Premature Ovarian Failure

from phenotype to genotype

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Colophon

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Premature Ovarian Failure

from phenotype to genotype

Prematuur Ovarieel Falen
van fenotype naar genotype
(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. J. C. Stoof, ingevolge het besluit van het college van promoties in het openbaar te verdedigen op woensdag 22 april 2009 des middags te 4.15 uur

door

Antonius Hendricus Knauff
geboren op 20 september 1974 te Zeist

Promotoren:

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Prof. dr. C. Wijmenga

Co-promotor:

Dr. A.J. Goverde

Porque también somos lo que hemos perdido[1]
(Omdat we ook zijn, wat we zijn kwijt geraakt)

Contents

POF: from phenotype to genotype (general introduction)

Chapter 1

POF phenotype and its impact on women's health

In 1672 Reinier De Graaf described the ovaries as female testicles that contained "bollekes" (bubbles), later named follicles. Five years later Antonie Van Leeuwenhoek, together with his student Johannes Ham, discovered the mammalian spermatozoa[2]. These two Dutch scientists stood at the cradle of what is nowadays called reproductive science. Their discoveries were among the first in exploring the motor driving evolution: the possibility to pass our genes on to the next generation.

For most women this possibility is set in a fixed time frame, lying between menarche and their late thirties in natural fertility populations all over the world, although fertility already starts to decrease early in the 4th decade of life[3, 4]. In relation to the decreasing fertility, the number of primordial follicles in the ovaries also declines, finally leading, in the general population, to exhaustion of the follicle pool at around the age of 50; this is referred to as menopause (the date of the last menstruation)[5]. However, around 1% of all women experience a spontaneous cessation of menses before 40 years of age, accompanied by high levels of gonadotrophins[i]. This condition is referred to as premature ovarian failure (POF)[6]. Their fertility is severely compromised and the only real option for achieving pregnancy is to make use of oocyte donation, although an estimated 5-10% of POF patients may still achieve a spontaneous pregnancy (these numbers are based on a meta-analysis of 29 case reports)[7]. Longitudinal follow-up studies to investigate spontaneous pregnancy rates are not available.

Understandably, the impossibility to conceive when a patient wants to have children, in combination with a postmenopausal hormonal status at a younger age, has a major impact on her psycho-sexual well-being[8,9]. Many other women's health issues are also related to an earlier age at menopause, e.g. osteoporosis[10-13], cardiovascular disease[14], cognition, Alzheimer, parkinsonism[15, 16], longevity[17] and breast cancer[18, 19].

The relationship between age at menopause and cardiovascular death[14] is a concern for POF patients and their doctors, but its aetiology is unknown. Several studies, along with basic research, have pointed in the direction of oestrogen-deprivation and cardiovascular health[20], whilst epidemiological studies have not been able to establish a clear relationship[21]. The POF phenotype may be comparable to a very early postmenopausal status and we felt it would therefore be valuable to investigate the distribution of established cardiovascular risk factors such as lipid profile in these young "postmenopausal" patients (see Chapter 3).

Definition of premature ovarian failure

Premature ovarian failure is defined in most of the literature as secondary amenorrhea with follicle stimulating hormone (FSH) levels exceeding 40 IU/L before age 40, although sometimes oestradiol (E_2) levels or lower FSH cut-off values are included[22]. The FSH cut-off value of 40 IU/L is probably based on the highly cited, initial paper of Coulam[6] who established this definition. However, from a biological point of view, it is reasonable to assume that an FSH level above 40 IU/L is a clear

i Protein hormones secreted by gonadotrophe cells of the pituitary gland of vertebrates. The two principal gonadotrophins are luteinizing hormone (LH) and follicle stimulating hormone (FSH).

sign of severely diminished ovarian reserve, since postmenopausal gonadotrophins are also in that range[23]. The duration of the amenorrhea for the diagnosis of POF is not generally well defined. True premature menopause should be set at 12 months' amenorrhea, but since hormone therapy is warranted in the majority of POF patients, such a time frame has its limitations. Furthermore, it is known that ovarian function may return for a short period in POF patients[24].

In the Netherlands the duration of absent menstruations for the definition of POF is set at 4 months[25]. In this thesis, we define POF as follows: *the absence of spontaneous menstruation for at least 4 months after spontaneous menarche, in combination with FSH >40 IU/L (independent of the assay used) before age 40.* Primary amenorrhea and iatrogenic POF (due to chemotherapy and/or pelvic radiotherapy or surgery) are beyond the scope of this thesis.

This Dutch definition of POF has its limitations, for example: women with an amenorrhea before age 40 but elevated FSH levels below 40 IU/L, or women with irregular cycles in combination with highly elevated FSH levels (and low E_2 levels), are excluded by this definition. In clinical practice, however, these patients will sometimes be treated and/or counselled as patients presenting with POF but without knowing their true ovarian reserve. Furthermore FSH, which is a product of the pituitary gland, is an indirect marker of ovarian function and is regulated via a multitude of feedback signals (primarily E_2 and the inhibins).

Nowadays, more direct ovarian markers are available to measure ovarian function or reserve: inhibin B, a product of the granulosa cells[ii] of the developing ovarian follicle; antral follicle count (AFC) (counting the visible intra ovarian follicle <5 mm via transvaginal ultrasonography), and anti-Müllerian hormone (AMH), also a product of the ovarian granulosa cells.

AMH has been examined in numerous studies and is an established ovarian marker for poor response to in vitro fertilization, polycystic ovary syndrome, hypogonadotropic hypogonadism and, likely, also age at menopause[26-28]. These ovarian function markers, especially AMH, may well give a more accurate estimation of the ovarian follicle quantity in young hypergonadotropic patients than FSH (see Chapter 2).

Physiology: the human ovarian follicle pool throughout life

Knowledge of the development and maintenance of the pool of follicles in the ovary is important because there are a multitude of 'genetically controlled' time points on the follicle's route through life: germ cell development and migration, mitotic multiplication, meiotic arrestment, granulosa cell envelopment and finally, atresia via apoptosis. A minor defect caused by an underlying genetic variation somewhere in this complex pathway of development, may have a tremendous effect on the size and function of the ovarian follicle pool and may well influence age at menopause or lead to POF.

ii Granulosa cells are somatic cells found closely associated with the developing oocyte in the ovary of mammals. In the primary ovarian follicle, and later in follicle development, they advance to form a multilayered cumulus oophorus surrounding the oocyte in the pre-ovulatory or Graafian follicle. The major functions of granulosa cells include the production of steroids, as well as a myriad of growth factors thought to interact with the oocyte during its development.

At approximately 5 weeks of gestation, the paired gonads are composed of primitive germ cells, coelomic surface epithelial cells and an inner core of medullary mesenchymal tissue. Together they form the gonadal ridges. It is still not entirely clear where these germ cells come from or how exactly they enter these gonadal ridges. Hereafter, rapid mitotic multiplication of these germ cells leads to the maximum number of germ cells available in a lifetime: around 6-7 million are generated between 16-20 weeks of gestation[29-31]. Meanwhile these oogonia enter the first meiotic division, arrest in the diplotene stage and are enveloped with a single layer of granulosa cells. They are called oocytes from then on. Resumption of meiosis may occur many years later, namely just before ovulation. However, more than 99.99% of these millions of oocytes will never reach ovulation, because they will be lost through irreversible atresia[iii] via apoptosis.

Massive atresia of fetal ovarian primordial follicles occurs already in the 2nd half of prenatal development leading to an estimated 1 million oocytes at birth 32, a reduction of already ~80% of the pool. Why this seemingly inefficient energy use takes place during foetal development is largely unknown. During childhood, atresia continues and at menarche between 300,000 – 500,000 germ cells survive, but only a few hundred of these enter the process of follicular growth and finally ovulation[33].

Recently, the paradigm that women are born with a finite number of oocytes was challenged[34-37]. A fundamental paradigm shift would take place in reproductive medicine if it was found that new follicle eggs may enter the ovarian pool later in life. However, no other research group has replicated their reported findings and there is extensive debate about this idea of neo-oogenesis[38-44].

During a woman's reproductive years, every month around ~1000 follicles still go into atresia. At the end of the 4th decade a final acceleration phase in the follicle pool's decline takes place, heralding the end of fertility. When the last follicles disappear from the ovary, the follicle pool is (nearly) exhausted and the last menstruation occurs: menopause.

Aetiology of POF

More than three centuries after De Graaf and Van Leeuwenhoek, the processes and factors determining the variation in the rate of decline of oocyte quantity and quality among individuals are still largely unknown. For instance, it is not clear whether females with early menopause or POF start with a smaller number of follicles or have an increased rate of atresia.

Family history is the most important predictor of early menopause, suggesting a major role for genetic factors. The highest reported overall incidence of familial cases among women with POF is around 30%[45-47]. In a large Italian study, one-third of the idiopathic POF patients showed an inherited pattern[48]. A subsequent Dutch study reported the incidence of familial cases to be 12.7%[49]. The variation between reported incidences might be explained by differences in the definition of familiar POF, by differences in population recruitment, and by selection and recall bias.

Pedigree studies on affected POF families show a mode of inheritance suggestive of autosomal

iii Degeneration and subsequent resorption of one or more immature ovarian follicles.

dominant, sex-limited transmission, or X-linked inheritance with incomplete penetrance[iv] [48, 50-53]. Moreover, the heritability[v] of age at menopause has been estimated to be ~40-70% based on studies in sisters and twins, implying that genetic factors are the most important determining factors[54-57]. However, environmental factors, like smoking, do contribute to the observed variation[58]. Therefore, menopausal age as a phenotypic trait can best be described as a complex genetic trait, in which multiple genetic and environmental factors play a role.

Genetics of POF

The search for genes associated with the POF phenotype or genes influencing age at menopause in general, has been an ongoing process. To date, multiple linkage regions and candidate genes have been suggested to be involved in these phenotypes (see table 1 and next paragraph). Unfortunately most of the studies report rare mutations with frequencies <1%, and have been performed in small cohorts and/or in insufficiently phenotyped patients[59, 60].

Our group performed a linkage analysis in 165 families with either early (before 46 years of age) or late menopause (e.g. after 55 years of age) or both. The highest LOD score[vi] of 3.1 showed suggestive linkage in the region Xp21.3[61]. More recently, two familial linkage studies identified POF loci on Xq21.1 - Xq21.3.3 (in a gene named *POF1B*) and recently on chromosome 5q14.1-5q15[62, 63].

The X chromosome seems to be important for ovarian development and maintenance. The following four arguments are in favour of 'the POF X factor':

1) POF is often found in combination with a (mosaic) Turner phenotype;
2) There are multiple case reports of X-chromosomal aberrations in POF patients, which has resulted in the critical region hypothesis;
3) There is a higher prevalence of premutation carriership of the fragile X mental retardation 1 gene (FRM1) in POF patients, the only established common genetic risk factor for POF;
4) POF families may show a deficit of males.

POF and (mosaic) Turner phenotype

The X-chromosome in relation to ovarian failure was one of the earliest findings in genetics as Turner syndrome[vii] as found to be due to the absence of one X-chromosome in females[64] and later also related to different deletions on the short arm of the X-chromosome[65]. Mosaic Turner patients

iv Penetrance is a term used to describe the proportion of individuals that carry a particular variation of a gene (an allele or genotype) that also expresses an associated trait (the phenotype).

v Heritability is the proportion of phenotypic variation in a population that is attributable to genetic variation among individuals.

vi The LOD score (logarithm (base 10) of odds) is a statistical test often used for linkage analysis in human populations. By convention, a LOD score greater than 3.0 is considered evidence for linkage. A score of 3.0 means the likelihood of observing the given pedigree if the two loci are not linked is less than 1 in 1000. On the other hand, a LOD score less than -2.0 is considered evidence to exclude linkage.

vii Turner syndrome (TS) or Ullrich-Turner syndrome is a chromosomal disorder affecting females in which all or part of one of the X chromosomes is absent. The syndrome occurs in 1 out of every 2500 girls and manifests itself in a number of ways. There are characteristic physical abnormalities, such as short stature, lymphoedema, broad chest, low hairline, low-set ears, and webbed neck. Girls with TS typically experience gonadal dysfunction with subsequent primary amenorrhea and no breast development.

(46,XX;45,X0) show a less severe phenotype: taller, some breast development and sometimes menarche[66]. Histological studies in Turner foetuses show initial ovarian development with primary follicles but secondary ovarian failure leading to so-called streak gonads. It is hypothesized that hemizygosity for one or more X-loci could impair ovarian function. The role of X-inactivation[viii] in this process is not fully understood yet, but is seems likely that one X-chromosome is necessary for ovarian development and that the other X chromosome plays a role in ovarian maintenance[67].

X-chromosomal aberrations in POF patients

Early cytogenetic studies of premature ovarian failure patients from before 1990 revealed sporadic X-chromosomal aberrations. The first reported familial X-chromosome defect was located on the long arm[68]. With increasing cytogenetic banding techniques, many more autosomal and X-translocations, deletions and inversions were found. The region Xq21-q25 with clustered aberrations on the long arm was named as critical region, i.e. the region which seems to be critical for ovarian function[65]. Schlessinger and colleagues reviewed the ovarian phenotype of 118 published, balanced X-translocations[69]. Fifty-one patients showed primary (n=36) or secondary (n=15) amenorrhea. Their study showed a clear overrepresentation in the karyograms of these amenorrheic patients of breakpoints in the critical regions, in particular Xq21.

Deletions on the X-chromosome in patients with secondary amenorrhea without Turner stigmata (n=11) were found only on the long arm of the X-chromosome. i.e. on Xq13, Xq21, Xq22, Xq24 and Xq26. Refining of the so-called critical region led to two separated loci: a more distal POF1 region on Xq27-27.3 and a proximal POF2 locus between Xq13-q21[70].

Increasing cytogenetic technology means there is still an important role for chromosomal analysis in the workup of a POF patient. Two comprehensive reviews were recently published, focusing on POF and the presence of chromosomal aberrations[69, 71].

FMR1-premutation (FRAXA)

One of the most investigated single mutations in POF lies on Xq27.3 and involves the FMR1 gene. Full mutation[ix] FMR1 carriers suffer from Fragile X syndrome, whilst female premutation FMR1 carriers - who carry between 50-200 CGG repeats within the FMR1 gene - show a POF phenotype in 30% of the cases[72]. Genetic epidemiology of the distribution of the presence of premutations among females with an early menopause (46-50 years) has not been performed yet, but could lead to important knowledge about genetics and pathways in premature, early or even normal menopause. Interestingly, increased CGG repeat length in premutation carriers is associated with an increased severity of POF[73].

Studies of POF families showing a deficit of males

One of the first studies which mentioned a relatively low number of males in families with early menopause and POF was reported by Cramer et al, suggesting an X-related problem due to the hy-

viii X-inactivation is a process by which one of the two copies of the X chromosome present in female mammals is inactivated, and it occurs so that the female, with two X chromosomes, does not have twice as many X chromosome gene products as the male, who only has a single copy of the X chromosome.

ix A full mutation in the FMR1 gene involves expansion of >200 CGG repeats.

pothetical mechanism that male embryos would have a smaller chance to survive in utero as they only have a deviant X chromosome compared to females[45]. In another study, although sample groups were small and thus lacked epidemiological power, a lower frequency of brothers in sibships of cases with a family history of early menopause was also found[74].

Non X-linked POF candidate genes

A multitude of loci and genes on the 22 autosomal genes are also linked to the POF phenotype. Most of these have been identified in single patients or families, small patient groups, isolated populations, or through animal knockout models. A comprehensive overview of both autosomal and X-linked candidate genes can be found in table 1.

Other aetiologies of POF

Iatrogenic

In patients who develop malignant diseases, pelvic radiotherapy and chemotherapy can also lead to POF. Pelvic surgery may damage the ovary by affecting its blood supply or causing inflammation in the area[75]. However, iatrogenic causes of POF are beyond the scope of this thesis.

Autoimmunity

Some cases of POF may be due to an abnormal self-recognition by the immune system. The most convincing evidence comes from the commonly observed association of POF with autoimmune disorders. In general, it is considered that about 20% of POF patients have a history of autoimmune disease, most commonly autoimmune thyroid disease. Occasional studies have reported this association in as many as 39% of women with chromosomally competent POF[76]. Both endocrine and non-endocrine autoimmune associations have been described in relation to POF; the endocrine include thyroid, hypoparathyroid, diabetes mellitus, hypophysitis, and the non-endocrine include chronic candidiasis, idiopathic thrombocytopenic purpura, vitiligo, alopecia, autoimmune haemolytic anaemia, pernicious anaemia, systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease, Sjogren syndrome, primary biliary cirrhosis and chronic active hepatitis[76, 77].

Clinically, autoimmune ovarian failure is broadly discussed in two scenarios: (a) in association with autoimmune Addison's disease and (b) isolated or associated with other autoimmune diseases. Of course, genetic mechanisms also play a major role in the aetiology of auto-immunity[78].

Galactosemia[x]

A homozygous GALT (galactose-1-phosphate uridylyltransferase) mutation on chromosome 9p13 leads to a severe disease in the newborn. Around 81% of galactosemia patients, e.g., homozygous GALT mutation carriers, develop ovarian failure[79]. There is uncertainty whether a heterozygous GALT mutation influences ovarian reserve or menopausal age, since three studies using questionnaires have shown conflicting evidence[80-82]. No studies have so far investigated the relationship between GALT carriership (heterozygosity) using serum and ultrasonographic markers for ovarian reserve (Chapter 5).

x Galactosemia is a rare genetic metabolic disorder which affects an individual's ability to properly metabolize the sugar galactose.

Genetic terms and techniques

Since one of the main aims of the current thesis focuses on the identification of genetic variants for the POF phenotype, a few of the most widely used genetic terms and techniques are explained below.

Genetic variation: sequencing, (tagging) SNPs, linkage disequilibrium and the HapMap project

DNA is built out of four nucleotides, A, C, G and T. A single human genome contains around 2.85 billion nucleotides spread over the 23 chromosome pairs. The order of these nucleotides is more than 99.9% identical in every human being. A vast amount of the human phenotypic variation can be explained by genetic variation in single nucleotides. If a nucleotide is substituted by another nucleotide in more than 1% of the population it is considered a single nucleotide polymorphism (SNP). Current estimates are that SNPs occur as frequently as every 100-300 bases. This implies that each human genome contains approximately 10 to 30 million potential SNPs. More than 3 million SNPs have been identified so far[83].

The HapMap describes all SNPs in the human genome as well as their pairwise correlations. The HapMap project is built upon the notion that DNA is not passed onto the next generation nucleotide by nucleotide, but in blocks. These DNA blocks are called haploblocks or haplotypes[84]. Nucleotides or SNPs within such a block are correlated to each other, i.e. they are in linkage disequilibrium (LD). Hence, a SNP within a haploblock can usually predict other closely correlated SNPs, i.e. it can 'tag' other SNPs within that block. Instead of genotyping all nucleotides within a certain area, referred to as sequencing, it is nowadays possible, using tag SNPs, to genotype a genome with a limited set of tag SNPs (currently usually between 100,000 and 1,000,000).

Genome-wide chips

Currently, genome-wide SNP arrays (mainly manufactured by companies as Illumina and Affymetrix) use a fixed set of SNPs throughout the human genome (in the case of Affymetrix these are densely spread SNPs, for Illumina they are tag SNPs). The most recent genotyping arrays contain 1,000,000 oligonucleotides[xi] per array. These chips can be used in a case-control setting[xii] which is often referred to as genome-wide association study (GWAS)[85], on multigenic or complex traits such as POF. A GWAS combines candidate studies (if the genes of interested are covered by the array) and may also identify new loci in the genome, e.g. those involved in regulating the ovarian follicle pool without making prior assumptions about candidate genes or regions[86].

GWA studies have already identified nearly 100 loci for some 40 common diseases and traits[87]. In Chapter 6 we describe using a genome-wide array to identify genetic variants associated to POF using a well phenotyped cohort of POF patients and controls.

xi An oligonucleotide is a short nucleic acid polymer, typically with 20 or fewer bases, it can readily bind to their respective complementary nucleotide, and is therefore often used as a probe for detecting a unique DNA sequence.

xii Case-control studies are used to identify factors that may contribute to a medical condition by comparing subjects who have that condition (the 'cases') with patients who do not have the condition but are otherwise similar (the 'controls').

A new genetic paradigm: copy number variation (and its potential role in POF)

After the completion of the human genome sequence in 2001, the initial hypothesis was that SNPs could explain most of the phenotypic differences between humans. In 2006, the discovery of multiple copy number variants (CNV) in the human genome dramatically expanded our understanding of the differences between individuals and initiated a paradigm shift in genetics[88-97]. CNVs are small deletions and duplications of between 1 kb to several mega bases in size. In other words: *SNPs change DNA quality (e.g. C instead of A), and CNVs change DNA quantity (e.g. fewer nucleotides at a locus due to a microdeletion).*

At the moment, large GWAS datasets are available, and besides this high throughput genotyping data, the oligonucleotide probes also generate intensity data, providing an estimation of how intensely DNA binds. Where DNA is deleted, intensities are low, where DNA is duplicated, intensities are high. Using appropriate software, these intensities can be used to detect CNVs in areas where the probes are placed[98]. For example, the study by Redon and colleagues 96 identified more than 1400 CNV regions (~12% of the genome) in the 270 HapMap individuals; they used an Affymetrix 500K chip together with a whole-genome tiling path comparative genomic hybridization (CGH) array that covered most of the euchromatic DNA.

In 2007 Illumina Inc. announced the Human-CNV-370-duo BeadChip. This array contains the same SNPs as its precursor, the Human 300K chip, but includes an extra ~55,000 markers specifically designed to detect around 11,000 copy number variants throughout the human genome. This additional content provides dense coverage of nearly 2700 CNV regions from the Database of Genomic Variants (http://projects.tcag.ca/variation/). In Chapter 7 we describe using this chip to study the role of CNVs on the X-chromosome in POF patients.

Aim and outline of the thesis 1

Aim

To further elucidate the phenotype of premature ovarian failure and to explore the genetic mechanisms underlying this phenotype.

Outline

Chapter 1 is the general introduction.

Chapter 2 provides reviews of the biology, genetics and assessment of female reproductive ageing in the social context that Western women are now delaying their childbearing.

Chapter 3 is a phenotype study which describes the distribution of novel ovarian reserve parameters (anti-Müllerian hormone, inhibin B and antral follicle count) in the complete spectrum of young women with elevated FSH levels.

Chapter 4 is another phenotype study and investigates the possible differences between the lipid profiles of premature ovarian failure (POF) patients and population controls, to further examine the relationship between increased cardiovascular disease risk and cessation of ovarian function.

In **Chapter 5** we investigate whether a genetic variant (GALT mutation carriership) influences the phenotype of ovarian reserve (measured via AMH) or age at menopause.

In **Chapter 6** a genome-wide association analysis to identify new genetic variants in POF is described.

Further genetic exploration was performed and in **Chapter 7** we describe the structural variation (copy number variants) of the X chromosome in POF patients.

Chapter 8 is a general discussion and proposes future research concepts.

Table 1. Overview of (potential) POF candidate genes.

Gene*	Gene-name	Locus in human	Animal / human	Reference	Summary
HSD3B2	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	**1p13.1**	Human	99	Arif et al. (1999) reported an association between premature ovarian failure, 3betaHSD autoimmunity, and a distinctive HLA-DQ molecule and proposed the hypothesis that autoantibodies to this steroid cell enzyme may be markers of autoimmune ovarian failure.
DDX20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20	**1p21.1-p13.2**	Rat	100	Follicle atresia, Transcriptional factor FOXL2 interacts with DP103 and induces apoptosis
Msh4	MutS homologue 4	**1p31**	Mouse	101	Profase I meiotic defects at zygotene/pachytene stage; resulting in germ cell loss within a few days post-partum
LHX8	LIM homeobox 8	**1p31.1**	Mouse / human	102 / 103	Lack of germcells in null-mice / Uncommon in Caucasian POF patients
TGFBR3	transforming growth factor, beta receptor III	**1p33-p32**	Human	104, 105	mutational report of the TGFBR3 gene in correlation with ovarian failure. Significant diversity of genotype distribution and haplotype analysis suggested susceptibility of the TGFBR3 gene for ovarian failure aetiology
Wnt4	Wingless-related MMTV integration site 4	**1p35**	Mouse	106	Ovaries depleted from oocytes
GJA4	Gap junction protein, ,alpha 4	**1p35.1**	Mouse	107	Late folliculogenesis defect. Associated with Perrault syndrome in human
Bmp8b	Bone morphogenetic protein 8b	**1p35-p32**	Mouse	108	Absent PGC population
Gpr3	G protein-coupled receptor 3	**1p36.1-p35**	Mouse / human	109 / 110	Role in meiotic arrest in the mouse oocyte / 0/82 POF women showed perturbations of significance
FIGLA	folliculogenesis specific basic helix-loop-helix	**2p13.3**	Mouse / human	111 / 112	No primordial follicles at birth and oocytes die./4 mutations in 100 POF women
LHR	Luteinizing Hormone Receptor (LHCGR)	**2p21**	Mouse	113, 114	Preantral folliculogenesis block in combination with underdeveloped sex organs

● ENTREZ gene name (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene) is chosen were more gene abbreviations are commonly used.

FSHR	FSH receptor	**2p21-p16**	Mouse / human	115 / 116	Female pre-antral block in folliculogenesis. The protein encoded by this gene belongs to family 1 of G-protein coupled receptors. It is the receptor for follicle stimulating hormone and functions in gonad development. Alternative splicing occurs at this locus and two transcript variants encoding distinct isoforms have been identified.
EIF5B	eukaryotic translation initiation factor 5B	**2q11.2**	Human	117	eIF2B mutations, already described in 93 cases of POF associated with white matter abnormalities, are an uncommon cause of pure spontaneous premature ovarian failure.
INHA	inhibin, alpha	**2q33-q36**	Human	118, 119	The Inhibin alpha subunit joins either the beta A or beta B subunit to form a pituitary FSH secretion inhibitor. Inhibin has been shown to regulate gonadal stromal cell proliferation negatively and to have tumour-suppressor activity. In addition, serum levels of Inhibin have been shown to reflect the size of granulosa-cell tumours and can therefore be used as a marker for primary as well as recurrent disease. However, in prostate cancer, expression of the inhibin alpha-subunit gene was suppressed and was not detectable in poorly differentiated tumour cells. Furthermore, because expression in gonadal and various extragonadal tissues may vary several fold in a tissue-specific fashion, it is proposed that inhibin may be both a growth/differentiation factor and a hormone.
Mlh1	MutL homologue 1	**3p21.3**	Mouse	120, 121	Meiotic arrest and genomic instability
DAZL	deleted in azoospermia-like	**3p24**	Mouse / human	122 / 123, 124	The DAZ (Deleted in AZoospermia) gene family encodes potential RNA binding proteins that are expressed in prenatal and postnatal germ cells of males and females. The protein encoded by this gene is localized to the nucleus and cytoplasm of fetal germ cells and to the cytoplasm of developing oocytes.
FOXL2	forkhead box L2	**3q23**	Mouse / human	125, 126 / 127 128, 129	Aborts proliferation of granulosa cells.
KIT	Kit receptor, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	**4q11-q12**	Mouse	130	White spotting null mutation causes PGC defects

Tert	Telomerase reverse transcriptase	**5p15.33**	Mouse	131	Fewer oocytes leading to progressive infertility
Fst	Follistatin	**5q11.2**	Mouse	132	TgfBETA BINDING PROTEIN
Adamts19	ADAM metallopeptidase with thrombospondin type 1 motif, 19	**5q31**	Mouse	133	Ovary specific metalloprotease upregulated during female gonadal development
GDF9	growth differentiation factor 9	**5q31.1**	Mouse / human	134, 135 / 136-139	Growth factors synthesized by ovarian somatic cells directly affect oocyte growth and function. Growth differentiation factor-9 (GDF9) is expressed in oocytes and is thought to be required for ovarian folliculogenesis. GDF9 is a member of the transforming growth factor-beta superfamily.
MSH5	mutS homolog5	**6p21.3**	Mouse / human	140 / 141	KO mice have normal ovaries at birth with decline form day 3 until adulthood. / In human 2/41 POF patients showed a mutation
POU5F1	POU class 5 homeobox 1	**6p21.31**	Mouse	142	Loss of Oct4 function leads to apoptosis of PGCs rather than to differentiation into a trophectodermal lineage, as has been described for Oct4-deficient ICM cells. These new results suggest a previously unknown function of Oct4 in maintaining viability of mammalian germline.
Foxo3a	FOXO subfamily 3a	**6q21**	Mouse / human	143, 144 / 145-147	Retarded oocyte growth and follicular development. Mutations cause a reduction in expression of BMP15, connexin 37 and connexin 43
ESR1	estrogen receptor α	**6q25.1**	Human	148	At a repeat in a promoter of the oestrogen receptor alpha (ESR1) gene, POF patients had fewer (<18) short repeat alleles than did controls (P=.004 vs. combined controls). Genotypes consisting of two short alleles were found in 36.4% of control women but only 5.5% of the 55 POF patients (P<.0001 vs. 134 combined controls). The ESR1 repeat may confer risk for POF in a simple dominant manner in which carriers of a long repeat have a relative risk of 9.7 (95% CI = 2.6 – 35.6).
EGFR	epidermal growth factor receptor	**7p12.3-p12.1**	Zebrafish	149	EGF is predominantly expressed in the oocytes whereas EGFR was expressed in the follicle cells, strongly suggest that EGF is likely a potential paracrine/justacrine factor from the oocytes to regulate the function of the follicle cells.

Ahr	Aryl-hydrocarbon receptor	**7p15**	Mouse	150	Early development of primordial follicles, decreased number of antral follicles
NOBOX	Newborn ovary homeobox gene	**7q35**	Mouse / human	151 / 152 153	Postnatal oocyte loss and abolishes the transition from primordial to growing follicles in mice. / In POF patients deletions are found but rare.
NR6A1	nuclear receptor subfamily 6, group A, member 1	**9q33-q34.1**	Mouse	154	NR6A1 directly regulates paracrine communication between the oocyte and somatic cells by regulating the expression of BMP-15 and GDF-9, to affect female fertility.
SOHLH1	spermatogenesis and oogenesis specific basic helix-loop-helix 1	**9q34.3**	Mouse	102	Sohlh1 disruption perturbs follicular formation in part by causing down-regulation of two genes that are known to disrupt folliculogenesis: Nobox and Figla. In addition Lhx8 is downstream of Sohlh1 and critical in fertility. Thus, Sohlh1 and Lhx8 are two germ cell-specific, critical regulators of oogenesis.
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	**10q11.1**	Mouse	155	Involved in guidance of Primordial Germ Cell Migration. The authors demonstrate that sdf-1 mRNA is expressed in locations where PGCs are found and toward which they migrate in wild-type as well as in mutant embryos in which PGC migration is abnormal. Knocking down SDF-1 or its receptor CXCR4 results in severe defects in PGC migration.
PTEN	phosphatase and tensin homolog	**10q23.3**	Mouse	156	Normal ovarian and follicle development at birth and early depletion in early adulthood.
BDNF	brain-derived neurotrophic factor	**11p13**	Mouse	157	Using gene-targeted mice lacking all TrkB isoforms, we show that the ovaries of these mice or those lacking both NT-4 and BDNF suffer a stage-selective deficiency in early follicular development that compromises the ability of follicles to grow beyond the primary stage. Proliferation of granulosa cells-required for this transition-and expression of FSH receptors (FSHR), which reflects the degree of biochemical differentiation of growing follicles, are reduced in trkB-null mice. Ovaries from these animals fail to sustain follicular growth and show a striking loss of follicular organization, preceded by massive oocyte death. These results indicate that TrkB receptors are required for the early growth of ovarian follicles and that they exert this function by primarily supporting oocyte development as well as providing granulosa cells with a proliferative signal that requires oocyte-somatic cell bidirectional communication.

FSHB	FSH hormone βsubunit	**11p13**	Mouse / human	158 / 159	Female pre-antral block in folliculogenesis
ATM	Ataxia telangiectasia	**11q22.3**	Mouse	160, 161	Germ cells generate; disruptions evident in meiosis I
KITLG	KIT ligand	**12q22**	Mouse	162	Steel defect mutation causes defect in PGC migration/survival
Foxo1a	Forkhead box 01	**13q14.1**	Mouse / human	163 / 145	Key regulator in G1/S transition in granulosa cells influencing their growth during follicle development
Rb1	Retinoblastoma	**13q14.1-q14.2**	Mouse	164	Knockout mice showed increased follicular atresia and apoptosis
BCL2L2	BCL2-like 2	**14q11.2-q12**	Mouse	165	Reduced PGC survival
Bmp4	Bone morphogenetic protein 4	**14q22-q23**	Mouse	166	Absent PGC population
ESR2	Estrogen Receptor β	**14q23.2**	Mouse	167	Subfertility in mouse
POLG	Polymerase, Dna, Gamma	**15q25**	Human	168	Dysfunction of mitochondrial POLG causes a severe progressive multisystem disorder including parkinsonism and premature menopause
cpeb1	cytoplasmic polyadenylation element binding protein 1	**15q25.2**	Mouse	169	Follicle arrest in meiotic profase I, with no germcells in the postnatal ovary.
SH2B1	SH2B adaptor protein 1	**16p11.2**	Mouse	170	Small anovulatory ovaries with reduced numbers of developing follicles
Ybx2	Y box binding protein 2	**17p11.2-p13.1**	Mouse / human	171 / 172	Female mice lacking Ybx2 protein have normal ovaries after birth but during life ovarian abnormalities emerge (e.g. follicle loss, sterility in human males and females)
NOG	Noggin	**17q22**	Human	173, 174	Because NOG is expressed in the ovary and interacts with bone morphogenetic proteins, which play an important role in the ovarian function, a NOG mutation may constitute one of the multiple susceptibility genes for the development of POF. / Screening for NOG mutations in 100 non syndromic POF did reveal one heterozogyzous nonsynonymous substitution

TAF4B	TATA box binding protein (TBP)-associated factor 4B	**18q11.2**	Mouse	175, 176	Follicular development defects
Bcl2	B-cell CLL/lymphoma 2	**18q21.3**	Mouse	177	Fewer primordial follicles in the post-natal ovary
NANOS3	Nanos homolog 3	**19p13.12**	Mouse / human	178 / 179	RNA binding protein. No germcells in male and female knock-out mice./ No perturbations identified in 80 Chinese and 88 American Caucasians
AMH	anti-Mullerian hormone	**19p13.3**	Mouse / human	180, 181	Early depletion of primordial follicles in mice
LHB	luteinizing hormone beta polypeptide	**19q13.32**	Human	182 183	Mutations in this gene are associated with hypogonadism which is characterized by infertility and pseudohermaphroditism./ Mutation in 2 hypogonadotropic brothers and one POF sister
NLRP5	NLR family, pyrin domain containing 5 (Mater)	**19q13.42**	Mouse	184	Development beyond the two cell stage is blocked; maternal effect gene
RFPL4A	ret finger protein-like 4A	**19q13.42**	Mouse	185	RFPL4 targets cyclin B1 for proteasomal degradation, a key aspect of oocyte cell cycle control during meiosis
Cdc25b	Cell division cycle 25 homolog B	**20p13**	Mouse	186	Oocytes arrested in meiotic prophase
BCL2L1	BCL2-like 1	**20q11.21**	Mouse	187	Primordial germ cell apoptosis regulating gene
BMP7	bone morphogenetic protein 7	**20q13**	Rat	188	Initiation of primordial follicle growth
SPO11	SPO11 homolog	**20q13.2-q13.3**	Mouse	189, 190	Meiotic defects resulting in post partum oocyte loss
AIRE	autoimmune regulator (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy)	**21q22.3**	Human	191	Ovarian failure is associated with autoantibodies to steroid hormone secreting cells in the adrenal cortex in these POF patients,
DMC1	Disrupted meiotic cDNA 1 homolog	**22q13.1**	Mouse / human	141, 192, 193	Chromosome synapsis defect during meiosis resulting in germ cell degeneration Oogenesis in mice knockout was disrupted. / In human 1/41 POF patients showed a perturbation

USP9X	Ubiquitin-Specific Protease 9	**Xp11**	Drosophila	194	In Drososophila this gene is required for oogenesis and maps in a highly POF susceptible region on the X chromosome
BMP15	bone morphogenetic protein 15	**Xp11.2**	Mouse / human	195/ 138, 196-199	The protein encoded by this gene is a member of the bone morphogenetic protein family which is part of the transforming growth factor-beta superfamily. The transforming growth factor-beta superfamily includes large families of growth and differentiation factors. It is thought that this protein may be involved in oocyte maturation and follicular development as a homodimer or by forming heterodimers with a related protein, Gdf9.
DDX3X	Dead box polypeptide 3, X linked	**Xp11.3-p11.23**	Mouse	200	Involved in germ cell metabolism / No studies in human
ZFX	zinc finger protein, X-linked	**Xp22.2-p21.3**	Mouse	201	Reduced germ cell numbers and maps in POF critical region
AR	Androgen receptor	**Xq11.2-q12**	Mouse	202	Impaired folliculogenesis
TAF1	TATA box binding protein (TBP)-associated factor	**Xq13.1**	Drosophila	203	Function in oocyte kinetochore formation and chromosome cohesion
DACH2	dachshund homolog 2 (Drosophila)	**Xq21**	Human	204 205	X/autosomal translocation in a POF patients. A recent did not find a high incidence of mutations in a cohort of 212 POF patients.
POF1B	premature ovarian failure, 1B	**Xq21.1-q21.2**	Human	62 205	A linkage study in a family with primary amenorrhea found homozygosity for R329Q in exon 10 and mapped distal to POF2. Replication in POF (n=207) failed.
DIAPH2	diaphanous homolog 2 (Drosophila)	**Xq21.33**	Drosophila / human	206 207 / 208	In flies dia perturbation causes sterility. / In humans an Xq21/ autosome translocation was found to have undergo intronic disruption. And in a recent study 1 of 100 patients showed a deletion CNV.
CENPI	Centromere protein I	**Xq22.1**	Rat	209	Protein involved in the response of gonadal tissues to follicle-stimulating hormone and a potential candidate for human X-linked disorders of gonadal development.

PGRMC1	Progesterone Receptor Membrane Component-1	**Xq22-q24**		210	Mutation screening of 67 females with idiopathic POF identified a patient with a missense mutation (H165R) located in the cytochrome b5 domain of PGRMC1. PGRMC1 mediates the anti-apoptotic action of progesterone in ovarian cells and it acts as a positive regulator of several cytochrome P450 (CYP)-catalyzed reactions. These findings suggest that mutant or reduced levels of PGMRC1 may cause POF through impaired activation of the microsomal cytochrome P450 and increased apoptosis of ovarian cells.
BHLHB9	basic helix-loop-helix domain containing, class B, 9	**Xq23**	Human	211	apoptosis gene in the POF Critical region only investigated in an Alzheimer patient
XPNPEP2	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	**Xq25**	Human	212	Translocation breakpoint in POF patient at XPNPEP2 locus.
POF1	premature ovarian failure 1	**Xq26-q28**	Human	213	Gene based on identified cytogenetic studies of X-chromosome aberrations in POF patients. Not annotated on genomic reference assembly
SOX3	SRY (sex determining region Y)-box 3	**Xq27.1**	Mouse	214	Female Sox3 null mice (-/-) developed ovaries but had excess follicular atresia, ovulation of defective oocytes, and severely reduced fertility.
FMR1	fragile X mental retardation 1	**Xq27.3**	Human	215	~16-20% of premutation carriers have POF
FMR2	fragile X mental retardation 2	**Xq28**	Human	216	microdeletions within FMR2 may be a significant cause of premature ovarian failure, being found in 1.5% of women with the condition, and in only 0.04% of the general female population, not confirmed in other populations
XIST	X (inactive)-specific transcript (non-protein coding)	**inactive Xq13.2**	Human	217-220	Although hypothetically interesting only one study reported increased X skewing in ovarian failure patients

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Female reproductive ageing: current knowledge and future trends

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Chapter 2

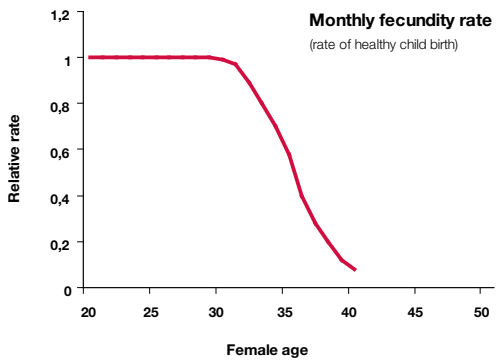
Introduction

The large scale availability of reliable methods for contraception from the 1960s onwards has greatly influenced the number of children born per family in Western societies[221]. Control of reproduction and growing economical wealth have afforded women the opportunity to increase their level of education and participation in the labour force. As a result, first child bearing was postponed in many women leading to a sharp increase in the mean female age at first childbirth 3 (see also http://epp.eurostat.ec.europa.eu).

Female reproductive ageing is a process that will increasingly reduce monthly fecundity (the ability to have a viable embryo implanted) after the age of 30 (figure 1). By postponing childbearing, a growing proportion of women attempting to conceive will fail in achieving that goal within a time frame of 12 months, a condition referred to as subfertility[222]. The struggle to become pregnant for women who start families in their thirties should be added to the impact of the voluntary reduction in the number of children per family. This resulted in a drop in the estimated average number of children born per family of ~3 in the early 1960s to ~1.5 in 2000 for the member countries of the European Union. For the USA the decline in total fertility rate from the 1960s onwards has halted and currently is just over 2 children per woman[221]. An increasing proportion of couples will depend on assisted reproduction technologies (ART) in order to achieve a pregnancy, solely on the basis of the postponement of childbearing. This trend has major implications for society[223].

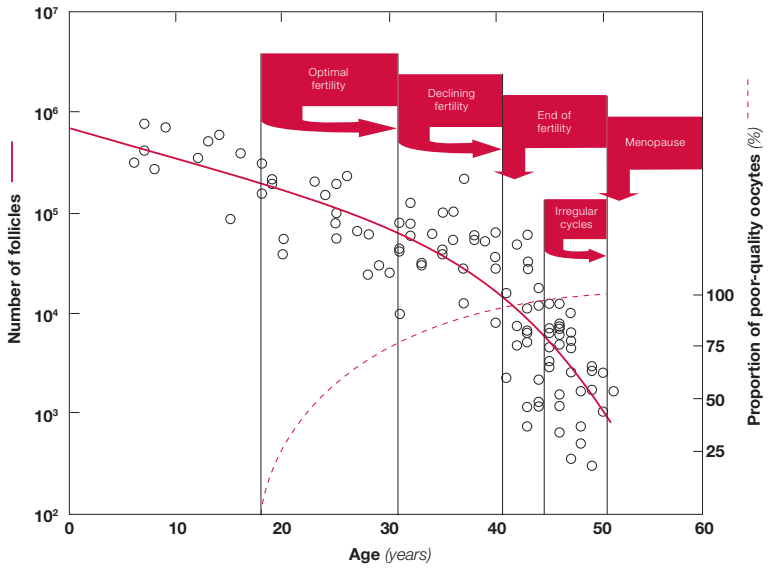
In this review the backgrounds of reproductive ageing are presented in the context of the biological and genetic factors involved in this process. Methods for assessing a woman's individual reproductive age status are also summarised along with current and future possibilities to use these tools in prediction of fertility potential.

Figure 1.



The decrease in monthly fecundity rate (rate of healthy child birth) relative to the fecundity rate of women in the age group of 20 – 30 years. The fall in fecundity is estimated to start at around 31 years (critical age), after which the probability of conception falls rapidly. (Redrawn with permission from [232]).

Figure 2.



The decline in follicle number (solid line) and the increase in poor quality oocytes (dotted line) in relation to reproductive events with increasing female age (reprint with permission from [284])

Biology of reproductive ageing

The reproductive ageing process is thought to be dictated by a gradual decrease in both the quantity and the quality of the oocytes held within the follicles present in the ovarian cortex 3. At the fourth month of fetal development the ovaries contain some 6-7 million oocytes surrounded by a layer of flat granulosa cells to form primordial follicles. Through a rapid transition of the majority of the primordial follicles via apoptosis at birth, only 1-2 million primordial follicles remain[224]. After birth this high rate of follicle loss slows down so that at menarche approximately 300,000 to 400,000 follicles remain (figure 2). During the reproductive years the decline in the number of primordial follicles remains steady at some 1000 follicles per month and accelerates after the age of 37. At the time of menopause, the number of remaining follicles has dropped clearly below 1000[225].

With the decay in follicle numbers oocyte quality also diminishes, at least after the age of 31 years when fecundity gradually decreases (figure 1 and 3). The loss of oocyte quality is believed to be established by an increase in meiotic non-disjunction leading to an increasing rate of aneuploidy in the early embryo at higher female ages. Several rea-

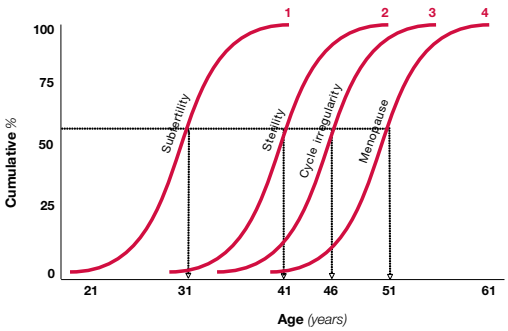
sons may underlie the age related decay in oocyte quality. They relate to possible differences between germ cells at the time they are formed during fetal life, accumulated damage of oocytes in the course of a woman's life or changes in the quality of the granulosa cells surrounding the oocyte 3.

The first noticeable clinical sign of progress in the reproductive ageing process is a shortening of the length of the menstrual cycle by 2-3 days, while regularity remains unaffected[226]. This is the result of a shortening of the follicular phase, owing to early selection and maturation of the dominant follicle[227, 228]. Elevated follicle stimulating hormone (FSH) levels are held responsible for this early start and result from decreased circulating levels of inhibin B as an expression of a decreased number of small antral follicles[229].

Despite the subtle changes in cycle length, regularity remains unaffected in a period in which already clear changes occur in follicle numbers[230]. At the same time profound changes occur at the oocyte level. Even with regular menstrual cycles during many years monthly fecundity becomes dramatically decreased (figure 2). From natural population studies it has been shown that the end of natural fertility already occurs at a mean age of 41[231]. From studies where both coital behaviour and the male factor have been controlled for, the same pattern of decline in female fecundity has become apparent, indicating that the fecundity decrease is mainly accounted for by the ovarian factor[232] (Figure 1).

It is only at the time when cycles become irregular that women first notice the signs of the ongoing reduction in follicle numbers. The occurrence of menopause eventually represents an almost exhausted follicle pool[233]. The mean age at menopause is 51 years with variation ranging

Figure 3.



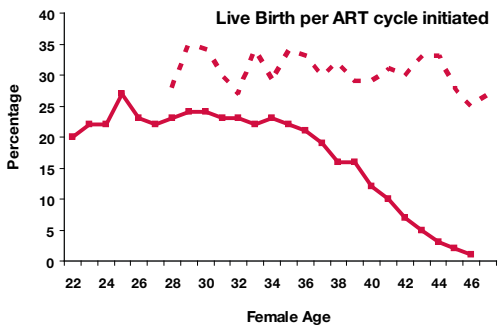
The distributions of age at the onset of subfertility (cumulative curve 1), at occurrence of natural sterility (cumulative curve 2), at transition into cycle irregularity (cumulative curve 3) and at occurrence of menopause (cumulative curve 4). Mean ages for these events are depicted on the X-axis. Curve 4 is based on data by Treloar and Broekmans[231, 234], curve 3 and its temporal relation to curve 4 is based on data from den Tonkelaar[235], curve 2 is based on data on last child birth in a 19th century natural fertility population[285] and curve 1 is a hypothetical construct based on the age distribution of related reproductive events as depicted in curve 2,3 and 4 and partially supported by data from Eijkemans[237].

from ~40 – ~60 years[234], but possibly even into younger ages[231]. This variation also holds for the occurrence of the irregular menstrual cycles preceding menopause at a mean age of 46 years[235]. Between the onset of cycle irregularity and the occurrence of menopause a fixed temporal relationship is believed to be present 3, although longitudinal data are scarce[236] (figure 3). As the age at which women become sterile shows the same degree of variation as observed for menopause[231], it is generally assumed that this event carries a fixed temporal relationship with cycle irregularity and menopause with a presumed interval of about 5 and 10 years, respectively. The same fixed relationship may also be true for the age at which women start to become subfertile (assumed mean age 31 years) (figure 3)[237, 238].

The human species can be considered as relatively subfertile compared to animal species[239, 240]. The average monthly fecundity rate of about 20% implies that among human couples trying to conceive many exposure months may be needed to achieve their goal, especially if monthly fecundity has dropped with increasing female age[222]. The proportion of subfertile couples (failing to achieve a vital pregnancy within one year) will amount to 10-20% in the age group of women over 35, compared to only 4% for women in their 20s. These subfertility rates may rise to 30-50% for only moderately fecund women of age 35 and over who have tried to conceive for several years[222, 241]. The maintenance of regular menstrual cycles until ages where natural fecundity has already been reduced to zero means that women are largely unaware of this process taking place.

An age related decline in fertility has also been shown in numerous reports on assisted reproduction technology (ART, i.e. In Vitro Fertilisation (IVF))

Figure 4.



Effect upon average singleton live birth rates of female age, showing a steady decrease after the age of 34 years. The dotted line represents the average singleton live birth rate after oocyte donation as a function of the recipient age. It underlines the potential of oocyte donation in the treatment of women who remained unsuccessful in previous IVF treatment. Data were drawn from the 2003 CDC ART report http://www.cdc.gov/ART/ART2003/section2a.htm#f12).

programs (figure 4). The chance of implantation for an IVF embryo is also dependent on the ovarian reserve status of an individual woman. A poor response to ovarian hyperstimulation in IVF, especially in those with abnormal ovarian reserve test results, is a strong predictor of poor prospects of becoming pregnant, and also of clearly reduced spontaneous fecundity and early menopause[242-244].

Age related subfertility is often considered a problem that can be easily solved by the application of ART. From studies by Leridon[221] it was recognised that postponing family building until well in the thirties will frequently lead to a definitive loss of a woman's own reproductive potential even after applying ART treatment. However, as shown in figure 4, it can be deduced that application in IVF of donor oocytes from younger women will greatly restore the reproductive prospects for women at ages at which they have little chance to obtain a pregnancy with their own oocytes.

Genetics of reproductive ageing

The variation of menopausal age is highly similar across populations and follows a Gaussian distribution with skewness to the younger ages[231]. In general, factors that dictate the variation in age at menopause are not known. Many environmental and lifestyle factors have been suggested to affect age at natural menopause, such as oral contraceptive use, parity and smoking[46, 245-247]. However, these factors do not fully explain the variation in menopausal age[46, 248, 249]. In recent years the role of genes as determinants of the trait menopausal age has received growing attention. Association between menopausal age of mothers and daughters and sister pairs has been convincingly demonstrated, implying indeed that genetic factors must be greatly involved in the reproductive ageing process. Estimates of heritability for age at menopause range from 30 to 85%[54, 238, 246, 250].

For a complex trait such as menopausal age association studies are suitable to identify related genetic determinants[251]. The principle is to study associations between a trait (menopausal age) and a candidate marker gene. Candidate genes may be chosen based on their role in fetal ovarian development, primordial follicle maturation, follicular apoptosis or ovarian vascularisation and preferably contain known genetic variants (polymorphisms) that may lead to the variation in the trait. Also, research in patients with premature ovarian failure (POF) may provide candidate genes. For instance, microdeletions involving GDF-9 and BMP-15 and FOXL2, factors that are involved in the transition from primordial follicles into early growing follicles, have shown to produce early ovarian arrest[125, 198, 199, 252]. Detailed information on single nucleotide polymorphisms (SNPs) for all of these genes can be obtained from databases in which the haplotypes for a certain population have been mapped[84, 253]. By identifying the SNPs that may be present in the candidate gene, further analysis on the relationship between the SNPs and the phenotypic variant can be carried out.

Recently, studies addressing this type of genetic analysis in the human have emerged. The presence of at least one mutant allele of Factor V Leiden and Apolipoprotein E (APOE) -2 was associated with age at natural menopause[254, 255]. Also, higher levels of clotting factor VII are associated with early menopause, indicating that vascular compromise may well be the cause for earlier menopause in stead of being the result of it[256, 257]. This indicates that genetically altered vascular support with accumulation of oxidative

stress may have long term effects on ovarian follicle depletion[254, 258-260]. A common polymorphism within the steroid 5-α-reductase type 2 gene, leading to reduced conversion of testosterone into 5-dihydro-testosterone, appeared to have no relation to menopausal age[261]. A decrease of influence by androgens upon follicle growth and wastage therefore seems unlikely. The oestrogen-inactivating CYP1B1-4 polymorphism was shown to be associated with a reduced age at natural menopause[262]. With this polymorphism higher levels of oestrogens throughout the reproductive life are believed to exist, but in which way this would affect ovarian follicular wastage remains to be elucidated. In older studies association was found with oestrogen receptor (ER) polymorphisms, where women carrying the homozygous mutant allele had a 1.1 year later onset of menopause[263]. These findings however could not be confirmed in Dutch and Japanese cohort studies[264, 265].

Another genetic approach to detecting genes involved in the trait menopause is linkage-based genome-wide scanning. In this type of analysis the presence of phenotypic similarity between sibling sister pairs for the trait of interest is used. In a linkage based genome scan in 165 Dutch families, ascertained using extremely selected sampling and genotyped for 417 scanning markers, two chromosomal regions showed suggestive linkage: 9q21.3 and Xp21.3[61]. The finding of the region on the X chromosome comes as no surprise, because of its widespread involvement in premature ovarian failure[266]. Cases with macrodeletions and translocations of the X chromosome, mutations in the BMP15 gene or increased CGG repeats in the promoter region of the FMR1 gene all show increased risk for early ovarian failure[267]. For the chromosome 9 it is of interest that one of the genes in the linkage region encodes for a member of the BCL2 family, which is involved in apoptosis[268, 269]. Apoptosis is the most common fate of the follicles in the ovaries and the rate of apoptosis of the follicle pool is believed to dictate the advent of menopause. Fine mapping of the linked regions will be the next step and will necessitate the availability of a large cohort of women with adequate information on the age of natural menopause[270].

Currently, genome wide association studies using a fixed set of SNP's densely spread throughout the whole human genome become available for studies on menopause variation. This kind of studies may identify new loci in the genome involved in regulation of the ovarian follicle pool without making prior assumptions on candidate genes or regions 86. Also, large scale DNA copy number variations (CNV) present throughout the genome may also be associated with variation in human traits and therefore become targets in future research[96]. So, future approaches to unravel the genetic regulation of menopause must, besides SNP's, also take into account the structural variation patterns within the human genome (such as common deletions) the role of which in the pathogenesis of complex genetic diseases becomes more and more clear[89].

Assessment of reproductive ageing

In view of the variability of reproductive performance among women of the same age group there has been a long lasting need to develop a test that provides crucial information in addition to chronological female age. Most tests examined in the literature are evaluated by their capacity to predict some defined outcome related to ovarian reserve. The preferred or gold standard outcome

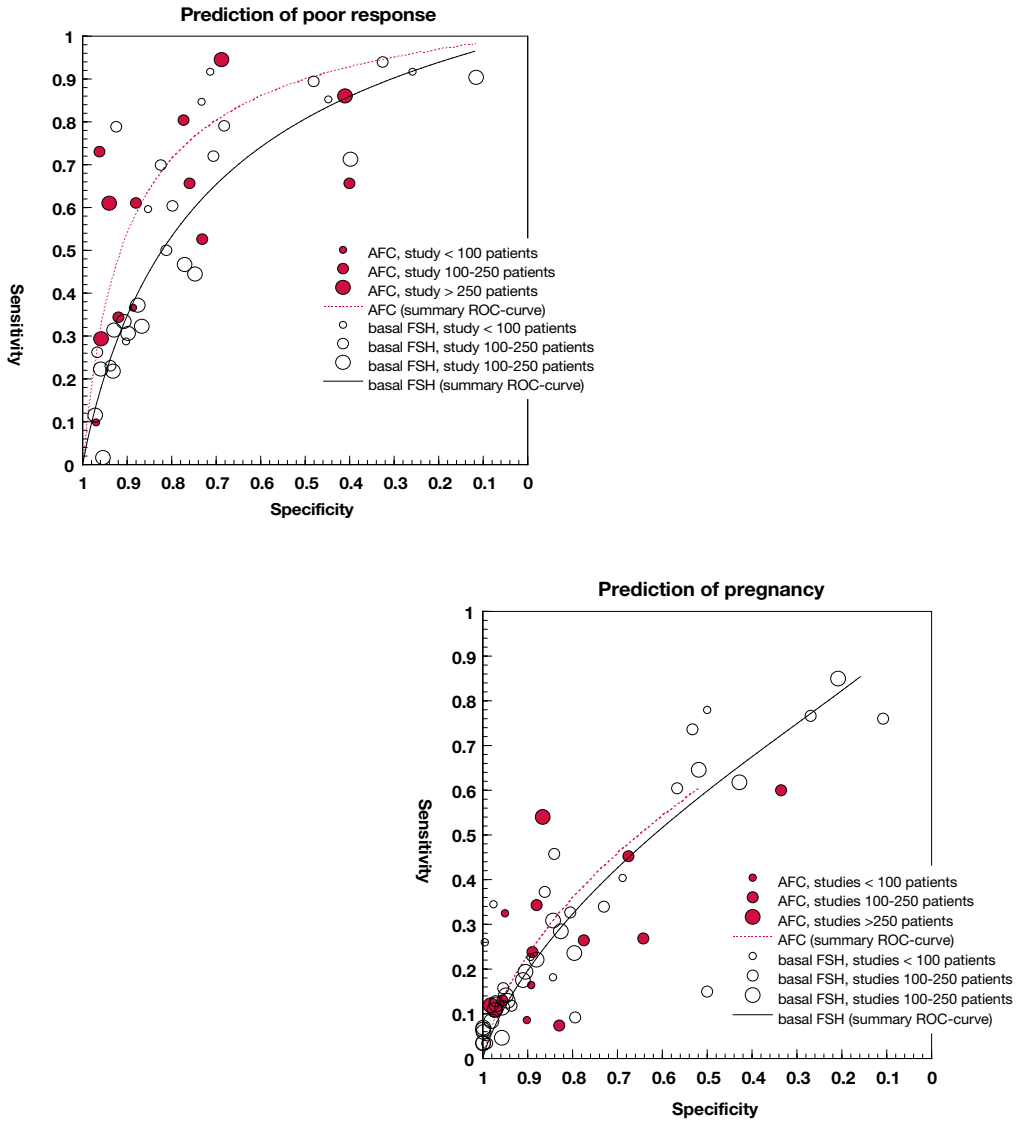
of prediction studies would be live birth after spontaneous exposure or ART, but other outcomes (such as oocyte yield or follicle number in IVF) are much more common.

Every ovarian reserve test relates to follicle cohort size. The antral follicle count (AFC) and ovarian volume (OVVOL) assessed by transvaginal ultrasonography provide direct measurements[271]. The endocrine markers anti-Müllerian hormone (AMH) and inhibin B which are released from antral follicles provide other direct markers of quantity[26, 272]. Basal FSH, extensively studied in the past decades, provides the most indirect marker. FSH levels will become increased with advancing age, by a reduction in the release of inhibin B, thereby reducing the negative feedback on FSH release from the pituitary[229]. High FSH levels therefore represent small cohort size. Endocrine challenge tests in which the growth of antral follicles is stimulated by endogenous or exogenous FSH and response is assessed in terms of output of oestradiol or inhibin B are also adequate markers of cohort size[26]. However, they are considered as too laborious for screening purposes and will not add much predictive value compared to static tests like AMH or the AFC[273, 274]. The same may be true for the clomiphene citrate (CC) challenge test, in which a CC induced rise in FSH levels is counteracted by release of oestradiol and inhibin B from growing antral follicles. The size of the antral follicle cohort will determine the amount of FSH suppression. Like the other challenge tests the CC challenge test does not provide much additional information compared to basal FSH[275, 276].

In a recent review the predictive performance of all these tests was analysed by using the approach of the systematic review and meta-analysis[26]. It was shown that most of the tests have quite adequate capacity to predict poor responders to ovarian hyper stimulation in IVF. If poor response was an endpoint of interest then the clinical value of these tests would be satisfactory (figure 5). Unfortunately, the predictive ability towards the occurrence of pregnancy after one IVF cycle was shown to be only marginal, as only very small proportions of the non pregnant cases were predicted correctly and false positives remained even with extreme cut offs for an abnormal test (figure 5). Even observed poor responders in the first treatment cycle fail to have such poor prospects in that cycle that prior prediction should lead to refusal of treatment. Only if a poor response occurs in cases with an otherwise unfavourable profile (female age over 38, abnormal ovarian reserve test, repeated poor response) does prognosis for subsequent cycles become cumbersome to an extent that justifies further denial of treatment[243, 244, 277]. In general, therefore, ovarian reserve testing for clinical practice has to be regarded as not useful. This is further shown in table 2, where the clinical value of several basal ovarian reserve tests in IVF treatment are depicted, based on a comprehensive meta-analysis of the existing literature[26].

Improvement of test performance in the identification of women with a reduced ovarian reserve for their age category may come from combining endocrine, imaging and genetic tests. Combination of several endocrine and imaging tests into predictive models has shown to improve the accuracy of poor response prediction[26]. With the possible finding of genetic markers for ovarian reserve status in the near future the performance of these models may improve further[278]. Currently, studies concerning AMH and ovarian ageing are rapidly accumulating[279-281]. In view of the high correlation between the AFC and AMH widespread availability

Figure 5.



Example of ovarian reserve test performance (AFC and FSH) showing receiver operator characteristic (ROC) curves for the prediction of poor response (upper panel) and non pregnancy (lower panel) in IVF. The solid circles represent the performance of the AFC, whereas the open circles represent the performance of basal FSH. Summary ROC-curves are given: the dotted line represents the summary ROC-curve for the AFC and the solid line represents the summary ROC-curve for basal FSH. Reprinted with permission from [271].

Table 2. The clinical value of several ovarian reserve tests for outcome prediction in IVF.

	Prediction of poor response in IVF (pre-test probability = 20%)		Prediction of non-pregnancy after IVF (pre-test probability = 80%)	
Test	Positive test rate (for the pLR value ≥4)	Post-test probability (of poor response)	Positive test rate (for the pLR value ≥4)	Post-test probability (of non-pregnancy)
FSH	5%	>50%	4%	>94%
Inhibin B	1%	>50%	2%	>94%
Estradiol	1%	>50%	0%	>94%
AFC	12%	>50%	2%	>94%
OVVOL	0%	>50%	0%	>94%

Shown is the occurrence of abnormal ovarian test results, given a positive likelihood ratio (pLR) value of ≥4, and the concomitant post-test probabilities of poor response and non-pregnancy, given a prevalence of poor response of 20% and non-pregnancy of 80%. Data were based on a recent meta-analysis on ovarian reserve tests 26.

In poor response prediction only for the AFC a reasonable proportion of positive tests is observed at cut-off levels with a moderate to good levels of the pLR, leading to a substantial change in the chance of producing a poor response in IVF. For non-pregnancy prediction the abnormal test rate is clearly low at the cut-off levels that lead to an appropriate overall test performance and probability of non pregnancy shifts moderately in case of such test result.

of the assay to measure AMH levels may lead to replacement of the AFC as the most direct test for quantitative reserve screening[282].

The true challenge for ovarian reserve tests lies in the possibility of identifying women with a reduced reproductive lifespan at such a stage in their lives that adequate action can be taken. In such test the preferable outcome variable to judge the test upon is the age at which a woman will become menopausal. The relation between menopausal age and the end of natural fertility has been hypothesized to be fixed 3. If a test existed that adequately predicts age at menopause, then family planning clinics where any young woman can be tested for her reproductive expectations or limits may become reality. On the basis of cross sectional data, however, such predictive test has not emerged, although some predictive ability has been attributed to the AFC, ovarian volume and AMH[231, 283]. First examples of longitudinal studies have shown increased early occurrence of the menopausal transition and menopause in poor responders in IVF (see table 3)[242, 243].

Conclusions

Age related female subfertility as a result of postponement of child bearing in Western societies can be mainly considered a problem for the couple itself when faced with involuntary childlessness. Yet, postponement of child birth also contributes to the reduction in family size in many European countries with halted population growth as the possible result. For several reasons this demographic development may be considered as undesired, as economical growth may become hampered and societal stability decreased.

Knowledge regarding the processes that dictate reproductive ageing is still limited. We understand the principles of reduction in follicle numbers with age but lack knowledge on how follicle reserve builds up in the fetal ovaries and is subsequently wasted. Thus, we cannot explain inter individual variation in this reduction process. We do know that oocytes lose or lack the competence to produce viable embryos with advancing age, but fail to understand the mechanisms behind it.

Our scarce knowledge at present prevents the possibility of assessing ovarian reserve or reproductive age in an adequate way and as such we are not capable of offering any preventive information on an individual basis. Identifying genetic markers of the processes that regulate follicle quantities and oocyte quality as well as longitudinal studies on the relationship between these markers and the occurrence of menopause seem needed to truly advance the field of assessing ovarian ageing and predicting reproductive potential on an individual basis.

Table 3.

Study group (IVF poor responders)					
Study	N	Median Follow Up Time	Cases (%) entered Menopause or Menopausal transition	FSH U/l	
Farhi, 1997 Case Report	12	9 months	100%	23-85	
De Boer, 2002/2003 Retrospective Cohort	636	6 years	22%	-	
Lawson, 2003 Retrospective Cohort	118	5 years	50%	-	
Nikolaou, 2002 Case Control	12	7 years	92%	-	

* Adjustments were carried our for age and/or smoking behaviour

	Control group (IVF normal responders)			Adjusted* Odds or Hazard ratio	Reference
	N	Median Follow Up Time	Cases (%) entered Menopause or Menopausal transition		
	-	-	-		286
	3675	5 years	7%	~3.1 (Odds)	242, 277
	265	5 years	16%	~3.1 (Hazard)	243
	24	7 years	17%	~5.3 (Odds)	287

Evidence from existing literature supporting the association between a poor response to ovarian hyperstimulation for IVF in women with regular cycles and the early occurrence of menopausal transition or menopause. Differences in the probability of entering menopause or the menopausal transition may arise from differences in the definition of poor responder (single or repeated occurrence) and study design (case-control versus cohort study).

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Anti-Müllerian hormone, inhibin B and antral follicle count in young women with ovarian failure

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Chapter 3

Introduction

Abnormal ovarian function is classified into three different subgroups, according to the World Health Organization (WHO). This classification is primarily based on serum levels of FSH and oestradiol[288]. FSH levels are regulated through negative feedback actions of inhibin and oestradiol, produced by the ovarian follicles[289]. A hypogonadotropic condition (WHO I) indicates disturbance at the hypothalamic-pituitary level, whereas a normogonadotropic oligo- or amenorrheic state (WHO II) is associated with a pituitary-ovarian dysbalance[290]. In contrast, a hypergonadotropic status (WHO III) coincides with ovarian dysfunction due to follicle pool exhaustion[291].

Idiopathic premature ovarian failure (POF) represents the most extreme phenotype of diminished ovarian reserve at young age. The most frequently applied definition of POF is the spontaneous absence of menses for at least 4 months in combination with FSH levels exceeding 40 IU/L before age 40. This condition occurs in approximately 1% of the female population[6]. It is not clear however, how women below the age of 40 with cycle disturbances and a hypergonadotropic hormonal status who do not fulfil the strict definition of POF should be counselled with regard to fertility treatment, nor whether they have a similar ovarian follicular status as POF patients. In the current study this intermediate group is referred to as transitional ovarian failure (TOF).

Incipient ovarian failure (IOF) or late reproductive aging (Stage 3) according to the Stages of Reproductive Aging Workshop (STRAW) classification, represents another subgroup characterized by elevated follicular phase FSH levels along with a regular menstrual cycle[292]. IOF precedes the onset of cycle irregularity and hence the menopausal transition by 3-10 years and may be considered an early sign of advanced ovarian aging in young women[293]. Thorough insight into the ovarian reserve profile of this heterogeneous and clinically important group is still lacking[294].

In western society, women are delaying starting a family until later in life. As a result, the number of female patients presenting with elevated FSH levels suggestive of reduced ovarian reserve – with or without cycle abnormalities – is increasing[295]. Therefore, a more thorough insight into the ovarian phenotype of these patients is warranted. Numerous studies indicate that FSH itself cannot be used as a predictive marker for deciding to start infertility treatment or for ovarian response prediction[296]. Recently more direct ovarian markers, such as anti-Müllerian hormone (AMH), inhibin B and antral follicle count (AFC) have become available.

AMH is a product of the granulosa cells that envelop the oocyte and continues to be expressed until the antral stage[297]. Inhibin B is produced by the cohort of developing preantral and early antral follicles, and its circulating concentrations are maximal during the early to midfollicular phase[298]. Early follicular inhibin B levels decrease during reproductive aging leading to increasing FSH concentrations[229]. Similarly, AFC decreases during reproductive aging in line with the contention that the number of visible antral follicles reflects the size of the primordial follicle pool[299]. In contrast to FSH, E_2, Inhibin B and AFC, AMH levels do not appear to vary with cycle day[300]. Moreover, AMH has a superior cycle-to-cycle reproducibility compared to inhibin B and FSH[301]. AMH levels show a decreasing trend with age, remaining relatively stable until age 30 but declining more steeply thereafter[230, 302].

Scant information exists with regard to AMH levels in patients presenting with a hypergonadotropic hormonal status at a young age. An earlier study compared patients with secondary amenorrhea with controls and identified a high percentage of very low or undetectable AMH levels in POF patients[303]. Another small study identified low AMH levels as marker of diminished ovarian reserve in IOF patients with consistently elevated FSH levels[304]. The present study aims to show the relationship between several direct markers of ovarian reserve and varying clinical degrees of ovarian failure based on FSH values and cycle disturbances in women under forty.

Materials and methods

Subjects

From October 2004 onwards, a nationwide standardized systematic screening protocol was applied for women with suspected diminished ovarian reserve visiting the infertility outpatient clinics of 10 Dutch hospitals. This protocol was approved by all local Institutional Review Boards and written informed consent was obtained from all participating women for standardized screening. This screening included a questionnaire regarding fertility, family history, climacteric complaints, as well as transvaginal ultrasonography and blood withdrawal.

Inclusion criteria for screeningwere: age between 25-40 years, increased FSH serum levels (>10.2 IU/L), a history of having experienced regular menstrual cycles (26-32 days), known last spontaneous menstruation date, no current use of hormone therapy and no history of radiotherapy/ chemotherapy or ovarian surgery. Women with regular cycles applying to these criteria were screened in the early follicular phase (cycle day 2-5), whereas women without a regular cycle were screened at random and progesterone levels were measured additionally. Serum was frozen in -20° C within 4 hours for further analysis. Increased baseline FSH was defined as >10.2 IU/L, which is the upper 95% reference value of the FSH assay used in the UMC Utrecht (ADVIA Centaur / Bayer Corporation, Tarrytown, NY, USA). This arbitrary cut-off was chosen because this assay was used in the control cohort, and secondly a Dutch study using another assay identified an upper value of 11.2 IU/L in regularly menstruating women below age thirty-five[305], conversion to the ADVIA Centaur assay led to a cut-off of 10.4 IU/L.

Incipient ovarian failure (IOF) was defined as regular cycles between 25-35 days with elevated FSH on cycle day 2-5. Transitional ovarian failure (TOF) patients had (a history of) transformation to irregular cycles (>35 days) with FSH levels exceeding 10.2 IU/L without fulfilling the POF criteria. POF was defined as at least one episode of secondary amenorrhea for more than 120 days (4 months) in combination with FSH >40 IU/L.

Proven fertile, regularly menstruating women from an earlier described cohort served as controls[230]. Women with early follicular FSH levels below 10.2 IU/L between 25-40 yrs. were selected for the current study.

Methods

All serum measurements in the hypergonadotropic patients were performed in the same laboratory using the same assays in a single run. FSH levels were measured using a chemoluminescence-based immunometric assay (Immulite 2000, Diagnostic Products Corporation, Los Angeles,

CA). The detection limit of the assay was 0.1 IU/L, inter- and intra-assay coefficients of variation were below 3.0 and 5.6%. Progesterone was measured on an ADVIA Centaur Immunoassay System (Bayer Corporation, Tarrytown, NY, USA). Inter-assay coefficients were 11%, 6% and 5% at 6, 30 and 95 nmol/L. Within run variation for values > 10 nmol/L was < 3% and at 5 nmol/L it was 7%. Luteal progesterone was set at a value of > 10 nmol/L (which coincides with the 2.5th percentile in 84 normo-ovulatory subjects). Levels of inhibin B were measured using enzyme-immunometric kits obtained from Oxford BioInnovation (Oxford, United Kingdom). Inter- and intra-assay coefficients of variation were below 7.0% and 14% at 240 ng/L. The detection limit was 10 ng/L. AMH levels were determined using the enzyme-immunometric assay (Diagnostic Systems Laboratories, Webster, TX, USA). Inter- and intra-assay coefficients of variation were below 5% at the level of 3 µg/L, and below 11% at the level of 13 µg/L. The detection limit of the assay was 0.026 µg/L. In controls AMH levels were measured using the Immunotech Coulter (Marseilles, France) enzyme-immunometric assay and converted to DSL assay values as described earlier[300].

AFC was defined as the total number of visible round or oval, intra-ovarian transonic structures with diameter between 2 and 10 mm. Ultrasound examinations were performed by experienced fertility specialists in each of the participating centre. If one or both ovaries could not be visualized, the AFC was marked as "not visible". Low AFC cut-off was set at less than 5 follicles since this number is associated with poor response and significantly lower rate of pregnancies in IVF[306]. The menopausal threshold for serum parameters were set as inhibin B less than 10 ng/L[307] and AMH below 0.086 µg/mL[28]. Furthermore lowered premenopausal AMH cut-offs were defined as below 5th percentile of the distribution within the normal population by age. This is for age 30: 0.3085; 35: 0.2365 and 40: 0.1036 µg/mL respectively (extracted from the original data of [28]).

Statistics

Continuous variables were expressed as mean +/- standard deviation (SD) and categorical variables as percentages. The ovarian reserve markers were logarithmically transformed in case of significant deviation from the normal distribution. This applied to AMH and Inhibin B. Therefore results for these ovarian markers were presented as medians and range. Between-group differences were tested with analysis of variance (ANOVA) for continuous parameters and Chi squared tests for categorical parameters, respectively. To assess a systematic change when moving from controls via IOF and TOF to POF, tests for linear trend were used. A separate analysis was performed to analyze the relationship between each ovarian reserve parameter and age: multiple linear regression was carried out with the ovarian reserve parameter as dependent and age and group as independent variable. Absolute differences between groups were assessed, as well as in the interaction between group and age, defined by the slope of the regression line were tested.

Results

Up to January 2007 a total of 408 patients between 25 and 40 years with idiopathic elevated FSH visited one of the participating clinics. For this study 62 current hormonal therapy users, 34 with unknown or unreliable last menses and 25 patients with a regular cycle who were not screened in the early follicular phase were excluded.

Additionally 28 samples were excluded because of insufficient amount or quality of serum for analysis. In total 342 women (68 IOF, 79 TOF, 112 POF patients and 83 controls) were included for the current study.

In Table 4 baseline and cycle characteristics at time of screening are outlined. No statistically significant differences existed in age (P = 0.11), BMI (P = 0.38), age at menarche (P = 0.36) and pack years of smoking (P = 0.86) between controls, IOF, TOF and POF cases. Significant differences (P < 0.001) were identified in menopausal complaints (flushes and night sweats). By definition all control and IOF patients had experienced a spontaneous menstruation within the previous 35 days, and this was also the case in 65% of the TOF patients. 31% of the POF patients had experienced a spontaneous bleed within the last 4 months. In 11% of POF patients, a second FSH measurement after diagnosis did not show an FSH level above 40 IU/L (Figure 6).

Table 4. Baseline characteristics (mean ± SD or percentages) of control women and hypergonadotropic patients.*

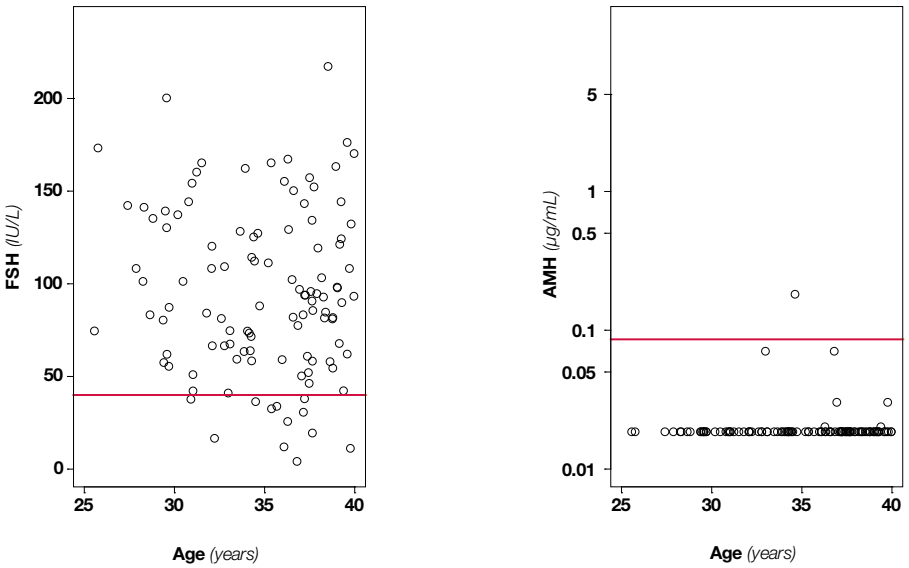
	Controls (n=83)	IOF (n=68)	TOF (n=79)	POF (n=112)
Age (yrs.)	34.2 ± 3.4	35.2 ± 3.1	34.0 ± 3.9	35.0 ± 3.7
BMI (kg/m2)	24.6 ± 4.3	24.2 ± 4.7	25.4 ± 6.2	24.3 ± 4.2
Pack years of smoking	4.2 ± 6.4	3.4 ± 6.0	3.9 ± 5.6	4.1 ± 6.2
Age at menarche (yrs)	13.2 ± 1.6	12.9 ± 1.4	12.8 ± 1.7	13.1 ± 1.4
Flushes (% of patients)*	2 %	8 %	40 %	71 %
Night sweats (% of patients)*	0 %	14 %	20 %	51 %
Cycle history characteristics at time of screening:				
Last menses < 35 days (% of patients)	100 %	100 %	65 %	10 %
Last menses 35 – 120 days (% of patients)	0 %	0 %	22 %	21 %
Last menses >120 days (% of patients)	0 %	0 %	14 %	69 %

* significantly (p < 0.001) different between all subgroups

Endocrine screening (Table 5) identified luteal progesterone in 12% and 3% of TOF and POF patients, respectively. All measured mean or median ovarian reserve parameters (FSH, AMH, inhibin B and AFC) differ between regular menstruating controls and the hypergonadotropic women ($P < 0.001$). AMH was detectable in 6% of the POF; all had AMH levels below the 5th percentile for their age (Figure 7). Median AMH value for IOF was 0.33 µg/mL and 0.02 µg/mL for TOF. Of the IOF patients 75% had AMH levels in the normal range (> P5 for her age) compared to 33% in the TOF group.

When comparing the direct ovarian parameters inhibin B, AFC and AMH (Figure 8), the slope of the regression lines against age were significant ($P < 0.0001$) for AFC and AMH, indicating age dependency. In contrast, inhibin B levels were not significantly associated with age ($P = 0.26$) (Figure 8). When comparing the regression lines by age for IOF and TOF, AFC failed to differentiate between these groups in the higher age groups whilst discrimination between these two groups on the basis of AMH levels was possible at every age group: the difference became more pronounced at advanced age (Figure 3).

Figure 6.



FSH (2nd measurement after diagnosis) and AMH levels (log scale) in relation to age in 112 POF patients; the lines indicate the cut-off value of 40 IU/L for FSH and the menopausal threshold (0.086 µg/mL) for AMH [28].

Table 5. Endocrine and ultrasonography assessment at screening (means ± SD)

	Controls (n=83)	IOF (n=68)	TOF (n=79)	POF (n=112)
FSH in study batch *(IU/L)*	N/A[a]	14.0 ± 7.8	29.0 ± 30.1	94.6 ± 44.7
% normal (≤10.2 IU/L)	N/A[a]	28%	8%	1%
% FSH > 10.2 IU/L	N/A[a]	72 %	92 %	99 %
% FSH > 40 IU/L	N/A[a]	2%	19 %	89 %
% Luteal Progesterone	N/A[a]	N/A	12 %	3 %
Ovarian reserve parameters				
AMH *(µg/mL)*[b]	3.51 (0.09-15.84)	0.33 (0.02-3.56)	0.02 (0.02-4.49)	0.02 (0.02-0.18)
% < P_5 for her age	2%	25%	66%	100%
% < P_5 at age 30 (0.3085)	5%	41%	73%	100%
% < P_5 at age 35 (0.2365)	4%	24%	66%	100%
% < P_5 at age 40 (0.1036)	1%	13%	60%	99%
% < mp threshold (0.086)	0%	13%	58%	99%
% undetectable[c]	0%	7%	52%	94%
Inhibin B *(ng/L)*[b]	93 (7-249)	85 (7-237)	17 (7-293)	11 (7-110)
% undetectable[c]	2%	6%	37%	44%
AFC *(no. of follicles in 2 ovaries)*	8.6 ± 5.7	4.4 ± 3.4	5.2 ± 6.8	1.2 ± 2.0
% AFC <5	24 %	63 %	63 %	91 %
% zero follicles	1 %	7 %	21 %	37 %
% not visible	0 %	13 %	23 %	37 %

mp = menopausal

N/A = not applicable

a In controls no 2nd FSH measurement was performed since this group was selected on FSH level; mean FSH ± SD in controls was 6.3 ± 1.7 IU/L.

b AMH and Inhibin B values were logarithmically transformed, therefore medians and ranges are presented.

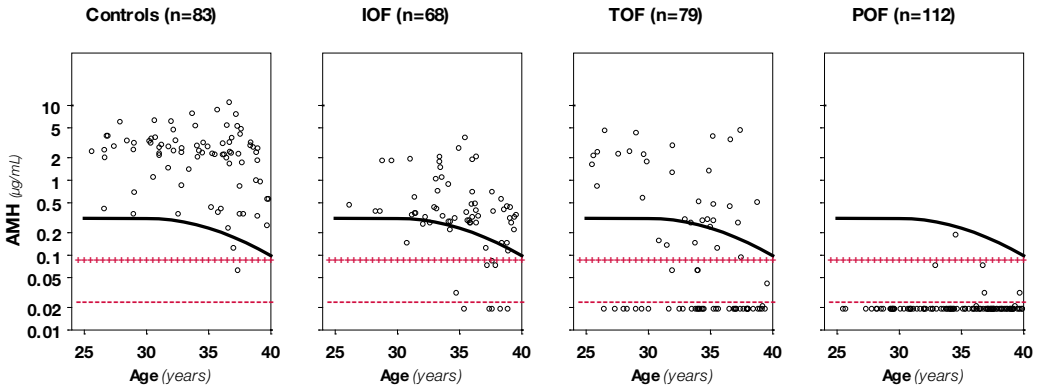
c Undetectable levels are <0.026 µg/mL for AMH and <7 ng/L for Inhibin B.

Discussion

The current study describes the direct ovarian reserve markers AMH, inhibin B and AFC in young women presenting with various degrees of hypergonadotropic ovarian failure. Although ovarian reserve is not well defined in the literature and no clinical endpoints such as successful IVF treatment and/or ongoing pregnancy are used in the current descriptive study, our data support the application of AMH in estimating the extent of follicle pool depletion in young hypergonadotropic women.

AMH is already a proven ovarian marker with regard to reproduction in non hypergonadotropic subjects[27]. In regular menstruating women, AMH appears to be more predictive of IVF outcome than other direct ovarian markers such as oestradiol and inhibin B[280]. Decreased AMH levels are clearly correlated to poor response in IVF, which is a functional outcome of diminished ovarian reserve[308]. AMH has also been presented recently as a useful marker of ovarian dysfunction and prediction of outcomes of intervention in other clinical conditions such as normogonadotropic anovulation (chiefly polycystic ovary syndrome)[309], anorexia nervosa[310] or chemotherapy-induced ovarian damage[311]. Furthermore, recent studies suggest that AMH levels may predict age at menopause, and in women approaching menopause extremely low AMH levels are observed[28,312]. However it has not yet been established whether data from the normal menopausal transition may be applied to hypergonadotropic ovarian failure at a much younger age.

Figure 7.

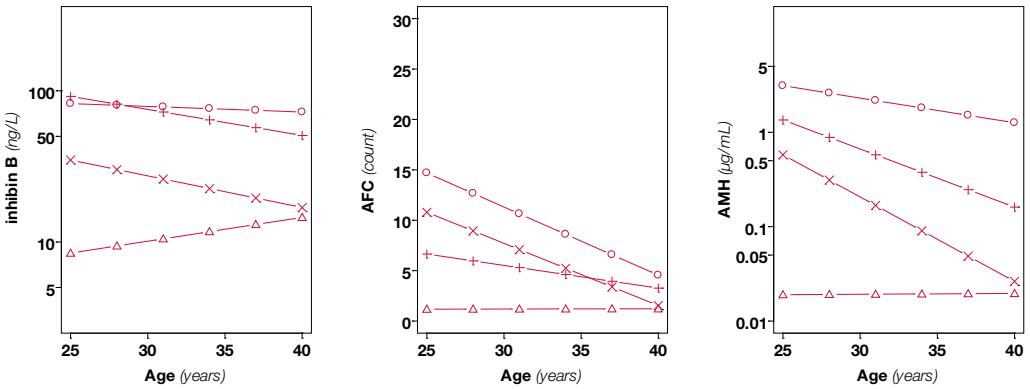


Individual AMH levels (log scale) in relation to age in regularly menstruating controls and 3 subgroups of patients with elevated FSH levels. The black lines indicate the P5 value for age.

The **+++++** lines indicates the menopausal threshold value for AMH (0.086 µg/mL)

The **--------** lines indicates the lower level of detection of the AMH assay (0.023 µg/mL) [28]

Figure 8.



Regression lines of inhibin B (log scale), AFC and AMH (log scale) values by age for subgroups of young hypergonadotropic women.

o-o-o regular menstruating controls

+-+-+ incipient ovarian failure (IOF)

x-x-x transitional ovarian failure (TOF)

Δ-Δ-Δ premature ovarian failure (POF)

Our data in POF patients show that AMH values are consequently below the menopausal threshold and in the vast majority even undetectable, despite fluctuations in FSH levels and incidental vaginal bleedings. This finding provides further evidence for the notion that infertility treatment is of no benefit in these patients with the exception of oocyte donation. In contrast, in patients with elevated FSH levels and regular cycles (IOF) or oligo/amenorrhea (TOF), we found that AMH may still be normal, suggesting the presence of a fair amount of follicles. We therefore suggest that AMH may be applied to identify women with a less abnormal ovarian reserve (i.e. AMH levels above the 5th percentile) and thus possibly a better reproductive potential. Young IOF and TOF patients present more frequently with 'normal' AMH levels for their age compared to older patients (P = 0.07 and P = 0.007, respectively). This observation supports the clinical finding of a normal response to ovarian stimulation in some women with elevated FSH levels[313]. When using AMH rather than FSH to differentiate between normal or diminished ovarian reserve, only a quarter of all IOF and two thirds of TOF patients would be labelled as abnormal. In other words, in 75% of IOF and one third of TOF patients normal AMH concentrations were

observed despite elevated FSH and cycle disturbances. However, before AMH can be applied clinically, longitudinal follow-up studies should prove the ability of AMH to predict clinical outcome in young hypergonadotropic patients.

Moreover, our data also suggest that AMH is more consistent than inhibin B or AFC as a measure to assess the extent of the follicle pool in these young hypergonadotropic patients, although the results of the regression analysis should be interpreted with caution given the transverse nature of the data. It is interesting however, that AFC and AMH, which are both "direct" markers of "ovarian reserve" differ, particularly between the IOF and TOF subjects. IOF subjects have lower (and more slowly falling) antral follicle counts than those with TOF. This finding may indicate that a milder follicle depletion pattern is present in IOF patients.

Although inhibin B is known to be decreased in older women[314] and is significantly decreased in TOF and POF patients, its capacity to differentiate between controls and IOF is absent in our cohort. Inhibin B is probably a mere marker of ovarian activity rather than of ovarian reserve. This may be due to its direct relationship with the cohort of growing small antral follicles following secondary follicle recruitment during the luteo-follicular transition[298]. A recent longitudinal study in a general population cohort demonstrated inhibin B was less predictive of menopause in the general population than AMH[315, 316]. Finally, inhibin B levels may also be affected by the waxing and waning of ovarian function often seen during ovarian aging as well as throughout the menstrual cycle[304].

Overall, the discriminative power of AFC to differentiate between various subgroups decreases significantly with increasing age. Earlier studies have shown strong correlations between AMH and early follicular phase AFC in the context of ovarian aging[230]. Despite this correlation, AFC may reflect the active cohort of growing follicles[317] rather than the pre-antral follicle pool. Our current observations in hypergonadotropic patients indicate that ultrasonography is not conclusive in 26% of the patients since one or both ovaries were not visible. Moreover AFC requires state-of-the-art ultrasound machines and experienced ultrasonographists[318]

The arbitrary cut-off value for FSH should also be considered. Different studies, using different outcomes have used different FSH cut-off levels[296]. A recent large Dutch multi-centre trial (using different assays) using FSH as continues variable identified fewer pregnancies with FSH levels exceeding 8 IU/L[319]. Moreover other factors may be involved in regulating absolute levels of FSH[305], including FSH receptor polymorphisms[320, 321]. Hence, factors different from ovarian reserve may also impact on absolute FSH concentrations.

In conclusion, the current prospective, cross sectional evaluation of ovarian reserve markers in young hypergonadotropic women indicates that AMH may represent a useful future marker to assess the extent of diminished ovarian reserve for a given patient. Moreover, our data further suggest that the classical role of serum FSH as the primary determinant for diagnosing premature follicle pool depletion - with premature ovarian failure (POF) as its extreme phenotype - may need to be revised. Before the widespread clinical application of AMH can be recommended, longitudinal follow-up data for the general population are needed[315]. It would be extremely useful to define age dependent AMH cut-off levels. Furthermore, studies using AMH as an ovarian reserve marker for clear clinical outcome measures may improve its predictive value.

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Lipid profile of premature ovarian failure patients

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Chapter 4

Introduction

Lipoproteins play a major role in the atherosclerotic processes leading to cardiovascular events. Around and after menopause women experience unfavourable changes in plasma lipids and lipoproteins, i.e. increases in total (total-C) and LDL-cholesterol (LDL-C) and triglyceride (TG) levels[322]. These changes are considered the result of (the interaction between) chronological ageing, weight gain mainly by increase in abdominal fat and loss of ovarian function during the fifth decade[323-325].

An earlier age at menopause is related to a higher incidence of cardiovascular events later in life[14, 326]. The beneficial effects of intact ovarian function on lipids and its lipoproteins are generally ascribed to endogenous oestrogens. Oestrogens up regulate LDL receptors in the liver and are associated with an increased clearance of LDL particles. The fall in oestrogen levels as seen after menopause is associated with a down regulation of the LDL receptor activity and can contribute to elevations in plasma LDL-chol and TG concentrations[327-329].

Elevations in plasma TG concentrations indirectly interfere with lipoprotein metabolism and are mainly associated with obesity and increased insulin resistance and several studies suggest that in women hypertriglyceridemia results in greater cardiovascular risk than in men[330-332]. Besides oestrogens also androgens levels might be associated with cardiovascular risk and high free-androgen index (FAI) and/or low SHBG levels might be markers for increased insulin resistance[333, 334]. Spontaneous loss of ovarian function before the age of 40 years is referred to as (idiopathic) premature ovarian failure (POF) which occurs in 1% of all women[6]. A postmenopausal hormonal status is already present at a young age in POF, although in some patients ovarian function may temporarily resume[335]. POF may be considered an appropriate in vivo model that allow us to study the effect of spontaneous cessation of ovarian function on lipid profile and any other cardiovascular risk factors independent from effects of advancing chronological age.

Scant information is available regarding lipid profiles in POF. A single study focusing on endothelial dysfunction compared 18 POF patients with 20 controls and found no difference in lipid profile[336]. We conducted a study in a large cohort of young women recently diagnosed with POF without recent HT use and population controls without oral contraceptive use in the same age range to investigate whether early changes in lipid profile can already be observed in POF. Furthermore the relationship between lipids and duration of oestrogen deprivation, oestrogen and androgen levels in POF women was studied.

Materials and methods

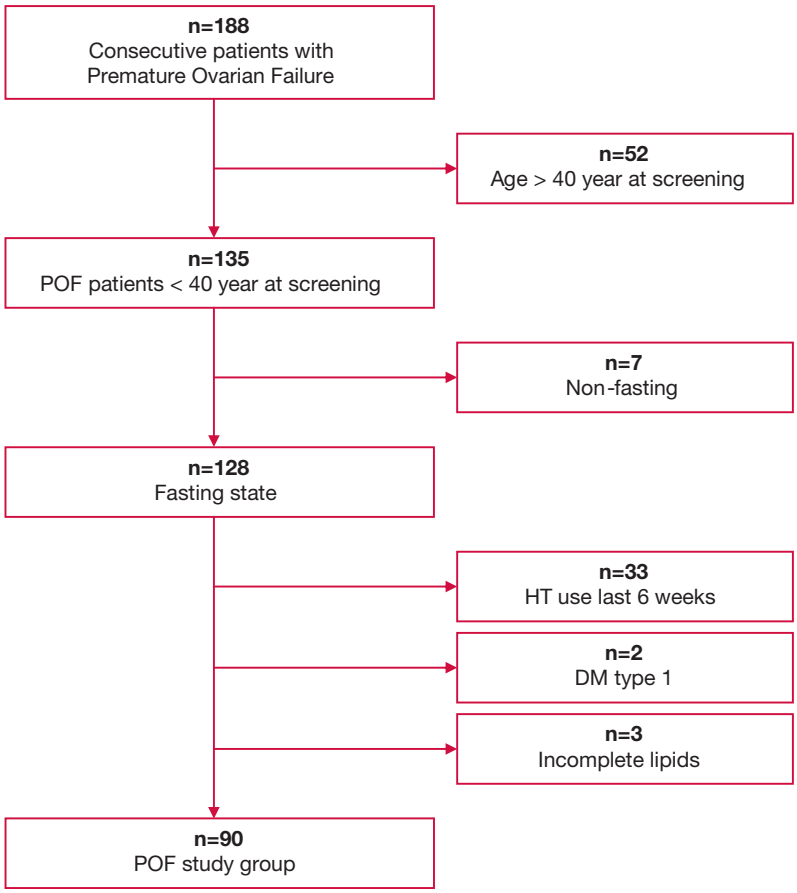
Cases

POF was defined as spontaneous cessation of menses for at least 4 months in women younger than 40 years in combination with a hypergonadotropic (FSH >40 IU/L) hormonal status (WHO III class)[6, 288]. All consecutive patients between October 2004 and January 2007 with a suspected WHO III class status who attended the outpatient clinics of the University Medical Centre Utrecht and the Erasmus Medical Centre Rotterdam (n=188) underwent standardized investigation in a fasting state to ascertain the diagnosis and exclude associated disease. Of every patient lipid profile (TG, Total-C and, HDL-C), E_2, Testosterone (T) and Sex Hormone Binding Globulin (SHBG) levels and a 2nd FSH level were determined. All patients

gave written informed consent for participation in this Institutional Review Board approved study.

For the current study, we selected only patients younger than age 40 at the time of screening (n=135) and those who visited our standardized investigation in a fasting state (n=128). After excluding patients with diabetes mellitus (n=2), incomplete lipid profile (n=3) and use of any hormonal therapy (HT) within the least 6 weeks (n=33) a total of 90 women were included for further analysis (Figure 9). Time of oestrogen deprivation was defined as

Figure 9. Flowchart patient selection



time since last menstruation or bleeding after discontinuing HT or oral contraceptives use.

Fasting triglycerides, Total-C and HDL-C concentrations were measured using a Vitros Dry Chemistry Analyzer 950 (Johnson & Johnson, Rochester, New York, USA). LDL-C was calculated using the Friedewald formula[337]. SHBG concentrations were quantified using an Immulite® platform (Diagnostic Products Corporation, Breda, the Netherlands). Testosterone levels were determined using in house radio immuno-assays. The FAI was calculated as T/SHBG * 100. E_2 concentrations were measured using the Roche E170 Modular (Roche, Basel, Switzerland). Intra- and inter- assay coefficients of variation for the analytical assays described above are all below 10%.

Controls

Population controls were selected from the Atherosclerosis Risk in Young Adults study. This

Table 6. Baseline characteristics and uncorrected parameters

	POF (n=90) Mean ± sd	Controls (n=198) Mean ± sd
Age *(years)*	33.8 ± 5.6	30.3 ± 2.9
BMI *(kg/cm2)*	24.9 ± 4.3	24.3 ± 4.8
WC *(cm)*	82.6 ± 10.2[a]	83.7 ± 12.0
Smoking status *(pack years)*	4.5 ± 6.3[b]	3.5 ± 5.8
TG *(mmol/L)*	1.03 ± 0.43	0.92 ± 0.49
Total-C *(mmol/L)*	4.80 ± 0.95	4.70 ± 0.86
HDL-C *(mmol/L)*	1.48 ± 0.48	1.49 ± 0.31
LDL-C *(mmol/L)*	2.86 ± 0.86	2.81 ± 0.81
Age at secondary amenorrhea *(years)*	31.8 ± 6.3	
Time of estrogen deprivation *(days)*	433 ± 686	
FSH *(IU/L)*	79.5 ± 42.3	
E_2 *(pmol/L)*	140 ± 152	
T *(nmol/L)*	1.03 ± 0.45	
SHBG *(nmol/L)*	52.5 ± 27.8	
FAI *(T/SHBG * 100)*	2.42 ± 1.39	

a n = 53

b n = 72

From SI units to conventional units multiply by conversion factor: TG (mg/dL) 88.495; Total- HDL-, LDL-C (mg/dL) 38.61; E_2 (pg/mL) 0.2724; T (ng/dL) 28.818; SHBG (µg/mL) 0.348.

Table 7. Lipid profile adjusted for age, BMI and smoking

	Estimated marginal means ± SEM		Difference POF vs. controls	95% CI
	POF (n=90)	Controls (n=198)		
ln (TG) *(mmol/L)*	-0.03a ± 0.05	-0.20a ± 0.04	0.17	0.06 - 0.29
Total-C *(mmol/L)*	4.67 ± 0.11	4.78 ± 0.08	-0.11	-0.35 – 0.12
HDL-C *(mmol/L)*	1.45 ± 0.04	1.51 ± 0.03	-0.07	-0.16 – 0.03
LDL-C *(mmol/L)*	2.76 ± 0.10	2.85 ± 0.07	-0.09	-0.31 - 0.13

To convert to conventional units (mg/dL) multiply TG by 88.495 and Total-, HDL-, LDL-C by 38.61

Institutional Review Board approved study was initiated to determine cardiovascular risk factors at an early stage in life. A total of 1011 young adults (562 women) born between 1963-1968 (mean age 34.3 yrs) and 1970-1973 (mean age 28.4 yrs) were recruited from two secondary schools in two large Dutch cities. From these women we selected non oral contraceptive users of whom fasting blood samples were available to serve as a comparison group (n=198).

Fasting triglycerides, Total-C and HDL-C concentrations were measured using automated methods (Vitros Dry Chemistry Analyzer 950 (Johnson & Johnson, Rochester, New York, USA) and Synchron LX 20 (Beckman Coulter, Fullerton, USA)). LDL-C was calculated using the Friedewald formula[337]. Additional information of this population cohort has been published elsewhere[338].

Statistical analysis

The differences between the two groups in mean levels of triglycerides, total-, HDL-, LDL-, cholesterol were statistically tested by Student's t-test. Analysis of covariance (ANCOVA) was used to adjust the differences between the groups for the confounding factors age, BMI and smoking status, and estimated marginal means were determined, which is preferable over a matched control group[339]. Because smoking status was missing in 18 patients, we used multiple imputation using the 'AregImpute' function in S-plus version 7.0 (Insightful Corp), to avoid loss of data and potential bias. Statistical analysis was performed using the statistical package for social sciences SPSS for Windows, version 12.1 (SPSS Inc., Chicago, IL, USA). Pearson correlation coefficients were determined and corrected for age. We calculated correlations coefficients between TG and HDL-C and duration of oestrogen deprivation, E_2, T, SHBG-levels and FAI.

Results

Baseline characteristics and uncorrected laboratory values of POF patients and controls are shown in Table 6. Lipid profile of POF patients and controls after adjustment for age, BMI and smoking

Table 8. Correlation coefficients (r) between TG and HDL-C and ovarian markers, adjusted for age

	r	p-value
TG vs FAI	+0.35	0.001*
TG vs T	+0.10	0.4
TG vs SHBG	-0.32	0.002*
TG vs time of oestrogen deprivation	+0.10	0.3
TG vs E2	-0.03	0.7
HDL-C vs FAI	-0.06	0.6
HDL-C vs T	+0.26	0.01*
HDL-C vs SHBG	+0.24	0.03*
HDL-C vs time of oestrogen deprivation	-0.03	0.8
HDL-C vs E2	-0.04	0.7

* p value <0.05 considered statistically significant

are presented in Table 7. Although all mean values in the POF cases are within the normal physiologic range, POF patients had significantly higher plasma triglyceride concentrations compared to controls and HDL values were borderline significantly lower in the POF group.

Significant positive correlation was identified between FAI and TG and a significant negative correlation was identified between SHBG and TG. Also HDL-C levels and androgens (higher T, lower SHBG) were significantly correlated. No significant correlation was found between E_2 levels or time of oestrogen deprivation and any of the investigated lipids (Table 8).

Discussion

The current study indicates that women presenting with POF and not using hormone replacement present with subtle but significant changes in lipid profile at a young age compared to controls. Interestingly, although POF patients exhibit a post-menopausal hormonal status, no difference in BMI and WC was observed compared to pre- menopausal controls of comparable age. This finding, together with a comparable smoking status, suggests that the observed differences in lipid profile are most likely related to the premature cessation of ovarian function. In our cohort no clear relationship between E_2 concentrations

or duration of oestrogen deprivation and severity of lipid profile could be established in POF patients. In contrast, we found similar LDL-C concentrations in POF patients and controls. In the absence of differences in LDL-C it is unlikely that the higher TG in POF can be attributed to lower LDL-receptor activity which would have been expected in a hypo-oestrogenic status as seen in menopause. However, in most studies POF is defined as a cessation of menstruation for at least 4 months whilst menopause is defined as an amenorrhea for at least 1 year. POF patients may also differ from women who experience menopause later in life because occasional ovarian function, thus intermittent E_2 exposition, may resume[335]. Of the 90 patients in the current study, 13 patients experienced one or more spontaneous menstruation after diagnosing POF and this may render observed differences between POF patients and population controls less pronounced.

Other pathophysiological mechanisms that could explain the findings of increased plasma TG concentrations in POF patients may include relative androgen excess as an early indication of decreased insulin sensitivity. The current study shows indeed a positive correlation between TG and FAI and a negative association between TG and SHBG. The few aetiological studies of involvement of androgens in the pathogenesis of dyslipidemias and other CVD risk factors point towards an indirect role of androgens via obesity and decreased insulin sensitivity as seen for example in hyperandrogenic polycystic ovarian syndrome patients and a recent study identified lower insulin sensitivity in a large cohort of POF patients[340, 341].

Spontaneous cessation of ovarian function in relation to POF is a heterogeneous condition involving karyotypic abnormalities (macrodeletions, translocations, mosaic Turner), FMR1 premutation and auto-immunity related aetiologies[76, 191]. However in the vast majority of women premature menopause is of unknown origin. Further exploration of the relation between lipid abnormalities and POF subgroups would potentially be very interesting, as there are some indications that effects are stronger in some phenotypes than in others. In addition to the earlier mentioned study[336], there have been two other studies investigating lipid profiles in women with early ovarian ageing. It has been described previously that Turner's syndrome patients have a more atherogenic lipid profile than POF women[342]. A study in 40 women with regular menstrual cycles in combination with elevated FSH levels as sign of ovarian ageing found higher Total-C and LDL-C levels and no difference in HDL-C or TG levels compared to women with normal FSH levels[343]. In this study it was not possible for all 90 patients to undergo routinely karyotyping, FMR1 premutation and auto-immunity screening. Therefore no analysis on POF subgroups was performed. Moreover, the numbers in the subgroups would probably have been too small for appropriate statistical analysis.

In conclusion, the current study suggests that spontaneous cessation of ovarian function occurring early in life (women presenting with POF) induces elevated TG concentrations, probably related to decreased insulin sensitivity. Besides additional clarification of the role of androgens in female cardiovascular health future follow-up studies should establish further deviation of lipid profile and other cardiovascular risk factors over time along with its implications for true cardiovascular events.

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Heterozygosity for the classical galactosemia (GALT) mutation does not affect ovarian reserve and menopausal age

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Chapter 5

Introduction

Classical galactosemia is an autosomal recessive disorder of galactose metabolism caused by a deficiency of the enzyme galactose-1-phosphate uridyltransferase. The coding region of this enzyme is located on chromosome 9p13 and among Caucasians; the gene frequency of the GALT mutations is estimated to be 0.34-0.37%. The estimated incidence of classical galactosemia is 1 in 47,000 births. Patients mostly present in the first two weeks of life with feeding problems, hypoglycemia, liver and kidney failure, sepsis and cataract. A dietary restriction of galactose is life saving.[344-346]

Most female patients with galactosemia suffer from hypergonadotropic primary amenorrhea or premature ovarian failure (POF).[79, 347, 348] However, the underlying mechanisms have not yet been fully elucidated. It is also uncertain whether heterozygosity for a GALT mutation may cause accelerated depletion of the pool of ovarian follicles resulting in early menopause. Indeed, earlier menopause and a history of infertility have been observed in women with a low GALT-activity.[80] Two more recent studies in GALT mutation carriers, however, could not confirm these findings.[81, 82] This ongoing debate was a cause for concern among the female relatives of Dutch patients with classical galactosemia. The Dutch Galactosemia Society proposed to investigate whether heterozygous GALT mutation carriers exhibit signs of diminished ovarian reserve or earlier menopause.

Materials and methods

This study was approved by the local Ethics Committee and written informed consent was obtained from all participants. Mothers of classical (homozygous) galactosemia patients (ie. known female carriers of galactosemia) were recruited via the Dutch Galactosemia Society. In addition, parents of the galactosemia patients were asked if they had any family members who were known to be carriers of the GALT mutation as a result of genetic counselling. These women were also invited to participate in the study. The volunteers received financial compensation for travel expenses. The control group (n=166) consists of healthy female volunteers (age range 25 to 46 years) with regular menstrual cycles, biphasic temperature curve, proven natural fertility with at least one pregnancy carried to term, pregnancies were established within one year, no (history of) endocrinological disease, ovarian surgery or abnormalities on ultrasound, no hormonal anticonception use for at least two months and has been described earlier in detail.[299].

Participants were requested to undergo a structured interview designed by the authors regarding fertility, smoking status and menopause. The interview was taken by telephone (by R.R.) and took approximately 45 minutes per person to complete. Age at menopause (defined as the date of the last menstruation followed by $\geq$ 1 year of amenorrhea) is considered the most distinct marker of the apparent exhaustion of the follicle pool. All women with a menstrual bleeding within the last year were invited to visit our outpatient clinic. Women with secondary oligomenorrhea, menopausal complaints and elevated FSH (>20 IU/L) levels in combination with low oestradiol (<200 pmol/L) were regarded as perimenopausal. Their menopausal age was estimated as their age at the interview plus 1 year. To determine age at menopause, the Kaplan-Meier method was used. The woman's age was the time variable and women who were

classified as premenopausal were censored at the age at which they had filled in the questionnaire.

In order to assess the extent of follicle pool depletion in pre- and perimenopausal women, baseline FSH, Inhibin B, anti-Müllerian hormone (AMH) levels and the AFC were assessed as tests for ovarian reserve.[295, 302] To find a difference in AMH values of 0.5 SD (Cohen's d) between GALT carriers and controls 42 GALT carriers were required (assuming a case:control ratio of 1:3) to obtain 80% power at $\alpha = 0.05$. All women using oral contraceptives (n= 10) were asked to stop, and blood specimens were taken during the early follicular phase of the

Figure 10. Flowchart patient selection

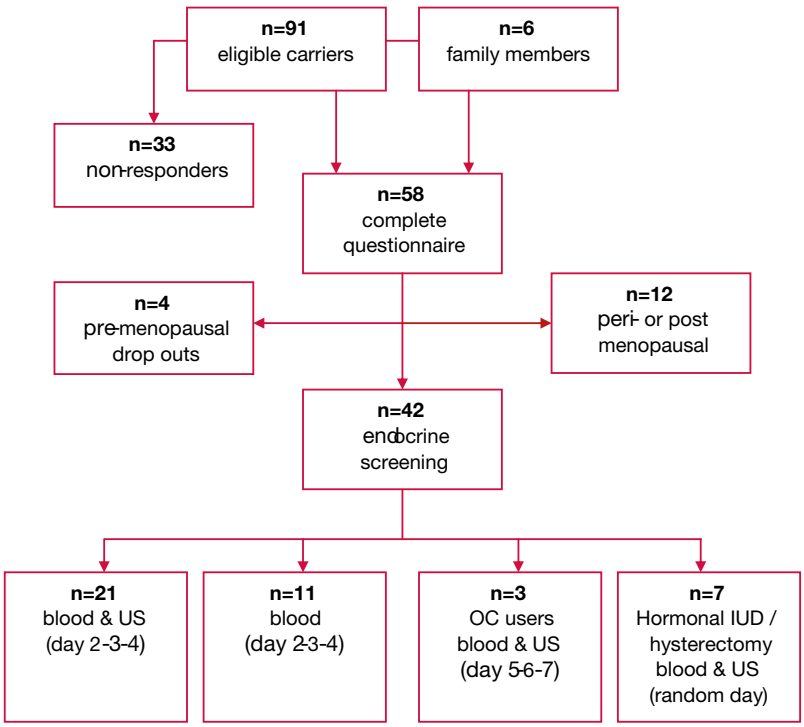


Table 9. Baseline characteristics pre-menopausal GALT carriers and controls (Mean ± sd)

	Premenopausal GALT carriers (n=42)	Controls (n=166)	p-value* (T-test)
Age *(years)*	39.3 ± 5.2	38.0 ± 5.5	0.14
Age at last childbirth *(years)*	31.3 ± 3.7	30.3 ± 9.5	0.49
Pack years	5.7 ± 7.6	4.8 ± 7.3	0.47

second spontaneous cycle after interruption of the oral contraceptive. Serum of women who could not stop for practical reasons (n=3) was obtained on day 5-7 of the pill-free interval to be as comparable as possible with early follicular levels.349 Hormonal intrauterine device (Mirena®) users with amenorrhea or women who had a hysterectomy (n=7) were sampled on a random day.

Blood sampling specimens were stored at –20 °C within one to six hours after retrieval. FSH was measured with a chemiluminescence FSH assay on the ADVIA Centaur® Automated System (Bayer Corporation, Tarrytown, NY, USA). Intra- and inter-assay coefficients of variation were < 6 and < 3%, respectively. Inhibin B was measured by enzyme-immunometric assay, obtained from Oxford Bioinnovation (Oxford, UK). Intra- and inter-assay coefficients of variation were below 15% and 14%, respectively. AMH levels were assessed using the enzyme-immunometric assay described earlier.[350] Inter- and intra-assay coefficients of variation were below 5% at the level of 3 ug/l, and below 11% at the level of 13 ug/l. The detection limit of the assay was 0.026 ug/l. Transvaginal sonography of the ovaries was carried out in the early follicular phase of the menstrual cycle (cycle day 2, 3 or 4). All scans were performed by the same observer (E.K.) using the transvaginal probe of a Voluson 530D (Kretz Technik, Austria). Round or oval echo-free structures of 2-5 mm in the ovaries were regarded as antral follicles. The sum of the numbers of counted follicles in both ovaries was the AFC.[317]

To compare laboratory and ultrasonographic values between heterozygote GALT carriers and controls, Student's t-test was used, after logarithmic transformation where appropriate. Correction for age and smoking status was done by analysis of covariance (ANCOVA). The relationship between AMH and age within each group was assessed by ordinary linear regression analysis, with the logarithm of AMH as dependent variable and age, group and the interaction age*group, to test for a difference in regression line between the two groups.

Results

A total of 58 women participated in the study. Of the 91 mothers of patients with classical galactosemia invited to participate in the study through the Dutch Galactosemia Society, 52 women completed the questionnaire, which is a response rate of 53%. Six women were family members of the fathers or mothers, and were previously proven to be carriers. Of this total of 58 women, 12 were peri -or postmenopausal 2 mothers were pregnant at the time of investigation and 2 GALT carriers did not consent to ultrasound or blood sampling due to personal or practical reasons. A total of 42 GALT carriers were enrolled in the endocrine screening and in 21 women, transvaginal ultrasonography was performed (Fig 10). In order to rule out selection bias towards a low frequency of earlier menopause, non-responders were asked by the Dutch galactosemia society to answer a very short questionnaire by telephone. Twenty of 33 non-responders were contacted and in this group, all women had regular menses.

The mean age at menopause in the GALT carrier group was 49.7 (95%CI 48.7-50.7) years. Table 9 shows baseline characteristics of the GALT carriers and controls. No significant differences were found in the mean difference or ratio of the ovarian reserve parameters between controls (n=166) and premenopausal GALT carriers (n=42) (Table 10). Since ovarian reserve parameters are strongly associated with age and, in a lesser extent, also influenced by smoking, we corrected for these parameters. Figure 11 demonstrates that the regression lines between AMH and age for GALT carriers and controls are essentially the same.

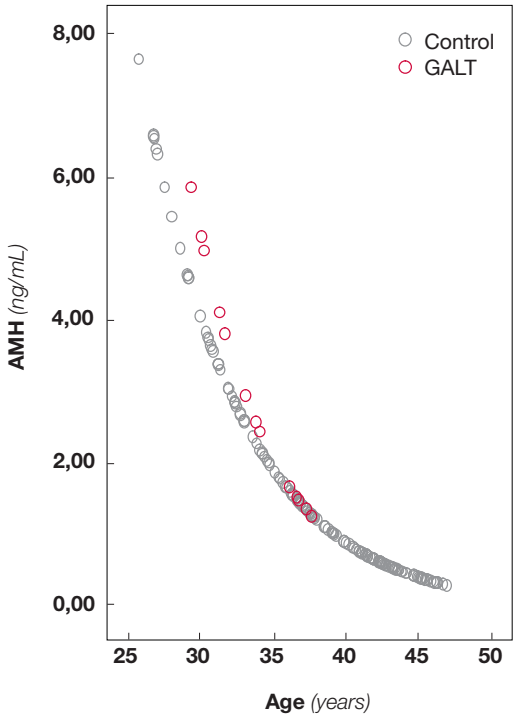
Table 10. Ovarian reserve parameters between pre-menopausal GALT carriers (n=46) and controls (n=16)

	Measured values		Uncorrected mean		Corrected[a] mean	
	GALT	Controls	difference [95% CI]	p-value	difference [95% CI]	p-value
FSH *(IU/L)*[b]	2.03 ± 0.46	1.97 ± 0.45	-0.06 [-0.21 / +0.09]	0.4	-0.02 [-0.17 / +0.13]	0.8
Inhibin B *(ng/L)*	76.8 ± 56.4	88.3 ± 51.1	11.5 [-6.3 / +29.3]	0.18	11.2 [-7.0 / +29.4]	0.23
AMH *(ng/mL)*[b]	-0.08 ±1.74	0.19 ±1.45	0.27 [-0.25 / +0.79]	0.3	0.04 [-0.39 / +0.47]	0.9
AFC	5.5 ± 4.4	6.0 ± 5.5	0.5 [-2.0 / +2.9]	0.8	0.36 [-1.4 / +2.2]	0.7

a corrected for age and smoking

b values are on a logarithmic scale (on a normal scale values must be exponentiated) resulting in ratio instead of difference.

Figure 11.



Regression curves for the relation between AMH and age in GALT carriers and controls

Discussion

The results of the present study indicate that there is no evidence of an effect of GALT mutation carriership on ovarian reserve and age at menopause. These findings are supported by the fact that earlier preliminary studies in women suffering from POF or infertility failed to demonstrate an increased frequency of the common GALT mutations.[158, 351-353] The published literature concerning a possible relationship between GALT carriership and fertility thus far was limited to observations on menstrual and fertility history. In the present study, we applied established endocrine and ultrasound parameters for ovarian reserve. None of the parameters used appeared to differ from the control group.

Mean age at menopause in a cohort of 4,686 postmenopausal women in the Netherlands was 50.16 years (95%CI 50.1-50.2).[46] This is not different from the mean age of menopause in our current study cohort. Our arbitrary decision to set the menopausal age in women in the menopausal transition at one year after blood sampling could lead to an underestimation of the age at

menopause in our study cohort, as the menopausal transition is nearly 4 years.[354] On the other hand recall bias of age at menopause may occur, although all retrospective studies involving age at menopause will suffer from this bias.

We used random blood samples of 7 GALT carriers who used a hormonal IUD or had undergone a hysterectomy. However, while FSH and Inhibin B levels are cycle dependent, accumulating evidence points towards AMH as a cycle independent ovarian reserve marker.[300, 355] Another limitation of our study is that we only evaluated ovarian reserve and age at menopause in GALT carriers with proven fertility. Thus our observations do not exclude the possibility that GALT carriers without offspring may have a different ovarian phenotype. To rule out dependency analysis was performed without the included family members and similar results were obtained (data not shown).

As classical galactosemia is a rare disorder with an average of 6 new patients per year in the Netherlands, the number of mothers of patients who are proven carriers participating in the study remains relatively small. Nevertheless, more than 50% of mothers who are known to the Dutch Galactosemia Society participated in the study. Because at the time of this study there was no neonatal screening for classical galactosemia in The Netherlands, all patients presented with a neonatal crisis and carry mutations causing a severe phenotype.[356]

Abnormal glycosylation of hormones, as well as toxic damage to the ovaries, have been reported in patients with classical galactosemia, and are suspected to be involved in the observed hypergonadotropic hypogonadism in these patients.[357-361] The toxic damage is attributed to the elevated levels of the metabolite galactitol, which always occurs in patients, despite a galactose restricted diet.[362] The abnormal galactosylation of hormones is most likely related to the accumulation of the metabolite galactose-1-phosphate, the precursor of UDP galactose, which is the substrate for galactose transferase, incorporating galactose into glycoproteins. Even in strictly controlled patients, red cell galactose-1-phosphate levels are elevated at all times, probably because of endogenous galactose production.[346, 363] However, in carriers of classical galactosemia, no elevation of urine galactitol or red cell galactose-1-phosphate can be detected. Therefore, in the absence of toxic effects or abnormal glycosylation, the occurrence of decreased fertility and/or a decreased ovarian reserve is unlikely in GALT carriers. Indeed, as our study failed to demonstrate such an association, we conclude that concern with regard to earlier menopause is not justified and that there is no indication for screening for GALT mutations in women with decreased fertility or POF.

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Genome-wide association study in premature ovarian failure patients suggests ADAMTS19 as a possible candidate gene

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§ These authors contributed equally to this manuscript.

Chapter 6

Introduction

Spontaneous Premature Ovarian Failure (POF) is a common disorder in women, with a prevalence of 1%[6]. POF is characterized by secondary amenorrhea before the age of 40 years along with postmenopausal gonadotrophin levels (FSH > 40 IU/L) and very low or undetectable AMH levels[364]. POF not only truncates the patients' fertile lifespan, but also has major implications for their long-term health (i.e. osteoporosis, cardiovascular health and cognition). POF is usually due to premature exhaustion of the primordial follicle pool. Although an association with auto-immunity and macroscopic genetic aberrations has been demonstrated, the aetiology of the great majority of spontaneous POF cases remains unknown[71]. Since family history has been shown to be the best predictor for early menopause and strong associations have been disclosed between the menopausal ages of mothers and daughters, sisters and twin pairs, idiopathic POF is most likely due to genetic factors[45, 47, 54-57]. The incidence of familial POF is reported to be between 4 and 31%[49, 365].

Cytogenetic abnormalities involving the X chromosome have been identified in some POF patients, in particular XO mosaicism and X chromosomal rearrangements (macrodeletions and translocations)[69]. The only common genetic risk factor (prevalence > 1%) described in POF is being a carrier of a Fragile X premutation (*FMR1*). Sixteen percent of these carriers suffer from POF, whereas in POF patients the prevalence of Fragile X carriership is reported to lie between 3% and 15%, depending on familial distribution[72, 215, 366].

Recently two familial linkage studies identified POF loci on Xq21.1 - Xq21.3.3 (in a gene named *POF1B*) and on 5q14.1-5q15[62, 63]. Linkage analysis in sibling pairs discordant for menopausal age previously performed by our group resulted in two suggestive linkage regions on 9q21.3 and again on Xp21.3 61 and many other POF candidate genes have been suggested. These were mostly identified in single patients or families, small patient groups, isolated populations, or through animal knock-out models (see Chapter 1 - Table 1).

Using high-density oligonucleotide genotyping platforms, it is now possible to screen up to millions of single nucleotide polymorphisms (SNPs) throughout the genome of a single individual or cohort – the so-called genome-wide association studies (GWAS). Via GWAS it is possible to identify common genetic variants contributing to susceptibility to genetically complex (or polygenic) diseases such as diabetes, hypertension, Crohn's disease, neurological and psychiatric disorders[87]. We designed a GWAS using SNP arrays to identify predisposing genetic risk factors in a well phenotyped set of POF patients and controls.

Materials and methods

Study population and sample collection

From October 2004 onwards, a nationwide, standardized systematic screening protocol has been applied to women with suspected POF visiting the outpatient clinics of 10 Dutch hospitals. This protocol was approved by all the local institutional review boards and written informed consent was obtained from all participants. Screening included a questionnaire regarding fertility, family history, climacteric complaints, transvaginal ultrasonography, and blood withdrawal. Blood samples were also collected in 10 ml EDTA tubes. DNA was isolated using a salting out procedure and frozen at 80°C until genotyping experiments began.

POF was defined as at least one episode of spontaneous secondary amenorrhea for more than 120 days (4 months) along with at two moments FSH levels > 40 IU/L, before age 40 yrs), and AMH levels below the menopause threshold of 0.086 µg/L[28]. All patients presented with spontaneous menarche, with no history of chemotherapy, pelvic radiotherapy/surgery, or other medical conditions known to be associated with POF. All patients were Caucasian. Their karyotypes were obtained and they all underwent FMR1 premutation screening. Those with an abnormal karyotype (also including low 45,X/46,XX mosaicism) were excluded from the current analysis. Patients with more than 40 CGG repeats in the promotor region of the FMR1 gene were also excluded.

DNA samples from population control women with an age at menopause above 53 years were selected from the GOAL (Genetics of ovarian ageing by linkage-analysis) study cohort, further referred to as OldMP[61]. These Caucasian women had at least 12 consecutive months of spontaneous secondary amenorrhea. Genotyping data from 181 healthy Dutch female controls (mean age 60.8 ± 10.3 yrs) were added from a GWAS in amyotropic lateral sclerosis to increase study power, further referred to as FemC[367]. No menstruation history of these women was obtained. These two groups formed the control cohort for the current study in Phase I.

For the replication study (Phase II), we genotyped the SNP identified in Phase I in 60 additional POF samples and 90 OldMP females. Of the POF cases, 19 were recruited from the ongoing research protocol in the UMC Utrecht and 9 from Phase I samples with low genome-wide call rates (<95%). An additional 32 POF samples were recruited from the GOAL cohort, in which women with a very early age at menopause had also been genotyped.

Genotyping methods

In Phase I, POF cases and OldMP women were genotyped using Illumina Infinium II Hapmap370 SNP duochips v.1.1 April 2007 (Illumina, San Diego, CA, US). All experiments in Phase I were carried out at the Complex Genetics Group in the UMC Utrecht according to the manufacture's protocol. In short, 750 ng of DNA per sample was whole-genome amplified, fragmented, precipitated and resuspended in the appropriate hybridization buffer. Denaturized samples were then hybridized on Illumina BeadChips at 48°C for a minimum of 16 hours. After hybridization, the BeadChips were processed for single base extension reaction and stained. Chips were then imaged using the Illumina Bead Array Reader.

For Phase II (replication) the most significant SNP from Phase I was genotyped in the Genetic Department, UMC Groningen, using Taqman allelic discrimination assays. PCR was carried out with mixes consisting of 15 ng of genomic DNA, 1 x AbSolute QPCR ROX mix (AbGene Mix, Thermo Scientific) and 1 x assay mix (Applied Biosystems, Foster City, Ca, US) and ultraPURE distilled water (Dnase, Rnase Free, Gibco) in a 5 µl reaction volume in 384-well plates (Applied Biosystems). PCR conditions were as follows: denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. Allelic PCR products were analyzed on the ABI Prism 7900HT sequence detecting system using SDS 2.3 software (Applied BioSystems). Primer sequences for *ADAMTS19* was TCTCTTGTCTCATTTGGGCACTTTA [G/T]AAATTTGTGGATGGCTATTTATTGG.

Statistical analyses and quality control

Once all samples for the GWA study had been genotyped, various quality control procedures

were employed. For each sample, normalized bead intensity data was used in BeadStudio v3.0. to call genotypes. Samples that had an overall call rate less than 95% were removed. SNPs that had a call rate less than 95%, a minor allele frequency (MAF) in the controls lower than 1%, or showed deviations from Hardy-Weinberg equilibrium (HWE) in the controls (Exact HWE P-Value < 0.001) were removed from subsequent analyses.

To test for association we used Prioritizer GWA[368], and employed a single marker test, comparing allele count frequencies between cases and controls. Significance of association was determined by using an allele count χ^2 test (1 df). As over 300,000 tests were performed, we corrected for multiple testing. First, Type I errors were ascertained by a quantile-quantile (Q-Q) plot, generated by plotting the observed ordered null-allele associations against the ordered expected associations (see figure 1). Then we fitted a line to the lower 90% of the distribution, of which the slope ($\lambda_{inflation}$) denotes either the inflation or deflation of the test statistic.

Subsequently we determined what nominal single SNP P-Value corresponded to a P = 0.05, after correction for multiple testing. A commonly used threshold for deeming a SNP association genome-wide significant is P = 5 x 10-7[369]. We established through 200 permutations of the affection status labels of our samples that a nominal P = 2.0 x 10-7 corresponds to a genome-wide significance of P = 0.05 on the Infinium II Hap370 platform.

For the replication analyses, we carried out Cochran-Mantel-Haenszel allele count χ^2 association tests using SPSS 16.0.1 with two clusters: Phase I (Infinium assay), Phase II (TaqMan assay). All P values are two-tailed. As we observed no

Table 11. Overall statistical power of study

MAF	OR	Power % (P=0.01)	Power % (P=2.0x10-7)
0.26	3	100	89
0.26	2.83	100	80
0.26	2.5	100	54
0.26	2	92	11

Power shown was calculated for 99 cases and 235 controls, assuming a minor allele frequency (MAF) of 0.26, which corresponds to the mean MAF of all SNPs present on the oligonucleotide array. Results are shown for four different odds ratios (OR) with the expected power: P = 0.01 or P = 2.0 x 10-7 (genome-wide significance).

evidence for bias in the test statistics as the Type 1 error rate ($\lambda_{inflation}$ = 1.017) was not inflated, we present uncorrected statistics throughout this paper.

Power calculations

Power calculations for the GWAS were performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) based on our sample size, the average observed minor allele frequency (MAF) for SNPs present on the Illumina HumanHap370 (26%), under the assumption of a multiplicative model and a POF prevalence of 1 per 100[6]. Using these parameters the genome-wide scan was 80% powered to detect an allelic association with P < 2.0 x 10-7 (which corresponds to a genome-wide significance of P = 0.05 when taking linkage disequilibrium (LD) into account, determined using permutations) and an odds ratio of 2.85 (see also Table 11).

Linkage regions analysis

Similar to the candidate gene analysis, we first determined the most significant SNP (allele count 1df χ_2 P value) for each of these loci and then performed a permutation analysis, assuming that only one variant was responsible for the observed linkage signal. By permuting affection status labels 500 times (which leaves the LD structure intact), we could empirically determine the significance of association for each of these SNPs, correcting for the fact that linkage regions can differ in size and in the number of SNPs that map in them.

We also employed another procedure (allelic heterogeneity), in which we assumed that multiple independent, but common, variants within each of these loci might have contributed to the observed linkage signal. For each of the loci we determined the product of the individual allele frequency P values for all of the SNPs that mapped in these loci. Subsequently we permuted the affection status labels 500 times, and in each permutation we compared the permuted product of allele frequency P values against the observed product P value, enabling us to determine a significance of P values, while assuming allelic heterogeneity.

Functional candidate gene analysis

We selected 74 candidate genes based on one of the three following criteria: (1) incidental finding in POF patients, (2) previously tested in POF patients, or (3) animal knock-out model showed a POF-like phenotype and the gene involved had a human homolog (see Supplementary Table I). For each of these candidate genes, we determined whether associated SNPs were present that either mapped within these genes or were in strong LD with SNPs within these genes (R2 > 0.25). SNPs which are in LD with SNPs within these genes were included, because these SNPs might tag for a causal variant that maps within these genes.

We then determined the most significant SNP for each gene (through an allele frequency P-value) and performed a permutation analysis, enabling us to empirically determine the significance of each candidate gene. The reason for this procedure was that the number of SNPs can differ considerably per gene, because genes vary in size and LD patterns differ. This is particularly true for SNPs that map within or very close to the major histocompatibility locus on chromosome 6, where LD patterns are very extensive and many SNPs might tag the same causal variant. As such, by permuting affection status labels 500 times (leaving the LD structure intact), we empirically determined the significance of association for each of these candidate genes while controlling for LD and the number of SNPs.

Table 12. POF patient phenotype characteristics (n=99)

	Mean SD	%
Age at screening	36.5 ± 7.3	
1st FSH *(IU/L)*	82.4 ± 29.5	
2nd FSH *(IU/L)*	79.7 ± 38.1	
Age at menarche *(yrs)*	13.2 ± 1.6	
Age at amenorrhea *(yrs)*	31.1 ± 8.1	
Familial clustering[a] *(%)*		19
46 XX karyotype *(%)*		100
FMR1 repeats n < 40 *(%)*		100
Caucasian *(%)*		100
AMH below menopause threshold[b] *(%)*		100
Undetectable AMH *(%)*		93
Positive anti-TPO antibodies *(%)*		25
Adrenal antibodies *(%)*		2

FSH = follicle stimulating hormone

FMR1 = Fragile X mental retardation 1

AMH = anti-Müllerian hormone

anti-TPO = anti-thyroid peroxidase

a Defined as at least 2 first- or second-degree female family members with POF (including the index patient)

b Menopause threshold for AMH is < 0.086 µg/mL 28.

Results

In total, 108 POF samples and 60 OldMP samples were genotyped using the Illumina HumanHap370 BeadChip. Nine POF samples and 6 OldMP samples were excluded from analysis because their call rates fell below 95%. Ninety-nine POF cases (Table 12) and 235 controls (54 OldMP and 181 FemC) were included for further analysis. All the POF patients had anti-Müllerian hormone (AMH) values below the menopause threshold, and most had undetectable AMH levels. No related individuals were identified after comparing all the samples. In total 309,158 SNPs passed our quality control (Exact HWE P-value > 0.001 in the controls, MAF in the controls > 0.01, call rates for controls > 95%

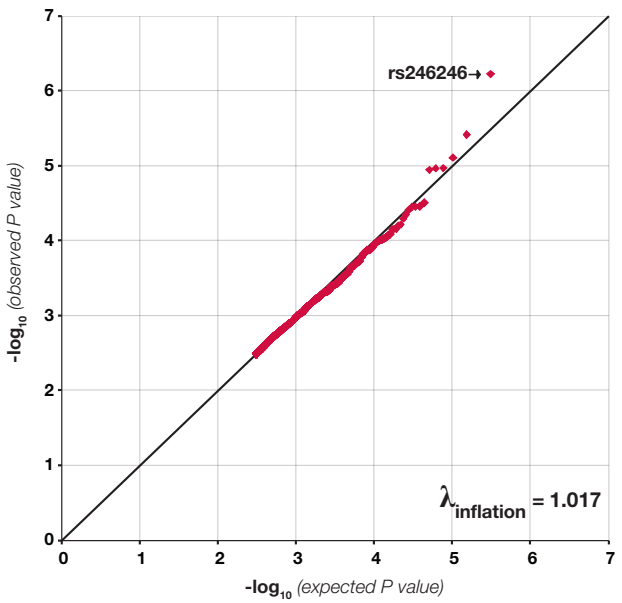
and for cases > 95%). Three SNPs were not genotyped correctly upon visual inspection, and were excluded from further analysis. A quantile-quantile (QQ) plot analysis indicated no inflation of the test statistics (Figure 12) as the $\lambda_{inflation}$ = 1.017.

One SNP achieved near genome-wide significance after correction for multiple testing (A P-value < 5 x 107 is considered to reflect genome-wide significance[369], permutation analysis of our data indicated a genome-wide significance threshold of P-value < 2 x 10-7). SNP rs246246 was associated with an allele frequency P-value of 5.98 x 10-7. rs246246 SNP maps to an intronic region of a gene named *ADAMTS19*, in a 200 kb block of LD on chromosome 5q31, which also includes the small *KIAA1024L* gene on 5q23.3 with unknown function (Figure 12).

Since thyroid peroxidase auto-antibodies (anti-TPO) were present in 25 percent of the POF samples we stratified for anti-TPO in relation to rs246246 genotype. No relation could be identified using a two tailed Fisher's exact test (P=0.283).

We performed a small-scale replication study in Phase II covering 60 additional POF cases and 90 additional controls. Apart from genotyping rs246246 in the replication cohort, we also genotyped six random individuals from Phase I to ensure both SNP genotyping platforms generated the same genotype for each individual (concordance rate = 100%). No significant difference in MAF between cases and controls from the replication cohort was observed (MAF-cases = 0.05 and MAFcontrols = 0.0556), resulting in P = 0.83. A joint analysis 370 of Phases I and II using

Figure 12.



Quantile-quantile plot of observed versus expected P values. $\lambda_{inflation}$ = 1.017, suggesting no inflation of the test statistic.

a Maentel-Haenszel test resulted in P = 4.05 x 10-5. Table 13 presents an overview of the descriptive statistics and results for SNP rs246246 in Phase I/Phase II and the joint analysis. An overview of all SNPs from Phase I with a P < 0.05 can be found in Supplementary Table II (not included in the thesis).

In each of the three POF associated linkage regions (Xq21.1 - Xq21.3.3, 5q14.1-5q15 and 9q21.3), SNPs were identified that had a nominal P value < 0.01. However, none of these findings remained significant after the permutation analysis, irrespective of the model assumed. Candidate analysis of the most significant SNP revealed 29 candidate genes with a nominal P value < 0.05. After permutation, five genes (*BDNF, CXCL12, LHR, USP9X* and *TAF4B*) showed a nominal P value < 0.05 on gene level, which is more than expected by chance (0.05 x 74 = 3.7) (Chapter 1 – Table 1).

Discussion

To our knowledge, this is the first reported genome-wide association study in POF. Our results suggest that the gene *ADAMTS19*, located on chromosome 5q31, may be involved in POF. This finding will need to be replicated in a larger and independent study population.

ADAMTS19 is a member of the large ADAMTS (a desintegrin-like and metalloprotease with thrombospondin type 1 motif) family of metalloproteases (metal-binding enzymes). ADAM proteins are responsible for the proteolytic cleavage of many transmembrane proteins and the release of their extracellular domain, and seem to play an important role in gonad formation and function[371, 372]. In a previous study on gene expression differences between embryonic XX and XY mouse gonads

Table 13. Descriptive statistics and results for SNP rs246246 (G/T) in Phase I / Phase II and joint analysis. Allele G is the susceptibility allele.

	cases (n)	controls (n)	MAF[a] cases	MAF[a] controls	HWE[b] cases	HWE[b] controls	P value[c]	OR[d]	95% CI
Phase I	99	235	0.15	0.04	0.09	0.25	5.98 x 10-7	4.31	(2.33 – 7.94
Phase II	60	90	0.05	0.05	0.68	0.15	0.83	0.89	(0.32 – 2.53)
Combined[e]	159	325	0.10	0.04	0.11	0.06	4.05 x 10-5	2.75	(1.65 – 4.59)

a MAF = minor allele G frequency;

b Hardy-Weinberg equilibrium P values;

c P values were calculated for each individual population using β2 test on allele counts;

d Odds ratios (OR) were calculated for the minor allele;

e P values and ORs were calculated using the Mantel-Haenszel method.

using cDNA subtraction, *ADAMTS19* was shown to be significantly upregulated in XX gonads at the moment of sex differentiation. Using whole-mount in situ hybridization abundant expression of *ADAMTS19* was noted during the embryonic phase of gonadal development[133]. These findings provide a biological plausibility to *ADAMTS19* as a possible candidate gene for POF. Other ADAMTS proteases are also widely involved in female reproduction; gonad formation is disrupted in C. elegans when the ortholog of *ADAMTS-9* and *20* is mutated, while female homozygous *ADAMTS-1* knock-out mice had a reduced number of ovarian follicles[373]. Furthermore, ADAMTS proteins seem to play a role in ovulation processes as well as in folliculogenesis[374, 375].

The near genome-wide significance of a SNP in a study with a relatively small number of patients and that it mapped to a plausible candidate gene like *ADAMTS19* (Phase I) were the reasons to perform a validation study in an independent subset of patients and controls (Phase II).

A weakness of our study is that for Phase I POF only cases had been included that had been carefully phenotyped. As such in Phase II, fewer cases were available with equally well defined phenotypes. Also some of the cases in Phase II were less extensively phenotyped than in Phase I, since these samples were taken from the GOAL cohort[61]. This group was only phenotyped as POF via their last recorded, spontaneous, menstruation date. It is possible that this phenotypic heterogeneity interferes with the results, although there was no statistical significant difference between the heterozygosity incidence. The 90 control samples in Phase II were again selected from the OldMP cohort and all had a history of spontaneous menstruations beyond age 52 years. Albeit this major shortcomings we feel encouraged by the initial robust Phase I results and think the novelty of these preliminary findings are of interest for the scientific community.

Since POF is considered a complex genetic condition involving multiple genes, our genome-wide SNP data allowed us to investigate associated SNPs in POF candidate genes identified earlier (see Supplementary Table I). After permutation analysis five candidate genes showed higher P values than expected. All five have been labelled as possible candidate genes via animal models showing a POF or POF-like phenotype. Brain-derived neurotrophic factor (*BDNF*) maps on chromosome 11p13. Ovaries of BDNF knock-out mice show loss of follicular organisation, preceded by massive oocyte death[157]. Chemokine (C-X-C motif) ligand 12 (*CXCL12*) maps on chromosome 10q11.1 and is involved in guiding primordial germ cell migration[155]. *LHR* (luteinizing hormone receptor) knock-out mice show a block in preantral folliculogenesis in combination with underdeveloped sex organs[113, 114]. Ubiquitin-specific protease 9 (*USP9X*) is a gene required for oogenesis in Drosophila and maps in humans in a highly POF susceptible region on the short arm of the X chromosome (Xp11)[194]. TATA box binding protein (TBP)-associated factor 4B (*TAF4B*) maps on chromosome 18q11.2, while heterozygous *TAF4B* mice have a reduced number of ovarian follicles[176]. Although SNPs in these genes did not show strong significance on a genome-wide level in our study, their biological relevance might warrant attention in future genome-wide association analyses.

In conclusion, this first-stage, genome-wide association study in a relatively small, homogeneous cohort of well-phenotyped POF patients did not reveal any common variants with genome-wide significance that confers risk to POF. However, *ADAMTS19* was identified as a potential candidate gene for POF. As-

suming that the Illumina HumanHap370 tags human genetic variation well[376], and taking into account the power calculations that we performed, we conclude that the contribution of common genetic variants to POF is modest (odds ratio < 2.83). However, in the current study, we only assessed common SNP variants; rare variants or common structural variants were not investigated. As such, additional, systematic, genome-wide analyses of POF patients using more extensive arrays, CNV detection algorithms, and larger sample sizes are warranted in the search for POF-associated genetic variants.

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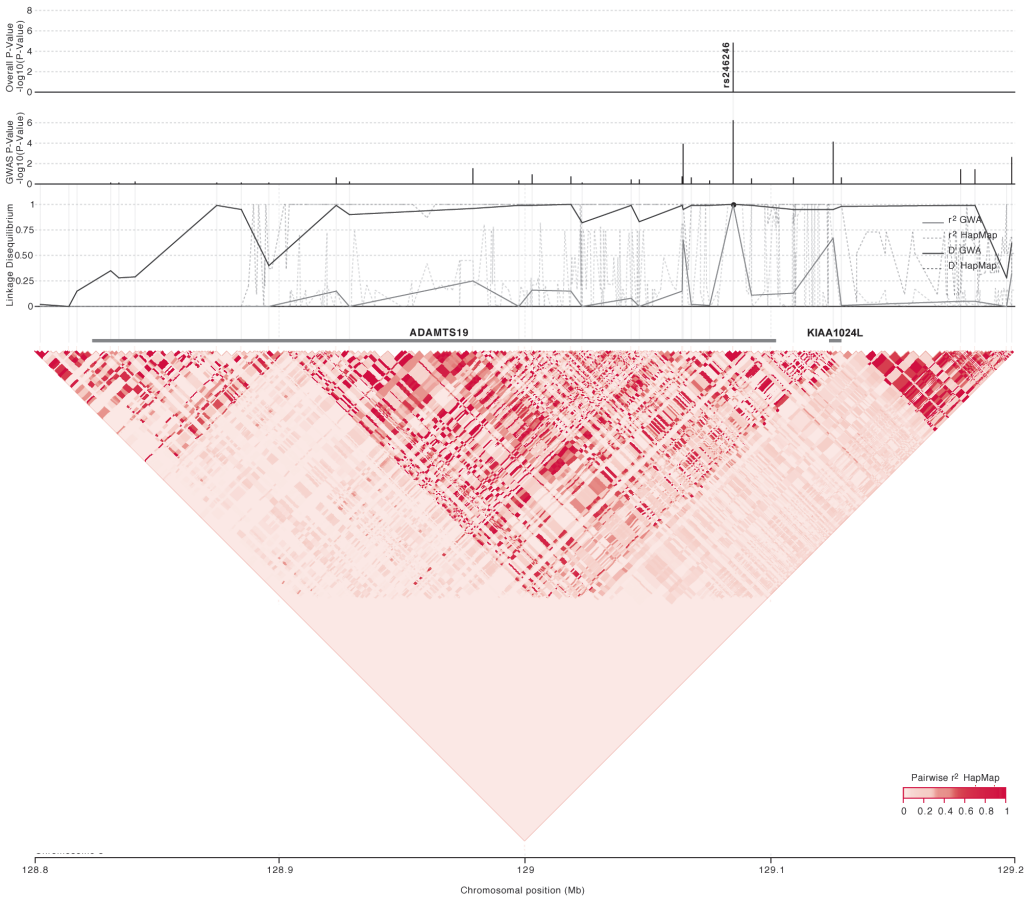
Figure 13.



Schematic 400 kb haploblock view on the long arm of chromosome 5 surrounding the rs246246 SNP and covering two genes: *ADAMTS19* and *KIAA1024L*.

A: indicates the combined Phase I and Phase II P-value for rs246246.

B: shows the observed P-values for the SNPs assayed in Phase I.

C: shows linkage disequilibrium for rs246246 with neighbouring SNPs, both for the SNPs in the genome-wide analysis and SNPs present within HapMap, based on the CEU population.

In preparation

Copy number variants on the X chromosome in premature ovarian failure patients

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Chapter 7

Introduction

Premature ovarian failure (POF) is a common complex disorder in females. This condition is characterized by a secondary amenorrhea before 40 years of age and associated with severely compromised fertility and multiple women's health issues[71]. Serum follicle stimulating hormone (FSH) levels are elevated above 40 IU/L and anti-Müllerian hormone (AMH) levels are below the menopause threshold or undetectable[364].

Menopause marks the endpoint of the continuous exhaustion of the ovarian follicle pool which is initiated before birth[29, 377, 378]. The age of menopause shows a Gaussian distribution with a median around 51 years (range 40 – 60 yrs) worldwide[3, 379]. The processes and factors that determine the variation among individuals in the rate of decline in oocyte quantity are largely unknown[238]. For instance, it remains unknown as yet whether females with an earlier menopause exhibit an initial low count of follicles or an increased rate of follicle depletion.

Since heritability for age at menopause is between 40-70% in sisters and twins[54-57] and no evident causal environmental factor seems to be associated with age at menopause, genetic factors seems to play a major role[295]. From a biological point of view the X chromosome seems to be a likely candidate involved in ovarian function[380]. Similarly, mutations in the Y chromosome have been shown to be associated with decreased male fertility[381]. Hemizygosity for the X chromosome, as seen in Turner (XO) syndrome leads to primary amenorrhea with streak ovaries. Additionally, in Turner foetuses ovarian follicles could be identified, implicating initial follicle development but a rapid decline thereafter[382]. Mosaic Turner patients often show a milder ovarian phenotype compared to patients with complete absence of one X chromosome. Sometimes menarche and even pregnancy is reported, suggesting a dose effect[383]. Furthermore, in POF families there is evidence for a female sex preponderance which might be due to a lethal genetic factor in male foetuses, possibly due to lack of a protective homozygous sex chromosomal state[74].

In the majority of POF patients no common genetic risk factor has been identified yet. However, in a subset of patients fragile X (FMR1) premutation or microscopic X chromosome aberrations can be identified. Besides 45X/46XX mosaicism, the vast majority of identified genetic aberrations involve the long arm of the X chromosome. In particular balanced Xq21 autosomal translocations[65, 69, 384], and terminal deletions are associated with POF (see Table 14). The vast majority of translocation breakpoints and deletions on the X chromosome cluster within two POF critical regions on its long arm (i.e.loci Xq13.3–Xq21 and Xq23-Xq27) 65. Also X chromosomal interstitial deletions[213, 384-386], inversions[387], Robertsonian translocations[385, 388] and short arm deletions[389] are described in relation to the POF phenotype. Recently a study in three patients with a terminal Xq deletion revealed a cryptic duplication on Xp, including the SHOX gene[390]. Genetic linkage studies in POF families or women discordant for menopausal age identified loci on 5q14.1-q15 (LOD score:2.4) 63, 9q21.3 (LOD score 2.6) and two times on chromosome X, Xp21.3 (LOD score 3.1)[61] and Xq21.1-21.3.3 (LOD score 2.7)[62].

Nowadays high throughout genotyping methods, using single nucleotide polymorphisms (SNPs) are available. These genome wide arrays contain between 300,000 and 1,000,000 probes, and have

Table 14. Overview of Xq deletions and translocations associated with POF

Reference	Age*	Karyotype
Deletions		
68		46,X,del(X)(pter-q21.3::q27-qter
390	33	46,X,del(X)(q21.3)
390	15	46,X,del(X)(q21.2)
390	26	46,X,del(X)(q22.3)
403	22	46,X,del(X)(q27)
385	33	46,X,del(X)(q26.2)
385	26	46,X,del(X)(q21.2)
404	30	46,X,del(X)(q26)
405	27	46,X,del(X)(q22)
406	28	46,X,del(X)(q28)
DPC	35	46,X,del(X)(q23)
DPC	15	46,X,del(X)(q22)
DPC	38	46,X,del(X)(q24)
DPC	30	46,X,del(X)(q22)
DPC	28	46,X,del(X)(q22.2) isoX(p)
DPC	28	46,X,del(X)(q22)
Translocations**		
DPC	34	46,X,t(X;5)(q22.1;q22.1)
DPC	18	46,X,t(X;1)(q2,2;34.1)
384	20	46,X,t(X;5) (q21;q35)
384	17	46,X,t(X;3) (q21;p21)
384	unknown	46,X,t(X;2) (q21;q23)
384	15	46,X,t(X;9) (q21;q33)
384	30	46,X,t(X;12) (q21;p13)
384	16	46,X,t(X;3) (q21.2;q12)

DPC: Dutch POF Consortium (not published)

* Age at last reported spontaneous menstruation

** Cases reported in the last 5 years.

helped to identify multiple genetic risk factors that are associated with complex genetic disorders[87]. Besides variation at the SNP level, it is now recognized that insertions, duplications or deletions of DNA sequence, also contribute to phenotypic variation and disease susceptibility[391]. These are referred to as copy number variants (CNVs) and typically range from 1kb to several mega bases in size, and usually are too small to be detected by conventional karyotyping techniques. CNVs may be involved in altering gene expression through disruption of regulatory elements or by changing dose effects[89, 392]. Currently, nearly 20,000 common CNVs (involving more than 6000 loci), covering over 20% of the genome, have been identified in healthy people[96, 393-394].

In addition to genotype data, high density SNP arrays also generate intensity data for each oligonucleotide probe, which enables quantification of input template DNA and, indirectly, detection of deletions and duplications. The high density of markers means that an enormous increase (x 100) in resolution could be obtained as compared to that of karyograms[395].

As mentioned, X chromosomal macrodeletions are related to POF. Therefore the current study is based on the on the premise that intensity data of a genome wide SNP array might reveal submicroscopic X chromosomal deletions (or duplications) in POF patients with a normal conventional karyogram.

Materials and methods

Patients:

Since October 2004 a nationwide phenotyping project of POF patients in the Netherlands was initiated by the department of Reproduction and Gynaecology of the UMC Utrecht. Sixteen Dutch fertility clinics participated (http://www.umcutrecht.nl/pof). Suspected of previously diagnosed POF patients under age 40 yrs all underwent standardized screening involving a questionnaire (medical and family history etc.), ultrasonography, hormonal profiling, karyotyping plus informed consent for a blood specimen for DNA extraction. The study has been approved by local ethics review committees of all participating hospitals. Patients with an evident history of POF but were older at the time of screening only filled in the questionnaire and donated blood for DNA extraction.

Only the following POF phenotype was used in this study; spontaneous secondary amenorrhea before age 40 yrs, Caucasian, normal karyotype, no (or low) XO mosaicism, no FMR1 premutation. All patients gave written informed consent.

Laboratory Procedures:

All DNA samples were isolated from fresh venous blood using a salting-out procedure and genotyped with Human-CNV-370-Duo v1.0 Bead Chips (Illumina, San Diego CA, US). This chip contains 370,404 markers with a median spacing of 5.0 kb and 12,556 X chromosome probes. By incorporating monomorphic probes this chip was specifically designed to detect CNVs by covering 3,034 CNV regions from the Database of Genomic Variants (DoGV)[394].

All procedures were performed according to the manufacturer's protocol. In short, 750 ng of genomic DNA was amplified, fragmented, and hybridized to the array, and products were fluorescently labelled and scanned on the Illumina Beadstation scanner. Raw intensity data were then uploaded into Beadstudio v3.0 (Illumina, CA, US).

CNV detection and quality control:

To reliably detect CNV segments we used PennCNV[396]. PennCNV uses a Hidden Markov Model to interrogate SNP intensity signals, and is capable of detecting CNVs if these typically span multiple SNPs. PennCNV uses B allele frequency (a measure of the allelic ratios) and Log R values (normalized intensity values) to call CNVs. For each SNPs these values are calculated based on raw intensity characteristics of all samples for that particular SNP. As two different batches of samples resulted in slightly different overall intensity characteristics, Log R and B allele frequencies were calculated separately for these two batches. PennCNV was subsequently run using default settings.

Table 15. Phenotype of 108 POF patients included in CNV analysis

	Mean SD	%
Age at screening	36.3 ± 7.2	
FSH *(IU/L)*	81.7 ± 30.6	
Age at menarche *(yrs)*	13.2 ± 1.6	
Age at amenorrhea *(yrs)*	31.1 ± 8.1	
Familial clustering[a] *(%)*		17
46 XX karyotype *(%)*		100
FMR1 repeats n < 40 *(%)*		100
Caucasian *(%)*		100
AMH below menopause threshold[b] *(%)*		100
Undetectable AMH *(%)*		93
Positive anti-TPO antibodies *(%)*		32
Adrenal antibodies *(%)*		3

FSH = follicle stimulating hormone
FMR1 = Fragile X mental retardation 1
AMH = anti-Müllerian hormone
anti-TPO = anti-thyroid peroxidase

a Defined as at least 2 first- or second-degree female family members with POF (including the index patient)

b Menopause threshold for AMH is < 0.086 µg/mL 28

Table 16. Detected CNVs on the X chromosome (after quality control)

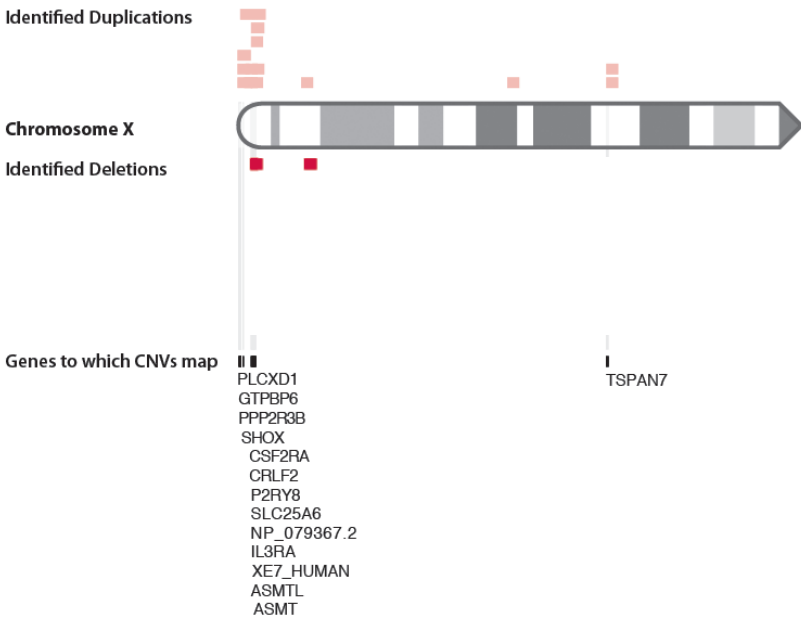
	All CNVs	Deletions	Duplications
Amount *(n)*	65	45	20
Mean size *(kb) – range*	423,033 bp (541 – 3706,479)	515,190 bp (541 – 3706,479)	215,679 bp (11573 – 1627,987)
Mean no. probes/ CNV – *range*	36 (10-248)	38 (10-248)	31 (10-168)
Mean amount *(n)***/ patient*** *(range)*	1.9 (1-6)	2.3 (1-6)**	1 (1-1)***
Xp *(n)*	19	4	15
Xq *(n)*	46	41	5

* 33 patients showed at least one CNV

** 20 patients showed at least one deletion

*** 20 patients showed (at least) one duplication

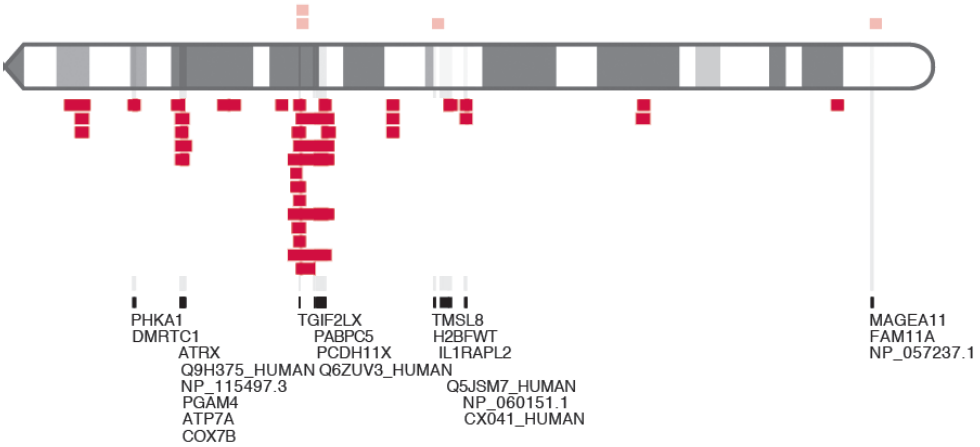
Figure 14. CNVs identified on the X chromosome in 108 POF patients.



To obtain high quality CNV calls we applied rigorous quality control measures. We discarded CNV calls when less than 10 consecutive probes showed significant intensity change as identified by PennCNV. CNVs spanning the centromeres were excluded from further analyses, since the low probe coverage in these regions precludes accurate CNV boundary estimation[397]. CNVs within <10 kb distance from each other within the same individual were considered as one CNV. After filtering all X chromosome deletions and duplications, these were visually inspected within Beadstudio using LogR and B allele frequency plots. Furthermore all CNVs were analyzed according to their cytogenetic band and genes within these CNV loci were listed.

Results

108 POF patients were included in the current analysis. Phenotypic characteristics are listed in Table 15. Before quality control PennCNV identified 310 X-CNVs in total (211 deletions and 99 duplications). Size varied between 100 basepairs and 1 Mb (mean size: 92 kb). In total 88 samples showed at least one CNV. After correction 33 patients showed a CNV, and their characteristics are listed in Table 16. Of the 20 identified duplications, 75% map on the short arm and 87% (13 out of 15) of these map on p22. On the long arm 63% (29 out of 46) of the CNVs map at locus q21, of these 90% involves a deletion (26 out of 29). Figure 14 shows the X chromosome with total amount



of CNVs (split by deletions and duplications) per cytogenetic band.

In 31 genes at least one CNV could be detected; in 20 genes at least one sample showed a duplication, and in 15 genes at least one sample showed a deletion. In 3 genes deletions as well a duplications could be identified, namely TGIF2LX, MAGT1 and ASMT. Genes with a CNV in at least 2 POF samples are listed in Table 17.

Discussion

Although our findings are preliminary our experiment is a clear proof-of-principle that new genetic techniques might identify new determinants, especially CNVs, which might play a role in the aetiology of idiopathic POF. It could well be possible that POF might be the results of multiple genetic risk factors, including SNPs, rare sequence variants and CNVs. The abundant overrepresentation of CNVs on Xq21 further substantiates the important role of this locus in the genetics of POF. The large amount of duplications on Xp22 may be due to overall low mean intensity levels.

On the X chromosomes only a small number of X genes are biologically plausible POF candidate genes since their function is explicitly implicated in follicle depletion processes and/or POF phenotype in humans or animal knockout models (Table 1, Chapter 1). None of these earlier identified POF candidate genes were interrupted by a quality- checked CNV in our cohort. However, in the identified smaller CNVs one duplication of 8 consecutive probes was identified in *DIAPH2* (data not shown). The absence of many CNVs in established X linked POF candidate genes can be explained by the fact that most candidate genes are identified on the basis of genotyping data (e.g. sequencing in search for SNPs) rather than intensity levels that might identify CNVs. Furthermore duplications and deletions might have another basis of interrupting gene transcription than SNPs. Three patients in our cohort showed a duplication in the *SHOX* gene (Table 5), which was earlier identified in a POF patient using bacterial artificial clone comparative genomic hybridization[390].

In the current study between 3-15% of the POF patients have a CNV in *PCDH11X*, *TGIF2LX*, *P2RY8*, *ASMTL* or *ATRX* (Table 17). Although unlikely from the current knowledge these genes might directly reveal new pathways in POF aetiology, these genes might be added to the already extensive list of potential candidate genes for POF. *PCDH11X* or protocadherin 11 X-linked has been scarcely investigated until very recently a polymorphism in this gene was linked to Alzheimer's disease[398, 399]. *TGIF2LX* or TGFB-induced factor homeobox 2-like, X-linked is very recently associated with male infertility and may be required for the regulation of spermatogonial stem cell specification and proliferation[400]. The function of *P2RY8* (purinergic receptor P2Y, G-protein coupled, 8) and *ASMTL* (acetylserotonin O-methyltransferase-like) is generally unknown. *ATRX* or alpha thalassemia/mental retardation syndrome X-linked undergoes X inactivation and seems to be is involved in gene regulation at interphase, as well as chromosomal segregation at mitosis during embryogenesis[401, 402].

There are some limitations of the current study. Although visual inspection was undertaken to validate the identified CNVs, different assays are required in order to validate these. Also replication in an independent cohort is needed to establish the true role of chromosome X CNVs in POF pathophysiology and to rule out population strati-

fication. Furthermore, the current study only investigated POF samples, and therefore most likely rare variants since all intensity data are normalized on the whole cohort. When adding a non-POF control cohort it might be possible to identify common variants and also more clearly identify POF specific variants, because it could well be possible that multiple CNVs might also show up as (common) CNVs in control samples.

In the near future identification of autosomal CNVs may be carried out. Also comparison of identified CNVs with earlier identified genome wide CNVs, for example those listed in the Database of Genomic Variants[394] may be performed, as well as genome wide CNV candidate gene analysis and POF linkage region analysis.

Our findings are in line with earlier published findings from cytogenetic studies and therefore might add new insights to the incidence of chromosome X abberations and genetic mechanisms influencing follicle pool depletion and/or (premature) menopause through disruption of DNA sequences on the X chromosome by submicroscopic deletions and duplications, especially on the Xq21 locus.

Table 17. Genes on the X chromosome with two or more CNVs in POF patients (n=108)

Gene	No. of POF samples	Percentage	CNV type
PCDH11X	16	14.8%	deletion
TGIF2LX	11	10.2%	deletion
P2RY8	7	6.5%	duplication
ASMTL	5	4.6%	duplication
ATRX	4	3.7%	deletion
GTPBP6	3	2.8%	duplication
PLCXD1	3	2.8%	duplication
SHOX	3	2.8%	duplication
Cxorf41	2	1.9%	deletion
NUP62CL	2	1.9%	deletion
MAGT1	2	1.9%	deletion
ASMT	2	1.9%	duplication
PPP2R3B	2	1.9%	duplication
TGIF2LX	2	1.9%	duplication
TSPAN7	2	1.9%	duplication
CXYorf3	2	1.9%	duplication

General discussion and future research concepts

Chapter 8

The Dutch POF consortium

Since 2004, sixteen Dutch fertility clinics have worked together and collected data, serum and DNA from young hypergonadotropic women, with the aim of further elucidating the phenotype, women's health implications, and genetics underlying premature ovarian failure. This thesis describes the first results from this national research project, which is ongoing. Without the help of our colleagues, we would not have been able to perform these studies.

Limitations of basal FSH

Follicle-stimulating hormone (FSH), measured during the early follicular phase of the menstrual cycle, is the most commonly used test to assess ovarian ageing. As reviewed in Chapter 2, elevated FSH levels are correlated to adverse outcome in terms of spontaneous conception changes as well as IVF treatment outcome. Despite the relatively limited predictive value of FSH, elevated baseline levels in subfertile patients make doctors and their patients feel uneasy. In clinical practice this leads to an undefined diagnosis, namely 'diminished ovarian reserve'. There is no clear cut-off value for FSH to define this condition. When using the 95th percentile of normally menstruating women and without considering individual variations in FSH receptor sensitivity, inter-cycle variability, and the different laboratory tests used, we generally take a baseline FSH concentration exceeding 10 IU/L as elevated. Recently age-specific levels of FSH have been published[407]. In Chapter 2, we reviewed the recent observations that anti-Müllerian hormone (AMH) has been presented as a promising and more stable direct ovarian marker for follicle quantity estimation than FSH.

Lack of clear ovarian failure phenotypes

We can distinguish four different ovarian reserve phenotypes using baseline FSH in combination with cycle pattern before age 40 years. Surprisingly, only three of them are generally known. Women with an FSH level below 10 IU/L and regular menses are considered 'normal'. An FSH level exceeding 10 IU/L in combination with regular menses is referred to as occult or 'incipient ovarian failure' (IOF). Finally, women presenting with amenorrhea and FSH levels exceeding 40 IU/L are referred to as POF. However, there is a fourth group, women with elevated FSH not fulfilling the definitions of POF or IOF (e.g. oligomenorrhea and elevated FSH; or amenorrhea and FSH values between 10 and 40 IU/L) who have not been investigated yet with regard to their ovarian phenotype nor their fertility potential. We looked at the cohort of consecutively recruited, hypergonadotropic patients used in Chapter 3 and found this fourth group makes up around 30% of the young hyper¬gonadotropic patients. We named this group 'transitional ovarian failure' (TOF), since this group is most likely to be in a phase of transition to a permanent hypergonadotropic amenorrhea (e.g. POF or menopause).

The added value of AMH

After setting these definitions, it was possible to investigate AMH levels in a cohort of 259 patients with elevated FSH levels, covering the entire spectrum of phenotypes associated with hypergonatropic ovarian failure and comparing them to controls. This is described in Chapter 3. One interesting finding was that we could identify some women with an age-appropriate AMH level despite their elevated FSH level, especially in the IOF and TOF groups. It may therefore be worth considering giving AMH a more prominent role in defining ovarian failure, rather than basing it solely on FSH levels. This may also have an impact on ovarian failure nomenclature and thus on future

pheno- and genotype studies. Additional follow-up studies should elucidate whether these women show a truly distinct, different fertility potential and menstrual cycle pattern in the years following diagnosis.

Despite these promising results with regard to AMH, so far no ovarian reserve test has shown sufficient reliability to allow us to refuse often intensive and expensive fertility treatment to some patients. Since Western women (and couples) often delay having their first child, women with a 'diminished ovarian reserve' will be seen more often in fertility clinics. At the moment, however, the most practical approach is to offer treatment and wait and see what happens. The need for an adequate ovarian reserve test seems to be greater than ever.

Educating young women as well as their (future) partners that their chances of conceiving are closely related to a woman's chronological age will hopefully bring out that couples will make a balanced decision and ultimately plan a (first) pregnancy earlier in life.

Counselling POF patients

POF is the most extreme phenotype of ovarian failure below 40 years of age. AMH levels are consequently below 0.1 µg/mL or undetectable (Chapter 3). With regard to fertility prognosis, it is widely regarded as very poor. Patients suffering from POF may find it very difficult to accept this prospect in addition to perimenopausal complaints. Sometimes it is necessary to consult a specialized social worker, psychologist or geneticist (e.g. in the case of familiar POF predisposition, fragile X carriership, or karyotype abnormalities). Despite spontaneous POF having a prevalence of 1% in all women above age 40 (~ 40,000 females in the Netherlands in 2008 – www.statlinecbs.nl), counselling of spontaneous POF patients with regard to their future health risks, as reviewed in Chapter 1, is mainly based on studies in iatrogenic POF. It is not clear if results from these studies may be extrapolated to spontaneous POF patients. Furthermore, we may question what percentage of the POF phenotype can be regarded as true menopause praecox, since it is possible that a subgroup of POF patients may be different to those in very early 'physiological' menopause.

A first exploration of cardiovascular risk parameters, namely lipid profiles, in POF patients is described in Chapter 4. We were surprised that we could not establish any clear correlation between the duration of oestrogen deprivation or level of serum E_2 levels and the severity of serum lipid deviations in POF patients. However, we were able to identify a relationship between androgens and triglycerides. In general, it can be concluded that lipid abnormalities in spontaneous POF are very modest, which is in contrast to studies performed in iatrogenic POF patients or in animal models[408-410]. From these first results, it does not seem necessary to measure lipid levels at a young age in POF patients. Age is probably a much greater risk factor than menopausal state. Future follow-up studies may yield more relevant information on cardiovascular risk factors in POF patients. Especially the role of androgens in relation to cardiovascular health in females needs to be clarified.

Nearly all the patients in our nationwide research project gave informed consent for future follow-up studies. Our current cohort of more than 300 POF patients and 600 hypergonadotropic non-POF patients (numbers in January 2009) will enable us to determine the true prevalence of auto-immune diseases, fragile X premutation, genetic aberra-

tions, familial distribution and hormonal therapy use in young hypergonadotropic patients. Such a large cohort will also make it possible to obtain longitudinal follow-up data on spontaneous fertility, outcome of fertility treatment, course of cycle disturbances after diagnosis, timeframe of transition from IOF to complete amenorrhea, psycho-sexual well-being, cognition, bone density, cardiovascular risk parameters and longevity.

Genetic studies in POF

As reviewed in Chapter 1 and 2, genetic factors play a major role in POF and age at menopause. Hence, POF may act as a genetic model for studying accelerated follicle pool depletion. Lessons learned from genetic research on the POF phenotype may lead to a better understanding of the genetic background of menopause in general.

In Chapter 5 we clearly demonstrated that carriership of the GALT mutations is unlikely to be a candidate gene for POF or menopause, and that the gonadal failure in galactosemia patients is therefore most likely to be due to direct toxic effects at the ovarian level.

By performing the first genome-wide association study in POF patients we were able to identify a common single nucleotide polymorphism (SNP) in the *ADAMTS19* gene. This was described in Chapter 6. Mouse cDNA subtraction experiments showed that *ADAMTS19* is significantly upregulated in XX gonads at the moment of sex differentiation, with abundant expression of *ADAMTS19* being noted during the embryonic phase in the gonads' development. Unfortunately, at the moment, there is no sufficiently large, independent, validation cohort available. In conclusion, our result in a relatively small cohort of POF patients (n=99), as well as the fact that the SNP is located in a biologically plausible gene, means our findings are exciting and hold promise for future genetic experiments and pathway analysis in POF.

After we had reviewed the literature on the huge number of microscopic X-chromosome aberrations that occur, it came as no surprise that we could identify multiple submicroscopic deletions or duplications (so-called copy number variants (CNVs)) on the X-chromosome in POF patients by using state-of-the-art techniques. Although the results of Chapter 7 are preliminary, they are promising and a proof-of-principle for the techniques used. Future studies will investigate the role of CNVs in the pathogenesis of common diseases, in general, and POF, in particular.

Future genetic studies in POF

High quality, well powered genetic studies for diseases with a relatively low prevalence like POF can only be performed if the following conditions can be met: 1) large, international consortia to obtain sufficiently large cohorts; 2) use of stringently defined and internationally accepted phenotypes; 3) with geneticists understanding the pathophysiology of the disease and appreciating the hurdles doctors encounter in obtaining DNA samples, and 4) with doctors having a better understanding of the genetics and the available techniques[411]. These kinds of studies are time-consuming and expensive, and require full support from medical doctors, geneticists and funding organizations, as well as from medical journals (e.g. multiple author papers), ethical committees (e.g. anonymous biobanking) and legislators (e.g. protection of identified findings).

Clinicians around the world have to deal daily with the problems of decreasing fertility potential with increasing age. Using whole-genome analysis,

it may be possible in the near future to identify genes involved in follicle pool depletion and menopausal age. It may even be feasible to design a genetic test for these two factors focusing on SNPs and CNVs in relevant genomic areas. Such a test could then predict at a young age whether a decrease in natural fertility might occur relatively early or late for any individual woman and this would help her in making important decisions concerning family planning or career opportunities. Such a test might also identify women with a high risk of suffering from early POF in the future, and these might eventually apply for ovarian cryopreservation or other fertility preservation techniques.

However, important ethical questions also remain. For example: does it do any good to provide women with a predictive test giving an outcome not as yes or no but as a statistical chance? How should we as doctors handle such risk estimations? Is commercial exploitation of genetic findings advisable? Who will be responsible for counselling women after taking such a test? What role will the insurance companies play?

Conclusions

In this thesis we have tried to define more specifically the phenotype of accelerated follicle depletion at a young age, a phenotype with tremendous negative impact on fertility and quality of life and that has not been well studied so far. In particular, AMH seems to be a promising useful marker to determine this phenotype more robustly. With regard to future cardiovascular disease risk, the lipid profiles of POF patients are not very different from control women.

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Summary

Chapter 1

We describe the phenotype of premature ovarian failure (POF). In particular, we focus on the impact of this diagnosis on women's health issues and the various definitions used for 'ovarian failure'. We have summarized the physiology of ovarian development and reviewed current knowledge on the aetiology and genetics of POF, including an overview of the majority of POF candidate genes that have been identified. We have also explained some of the genetic concepts and techniques used in this thesis.

Chapter 2

In the past few decades, postponement of childbearing has led to a decrease in family size and increased rates of age-related female subfertility. Reproductive ageing in the female is almost exclusively based on changes in the ovaries. Age-related decreases in follicle numbers and decay in oocyte quality influence the natural loss of fecundity and ultimately the start of menopause. The decline in fertility with age has been described in many natural and contemporary populations, as well as in assisted reproduction technology (ART) programs.

The rate of ovarian ageing is highly variable among women, so it is clinically relevant to be able to identify women who have severely decreased ovarian reserve for their age. Current tests relate mainly to the quantitative aspect of ovarian reserve, but the ability to predict the chances for pregnancy in ART are still very limited. As menopause and the preceding definitive loss of natural fertility are reproductive events with a fixed time interval, tests that predict age at menopause may be useful in assessing reproductive lifespan. In addition to hormonal and imaging tests for ovarian reserve, we may be able to identify genetic factors regulating the size of the follicle pool and the rate of its depletion and this will aid the accurate prediction of a woman's reproductive lifespan.

Chapter 3

Classically, ovarian dysfunction is categorized on the basis of cycle history and follicle-stimulating hormone (FSH) and oestradiol levels. Novel ovarian markers (anti-Müllerian hormone (AMH), inhibin B, and antral follicle count (AFC)) may provide more direct insight into follicle quantity in hypergonadotropic women. We wanted to investigate the distribution of these markers in young hypergonadotropic women compared to

normogonadotropic, regularly menstruating, women in a Dutch nationwide, prospective, cohort study.

Three ovarian failure phenotypes seen in women younger than 40 years of age were included: regular menstrual cycles and elevated FSH (incipient ovarian failure (IOF); n=68), oligomenorrhea and elevated FSH (transitional ovarian failure (TOF); n=79), or at least 4 months' amenorrhea together with FSH levels exceeding 40 IU/L (POF); n=112). Women with regular menses and normal FSH served as controls (n=83).

All POF patients showed AMH levels below the 5th percentile (p5) of normo-ovulatory women. Normal AMH levels (> p5) could be identified in 75% of IOF, in 33% of TOF patients, and in 98% of regular menstruating controls. AFC and AMH levels changed with increasing age (P<0.0001), whereas inhibin B remained constant (P=0.26). AMH levels were significantly different between TOF and IOF over the entire age range, whereas AFC became similar for TOF and IOF at higher ages.

Compared to inhibin B and AFC, AMH was more consistently correlated with the clinical degree of follicle pool depletion in young women presenting with elevated FSH levels. Thus AMH may provide a more accurate assessment of the follicle pool in young hypergonadotropic patients, especially in the clinically challenging subgroups of patients with elevated FSH and regular menses (i.e. IOF) and in hyper¬gonadotropic women with cycle disturbances but not fulfilling the POF diagnostic criteria (i.e. TOF).

Chapter 4

Early menopause is associated with a higher incidence of cardiovascular events later in life. Concurrent with the ages of menopausal transition, there is a shift in lipid profile. POF or premature menopause provides a route to studying the effect of cessation of ovarian function on lipid profile independent of the effects of advanced chronological age.

Fasting triglycerides (TG) and total-, HDL- and LDL-cholesterol levels were measured in 90 POF patients not using any hormone therapy and in 198 population controls not taking oral contraceptives. Furthermore we determined correlations between lipids and ovarian function parameters.

After correction for age, BMI and smoking, POF women presented with significantly higher TG levels (mean difference 0.17 LN-mmol/l [95%CI 0.06 - 0.29]). HDL-C levels were borderline significantly lower in POF patients. No age-corrected correlation between TG or other lipids and oestradiol levels or time of oestrogen deprivation could be identified. However, the free androgen index (FAI), SHBG and testosterone concentrations showed significant correlations with TG and/or HDL-C concentrations.

Loss of ovarian function at a very young age (POF) coincides with subtle changes in lipid profile (higher TG levels, marginal lower HDL). Androgens (increased FAI and T, and decreased SHBG) are better markers for unfavourable lipid changes than oestrogen levels or duration of oestrogen deprivation in POF patients. Elevated TG in combination with increased (free) androgens may be an early manifestation of reduced insulin sensitivity.

Chapter 5

Women with classical galactosemia (galactose-1-phosphate uridyltransferase (GALT) deficiency) frequently suffer from POF, despite treatment with a galactose-restricted diet. Earlier research has suggested an association between heterozygosity for GALT mutations and early menopause. We undertook a study to evaluate the effect of being a carrier for classical galactosemia on women's ovarian reserve and menopausal age.

Proven female carriers of classical galactosemia were recruited via the Dutch Galactosemia Society. All 58 participants underwent a structured interview regarding their fertility, smoking status and menopause. We determined ovarian reserve in 42 pre-menopausal GALT carriers, by measuring AFC by transvaginal ultrasound and sampling early follicular phase blood to measure FSH, inhibin B and AMH hormone levels. These ovarian reserve parameters were compared with a cohort of proven fertile women (n=166).

The mean age at menopause in GALT carriers was 49.7 years, which is no different to the mean age at menopause in the general female population in the Netherlands. There was no difference in FSH, inhibin B and AMH levels, nor in the AFC (when corrected for age and smoking status) between 42 pre-menopausal GALT carriers and population controls. We found no evidence that GALT mutation carriership affects ovarian reserve or menopausal age.

Summary

Chapter 6

Spontaneous POF occurs in 1% of women worldwide and has major implications for their fertility and health. Besides X chromosomal aberrations and fragile X premutations, no common genetic risk factors have been discovered so far in POF. Using high-density single nucleotide polymorphism (SNP) arrays, we set out to identify new genetic variants involved in this condition.

A genome-wide association study involving 309,158 SNPs was performed in 99 unrelated, idiopathic, Caucasian POF patients and 235 unrelated, healthy Caucasian female controls. We specifically focussed on chromosomal areas and candidate genes previously implicated in POF and performed a replication study on the most significant finding.

Suggestive genome-wide significant association was observed for rs246246 (allele frequency P=6.0 x 10-7), which mapped to an intron of *ADAMTS19*, a gene known to be upregulated in female mouse gonads during sexual differentiation. However, replication in an independent Dutch cohort (60 POF patients and 90 controls) did not confirm a clear association (P=4.1 x 10-5 in a joint analysis). We did not observe strong evidence for any of the 74 selected POF candidate genes or linkage regions being associated with idiopathic POF in Caucasian females, although suggestive association (P<0.005) was observed for SNPs that mapped to the *BDNF*, *CXCL12*, *LHR*, *USP9X* and *TAF4B* genes.

However, we did observe a possible association between POF and a SNP in a biologically plausible candidate gene, *ADAMTS19*. This finding warrants a follow-up study to investigate it as a possible candidate gene for POF.

Chapter 7

Besides SNPs, the human genome contains multiple copy number variants (CNVs), small, sub-microscopic deletions and duplications. POF is associated with macroscopic deletions, in particular on the Xq arm. These aberrations are detected by karyotyping. Using the intensities of high-density, genome-wide oligonucleotide arrays, it is now possible to obtain an increase in resolution of more than 100 times compared to conventional karyograms, and it is thus possible to detect CNVs.

Summary

We investigated the incidence, size and location of CNVs on the X chromosome in 108 Caucasian, spontaneous POF patients, with normal karyotypes and absence of fragile X premutation. We used PennCNV software. After quality control, 33 patients (31%) showed at least one CNV; sizes varied between 500 bp and 3 Mb (mean size 423 kb). On the long arm, 63% (29/46) of the CNVs mapped to locus q21 and of these 90% involved a deletion (26/29). At least one CNV could be detected in 31 genes. 3% to 15% of the POF patients showed a CNV in the *PCDH11X*, *TGIF2LX*, *P2RY8*, *ASMTL* or *ATRX* gene.

The current X chromosome CNV analysis in POF patients is a proof-of-principle; it shows that new genetic techniques might identify new factors that play a role in the aetiology of idiopathic POF. Our findings are preliminary although the abundant over-representation of CNVs on Xq21 further substantiates the important role of this locus in the genetics of POF.

Chapter 8

General discussion and future research concepts.

Nederlandse samenvatting

In **hoofdstuk 1** wordt het fenotype van prematuur ovarieel falen (POF) beschreven; het spontaan stoppen van de menstruaties (amenorrhoe) voor het 40e levensjaar in combinatie met FSH (follikel stimulerend hormoon) concentraties van meer dan 40 IU/L. Eenduidigheid in de nomenclatuur van het continuüm[xii] van ovarieel falen op jonge leeftijd ontbreekt.
In dit hoofdstuk geven we een samenvatting van de fysiologie van de ovariële follikel pool door het leven heen en de huidige beschikbare wetenschappelijke kennis over POF. De diagnose POF heeft een grote impact op het psychosociale welbevinden van patiënten. Naast onvruchtbaarheid zijn er talloze gezondheidsrisico's verbonden aan een vroege menopauzeleeftijd. We gaan meer uitgebreid in op de genetische mechanismen die wellicht een rol spelen bij POF en we geven een opsomming van de kandidaat-genen die tot op heden zijn geassocieerd met dit ziektebeeld. Tot slot leggen we in dit hoofdstuk een paar genetische basisconcepten en technieken uit die we hebben gebruikt voor de studies in dit proefschrift.

Hoofdstuk 2 beschrijft het verloop van de vruchtbaarheid van de vrouw gedurende haar leven in detail. Gezien het feit dat steeds meer vrouwen steeds later in het leven kinderen krijgen zien artsen steeds meer subfertiliteit die gerelateerd is aan de leeftijd van de vrouw.
De vruchtbaarheid van de vrouw wordt grotendeels bepaald door de eierstokfunctie. Vooral de afname van het aantal follikels / eicellen en de afname van de kwaliteit van de eicellen spelen hierbij een belangrijke rol.
De snelheid waarmee de eierstokken hun follikels verliezen varieert tussen vrouwen. Het identificeren van vrouwen die voor hun leeftijd een verminderde hoeveelheid eicellen hebben is voor de klinische praktijk belangrijk. Hiervoor zijn zogenaamde ovariële reserve testen ontwikkeld. Helaas zeggen deze testen weinig over de kwaliteit van de eicellen en zijn deze ook niet geschikt om kansen op zwangerschap te voorspellen. Uit de literatuur blijkt dat er tussen de leeftijd waarop een vrouw haar laatste kans op zwangerschap heeft en de leeftijd van haar laatste menstruatie (menopauze) een min-of-meer vast tijdsinterval zit. Het vooraf voorspellen van de menopauzeleeftijd van de vrouw zou dus wat kunnen zeggen over haar vruchtbaarheidsduur. Hormonale en genetische testen naar menopauzeleeftijd kunnen hieraan wellicht een belangrijke bijdrage leveren.

xiii De transitie van een regelmatige cyclus met een laag FSH naar afwezigheid van de menstruaties (amenorrhoe) en hoge FSH concentraties.

Nederlandse samenvatting

Ovariële reserve wordt vooralsnog gecategoriseerd op basis van het menstruatiepatroon en de waarde van de FSH concentratie. In **hoofdstuk 3** worden de waarden van nieuwe hormonale (anti-Müllerian hormoon (AMH) en inhibine B) en echografische (antrale follikel telling (AFC)) markers in vrouwen met een verhoogd FSH beschreven. We vergeleken deze waarden met de waarden van vrouwen met een regelmatige cyclus en normale FSH waarden.

Vrouwen bij wie het FSH verhoogd is kunnen in drie subgroepen (fenotypes) worden ingedeeld. Ten eerste de subgroep van vrouwen met een regelmatige cyclus en een verhoogd FSH, er is sprake van imminent ovarian failure (IOF) (n=68); ten tweede de vrouwen met een onregelmatige cyclus (oligomenorrhoe) en verhoogd FSH, wij benoemden deze groep transitional ovarian failure (TOF) (n=79); en als laatste vrouwen met tenminste 4 maanden amenorrhoe en een FSH waarde hoger dan 40 IU/L, deze worden in de literatuur benoemd als POF (n=112).Vrouwen met een regelmatige cyclus en bij wie de FSH concentratie normaal is fungeerden als controles (n=83). Alle vrouwen in deze studie waren jonger dan 40 jaar.

Zowel AMH, als Inhibine B en AFC laten een afname zien bij toename van de leeftijd in de gehele groep. In tegenstelling tot inhibine B (P=0.26) veranderden de AFC en AMH ($p<0.0001$) waarden voor alle vier de subgroepen met stijging van de leeftijd. Bij alle POF vrouwen bleek de AMH waarde lager dan de laagste 5e percentiel van onafhankelijke controlevrouwen uit Nederlandse populatie, gecorrigeerd voor leeftijd (P5). In geval van IOF was bij 75% van deze vrouwen de AMH waarde hoger dan de P5, en bij vrouwen met TOF was dit percentage 33%. Als AMH en AFC onderling werden vergeleken bleven de AMH waarden significant anders tussen de TOF en IOF subgroepen terwijl de AFC minder discriminerend werd bij de oudere TOF en IOF patiënten.

In onze studie lijkt AMH beter gecorreleerd met de follikelvoorraad in de vier klinische fenotypes gebaseerd op FSH en menstruatiepatroon dan inhibine B en AFC. Het lijkt er dan ook op dat AMH een goede inschatting kan geven van de grootte van de eicelvoorraad in de eierstokken bij vrouwen met een verhoogd FSH. Vooral voor de IOF en TOF patiënten is AMH mogelijk van toegevoegde waarde. Toekomstige studies moeten de voorspellende waarde van AMH met betrekking tot kans op (spontane) zwangerschap bij deze fenotypes verder bewijzen.

Menopauze op een jonge leeftijd lijkt gerelateerd aan een hogere kans op cardiovasculaire ziekten later in het leven. Rondom de menopauze veranderen bij vrouwen de lipidenwaarden (triglyceriden, totaal-, HDL- en LDL cholesterol).

Bij vrouwen met POF is er sprake van een vervroegde uitval van de ovariële functie op zeer jonge leeftijd. Men mag dus stellen dat we bij POF patiënten menopauze effecten kunnen onderzoeken waarbij de chronologische leeftijdscomponent in vasculaire veroudering een minder prominente rol speelt.

In **hoofdstuk 4** hebben we bij 90 POF patiënten de lipiden vergeleken met die van een controlegroep bestaande uit 198 vrouwen uit de Nederlandse populatie in dezelfde leeftijdgroep. Geen van de vrouwen in deze studie gebruikten exogene hormonen. Daarnaast probeerden we binnen de POF patiënten een correlatie te vinden tussen de lipidenwaarden en ovariële functie parameters.

Na statistische correctie voor leeftijd, Quetelet index en roken waren de triglyceriden waarden van POF patiënten significant hoger dan bij de controles (gemiddeld verschil 0.17 LN-mmol/L). Voor het HDL cholesterol was er een duidelijke trend waarneembaar (HDL lager bij POF patiënten). Er bleek geen verband te zijn tussen de waarden van de lipiden en het oestradiol en/of de duur dat de POF patiënten niet meer blootgesteld waren geweest aan endo- of exogene oestrogenen. Wel was sprake van een significant verband tussen de concentraties van triglyceriden of HDL-cholesterol en die van androgenen (vrije androgeen index, sex-hormoon bindend globuline en testosteron).

Wij kunnen concluderen dat bij POF patiënten de lipiden minimaal veranderen. Androgenen lijken betere voorspellers voor afwijkende lipidenwaarden dan oestradiol concentraties of duur van oestrogeendeprivatie. Verhoogde triglyceriden in combinatie met verhoogde vrije androgenen zouden een eerste aanwijzing kunnen zijn voor verminderde insulinegevoeligheid bij POF patiënten.

In **hoofdstuk 5** onderzochten we de rol van dragerschap van de klassieke galactosemie mutatie (GALT) in relatie tot ovariële reserve en menopauze leeftijd. Eerder wetenschappelijk onderzoek heeft een relatie beschreven tussen heterozygoot dragerschap en vervroegde overgang/verminderde vruchtbaarheid. Personen die homozygoot zijn voor deze mutatie (galactosemie patiënten) lijden in overgrote meerderheid aan POF. Via de Galactosemie Vereniging Nederland werden vrouwen met dragerschap gerekruteerd, voornamelijk moeders van klassieke galactosemie patiënten. Alle 58 vrouwen werden gevraagd naar hun vruchtbaarheid, menopauzeleeftijd en rookgedrag. Van de 42 vrouwen die nog pre-menopauzaal waren werd de ovariële reserve bepaald met behulp van FSH, AMH, inhibine B en AFC. De waarden van deze ovariële reservetesten werden vergeleken met een Nederlands populatiecohort vruchtbare vrouwen (n=166). De gemiddelde leeftijd van menopauze van de GALT mutatie draagsters was met 49.7 jaar niet statistisch significant verschillend van de menopauzeleeftijd in Ne-

derland. Ook de waarden van de hormonale en echografische ovariële reservetesten waren na correctie voor leeftijd en rookgedrag niet significant verschillend tussen GALT draagsters en controles. Er lijkt derhalve geen bewijs te zijn dat GALT dragerschap ovariële reserve of menopauzeleeftijd beïnvloedt.

In **hoofdstuk 6 en 7** hebben wij getracht genetische oorzaken bij POF te identificeren. Eén procent van de vrouwen lijdt aan POF. Behoudens karyogram afwijkingen op het (de lange arm van het) X chromosoom (deleties en translocaties) en fragiele X syndroom premutaties zijn er (nog) geen veel voorkomende genetische afwijkingen beschreven bij POF.

Met behulp van genoomwijde chips, waarmee duizenden single nucleotide polymorfismen (SNPs) worden getypeerd, is het mogelijk om genetische risicofactoren te identificeren bij POF patiënten. Wij voerden in **hoofdstuk 6** een genoomwijde associatie studie uit met behulp van 309.158 SNPs waarbij het gehele genoom van 99 Nederlandse POF patiënten werd vergeleken met dat van 235 Nederlandse controle vrouwen. Daarnaast richtten wij ons specifiek op de gebieden en kandidaat-genen die al eerder met POF zijn geassocieerd. Onze meest significante bevinding werd gerepliceerd in een onafhankelijk cohort.
Een zeer sterke associatie werd gevonden voor een SNP genaamd rs246246 op de lange arm van chromosoom 5. De allel frequentie P-waarde bedroeg 6.0 x 10-7 wat bijna genoomwijd significant is. Deze SNP ligt in een intron (een stuk DNA dat wordt afgelezen) van het gen *ADAMTS19*. Van dit gen is bekend dat het bij de muis sterk tot expressie komt tijdens de embryonale ontwikkeling van de gonaden in vrouwelijke richting. Echter, de replicatie studie (in 60 POF patiënten en 90 controles) liet geen sterke associatie zien waardoor de P-waarde na samenvoeging van beide associatie studies nog minder significant werd (P = 4.1 x 10-5).
Geen van de 74 onderzochte POF kandidaat-genen of eerder gerapporteerde koppelingsonderzoek regio's was sterk geassocieerd met POF. Een P-waarde van <0.005 werd gevonden voor de volgende vijf kandidaat-genen: BDNF, CXCL12, LHR, USP9X en TAF4B.
Ondanks bovenstaande bevindingen mag niet gesteld worden dat wij een genetische associatie hebben gevonden van *ADAMTS19* met het POF fenotype. Echter de identificatie van deze SNP in een biologisch plausibel POF gen rechtvaardigen wel een follow-up studie in een veel groter cohort POF patiënten.

Behoudens SNPs lijkt de variatie tussen mensen verklaard te worden door vele microdeleties en duplicaties in het genoom; zgn. copy number variants (CNVs). Door gebruik te maken van de intensiteiten van het DNA dat zich bindt aan de genoomwijde chips is het mogelijk om deze CNVs te identificeren. De resolutie ten opzichte van een conventioneel karyogram wordt hierdoor verhoogd met ongeveer een factor 100.
Omdat het X chromosoom een belangrijke rol lijkt te spelen bij de etiologie van POF onderzochten wij in **hoofdstuk 7** het voorkomen, de grootte en de ligging van CNVs op het X chromosoom bij deze patiënten. Hiervoor gebruikten wij PennCNV software. Alle 108 kaukasische POF patiënten in deze studie hadden een normaal karyogram en er was geen sprake van fragiele X premutatie dragerschap.
Na kwaliteitscontrole bleek 31% (33 van de 108) van de patiënten tenminste 1 CNV op het X chromosoom te herbergen met een grootte tussen de 500 bp en 3Mb (gemiddeld 423 kb). Op de lange arm van het X chromosoom bleek 63% (29 van de 46) van de CNVs zich te bevinden op het q21 locus. Hiervan was 90% (26/29) een deletie. In 31 X chromosoom genen werd tenminste één CNV gevonden. Drie tot vijftien procent van de POF patiënten had een CNV in één van de volgende genen: PCDH11X, TGIF2LX, P2RY8, ASMTL of ATRX.
Deze studie bewijst dat het mogelijk is om met behulp van nieuwe technieken de genetische achtergrond van POF te verduidelijken. De bevindingen van deze studie zijn nog onder voorbehoud maar de clustering van deleties op de lange arm van het chromosoom op locus q21 bevestigt de reeds eerder gerapporteerde potentiële rol van dit gebied in de etiologie van POF.

Hoofdstuk 8 is een algemene en maatschappelijke beschouwing van de bevindingen van de studies in dit proefschrift en geeft suggesties voor toekomstig onderzoek naar POF in het algemeen.

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Over de auteur

Erik Knauff werd geboren op 20 september 1974 in Zeist waar hij opgroeide als oudste in een gezin met vier kinderen. In 1994 behaalde hij zijn VWO examen aan het Christelijk Lyceum in Zeist. Na een jaar geneeskunde te hebben gestudeerd aan de Universiteit Gent werd Erik in 1995 wel ingeloot en kon hij zijn studie geneeskunde aan de Universiteit van Amsterdam vervolgen. Naast deze studie wist Erik voldoende tijd vrij te maken voor de sociale facetten van het studentenleven. Tijdens zijn studie deed hij een wetenschappelijke stage aan Imperial College in Londen waar zijn interesse voor basaal wetenschappelijk onderzoek werd gewekt. Vervolgens werd de keuze voor het vak gynaecologie en verloskunde tijdens de co-schappen definitief. Na het behalen van het arts-examen in 2003 werkte hij een jaar als arts-assistent-niet-in-opleiding in het Medisch Centrum Alkmaar (opleiders Dr. J.B. Maathuis / Dr. Y.M. van Kasteren). Daar werd zijn keuze voor het vakgebied bevestigd en werd hij ook geprikkeld tot het verrichten van wetenschappelijk onderzoek. Dit resulteerde in een onderzoeksplaats in de groep van Prof. dr. B.C.J.M. Fauser in het UMC Utrecht waar Erik tevens aan de basis stond van het COLA[xiv]-spreekuur. Naast zijn onderzoek startte Erik op 1 januari 2008 met de opleiding tot gynaecoloog in het Sint Elisabeth Ziekenhuis te Tilburg (opleider Dr. H.A.M. Vervest) en sinds september 2008 in het UMC Utrecht (opleider Prof. dr. G.H.A.Visser). Erik woont momenteel in Amsterdam samen met Klaartje.

Nicole Horrée & Marieke Verberg,
paranimfen

xiv COLA: cyclusstoornissen, oligomenorrhoe, amenorrhoe

309,158 letters from the DNA of Erik Knauff generated by the Illumina BeadChip 370k v.1.1

White Homozygous wild-type allele
100% red Heterozygous
50% red Homozygous rare allele

Dankwoord

Mijn dank gaat op de eerste plaats uit naar alle patiënten die bereid waren deel te nemen aan dit onderzoek.

Daar dit proefschrift zoveel verschillende vakgebieden omvat en door de (vaak geheel belangenloze) inzet van verschillende medewerkers en klinieken (zie blz. 133) tot stand is gekomen, is het vrijwel onmogelijk hier een ieder te noemen. Desalniettemin zijn al die mensen van groot belang geweest dit project tot een goed einde te brengen. U bent het DNA van dit proefschrift. Ik heb getracht u allen te vermelden op de achterkaft maar realiseer me op voorhand dat ik sommigen van u per ongeluk ben vergeten.

Enkele betrokkenen wil ik nog persoonlijk noemen:

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Lude, jonge doctor Franke. Zonder jou was er niets van hoofdstuk 6 en 7 terecht gekomen. Je bent een fantastischgeniaalmultitalent.

Frank, wat zou dit onderzoek zijn zonder *Dr. Ovarian Reserve and his fabulous controls*? Jouw bijdrage getuigde immer van een gedegen wetenschappelijke visie. Je was er steeds met briljante ingevingen en zinnig commentaar en dat altijd met een grote lach en opgewektheid.

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Erik,

Amsterdam, april 2009