-0.06, 0.20	0,067	0,507	0,013	-0.04, 0.07	0,028	0,507	-0,012	-0.25, 0.23	0,123	0,507	0,095	-0.15, 0.35	0,128	0,02	0,361
rs13266634	8	SLC30A8	t	c	0,296	0,063	-0.08, 0.21	0,073	0,310	-0,045	-0.11, 0.02	0,032	0,291	0,135	-0.13, 0.40	0,136	0,289	0,037	-0.24, 0.31	0,139	-0,02	0,524
rs10811661	9	CDKN2A, CDKN2B	t	c	0,825	0,011	-0.16, 0.18	0,086	0,817	0,009	-0.07, 0.09	0,039	0,817	-0,167	-0.48, 0.15	0,160	0,824	0,031	-0.31, 0.37	0,173	0,00	0,949
rs1111875	10	HHEX	t	c	0,399	-0,064	-0.20, 0.07	0,068	0,416	-0,030	-0.09, 0.03	0,029	0,391	0,025	-0.22, 0.27	0,127	0,382	-0,069	-0.33, 0.20	0,136	-0,03	0,192
rs12779790	10	CDC123, CAMK1D	a	g	0,824	-0,058	-0.25, 0.13	0,096	0,809	-0,010	-0.09, 0.06	0,038	0,814	0,021	-0.30, 0.34	0,162	0,810	-0,143	-0.46, 0.17	0,162	-0,02	0,542
rs4506565	10	TCF7L2	a	t	0,684	0,003	-0.14, 0.14	0,071	0,686	-0,028	-0.09, 0.03	0,032	0,663	-0,151	-0.41, 0.11	0,132	0,629	0,160	-0.11, 0.43	0,136	-0,02	0,450
rs7901695	10	TCF7L2	t	c	0,687	0,047	-0.10, 0.19	0,076	0,690	-0,044	-0.11, 0.02	0,033	0,665	-0,162	-0.42, 0.10	0,133	0,632	0,166	-0.10, 0.43	0,135	-0,03	0,355
rs7903146	10	TCF7L2	t	c	0,295	0,013	-0.13, 0.16	0,072	0,299	0,031	-0.03, 0.09	0,032	0,317	0,158	-0.10, 0.42	0,134	0,353	-0,147	-0.41, 0.12	0,136	0,03	0,343
rs1552224	11	ARAP1	a	c	0,827	-0,046	-0.22, 0.13	0,088	0,844	0,012	-0.07, 0.09	0,041	0,855	-0,276	-0.62, 0.07	0,175	0,860	-0,122	-0.48, 0.23	0,180	-0,01	0,683
rs231362	11	KCNQ1	a	g	0,486	-0,023	-0.16, 0.11	0,067	0,513	-0,026	-0.09, 0.04	0,032	0,487	-0,138	-0.38, 0.10	0,124	0,488	-0,195	-0.45, 0.06	0,131	-0,04	0,160
rs5215	11	KCNJ11	t	c	0,635	-0,057	-0.19, 0.08	0,069	0,629	-0,007	-0.07, 0.05	0,030	0,626	-0,050	-0.30, 0.20	0,126	0,609	-0,001	-0.26, 0.26	0,133	-0,02	0,548
rs9300039	11	Intergenic	a	c	0,093	0,253	0.03, 0.47	0,113	0,089	-0,002	-0.10, 0.10	0,052	0,093	-0,055	-0.47, 0.36	0,211	0,087	0,022	-0.42, 0.47	0,228	0,04	0,412
rs7957197	12	HNF1A	a	t	0,197	-0,162	-0.32, 0.00	0,082	0,212	-0,063	-0.14, 0.01	0,037	0,205	-0,223	-0.52, 0.08	0,154	0,186	-0,209	-0.53, 0.11	0,163	-0,09	0,005
rs7961581	12	TSPAN8, LGR5	t	c	0,718	-0,060	-0.21, 0.09	0,076	0,734	-0,010	-0.08, 0.06	0,034	0,695	0,212	-0.05, 0.47	0,133	0,727	0,133	-0.15, 0.42	0,146	0,00	0,974
rs11642841	16	FTO	a	c	0,403	-0,038	-0.17, 0.10	0,068	0,420	-0,032	-0.09, 0.03	0,032	0,404	0,110	-0.14, 0.35	0,125	0,559	-0,242	-0.02, 0.50	0,133	-0,01	0,599
rs8050136	16	FTO	a	c	0,401	-0,011	-0.14, 0.12	0,068	0,402	-0,026	-0.08, 0.03	0,029	0,411	-0,012	-0.25, 0.23	0,124	0,429	0,245	-0.01, 0.50	0,128	-0,01	0,639
rs3794991	19	GATAD2A	t	c	0,084	-0,105	-0.34, 0.13	0,119	0,084	0,016	-0.09, 0.12	0,054	0,084	0,212	-0.22, 0.65	0,222	0,089	-0,064	-0.50, 0.37	0,223	0,00	0,959

T2D

For T2D, a total of 28 SNPs in 23 loci were included in our analysis. As shown in table 3, none of the SNPs reached the significance threshold of 0.002. However, two SNPs were suggestively associated with age at natural menopause. Women with a T allele at rs7957197 reached menopause on average approximately 1 month earlier (β=-0.09; *P*=0.005). This SNP is located on chromosome 12 near the *Hepatocyte Nuclear Factor-1-Alpha* (*HNF1A*) gene.

Next, rs10923931, which is located on chromosome 1 near *Notch homolog 2* (*NOTCH2*) gene, was suggestively associated with age at menopause. Women carrying a G allele experienced menopause 1 month later than women who did not (β=0.09; *P*=0.028).

DISCUSSION

In the current study, we investigated the association of 18 genome-wide significant CAD SNPs and 28 genome-wide significant T2D SNPs with on timing of menopause in nearly 50,000 women. CAD associated SNPs were not statistically significantly associated with menopausal age. For the SNPs associated with T2D, two suggestive associations were identified in timing of menopause (rs7957197 and rs10923931).

As none of the studied SNPs involved in CAD were associated with timing of menopause, we could not support a previous finding that women with an increased risk of CAD experience an earlier onset of menopause [15]. Since it is well known that experiencing an early menopause is associated with an increased risk of CVD later in life [1-5], possibly, the rate of ovarian ageing and its endpoint (i.e. menopause) is an expression of the rate of general ageing as a whole, and not specific vascular ageing. Accelerated general ageing could thus be reflected by accelerated ovarian ageing and a subsequent earlier menopause, together with an increased risk of CVD [15].

Of the T2D-related SNPs, two SNPs were suggestively associated with age at natural menopause. The strongest suggestive association was seen in rs7957197, which is located near the *HNF1A* gene on chromosome 2. Besides T2D, this region has been associated with many other traits, including CAD [38], gamma glutamyl transferase [39], C-reactive protein (CRP) levels [40] and also longevity [41]. Particularly the latter trait would make this region interesting for involvement in timing of menopause since , as stated above, timing of natural menopause may be seen as a marker of general ageing and health. A later onset of menopause has been associated with a greater life expectancy and a reduced risk of overall mortality [42,43]. Another indication for the relation between ovarian ageing and overall ageing is the fact that women suffering from syndromes causing accelerated ageing, like Down's syndrome or Werner's Syndrome, have an early menopause or are infertile [44,45]. So maybe the effect of rs7957197 on menopausal age actually flows through its effect on longevity instead of through T2D. The second SNP associated with

menopause is rs10923931, located on chromosome 1 near the *NOTCH2* gene. This gene is involved in pancreatic organogenesis [46]. A role for the two abovementioned suggestive associated genes in timing of menopause has not been reported before and is unclear.

Remarkably, for both suggestively associated T2D SNPs, the allele that was associated with an increased T2D risk in GWAS, was in our study consistently associated with a later age at menopause. These findings contradict the hypothesis that T2D related vascular damage causes earlier onset of menopause [47]. Furthermore, T2D has previously been associated with accelerated ovarian ageing as premenopausal women with T2D had a lower ovarian reserve than healthy controls [16]. However, in a large European study from our group, women diagnosed with diabetes after 50 years of age experienced a later onset of menopause [17]. Why women with late-onset T2D would suffer from a later menopause, however, remains unclear.

One of the strengths of our study lies in the fact that studied SNPs were genome-wide significantly associated with CAD or T2D and that these results were replicated afterwards. This implies that a true association with these diseases can be assumed. For that reason, these SNPs are suitable to test whether CAD and T2D are causally related to age at menopause.

Furthermore, this study included a very large sample-size of nearly 50,000 women. Given an alpha level of 0.002, we had 80% power to detect effect sizes ranging from 0.128 (6.7 weeks) to 0.213 (11.1 weeks) for minor allele frequencies ranging from 0.1 to 0.5. In the most recent GWAS on menopause, the reported effect sizes ranged from 0.16 to 0.95 (or -0.16 to -0.42). Thus, although we could detect reasonably small effect sizes, used a less stringent correction for multiple testing and had a larger sample size than the largest GWAS to date, our study was still underpowered to detect very small effect sizes. On the other hand, one might speculate whether even smaller effect sizes can be picked-up at all in studies based on self-reported age at menopause in postmenopausal women, which is prone to recall-bias. Previous studies have shown that the validity and reproducibility of self-reported age at menopause are fairly good [48-51]. Nonetheless, studies on the timing of menopause usually record age at menopause in whole years, possibly resulting in random misclassification. This will reduce power to detect very small effects.

Our study also has some limitations that should be mentioned. As we started our search of vascular and T2D related SNPs (or proxies) available on the IBC chip, we did not cover all SNPs that were genome-wide significantly associated with CAD or T2D. However, the SNPs present on the IBC chip were chosen for their relatedness to CVD, inflammatory and metabolic regions and included for the strongest GWAS signals for both diseases. It is possible, though, that we failed to identify CAD and T2D SNPs associated with timing of menopause because they were not available on the IBC chip and we consequently did not include them in our study.

Also, we used proxies for a few of the originally genome-wide associated SNPs for T2D and CAD in the studies from the CARe consortium and the women in the InterAct study that were genotypes on the Metabochip. This may have reduced power for these SNPs as power reduces

if the correlation between a proxy SNP and the original SNP lowers. However, we only included proxies with an $r^2 \geq 0.80$. Thus, the effect of using proxies on the power of our study is probably limited.

Next, calculating genetic risk scores for the CAD SNPs and theT2D SNPs might enhance power to detect a possible causal role for CAD and T2D SNPs on menopausal timing. As we only had the look-ups of SNPs and their effect sizes at our disposal we could not perform these analyses.

In conclusion, two T2D-related SNPs were suggestively associated with timing of menopause, implying a role for this metabolic disease in timing of menopause. These associations imply that T2D delays menopausal age, rather than the reverse. The biological mechanisms underlying this possible association remain unclear.

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Chapter

Six

The association of CGG repeats in the FMR1 gene and timing of natural menopause

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Hum Reprod. 2013, 28(2):496-501

ABSTRACT

Title

The association of CGG repeats in the *FMR1* gene and timing of natural menopause.

Study question

Is there an association between the number of CGG repeats in the *FMR1* gene in the normal and intermediate range and age at natural menopause?

Summary answer

The number of CGG repeats in the normal and intermediate range in the *FMR1* gene was not associated with age at natural menopause.

What is known already

Excessive triple CGG repeats in the FMR1 gene have been widely associated with primary ovarian insufficiency. Recently, the number of CGG repeats in the normal and intermediate range (up to 55 repeats) was found to be associated with serum levels of AMH and FSH, as markers for ovarian ageing. This suggests that repeats in the normal and intermediate range could be involved in the rate of exhaustion of the ovarian primordial follicle pool and ultimately the timing of menopause.

Study design, size

Cross-sectional study in a population-based sample of 3611 Caucasian women with natural menopause.

Participants/materials, setting, methods

The *FMR1* CGG repeat number was determined by PCR amplification in 3611 women with a known age at natural menopause. A possible relation between CGG repeats in the normal and intermediate range (up to 55 repeats) and menopausal age were analyzed in various ways, including linear regression analysis and analysis of variance (ANOVA).

Main results and the role of chance

The number of CGG repeats in the normal and intermediate range in the *FMR1* gene was not associated with age at natural menopause. The mean age at menopause was 50.30 (± 4.2) years for women with <45 repeats and 50.64 (± 3.4) years for women with intermediate sized repeats (P= 0.37). Linear regression analysis of the number of CGG repeats showed no association with menopausal age (β= 0.019, P=0.16).

Limitations, reasons for caution

In our cohort, age at menopause was self-reported and determined retrospectively.

Wider implications of the findings

Earlier observations suggesting that the number of FMR1 CGG repeats in the normal and intermediate range is associated with the individual variation of the ovarian ageing process could not be confirmed in the current, large sample size study. A relation between the number of CGG repeats in the normal and intermediate range and age at natural menopause appeared to be absent. This finding questions the role of CGG repeat sizes in the ovarian ageing process.

INTRODUCTION

Excessive triple CGG repeats in the *fragile X mental retardation* (*FMR1*) gene have been associated with early ovarian failure. The *FMR1* gene is located in the 5' untranslated region of the X chromosome and normally contains less than 55 copies of the CGG repeat. Alleles with <45 repeats are stably inherited. CGG repeats in the intermediate range, 45-55 repeats, however, may become unstable during transmission and have the ability to expand to a premutation (55-200 repeats), which can further expand to a full mutation (>200 repeats) causing the fragile X syndrome in the subsequent generation [1,2].

CGG repeats in the premutation range have been associated with primary ovarian insufficiency (POI, i.e. menopause before the age of 40 years, also known as POF, premature ovarian failure), referred to as fragile X-associated POI (FXPOI) [3-5]. The prevalence of POI in women carrying the *FMR1* premutation is estimated to be between 13 and 26% [4,6]. The association between the number of repeats in the premutation range and the risk of POI is believed to be nonlinear and suggests that carriers of premutations in the mid-size range (80-100 repeats) are at greatest risk for POI [7]. Furthermore, premutation carriers experience menopause on average 5 years earlier than non-carriers [6,8]. Repeat sizes in the intermediate (45-54 repeats) and higher end of the normal range (35-44 repeats) have also been associated with POI [9,10]. However, this association has not been confirmed in a large group of POI cases [11].

Recently, Gleicher and colleagues reported an association between the number of CGG repeats in both the high end of the normal range and in the intermediate range (35-55 repeats) with Anti-Mullerian hormone (AMH) levels in premenopausal women. Furthermore, with increasing numbers of CGG repeats, the risk for early ovarian senescence, defined as elevated levels of follicle-stimulating hormone (FSH), increased [12]. In addition, women carrying either alleles with <28 repeats or alleles with >33 repeats presented with reduced AMH levels, suggesting diminished ovarian reserve in these CGG repeat ranges [13]. The results of these and a few other

studies [14-16] suggest that the number of CGG repeats in the normal and intermediate range might also affect the ovarian ageing process over time and thereby the timing of menopause. Therefore, we aimed to investigate the association between the number of CGG repeats in the normal and intermediate range (up to 55 repeats) and age at natural menopause using a large cohort of Dutch postmenopausal women.

MATERIALS AND METHODS

Study population

The Prospect-EPIC cohort is one of the two Dutch contributions to the European Prospective Investigation into Cancer and Nutrition (EPIC). The design and rationale of this study has been described previously [17]. In brief, this cohort consists of 17357 white women living in Utrecht and surroundings, The Netherlands, aged 49 – 70 years, who were invited to participate in the study through the national breast cancer screening program between 1993 and 1997. All women filled out detailed questionnaires about dietary, reproductive, and medical history and underwent a physical examination at enrolment. In addition, women donated a 30-mL non-fasting blood sample which was fractioned into serum, citrated plasma, buffy coat and erythrocyte aliquots of 0.5 mL each. The samples were stored under liquid nitrogen at -196^{0}C for future research.

Natural menopause was defined according to the Word Health Organization as amenorrhea for at least 12 consecutive months without other obvious reasons. A total of 3497 women were premenopausal or perimenopausal at time of enrolment and therefore excluded. All women who experienced a surgical menopause (N=4449), used hormones during the menopausal transition (N=2161) or women with an unknown menopausal status or age (N=1194) were excluded. Next, all women who were younger than 58 years at inclusion in the Prospect-Epic cohort were excluded to avoid bias due to differential inclusion of women with an early menopause N=2248). Finally, 197 women were excluded because of missing buffy coat samples or failed DNA extraction, leaving a total of 3611 women available for analysis.

DNA extraction and genotyping

Genomic DNA was extracted from buffy coat aliquots by KBioscience using their own in-house silica based systems for buffy extraction (http://www.kbioscience.co.uk/lab%20services/DNA%20extraction/Ext_services_intro.html). Genotyping was performed at the Department of Medical Genetics, University Medical Center Utrecht, The Netherlands. The FMR1 CGG repeat number was determined by PCR amplification using Platinum *Pfx* DNA Polymerase according to the instruction of the manufacturer (Invitrogen). Primers for the FMR1 gene were: 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' and 5'- AGCCCCGCACTTCCACCACCAGCTCCTCCA-3'.

PCR conditions are available upon request. Repeat sizes were measured using an ABI prism 3730 DNA Analyzer (Applied Biosystems) and quantified using Genemapper Analysis Software v3.7 (Applied Biosystems).

As quality-control tests, plate-identifying blank wells and blind duplicates were used. Random duplicates analysed in the Genome Diagnostics Section of the Department of Medical Genetics laboratory, UMC Utrecht, The Netherlands were used as additional quality-control tests. The PCR analyses failed in 105 (2.9%) of our 3611 samples. Furthermore, 335 (9.2%) samples showed a homozygous pattern after PCR amplification. For 72 of these 335 presumed homozygous women, we could not clearly identify 2 alleles. This could indicate homozygosity for the small allele, or a larger allele (>100 repeats) may have been present but was not picked up by PCR analyses. Patient characteristics of these 72 samples did not differ from the total cohort. Therefore, we were confident that we could safely remove these samples from the analyses. Because we studied the association between the number of CGG repeats and age at natural menopause in the normal and intermediate range (up to 55 repeats) we excluded 11 samples because the largest allele counted ≥55 CGG repeats (premutation range). Thus, in our final data set, we had a total of 3423 women with repeat sizes between 20 and 55 CGG repeats (normal and intermediate range).

Data analysis

Characteristics of the study population were described using means and standard deviations for normally distributed continuous variables and frequencies and percentages for categorical variables. Alleles with the lower number of CGG repeats were designated as allele 1 and alleles with the higher number as allele 2. Women carrying <45 repeats on allele 2 were considered 'normal' and those carrying between 45-54 repeats on allele 2 are considered to be within the intermediate range. We used analysis of variance (ANOVA) to test differences in age at menopause between these two groups.

The linearity assumption between CGG repeat sizes on allele 2 was tested using restricted cubic splines composed of three polynomial segments in R (version 2.13.2; http://www.r-project.org), using the following libraries: foreign, Hmisc and Design. This analysis did not indicate deviations from linearity (P=0.275). Next, a linear regression analysis was performed with the number of CGG repeats for allele 2 as the independent and age at menopause as the dependent variable. This analysis was adjusted for the number of repeats on allele 1 by inclusion of the repeat sizes on allele 1 in the multivariate linear regression analysis.

The most frequent repeat sizes on allele 2 range from 29 to 34 repeats (75,7% of all women). For further analysis we considered these alleles 'common'. The repeat sizes to the right and left of these common alleles were considered uncommon. We used ANOVA to test differences in age at menopause between the three groups, i.e. group 1 <29 repeats, group 2 29-34 repeats (common) and group 3 >34 repeats.

To explore whether possible associations between repeat sizes and timing of menopause are driven by both alleles on the FMR1 gene, we performed analysis based on both alleles, organised in six categories. Again, the 'common' group was formed by women with both alleles between 29 and 34 repeats. The 'small' group consisted of women with both alleles <29 repeats. Women with heterozygous alleles with allele 1 <29 and allele 2 in the 'common' range (between 29-34 repeats), or allele 1 <29 and allele 2 >34 repeats, or allele 1 in the 'common' range and allele 2 >34 repeats were considered 'heterozygous S', 'heterozygous M', or 'heterozygous L', respectively. Finally, the 'large' group consisted of women with both alleles >34 repeats. Association analysis between the six groups and age at menopause was tested using ANOVA.

To assess if possible associations between CGG repeat sizes and age at menopause are driven by the POI cases in our study cohort, we performed sensitivity analyses by excluding women with a menopausal age under 40 yrs (i.e. POI, N=55). Statistical analyses were performed using SPSS for Windows (version 17.0; SPSS Inc., Chicago IL) and R (version 2.13.2; http://www.r-project.org). A *P* value of ≤0.05 was considered statistically significant.

RESULTS

Table 1 summarizes general characteristics of the women in our study cohort. Out of the total of 3423 women studied, 123 (3.6%) carried CGG repeats within the intermediate range (45-54 repeats) for allele 2. When comparing age at menopause between women carrying intermediate alleles and women carrying less than 45 CGG repeats, we found no difference in menopausal age. The mean age at menopause was 50.30 (± 4.2) years for women with <45 repeats and 50.64 (± 3.4) years for women with intermediate sized repeats (*P*= 0.373).

Linear regression analysis of the number of CGG repeats (allele 2) showed no association with age at menopause (β= 0.019, *P*=0.157). Adjustment for the number of repeats on allele 1 did not alter these results.

There was no difference in the mean age at menopause when comparing menopausal age of women carrying less than 29 repeats (49.90±4.5 year), between 29 and 34 repeats (50.33±4.2 year) and over 34 repeats (50.34±4.1 year), no difference between these three groups was present (table 2, *P*=0.34).

Table 3 presents an analysis of menopausal age of six genotype categories classified by CGG repeat sizes on both *FMR1* alleles present in women. Again, no statistical difference in age at menopause was present among the six groups (*P*=0.73).

Exclusion of POI cases (N= 55) from the study cohort did not alter the results of any of the analyses described in the aforementioned.

Table 1 Population characteristics (n = 3423)

Age at inclusion (yrs, mean ± SD)	63.0 ± 3.41
Age at natural menopause (yrs, mean ± SD)	50.31 ± 4.21
Range (yrs)	18 – 64
No women that were ever pregnant	2923 (85.4%)
Parity (mean ± SD)	2.65 ± 1.84
Nulliparous	521 (15.2%)
Parous	2902 (84.8%)
No women that ever used oral contraception	1180 (34.5%)
No women that ever used HRT [a]	226 (6.6%)
Smoking Never	1749 (51.1%)
Current or past	1674 (48.9%)
Body Mass Index (kg/m^2, mean ± SD)	26 ± 4
Waist circumference (cm)	85 ± 10
Systolic Blood Pressure (mmHg)	138 ± 21
Diastolic Blood Pressure (mmHg)	80 ± 10
No of CGG repeats allele 1 (mean ± SD)	27 ± 4.5
Range	7 – 47
No of CGG repeats allele 2 (mean ± SD)	33 ± 4.9
Range	20 – 54

a HRT, hormone replacement therapy

Table 2 CGG repeat sizes and age at menopause

	Group 1 [a]	Group 2 [b]	Group 3 [c]	Association analysis P [d]
No women (%)	164 (4.8)	2592 (75.7)	667 (19.5)	
Age at natural menopause (yrs, mean ± SD)	49.90 ± 4.5	50.33 ± 4.2	50.34 ± 4.1	0,43

a Group 1: allele 2 <29 repeats

b Group 2: allele 2 29-34 repeats (common)

c Group 3: allele 2 >34 repeats

Table 3 CGG categories and age at menopause

	Small [a]	Common [b]	Heterozygous S [c]	Heterozygous M [d]	Heterozygous L [e]	Large [f]	Association analysis P [g]
No women (%)	164 (4.8)	1507 (44.0)	1085 (31.7)	181 (5.3)	447 (13.1)	39 (1.1)	
Age at menopause (yrs, mean ± SD)	49.90 ± 4.54	50.33 ± 4.20	50.32 ± 4.20	50.08 ± 3.97	50.42 ± 4.27	50.69 ± 3.44	0,73

a Group Small: both alleles <29 repeats
b Group Common: both alleles 29-34 alleles
c Group Heterozygous S: allele 1 <29 and allele 2 29-34 repeats
d Group Heterozygous M: allele 1 <29 and allele 2 >34 repeats
e Group Heterozygous L: allele 1 29-34 and allele 2 >34 repeats
f Group Large: both alleles >34 repeats
9 ANOVA

DISCUSSION

Our study investigated the possible association between the number of CGG repeats in the *FMR1* gene and timing of natural menopause in a large cohort of Dutch postmenopausal women. We found that the number of CGG repeats in the normal and intermediate range (up to 55 repeats), analysed in various ways, was not associated with age at natural menopause.

To our knowledge, our study is the first to investigate a possible relation between normal and intermediate CGG repeat sizes in the *FMR1* gene and age at natural menopause in a large population-based cohort. This work basically builds on the studies where variations in the *FMR1* CGG repeat sizes within the normal and intermediate range were associated with the level of basal FSH and serum AMH, suggesting a further role of the FMR1 gene in the mechanisms of ovarian ageing [13-16]. In case these normal range variations would indeed affect follicle wastage, the endpoint of this wastage process (i.e. menopause), could well be influenced by these variations. This would also be in line with studies on non-normal variation in the CGG repeats sizes (i.e. premutation carriers; 55-200 repeats), where large effect sizes have been reported regarding earlier age at menopause [6-8].

In exploring the relation between normal ranged CGG repeats and markers for ovarian reserve, an association was previously demonstrated between the number of CGG repeats in the range of 35-55 repeats and AMH levels in a small pilot study of 40 premenopausal women [12]. Furthermore, they found that with increasing numbers of CGG repeats, FSH concentrations increased [12]. In addition, a study in 316 infertility patients reported that both alleles with <28 repeats and alleles with >33 repeats correlated with lower AMH levels, suggesting diminished ovarian reserve in these specific CGG repeat ranges [13]. In another study, the frequency of intermediate

alleles (45-55 repeats) was higher in 535 women with elevated FSH levels or with poor response to gonadotrophin stimulation (i.e. occult POI or imminent ovarian failure, IOF) compared to women in the control group (n=521) composed of oocyte donors and infertile women with no evidence of occult POI (*P*=0.046) [16]. FSH concentrations were also found to be significantly raised (P<0.0001) in women carrying 31-40 repeats compared to those carrying fewer than 30 repeats in a study population composed of 80 Indian POI cases and 70 Indian controls [15].

The observed associations between CGG repeats in the normal and intermediate range and proxies for (early) ovarian senescence in the above mentioned studies suggest a role for the FMR1 gene in ovarian ageing. However, we were not able to detect a relationship between FMR1 CGG repeats and the ultimate long-term outcome of the ovarian ageing process, i.e. age at onset of menopause. Following the analytical steps from these earlier studies did not yield any association with timing of menopause in our large study of postmenopausal women. Several explanations can be proposed to substantiate this seemingly inconsistent finding.

First, age at natural menopause may not accurately represent the ovarian ageing process taking place in the decades prior to menopause. Possibly, *FMR1* related ovarian ageing is not due to an inherently smaller follicle pool, but to a diminished recruitment rate from the primordial follicle pool. It was suggested that this diminished recruitment rate reflects lower circulating AMH levels in young women, but not an early menopause due to preservation of ovarian reserve into older age [14]. Thus, it could be that the numbers of CGG repeats in the normal and intermediate range have an effect on the rate of decline of ovarian reserve and not the endpoint of ovarian ageing (i.e. menopause) [14]. However, studies showing that AMH is highly predictive for timing of menopause would contradict this hypothesis [18-21]. The same holds true for poor response to gonadotrophin stimulation and prediction of menopausal age. Since it has been found that women with poor response to gonadrophin stimulation have an increased chance of early natural menopause [22], the possible increased frequency of intermediate CGG repeat sizes in these women would also be reflected in an earlier age at natural menopause. In fact, in a recently published study by Lledo et al., no relationship was found between normal and intermediate-sized CGG repeats and ovarian stimulation in 204 oocyte donors, suggesting no negative effect of CGG repeat in these ranges and ovarian reserve [23].

Secondly, the previous associations were found in relatively small studies, varying from 40 to 535 women whereas we investigated a population-based sample of 3,423 postmenopausal women. We cannot exclude the possibility that the earlier found associations between normal ranged repeat sizes and proxies for ovarian ageing (i.e. FSH and AMH) were in fact false-positive findings. Confirmation of the associations with FSH and AMH levels in large cohorts is therefore urgently needed.

The inconsistent findings could also be due to differences in ethnicities in the studied populations. A recent study demonstrated differences in CGG repeat distributions amongst various

racial/ethnic groups [24]. Moreover, it could be possible that the association between CGG repeats and (markers for) ovarian ageing varies between ethnicities [25]. So the observed discrepancy with other studies could be explained by the fact that the current study was performed on a Caucasian population, leaving the possibility open that in other ethnic groups the relation between CGG repeat distribution and the ovarian ageing process is fundamentally different.

Another explanation may stem from the fact that the populations in the previous studies consisted, at least partly, of infertile women and/or women with (occult) POI, whereas we investigated a population-based sample. Possibly, the numbers of CGG repeats are in fact associated with timing of menopause in an infertile population experiencing early ovarian senescence, rather than women in the general population who experience normal menopause (i.e. menopause between 45-55 yrs). However, subgroup analysis of nulliparous women (N=521) and infertile women (women who were ever diagnosed with infertility by a medical doctor, N=80) within our population also lacked an association between the number of CGG repeats and age at menopause (data not shown).

Next to markers for ovarian ageing, CGG repeats in the normal and intermediate range have been also associated with susceptibility to POI, albeit not consistent. An association with POI has been demonstrated in three studies comprising 53, 190 and 128 POI cases [9,10,26]. Importantly, the findings could not be replicated in the largest study performed so far, which studied 366 women with POI [11]. Whether POI cases with no obvious cause (idiopathic POI) may be considered as a separate genetic entity or to be part of the variation towards the entire distribution of natural menopause remains unclear. Therefore, it could be that the number of CGG repeats is primarily associated with early menopause (POI) and possibly has a less important or even no role in determining normal menopausal variation.

When interpreting our results, some strengths and limitations should be kept in mind. First we conducted a study in a large, well phenotyped cohort of natural menopausal women, which provides power to detect possible small effect sizes. Next, in studies associating CGG repeat lengths with ovarian ageing, no standard method is consistently used. Most studies focus on the allele with the higher number of CGG repeats (allele 2), although a solid biological rationale is lacking. Linear regression analysis of the number of CGG repeats on allele 1 or allele 1 and 2 combined , however, did not change our results ($\beta_{allele1}$= 0.026, P=0.122 and $\beta_{allele1+allele2}$= 0.029, P=0.092). Furthermore, it has been previously suggested that associations between CGG repeat sizes and ovarian reserve are dependent on heterozygous or homozygous expression of the genetic abnormality [14]. Again, as showed in table 3 no association with ANM was found in any of the categories. A limitation might be that in the Prospect-EPIC cohort, as in most studies conducted in timing of menopause, age at menopause was self-reported and determined retrospectively. Self-reported menopausal age could be susceptible to bias [27,28]. However, the reproducibility of self-reported menopause is high [29]. Also, it is unlikely that misclassification due

to recall bias differs across genotypes. However, we cannot fully exclude the possibility that non-differential bias precluded us from picking up a very small association.

In conclusion, in our comprehensive genetic study on the relation between the FMR1 gene and ovarian ageing, we observed no association between the numbers of CGG repeats in the normal to intermediate range and age at natural menopause. The evidence from recent studies for involvement of CGG repeat sizes up to 55 repeats in ovarian ageing process could thereby not be confirmed by the current study.

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Chapter

Seven

The significance of FMR1 CGG repeat sizes in the normal and intermediate range in women with Primary Ovarian Insufficiency

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Submitted

ABSTRACT

Context

CGG repeats in the *FMR1* gene in the premutation range (55-200 repeats) have been consistently associated with primary ovarian insufficiency (POI). Intermediate ranged CGG repeats have been considered for a potential association with POI.

Objective

To investigate whether the *FMR1* CGG repeats in the normal and intermediate range (up to 55 repeats) are associated with POI in a large case-control study.

Design

Case-control study

Setting and participants

375 well-phenotyped Dutch women diagnosed with POI and 3368 controls with natural menopause ≥ 40 years of age.

Main outcome measure

Intermediate sized *FMR1* CGG repeats in POI

Results

The frequency of intermediate sized CGG repeats on the allele with the longest triple repeat number was not statistically significantly different between POI cases and controls (resp. 2.7% and 3.8%, OR 0.72, 95% CI: 0.38-1.39, P=0.38). In women with POI, linear regression analysis for age at POI diagnosis and CGG repeat size also failed to show any association (β=-0.018, P = 0.74).

Conclusion

We found no association between intermediate sized CGG repeats and POI compared to controls. Therefore, the role of *FMR1* CGG repeat sizes up to 55 repeats in the ovarian ageing process may be questioned. Moreover, there seems limited value in the evaluation of normal- and intermediate *FMR1* repeat size in the diagnostic work-up of women affected by POI, or for prognostic purposes in women at risk of developing POI.

INTRODUCTION

Menopause typically occurs around 51 yrs of age [1]. However, approximately 1-2% of women experience the cessation of menses before age 40 yrs [2]. This condition – currently referred to as primary ovarian insufficiency (POI) (previously premature ovarian failure) – is characterized by amenorrhea for at least 4 months occurring prior to the age of 40 yrs, in combination with repeatedly elevated follicle-stimulating hormone (FSH) concentrations above 40 IU/l and with decreased estradiol (E_2) levels [3,4].

The high heritability of age at menopause [5,6] and the tendency for POI to run in families [7,8], imply a strong genetic component underlying POI. In fact, women were approximately 5 to 6 times more likely to reach menopause early if their mother or sister had experienced an early menopause (i.e. menopause before 45 years) [6]. Furthermore, environmental factors, such as smoking, have also shown to contribute to the susceptibility to develop POI [9,10]. POI is therefore considered a multifactorial heterogeneous condition for which the specific cause remains unknown in the majority of cases [11,12]. Genetic causes include numerical or structural chromosomal abnormalities including (full or mosaic) monosomy X [13], or relatively rare monogenetic causes such as mutations in the *FOXL2*, *BMP15*, *FSHR*, or *GDF9* gene [14-17]. The most frequent monogenic cause of POI is the premutation of the *fragile X mental retardation gene 1* (*FMR1*) with an incidence reported between 0.8-13% [18].

The *FMR1* gene is located on the distal long arm of the X chromosome (Xq27.3) and consists of 17 exons (OMIM ID number 309550). Dysfunction of the *FMR1* gene is caused by the amplification of an unstable CGG triplet in the 5' untranslated region of the first exon [19]. CGG repeat sizes exceeding 200 repeats is considered a full mutation, which leads to hypermethylation and subsequent silencing of the *FMR1* gene, and thereby results in the loss of fragile X related mental retardation protein (FMRP) [20]. In these cases the fragile X syndrome develops, which is characterized by mental retardation, characteristic facial features, and behavioural problems such as autism and attention deficit disorder (OMIM ID number 300624).

The full *FMR1* mutation may result from the expansion of a premutation in maternal transmission from one generation to the next [21]. The *FMR1* premutation is an amplification of the CGG repeat length to 55-200 repeats (normal range <45 repeats) [22]. CGG repeats in the premutation range have repeatedly been associated with POI, referred to as fragile X-associated POI (FXPOI) [23-27]. FXPOI accounts for approximately 1 to 8% of all cases of POI [18]. The severity of ovarian dysfunction depends on the CGG repeat size in a non-linear fashion: women with a mid-range number of repeats (80-100) experience POI earlier and more frequently than other carrier groups [27,28].

The intermediate CGG repeat length (45-54 repeats) [22,29] may expand to a full mutation after two or more generations [21]. Several recent studies investigated the role of intermediate CGG repeat size in idiopathic POI and yielded varying results. Some studies identified an increased

frequency of intermediate alleles in (occult) POI [30-35], whereas these findings have not been confirmed in a study with a much larger sample size [36].

A recently published study from our group in 3611 women with a known age at natural menopause, demonstrated no association between CGG repeats in the normal and intermediate range and age at natural menopause [37]. However, whether POI cases without an obvious cause (idiopathic POI) may be considered as a separate genetic entity or whether they represent variation within the entire distribution of natural menopause remains unclear. Therefore, the number of CGG repeats may primarily be associated with POI and may play a less important or even no role at all in determining normal menopausal variation.

In addition, there is no consensus on the definition of intermediate CGG repeat length [22, 29, 38], and the lower boundary of *FMR1* repeat sizes which alter ovarian function has not yet been defined [32]. Therefore, the current study was undertaken to assess whether the risk of POI is associated with CGG repeat length of normal and intermediate *FMR1* alleles in a large case-control study of Dutch women with idiopathic POI. Moreover, we aimed to investigate a possible association of number of CGG repeats with age at POI as a parameter of POI severity.

METHODS

Study population

POI cases

POI cases were recruited from two cohorts. 1) Since 2005, women suspected to suffer from a hypergonadotropic status presenting with either a regular menstrual cycle, oligomenorrhea, or amenorrhea are systematically evaluated in a standardised fashion in the outpatient clinics of sixteen fertility clinics in the Netherlands, as has been described in previous studies from our group [12, 39]. For the current study, all consecutive women in this cohort diagnosed with idiopathic POI, and in whom data of the *FMR1* gene are known were included (n = 159). For an additional 165 women with POI genotyping was performed to determine the *FMR1* CGG repeat numbers. POI was defined as spontaneous cessation of menses for at least 4 months in women younger than 40 years of age, along with repeated FSH concentrations exceeding 40 IU/l [4]. Women with primary amenorrhea were also included (n = 17). All women had a normal karyotype. The study protocol was approved by the institutional review board of the University Medical Centre Utrecht, the Netherlands, and all women gave written informed consent (clinicaltrials.gov identifier: NCT01411644). 2) In addition, 55 women with a self-reported natural age at menopause before 40 years, repeat sizes < 55 CGG repeats on allele 2, and a normal karyotype were included from the Prospect-EPIC cohort (the Prospect-EPIC cohort is described below).

Controls

The Prospect-EPIC cohort is one of two contributions to the European Investigation into Cancer and Nutrition (EPIC). The design and rationale of this study has been described previously [40]. In brief, this cohort consists of 17,357 women living in Utrecht and surroundings, The Netherlands, aged 49 – 70 years, who were invited to participate in the study through the national breast cancer screening program between 1993 and 1997. All women filled out detailed questionnaires about dietary, reproductive, and lifestyle factors and about medical history. Furthermore, they underwent a physical examination at enrolment. In addition, women donated a 30-mL non-fasting blood sample which was fractioned into serum, citrated plasma, buffy coat and erythrocyte aliquots. The samples were stored under liquid nitrogen at -196 °C for future research. All participants gave written informed consent and the study was approved according to the guidelines of the Helsinki Declaration by the Medical Ethics Committee of the Netherlands Organization of Applied Scientific Research

Natural menopause was defined according to the Word Health Organization as amenorrhea for at least 12 consecutive months without other obvious reasons. For the current study we used a selection of 3434 women from the Prospect-EPIC cohort with a known age at natural menopause and available data on *FMR1* CGG repeat numbers genotyped for a previous study [37]. We excluded women with an age at menopause before 40 years (n=55) and 11 cases because the largest allele counted ≥ 55 CGG repeats (premutation range). Thus, finally our control group consisted of 3,368 women with a known age at natural menopause ≥ 40 years and with *FMR1* CGG repeat sizes between 20 and 55 (normal and intermediate range).

Laboratory Analyses

POI cases

For each patient, a blood sample was collected in a 10 mL EDTA tube, and high molecular weight genomic DNA was extracted using established procedures. Amplification was performed by PCR of the CGG-repeat region of the *FMR1* gene using Platinum *Pfx* DNA Polymerase according to the instruction of the manufacturer (Invitrogen). As forward primer PK2038 (5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3') and as reversed primer 5' FAM labeled PK2039 (5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3') were used. Fragment length determination was performed on an ABI 3130 sequencer (Applied Biosystems) using the GeneMarker genotype analysis software (SoftGenetics, State College, PA).

Whenever a single allele length was observed in the 159 already genotyped POI cases, a Southern Blot analysis was performed on Hind III en EclXI restricted DNA to confirm homozygosity for the number of CGG-repeats. For the additional 165 genotyped women with POI, a total of 46 samples showed a homozygous pattern after PCR amplification. This could indicate homozygosity for the small allele, or a larger allele (>100 repeats) may have been present but was not picked up

by PCR analyses. Patient characteristics of these 46 samples did not differ from the other POI cases, we choose to include these samples as homozygous in our analysis. Also sensitivity analysis excluding these 46 samples did not alter any of the results (data not shown). Because we studied the association between the number of CGG repeats and POI in the normal and intermediate range (up to 55 repeats) we excluded 4 samples because the largest allele counted ≥55 CGG repeats (premutation range).

Controls and POI cases from the Prospect-EPIC cohort

Genomic DNA was extracted from buffy coat aliquots by KBioscience using their own in-house silica based systems for buffy extraction (http://www.kbioscience.co.uk/lab%20services/DNA% 20extraction/Ext_services_intro.html). Genotyping was performed at the Department of Medical Genetics, University Medical Center Utrecht, The Netherlands. The *FMR1* CGG repeat number was determined by PCR amplification using Platinum Pfx DNA Polymerase according to the instruction of the manufacturer (Invitrogen). Primers for the *FMR1* gene were: 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' and 5'- AGCCCCGCACTTCCACCACCAGCTCCTCCA-3'. Repeat sizes were measured using an ABI prism 3730 DNA Analyzer (Applied Biosystems) and quantified using ABI prism Genemapper Analysis Software v3.7 (Applied Biosystems). Of the samples that showed a homozygous pattern after PCR amplification, in 72 samples we could not clearly identify 2 alleles. As mentioned above, these samples could be homozygous for the small allele, or a larger allele (>100 repeats) could not be picked up by PCR analyses. Patient characteristics of these 72 samples again did not differ from the total cohort. Therefore, we were confident that we could safely remove these samples from the analyses. 11 Samples were excluded because the largest allele counted ≥55 CGG repeats (premutation range). In total, our control group existed of 3368 women with an age at natural menopause ≥40 years and repeat sizes below 55 CGG repeats (normal and intermediate range). Additionally, 55 women with a menopausal age <40 years and repeat sizes <55 CGG repeats were added to the POI cases.

As quality-control tests, plate-identifying blank wells and blind duplicates were used. As an additional quality control test, random duplicates were genotyped in both the Genome Diagnostics Section of the Department of Medical Genetics laboratory and the Department of Medical Genetics, UMC Utrecht, The Netherlands. We found no differences in the number of CGG repeats of the samples genotyped in both laboratories.

Statistical Analyses

Characteristics of the two study populations were described using means and standard deviations (SD) for continuous variables, and frequencies and percentages for categorical variables. The allele with the lowest triple repeat number was referred to as allele 1, while allele 2 was destined to represent the longest of both. A normal CGG repeat count was defined as <45 repeats and the

intermediate allele was defined as 45-54 CGG repeats [22, 29]. We used analysis of variance (ANOVA) to test differences in mean CGG repeat lengths on allele 1 and allele 2 between POI cases and controls.

The most frequent repeat sizes on allele 2 ranged from 29 to 34 repeats (78.1% of all POI cases and 75,7% of all controls). For further analysis we considered these alleles 'common'. The repeat sizes to the right and left of these common alleles were considered uncommon. We used Chi square to compare the proportion of women in the three groups, i.e. group 1 <29 repeats, group 2 29-34 repeats (common) and group 3 >34 repeats between POI cases and controls.

As stated earlier, the association between premutation ranged CGG repeats and age at menopause is non-linear, in which women with a mid-range number of repeats (80-100) are more susceptible to (early) POI in comparison to other carrier groups [27, 28]. To identify a possible non-linear association of number of CGG repeats in the allele with the longest triple repeat number (allele 2) in the normal- and intermediate range, and age at menopause in the POI cases, 3-knot restricted cubic splines were used. The restricted cubic splines did not indicate deviations from linearity (P=0.153). Next, a linear regression analysis was performed to investigate the association between the number of CGG repeats in allele 2 *vs*. age at POI (in 344 POI cases with a known age at onset of POI). To adjust for the number of repeats on allele 1 we included the repeat sizes on allele 1 in the multivariate linear regression analysis. Statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL) and R version 2.9.0. (http://www.r-project.org/). A P-value of ≤0.05 was considered statistically significant.

RESULTS

A total of 375 POI cases and 3,368 controls with natural menopause ≥ 40 years were included for the current study. General population characteristics of both groups are summarized in Table I.
Details for the *FMR1* CGG repeat lengths are reported in Table II. No statistically significant difference in the frequency of intermediate sized CGG repeats on allele 2 between POI cases and controls was found (resp. 2.7% and 3.8%; OR 0.72, 95% CI: 0.38-1.39, P = 0.38). The distributions of the CGG repeat size on allele 2 and allele 1 for each group are shown in Figure 1 and Figure 2 respectively. When comparing the distributions of normal and intermediate sized CGG repeats (up to 55 repeats) on allele 2, we did observe a statistically significant difference between the two groups (P < 0.001), with the most common repeat size for POI cases being 30 (37.3%) and the most common repeat size in controls being 31 CGG repeats (35.7%). The distributions of repeats on allele 1 did not differ between POI cases and controls (P = 0.55).

Table III presents the frequencies of women carrying less than 29 repeats, between 29-34 repeats, and over 34 repeats in the POI cases and controls. No statistically significant difference between these three groups was observed (P = 0.19).

Linear regression analysis for age at first manifestation of POI in the POI cases *vs.* CGG repeat size of allele 2 did not reveal any association (β = -0.018, *P* = 0.74) and is shown in Figure 3. Adjustment for the number of repeats on allele 1 did not alter the results.

Table I Population characteristics of the POI cases and controls

Population characteristics	POI N=375	Controls N=3368
Age at first manifestation of POI / natural menopause (years, mean ± SD)	32.2 ± 6.4	50.6 ± 3.7
Range (years)	14 - 39	40 - 64
Number of women who were ever pregnant	169/370 (45.7%)	2866 (85.1%)
Parity (mean ± SD)	0.9 ± 1.3	2.67 ± 1.8
Smoking never	199/348 (57.2%)	1718 (51%)
current or past	149/348 (42.8%)	1650 (49%)
Body mass index (kg/m^2, mean ± SD)	24.7 ± 4.7	26.3 ± 4.0
Systolic bloodpressure (mmHg)	128.6 ± 17.3	137.9 ± 20.8
Diastolic blood pressure (mmHg)	80.1 ± 10.47	79.4 ± 10.4

Table II CGG repeat sizes in POI cases and controls

	POI n = 375	controls n = 3368		P [a]
FMR1 CGG repeat size				
No of CGG repeats allele 1	26.96 ± 4.5	27.11 ± 4.5		0,55
Median (range)	29 (8 - 40)	30 (7 - 47)		
No of CGG repeats allele 2	31.79 ± 4.5	32.86 ± 4.9		<0.001
Median (range)	31 (19 - 53)	31 (20 -54)		
Prevalence of FMR1 allelic form			OR (95% CI)	P [b]
Intermediate (45-54 repeats)	10 (2.7%)	123 (3.7%)	0.72 (0.38-1.39)	0,38
Normal range (<45 repeats)	365 (97.3%)	3245 (96.3%)		

Data shown as mean ± SD, median (range), or n(%)
a ANOVA
b Fisher's exact test

Table III Categories of CGG repeat sizes on allele 2 in POI cases and controls

	<29 repeats	29-43 repeats	>34 repeats	Pa
POI cases	22 (5.9%)	293 (78.1%)	60 (16%)	
controls	161 (4.8%)	2548 (75.7%)	659 (19.6%)	0,19

Figure 1 Distribution of the number of CGG repeats on allele 2 in POI cases and controls

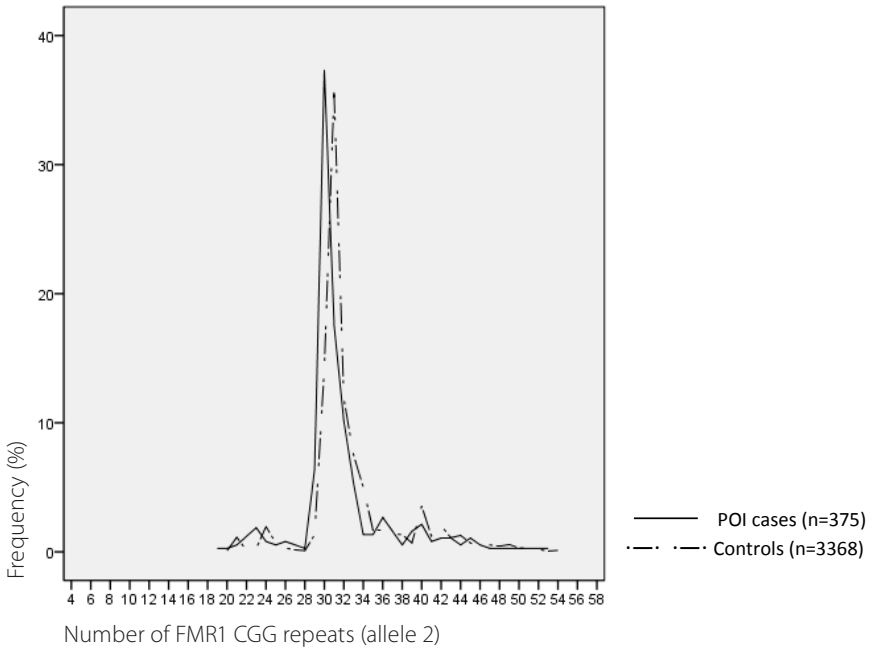


Figure 2 Distribution of the number of CGG repeats on allele 1 in POI cases and controls

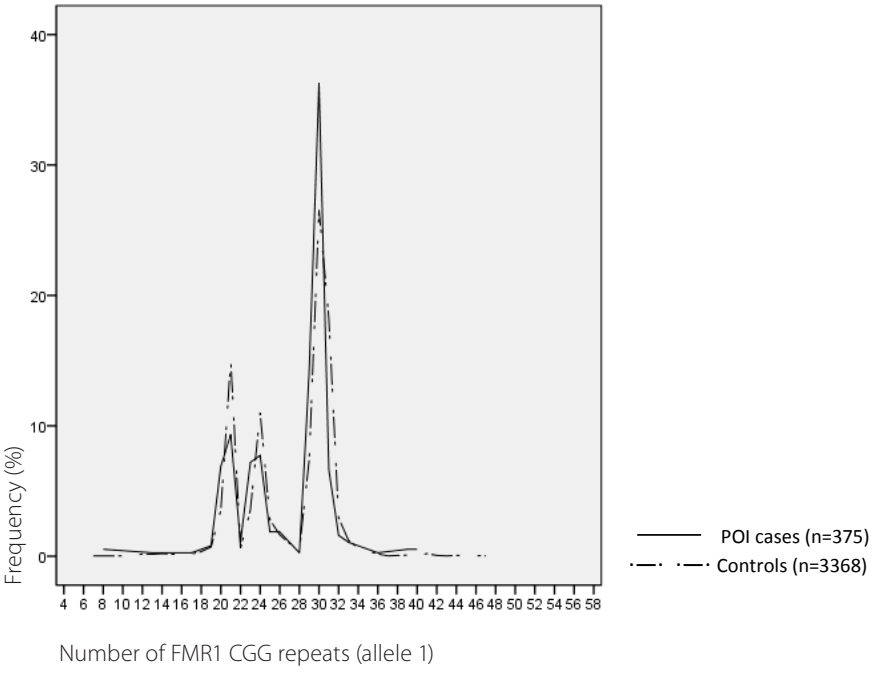
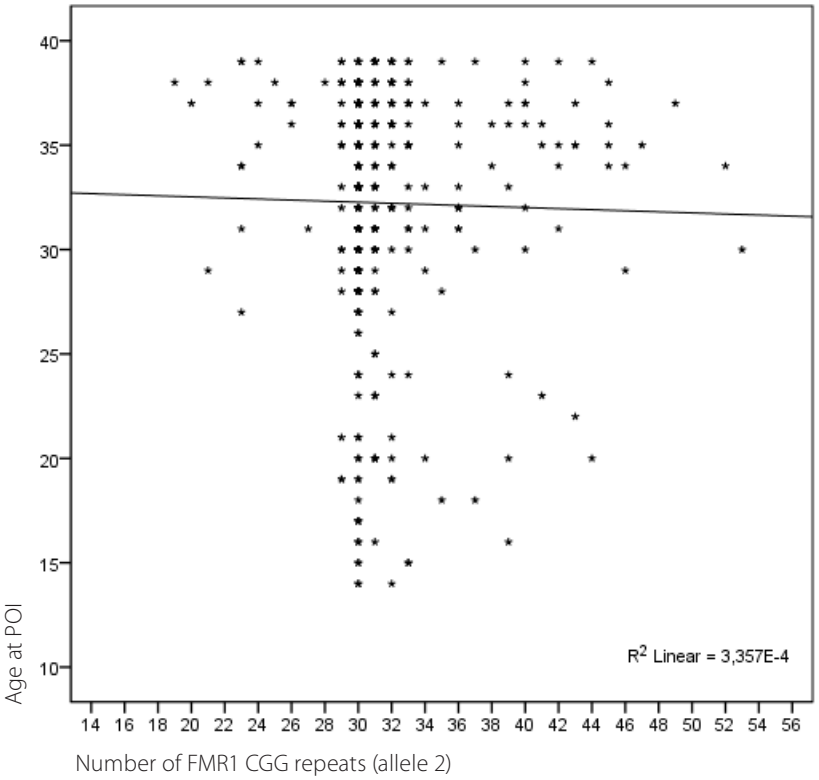


Figure 3 Linear regression analysis for age at POI vs. FMR1 CGG repeat lengths on allele 2



DISCUSSION

The current study was undertaken to investigate the prevalence of *FMR1* intermediate alleles in a considerable-sized cohort of well-phenotyped Dutch women with idiopathic POI compared to controls. Our data suggest that CGG repeats length in the intermediate range is not associated with the risk of POI, as the frequency of intermediate sized CGG repeats did not significantly differ between POI cases and controls. Furthermore, an association between the number of CGG repeats and age at first POI manifestation could not be established, not even when a nonlinear relationship was considered.

In contrast to the well-known association between *FMR1* CGG premutations and POI, the association of intermediate range CGG repeats number and susceptibility to POI is less consistent [30, 31, 34, 36]. The number of women carrying intermediate sized CGG repeats (between 45-54 repeats) did not differ between the POI cases and controls in our study. Up to now, only one study has used identical definitions to the current study, and observed an increased incidence of intermediate alleles in occult POI (i.e. imminent ovarian failure, IOF) compared to controls [34]. Most previous publications on POI applied different definitions for intermediate alleles, although not concurrent with the existing guidelines [22, 29, 38], hampering direct comparisons. For the sake of comparison with these publications, we converted our findings into the reported definitions of intermediate sized repeats. A rather wide definition of 34-54 repeats resulted in 16% in POI cases and 19.6% in controls in our study sample (OR 0.8, 95% CI 0.6 - 1.1, $P = 0.1$). This finding is consistent with the results of the largest study published so far in which no association was found between the number of alleles between 35 and 54 repeats amongst 366 POI cases and 1,389 controls [36]. When intermediate CGG repeats are defined as 41-58 repeats [31], we found a marginal statistical difference in the frequency of intermediate alleles between POI cases and controls, where intermediate repeats were less frequent in POI cases than controls in our study (6.1% and 9%, OR 0.64, 95% CI: 0.4 - 1.0, $P = 0.05$). While intermediate sized repeats of 41-58 were more common in a small study with 53 POI cases compared to 182 controls (OR 2.4, 95% CI: 1.7 - 7.7, P=0.01) [31], this association could not be replicated in a cohort of 366 POI cases (OR 1.3, 95% CI: 0.8 - 2.0, P=0.23) [36]. Thus, we could not replicate previously reported associations between CGG repeat sizes in the intermediate range and POI. In fact, although not statistically significant, the prevalence of intermediate sized CGG repeats was even higher in controls than POI cases in our study (resp. 3.8% and 2.7%).

The second objective of the current study was to examine a possible association between CGG repeat size and age at first manifestation of POI as a measurement for the severity of POI. No significant association could be identified. This finding is in contrast with the results of a study in Asian women, that suggested that having >38 CGG repeats was associated with younger age at onset of amenorrhea [35]. However, the number of CGG repeats in Asians has a different

distribution compared to other ethnicities, with a secondary peak of 34-36 repeats in addition to the most frequent peak of 30 or 31 repeats [41-44]. In the current study, the possibility of a non-linear relationship between CGG repeat length and age at diagnosis was excluded by applying 3-knot restricted cubic spline analysis.

The absence of an association between CGG repeats in the normal and intermediate range and POI in the current study is in accordance with the largest study published to date [36]. Non-replication of previous other studies could be explained by a number of reasons. Firstly, previous associations between CGG repeat lengths in the range up to 55 repeats and POI were found in relatively small studies with POI cases varying from 27 to 190 women. In the study by Bennett and colleagues in 366 POI cases no association with CGG repeats in this range was found. Possibly, the earlier found associations in the small studies could be considered false-positive.

Secondly, ethnic differences in the study populations could also be a cause for the inconsistent findings, since the distribution of CGG repeat lengths seems to differ significantly between different ethnic groups [45, 46]. In addition, possible effects of CGG repeat distributions on susceptibility to POI may vary between different ethnic groups [47] and therefore could not be picked up in our cohort of mainly Caucasian POI cases.

A third explanation may stem from the fact that susceptibility to POI is possibly influenced not by *FMR1* CGG repeats distributions alone, but by CGG repeat lengths in interaction with other genes and/or environmental factors. A recent study suggesting that the earlier found association between BRCA1 carriers and occult POI [48] is actually *FMR1*-mediated [49], strengthens this hypothesis.

The current study has some limitations that should be mentioned. Firstly, *FMR1* CGG repeat lengths in our POI cases (except the 55 POI cases from the Prospect cohort) and controls were genotyped in two different laboratories, which may lead to divergent results. However, random duplicates genotyped in both laboratories, showed no differences (data not shown). Also, when comparing CGG repeat lengths of the 320 POI cases included in the outpatient clinics of 16 Dutch fertility clinics with those of the 55 POI cases from the Prospect cohort, we found no statistically significant differences ($P = 0.67$).

Next, as mentioned above, distributions of CGG repeats may vary among different ethnical populations. In the current study, POI cases consisted of women with different ethnicities (although 84% of the POI cases were Caucasian), whereas our controls were all Caucasian. To assess whether possible associations between CGG repeats and POI were altered by the presence of non-Caucasian women in our POI cohort, a sensitivity analyses by excluding non-Caucasian POI cases (n = 60) was performed. Exclusion of non-Caucasian POI cases did not alter the results of our performed analyses (data not shown), except that the marginal statistically significant finding when comparing intermediate CGG repeats defined as 41-58 repeats between POI cases and controls disappeared (OR 0.7, 95% CI: 0.44 – 1.11, $P = 0.15$).

FMR1 premutation carrier ship has been consistently associated with POI during the last decade [27, 28, 31, 34, 36]. In *FMR1* premutation carriers gene transcription is significantly increased. The resulting increased *FMR1* mRNA levels probably contribute to the pathogenesis of FXTAS through toxic gain-of-function effect [50]. Up until now, a similar contribution of the *FMR1* premutation or intermediate repeat size to the pathogenesis of FXPOI has only been suggested [51]. However, *FMR1* mRNA transcript levels are highly variable in CGG repeat numbers at the lower end of the premutation range [52], and this variability does not correlate to the variance of CGG triplet numbers in women with POI [53]. Destabilization of the intermediate CGG stability in POI resulting from CGG repeats without AGG interspersions could be another potential biological pathway [31]. However, the finding that AGG interruptions do not seem to decrease *FMR1* mRNA levels pleads against this mechanism [54]. Clearly, the possible biological mechanism of intermediate (if any) and premutation sized CGG repeats in the pathogenesis of POI needs further study.

In conclusion, we observed no association between intermediate sized CGG repeats and POI compared to controls with an age at natural menopause above 40 years. Next, no statistically significant association between CGG repeat size and age at first manifestation of POI could be identified. Our results are in line with a previous study in 366 POI cases that found no association between CGG repeats up to 55 repeats and POI. Therefore, there seems limited value in the evaluation of normal- and intermediate *FMR1* repeat size in the diagnostic work-up of women affected by POI, or for prognostic purposes in women at risk for developing POI. Together with our earlier published data in which no association was found between normal and intermediate sized CGG repeats and age at natural menopause in a large cohort of Caucasian women, the role of *FMR1* CGG repeat sizes up to 55 repeats in the ovarian ageing process could be questioned.

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Chapter

Eight

Genome-wide association study in Primary Ovarian Insufficiency and Early Menopause

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Preliminary results

INTRODUCTION

Age at natural menopause varies roughly from 40 to 60 years with a mean of 51 years of age [1,2]. Early menopause (EM), occurring between age 40 and 45 years, affects around 6% of all women. For approximately 1% of women, the onset of menopause occurs before the age of 40 years [3]. This condition is referred to as primary ovarian insufficiency (POI) [2,4]. Besides an enormous effect on a woman's fertile lifespan, POI also has implications for general health, for example osteoporosis and cardiovascular health [4-6].

The diagnosis of POI is based on primary or secondary amenorrhoea together with elevated levels of follicle stimulating hormone (FSH >40 IU/l) before the age of 40 years [3]. POI can be caused by a number of monogenetic mutations, such as premutations of the fragile-X mental retardation 1 (*FMR1*) gene and mutations in the forkhead transcription factor L2 (*FOXL2*) gene, auto-immune diseases (most commonly auto-immune thyroid disease), or can be iatrogenic (i.e. after surgery, radiation- or chemotherapy). However, in most cases there is no conclusive factor causing POI (i.e. idiopathic POI) [4].

Whether idiopathic POI is part of the variation towards the lower end of the distribution of natural menopause or that it should be considered a separate genetic entity, is unclear. Over the last two decades, huge efforts have been made to discover the genetic variants that determine timing of menopause. Several candidate gene association studies, linkage studies and - more recent - genome wide association studies (GWAS) have identified a number of genetic variants underlying both variation of age at menopause and POI [7-17]. However, these genetic variants account for only a small fraction of the overall heritability in timing of menopause [16,17].

Recently a GWAS comparing cases with a self-reported age at menopause before 45 years with controls that reached menopause between 50 and 60 years of age was conducted [17]. This GWAS revealed four genome-wide significant loci associated with menopause before 45 years. Interestingly, all four of these loci were also identified in the GWAS in normal age at natural menopause, suggesting that early menopause and POI at least partly share a common genetic background [11,12,15,17]. No new genetic variants associated with menopause <45 years were detected. However, the early menopause cases in the GWAS by Perry and colleagues had considerable overlap with the samples used in the GWAS of normal menopausal age variation, and therefore the a priori chance of finding overlapping results was high [17].

In the current study, we conducted a GWAS in a very well-phenotyped cohort of European idiopathic POI and EM cases and compared these cases to controls with an age at natural menopause over 45 years. With our study we aim to contribute to the identification of genetic risk factors of POI and early menopause and thereby help elucidating further the hypothesis that POI is part of and shares the same genetic aetiology as the normal menopause distribution.

METHODS

A total of 966 POI and 115 EM cases from four different clinical center or research groups were included in the current study (table 1).

513 POI and 79 EM cases were included in **outpatient clinics of hospitals in the Netherlands** (participating in a nationwide, multicentre study of hypergonadotropic oligomenorrhoea/ amenorrhoea). All patients have been diagnosed with early ovarian ageing as determined by routine, standardized phenotyping according to a preset approach. POI was defined as: primary amenorrhea or at least 4 months of secondary amenorrhea before 40 years of age and an elevated basal FSH > 40 IU/l. EM was defined as: at least 4 months of secondary amenorrhea between 40 and 45 years of age and an elevated basal FSH > 40 IU/l. Phenotyping included a physical examination, assessment of physical and chemical signs of hyperandrogenism and/or insulin resistance, a transvaginal sonography for assessment of ovarian morphology and antral follicle count (2-5 and 6-10 mm diameter) and analysis of the pedigree premature ovarian ageing or early menopause. All patients were phenotyped in the same manner and all had DNA and blood serum samples taken after phenotyping was completed. All women with an abnormal karyogram (also including low 45,X/46,XX mosaicism) were excluded. Approval for this standard work-up including DNA sampling had been prior obtained from the Institutional Board for the Ethics of Research on Humans.

A total of 248 POI cases were recruited from the **Reproductive Endocrine Unit**, **Hospital St. Antoine in Paris, France**. Inclusion criteria were amenorrhoea for >4 months occurring before the age of 40 years or primary amenorrhoea, with an FSH serum level >40 UI/L. women were white and had a normal karyogram.

A total of 257 POI (n=237) and EM (n=20) cases were included in the **department of Medical Sciences, University of Milan, Italy**, Division of Endocrinology and Metabolic Diseases & Laboratory of Experimental Endocrinology. The POI status was defined as the natural cessation of ovarian function for a period of >6 months, before or at the age of 40 years, and FSH ≥ 40 IU/L detected on two different occasions. Subjects underwent complete medical assessment which included gynaecological and obstetric history. They also underwent clinical and gynaecological examination, ultrasound pelvic evaluation and karyotyping. All POI cases included in our study were white, were phenotypically normal and had a normal karyogram.

Finally, 63 women with a self-reported natural menopausal age younger than 45 years were included (menopause <40 years n=47 and EM n=16) from the **Prospect-EPIC cohort**. The Prospect-EPIC cohort is one of the two Dutch contributions to the European Prospective Investigation into Cancer and Nutrition (EPIC). The Prospect-EPIC study is coordinated by the Julius Center for Health Sciences and Primary Care, UMC Utrecht and comprises 17,357 healthy women aged 49-70 and living in Utrecht and surroundings. All participants were recruited through an

existing regional breast cancer screening project, to participate in the study between 1993 and 1997. At enrolment, all women underwent a physical examination and all participants filled out detailed questionnaires about dietary, reproductive and medical history. In addition, participants donated a 30-ml non-fasting blood sample, which was fractionated into serum, citrated plasma, buffy coat and erythrocyte aliquots of 0.5 ml each. Age at menopause was self-reported and questioned at the screening visit. Natural menopause was defined as 12 consecutive months of amenorrhea without other obvious reasons (i.e. surgical or chemical menopause). Women using hormone therapy during the menopausal transition were excluded.

Table 1 Study populations POI

Samples		Total number of samples	POI	EM
Dutch multi-center study in POI and EM,		513	434	79
Hospital St. Antoine in Paris, France		248	248	0
University of Milan, Italy	Division of Endocrin and Metab Diseases	67	67	0
	Lab. of Experimental Endocrinology	190	170	20
Prospect-EPIC cohort, The Netherlands		63	47	16
Total		1081	966	115

Controls

2214 Caucasian women with a self-reported age at natural menopause above 45 years were selected from the Rotterdam Study: RSI, RSII and RSIII to be included as controls. **Rotterdam Study I, II,** and **III** (**RSI, RSII, RSIII**) are ongoing prospective population-based cohort studies of Caucasian subjects aged 45 years and over, living in the Ommoord district of Rotterdam, the Netherlands. The study was designed to investigate the incidence and determinants of chronic disabling diseases in the elderly. Rationale and design have been described previously [18]. For RSI, all 10,275 inhabitants aged 55 years and over were invited for baseline examination between August 1990 and June 1993, of those, 7,983 participated. In 1999, 3,011 participants (out of 4,472 invitees) who had become 55 years of age or moved into the study district since the start of the study were added to the cohort (RSII). In 2006, a further extension of the cohort was initiated in which about 6,000 subjects aged 45–54 years, living in the Ommoord district, were invited, of which 3,932 participated (RSIII). Questionnaires including menopause related questions were filled out by study nurses during the home interview, while blood samples were taken of over 70% of the participants at the research center. The Rotterdam Study was approved by the medical ethics committee of the Erasmus University Medical School, and written informed consent was obtained from each subject.

GWAS genotyping and statistical analyses

We genotyped 1081 POI and EM cases using the Illumina Human 660W Quad array (Illumina, San Diego, CA, USA). The controls were genotyped with the Illumina 550K (RS1 and RS2) and Illumina 660W Quad array (RS3) (Illumina,, San Diego CA, USA). All genetic experiments were carried out in the Genetics Laboratory at the Department of Internal Medicine of the Erasmus Medical Centre in Rotterdam, the Netherlands according to the manufacturer's protocol.

After genotyping, samples with a call rate < 97.5% were excluded from further analysis. Genotyping quality control checks based on duplicate sample genotyping, SNP call rate (>95%), Hardy-Weinberg equilibrium (*P*-value <$1x10^{-6}$), Mendelian inconsistencies, and sex mismatch. After quality control 479,965 SNPs were left for further analysis. Identity by state (IBS)-clustering, identity by descent (IBD)-clustering and removal of X-chromosomal abnormalities left 901 samples (791 POI and 110 EM samples) for final analysis.

IBS-clustering showed that the Dutch, French and Italian white Caucasian samples did not cluster together. Therefore, we applied the linear model to the binary phenotype as implemented in the EMMAX software [19] to correct for population stratification. Inflation of the type I error rate was determined using a quantile-quantile (Q-Q) plot (figure 1). A *P*-value <$5x10^{-8}$ was considered genome-wide significant. However, as power is low in this study, all SNPs with a *P*-value <$0.5x10^{-5}$ will be reported and selected for further replication.

RESULTS

We present the data of the discovery GWAS in 791 POI and 110 EM cases of European descent compared to 2209 controls with an age at menopause >45 years.

No additional population stratification was observed, as can be seen from the QQ-plot, where no early deviation from the diagonal was seen (figure 1). No SNPs reached genome-wide significance (*P*-value < $5x10^{-8}$) (figure 2). The strongest association signals identified were rs2384687 (*P*-value = $8.28x10^{-7}$) near the *BR Serine/Threonine Kinase 1* (*BRSK1*) gene on chromosome 19, rs7939346 (*P*-value = $2.35x10^{-6}$) located in the intron region of the *NEL-LIKE 1* (*NELL1*) gene on chromosome 11, and rs2648718 and rs11186505 both located in the intron region of the *PolyComb Group ring Finger 5* (*PCGF5*) gene on chromosome 10 (*P*-value resp. $4.32x10^{-6}$ and $4.89x10^{-6}$). A total of 40 SNPs from 30 loci with *P*-values <$5x10^{-5}$ are presented in table 2 to be followed up in a replication cohort in a subsequent study.

Figure 1

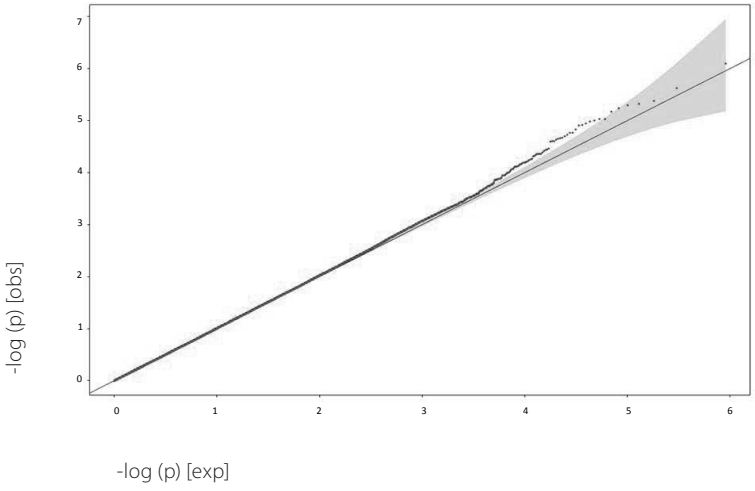
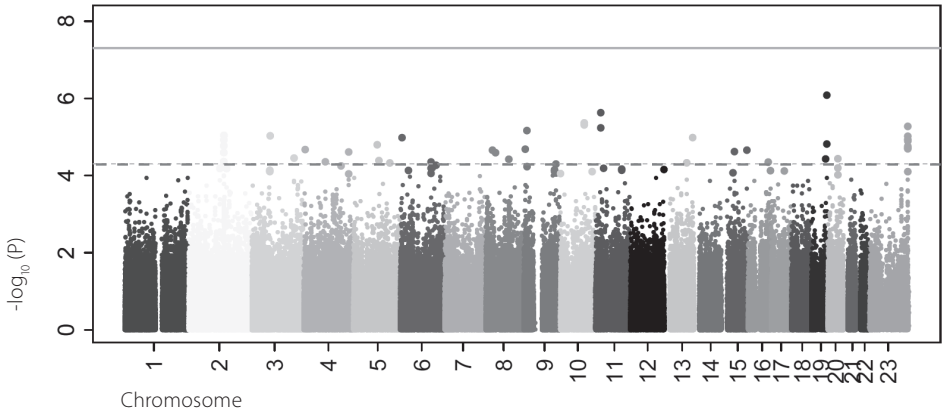


Figure 2 Manhattan Plot



Manhattan plot showing P-values for the POI and early menopause cases (n=901). The genom-wide Bonferroni adjusted threshold of $5x10^{-8}$ is marked by the top grey line, and the dashed grey line marks a P-value of $5x10^{-5}$.

Table 2 SNPs with a P-value <5x10^{-5}

SNP	Chr	Position	Gene	Minor allele	MAF	OR[a]	95% CI	Pvalue
rs2384687	19	60523000	BRSK1/ TMEM150B	C	0,376	1,049	1.03 - 1.07	8.28x10^{-7}
rs1172822	19	60511657	BRSK1	T	0,369	1,044	1.02 - 1.06	1.52x10^{-5}
rs722036	19	55922302	intergenic	T	0,016	1,046	1.02 - 1.07	3.71x10^{-5}
rs7939346	11	21313890	NELL1	T	0,309	0,950	0.93 - 0.97	2.35x10^{-6}
rs10833509	11	21311726	NELL1	A	0,303	0,952	0.93 - 0.97	5.81x10^{-6}
rs2648718	10	93001750	PCGF5	C	0,244	1,053	1.03 - 1.07	4.32x10^{-6}
rs11186505	10	92972888	PCGF5	A	0,255	1,051	1.03 - 1.07	4.89x10^{-6}
rs222347	X	149519155	MTM1	T	0,071	1,084	1.05 - 1.12	5.32x10^{-6}
rs1533423	X	149543123	MTM1	T	0,414	0,957	0.94 - 0.97	9.58x10^{-6}
rs5925391	X	149516226	MTM1	C	0,416	0,958	0.94 - 0.97	1.16x10^{-5}
rs3747313	X	149646677	MTMR1	T	0,431	1,043	1.02 - 1.06	1.28x10^{-5}
rs222378	X	149611773	MTMR1	C	0,511	0,959	0.94 - 0.98	1.74x10^{-5}
rs222364	X	149529777	MTM1	C	0,372	1,043	1.02 - 1.06	1.91x10^{-5}
rs222410	X	149577161	MTM1	G	0,530	0,959	0.94 - 0.98	2.03x10^{-5}
rs10124086	9	12539675	intergenic	A	0,192	1,056	1.03 - 1.08	6.83x10^{-6}
rs1929994	9	6214308	IL33	G	0,021	1,135	1.11 - 1.15	2.07x10^{-5}
rs1446585	2	136123949	R3HDM1	C	0,273	1,053	1.03 - 1.08	9.09x10^{-6}
rs1561277	2	135808531	ZRANB3	A	0,286	1,052	1.03 - 1.07	1.19x10^{-5}
rs6730157	2	135623558	RAB3GAP1	G	0,315	1,050	1.03 - 1.07	1.70x10^{-5}
rs7570971	2	135554376	RAB3GAP1	A	0,321	1,049	1.03 - 1.07	2.53x10^{-5}
rs2166480	2	135353808	ACMSD	A	0,408	1,044	1.02 - 1.06	4.35x10^{-5}
rs17008474	3	71432065	FOXP1	T	0,026	1,125	1.07 - 1.18	9.33x10^{-6}
rs9883680	3	163244321		A	0,161	1,055	1.03 - 1.08	3.52x10^{-5}
rs443079	13	108303141	---	C	0,482	0,958	0.94 - 0.98	1.04x10^{-5}
rs9547445	13	85403212	intergenic	G	0,264	0,954	0.94 - 0.97	4.67x10^{-5}
rs9505270	6	7688662	BMP6	A	0,243	1,051	1.03 - 1.07	1.04x10^{-5}
rs17083163	5	92793434	FLJ42709	A	0,036	0,883	0.83 - 0.93	1.59x10^{-5}
rs1542520	5	99376285	intergenic	C	0,371	1,043	1.02 - 1.06	4.09x10^{-5}
rs2338874	5	141447110	intergenic	C	0,306	0,956	0.94 - 0.98	4.71x10^{-5}
rs7681358	4	6817771	intergenic	A	0,087	0,928	0.90 - 0.96	2.12x10^{-5}
rs11721460	4	174176963	GALNTL6	T	0,369	0,957	0.94 - 0.97	2.45x10^{-5}
rs7679817	4	84339729	intergenic	A	0,041	1,094	1.05 - 1.14	4.40x10^{-5}
rs12437601	15	96504853	intergenic	C	0,073	1,080	1.04 - 1.11	2.19x10^{-5}
rs3098183	15	48593761	intergenic	T	0,338	0,955	0.93 - 0.97	2.39x10^{-5}
rs1048527	8	26285610	PPP2R2A	A	0,131	1,083	1.04 - 1.12	2.20x10^{-5}
rs2517388	8	38096889	ASH2L	G	0,194	0,949	0.93 - 0.97	2.56x10^{-5}
rs1477917	8	89199069	MMP16	G	0,050	1,089	1.05 - 1.13	3.78x10^{-5}
rs6072317	20	39314931	ZHX3	T	0,144	1,058	1.03 - 1.08	3.66x10^{-5}
rs4946385	6	119348111	FAM184A	C	0,345	1,043	1.02 - 1.06	4.44x10^{-5}
rs4267316	16	78459719	interenic	C	0,053	1,086	1.04 - 1.12	4.47x10^{-5}

a ORs were calculated for the minor allele

DISCUSSION

In the current study, no SNPs reached genome-wide significance (P < 5x108). However, 40 SNPs had a P-value <5 x 10^{-5} and are therefore promising genetic variants for follow up in a larger cohort of women with POI or EM.

Even though no genome-wide significant SNPs were discovered in our GWAS, our study did yield some interesting candidate genes for POI and EM. The strongest association was seen in rs2384687 which is located near the *BRSK1* gene on chromosome 19. Interestingly, this locus was previously identified in GWAS in natural menopause above 40 years [11,15]. Furthermore, in a recent GWAS in women with a self-reported early menopause (i.e. menopause before 45 years) this locus was also identified [17]. In humans, the highest expression of *BRSK1* is located in brain where it is required for neuronal polarization [20]. A possible biological mechanism for the role of the *BRSK1* gene in ovarian ageing is unidentified as yet.

Two other strong associations were found in rs7939346 and rs10833509 on chromosome 11, located within the *NELL1* gene. *NELL1* plays a key role in ossification during early development and in adult tissues and has been shown to repress adipogenic differentiation [21]. Genetic variants in the *NELL1* gene have been associated with Crohn's disease [22]. Next, on chromosome 10, two SNPs, rs2648718 and rs11186505, within the *PCGF5* gene showed a strong signal. Finally, two other potentially interesting candidate genes are the *Myotubularin Myopathy 1* (*MTM1*) and *Myotubularin Myopathy-Related Protein 1* (*MTMR1*) located on the X chromosome. Seven variants within these genes had P-values < 2x10-5. The *MTM1* and *MTMR1* genes are involved in muscle cell differentiation [23,24]. Besides *BRSK1*, none of the above described loci were previously associated with timing of menopause nor with susceptibility for POI. Replication of these loci in additional POI cases is necessary to determine whether they in fact play a role in risk of POI.

In a previous GWAS from our group in 99 women with a clinical diagnosis of POI compared to 235 controls, one SNP in the *ADAMTS19* gene (SNP rs246246) was borderline genome-wide significant (OR: 4.31 95% CI: 2.3-7.9, P-value = 5.98 x 10^{-7}) and was therefore suggested to play a role in POI [13]. However, after replication in a small-scale replication study in 60 POI cases and 90 controls the association attenuated in joint analysis of discovery and replication study (OR: 2.75 95% CI: 1.7-4.6, P-value = 4.05 x 10^{-5}). In our discovery GWAS, rs246246 or 2 SNPs with an R^2 <0.8 of rs246246, showed no strong association with POI and EM (rs246246, OR: 1.064 95% CI: 1.03-1.10, P-value = 1.52 x 10^{-3}). Consequently, our results could neither substantiate nor exclude a role for *ADAMTS19* in POI.

In a recent meta-analysis of GWAS in 3493 cases with a self-reported early menopause (< 45 years) compared to 13,598 controls [17], two other promising candidate genes were identified. In chromosome 1, rs1867631 located in the *SGIP1* gene and rs1473307 near the NYAP2 gene at chromosome 2 were borderline genome-wide significant (P-value = 2.42 x 10^{-7} and 3.31 x 10^{-7}),

although after follow up in replication cohorts, neither of the SNPs reached genome-wide significance (P-value = 0.31 and 0.68). We could not confirm an association for rs1867631 and rs1473307 with POI and EM in the current study (P-values respectively: 0.95 and 0.19).

The fact that we were not able to detect any genome-wide significant SNPs could well be due to a lack of power caused by limited sample size, even though this is the largest GWAS conducted in a well-phenotyped cohort of POI and EM cases to date. Strict correction for multiple testing in GWAS decreases the false-positive rate, but at the same time also increases the false-negative rate. Large study populations are thus needed to detect associated genetic variants with small effects. In this regard, the foremost problem is to gather a large sample of POI cases since the prevalence of POI is low. By genotyping the loci that that showed an association with POI and EM in the current study, albeit not genome-wide significant (with P-values $<5x10^{-5}$), in a subsequent study in POI and EM cases, we aim to increase power to detect real effects.

Since long, the question exists whether POI is part of and shares the same genetic aetiology as the normal menopause distribution (I.e. menopause between 45 and 60 years). Because our strongest association signal is the same as a previously identified locus in normal menopause, this strengthens the hypothesis that normal menopause, POI and EM at least share part of the same genetic background. The study in early menopause by Perry et al. referred to above, also supports this hypothesis [17]. However, other strong association signals identified in the current study have not been reported in normal menopause before. If these loci indeed proof to be associated with POI in a subsequent replication study, this could indicate a specific role for these loci in POI.

When searching for genetic variants underlying POI, however, it could be that specific genetic factors underlying POI are concealed and not picked up when studying women with an age at menopause under 45 years, due to too much overlap with menopause in the normal range. In fact, the study by Perry which included 3493 cases with menopausal onset before 45 years, only 1368 women had a reported age at menopause under the age of 40. The study by Perry et al. could have lacked the power to detect true genetic variants underlying POI, as well as the current study. Also, since POI can arise from a number of known causes (e.g. monogenic, iatrogenic and auto-immune causes), it is important to assess POI cases according to standard definitions in order to filter out non-idiopathic POI cases and thereby obtaining a homogenic cohort to increase power to detect underlying genetic loci of POI.

In conclusion, although we did not identify new genetic loci in our GWAS, our data supports the evidence that POI, EM and normal menopause share at least a proportion of their genetic aetiology. It therefore seems likely that POI and EM cases are part of the variation towards the entire distribution of natural menopause. A number of potentially interesting specific loci for POI and EM were identified, including *NELL1*, *PGCF5*, *MTM1* and *MTMR1*. It is pivotal that these loci are replicated in a larger cohort of POI cases before any conclusions can be drawn on their actual involvement in POI and EM.

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Chapter

Nine

General discussion

Timing of menopause greatly impacts women's fertility and general health. In this thesis we aimed to identify and validate genetic variants that are involved in ovarian ageing. We studied the association of genetic variants with timing of natural menopause in a general population. In addition we studied cases of primary ovarian insufficiency (POI) and early menopause (EM) compared to controls with a normal age at natural menopause (> 45 years) in order to identify genetic variants that are associated with these conditions.

In the chapter at hand, we discuss the current knowledge of genetic variants involved in timing of menopause and risk of POI in relation to the findings of this thesis. Next, recommendations for future genetic research in variation of menopausal age are proposed. Finally, we elaborate on possible implications for clinical practice for an individual woman.

GENES INVOLVED IN NATURAL MENOPAUSE - WHAT IS THE CURRENT STATUS?

In the search of genetic variants involved in timing of menopause in the last two decades, three approaches were used: linkage analysis, association studies on candidate genes and genome-wide association studies (GWAS). The roles of the latter two approaches, their contribution to the search of menopause-associated genetic variants and the utilization of these approaches in the studies performed in this thesis are discussed below.

Candidate gene association studies on age at natural menopause

A candidate gene association study is a hypothesis-based study in which candidate genes are chosen based on knowledge of the physiology of the investigated trait. However, little is known about the mechanisms underlying variation of menopause, which pinpoints exactly the difficulty of this approach when trying to find genetic variants associated with timing of menopause.

It is known that coronary artery disease (CAD) or, more generally, cardiovascular disease (CVD) is related to age at menopause. First, an increased risk of CVD is associated with an earlier onset of menopause [1]. This is substantiated by the fact that smoking during menopausal transition, elevated blood pressure, a high body mass index (BMI) and type 2 diabetes (T2D), which are important risk factors for cardiovascular disease, incline towards early vascular damage and a younger age at menopause [2-4].

Secondly, and in contrast to the fact that CVD may influence timing of menopause, women experiencing an early menopause are at increased risk of cardiovascular disease (CVD) later in life [5-10]. It was proposed that this increased risk of CVD after (early) menopause is due to a decline of circulating estrogens after menopause, since estrogens have beneficial effects on endothelial function and blood lipids. Therefore, in the search of genetic variants underlying

menopausal age using candidate gene association studies, emphasis has been put on two major biological pathways: vascular-function related genes and genes involved in steroid pathways (reviewed in chapter 2).

Vascular-function related genes

Studies that focused on vascular-related candidate genes are based on the hypothesis that vascular ageing and reproductive ageing are connected as described above. To date, the vascular related genes *APOE*, *F5* and *F7 and MTHFR* have been associated with menopausal age, albeit not consistently [11-15]. A candidate gene association study in 12,723 naturally postmenopausal women found no statistically significant associations in 2072 SNPs in 32 genes in the thrombophilia and vascular homeostasis pathway, including *APOE* and *F5*, and timing of natural menopause [16].

As shown in chapter 5, we investigated 18 SNPs that were genome-wide significantly associated with CAD in earlier GWAS, in relation to age at menopause in nearly 50,000 women. Next to the CAD associated SNPs, we extended our search of vascular related genetic variants by studying 28 genome-wide associated T2D SNPs, since T2D is recognized as a major risk factor for CVD and early menopause has been reported to be associated with the risk of T2D [17,18]. Because these 46 SNPs have been associated with CAD and T2D in GWAS and replicated afterwards, a true association with CAD can be assumed, which cannot always be said for the supposed vascular related genes used in earlier candidate gene studies. We therefore believe that these genome-wide associated SNPs in CAD are suitable to test the hypothesis that vascular ageing is causally related to timing of menopause as a proxy for reproductive ageing.

In our study, no statistically significant associations between the SNPs in CAD and T2D, and menopausal age were found. This suggests that vascular ageing is not causally related to timing of menopause. Similar large studies investigating the association of menopause SNPs on CAD risk are needed to test whether the reverse association is true. Another possibility is that CAD and menopausal timing are actually two expressions of general ageing. To test this hypothesis one could design studies that test the association of general ageing SNPs with both CAD and age at natural menopause.

Genes involved in steroid pathways

Among the studied candidate genes involved in steroid pathways, estrogen-related genes take up for the majority of genes that were investigated for their role in timing of menopause in this pathway (chapter 2). These estrogen-related candidate genes were chosen based upon the effects of estrogen on growth, differentiation and function of reproductive tissues. However, a well-founded biological rationale as to how these effects of estrogen would influence the ovarian ageing process, and thus timing of menopause, is lacking. The most commonly reported steroid associated genes that may influence variation in menopausal age are *CYP1B1*, *CYP19A1*,

ESR1, *ESR2*, *FSHB*, *HSD17B1*, *LHCGR*, *PGR* and *SRD5A1*, although again, these associations have not all been consistent [19-29]. The inconsistent results of the different studies and the fact that most associations were found in small study populations (typically in cohorts of several hundreds of women), have not been reassuring of an irrefutable role of these genes in determining age at menopause. However, in a large-scale candidate gene study in 12,723 women, an excess of statistically significant gene associations for age at natural menopause was reported in 38 genes in the steroid-hormone metabolism and biosynthesis pathway [16]. These data could suggest a role in timing of menopause of genes involved in steroid pathways, although a clear foundation for the assumed involvement of these genes on timing of menopause still remains absent.

Genes involved in formation and depletion of the ovarian follicle pool

A pathway that has been underexposed in the search of genes underlying menopausal age was, as far as we are concerned, the formation and subsequent wastage of the ovarian follicle pool. Women reach menopause when their ovarian follicle pool becomes exhausted and is insufficient to maintain menstrual cycles [30]. The primordial follicle pool is formed during fetal life and contains a peak of 6 to 7 million oocytes at 20 weeks of gestation [31]. Subsequently, the primordial follicle pool decreases dramatically due to atresia, until there are approximately 300,000-400,000 remaining oocytes at birth [32,33]. Constant recruitment of primordial follicles into the growing follicle pool takes place throughout life, which is referred to as initial recruitment. During reproductive years ongoing growth of follicles into antral stage and loss of follicles due to atresia lead to gradual decrease of the original follicle pool [34]. Finally, when the ovarian follicle pool becomes exhausted, containing less than 1000 follicles, women face menopause [35]. Genes involved in either the regulation of the quantity of follicles that are founded during embryogenesis, or genes that influence the rate of depletion of the follicle pool are most likely also associated with timing of menopause.

One of the factors playing an important role in initial follicle recruitment is the anti-Müllerian hormone (AMH). A study in *AMH* knock-out mice showed that in the absence of AMH, primordial follicle recruitment is accelerated and consequently the primordial follicle pool is exhausted at a faster rate [36]. This inhibitory effect of AMH on primordial follicle recruitment was confirmed in in vitro studies [37,38]. In this regard, the *AMH* gene and its specific receptor (*AMHR2*) gene could be of interest when searching for genes underlying menopause. In chapter 3 we sought to replicate a previously reported association between the *AMHR2* gene and age at natural menopause [39]. Besides the *AMHR2* gene, we extended our search for genes underlying variation of menopausal age with four additional genes involved in initial follicle recruitment: *AMH*, *BMP15*, *GDF9* and *FOXL2*. In this study we found an association between *BMP15* and timing of menopause, although the association was merely driven by the women with an age at menopause before 40 years (POI). More importantly, we showed that the *AMHR2* gene is associated with menopausal age in interaction with parity, confirming an earlier finding [39]. In a study that tested 16 SNPs in the

AMHR2 gene for association with onset of menopause, no statistically significant associations were found [16]. Unfortunately, this study did not investigate the interaction with parity. As demonstrated in many epidemiological studies, parity is related to timing of menopause, with parous women being older at onset of menopause compared to nulliparous women [2,40]. Still it remains unclear how the relation between age at menopause and parity is influenced by the *AMHR2* gene. Possibly, *AMHR2* is actually a fertility-related gene, or the expression of this specific receptor subtype is altered by changes in hormone levels. The latter could result in a stronger inhibition of primordial follicle recruitment during pregnancy resulting in a later menopause.

It is likely that complex traits - like menopausal age - are influenced not through single genetic variants alone, but also through gene-gene and gene-environment interaction [41,42]. Our finding of an association between *AMHR2* gene and age at menopause in interaction with parity fits this hypothesis well. In chapter 4, we extended our previous study in five genes involved in initial follicle recruitment, by searching for pair-wise interactions between the SNPs in these five genes and by further exploring gene-environment interactions between these genes and parity, smoking and BMI. In this study, we were not able to validate the interaction with parity using a model-based multifactor dimensionality reduction (MB-MDR). This non-replication could be due to different statistical methods used, to the very strict correction for multiple testing used with MB-MDR or the found interaction between the *AMHR2* gene and parity was actually a false positive result. We did however find an interaction between SNPs in the *AMH* and *AMHR2* gene in association with menopausal timing, suggesting a role of the *AMH* signaling pathway in this trait. More research is needed to validate this interaction, although a major challenge in finding these gene-gene and gene-environment interactions is the extreme high number of subjects that are required to identify these complex interactions.

Genes involved in Primary Ovarian Insufficiency

As it is conceivable that idiopathic POI cases represent the left tail of the entire menopause distribution, genes found in POI could also have an influence on normal age at menopause. It may well be possible that rarer variants with larger effects, which are not well captured by the SNP arrays used in the GWAS, are also involved in POI [43]. Also, it has been proposed that while severe gene mutations could be associated with the extreme phenotype of natural menopause (i.e. POI), the more common and concurrently less extreme variants of such gene mutations may influence normal variation in menopausal age [44].

The *FMR1* gene is a plausible candidate gene for timing of menopause since consistent evidence exists that excessive CGG repeats (over 55 repeats; i.e. premutations) on the *FMR1* gene are associated with POI. This is referred to as FXPOI [45-49]. It has been hypothesized that CGG repeats in the normal and intermediate range (up to 55 repeats) could also influence timing of menopause and we aimed to test this hypothesis [50,51]. First, as shown in chapter 6, we found no

association between the number of *FMR1* CGG repeats in the range up to 55 repeats and timing of natural menopause. Next, in chapter 7 we studied the role of *FMR1* CGG repeats in the normal and intermediate range in association with POI compared to controls with an age at natural menopause above 40 years. Again, we found no association, suggesting that *FMR1* CGG repeats in the range up to 55 repeats are not associated with POI and timing of menopause. Together, these results do not indicate a role for *FMR1* CGG repeats in the normal and intermediate range and onset of menopause. It is unknown why women carrying the premutation of the FMR1 gene are susceptible to POI, since a solid biological rationale for the increased risk for POI in these women is absent. A recent study in a *FMR1* mouse model reported that mice carrying the permutation initially have a normal primordial follicle pool but show a more rapid decline in follicle number with age than mice without the premutation [52]. Further research on the molecular basis of this presumed accelerated depletion of the follicular pool in premutation carriers may ameliorate our understanding of the impact of all *FMR1* CGG repeat numbers in ovarian ageing.

Overall, some consistent associations with timing of menopause were found using candidate gene association studies, mainly in genes involved in steroid pathways and in genes involved in formation and depletion of the ovarian follicle pool. Whether or not, since the wide availability of GWAS, candidate gene association studies will still be of importance in the search of genes involved in menopausal timing will be discussed hereafter.

GENOME-WIDE ASSOCIATION STUDIES IN AGE AT NATURAL MENOPAUSE

In contrast to a candidate gene study, a GWAS is an agnostic search for associations between several 100,000s common genetic variants across the entire genome and a trait or disease. This study approach became generally applied as from the mid-2000s. To date, three GWAS in age at menopause have been conducted that together have identified 17 loci that underlie this trait. In 2009, four loci associated with timing of menopause were reported in two simultaneously published GWAS [53,54]. A recent large meta-analysis of 22 studies with available GWAS data, in a total of nearly 39.000 women was performed and identified an additional 13 loci [55]. The 17 associated loci operate in diverse pathways including neuroendocrine pathways of ovarian function regulation, immune function and DNA repair. This suggests that the process of ovarian ageing is involved in both somatic and germ line ageing, although the exact contribution of these pathways to menopause remains unclear [55]. More in-depth analysis of the associated loci of GWAS will be necessary for the identification of the actual causal variants and gene. Further studies on the exact biological mechanisms (e.g. in animal knock out models) are needed to fully understand in what way these causal variants contribute to the determination of age at menopause.

None of the genetic variants that were associated with menopause in candidate gene studies, or loci in linkage disequilibrium (LD) with these genetic variants, showed overlap with the loci identified in the three conducted GWAS. As addressed in chapter 2, a number of reasons can be proposed for the fact that genetic variants associated with onset of menopause in candidate gene studies were not detected in GWAS. The major reason for non-replication of associations found in candidate gene studies in GWAS, is the strict correction for multiple testing in GWAS. By controlling the false positive rate in GWAS, the false negative rate will also increase and actual associated variants with small effect sizes can therefore not be detected in GWAS.

Is there still room for the candidate gene study approach in finding genetic variants underlying onset of menopause since the general availability of GWAS? We believe that there is. First, numerous loci underlying many traits and diseases are now identified by GWAS. These loci can be further explored using a study design known as 'Mendelian randomization' [56,57]. Mendelian randomization provides an opportunity to test causality between a risk factor with a disease or trait. By investigating the loci that were confirmed to be associated with a risk factor in GWAS with the trait or disease of interest, one can test whether that risk factor could causally be related to this trait or disease. As described previously, we applied this study design in chapter 5.

Secondly, by selecting an appropriate candidate gene, one is able to find an association with a large effect that would not have been picked up in GWAS because of low frequency of the mutation in the general population. A recent study proving this principle is a study in the *BRCA1* and *BRCA2* genes, which are both genes involved in DNA Double-Strand Break (DSB) repair. DNA DSB repair is identified as a cause of ageing in mouse and human oocytes [58]. Since *BRCA1* and *BRCA2* are key DNA DSB repair genes, these are plausible candidate genes for ovarian ageing. Recently, a case-control study was published in which the *BRCA1* and *2* gene were found to be associated with timing of menopause, showing that white women (n = 382) carrying the *BRCA1* or *BRCA2* gene reach natural menopause a striking 3 to 4 years earlier than women without that mutation (n = 765) [59]. These results were confirmed in 908 matched pairs of *BRAC1* and *2* carriers and noncarriers, although the reported difference in age at menopause was smaller (1.5 and 1.1 years earlier menopause in respectively *BRCA1* and *BRCA2* carriers compared to controls) [60].

GENETICS IN POI - COULD POI BE SEEN AS A CONTINUUM OF NORMAL MENOPAUSE?

Although POI can be caused by a number of monogenetic mutations and auto-immune diseases or can be iatrogenic, in most POI cases no single cause can be identified (idiopathic POI). Today, idiopathic POI is considered a complex trait. In this respect, the question whether POI is a continuum of normal menopause, representing the extreme left tail of the whole menopausal

age distribution, or is a separate entity, is still unanswered. The last couple of years, evidence has grown that POI, early menopause (EM, menopause between 40 and 45 years) and menopause in the normal range (>45 years) at least partly share the same etiology [43,44,61]. A recent meta-analysis of GWAS in 3493 cases with a self-reported early menopause (< 45 years) compared to 13,598 controls (age at menopause between 50 and 60 years), identified four SNPs that were all previously discovered in GWAS on natural menopause between 40 and 60 years [43]. In this meta-analysis, it was demonstrated that the joint effect of multiple genes involved in determining the age at normal menopause also affect menopause before the age of 45 years [43].

Though preliminary, the results of our GWAS in 901 well-phenotyped POI and EM cases as described in chapter 8 did not identify any genome-wide significant SNPs associated with POI and EM compared to controls. However, the most significant SNP (rs2384687), with a P-value of $8.1x10^{-7}$ was previously reported to be associated with age at menopause [53]. This finding strengthens the evidence that genes underlying natural age at menopause also influence POI and EM. However, we also found some suggestive evidence for an association with loci that were not previously detected in timing of normal menopause, suggesting that these loci play a specific role in POI. The fact that we were not able to detect any genome-wide significant SNPs is probably due to a lack of power caused by limited sample size. In this regard, the foremost problem is to gather a large cohort of POI cases since the prevalence of POI is low. In 2004, the Dutch POI consortium was established and consists of 14 participating centers across the Netherlands. This consortium currently has at its disposal a cohort of approximately 600 POI cases and is one of the world's largest POI cohorts. However, in order to reach sufficient power for genetic association studies, more POI cases are needed. For that reason, international collaboration is required to form a large enough cohort. Hence, for our GWAS we collaborated with groups in Italy and France.

The abovementioned studies show that genes underlying POI, as an extreme of normal menopausal age, may also influence menopause variation in the general population. So, it seems likely that idiopathic POI cases are part of the variation towards the entire distribution of natural menopause. It is unclear, though, to what extent the genetic background of POI and that of normal menopause show an overlap.

MISSING HERITABILITY - PROPOSED FUTURE RESEARCH

Although heritability estimates for age at menopause are high (up to 85%), only between 2.5% and 4.1% of the population variation in natural menopausal age is explained by the 17 loci associated with timing of menopause from the performed GWAS [55]. The conducted candidate gene association studies in menopausal age probably cannot contribute much to this figure. Given the high estimates for heritability of menopausal age, it could be that many more genetic

variants are yet to be discovered (referred to as missing heritability). However, it might well be that that heritability in menopausal age is in fact overestimated. Heritability estimates cannot be used to assess the magnitude of the genetic contribution to a complex trait since the components of the variance, i.e. genes and environment, are often not independent, but interactive (i.e. epistasis and gene-environment interactions) [62-66]. In other words, one should not expect that because only around 4% of the variation in menopausal age can be explained by the associated genetic variants found so far, there are still genetic variants accounting for the remaining 81% of heritability left to be found when assuming the total heritability is 85%. Nevertheless, in order to find the missing heritability in complex traits, like age at menopause, a number of strategies have been proposed. Firstly, it is expected that common genetic variants (minor allele frequency (MAF) >5%) have small effects sizes and thus individually explain only a small part of the variance in complex traits. Because of strict correction for multiple testing (the p-value threshold for a significant association in GWAS is $5x10^{-8}$), large study populations are needed to detect associated genetic variants with small effects. The largest meta-analysis of GWAS in menopausal age conducted so far, containing nearly 39.000 women, identified 17 loci with effect sizes ranging from 0.167 years to 0.971 years [55]. So, theoretically a GWAS in over 40.000 women would be able to detect additional genetic variants with effect sizes smaller than 0.167 years (8.7 weeks). It is also possible that the effects of single genetic variants are too small to be picked up in GWAS even in very large cohorts, since strict correction for multiple testing decreases the false-positive rate, but at the same time also increases the false-negative rate. This implies that it might be possible that we cannot detect common variants on an individual basis, whereas the genetic effects predicted by heritability estimates may be explained by the joint effect of numerous common variants with vary small effect sizes [65].

Secondly, genetic association studies have focused on common variants based on the assumption that common variants underlie common traits. In addition to these common variants, a significant proportion of the genetic variants influencing complex traits may consist of rare variants (with MAF between 0.1 and 5%), known as the rare variant hypothesis [67,68]. Up until a few years ago, association studies were not capable of finding these rare variants. However, since the recent release of the 1000 Genomes Project, that captures up to 98% of SNPs with a frequency of 1%, detection of rare variants in GWAS have become feasible [69,70]. Also, the number of genetic variants in LD with each GWAS signal is over 2 times greater in the 1000 Genomes Project data compared to the HapMap data. So, by imputation of existing GWAS with the 1000 Genomes Project data, new associations of both rare and common variants could be detected [71,72]. Although for the latest release of the 1000 Genomes Project already 1092 individuals from 14 populations worldwide were sequenced, for the final phase of the project a further 1500 individuals will be sequenced. This will probably provide us with even more data on genetic variants in the human genome and thus could lead to the identification of more genetic variants underlying variation

of age at menopause. Also, next-generation (or high-throughput) sequencing technologies could be used to detect rare variants [73]. As the technologies of complete genomic sequencing are developing rapidly and becomes more affordable, it will become feasible to apply next generation sequencing technologies on large study cohorts. On the condition of computationally efficient methods to analyze the whole-genome sequence data, it will be elucidated to what extend rare variants contribute to what is now considered missing heritability. Furthermore, candidate gene association studies can contribute to the identification of rare variants in complex traits, as was demonstrated by an association study between the *BRCA1* and *2* genes and timing of menopause, described above [57].

Next, copy number variations (CNVs), which are structural genetic variants including insertions, deletions and duplications, are believed to contribute to heritability of complex traits [74,75]. In the 1000 Genomes Project, in addition to 38 million SNPs, 1.4 million short deletions and insertions, and over 14.000 larger deletions are mapped [70]. This enables researchers to identify CNVs in GWAS. However, it is believed that common CNV's will not likely contribute much to the 'missing heritability' because common CNVs are in LD with SNPs [76-78]. Therefore, identified loci in GWAS would have already indirectly screened for the potential effect of CNVs [77]. However, it is possible that common CNVs that have previously not been genotyped and were poorly tagged have a much higher effect on disease risk than the well-tagged common CNVs. To assess the actual contribution of CNVs to the missing heritability, further large-scale association studies that directly capture CNVs are needed [77].

Finally, epigenetics may help us to elucidate genetic factors underlying complex traits. Epigenetics focuses on heritability that is not related to DNA sequence and that is involved in regulation of gene expression, like methylation of cytosine nucleotides at CpG sites and histone protein modification. In contrast to germline alterations of the DNA sequence, epigenetic scripts can change in response to environmental exposures, such as diet or hormones. Epigenetic variation may help to explain the quantitative nature of complex traits and diseases as well as the role of environment in their development [79,80].

IMPLICATIONS FOR CLINICAL PRACTICE

After two decades of genetic research in order to find genes underlying the variation of age at menopause, a number of genetic variants have been identified. Although the field of genetics is rapidly developing and it is to be expected that still more genes will be discovered in timing of menopause, emphasis could now be put on contemplating what the meaning of these already identified genes in the underlying biological mechanisms of age at menopause exactly is. Studies on gene expression of the identified genetic variants in specific tissues (e.g. ovaries), and studies

on knock out animal models could help elucidating the exact effect of these genetic variants on ovarian ageing.

A better understanding of these biological mechanisms behind ovarian ageing can lead to the identification of possible prognostic markers for fertility reasons. This would be especially beneficial for women with an increased risk of POI. These women can then make informed choices about family planning or, given the possibility of oocyte vitrification has become feasible, safeguard their future fertility. Ultimately, knowing the exact mechanisms of ovarian ageing, can lead to the development of therapeutic measures. As knowledge of the underlying mechanisms of ovarian aging increases, it could become feasible to for instance, manipulate the recruitment of follicles from the primordial follicle. However, the implementation of genetic research into clinical practice is a slow and cumbersome process and at this moment, there is no immediate implication of genetic markers for clinical practice, except for FMR1 premutations and a few monogenic causes of POI. So, in the meantime, as people are not aware of the rapid decline in women's fertility after the age of 30, we strongly advocate education (campaign) of the general public (women and men) about women's age-related infertility and its consequences. If the public is more aware of the drastic age-related decline of women's fertility, couples should be able to make informed choices in postponing childbirth.

As regards the health risks associated with menopause, the greatest gain in the identification of menopause-related genetic variants lies in increasing the understandings of the mechanisms involved in the development of these health risk. Ultimately, the results of genetic research could contribute to early detection of, and possible medical or lifestyle interventions for the menopause related diseases.

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Chapter

Ten

Summary / Samenvatting

SUMMARY

Chapter 1

Menopause is the endpoint of a process referred to as ovarian ageing. In the last decades the interest in the mechanisms behind ovarian ageing and the timing of natural menopause has increased since menopause has great implications for women's fertility and health (e.g. osteoporosis and cardiovascular disease). Although the exact physiological processes underlying the timing of menopause are far from elucidated at present, genetic factors have proven to play a major role in determining this variation in menopausal age as demonstrated in several mother-daughter, twin and sib-pair studies. The identification of genetic variants associated with (early) ovarian ageing and thereby infertility and consequences for health later in life, constitutes the core of this thesis. Once genetic variants associated with timing of menopause have been identified, studies can be carried out to unravel the role for these variants in processes that may direct the variation of ovarian ageing. Finally, these studies may yield genetic markers that will be analysed for their possible role in preventive management of age related infertility and menopause related diseases.

Chapter 2

Over the last two decades, several research groups have carried out genetic studies to identify genetic variants that influence the variation in timing of menopause. In this chapter we provided a systematic review of the genetic studies on age at natural menopause published until September 2009. A total of 28 papers emerged from our search: two papers on genome-wide linkage analysis, 23 papers on candidate gene association studies and three papers on GWAS. Some interesting new genetic loci were detected in GWAS, containing two promising candidate genes (*BRSK1* and *MCM8*). In candidate gene association studies, however, few consistent associations with menopausal age were found, if any. The latter could, amongst other things, be due to the fact that studies had been focusing on the wrong genes and that sample sizes had been small. Finally, possible new strategies for future genetic studies in timing of menopause are described.

Chapter 3

Menopause occurs when the follicle pool in the ovaries has become exhausted. Therefore genes that are involved in primordial follicle recruitment can be considered candidate genes for age at menopause. This chapter describes a candidate gene association study in 23 tagging SNPs in five genes involved in primordial follicle recruitment (*AMH*, *AMHR2*, *BMP15*, *FOXL2* and *GDF9*) for association with timing of menopause in 3616 women. The *AMHR2* gene was associated with age at natural menopause in interaction with parity, which confirmed an earlier finding in this respect. Next, a SNP in the *BMP15* gene was associated with timing of menopause, although this

association was merely driven by the women with an age at menopause before 40 (POI). Together, our findings suggest a role for genes involved in recruitment of the primordial follicle pool in the process of ovarian ageing and may thereby provide more insight in the biological mechanisms underlying ovarian ageing.

Chapter 4

To extend our previous study in genes involved in recruitment of the primordial follicle pool and timing of menopause, which is described in chapter 3, we searched for pairwise interactions between 23 tagging SNPs in the five genes previously selected (*AMH*, *AMHR2*, *BMP15*, *FOXL2*, *GDF9*). All possible pairwise interactions (n=210) were investigated. Based on the model-based multifactor dimensionality reduction (MB-MDR) test with a permutation-based maxT correction for multiple testing, we found a statistically significant interaction between rs10407022 in *AMH* and rs11170547 in *AMHR2* associated with age at natural menopause. Rs10407022 is an eQTL SNP that has been shown to influence mRNA expression levels in lymphoblastoid cell lines. This study provides additional insights into the genetic background of age at natural menopause and suggests a role of the *AMH* signaling pathway in the onset of natural menopause. However, these results remain suggestive and replication by independent studies is necessary.

Chapter 5

Women with early menopause are at an increased risk of cardiovascular disease (CVD) later in life. Besides an association with CVD, early menopause is also associated with an increased risk of type 2 diabetes (T2D), which is one of the major risk factors of CVD. To investigate whether coronary artery disease (CAD) and T2D are causally related to age at menopause, we have tested the association of previously reported GWAS hits in CAD and T2D for an association with age at natural menopause in nearly 50,000 women, exploiting the principle of Mendelian randomization. A total of 18 GWAS hits for CAD and 28 for T2D were selected and analysed to study a possible association with age at natural menopause. We found no statistically significant associations for any of the SNPs for CAD with age at menopause, and we could not support a previous finding that women with an increased risk of CAD experience an earlier onset of menopause. For the SNPs associated with T2D, two suggestive associations with menopausal age were identified located near the *HNF1A* gene and near the *NOTCH2* gene. These results imply a role for this metabolic disease in timing of menopause.

Chapter 6

Excessive triple CGG repeats in the *FMR1* gene have been widely associated with primary ovarian insufficiency (POI). Since it was hypothesized that *FMR1* CGG repeats in the normal and intermediate range (up to 55 repeats) could also influence timing of menopause, in this chapter

we aimed to test this hypothesis in a population-based sample of 3611 postmenopausal women. We found no association of the number of CGG repeats in the normal and intermediate range in the *FMR1* gene with age at natural menopause. Therefore, earlier observations suggesting that the number of CGG repeats in the normal and intermediate range is associated with the individual variation of the ovarian ageing process could not be confirmed. This finding questions the role of *FMR1* CGG repeat sizes in the ovarian ageing process.

Chapter 7

FMR1 CGG repeats in the permutation range have repeatedly been associated with POI, referred to as fragile-X-related POI (FXPOI). FXPOI accounts for approximately 1 to 8% of all cases of POI. Several studies investigated the role of intermediate CGG repeat size (45-54 repeats) in POI and yielded varying results. In chapter 6, we already showed that no association between *FMR1* CGG repeat sizes up to 55 repeats and timing of normal menopause exists. To assess the role of these normal and intermediate sized CGG repeats in POI, we investigated whether the risk of POI is associated with these sized CGG repeats in a case-control study of 375 women with idiopathic POI and 3368 controls with natural menopause above 40 years of age. The frequency of having intermediate sized number of CGG repeats was not statistically significantly different between POI cases and controls. Among women with POI, linear regression analysis for age at POI diagnosis and CGG repeat size also failed to show any association. The results of chapters 6 and 7 together suggest that a role of *FMR1* CGG repeat sizes up to 55 repeats in the ovarian ageing process may be questioned. Moreover, there seems limited value in the evaluation of normal- and intermediate *FMR1* repeat size in the diagnostic work-up of women affected by POI, or for prognostic purposes in women at risk of developing POI.

Chapter 8

POI has major implications for fertility and general health. Although POI can be caused by a number of monogenetic mutations, auto-immune diseases, or can be iatrogenic, in most cases there is no conclusive factor causing POI (i.e. idiopathic POI). In order to contribute to the identification of genetic risk factors of POI and early menopause (EM) we conducted a genome-wide association study (GWAS) in a cohort of 901 European idiopathic POI and EM cases and compared these cases to 2209 controls with an age at natural menopause over 45 years. In our study, no SNPs reached genome-wide significance ($P < 5x10-8$), although 40 SNPs had a P-value $<5 \times 10-5$ and are therefore promising genetic variants for follow up in a larger cohort of women with POI or EM. The strongest association was seen in a locus near the BRSK1 gene on chromosome 19. Interestingly, this locus was previously identified in GWAS in natural menopause above 40 years. So, although we did not identify new genetic loci in our GWAS, our data supports the evidence that POI, EM and normal menopause share at least a proportion of their genetic aetiology. It therefore seems likely that POI

and EM cases are part of the variation towards the entire distribution of natural menopause. In addition, a number of potentially interesting specific loci for POI and EM were identified, including NELL1, PGCF5, MTM1 and MTMR1. It is pivotal that these loci are replicated in a larger cohort of POI cases before any conclusions can be drawn on their actual involvement in POI and EM.

Chapter 9

In the final chapter of this thesis, we discuss the current knowledge of genetic variants involved in timing of menopause and risk of POI in relation to the findings of this thesis. Next, the 'missing heritability' is addressed and recommendations for future genetic research in variation of menopausal age are proposed. Finally, possible implications for clinical practice for an individual woman are discussed.

SAMENVATTING

Hoofdstuk 1

Menopauze is het eindpunt van ovariële veroudering. Omdat men steeds meer kennis heeft gekregen over de gevolgen van de menopauze op de vruchtbaarheid van een vrouw en haar algemene gezondheid (bijvoorbeeld osteoporose en hart- en vaatziekten), is de interesse in de onderliggende mechanismen van ovariële veroudering en het moment waarop een vrouw in de menopauze komt in de laatste decennia toegenomen. Hoewel het exacte fysiologische mechanisme dat ten grondslag ligt aan de timing van de menopauze nog grotendeels onbekend is, blijkt uit verschillende erfelijkheidsstudies dat genetische factoren een belangrijke rol spelen bij de variatie in menopauzeleeftijd. De identificatie van genetische varianten die geassocieerd zijn met (vroege) ovariële veroudering en de daarmee geassocieerde onvruchtbaarheid, evenals de gevolgen voor de algemene gezondheid van een vrouw, vormt de kern van dit proefschrift. Uiteindelijk kunnen deze studies een bijdrage leveren aan de identificatie van genetische varianten die de menopauzeleeftijd beïnvloeden. Deze varianten kunnen daarna verder geanalyseerd worden op hun mogelijke rol in preventie en behandeling van leeftijdsgebonden onvruchtbaarheid en menopauze-gerelateerde ziekten.

Hoofdstuk 2

In de afgelopen twintig jaar hebben verschillende onderzoeksgroepen studies uitgevoerd om genetische varianten te identificeren die de variatie in menopauzeleeftijd beïnvloeden. Hoofdstuk twee beschrijft een systematisch review van de genetische studies die zich hebben gericht op timing van de natuurlijke menopauzeleeftijd (gepubliceerd tot en met september 2009). Onze zoektocht heeft in totaal heeft 28 artikelen opgeleverd: twee artikelen over genoom-wijde linkage analyse, 23 artikelen over kandidaat-gen associatie studies en drie GWAS-artikelen. In deze laatstgenoemde artikelen werden enkele interessante nieuwe genetische loci ontdekt, gelegen in twee veelbelovende kandidaat-genen (het *BRSK1* en *MCM8* gen). In de kandidaat-gen associatiestudies werden echter weinig consistente associaties met menopauzeleeftijd gevonden. De oorzaak hiervoor kan onder andere liggen in het feit dat studies zich richtten op de verkeerde kandidaat-genen en doordat de studiepopulaties te klein waren. In de discussie worden mogelijke nieuwe strategieën beschreven voor toekomstige genetische studies op het gebied van timing van menopauze.

Hoofdstuk 3

Menopauze treedt op wanneer de voorraad follikels in de ovaria is uitgeput. Genen die betrokken zijn bij de rekrutering van primordiale follikels kunnen daarom worden beschouwd kandidaat-genen voor de timing van de menopauze. Dit hoofdstuk beschrijft een kandidaat-gen

associatiestudie in 23 polymorfismen (SNPs) die gelegen zijn in vijf genen die betrokken zijn bij het rekruteren van de primordiale follikels (*AMH*, *AMHR2*, *BMP*, *FOXL2* en *GDF9*). De eventuele rol van deze 23 SNPs in de variatie van menopauzeleeftijd werd onderzocht. In deze studie was het *AMHR2* gen geassocieerd met menopauzeleeftijd in interactie met pariteit en hiermee werd een eerdere gelijke bevinding bevestigd.

Naast het *AMHR2* gen werd een associatie gevonden tussen een SNP in het *BMP15* gen en timing van de menopauze, hoewel deze associatie grotendeels gebaseerd was op vrouwen met een vervroegde menopauze (primaire ovariële insufficiëntie, POI). Samen lijken onze bevindingen een rol te suggereren voor genen die betrokken zijn bij de rekrutering van de primordiale follikel in het proces van ovariële veroudering. Hierdoor kan meer inzicht worden verkregen in de biologische mechanismen die ten grondslag liggen aan ovariële veroudering.

Hoofdstuk 4

In aanvulling op de in hoofdstuk 3 beschreven studie naar genen die betrokken zijn bij de rekrutering van de primordiale follikels, hebben we gezocht naar alle mogelijke paarsgewijze interacties tussen de 23 SNPs in de vijf eerder geselecteerde genen (*AMH*, *AMHR2*, *BMP15*, *FOXL2*, *GDF9*) in associatie met menopauzeleeftijd. Op basis van de 'model-based multifactorial dimensionality reduction' test (MB-MDR) met een permutatie op basis van MaxT-correctie voor meervoudig testen, werd een statistisch significante interactie gevonden tussen rs10407022 in het *AMH* gen en rs11170547 in het *AMHR2* gen voor associatie met menopauzeleeftijd. Rs10407022 is een eQTL SNP waarvan is aangetoond dat het de mRNA expressie beïnvloedt in lymfoblastoïde cellijnen. De studie beschreven in dit hoofdstuk biedt aanvullende inzichten in de genetische achtergrond van menopauzeleeftijd en suggereert een rol voor de AMH signaleringsroute hierin. Replicatie van onze resultaten in onafhankelijke studiepopulaties is echter noodzakelijk.

Hoofdstuk 5

Vrouwen met een vroege menopauze hebben een verhoogd risico op hart- en vaatziekten (HVZ). Naast een associatie met HVZ, is vroege menopauze ook geassocieerd met een verhoogd risico op type 2 diabetes (T2D), dat één van de belangrijkste risicofactoren van HVZ is. Om te onderzoeken of HVZ en T2D causaal gerelateerd zijn aan menopauzeleeftijd, hebben we eerder gerapporteerde GWAS hits in HVZ en T2D onderzocht voor een eventuele associatie met menopauzeleeftijd in een studiepopulatie van bijna 50.000 vrouwen. Hierbij is gebruik gemaakt van het principe van de Mendeliaanse randomisatie. In totaal werden 18 GWAS hits voor HVZ en 28 hits voor T2D geselecteerd en daarna onderzocht op een mogelijke associatie met menopauzeleeftijd. Hierin zijn geen statistisch significante associaties gevonden tussen de SNPs in HVZ en menopauzeleeftijd. Met dit gegeven kon een eerdere bevinding dat vrouwen met een verhoogd risico voor HVZ een vroegere menopauzeleeftijd hadden niet worden ondersteund. In de onderzochte SNPs in T2D

werden twee suggestieve associaties met menopauzeleeftijd gevonden die gelegen zijn in de buurt van de *HNF1A* gen en van het *NOTCH2* gen. Deze resultaten impliceren een rol voor deze metabole ziekte in timing van de menopauze.

Hoofdstuk 6

Een premutatie (>55 CGG repeats) van het fragiele X mentale retardatie gen 1 (*FMR1*) is een bekende oorzaak van primaire ovariële insufficiëntie (POI). Omdat eerder is verondersteld dat FMR1 CGG repeats in de normale en intermediate range (tot 55 repeats) de timing van de menopauze ook beïnvloeden, hebben wij in dit hoofdstuk deze hypothese getest in een studiepopulatie van 3611 postmenopauzale vrouwen. In onze studie vonden wij echter geen associatie tussen het aantal CGG repeats in de normale en intermediate range in het *FMR1* gen en menopauzeleeftijd. Hiermee konden eerdere observaties die suggereerden dat het aantal CGG repeats in de normale en intermediate range geassocieerd zijn met de variatie van ovariële verouderingsproces niet worden bevestigd. Ook stellen onze resultaten hiermee de rol van normale en intermediate *FMR1* CGG repeats in ovariële veroudering in het algeheel ter discussie.

Hoofdstuk 7

FMR1 CGG repeats in de permutatie range zijn herhaaldelijk in verband gebracht met POI, dat wordt aangeduid als fragiele-X-gerelateerde POI (FXPOI). FXPOI is verantwoordelijk voor ongeveer 1 tot 8% van alle gevallen van POI. Verschillende studies onderzochten de rol van intermediate CGG repeats (45-54 repeats) in POI, met wisselende resultaten. In hoofdstuk 6 hadden we al aangetoond dat er geen verband is tussen *FMR1* CGG lengtes tot 55 repeats en de timing van de normale menopauzeleeftijd. Om de rol van deze normale en intermediate CGG repeats in POI te bepalen, hebben we onderzocht of het risico van POI is geassocieerd met deze CGG repeats in een case-control studie van 375 vrouwen met idiopathische POI en 3368 controles met een menopauzeleeftijd boven de 40 jaar. De frequentie van vrouwen met CGG repeats in de intermediate range verschilde niet statistisch significant tussen POI patiënten en controles. Verder liet lineaire regressie analyse in de groep vrouwen met POI geen associatie zien tussen de leeftijd bij diagnose van POI en het aantal CGG repeats. De resultaten van de hoofdstukken 6 en 7 samen suggereren dat een rol van *FMR1* CGG repeats tot 55 repeats in ovariële veroudering in twijfel kan worden getrokken. Voor de diagnostiek bij vrouwen met POI en voor voorspellende doeleinden bij vrouwen met een verhoogd risico op POI, lijkt de evaluatie van normale en intermediate *FMR1* CGG repeats van geen waarde te zijn.

Hoofdstuk 8

Ongeveer 1% van alle vrouwen wordt menopauzaal voor haar 40ste levensjaar (POI). POI heeft grote gevolgen voor de vruchtbaarheid en voor de algemene gezondheid. Hoewel POI door een

aantal mono-genetische oorzaken, auto-immuun ziekten of iatrogeen kan worden veroorzaakt, wordt er in de meeste gevallen van POI geen duidelijke oorzaak gevonden (idiopatische POI). In de zoektocht naar genetische risicofactoren voor POI en vroege menopauze (tussen 40 en 45 jaar; early menopause; EM) hebben wij een genoomwijde associatiestudie (GWAS) uitgevoerd in 901 Europese vrouwen met idiopatische POI of EM. Deze 901 vrouwen werden vergeleken met 2209 controlevrouwen met een natuurlijke menopauzeleeftijd van 46 jaar of ouder. Hoewel in onze studie geen SNPs gevonden werden die genoomwijd significant geassocieerd waren met POI (met een P-waarde < $5x10^{-8}$), hadden 40 SNPs een P-waarde < 5×10^{-5} en kunnen daarom beschouwd worden als veelbelovende SNPs voor vervolgonderzoek in een grotere groep vrouwen met POI of EM. De sterkst gevonden associatie was gelegen in een locus dichtbij het *BRSK1* gen op chromosoom 19. Het interessante aan deze bevinding is het feit dat deze locus eerder geïdentificeerd is in een GWAS naar natuurlijke menopauzeleeftijd in vrouwen met een menopauzeleeftijd boven de 40 jaar. Onze bevinding versterkt het recent gepubliceerde bewijs dat POI, EM en normale overgang op z'n minst een deel van hun onderliggende genetische varianten delen. Het ligt daarom voor de hand dat (idiopathische) POI en EM deel uitmaken van de normale variatie van menopauze en geen aparte entiteit vormen. Naast de locus in de buurt van het *BRSK1* gen werden nog een aantal potentieel interessante loci geïdentificeerd in onder andere het *NELL1*, *PGCF5*, *MTM1* en *MTMR1* gen. Echter, voordat we kunnen concluderen dat deze loci daadwerkelijk van invloed zijn op POI en/of EM is het belangrijk dat ze gerepliceerd worden in een groter cohort.

Hoofdstuk 9

In het laatste hoofdstuk van dit proefschrift wordt de huidige stand van de kennis van genetische varianten die van invloed zijn op timing van de menopauze en het risico op POI in relatie tot de bevindingen in dit proefschrift besproken. Vervolgens wordt de ´missing heritability´ behandeld en worden aanbevelingen gedaan ter zake van genetisch onderzoek in de toekomst. Tot slot worden enkele mogelijke implicaties voor de klinische praktijk voor de individuele vrouw besproken.

Chapter

Eleven

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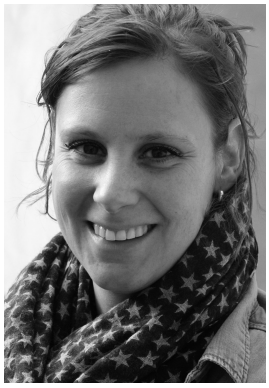
CURRICULUM VITAE – OVER DE AUTEUR



Marlies Voorhuis werd op 6 februari 1981 geboren in het ziekenhuis in Oldenzaal. Zij beleefde een fijne jeugd in Weerselo, een dorp in Twente, samen met haar ouders, oudere zus en broer.
Na het behalen van haar VWO-diploma aan het Thijcollege te Oldenzaal startte zij met de opleiding Geneeskunde in Groningen. Gedurende haar co-schap gynaecologie in het Deventer Ziekenhuis en een stage bij een verloskundigenpraktijk in Deventer werd haar gevoel bevestigd dat zij zich wilde specialiseren in de gynaecologie. Volgend op haar co-schappen deed zij haar keuze co-schap en wetenschappelijke stage op de afdeling gynaecologie en verloskunde in het St. Lucas Andreas Ziekenhuis in Amsterdam (prof. Scheele). Zij behaalde haar artsexamen (cum laude) in januari 2007. Vervolgens vertrok zij voor ruim 3 maanden naar Ghana, waar zij in het Holy Family Hospital in Berekum werkte. Na terugkomst in Nederland startte zij als arts-assistent niet in opleiding (ANIOS) gynaecologie en verloskunde in het St. Antonius Ziekenhuis te Nieuwegein (toenmalig opleider dr. The).
In oktober 2008 kreeg Marlies de mogelijkheid om promotieonderzoek te doen binnen een samenwerkingsverband tussen de afdeling Voortplantingsgeneeskunde (prof. Broekmans) en het Julius Centrum (prof. van der Schouw), beide onderdeel van het Universitair Medisch Centrum Utrecht (UMCU). Voor dit onderzoek werd door ZonMW (NWO) een AGIKO-beurs toegekend. Het resultaat van dit onderzoek ligt nu voor u.
Naast het afronden van haar promotieonderzoek is zij per 1 januari 2012 met heel veel plezier gestart met de opleiding tot gynaecoloog in het St. Antonius Ziekenhuis te Nieuwegein (opleider dr. J. Schagen van Leeuwen).
Marlies woont erg gelukkig samen met Guus en hun zoon Olivier in Amsterdam.

DANKWOORD

Klaar!

Ik ben heel veel mensen dankbaar voor hun hulp bij mijn onderzoek, maar een aantal wil ik hierbij graag persoonlijk bedanken.
Als eerste gaat mijn dank uit naar alle vrouwen die belangeloos hebben deelgenomen aan de Prospect-studie en aan de vrouwen die hebben deelgenomen aan de COLA-studie. Zonder hen was dit onderzoek niet mogelijk geweest.

Geachte prof. Broekmans, beste Frank. Ik ben je heel erg dankbaar dat je me in september 2008 hebt aangenomen voor dit promotieonderzoek ondanks dat ik destijds geen idee had waar het onderzoek over zou gaan en ik eerlijk gezegd niets wist van (complexe) genetica. Je onuitputtelijke enthousiasme en energie werkten (meestal) aanstekelijk. En hoewel jouw quick-and-dirty motto soms wat lastig te combineren was met mijn check-check-dubbel-check werkwijze, heeft het uiteindelijk voor een goede balans gezorgd. Bedankt voor je betrokken begeleiding en het vertrouwen in mij.

Geachte prof. van der Schouw, beste Yvonne. Je bewaarde het overzicht, legde vaak de vinger op de zere plek en mijn stukken werden altijd razendsnel en minutieus gecorrigeerd (ook tijdens mijn behoorlijk krappe tijdsplanning aan het eind). Ik heb inmiddels wel geleerd dat zaken niet altijd binnen een week geregeld kunnen en hoeven worden! Bedankt ook voor je lieve en opbeurende mails op kritieke momenten.

Geachte dr. Onland-Moret, beste Charlotte. Geweldig dat je mij als epidemiologische no-no vanaf het begin bij de hand hebt genomen. Jouw optimisme en vertrouwen waren voor mij (als soms gezonde pessimist) een grote steun. Daarnaast waren onze bijna wekelijkse overleg-uurtjes ook altijd erg gezellig. Dankjewel voor al je hulp!

Geachte prof. Fauser, beste Bart, bedankt voor je bijdrage aan de stukken in mijn proefschrift. Ik bewonder je directheid en je analyserend vermogen en ik weet zeker dat het POI-project uiteindelijk tot een goed einde gebracht wordt.

Geachte prof. Peeters, prof. van Bel, prof. de Bakker, prof. Laven en prof. Lambalk, bedankt dat u zitting hebt willen nemen in mijn leescommissie.

Geachte leden van de Prospect-EPIC stuurgroep, hartelijk dank voor het beschikbaar stellen van de data en het materiaal van de vrouwen in mijn studie.

Geachte prof. Uitterlinden en prof. Laven van het Erasmus Medisch Centrum Rotterdam, bedankt voor jullie hulp en de verhelderende meetings over het POI project.

Dr. Persani, dr. Marozzi, dr. Christin-Maitre and prof. Bouchard, although we have never met in person, it was very pleasant to have worked with you and I look forward to working with you in the future and to bring the POI project to a good end.

Alle co-auteurs die ik nog niet genoemd heb, hartelijk bedankt voor jullie bijdragen aan de manuscripten.

Lisette en Yvonne, mijn genetische heldinnen uit het Erasmus in Rotterdam. Vanaf mijn eilandje in Utrecht kon ik altijd bij jullie terecht voor mijn vele, vele, vele vragen. Heel erg bedankt voor al jullie hulp én voor de gezelligheid. Lisette, ik zal je vast nog een aantal keren 'stalken' voor de afronding van het POI-project (sorry alvast daarvoor). Yvonne, heel veel succes met de laatste loodjes van jouw onderzoek en leuk dat wij elkaar straks als AIOS vast weer tegenkomen.

Marie, bedankt voor je hulp bij de (voor mij soms onbegrijpelijke) analyses, pfff. Heel veel succes met het afronden van je proefschrift en voor je het weet is het voor jou ook zover.
Clara, heel fijn dat je me geholpen hebt met het soms onmogelijke samples-zoekwerk en mij wegwijs hebt gemaakt in het lab.

Geachte dr. Eijkemans, beste René. Hoewel wij officieel maar aan één gezamenlijk project hebben gewerkt, ben je de afgelopen jaren vaak een redder in nood geweest op kritieke SPSS en R momenten. Dankjewel daarvoor.

Ook veel dank aan alle lab medewerkers die mij met verschillende projecten hebben geholpen: het Genetisch Laboratorium Inwendige Geneeskunde, Erasmus MC (in het bijzonder Mila, Michael en Pascal) en van de Medische Genetica, UMCU (in het bijzonder Karen).

Gynaecologen, fertiliteitsartsen, polizusters en medewerksters van receptie 27, bedankt voor jullie hulp bij de COLA's en de gezelligheid. Tot snel in het AZU!

Secretaresses van het hooglerarensecretariaat, lieve Ellis, Ingrid en Tessa. Jullie zijn geweldig! Gezellig, altijd goed op de hoogte van de hele afdeling en redders in afspraken-nood.

Wonderbaarlijk hoe jullie toch nog altijd een afspraak weten te plannen in de overvolle agenda's van de hoogleraren. Bedankt voor al jullie hulp, steun en gezelligheid!

Lieve onderzoekers van het AZU en van de overkant (het waren er de afgelopen jaren nogal wat). Jullie maakten het onderzoek vaak wat draaglijker en vierden vrolijke momenten mee. Dat er nog maar veel acceptatie-kroketten gegeten mogen worden! Ook nog mijn hartelijke dank dat jullie mijn maandag-maisbrood-campagne volledig steunden, want als je niet luncht ga je dood. Allemaal heel veel succes en sterkte bij jullie onderzoek. Gezellig dat we elkaar straks als AIOS weer tegenkomen!
Felicia, bedankt voor je hulp bij het POI-labwerk in het Erasmus en heel veel succes met het afronden van de OVADIA en IOF studie.
Claar, heel moedig dat je de laatste maanden van mijn promotie met mij wilde carpoolen en mij door mijn meest kritieke promotietijd heen hebt geloodst. In het Mintje of het Dropje, op de weg haalde wij het slechtste (of beste?) van elkaar naar boven. Jawohl!

Lieve echoscopistes van het AZU, Hannah en Rommy. Bedankt dat jullie altijd (écht altijd) bereid waren om te helpen bij de echo's van COLA-patiënten. Daarnaast vaak even gezellig bij gekletst of stoom afgeblazen in de echokamer. En natuurlijk heel erg bedankt voor Oliviers prenatale eregalerij!

Olaf Schulte, dankjewel voor je hulp bij mijn download-, account-, wachtwoorden-, verdwenen inboxen-, geheugen-, trage computer-, en alle andere ICT-ellende.

Gynaecologen, verloskundigen en verpleegkundigen van het St. Antonius ziekenhuis Nieuwegein. Wat een fantastische plek om te beginnen als ANIOS, als eerstejaars AIOS en nu als tweedejaars AIOS. Voor de derde keer ben ik weer in een warm bad terechtgekomen, ontzettend bedankt daarvoor.
Lieve collega's-A(N)IOS van het Antonius nu en in de goede oude tijd. Wat is het fijn om zulke lieve en leuke mensen je heen te hebben en gezellig dat ik zoveel van jullie nog vaak zie.

Lieve Nicole, wat een super tijd hebben we gehad in het Antonius! Zo relaxed dat ik met jou de leuke, maar soms ook ellendige dingen en frustraties kon delen. Fijn dat we elkaar sinds die tijd nog altijd vaak spreken en zien. Verder ben je op z'n minst medeverantwoordelijk geweest voor de start van mijn promotie-baan (bedankjewel!) en ik vind het daarom geweldig dat je op 28 juni mijn paranimf bent.

De van Doet in Deventer, lieve Yvo, Laetitia, Jorunn en huisvriend Sybrand. Wat een briljante co-tijd! En wie had er nou gedacht dat of all people ík zou gaan promoveren… Bedankt voor jullie lieve interesse en ik hoop op nog vele briljante avondjes in de toekomst.

Lieve Cougars, ruim partij de afgelopen 13 jaar! Ik hoop dat we elkaar in de toekomst nog regelmatig blijven zien!

Lieve Caro en Lau, supervriendinnetjes! Niets werkt beter tegen promotie-stress dan een avondje wijntjes. Aan een half woord genoeg. Fijn dat jullie er altijd voor me zijn. Jullie zijn lief!

Lieve schoonfamilie Loes en Dick, Michiel en Nelleke, Marieke en Mark. Loes en Michiel, bedankt dat jullie zo'n fantastische zoon op de wereld hebben gezet. Altijd warm en gezellig bij de Haagse, maar vooral Bourgondische schoonfamilie. Wijn, kaasjes, Frankrijk, goûtjes en altijd geïnteresseerd in mijn onderzoek en opleiding. Marieke, gezellig dat ik bij Guus zo'n top-schoonzusje cadeau kreeg! Ook ontzettend bedankt voor jullie lieve zorg voor Olivier. Ik kan me geen betere schoonfamilie wensen.

Lieve Aniek, grote zus, wat fijn dat we de afgelopen steeds meer naar elkaar toe gegroeid zijn en we naast zusjes nu ook vriendinnen zijn. Ik ben er super trots op dat je de 28ste naast me wil staan. Lieve broer Bart en Marije, ik hoop dat we elkaar na jullie reis en mijn drukke periode weer vaker zien. Bedankt voor jullie lieve belangstelling voor mijn onderzoek en ik kan niet wachten om in augustus mijn nieuwe neefje vast te houden. Wat een feest!

Lieve papa en mama, bedankt voor jullie liefde. Altijd gestimuleerd, alle kansen geboden en gesteund om te doen wat mij gelukkig maakt (nou, ja liever geen kunstacademie dan…). Wat is het fijn om altijd en overal ter wereld te weten dat er een veilige haven is om op terug te kunnen vallen. Ook fantastisch om te zien dat jullie zo'n geweldige opa en oma voor Olivier zijn. Ik hou van jullie.

Lieve Guus, mijn allerliefste lief! Al meer dan 12 jaar ontzettend gelukkig met jou. Ik kan je niet genoeg bedanken voor al je hulp en steun de afgelopen jaren (zonder jou was me het nooit gelukt), maar vooral omdat je er altijd voor Olivier en mij bent. Ik ben dol op je! Lieve Olivier, wat ben jij een ongelofelijk lief, gezellig, relaxed en knap ventje. Je bent het mooiste dat me ooit is overkomen.
Samen met jullie is het leven zó mooi!