THE ROLE OF ANTI-MÜLLERIAN HORMONE IN OVARIAN FUNCTION, DYSFUNCTION AND AGING

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The Role of Anti-Müllerian Hormone in Ovarian Function, Dysfunction and Aging

De rol van anti-Müllers hormoon in ovariële functie, disfunctie en veroudering

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CONTENTS

Chapter 1
General Introduction

1.1 INTRODUCTION

The female gonad, the ovary, is of major importance for both reproduction and the endocrine status of women. The ovary ensures the differentiation and release of the mature oocyte for fertilization and the production of sex steroid hormones. Sex steroid hormones allow the development of female secondary sexual characteristics and support pregnancy, but also have widespread biological effects beyond the reproductive system [1]. During reproductive life, ovarian aging results in a gradual decrease of fertility and eventually leads to cessation of ovarian function, which is marked by menopause and causes an almost complete absence of female sex steroid production by the ovaries [2]. The decline in sex steroid levels after the menopausal transition has a major impact on healthy aging of women and their quality of life. It results in an increased risk for several health problems, such as osteoporosis and cardiovascular disease. Hence, the risk for developing these diseases is higher in women with an early onset of menopause [3, 4]. On the other hand, the higher exposure to sex steroids before menopause increases the risk for developing breast cancer, resulting in an increased breast cancer risk in women with a later onset of menopause [5].

1.2 OVARIAN PHYSIOLOGY AND AGING

1.2.1 Primordial follicle recruitment and early follicular development

The onset of decreasing fertility and menopause is dictated by the quantity and quality of the primordial follicle pool [6]. In women and rodents, the primordial follicle pool is already established before birth. During early fetal life 1000-2000 primordial germ cells migrate from the allantois to the gonadal ridge to populate the ovary. These germ cells divide until the maximum number of about 6 to 7 million oocytes is reached at about 20 weeks of gestation. Subsequently, when a single layer of pregranulosa cells surrounds each oocyte, primordial follicle formation starts. Oocytes not surrounded by granulosa cells are lost, probably via apoptosis [1], resulting in a dramatic fall in oocyte numbers with only one million primordial follicles remaining at birth [2]. At menarche, only 300.000 follicles are left and during reproductive life, follicle depletion occurs bi-exponentially with a sharp increase after the age of 35 years [7, 8]. At the mean age of 50-51 years, when the primordial follicle pool is exhausted, menopause is reached [9, 10] (Figure 1.1).

After follicle formation, the gradual decrease in the number of primordial follicles is caused by continuing apoptosis of these follicles and recruitment

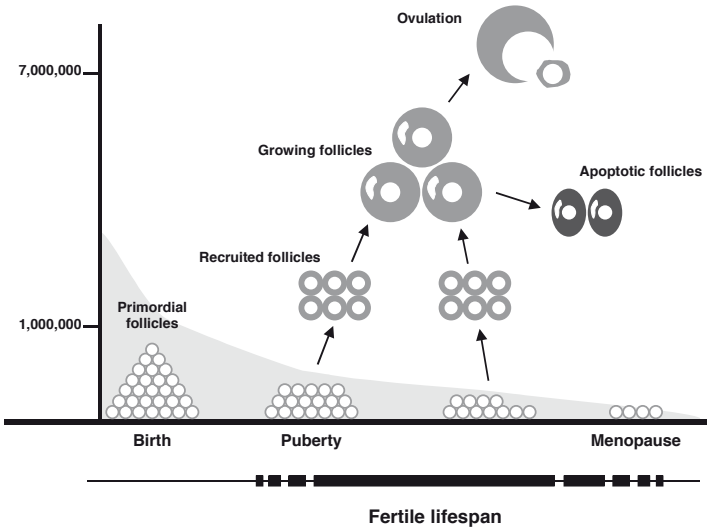

Figure 1.1 Life history of the primordial follicle pool

of follicles into the growing follicle pool, a process called initial recruitment [1]. Following initial recruitment, most growing follicles progress through primary and secondary stage to the antral stage, at which point they inevitably undergo atresia. After the onset of puberty, only a small number of antral follicles is rescued from atresia by gonadotropins to continue growth [1], a process which is called cyclic recruitment and will be discussed later in this chapter.

Initial recruitment is regulated by a decrease of inhibitory influences and/or an increase of stimulating factors that may allow the initiation of follicle growth. As follicle stimulating hormone (FSH) did not stimulate primordial follicle recruitment in *in vitro* experiments [11-13] and functional gonadotropin receptors are not yet expressed in primordial follicles, gonadotropins are not likely to play a role in this process [1, 14-16]. Hence, initial recruitment appears to be mainly regulated by intraovarian factors. These intraovarian factors also regulate progression through the following stages of follicle development and they exert their effect via bi-directional communication between the different celltypes of the follicle; the innermost oocyte, the surrounding granulosa cells and the outer layer of theca cells [17, 18] (Figure 1.2). Although several additional intraovarian factors, such as kit ligand, leukaemia inhibitory factor and fibroblast growth factor-2 and 7 have been shown to play a role in initial recruitment [18, 19], only members of the transforming growth factor-β (TGFβ) superfamily that are involved in this process will be discussed in more detail.

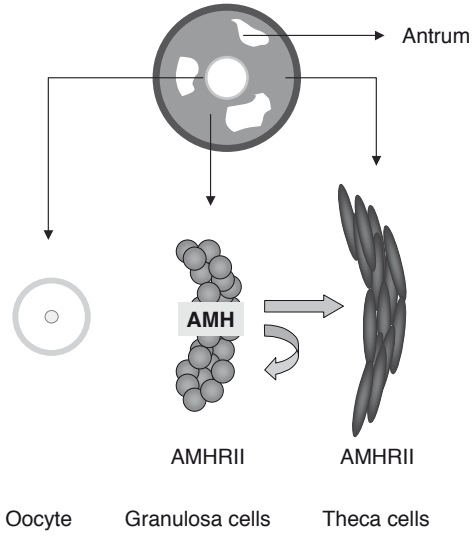

Figure 1.2 Structural organisation of an ovarian follicle

Role of the TGFβ superfamily in initial recruitment

The TGFβ superfamily, the largest family of growth and differentiation factors, is widely distributed throughout the body and involved in numerous physiological processes. This family can be divided into several subfamilies including the TGFβs, the bone morphogenetic proteins (BMPs), the growth and differentiation factors (GDFs), the activins/ inhibins and several additional members such as anti-Müllerian hormone (AMH) [18, 20, 21]. Although knowledge concerning the role of TGFβ family members in initial recruitment has emerged during the past decade, it should be said that most of this knowledge is obtained using rodent models and there is a relative paucity of data on other species including man [18]. This is in particular important because the effects of many TGFβ family members may differ between species.

BMP4 and BMP7 are TGFβ family members that are strongly expressed in the theca cells from primary follicles onwards in rats [22, 23]. These factors both signal via the BMP type II receptor (BMPRII), which is expressed in the granulosa cells and the oocytes [22, 23], and both stimulate primordial follicle recruitment. In vitro exposure of neonatal rat ovaries to BMP4 raised the proportion of developing primary follicles and lowered the number of primordial follicles [24]. Similarly, in vivo treatment of BMP7 in rats decreased the number of resting follicles, yet increased the number of primary, pre-antral and antral follicles [25].

GDF9 and BMP15 (also known as GDF9B), two very homologues members of the TGFβ family, may also have a role in initial recruitment. These factors are mainly expressed by oocytes from early-stage follicles onwards in rodents and

women [26-29]. The receptors through which these ligands can signal are expressed by the pregranulosa/granulosa cells of the corresponding early follicle stages [18, 23]. In vivo treatment of rats with GDF9 enhances the progression of primordial and primary follicles into small preantral follicles [13]. However, in mice deficient for GDF9, primordial follicles are activated to grow, demonstrating that GDF9 is not essential for initial follicle recruitment. Nevertheless, these mice are infertile and show arrested follicle development at the primary stage, indicating that GDF9 is required for proliferation and differentiation at subsequent follicular stages [30, 31]. Whether BMP15 regulates initial recruitment remains to be elucidated [19] but it is known that BMP15 promotes progression of folliculogenesis from the primary stage till the FSH-dependent stage [32]. Indeed, naturally occurring point mutations of the BMP15 (and GDF9) gene in sheep result in impaired follicular development beyond the primary stage and affect fertility profoundly [33, 34]. In addition, in women, mutations in the BMP15 (and GDF9) gene are associated with premature ovarian failure [35-39]. However, mice deficient for BMP15 have only minimal effects on follicle development and fertility [40], showing the species variation in the role of this factor.

Role of anti-Müllerian hormone in initial recruitment

Another member of the TGFβ family, which is involved in initial recruitment is anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS). The role of AMH signaling in follicle development will be the focus of this thesis and therefore, this hormone will be described in more detail.

Originally, AMH was identified because of its role in male sex differentiation. It is expressed in the Sertoli cells of the fetal testis and induces the regression of the Müllerian ducts, the anlagen of the female reproductive tract [41, 42]. During

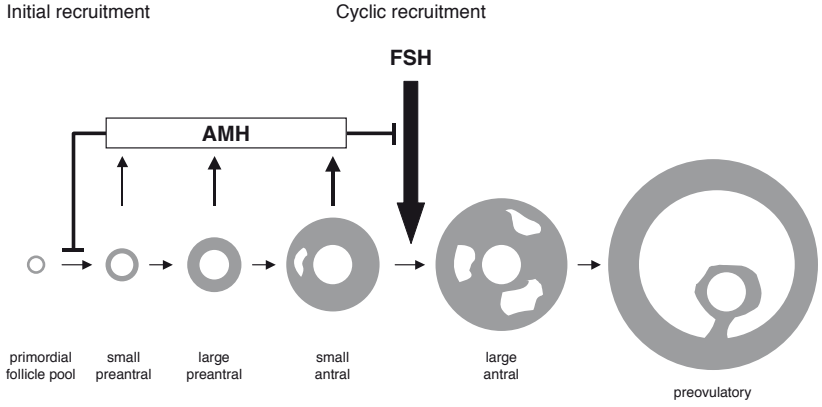

Figure 1.3 Stages of follicle development including AMH expression pattern and function in folliculogenesis

female sex differentiation AMH is not expressed in the ovary, but as from a few days after birth AMH is detected in the granulosa cells of rodent ovaries [43, 44]. In mice, AMH expression starts in the granulosa cells of primary follicles, is highest in granulosa cells of preantral and small antral follicles and gradually diminishes in the subsequent stages of follicle development [45] (Figure 1.3). The AMH type II receptor (AMHRII), which is specific and necessary for AMH signaling [46] (see paragraph 1.3), colocalizes with AMH in the granulosa cells of mouse and rat ovaries, showing a similar expression window [45, 47]. In addition, the AMHRII receptor is expressed in the theca cells of preantral and small antral follicles in rat ovaries [48] (Figure 1.2). The initiation of AMH and AMHRII expression as soon as primordial follicles are recruited for growth suggests that AMH may regulate this important step in follicle development. Indeed, studies of our group in AMH-deficient (AMHKO) mice demonstrated that AMH inhibits initial recruitment. Although these AMHKO mice are fertile, their ovaries are depleted earlier from their primordial follicles than control mice, as ovaries of adult and aged mice contain fewer primordial follicles than do ovaries of their wild-type littermates. Also, more preantral and small antral follicles are found in prepubertal and adult AMHKO mice. Therefore, the decrease in the primordial follicle pool is caused by increased recruitment of primordial follicles, indicating that in the absence of AMH, primordial follicles are recruited at a faster rate [49] (Figure 1.3).

The hypothesis that AMH regulates initial recruitment was also tested in an *in vitro* study, in which neonatal mouse ovaries were cultured in the presence or absence of AMH. AMH caused a 40-50% decrease in the number of growing follicles after 2 and 4 days of culture, showing that indeed, AMH inhibits initial follicle recruitment [44]. This inhibition is probably the result of a paracrine effect of AMH on the pre-granulosa cells that surround the oocyte in the primordial follicle. Indeed, the AMH type II receptor is present in ovaries of 2-day-old mice [44]. However, attempts to elucidate the specific expression pattern of the AMHRII in neonatal ovaries have not been successful yet.

In women, AMH has a similar expression pattern as in mice, with expression first appearing in primary follicles, being strongest in preantral and small antral follicles (≤ 4 mm) and gradually disappearing in larger antral follicles (4-8 mm) [50]. This specific expression pattern in the growing follicles is reflected by serum AMH levels, since these levels correlate strongly with the number of antral follicles detected by transvaginal ultrasound [51, 52]. As discussed above, the quantative aspect of ovarian aging is reflected by a decline in the size of the primordial follicle pool. Consequently, the number of growing follicles also decreases with aging [53]. Therefore, serum AMH levels probably not only reflect the growing follicle pool but also, indirectly, the primordial follicle pool. Indeed, AMH levels

decline with age and are undetectable in postmenopausal women [51, 54], making AMH an ideal marker for ovarian aging [55].

1.2.2 Cyclic recruitment and late follicular development

Before the onset of puberty, the normal fate of growing follicles is atretic demise. After puberty, growing follicles may be rescued by FSH, which is required for development beyond the early antral stage. As a result of the rise in FSH levels during the perimenstrual period, a cohort of antral follicles will be selected to continue growth, a process which is referred to as cyclic recruitment [1]. Among this cohort of selected follicles, only one follicle will gain dominance and ovulate (Figure 1.1). Although the exact reasons why one follicle emerges as dominant are unclear, this follicle is likely to be more sensitive to FSH than other antral follicles [1, 56]. Because FSH stimulates granulosa cell proliferation and antrum formation, this follicle will grow faster than the rest. However, FSH also stimulates luteinizing hormone (LH) receptor expression [57], inhibin B production [58], and induces aromatase activity in the granulosa cells, which converts the theca-cell derived substrate androstenedione into estradiol [57, 59, 60]. Therefore, the most FSH-sensitive, *i.e.* the dominant follicle, produces the highest levels of inhibin B and estradiol. Since inhibin B and estradiol both suppress pituitary FSH production, the formation of a dominant follicle indirectly results in a decline in serum FSH levels. Hence, the remaining growing follicles are deprived of adequate FSH stimulation required for survival and will become atretic [1]. Eventually, the rise in estradiol levels will induce an LH surge by the pituitary, resulting in the ovulation of the remaining dominant follicle.

Follicular development from the primary until the early antral stage is FSH-responsive whereas development beyond the antral stage is FSH-dependent [1]. However, the development of ovarian follicles is not regulated by FSH alone. Intraovarian factors play an important role in the determination of the individual FSH threshold of each follicle and hence, in cyclic recruitment. Similar to the factors involved in initial recruitment, these factors exert their effect via autocrine and paracrine communication in the ovary. Also, many of the factors involved in cyclic recruitment are members of the TGFβ family and their effects, in particular those with respect to steroidogenesis, will be described in this chapter.

Role of the TGFβ superfamily in cyclic recruitment

GDF9, BMP15 and BMP6, the TGFβ family members expressed in the oocyte, appear to attenuate FSH action, as has been demonstrated in rodent models. BMP15 inhibits FSH receptor expression and thereby FSH-induced steroidogenesis in rat granulosa cells [61, 62]. BMP6 also inhibits FSH signaling in rats but

downstream of the receptor by suppressing adenylate cyclase activity [63]. GDF9 suppresses FSH action by inhibition of FSH-dependent LH receptor expression and cAMP production [64]. BMP15, BMP6 and GDF9 all suppress FSH induced progesterone secretion but only GDF9 inhibits FSH-induced aromatase expression and estradiol production in rat granulosa cells [18, 63].

The theca-cell-derived BMP4 and BMP7 both attenuate FSH-stimulated progesterone and enhance FSH-induced estradiol production in rats [22, 25]. However, species differences are evident as in bovine granulosa cells, BMP4 and BMP7 do not stimulate FSH-induced estradiol secretion and even suppress progesterone secretion [65]. In addition, the granulosa-cell-derived BMP2 was shown to promote estradiol production from cultured ovine granulosa cells [66], whereas it reduced FSH-stimulated progesterone production in porcine cells [67].

Also, the TGFβ subfamily may have a role in cyclic recruitment. Three isoforms of the TGFβ subfamily have been shown to be produced by the ovary; TGFβ1, TGFβ2 and TGFβ3, which appear to induce similar effects [68] and therefore, will be described together. Nevertheless, the expression pattern and function of TGFβ seem to be highly species-dependent. In rodents and humans, expression is first detected in the granulosa and theca cells of preantral follicles and intensifies throughout subsequent stages of follicular development [68-71]. In rat granulosa cells TGFβ can stimulate FSH receptor expression [72] and amplify FSH-induced LH receptor expression, aromatase activity and estradiol, progesterone and inhibin production [73-75]. However, no effect of TGFβ was observed on aromatase expression in human granulosa cells [76]. In addition, in sheep and cows, the effects of TGFβ on steroidogenesis appear to be only mildly stimulatory or even inhibitory [68, 77].

Furthermore, inhibin and activin, also members of the TGFβ family, have been proposed to modulate FSH-action. Besides the regulatory effect of in particular inhibin on the FSH production of the pituitary, these hormones may have a local autocrine-paracrine effect in the ovary [78]. Inhibins are dimers of a unique α subunit linked to either a βA or βB subunit to generate inhibin A (α-βA) or inhibin B (α-βB). Activin arises from dimerisation of β subunits alone, resulting in three isoforms of activin, referred to as activin A (βA-βA), activin AB (βA-βB) and activin B (βB-βB) [78]. As many studies do not discriminate between the different activin isoforms, they will be described together. Activins are produced by granulosa cells from an early stage of follicle development onwards of the human and rat ovary [18, 79, 80]. In rat granulosa cells, it has been shown that activin stimulates FSH and LH receptor expression [81]. Furthermore, in rat, bovine and marmoset granulosa cells activin stimulates FSH-induced aromatase activity and estradiol production, regardless of the developmental stage. However, activin

promotes FSH-stimulated progesterone production in undifferentiated granulosa cells, whereas it suppresses this effect in partially differentiated granulosa cells [80-83]. The response of activin is under control of the activin-binding protein follistatin, which reverses its action.

Inhibin B is predominantly secreted by granulosa cells of the pre-antral and small antral follicles of the human ovary whereas inhibin A is predominantly secreted by granulosa cells of the preovulatory and dominant follicle [84-86]. In contrast to the endocrine role of inhibin, little is known about the autocrine role of inhibin A and B in the human ovary. In general, inhibin acts by antagonizing the effect of activin [87] and therefore, the intrafollicular balance between inhibin and activin is important in modulating FSH action [18]. Inhibin A may also independently affect FSH-induced estradiol secretion but data supporting this are rather inconsistent [18].

Role of AMH in cyclic recruitment

Last but not least, AMH is an important factor in fine-tuning the sensitivity of growing follicles to FSH. Despite a lower serum FSH concentration, ovaries of adult AMHKO mice contain more growing follicles than do ovaries of their wild-type littermates [49], indicating that AMH may inhibit FSH-induced follicle growth. This hypothesis was confirmed in cultures of mouse preantral follicles *in vitro*. AMH inhibited the FSH-dependent follicle growth in a time-dependent manner, as follicles cultured in the presence of AMH and FSH had a smaller diameter than follicles cultured with FSH alone [88]. This effect of AMH was mainly the result of reduced granulosa cell proliferation and is consistent with another *in vitro* study, in which was shown that exogenous AMH inhibited FSH-induced aromatase activity and LH receptor expression in cultured granulosa cells [89]. In contrast, in *in vitro* cultured rat follicles a stimulatory effect of AMH on FSH-induced follicle growth was found [90], although this may be explained by species variation and differences in culture conditions (presence/absence of serum and thus other growth factors) [45]. The effect of AMH on FSH-sensitivity of follicles was further tested *in vivo* by comparing the follicle dynamics of wild-type and AMHKO mice in the presence of low (after gonadotropin releasing hormone antagonist treatment) and high serum FSH concentrations. This study showed that irrespective of the FSH levels (high or low), more growing follicles were found in AMHKO than in wild-type animals, again indicating that AMH inhibits FSH sensitivity of the follicles [88]. Nevertheless, the higher number of growing follicles in AMHKO ovaries does not lead to a change in the number of preovulatory follicles because of an increased loss of follicles during the transition from the small preantral to the large preantral stage [91], indicating that there is no differ-

ence in the FSH sensitivity of large growing follicles between these animals. This may be explained by the specific window of expression of the AMHRII [91]. Since this receptor is not expressed in the large antral follicles, AMH signaling cannot influence the FSH threshold at this follicular stage. Hence, these mice studies demonstrate that AMH inhibits FSH sensitivity in small antral follicles but not in large antral follicles, thereby attenuating the FSH threshold for cyclic recruitment but not for selection of dominance (Figure 1.3).

1.3 AMH SIGNALING PATHWAY

Members of the TGFβ family signal through a serine/threonine kinase receptor complex consisting of ligand-specific type II receptors and more general type I receptors, also known as activin receptor-like protein kinases (ALKs). An activated receptor complex phosphorylates and activates cytoplasmic Smad proteins that translocate to the nucleus and directly or indirectly affect gene expression. Although all TGFβ family members share this molecular mechanism, two distinct modes of signaling exist, one represented by the TGFβs and activins and the other by the BMPs, GDFs and AMH [92]. Since AMH is the focus of this thesis, only AMH signaling will be discussed in detail.

For AMH one type II receptor has been identified (AMHRII) [93, 94] and shown to be specific and necessary for AMH signaling [46]. ALK2, ALK3 and ALK6, three

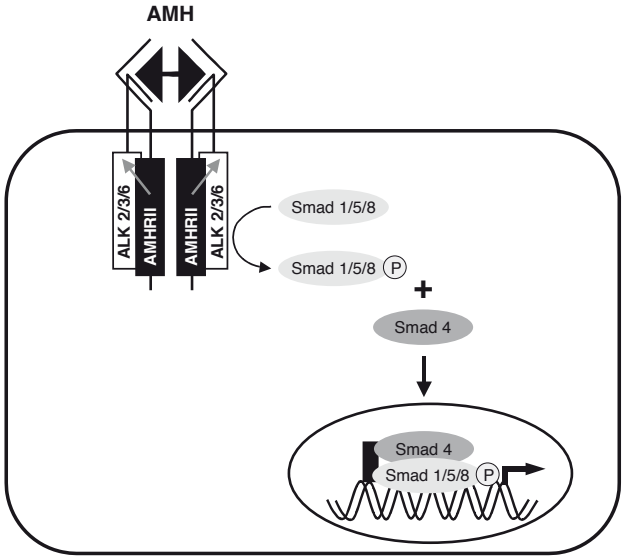

Figure 1.4 AMH signaling pathway

BMP type I receptors that also mediate the effect of other BMP ligands, have been identified as candidate AMH type I receptors [95-97] (Figure 1.4). The involvement of ALK2 in AMH signaling has been shown in the Müllerian ducts using an antisense approach. Thus, in an *in vitro* Müllerian duct regression assay, treatment with ALK2 oligonucleotides inhibited AMH-induced regression, whereas treatment with control or antisense ALK6 oligonucleotides did not affect regression [96]. The involvement of ALK2 in AMH signaling was also confirmed in cell lines [96, 97]. With respect to ALK6, a ligand-dependent interaction between this receptor and AMHRII has been shown *in vitro* [95]. However, ALK6 null mice do not display abnormalities in Müllerian duct regression [97], suggesting that ALK6 is not essential in the latter process but may be an AMH type I receptor involved in gonadal functions. In contrast, targeted disruption of ALK3 in the Müllerian ducts of male mice results in retention of the ducts, indicating that ALK3 is necessary for AMH signaling in the Müllerian ducts [98]. Nevertheless, as suggested by the antisense ALK2 experiments, ALK2 also appears to be important in AMH-mediated Müllerian duct regression [96]. A role for both type I receptors in this process could be explained through the formation of a heteromeric ALK2/ALK3 receptor complex, in which one type I receptor cannot signal in the absence of the other [99]. Alternatively, ALK2 and ALK3 may act sequentially as type I receptors for AMH signaling in the Müllerian ducts, as indicated by the spatiotemporal expression patterns of both receptors [100].

Hence, the use of ALK2, ALK3, and also ALK6, or a combination of each, in AMH signaling may be tissue- and/or cell-specific and little is known about the contribution of these receptors to AMH signaling in the ovary. So far, only the expression pattern of these receptors in the ovary is studied. Our preliminary experiments showed that ALK2 is expressed in the granulosa cells of small growing follicles [101]. ALK3 and ALK6 are expressed in granulosa cells and oocytes in the mouse ovary, but mostly in antral follicles [23, 102].

Similar to other members of the BMP family, downstream signaling of AMH is mediated through Smad1, Smad5 and Smad8 [95, 96, 101]. Upon activation of these receptor-specific Smads, a common complex with Smad4 is formed, which translocates to the nucleus and regulates gene transcription (Figure 1.4). For AMH, only a few target genes have been recognized. In the Müllerian ducts, matrix metalloproteinase 2 (MMP2), an enzyme involved in the degradation of extracellular matrix, was identified as an AMH-target gene [103], whereas in the ovary and testis, AMH downregulates the expression of the LH receptor, aromatase and certain steroidogenic enzymes [89, 104, 105].

1.4 GENETICS OF OVARIAN DYSREGULATION

As described in paragraph 1.2, initial and cyclic recruitment are key regulatory processes in folliculogenesis. Disturbance of these processes may result in failure of ovarian function and infertility. For example, premature ovarian failure (POF), which is characterized by the onset of menopause before the age of 40 years [106], may be caused by disordered establishment (gonadal dysgenesis) or excessive wastage of the primordial follicle pool [107]. Hence, amongst others, factors that regulate initial recruitment and thus, the usage of the primordial follicle pool may be involved in the pathophysiology of this disorder. For example, genetic variations in the TGFβ family members BMP15, GDF9 and Inhibin α have been shown to be associated with POF [35, 36, 38, 39, 108-111]. Under normal physiological conditions, the onset of menopause varies widely between women (range 40-60 years), indicating a large inter-individual variation in the efficiency of the usage of the ovarian follicle pool [2]. The wide range in age at menopause has several consequences for health and quality of life in individual women, as is discussed in paragraph 1.1.

The disturbance of cyclic recruitment and/or dominant follicle selection is the main feature of a very common disorder in women of reproductive age that causes infertility: Polycystic Ovary Syndrome (PCOS). According to the Rotterdam 2003 consensus, PCOS is characterized by at least two out of the three following criteria: oligo- or anovulation, hyperandrogenism or polycystic ovaries [112]. Women with polycystic ovaries display multiple follicles on transvaginal ultrasound. Despite normal serum FSH levels [113], none of these follicles become dominant and ovulate, resulting in oligo- or anovulation. This indicates that in these women, the individual FSH setpoint of these follicles is disturbed, resulting in aberrant follicle selection. Indeed, in most of these women ovulation can be induced by exogenous administration of high doses FSH [114, 115]. Intraovarian factors produced by growing follicles, such as AMH, may be involved in the regulation of FSH sensitivity and thereby, the aberrant follicle selection. Interestingly, PCOS women display a two to threefold increase in serum AMH levels compared to normo-ovulatory women [116, 117].

Complex traits and genetic association studies

Both age at menopause and PCOS are complex traits with a strong genetic component [118-121]. The etiology of complex traits is determined by multiple environmental and genetic factors. To identify the genes involved in these complex traits a candidate gene approach is often used. This implies that within a candidate gene, polymorphisms are identified and tested for association with

the phenotype of interest [122]. Gene polymorphisms are DNA sequence variants that have a minor allele frequency of more than 1% in the population. About 90% of the polymorphisms are Single Nucleotide Polymorphisms (SNPs), which represent variation in a single base. SNPs occur in the order of 1/1000 basepairs [123, 124] and across the whole genome there could be about 10 million common SNPs [125]. Gene polymorphisms resulting in an amino acid change are most likely to have a functional effect on the bioactivity of the protein and therefore, these polymorphisms are preferentially selected for genetic association studies [123, 124]. However, the functional importance of a SNP can also result from an effect on specific sites in noncoding DNA (e.g. regulatory sites) or RNA structures [126, 127].

Nevertheless, an observed genotype-phenotype relationship does not automatically indicate that the investigated polymorphism is functional. The association may also be driven by another SNP, which is located in proximity on the chromosome. The existence of association between two SNPs, which arises because of a shared population ancestry, is termed Linkage Disequilibrium (LD) [128, 129]. LD is quantified by the pair-wise linkage disequilibrium coefficient (D') and the correlation coefficient (r^2) [122]. The combination of polymorphic alleles at different loci along a single chromosome is called a haplotype [122, 123].

Until a few years ago, to identify polymorphisms in a candidate gene, sequencing of this gene was necessary. However, recently, the genetic variation of the complete human genome has been identified by the HapMap consortium [125, 130], allowing selection of SNPs in the gene of interest from publicly available data. In addition, this consortium has provided methods for estimation of the LD between SNPs and the haplotype structure of the genes. Using this information, it has now become relatively easy to cover the complete genetic variation of a candidate gene by the selection of so called 'tagging' SNPs [122].

1.5 AIMS AND SCOPE OF THIS THESIS

In this thesis, the role of AMH signaling in ovarian physiology, pathophysiology and aging will be described. Previous studies of our group have shown that AMH inhibits initial and cyclic recruitment in the mouse ovary. In Chapter 2 of this thesis we extended upon these studies and investigated the exact role of the AMH type II receptor in ovarian physiology and aging using a knockout mice model for this receptor. In Chapter 3 we examined whether AMH is a useful marker for ovarian reserve, reflecting the quantity of the primordial follicle pool. As the number of primordial follicles cannot be determined in women,

we studied the correlation of AMH levels with the size of the growing and the primordial follicle pool in mice.

Little is known about the role of AMH in the human ovary. Based on the similar expression pattern, we hypothesized that in women, AMH has a similar role in ovarian function as in mice. We investigated whether AMH regulates the usage of the primordial follicle pool and the sensitivity of the ovary to FSH in women using a candidate gene approach. Of the genes involved in the AMH signaling pathway, we have focussed on AMH, AMHR2 and ACVR1 (which encodes the protein ALK2). In Chapter 4 polymorphisms in the AMH and AMHR2 gene were identified. Subsequently, the association of these polymorphisms with FSH-sensitivity, reflected by estradiol levels, was investigated in two groups of normo-ovulatory women. In Chapter 5 these polymorphisms were studied in relation to parameters of ovarian aging, *i.e.* age at menopause, in two cohorts of postmenopausal women. In Chapter 6 the genetic contribution of the AMH and AMHR2 gene to the etiology of PCOS is discussed. Since PCOS women have elevated serum AMH levels, AMH may play a role in the disturbed FSH sensitivity of this syndrome. In addition, the possible functional significance of the studied polymorphism in the AMH gene was investigated. In Chapter 7 the genetic variation in one of the AMH type I receptors, ALK2 (encoded by the gene ACVR1), was characterized using a tagging SNP approach and investigated in normo-ovulatory and PCOS women. Finally, in the general discussion, the findings of this thesis are discussed in a general perspective and some directions for future research are given.

REFERENCES

1. **McGee EA, Hsueh AJ** 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-14
2. **te Velde ER, Scheffer GJ, Dorland M, Broekmans FJM, Fauser BCJM** 1998 Developmental and endocrine aspects of normal ovarian aging. Mol Cell Endocrinol 145:67-73
3. **Kritz-Silverstein D, Barrett-Connor E** 1993 Early menopause, number of reproductive years, and bone mineral density in postmenopausal women. Am J Public Health 83:983-8
4. **van der Schouw YT, van der Graaf Y, Steyerberg EW, Eijkemans JC, Banga JD** 1996 Age at menopause as a risk factor for cardiovascular mortality. Lancet 347:714-8
5. **Collaborative Group on Hormonal Factors in Breast Cancer** 1997 Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Lancet 350:1047-59
6. **te Velde ER, Pearson PL** 2002 The variability of female reproductive ageing. Hum Reprod Update 8:141-54
7. **Macklon NS, Fauser BC** 1999 Aspects of ovarian follicle development throughout life. Horm Res 52:161-70
8. **Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF** 1992 Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod 7:1342-6
9. **te Velde ER, Dorland M, Broekmans FJ** 1998 Age at menopause as a marker of reproductive ageing. Maturitas 30:119-25
10. **Treloar AE** 1981 Menstrual cyclicity and the pre-menopause. Maturitas 3:249-64
11. **Eppig JJ, O'Brien MJ** 1996 Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 54:197-207
12. **Mayerhofer A, Dissen GA, Costa ME, Ojeda SR** 1997 A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. Endocrinology 138:3320-9
13. **Vitt UA, McGee EA, Hayashi M, Hsueh AJ** 2000 In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. Endocrinology 141:3814-20
14. **Oktay K, Briggs D, Gosden RG** 1997 Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. J Clin Endocrinol Metab 82:3748-51
15. **O'Shaughnessy PJ, McLelland D, McBride MW** 1997 Regulation of luteinizing hormone-receptor and follicle-stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. Biol Reprod 57:602-8
16. **Rannikki AS, Zhang FP, Huhtaniemi IT** 1995 Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. Mol Cell Endocrinol 107:199-208
17. **Matzuk MM, Burns KH, Viveiros MM, Eppig JJ** 2002 Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178-80
18. **Knight PG, Glister C** 2006 TGF-beta superfamily members and ovarian follicle development. Reproduction 132:191-206
19. **Skinner MK** 2005 Regulation of primordial follicle assembly and development. Hum Reprod Update 11:461-71

20. **Piek E, Heldin CH, Ten Dijke P** 1999 Specificity, diversity, and regulation in TGF-beta superfamily signaling. Faseb J 13:2105-24
21. **Massagué J** 1998 TGF-beta signal transduction. Annu Rev Biochem 67:753-91
22. **Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ, Erickson GF** 1999 A functional bone morphogenetic protein system in the ovary. Proc Natl Acad Sci U S A 96:7282-7
23. **Erickson GF, Shimasaki S** 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod Biol Endocrinol 1:9
24. **Nilsson EE, Skinner MK** 2003 Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 69:1265-72
25. **Lee WS, Otsuka F, Moore RK, Shimasaki S** 2001 Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. Biol Reprod 65:994-9
26. **Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E, Heikinheimo M, Ritvos O** 1998 A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. Mech Dev 78:135-40
27. **Jaatinen R, Laitinen MP, Vuojolainen K, Aaltonen J, Louhio H, Heikinheimo K, Lehtonen E, Ritvos O** 1999 Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B. Mol Cell Endocrinol 156:189-93
28. **Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L, Ritvos O** 1999 Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab 84:2744-50
29. **Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM** 1998 The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol 12:1809-17
30. **Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM** 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383:531-5
31. **Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF** 1998 Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 204:373-84
32. **Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S** 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. J Biol Chem 275:39523-8
33. **Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O** 2000 Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 25:279-83
34. **Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM** 2004 Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). Biol Reprod 70:900-9
35. **Di Pasquale E, Beck-Peccoz P, Persani L** 2004 Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet 75:106-11

36. **Dixit H, Rao LK, Padmalatha V, Kanakavalli M, Deenadayal M, Gupta N, Chakravarty B, Singh L** 2005 Mutational screening of the coding region of growth differentiation factor 9 gene in Indian women with ovarian failure. Menopause 12:749-54
37. **Dixit H, Rao LK, Padmalatha VV, Kanakavalli M, Deenadayal M, Gupta N, Chakrabarty B, Singh L** 2006 Missense mutations in the BMP15 gene are associated with ovarian failure. Hum Genet 119:408-15
38. **Laissue P, Christin-Maitre S, Touraine P, Kuttenn F, Ritvos O, Aittomaki K, Bourcigaux N, Jacquesson L, Bouchard P, Frydman R, Dewailly D, Reyss AC, Jeffery L, Bachelot A, Massin N, Fellous M, Veitia RA** 2006 Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure. Eur J Endocrinol 154:739-44
39. **Di Pasquale E, Rossetti R, Marozzi A, Bodega B, Borgato S, Cavallo L, Einaudi S, Radetti G, Russo G, Sacco M, Wasniewska M, Cole T, Beck-Peccoz P, Nelson LM, Persani L** 2006 Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. J Clin Endocrinol Metab 91:1976-9
40. **Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM** 2001 Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15:854-66
41. **Josso N, Cate RL, Picard JY, Vigier B, di Clemente N, Wilson C, Imbeaud S, Pepinsky RB, Guerrier D, Boussin L, Legeai L, Carre-Eusebe D** 1993 Anti-mullerian hormone: the Jost factor. Recent Prog Horm Res 48:1-59
42. **Lee MM, Donahoe PK** 1993 Mullerian inhibiting substance: a gonadal hormone with multiple functions. Endocr Rev 14:152-64
43. **Ueno S, Kuroda T, Maclaughlin DT, Ragin RC, Manganaro TF, Donahoe PK** 1989 Mullerian inhibiting substance in the adult rat ovary during various stages of the estrous cycle. Endocrinology 125:1060-6
44. **Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 2002 Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076-84
45. **Durlinger ALL, Visser JA, Themmen APN** 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124:601-9
46. **Mishina Y, Rey R, Finegold MJ, Matzuk MM, Josso N, Cate RL, Behringer RR** 1996 Genetic analysis of the Mullerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. Genes Dev 10:2577-87
47. **Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen APN, Grootegoed JA** 1995 Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951-62
48. **Ingraham HA, Hirokawa Y, Roberts LM, Mellon SH, McGee E, Nachtigal MW, Visser JA** 2000 Autocrine and paracrine Mullerian inhibiting substance hormone signaling in reproduction. Recent Prog Horm Res 55:53-67; discussion 67-8
49. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-96
50. **Weenen C, Laven JSE, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BCJM, Themmen APN** 2004 Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 10:77-83

51. **de Vet A, Laven JSE, de Jong FH, Themmen APN, Fauser BCJM** 2002 Antimullerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril 77:357-62
52. **van Rooij IAJ, Broekmans FJM, te Velde ER, Fauser BCJM, Bancsi LF, de Jong FH, Themmen APN** 2002 Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod 17:3065-71
53. **Scheffer GJ, Broekmans FJM, Dorland M, Habbema JDF, Looman CWN, te Velde ER** 1999 Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. Fertil Steril 72:845-51
54. **van Rooij IA, Tonkelaar I, Broekmans FJ, Looman CW, Scheffer GJ, de Jong FH, Themmen APN, te Velde ER** 2004 Anti-mullerian hormone is a promising predictor for the occurrence of the menopausal transition. Menopause 11:601-6
55. **Visser JA, de Jong FH, Laven JSE, Themmen APN** 2006 Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 131:1-9
56. **Fauser BCJM, Van Heusden AM** 1997 Manipulation of human ovarian function: physiological concepts and clinical consequences. Endocr Rev 18:71-106
57. **Richards JS, Ireland JJ, Rao MC, Bernath GA, Midgley AR, Jr., Reichert LE, Jr.** 1976 Ovarian follicular development in the rat: hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone. Endocrinology 99:1562-70
58. **Lee VW, McMaster J, Quigg H, Leversha L** 1982 Ovarian and circulating inhibin levels in immature female rats treated with gonadotropin and after castration. Endocrinology 111:1849-54
59. **Hillier SG, Reichert LE, Jr., Van Hall EV** 1981 Control of preovulatory follicular estrogen biosynthesis in the human ovary. J Clin Endocrinol Metab 52:847-56
60. **Short RV** 1962 Steroids in the follicular fluid and the corpus luteum of the mare. A 'two-cell type' theory of ovarian steroid synthesis. J Endocrinol 24:59-63
61. **Otsuka F, Yamamoto S, Erickson GF, Shimasaki S** 2001 Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem 276:11387-92
62. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72-101
63. **Otsuka F, Moore RK, Shimasaki S** 2001 Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J Biol Chem 276:32889-95
64. **Vitt UA, Hayashi M, Klein C, Hsueh AJ** 2000 Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol Reprod 62:370-7
65. **Glister C, Kemp CF, Knight PG** 2004 Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. Reproduction 127:239-54
66. **Souza CJ, Campbell BK, McNeilly AS, Baird DT** 2002 Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. Reproduction 123:363-9
67. **Brankin V, Quinn RL, Webb R, Hunter MG** 2005 Evidence for a functional bone morphogenetic protein (BMP) system in the porcine ovary. Domest Anim Endocrinol 28:367-79
68. **Juengel JL, McNatty KP** 2005 The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum Reprod Update 11:143-60

69. **Chegini N, Flanders KC** 1992 Presence of transforming growth factor-beta and their selective cellular localization in human ovarian tissue of various reproductive stages. Endocrinology 130:1707-15
70. **Teerds KJ, Dorrington JH** 1992 Immunohistochemical localization of transforming growth factor-beta 1 and -beta 2 during follicular development in the adult rat ovary. Mol Cell Endocrinol 84:R7-13
71. **Schmid P, Cox D, van der Putten H, McMaster GK, Bilbe G** 1994 Expression of TGF-beta s and TGF-beta type II receptor mRNAs in mouse folliculogenesis: stored maternal TGF-beta 2 message in oocytes. Biochem Biophys Res Commun 201:649-56
72. **Dunkel L, Tilly JL, Shikone T, Nishimori K, Hsueh AJ** 1994 Follicle-stimulating hormone receptor expression in the rat ovary: increases during prepubertal development and regulation by the opposing actions of transforming growth factors beta and alpha. Biol Reprod 50:940-8
73. **Adashi EY, Resnick CE, Hernandez ER, May JV, Purchio AF, Twardzik DR** 1989 Ovarian transforming growth factor-beta (TGF beta): cellular site(s), and mechanism(s) of action. Mol Cell Endocrinol 61:247-56
74. **Zachow RJ, Weitsman SR, Magoffin DA** 1999 Leptin impairs the synergistic stimulation by transforming growth factor-beta of follicle-stimulating hormone-dependent aromatase activity and messenger ribonucleic acid expression in rat ovarian granulosa cells. Biol Reprod 61:1104-9
75. **Zhang ZW, Findlay JK, Carson RS, Herington AC, Burger HG** 1988 Transforming growth factor beta enhances basal and FSH-stimulated inhibin production by rat granulosa cells in vitro. Mol Cell Endocrinol 58:161-6
76. **McAllister JM, Byrd W, Simpson ER** 1994 The effects of growth factors and phorbol esters on steroid biosynthesis in isolated human theca interna and granulosa-lutein cells in long term culture. J Clin Endocrinol Metab 79:106-12
77. **Juengel JL, Bibby AH, Reader KL, Lun S, Quirke LD, Haydon LJ, McNatty KP** 2004 The role of transforming growth factor-beta (TGF-beta) during ovarian follicular development in sheep. Reprod Biol Endocrinol 2:78
78. **Knight PG, Glister C** 2001 Potential local regulatory functions of inhibins, activins and follistatin in the ovary. Reproduction 121:503-12
79. **Yamoto M, Minami S, Nakano R, Kobayashi M** 1992 Immunohistochemical localization of inhibin/activin subunits in human ovarian follicles during the menstrual cycle. J Clin Endocrinol Metab 74:989-93
80. **Hillier SG, Miro F** 1993 Inhibin, activin, and follistatin. Potential roles in ovarian physiology. Ann N Y Acad Sci 687:29-38
81. **Xiao S, Robertson DM, Findlay JK** 1992 Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131:1009-16
82. **Miro F, Smyth CD, Hillier SG** 1991 Development-related effects of recombinant activin on steroid synthesis in rat granulosa cells. Endocrinology 129:3388-94
83. **Lin SY, Morrison JR, Phillips DJ, de Kretser DM** 2003 Regulation of ovarian function by the TGF-beta superfamily and follistatin. Reproduction 126:133-48
84. **Welt CK, Smith ZA, Pauler DK, Hall JE** 2001 Differential regulation of inhibin A and inhibin B by luteinizing hormone, follicle-stimulating hormone, and stage of follicle development. J Clin Endocrinol Metab 86:2531-7
85. **Welt CK** 2002 The physiology and pathophysiology of inhibin, activin and follistatin in female reproduction. Curr Opin Obstet Gynecol 14:317-23
86. **Laven JSE, Fauser BCJM** 2004 Inhibins and adult ovarian function. Mol Cell Endocrinol 225:37-44

87. **Martens JWM, de Winter JP, Timmerman MA, McLuskey A, van Schaik RH, Themmen APN, de Jong FH** 1997 Inhibin interferes with activin signaling at the level of the activin receptor complex in Chinese hamster ovary cells. Endocrinology 138:2928-36
88. **Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-9
89. **di Clemente N, Goxe B, Remy JJ, Cate R, Josso N, Vigier B, Salesse R** 1994 Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine 2:553-558
90. **McGee EA, Smith R, Spears N, Nachtigal MW, Ingraham H, Hsueh AJ** 2001 Mullerian inhibitory substance induces growth of rat preantral ovarian follicles. Biol Reprod 64:293-8
91. **Visser JA, Durlinger ALL, Peters IJ, van den Heuvel ER, Rose UM, Kramer P, de Jong FH, Themmen APN** 2007 Increased Oocyte Degeneration and Follicular Atresia during the Estrous Cycle in Anti-Mullerian Hormone Null Mice. Endocrinology 148:2301-8
92. **Shi Y, Massagué J** 2003 Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685-700
93. **di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard JY, Vigier B, Josso N, Cate R** 1994 Cloning, expression, and alternative splicing of the receptor for anti-Mullerian hormone. Mol Endocrinol 8:1006-20
94. **Baarends WM, van Helmond MJL, Post M, van der Schoot PJCM, Hoogerbrugge JW, de Winter JP, Uilenbroek JTJ, Karels B, Wilming LG, Meijers JHC, Themmen APN, Grootegoed JA** 1994 A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the mullerian duct. Development 120:189-97
95. **Gouedard L, Chen YG, Thevenet L, Racine C, Borie S, Lamarre I, Josso N, Massague J, di Clemente N** 2000 Engagement of bone morphogenetic protein type IB receptor and Smad1 signaling by anti-Mullerian hormone and its type II receptor. J Biol Chem 275:27973-8
96. **Visser JA, Olaso R, Verhoef-Post M, Kramer P, Themmen APN, Ingraham HA** 2001 The serine/threonine transmembrane receptor ALK2 mediates Mullerian inhibiting substance signaling. Mol Endocrinol 15:936-45
97. **Clarke TR, Hoshiya Y, Yi SE, Liu X, Lyons KM, Donahoe PK** 2001 Mullerian inhibiting substance signaling uses a bone morphogenetic protein (BMP)-like pathway mediated by ALK2 and induces SMAD6 expression. Mol Endocrinol 15:946-59
98. **Jamin SP, Arango NA, Mishina Y, Hanks MC, Behringer RR** 2002 Requirement of Bmpr1a for Mullerian duct regression during male sexual development. Nat Genet 32:408-10
99. **Visser JA** 2003 AMH signaling: from receptor to target gene. Mol Cell Endocrinol 211:65-73
100. **Zhan Y, Fujino A, MacLaughlin DT, Manganaro TF, Szotek PP, Arango NA, Teixeira J, Donahoe PK** 2006 Mullerian inhibiting substance regulates its receptor/SMAD signaling and causes mesenchymal transition of the coelomic epithelial cells early in Mullerian duct regression. Development 133:2359-69
101. **Visser JA, Themmen APN** 2005 Anti-Mullerian hormone and folliculogenesis. Mol Cell Endocrinol 234:81-6

102. **Yi SE, LaPolt PS, Yoon BS, Chen JY, Lu JK, Lyons KM** 2001 The type I BMP receptor BmprIB is essential for female reproductive function. Proc Natl Acad Sci U S A 98:7994-9
103. **Roberts LM, Visser JA, Ingraham HA** 2002 Involvement of a matrix metalloproteinase in MIS-induced cell death during urogenital development. Development 129:1487-96
104. **Racine C, Rey R, Forest MG, Louis F, Ferre A, Huhtaniemi I, Josso N, di Clemente N** 1998 Receptors for anti-mullerian hormone on Leydig cells are responsible for its effects on steroidogenesis and cell differentiation. Proc Natl Acad Sci U S A 95:594-9
105. **Teixeira J, Fynn-Thompson E, Payne AH, Donahoe PK** 1999 Mullerian-inhibiting substance regulates androgen synthesis at the transcriptional level. Endocrinology 140:4732-8
106. **Coulam CB, Adamson SC, Annegers JF** 1986 Incidence of premature ovarian failure. Obstet Gynecol 67:604-6
107. **Schlessinger D, Herrera L, Crisponi L, Mumm S, Percesepe A, Pellegrini M, Pilia G, Forabosco A** 2002 Genes and translocations involved in POF. Am J Med Genet 111:328-33
108. **Chand AL, Ponnampalam AP, Harris SE, Winship IM, Shelling AN** 2006 Mutational analysis of BMP15 and GDF9 as candidate genes for premature ovarian failure. Fertil Steril 86:1009-12
109. **Harris SE, Chand AL, Winship IM, Gersak K, Nishi Y, Yanase T, Nawata H, Shelling AN** 2005 INHA promoter polymorphisms are associated with premature ovarian failure. Mol Hum Reprod 11:779-84
110. **Dixit H, Deendayal M, Singh L** 2004 Mutational analysis of the mature peptide region of inhibin genes in Indian women with ovarian failure. Hum Reprod 19:1760-4
111. **Shelling AN, Burton KA, Chand AL, van Ee CC, France JT, Farquhar CM, Milsom SR, Love DR, Gersak K, Aittomaki K, Winship IM** 2000 Inhibin: a candidate gene for premature ovarian failure. Hum Reprod 15:2644-9
112. **The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group** 2004 Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 19:41-7
113. **Fauser BCJM, Pache TD, Lamberts SWJ, Hop WC, de Jong FH, Dahl KD** 1991 Serum bioactive and immunoreactive luteinizing hormone and follicle-stimulating hormone levels in women with cycle abnormalities, with or without polycystic ovarian disease. J Clin Endocrinol Metab 73:811-7
114. **Jonard S, Dewailly D** 2004 The follicular excess in polycystic ovaries, due to intraovarian hyperandrogenism, may be the main culprit for the follicular arrest. Hum Reprod Update 10:107-17
115. **Laven JSE, Imani B, Eijkemans MJC, Fauser BCJM** 2002 New approach to polycystic ovary syndrome and other forms of anovulatory infertility. Obstet Gynecol Surv 57:755-67
116. **Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, Dewailly D** 2003 Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. J Clin Endocrinol Metab 88:5957-62
117. **Laven JSE, Mulders AGMGJ, Visser JA, Themmen APN, De Jong FH, Fauser BCJM** 2004 Anti-Mullerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age. J Clin Endocrinol Metab 89:318-23

118. **Urbanek M** 2007 The genetics of the polycystic ovary syndrome. Nat Clin Pract Endocrinol Metab 3:103-11
119. **Kok HS, van Asselt KM, van der Schouw YT, Peeters PHM, Wijmenga C** 2005 Genetic studies to identify genes underlying menopausal age. Hum Reprod Update 11:483-93
120. **van Asselt KM, Kok HS, Pearson PL, Dubas JS, Peeters PH, Te Velde ER, van Noord PA** 2004 Heritability of menopausal age in mothers and daughters. Fertil Steril 82:1348-51
121. **Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI** 2006 Heritability of polycystic ovary syndrome in a Dutch twin-family study. J Clin Endocrinol Metab 91:2100-4
122. **Balding DJ** 2006 A tutorial on statistical methods for population association studies. Nat Rev Genet 7:781-91
123. **Burton PR, Tobin MD, Hopper JL** 2005 Key concepts in genetic epidemiology. Lancet 366:941-51
124. **Brookes AJ** 1999 The essence of SNPs. Gene 234:177-86
125. **The International HapMap Consortium** 2003 The International HapMap Project. Nature 426:789-96
126. **Sunyaev S, Lathe W, 3rd, Bork P** 2001 Integration of genome data and protein structures: prediction of protein folds, protein interactions and "molecular phenotypes" of single nucleotide polymorphisms. Curr Opin Struct Biol 11:125-30
127. **Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, Gottesman MM** 2007 A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 315:525-8
128. **Zondervan KT, Cardon LR** 2004 The complex interplay among factors that influence allelic association. Nat Rev Genet 5:89-100
129. **Cordell HJ, Clayton DG** 2005 Genetic association studies. Lancet 366:1121-31
130. **The International HapMap Consortium** 2005 A haplotype map of the human genome. Nature 437:1299-320

Chapter 2

Anti-Müllerian hormone type II receptor null mice display a more severe ovarian phenotype than AMH null mice

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ABSTRACT

Anti-Müllerian hormone (AMH) null female mice display an increased rate of primordial follicle recruitment, and as a consequence, depletion of their pool at an earlier age. Similar to AMH, its specific type II receptor (AMHRII) is expressed in granulosa cells of non-atretic growing follicles. To determine the ovarian phenotype of AMHRII null mice and whether this phenotype resembles that of AMH null mice, we have analyzed the follicle pool of AMHRII null mice and compared it to the pool in AMH null mice.

In 13-month-old AMHRII null mice, the primordial follicle pool was completely depleted and, as a consequence, significantly less growing follicles (10-fold) were present compared to wild-type mice. Ovaries of 4-month-old mice displayed a 3-fold increase in number of growing follicles whereas the number of primordial follicles was decreased compared to wild-type mice. These results suggest that primordial follicle recruitment is enhanced in AMHRII null mice, resembling the AMH null ovarian phenotype. The change in numbers of growing follicles at 4 and 13 months was reflected by serum AMH levels.

Interestingly, the decrease in primordial follicle number was more pronounced in AMHRII than AMH null mice. Furthermore, whereas in AMH null mice the number of primordial follicles did not differ between wild-type and null mice at 25 days of age, ovaries of AMHRII null mice contained significantly less primordial follicles. This suggests that AMHRII may have an AMH-independent role in the ovary.

In conclusion, AMHRII null mice display a more severe ovarian phenotype than AMH null mice.

INTRODUCTION

Anti-Müllerian hormone (AMH), a member of the TGFβ superfamily, is known for its role in male sex differentiation. AMH induces the regression of the Müllerian ducts, the anlagen of the female reproductive tract [1, 2]. During fetal development, AMH is solely expressed by the fetal testis [3]. Postnatally, this sex-dimorphic expression pattern is lost and AMH is expressed in both the testis and ovary (reviewed in [4, 5]). Insight into the role of AMH in gonadal function was obtained from analysis of AMH null mice. Testes of male AMH null mice displayed Leydig cell hyperplasia, suggesting an inhibitory role of AMH in Leydig cell proliferation [6]. Ovaries of AMH null mice displayed increased primordial follicle recruitment, resulting in a decreased number of primordial follicles and an almost three-fold increase in the number of small growing follicles compared to wild-type mice [7]. In addition, studies revealed that, in the absence of AMH, follicles have become more sensitive to follicle stimulating hormone (FSH). AMH null mice have an increased and earlier cyclic recruitment of growing follicles despite a blunted FSH surge at estrus [8, 9]. Furthermore, FSH-induced growth of mouse preantral follicles was inhibited by AMH in an *in vitro* culture system [8]. Hence, in the ovary AMH functions as a negative regulator of initial and cyclic follicle recruitment.

At present it is uncertain whether the same molecular machinery mediates all biological effects of AMH in both the embryo and adult gonads. Compared to other TGFβ family members, the AMH signaling pathway is relatively simple. AMH signals through one type II receptor and three type I receptors (reviewed by [10, 11]). Although these AMH type I receptors, ALK2, ALK3, and ALK6 [12-15], are shared by bone morphogenetic proteins (BMPs) [16], signaling specificity is guaranteed by the exclusive AMH type II receptor (AMHRII). Mutations in both the AMH and AMHRII gene result in a similar phenotype in men, *i.e.* persistent Müllerian duct syndrome [17]. Also male AMHRII null mice are a phenocopy of male AMH null mice, displaying persistence of Müllerian ducts, seminiferous tubule atrophy, and Leydig cell hyperplasia [18]. Furthermore, male mice null for both AMH and AMHRII are indistinguishable from either of the single null mice [18]. Whether AMHRII null mice mimic the ovarian phenotype of AMH null mice is not known yet.

In the ovary, AMH and AMHRII do not always have a similar temporal expression pattern. In the adult ovary, AMH and AMHRII expression colocalize in granulosa cells of nonatretic preantral and small antral follicles [19-25]. Furthermore, AMHRII is expressed in theca cells of these follicles [26]. However, whereas AMH expression only starts in the columnar granulosa cells of primary follicles immediately after differentiation from flattened pre-granulosa cells of primordial

follicles [3, 27, 28], AMHRII is expressed prior to that. In situ hybridization revealed that AMHRII is expressed in the neonatal ovary, although the exact localization to specific cell types could not be determined [5, 19, 27]. Furthermore, AMHRII is expressed in the fetal ovary in the absence of AMH expression [24, 25, 29]. The lack of AMH expression in the fetal ovary is not only crucial for Müllerian duct development, but also for normal ovarian development. Overexpression of AMH in transgenic mice results in germ cell loss in fetal ovaries, subsequent development of somatic cells into seminiferous cord-like structures, and eventually degeneration of the ovary in adult females [30, 31]. Furthermore, aromatase activity was decreased in fetal ovaries of transgenic mice and in fetal ovaries exposed to AMH *in vitro* [31, 32]. These studies indicate that the AMHRII present in fetal ovaries is a functional receptor. Whether the AMH type II receptor also may have a function in the ovary in the absence of AMH is not known.

To investigate the role of AMHRII in the ovary we have analyzed the entire follicle population in wild-type and AMHRII null mice. In addition, these follicle numbers were compared to numbers in AMH null mice to determine whether female AMHRII null mice, similar to male AMHRII null mice, are a phenocopy of AMH mice.

MATERIAL & METHODS

Animals

Wild-type and AMHRII null female mice on a C57Bl/6J background were generated as described previously [33]. The animals were obtained from the Animal Facility of the Erasmus MC in Rotterdam (The Netherlands) and were kept under standard animal housing conditions in accordance with the NIH guidelines for the Care and Use of Experimental Animals. The animals were sacrificed at 25 days, 4 and 13 months of age. The 4- and 13-months-old mice were sacrificed on the day of estrus, which was determined as described previously [7]. For each time point, ovaries from six to eight wild-type (wt), heterozygous (het), and homozygous AMHRII (knockout, ko) null females were collected. Animals were sacrificed at 1400 h by decapitation. Blood samples were collected and serum was isolated as described previously [7]. Serum samples were stored at –20 C until assayed for AMH. Ovaries and uteri were removed and weighed. The ovaries were fixed overnight in Bouin's fluid at room temperature. For histological examination of the follicle population, fixed ovaries were embedded in paraffin, and after routine histological procedures, 8 μm sections were mounted on slides and stained with hematoxylin and eosin.

Ovarian histology and follicle counting

Follicle count was performed as described previously [7], using serial sections of both ovaries. All follicles were counted in every fifth section. Growing follicles were divided into four groups based on their mean diameter, which was determined by measuring two perpendicular diameters in the section in which the nucleolus of the oocyte was present: small preantral follicles (20-170 µm), large preantral follicles (171-220 µm), small antral follicles (221-310 µm) and large antral follicles (> 311 µm) [7, 9]. For this study, growing follicles with a diameter smaller than 310 µm were combined. Atretic follicles were identified by the presence of pyknotic nuclei in granulosa cells [34, 35]. Follicles in stage 1a and stage 1b of atresia are defined as early atretic follicles.

To compare follicle numbers in AMH- and AMHRII null mice, numbers were expressed as a percentage of follicle numbers observed in wild-type mice (wild-type = 100%), since follicles were counted every second (primordial) or every (growing follicles) section in AMH null mice and every fifth section in AMHRII null mice. Data on follicle numbers in AMH null mice were taken from Durlinger *et al.* [7].

Hormone measurements

AMH serum levels were determined by an in house AMH ELISA assay [9, 36]. The inter- and intra-assay coefficient of variation was less than 10% and less than 5%, and all samples of the same age group were measured in one assay.

Statistical analysis

Results are presented as the mean ± SEM. Differences in ovarian parameters between genotype groups were tested using one-way ANOVA, followed by Duncan's new multiple range test. All analyses were performed using SPSS, Inc.11.0.1 (SPSS, Inc, Chicago, IL, USA). $P < 0.05$ was considered to be significant.

RESULTS

Weight of ovaries and uterus

Ovarian and uterine weights were determined as a first indication of a functional effect of loss of AMHRII expression. At 25 days and 13 months, no difference in ovarian weight was observed between the three genotype groups (Table 2.1). However, at 4 months of age the ovarian weight of AMHRII null mice was significantly increased (1.7 fold, $P < 0.001$) (Table 2.1) compared to heterozygous

Table 2.1 Ovarian and uterine weight in wild type (wt), heterozygous (het), and homozygous (ko) AMHRII null female mice of 25 days, 4 months, and 14 months of age.

Age	Genotype	Ovarian weight (mg)	Uterine weight (mg)
25 days	wt	5.63 ± 0.24	11.43 ± 1.76
	het	5.31 ± 0.51	8.51 ± 1.69
	ko	6.10 ± 0.24	7.63 ± 1.47
4 months	wt	8.73 ± 0.55	89.82 ± 6.69
	het	8.82 ± 0.44	96.39 ± 14.34
	ko	14.32 ± 1.37***	74.83 ± 15.01
13 months	wt	6.65 ± 0.48	108.12 ± 4.91
	het	7.08 ± 1.87	129.33 ± 13.34
	ko	5.38 ± 0.62	154.10 ± 21.10

The combined weight of both ovaries is given. Data represent means ± SEM (n = 6-8 mice). ***, Significantly different from wild-type and heterozygous mice, $P < 0.001$.

and wild-type mice. No statistical differences were observed in uterine weight between the three genotype groups at all ages studied (Table 2.1).

Follicle numbers in AMHRII null mice

The entire follicle pool was determined in the AMHRII null mice. At all ages studied, the primordial follicle pool was significantly smaller in homozygous AMHRII null mice compared to heterozygous and wild-type mice ($P < 0.001$) (Figure 2.1). In fact, in 13-month-old AMHRII null females, the primordial follicle pool was completely depleted whereas ovaries of wild-type female mice still contained a significant number of primordial follicles. At almost all ages, heterozygous AMHRII null female mice had an intermediate number of primordial follicles compared to wild-type and homozygous AMHRII null mice ($P < 0.05$) (Figure 2.1).

At 25 days of age, the number of non-atretic growing follicles did not differ between the three genotype groups. However, at 4 months of age, ovaries of homozygous AMHRII null mice contained nearly 3 times more growing follicles compared to heterozygous and wild-type mice ($P < 0.001$) (Figure 2.1). This increase in follicle number was also observed for the atretic follicles ($P < 0.01$) (Figure 2.1). In contrast, ovaries of 13-month-old homozygous AMHRII null mice contained significantly less non-atrectic and atretic follicles than ovaries of wild-type mice ($P < 0.001$ and $P < 0.01$ respectively) (Figure 2.1). At this age, also ovaries of heterozygous mice contained less healthy growing follicle compared to wild-type mice ($P < 0.01$) (Figure 2.1).

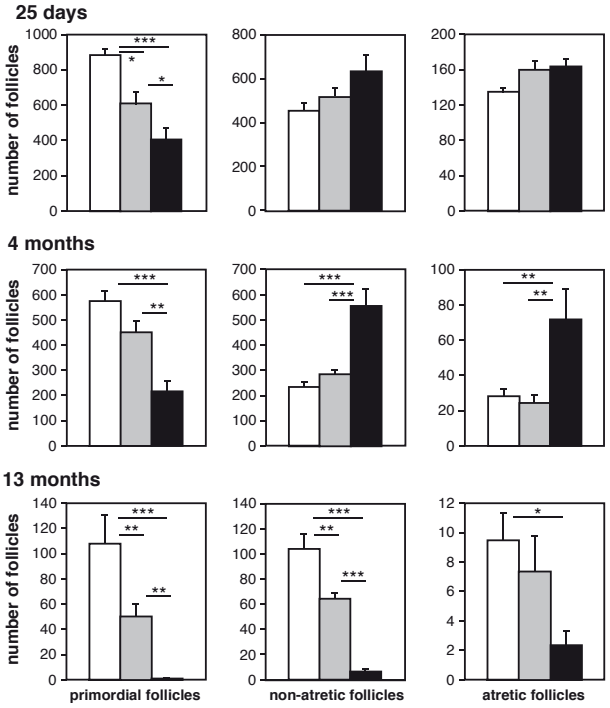

Figure 2.1 Follicle population in AMHRII null mice. The number of primordial, non-atretic and early atretic follicles were determined at 25-day-, 4-month-, and 13-month-old wild-type (*open bars*), heterozygous (*grey bars*), and homozygous (*black bars*) AMHRII null mice. Data represent means ± SEM (n = 6-8). *, **, ***, Significant different numbers of follicles, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

AMH levels in AMHRII null mice

Previously, we have shown that serum AMH levels strongly correlate with the number of growing follicles in mouse ovaries [36]. Therefore, it is anticipated that the changed follicle pool in AMHRII null mice will be reflected by altered

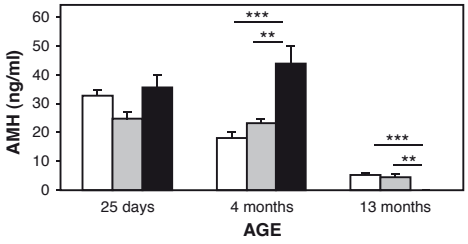

Figure 2.2 AMH levels in 25-day-, 4-month-, and 13-month-old mice. Serum levels were determined in wild-type (*open bars*), heterozygous (*grey bars*), and homozygous (*black bars*) AMHRII null mice. Data represent means ± SEM (n = 6-8). **, ***, Significant difference at $P < 0.01$, $P < 0.001$, respectively.

serum levels. At 25 days of age, serum AMH levels did not differ between the genotype groups (Figure 2.2). However, AMH levels were significantly increased in 4-month-old homozygous AMHRII null mice compared to heterozygous and wild-type mice ($P < 0.001$) (Figure 2.2). In contrast, at 13 months of age, AMH levels were undetectable in homozygous AMH null mice, whereas AMH could still be measured in serum of heterozygous and wild-type mice (Figure 2.2).

Comparison of AMH and AMHRII null mice

In male mice, AMHRII deficiency yields a phenotype that is a phenocopy of AMH deficiency. To determine whether this also holds true for the ovarian phenotype of female mice, we compared follicle numbers of both genetic strains. The data on follicle number in AMH null mice were taken from Durlinger *et al.* [7]. The numbers of primordial and non-atretic growing follicles were expressed relative to the numbers observed in wild-type mice. No difference in the number of non-atretic growing follicles was observed between AMH and AMHRII null

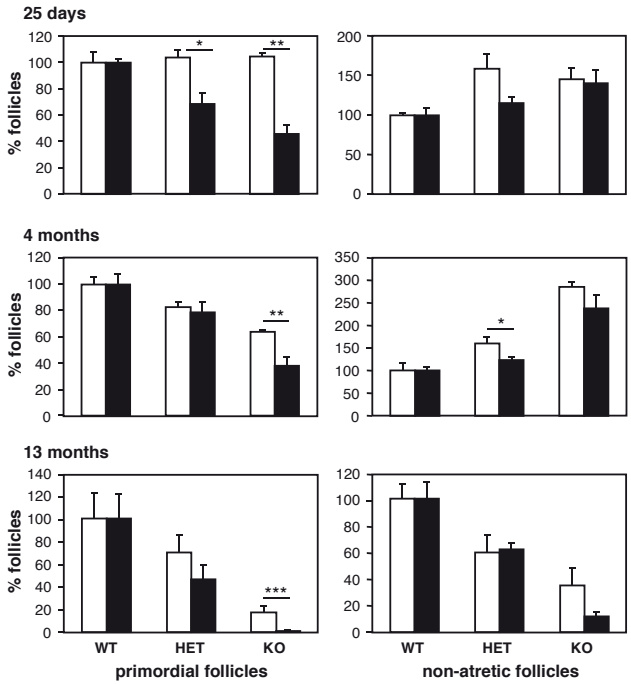

Figure 2.3 Comparison of follicle numbers in AMH null and AMHRII null mice. The percentage of primordial and non-atretic growing follicles were compared between AMH null mice (*open bars*) and AMHRII null mice (*black bars*) at 25 days, 4 months, and 13 months of age. Follicle numbers are expressed as percentage of numbers in wild-type mice. AMH null mice, n = 4-5; AMHRII null mice, n = 6-8. *, **, ***, Significantly different from AMH null mice, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

mice (Figure 2.3). The increase in the number of growing follicles at 4 months of age, and decrease at 13 months of age were similar in both strains. In contrast, the decline in primordial follicle number was more pronounced in AMHRII null mice compared to AMH null mice at 4 and 13 months of age ($P < 0.01$ and $P < 0.001$, respectively) (Figure 2.3). Furthermore, whereas the number of primordial follicles in AMH null mice did not differ from wild-type mice at 25 days of age, ovaries of AMHRII null mice already contained significantly less primordial follicles ($P < 0.01$) (Figure 2.3).

DISCUSSION

AMH has been identified as one of the intraovarian growth factors that regulate the rate at which primordial follicles are recruited for further growth [8, 9, 27]. The effects of AMH are mediated through its specific type II receptor (AMHRII) and three candidate type I receptors [10, 11]. In this study, we determined the ovarian phenotype of AMHRII null mice. We observed that AMHRII null mice display an increased number of growing follicles, and concurrently, a reduced number of primordial follicles. As a consequence of this enhanced recruitment, AMHRII null mice show advanced ovarian aging, with a completely depleted primordial follicle pool at 13 months of age.

The changes in the follicle pool are reflected by the serum AMH levels in AMHRII null mice. Previously, we have shown that serum AMH levels strongly correlate with the number of growing follicles [36]. Indeed, the increased number of growing follicles at 4 months of age in AMHRII null mice resulted in increased AMH levels, whereas in aged AMHRII null mice serum AMH levels are undetectable, in agreement with the nearly complete absence of growing follicles. These results suggest that AMH expression in not under the control of a strong positive or negative feedback system, although subtle regulatory mechanisms cannot be excluded.

The enhanced primordial follicle recruitment in AMHRII null mice resembles the phenotype observed in AMH null mice [7]. Nonetheless, female AMHRII null are not an exact phenocopy of female AMH null mice, in contrast to what has been observed for the male null mice [18]. Whereas in female AMH null mice the primordial follicle pool does not differ from the pool in wild-type mice at 25 days of age, the number of primordial follicles is reduced in AMHRII null mice at this age. As a consequence of the diminished primordial follicle pool, depletion of the pool is more dramatic in the aged AMHRII null mice than in aged AMH null mice. The finding that the number of growing follicles does not differ

between AMH and AMHRII null mice at 4 months of age, suggests that the rate of primordial follicle recruitment is enhanced to a similar extent in both strains. The smaller primordial follicle pool in AMHRII null mice, therefore, suggests that either in AMHRII null mice primordial follicle recruitment is enhanced at an earlier age compared to AMH null mice, or that AMHRII null mice have a smaller pool to start with.

Both explanations imply that AMHRII has an AMH-independent role in the ovary. The more severe phenotype in AMHRII null mice could simply reflect the loss of basal receptor activity, which remains present in AMH null mice. Interestingly, basal activity of AMHRII requires the presence of a type I receptor [26]. In contrast to the TGFβ signaling, where the type II receptor recruits the type I receptor upon ligand binding, BMP receptors can form ligand-independent receptor complexes [16]. AMHRII, whose signaling resembles the BMP signaling pathway, can also interact with type I receptors in the absence of ligand, although the latter was observed in *in vitro* overexpression studies [10, 12].

Alternatively, other ligands may signal through AMHRII during early ovarian development. A high degree of crosstalk or promiscuity exists for ligands and receptors of the TGFβ family. For instance, a receptor, type II and type I, can interact with multiple ligands, as was illustrated by the different phenotypes in mice null for activin ligands or the activin type II receptor [37-39]. Similar to AMH- and AMHRII null mice, BMP2 and BMP4 null mice are not exact phenocopies of the BMPRII null or BMPR1A null mice [40-43]. Since AMHRII interacts with BMP type I receptors, BMP ligands are potential candidate ligands for AMHRII. It is known that BMPs regulate the formation and early proliferation of primordial germ cells (PGCs). Mice lacking BMP ligands such as BMP2, BMP4, BMP8b, the type I receptor ALK2, or downstream Smads, such as Smad1 and Smad5, have reduced numbers of primordial germ cells [42, 44-49]. These BMP-related defects occur during early embryonic development affecting predominantly PGC migration. However, AMHRII mRNA expression is first detected at embryonic day 10.5 (E10.5) [50; Visser and Themmen, unpublished results], at a time when PGCs have arrived in the gonad and have formed germ cell cysts, which is accompanied by mitotic divisions. Around E13.5, germ cells or oogonia enter meiosis and eventually arrest in the diplotene stage. After birth, germ cell cysts break down, and pre-granulosa cells enclose individual oocytes to form primordial follicles [51-54]. This process of germ cell breakdown is accompanied by a massive loss of oocytes, particularly oocytes that have not become encapsulated by pre-granulosa cells [54]. AMHRII is expressed throughout germ cell cysts formation and primordial follicle formation, and could potentially play a role during this process and thus in the establishment of the primordial follicle pool.

Analysis of ovaries at younger ages will reveal at which stage of the formation of the primordial follicle pool AMHRII plays a crucial role. These ongoing studies will give insight into the AMHRII signaling effects, but will also give a better understanding of the mechanisms involved in establishment of the size of the primordial follicle pool. This may be of importance to identify genes involved in premature ovarian failure.

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REFERENCES

1. **Josso N, Cate RL, Picard JY, Vigier B, di Clemente N, Wilson C, Imbeaud S, Pepinsky RB, Guerrier D, Boussin L, Legeai L, Carré-Eusèbe D** 1993 Anti-mullerian hormone: the Jost factor. Recent Prog Horm Res 48:1-59
2. **Lee MM, Donahoe PK** 1993 Mullerian inhibiting substance: a gonadal hormone with multiple functions. Endocr Rev 14:152-64
3. **Munsterberg A, Lovell-Badge R** 1991 Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. Development 113:613-24
4. **Teixeira J, Maheswaran S, Donahoe PK** 2001 Mullerian inhibiting substance: an instructive developmental hormone with diagnostic and possible therapeutic applications. Endocr Rev 22:657-74
5. **Durlinger ALL, Visser JA, Themmen APN** 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124:601-9
6. **Behringer RR, Finegold MJ, Cate RL** 1994 Mullerian-inhibiting substance function during mammalian sexual development. Cell 79:415-25
7. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-96
8. **Durlinger ALL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen APN** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-9
9. **Visser JA, Durlinger ALL, Peters IJJ, van den Heuvel ER, Rose UM, Kramer P, de Jong FH, Themmen APN** 2007 Increased oocyte degeneration and follicular atresia during the estrous cycle in anti-Mullerian hormone null mice. Endocrinology 148:2301-8
10. **Visser JA** 2003 AMH signaling: from receptor to target gene. Mol Cell Endocrinol 211:65-73
11. **di Clemente N, Josso N, Gouedard L, Belville C** 2003 Components of the anti-Mullerian hormone signaling pathway in gonads. Mol Cell Endocrinol 211:9-14
12. **Visser JA, Olaso R, Verhoef-Post M, Kramer P, Themmen APN, Ingraham HA** 2001 The serine/threonine transmembrane receptor ALK2 mediates Mullerian inhibiting substance signaling. Mol Endocrinol 15:936-45
13. **Clarke TR, Hoshiya Y, Yi SE, Liu X, Lyons KM, Donahoe PK** 2001 Müllerian-inhibiting substance signaling uses a bone morphogenetic protein (BMP)-like pathway mediated by ALK2 and induces SMAD6 expression. Mol Endocrinol 15:946-59.
14. **Gouédard L, Chen YG, Thevenet L, Racine C, Borie S, Lamarre I, Josso N, Massagué J, di Clemente N** 2000 Engagement of bone morphogenetic protein type IB receptor and Smad1 signaling by anti-Müllerian hormone and its type II receptor. J Biol Chem
15. **Jamin SP, Arango NA, Mishina Y, Hanks MC, Behringer RR** 2002 Requirement of Bmpr1a for Müllerian duct regression during male sexual development. Nat Genet 32:408-10
16. **Massague J, Chen YG** 2000 Controlling TGF-beta signaling. Genes Dev 14:627-44
17. **Belville C, Josso N, Picard JY** 1999 Persistence of Mullerian derivatives in males. Am J Med Genet 89:218-23
18. **Mishina Y, Rey R, Finegold MJ, Matzuk MM, Josso N, Cate RL, Behringer RR** 1996 Genetic analysis of the Mullerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. Genes Dev 10:2577-87

19. **Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen APN, Grootegoed JA** 1995 Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951-62
20. **Ueno S, Takahashi M, Manganaro TF, Ragin RC, Donahoe PK** 1989 Cellular localization of mullerian inhibiting substance in the developing rat ovary. Endocrinology 124:1000-6
21. **Ueno S, Kuroda T, Maclaughlin DT, Ragin RC, Manganaro TF, Donahoe PK** 1989 Mullerian inhibiting substance in the adult rat ovary during various stages of the estrous cycle. Endocrinology 125:1060-6
22. **Hirobe S, He WW, Lee MM, Donahoe PK** 1992 Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 131:854-62
23. **Hirobe S, He WW, Gustafson ML, MacLaughlin DT, Donahoe PK** 1994 Mullerian inhibiting substance gene expression in the cycling rat ovary correlates with recruited or graafian follicle selection. Biol Reprod 50:1238-43
24. **di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard JY, Vigier B, Josso N, Cate R** 1994 Cloning, expression, and alternative splicing of the receptor for anti-Mullerian hormone. Mol Endocrinol 8:1006-20.
25. **Teixeira J, He WW, Shah PC, Morikawa N, Lee MM, Catlin EA, Hudson PL, Wing J, Maclaughlin DT, Donahoe PK** 1996 Developmental expression of a candidate mullerian inhibiting substance type II receptor. Endocrinology 137:160-5
26. **Ingraham HA, Hirokawa Y, Roberts LM, Mellon SH, McGee E, Nachtigal MW, Visser JA** 2000 Autocrine and paracrine Mullerian inhibiting substance hormone signaling in reproduction. Recent Prog Horm Res 55:53-67; discussion 67-8
27. **Durlinger ALL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen APN** 2002 Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076-84
28. **Taketo T, Saeed J, Manganaro T, Takahashi M, Donahoe PK** 1993 Mullerian inhibiting substance production associated with loss of oocytes and testicular differentiation in the transplanted mouse XX gonadal primordium. Biol Reprod 49:13-23
29. **Baarends WM, van Helmond MJ, Post M, van der Schoot PJ, Hoogerbrugge JW, de Winter JP, Uilenbroek JT, Karels B, Wilming LG, Meijers JH, Themmen APN, Grootegoed JA** 1994 A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the mullerian duct. Development 120:189-97
30. **Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL** 1990 Abnormal sexual development in transgenic mice chronically expressing mullerian inhibiting substance. Nature 345:167-70
31. **Lyet L, Louis F, Forest MG, Josso N, Behringer RR, Vigier B** 1995 Ontogeny of reproductive abnormalities induced by deregulation of anti-mullerian hormone expression in transgenic mice. Biol Reprod 52:444-54
32. **Vigier B, Forest MG, Eychenne B, Bezard J, Garrigou O, Robel P, Josso N** 1989 Anti-Mullerian hormone produces endocrine sex reversal of fetal ovaries. Proc Natl Acad Sci U S A 86:3684-8
33. **Arango NA, Lovell-Badge R, Behringer RR** 1999 Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 99:409-19

34. **Byskov AG** 1974 Cell kinetic studies of follicular atresia in the mouse ovary. J Reprod Fertil 37:277-85
35. **Osman P** 1985 Rate and course of atresia during follicular development in the adult cyclic rat. J Reprod Fertil 73:261-70
36. **Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BMN, de Jong FH, Groome NP, Themmen APN, Visser JA** 2006 Serum anti-Müllerian hormone levels reflect the size of the primordial follicle pool in mice. Endocrinology 147:3228-34
37. **Matzuk MM, Kumar TR, Bradley A** 1995 Different phenotypes for mice deficient in either activins or activin receptor type II. Nature 374:356-60
38. **Matzuk MM, Kumar TR, Vassalli A, Bickenbach JR, Roop DR, Jaenisch R, Bradley A** 1995 Functional analysis of activins during mammalian development. Nature 374:354-6
39. **Vassalli A, Matzuk MM, Gardner HA, Lee KF, Jaenisch R** 1994 Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. Genes Dev 8:414-27
40. **Mishina Y, Suzuki A, Ueno N, Behringer RR** 1995 Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev 9:3027-37
41. **Winnier G, Blessing M, Labosky PA, Hogan BL** 1995 Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9:2105-16
42. **Zhang H, Bradley A** 1996 Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977-86
43. **Beppu H, Kawabata M, Hamamoto T, Chytil A, Minowa O, Noda T, Miyazono K** 2000 BMP type II receptor is required for gastrulation and early development of mouse embryos. Dev Biol 221:249-58
44. **Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL** 1999 Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13:424-36
45. **Ying Y, Zhao GQ** 2001 Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. Dev Biol 232:484-92
46. **Ying Y, Liu XM, Marble A, Lawson KA, Zhao GQ** 2000 Requirement of Bmp8b for the generation of primordial germ cells in the mouse. Mol Endocrinol 14:1053-63
47. **de Sousa Lopes SM, Roelen BA, Monteiro RM, Emmens R, Lin HY, Li E, Lawson KA, Mummery CL** 2004 BMP signaling mediated by ALK2 in the visceral endoderm is necessary for the generation of primordial germ cells in the mouse embryo. Genes Dev 18:1838-49
48. **Tremblay KD, Dunn NR, Robertson EJ** 2001 Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. Development 128:3609-21
49. **Chang H, Matzuk MM** 2001 Smad5 is required for mouse primordial germ cell development. Mech Dev 104:61-7
50. **Klattig J, Sierig R, Kruspe D, Besenbeck B, Englert C** 2007 Wilms' tumor protein Wt1 is an activator of the anti-Mullerian hormone receptor gene Amhr2. Mol Cell Biol 27:4355-64
51. **McLaren A** 2000 Germ and somatic cell lineages in the developing gonad. Mol Cell Endocrinol 163:3-9
52. **Pepling ME, Spradling AC** 1998 Female mouse germ cells form synchronously dividing cysts. Development 125:3323-8

53. **Pepling ME, Spradling AC** 2001 Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev Biol 234:339-51
54. **Pepling ME** 2006 From primordial germ cell to primordial follicle: mammalian female germ cell development. Genesis 44:622-32

Chapter 3

Serum anti-Müllerian hormone levels reflect the size of the primordial follicle pool in mice

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ABSTRACT

Reproductive aging is the decline of female fertility with age. It is caused by the decrease in the number of growing follicles, resulting from primordial follicle pool depletion. Recently, we have shown that anti-Müllerian hormone (AMH) is produced by growing follicles, and studies in women indicate that serum AMH levels decrease with age and correlate with antral follicle count. However, whether serum AMH levels correlate directly with the size of the primordial follicle pool cannot be determined in women.

In this work, we describe studies in mice in which we determined the dynamics of ovarian follicles during aging. Furthermore, we describe the development of a mouse AMH ELISA, allowing us to measure AMH levels in mice, for the first time. We observed that serum AMH levels decline with increasing age, whereas expression of AMH in individual growing follicles, studied by immunohistochemistry, did not change with age. Thus, the decline in serum AMH correlates directly with the decline in number of growing follicles ($r = 0.86$, $P < 0.0001$). We observed that the number of growing follicles correlated with the number of primordial follicles ($r = 0.93$, $P < 0.0001$). Similarly, we found a strong correlation between AMH levels and number of primordial follicles ($r = 0.83$, $P < 0.0001$).

In conclusion, serum AMH levels reflect the size of the primordial follicle pool in aging mice. Therefore, AMH is an excellent marker to assess the quantitative aspect of ovarian reserve, which may be useful for women at risk for early ovarian aging such as survivors of childhood cancers.

INTRODUCTION

In women, menopause indicates the absolute end of reproductive life. However, a decline in fertility is already apparent 20 yr before menopause, and 10 yr before menopause the ability to conceive is extremely low [1-3]. Ovarian aging is a major determinant of this age-related decrease in female fertility, and is related to a decrease in the size of the ovarian follicle pool and the quality of the oocytes therein [4]. The size of the follicle pool is established before (primates) or directly after (mice) birth. During embryonic development, germ cells populate the ovary and become surrounded by pre-granulosa cells forming the primordial follicles. During early childhood, many oocytes degenerate resulting in a stock of 300.000-500.000 primordial follicles at menarche [5]. This concept of a nonrenewable primordial follicle pool was recently challenged by Johnson *et al* [6, 7] with the identification of bone marrow and blood-derived germ cells that may add to the primordial follicle pool. Nevertheless, at the age of menopause, the ovary is devoid of follicles due to the exponential decline in the number of primordial follicles throughout life. Similarly, the number of follicles that initiate growth to the antral stage decreases with age, and appears to be primarily related to the number of follicles in the primordial follicle pool [8].

Assessment of the ovarian reserve is important in the infertility clinic, where ovarian aging is characterized by decreased ovarian responsiveness to exogenous gonadotropin administration and poor pregnancy outcome. Currently, early follicular phase serum levels of FSH, inhibin B, and estradiol (E_2) are measured to assess the ovarian reserve in women. Upon ovarian aging, serum levels of inhibin B and E_2 decline and subsequently FSH levels rise [9]. However, these markers constitute the classical hypothalamus-pituitary-gonadal feedback loop and, therefore, are not independent of each other. Furthermore, changes in serum levels of FSH, inhibin B, and E_2 occur relatively late in the reproductive aging process [10]. Therefore, in addition to these hormones, the number of antral follicles [antral follicle count (AFC)] is determined by ultrasonography, because the AFC gives a better prediction of the ovarian reserve [11]. Recently, measurement of serum anti-Müllerian hormone (AMH) levels has been added to the panel of markers for ovarian aging [12-14].

AMH, also known as Müllerian-inhibiting substance (MIS), is expressed in granulosa cells of growing follicles [15-19]. Detailed studies in rodents have shown that AMH expression is flanked by two major regulatory steps of folliculogenesis, *i.e.* initial follicle recruitment and cyclic selection for dominance [20, 21]. AMH expression starts in the granulosa cells of primary follicles, directly after differentiation from flattened pre-granulosa cells of primordial follicles. Highest

expression is observed in granulosa cells of preantral and small antral follicles, whereas expression is absent during the FSH-dependent final stages of follicle growth [16, 17, 19, 21, 22]. In the human ovary, AMH is expressed in a similar pattern, with expression first appearing in granulosa cells of primary follicles and being strongest in preantral and small antral follicles. AMH expression disappears in follicles of increasing size and is lost in large antral follicles, where weak staining only remains present in the granulosa cells of the cumulus [23].

This specific expression pattern of AMH in growing non-selected follicles has lead us and others to study whether serum AMH levels are indicative for the number of growing follicles (reviewed in [24]). Indeed, in women, serum AMH levels decline with increasing age and changes in serum AMH levels were apparent before changes in other serum markers of ovarian aging, such as FSH and inhibin B, were present. Furthermore, AMH levels correlated strongly with the AFC [12, 13]. In contrast with other serum markers, AMH levels remain relative constant during the menstrual cycle [25, 26]. Furthermore, studies suggest that serum AMH levels are not influenced by the gonadotropic status, and only reflect the follicle population [13, 27, 28].

Despite accumulating data on the use of AMH serum levels as a marker for ovarian reserve, the relationship between AMH levels and the size of the primordial follicle pool has not been studied directly, because direct assessment of the size of this pool is not possible in women.

With the development of a mouse AMH ELISA, we have addressed this question in aging mice. In this study, we show that, similar to women, serum AMH levels decline with increasing age in mice. The decline in AMH levels correlates with the decrease in the number of growing follicles with aging, and most importantly, with the size of the primordial follicle pool. These findings show that serum AMH levels reflect the quantitative aspect of ovarian reserve in rodents.

MATERIALS AND METHODS

Antibody production

AMH antibodies were generated as described previously [29]. The initial screening was done by ELISA using microplates coated with recombinant human AMH with a secondary screening with plates coated with rat AMH. Recombinant human and rat AMH were produced as described previously [23, 30]. Selected cell lines were recloned, and cells producing antibodies for purification were grown in tissue culture medium using low IgG fetal calf serum (Invitrogen, Paisley, UK). Antibodies were purified on columns of protein G by standard methods (Prosep-

G; Millipore, Watford, UK). Ten antibodies selected for further immunoassay work were all isotyped as IgG1 (mouse monoclonal antibody isotyping test kit; Serotec, Oxford, UK). All antibodies were biotinylated using EZ-link Sulfo-NHS-LC-Biotin (Perbio Science, Cramlington, UK) adopting the recommended protocol. The specificity of the antibodies was further tested by Western blot analysis against recombinant rat and human AMH as described previously under reducing and nonreducing conditions [23].

Two-site immunoassay development

To identify an optimum pair of antibodies to allow sensitive detection of AMH, every antibody was tested as capture antibody in combination with all other biotinylated detection antibodies. Ten 96-well microplates (Nunc Maxisorb; SLS, Nottingham, UK) were coated with each of the different antibodies (raised against human AMH, also reacting with rat AMH) at 2 µg/ml in 0.05 M bicarbonate buffer (pH 9.4) and incubated at 4 C overnight, before incubation with a blocking buffer containing 0.5% (wt/vol) casein (Mast Group Ltd., Bootle, UK; Ref. M2052) and 6% (wt/vol) sucrose in PBS for 1 h. Next, plates were emptied, dried, and stored in aluminum pouches with desiccant. Recombinant human AMH, recombinant rat AMH, and various mammalian sera were diluted in high-performance ELISA (HPE) immunoassay buffer (Mast Group Ltd; Ref. M1940) and 50 µl/well was added to duplicate wells. Sera were diluted at 1:5 in HPE immunoassay buffer, 50 µl/well of each sample was added to duplicate wells on the plate, and plates were shaken at room temperature for 2 h. After washing with PBS/Tween (0.005% Tween-20) three to five times, 50 µl/well of each of the 10 biotinylated monoclonal antibodies was added at a concentration of 0.3 µg/ml diluted in 1% (wt/vol) casein buffer and incubated at room temperature for 1 h. Plates were again washed five times with PBS/Tween and incubated for 30 min with poly HRP-streptavidin conjugate (Mast Group Ltd.; Ref. M2051), added at a 1:10000 dilution in 1% (wt/vol) casein buffer. After a thorough wash with PBS/Tween followed by deionized water, the plates were developed with the tetramethylbenzidine substrate (TMB) (Insight Biotechnology International, Wembley, UK). The best combination of detector and capture antibodies to allow detection of AMH in mouse serum was selected. This was F2B/7A for detection and F2B/12H for capture. This combination also provided highly sensitive assays for rat and human AMH. The assay is available through DSL Inc. (DSL-10-14400).

Animals

C57Bl/6J wild type mice were obtained from the Animal Facility of the Erasmus Medical Center (Rotterdam, The Netherlands) and were kept under standard animal housing conditions in accordance with the National Institutes of Health guidelines for the Care and Use of Experimental Animals. Animals were killed at various ages ranging from 4-18 months of age, when possible at estrus. For each age group, ovaries from eight to 10 mice were collected. To determine the day of the cycle, daily vaginal smears were taken for a period of at least 2 wk and examined as described previously [20]. Mice were killed at 1400 h by decapitation, blood samples were collected, and serum was isolated as described previously [20]. Serum samples were stored at –20 C until assayed for AMH. Ovaries were removed and fixed overnight in Bouin's fluid at room temperature. For histological examination of the follicle population, fixed ovaries were embedded in paraffin, and after routine histological procedures, 8-µm sections were mounted on slides and stained with hematoxylin and eosin.

Follicle counting

Follicle count was performed as described previously [20], using one ovary per animal. Follicles were classified based on their mean diameter, which was determined by measuring two perpendicular diameters in the section in which the nucleolus of the oocyte was present. Primordial follicles (diameter <20 µm) were counted in every 10th section in all mice, whereas growing follicles were counted in every fifth section in four randomly selected mice per age group.

Immunohistochemistry

For immunohistochemical staining, sections were mounted on 3-aminopropyltriethoxysilane (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands)-coated slides. After deparaffinization, sections were quenched for 20 min in 3% H_2O_2 / methanol solution to block endogenous peroxidase activity, washed with water, and transferred to PBS. Sections were subjected to heat-induced antigen retrieval for 3 x 5 min at 700 W in 0.01 M citric acid buffer, pH 6.0 (Merck, Darmstadt, Germany), in a microwave oven, cooled down to room temperature, rinsed in PBS, subsequently incubated with a biotinylated AMH mouse monoclonal antibody (antibody 5/6A, MCA2246; Serotec; Ref. 23), and diluted 1:100 at 4 C overnight followed by a wash step with PBS. Next, sections were incubated for 30 min at room temperature with streptavidin-biotin-peroxidase complex (ABC; diluted 1:200 in PBS; Dako, Glostrup, Denmark), and washed three times with PBS, and the peroxidase activity was developed with 0.07% 3,3'-diamin-

obenzidine tetrahydrochloride (DAB; Sigma-Aldrich). Finally, all sections were counterstained with hematoxylin.

Quantitative analysis of the AMH staining intensity in four to five follicles per age group was performed using the ImageJ software 1.35p (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

To biotinylate the 5/6A antibody, the antibody was concentrated to approximately 1.5 mg/ml and dialyzed against 0.1 M $NaHCO_3$ with several changes overnight. Sulfo-NHS-LC-Biotin (Pierce, Perbio Sciences Nederland B.V., Etten-Leur, The Netherlands) was dissolved in water to give a 2-mg/ml solution, and 50 µl were added to 1 ml of antibody (at 1 mg/ml) and incubated for 2 h at 4 C. The biotinylation reaction was stopped by adding 0.1 ml of 1 M NH_4Cl. Next, the solution was dialyzed against PBS with two changes over 2 d. A 0.1% sodium azide solution was prepared, and the biotinylated antibody was stored at 4 C.

AMH ELISA procedure

A standard line was included for each plate. Human AMH standards made in working strength HPE buffer and mouse serum samples diluted 1:41 in HPE buffer were added to duplicate wells (50 µl/well) on F2B12/H antibody-coated microplates and incubated for 2 h at room temperature. After washing with PBS/Tween (300 µl/well three to five times), 50 µl of biotinylated AMH monoclonal antibody F2B7/A was added at a 1:3000 dilution in 1% (wt/vol) casein buffer. After 1 h incubation at room temperature, the plate was washed five times with PBS/Tween (300 µl/well). Next, the wells were incubated for 30 min at room temperature with the poly HRP conjugate (50 µl /well) at a 1:20.000 dilution in 1% (wt/vol) casein buffer. After washing with PBS/Tween (300 µl/well, five to seven times), followed by washing with deionized water, TMB substrate (100 µl/well) was added. After 10 min of incubation at room temperature in the dark, the chromogenic reaction was stopped by adding 100 µl of 6% (vol/vol) phosphoric acid to each well, and absorbances were read at 450 nm with a reference wavelength set at 655 nm using a micro plate reader (Bio-Rad, Hemel Hempstead, UK).

Statistical analysis

Data were analyzed with SSPS11 (SPSS Inc., Chicago, IL) and expressed as mean ± SEM. Data were evaluated for statistical differences by one-way ANOVA, followed by Duncan's new multiple range test. Differences were considered significant at $P < 0.05$. The correlations between different parameters were assessed using Spearman's correlation coefficients.

RESULTS

Specificity

Out of the antibody combinations tested for the mouse AMH immunoassay antibody F2B/12H was selected for plate coating and F2B/7A for biotinylation. This combination gave the highest sensitivity for mouse AMH and was one of the best for detecting immunoreactivity in human serum and rat recombinant AMH samples as well. The assay did not recognize other TGFβ family members, such as BMP4, TGFβ, and activin. Furthermore, serum from AMH null mice gave no signal, confirming the specificity of the assay for AMH (results not shown).

Western blot analysis of human and rat recombinant AMH showed that the F2B/7A antibody recognizes epitopes in the pro-region under both reducing (Figure 3.1) and nonreducing (results not shown) conditions, whereas antibody F2B/12H recognizes epitopes in the mature region of AMH under nonreducing conditions only (Figure 3.1). Based on the recognition of both regions, the assay is expected to measure total AMH.

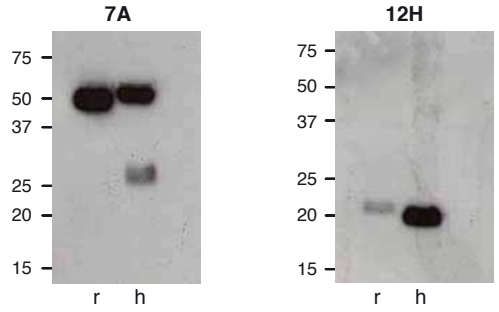

Figure 3.1 Western blot analysis of rat and human recombinant AMH using the detector (7A) and capture (12H) monoclonal antibodies. Antibody 7A recognizes the full-length 57-kD N-terminal pro-region, and a second 30-kD subunit (as a result of a possible second cleavage site in human AMH) under reducing conditions. Antibody 12H recognizes the mature region under nonreducing conditions only. r, Recombinant rat AMH; h, recombinant human AMH.

Stability of immunoreactivity

Studies with an earlier AMH assay developed by our group [29] showed that the human recombinant standard preparation was unstable unless stored frozen. This was shown to be due to the sensitivity of epitopes in the pro-region to proteases in the sample. In contrast, in the present assay, which uses different antibodies, recombinant human AMH could be incubated for 7 d at 37 C with no change in the apparent concentration. AMH immunoreactivity in serum samples in both assays was also stable for several days at room temperature and

after repeated freeze thaw cycles, whereas heat-inactivation of serum samples resulted in a reduction of immunoreactivity (data not shown).

Assay range, sensitivity, and specificity

The range of the AMH standards used in this assay was from 5-0.037 ng/ml (Figure 3.2). The detection limit, defined as the mean of the absorbance of the blank replicates + 2 SDS, was 6.3 pg/ml. The mean interassay and intraassay coefficients of variation were less than 10% and less than 5%, respectively.

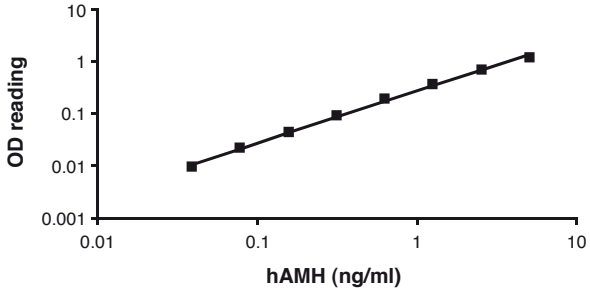

Figure 3.2 Standard curve of the AMH ELISA. The error bars are too small to be visible in the graph.

Parallelism and recovery

To test the parallelism of the assay, four mouse serum samples and a recombinant rat AMH sample were serially diluted with HPE buffer and assayed. The dilution curve for each sample was linear ($r^2 > 0.99$), and showed parallelism with the standard curve (Figure 3.3). In addition, six serum samples were spiked with a known amount of recombinant rat AMH. The average percentage recovery of recombinant AMH from mouse serum samples was 103.9 ± 3.0%.

AMH levels in aging mice

Serum AMH levels were determined in mice of various ages. With increasing age, serum AMH levels declined significantly ($r = -0.84$, $P < 0.0001$). Mice could be subdivided into three groups reflecting the reproductive status of the mice based on their AMH levels. The first group of 4-8 months of age with an average serum AMH level of 28.34 ± 7.12 ng/ml were all fertile mice. The second group of 10-12 months of age contained mice with an irregular cycle, in which AMH levels had declined significantly to 20.82 ± 5.35 ng/ml ($P < 0.05$). A further significant decline in AMH levels (5.62 ± 3.78 ng/ml, $P < 0.05$) was observed in the third group of 14-18 months of age, which represents mice at anestrus (Figure 3.4A).

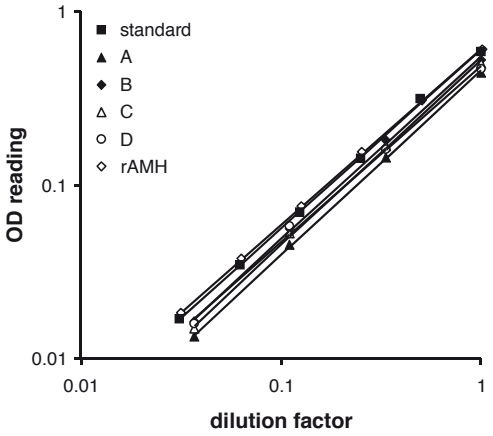

Figure 3.3 Parallelism of dilution curves for mouse serum samples. Data presented are the results of serial dilutions of the standard, a recombinant rat AMH sample, and four mouse serum samples.

Number of primordial and growing follicles in aging mice

Analysis of the follicle dynamics revealed that the number of both primordial and growing follicles declined with increasing age (r = -0.89, $P < 0.0001$, and r = -0.94, $P < 0.0001$, respectively). The number of primordial follicles decreased progressively during the period between 4-8 months of age, as the numbers of primordial follicles in the 4-, 6-, and 8-month-old groups differed significantly ($P < 0.05$). In older mice, the decline of primordial follicle numbers had decelerated, because the number of primordial follicles was not different between mice of 10-12 months of age, and between mice aged 14-18 months. The number of primordial follicles was significantly different between these groups ($P < 0.05$) (Figure 3.4B). In contrast, the number of growing follicles did not decline in mice of 4-8 months of age, and their decline only became evident in older mice, with numbers in mice of 10-14 months of age being significantly different from those in mice of 16-18 months of age ($P < 0.05$) (Figure 3.4B).

Spearman's correlation coefficient showed that the numbers of growing follicles correlated strongly with the numbers of primordial follicles in the same animal (r = 0.93, $P < 0.0001$) (Figure 3.4C).

AMH expression in aging mice

The decrease in serum AMH with increasing age might be caused by a decline in AMH expression per follicle. Therefore, we performed immunohistochemistry to determine the expression pattern of AMH in ovaries of 4- to 16-month-old mice (Figure 3.5). At 4 months of age, AMH staining was found in granulosa cells of small growing follicles up to the small antral stage. Weak staining was observed in primary follicles with a single layer of granulosa cells. Strongest expression

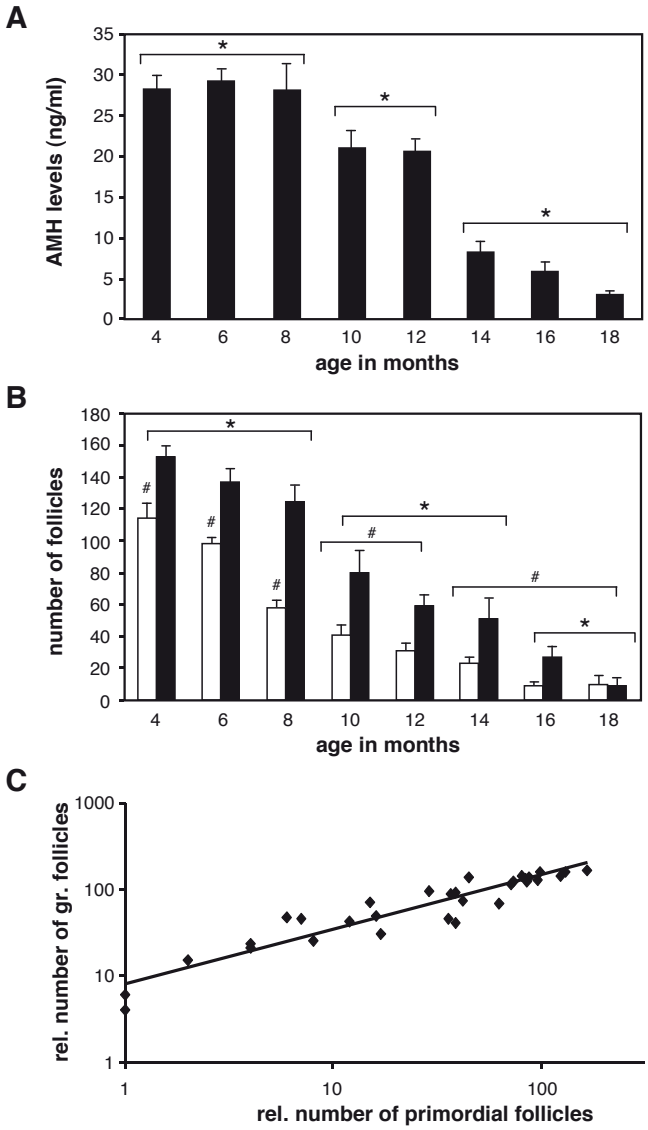

Figure 3.4 Serum AMH levels and follicle numbers in aging mice. A, Serum AMH levels declined in aging mice (n = 8-10 per age group, r = -0.84, P < 0.0001). *, Statistically significant groups, P < 0.05. B, The relative numbers of primordial (open bars, n = 8-10 mice per age group) and growing follicles (closed bars, n = 4 mice per age group) declined in mice of increasing age (r = -0.89, P < 0.0001, and r = -0.94, P < 0.0001, respectively). # and *, Age-groups of mice with statistically significant numbers of primordial follicles and number of growing follicles, respectively, P < 0.05. C, Correlation between the relative numbers of growing follicles with the relative numbers of primordial follicles (r = 0.93, P < 0.0001, n = 4 mice per age group).

was observed in large preantral follicles with several layers of granulosa cells, whereas expression decreased in small antral follicles. AMH expression was absent in large antral follicles. Ovaries of aging (8 and 12 months old) and aged (16 months old) mice showed a similar expression pattern, with AMH expression being strongest in the large preantral follicles. Furthermore, the AMH staining intensity of similar sized follicles in aging and aged mice appeared to be similar to those of 4-month-old mice (Figure 3.5). Indeed, quantitative analysis of the

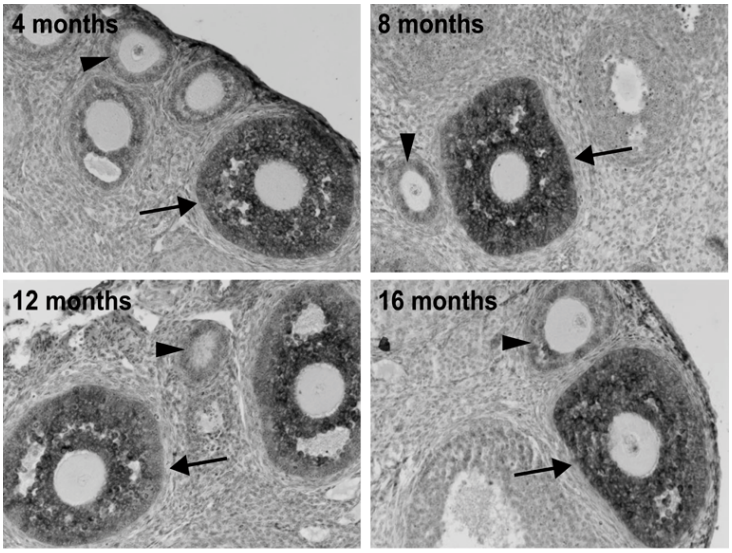

Figure 3.5 Immunohistochemical analysis of AMH expression in aging mice. AMH is expressed in granulosa cells of growing follicles and staining intensity does not differ between mice of 4, 8, 12, or 16 months of age. Arrowheads indicate small preantral follicles; arrows indicate large preantral follicle.

immunolabeling using ImageJ software showed no difference in the staining intensities (results not shown).

AMH levels correlate with the primordial and growing follicles pool

Because AMH expression within a follicle class did not change with age, we next examined whether the decline in serum AMH levels reflects the decrease in number of follicles with age. Indeed, a strong correlation was observed between AMH levels and numbers of growing follicles ($r = 0.86$, $P < 0.0001$) (Figure 3.6A). In addition, AMH levels correlated strongly with the numbers of primordial follicles ($r = 0.83$, $P < 0.0001$) (Figure 3.6B).

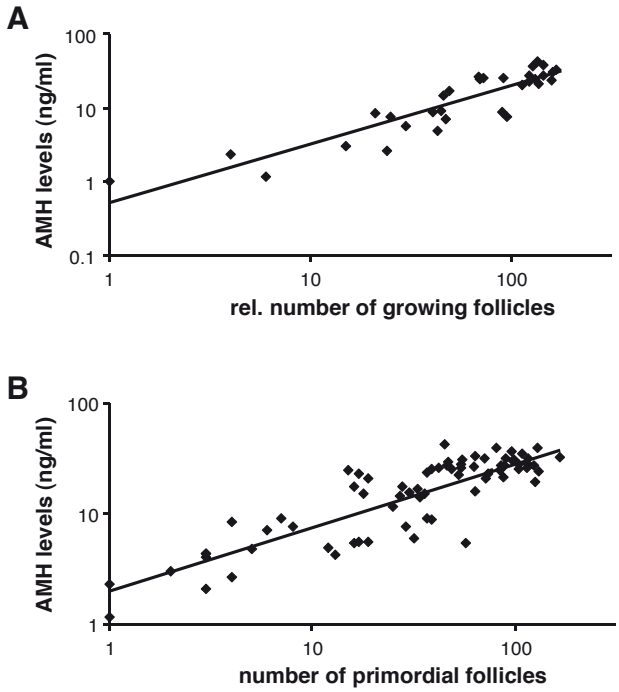

Figure 3.6 Correlations between serum AMH levels and relative numbers of growing (A) (n = 4 mice per age group) and primordial (B) follicles (n = 8-10 mice per age group) (r = 0.86, P < 0.0001 and r = 0.83, P < 0.0001, respectively).

DISCUSSION

At the end of reproductive life, women enter menopause, whereas rodents enter a permanent vaginal estrous or diestrous phase, also known as anestrus. In both species the decline in fertility with increasing age is related to the concomitant decline of the primordial follicle pool [4, 31]. AMH has been identified as one of the intraovarian growth factors that regulate the rate at which primordial follicles are recruited for further growth. In neonatal ovaries cultured in the presence of AMH, the recruitment of primordial follicles was inhibited [30]. In mice deficient of AMH, more primordial follicles are recruited, resulting in a larger pool of growing follicles. As a consequence of the increased rate of recruitment, the primordial follicle pool is depleted at an earlier age than wild-type mice [20].

Recently, serum levels of AMH have been shown to be a promising marker for ovarian aging in women (reviewed in [24]). In this study, we show that, similar to the situation in women, AMH levels decrease in mice of increasing age. This decline in serum AMH is not reflected by a similar change in AMH expression level. Using immunohistochemical analysis, we show that the expression level of

AMH within growing follicles remains similar with increasing age, indicating that the expression of AMH is independent of other aging markers such as FSH and inhibin B. Furthermore, the constant AMH expression per follicle suggests that the decrease in serum AMH levels in aging mice directly reflects the decrease in number of AMH-expressing follicles. Indeed, serum AMH levels correlated strongly with the number of growing follicles in this study. This finding is in agreement with results obtained in women in whom serum AMH levels correlated with AFC [12, 14].

It has been suggested that the size of the primordial follicle pool is reflected by the number of growing follicles, in both women and mice [11, 32]. This suggestion was confirmed in the current study in which we observed a high correlation between numbers of growing and primordial follicles. Moreover, we observed that serum levels of AMH were strongly correlated with the number of primordial follicles. Both AMH levels and the number of primordial follicles declined with increasing age. However, whereas size of the primordial follicle pool decreased from the age of 4 months onward, AMH levels did not change initially. Similarly, the number of growing follicles remained constant during the early reproductive period in mice, which explains the constant levels of AMH during this period. This suggests that compensatory mechanisms are present to maintain the number of growing follicles, and therefore, serum AMH levels, at a constant level despite a declining primordial follicle pool. It has been suggested that protection from oocyte degeneration and atresia may play a role in the preservation of the growing follicle pool [32]. It is also possible that relatively more follicles are recruited from the declining primordial follicle pool to establish a constant number of growing follicles. However, in unilateral ovariectomized mice and rats, in which the primordial follicle pool is reduced by 50%, an increased recruitment was only observed immediately after ovariectomy to establish a normal number of ovulations. In the long-term, a reduction of the number of atretic antral follicles appears to be the main mechanism to obtain the appropriate number of preovulatory follicles [33, 34]. Interestingly, these compensatory ovulations are absent in aged unilateral ovariectomized animals as they show an earlier onset of irregular cyclicity and infertility than control mice [35, 36].

Although little is known about the mechanisms that regulate follicle recruitment and early follicle growth, it is likely that both stimulatory and inhibitory signals are involved. These may be factors produced by both oocyte and granulosa cells acting in an autocrine and paracrine fashion. AMH has been identified as one of the factors that inhibit follicle recruitment [20]. However, despite the increased AMH levels relative to the size of the primordial follicle pool, primordial follicle recruitment, as reflected by the number of remaining follicles, is not decelerated

during early reproductive life. Thus, our present results on serum levels of AMH suggest that AMH is not a dominant factor in the regulation of follicle numbers in aging mice. However, a strong conclusion may not be drawn from this data, since serum AMH levels may not give a proper reflection of the intraovarian action of AMH.

From 8 months of age onwards, both serum AMH levels and the number of growing follicles showed a steady decline, suggesting that after the size of the primordial pool has reached a certain threshold level, the compensatory mechanisms to maintain the pool of growing follicles at a fixed number may no longer be sufficient. Interestingly, toward the end of reproductive life, the depletion of the primordial follicle pool slowed down, whereas the gradual decline in the number of growing follicles did not change. This suggests that, during early reproductive life, the use of primordial follicles is relatively wasteful, whereas, at the end of reproductive life, primordial follicles are used more efficiently.

In conclusion, our results indicate that serum AMH is an excellent marker for the size of the primordial follicle pool and, therefore, for ovarian aging in mice. Our results also imply that AMH levels may only have predictive value when changes in fertility are already apparent because changes in serum levels were only evident in mice that already displayed cycle irregularities.

Similarly, also in women, serum AMH levels are predictive when changes in fertility are present. In women, cycle irregularities are preceded by a decline in fertility [2], and indeed changes in serum AMH levels are present before cycle irregularities occur [12, 37]. Studies indicate that AMH is an equally good predictor as AFC for the decline in fertility [38]. However, when after adjusting for age, only serum AMH levels remains predictive for cycle irregularities [37]. Nevertheless, more studies are necessary to determine the relationship between serum AMH levels and status of fertility, preferably in a prospective study. Nevertheless, studies in women have shown that, currently, serum AMH levels are the earliest marker for ovarian aging, and give a better prediction than serum levels of FSH or inhibin B [12, 37, 38], which may be particularly important for women at risk of early ovarian aging, such as survivors of childhood cancer.

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REFERENCES

1. **den Tonkelaar I, te Velde ER, Looman CW** 1998 Menstrual cycle length preceding menopause in relation to age at menopause. Maturitas 29:115-23
2. **te Velde ER, Dorland M, Broekmans FJ** 1998 Age at menopause as a marker of reproductive ageing. Maturitas 30:119-25
3. **te Velde ER, Pearson PL** 2002 The variability of female reproductive ageing. Hum Reprod Update 8:141-54
4. **te Velde ER, Scheffer GJ, Dorland M, Broekmans FJ, Fauser BC** 1998 Developmental and endocrine aspects of normal ovarian aging. Mol Cell Endocrinol 145:67-73
5. **Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF** 1992 Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod 7:1342-6
6. **Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL** 2004 Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428:145-50
7. **Johnson J, Bagley J, Skaznik-Wikiel M, Lee HJ, Adams GB, Niikura Y, Tschudy KS, Tilly JC, Cortes ML, Forkert R, Spitzer T, Iacomini J, Scadden DT, Tilly JL** 2005 Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 122:303-15
8. **Gougeon A** 1996 Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 17:121-55
9. **Burger HG, Dudley EC, Hopper JL, Shelley JM, Green A, Smith A, Dennerstein L, Morse C** 1995 The endocrinology of the menopausal transition: a cross-sectional study of a population-based sample. J Clin Endocrinol Metab 80:3537-45
10. **Burger HG, Dudley EC, Hopper JL, Groome N, Guthrie JR, Green A, Dennerstein L** 1999 Prospectively measured levels of serum follicle-stimulating hormone, estradiol, and the dimeric inhibins during the menopausal transition in a population-based cohort of women. J Clin Endocrinol Metab 84:4025-30
11. **Scheffer GJ, Broekmans FJ, Looman CW, Blankenstein M, Fauser BC, de Jong FH, te Velde ER** 2003 The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. Hum Reprod 18:700-6
12. **de Vet A, Laven JS, de Jong FH, Themmen APN, Fauser BC** 2002 Antimullerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril 77:357-62
13. **van Rooij IA, Broekmans FJ, te Velde ER, Fauser BC, Bancsi LF, de Jong FH, Themmen APN** 2002 Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod 17:3065-71
14. **Fanchin R, Schonauer LM, Righini C, Guibourdenche J, Frydman R, Taieb J** 2003 Serum anti-Mullerian hormone is more strongly related to ovarian follicular status than serum inhibin B, estradiol, FSH and LH on day 3. Hum Reprod 18:323-7
15. **Vigier B, Picard JY, Tran D, Legeai L, Josso N** 1984 Production of anti-Mullerian hormone: another homology between Sertoli and granulosa cells. Endocrinology 114:1315-20
16. **Ueno S, Takahashi M, Manganaro TF, Ragin RC, Donahoe PK** 1989 Cellular localization of mullerian inhibiting substance in the developing rat ovary. Endocrinology 124:1000-6
17. **Ueno S, Kuroda T, Maclaughlin DT, Ragin RC, Manganaro TF, Donahoe PK** 1989 Mullerian inhibiting substance in the adult rat ovary during various stages of the estrous cycle. Endocrinology 125:1060-6

18. **Munsterberg A, Lovell-Badge R** 1991 Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. Development 113:613-24
19. **Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen APN, Grootegoed JA** 1995 Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136:4951-62
20. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-96
21. **Durlinger ALL, Visser JA, Themmen APN** 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124:601-9
22. **Hirobe S, He WW, Lee MM, Donahoe PK** 1992 Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 131:854-62
23. **Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC, Themmen APN** 2004 Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 10:77-83
24. **Visser JA, de Jong FH, Laven JS, Themmen APN** 2006 Anti-Müllerian Hormone: a new marker for ovarian function. Reproduction 131:1-9
25. **Cook CL, Siow Y, Taylor S, Fallat ME** 2000 Serum mullerian-inhibiting substance levels during normal menstrual cycles. Fertil Steril 73:859-61
26. **La Marca A, Malmusi S, Giulini S, Tamaro LF, Orvieto R, Levratti P, Volpe A** 2004 Anti-Mullerian hormone plasma levels in spontaneous menstrual cycle and during treatment with FSH to induce ovulation. Hum Reprod 19:2738-41
27. **La Marca A, Giulini S, Orvieto R, De Leo V, Volpe A** 2005 Anti-Mullerian hormone concentrations in maternal serum during pregnancy. Hum Reprod 20:1569-72
28. **Fanchin R, Schonauer LM, Righini C, Frydman N, Frydman R, Taieb J** 2003 Serum anti-Mullerian hormone dynamics during controlled ovarian hyperstimulation. Hum Reprod 18:328-32
29. **Al-Qahtani A, Muttukrishna S, Appasamy M, Johns J, Cranfield M, Visser JA, Themmen APN, Groome NP** 2005 Development of a sensitive enzyme immunoassay for anti-Mullerian hormone and the evaluation of potential clinical applications in males and females. Clin Endocrinol (Oxf) 63:267-73
30. **Durlinger ALL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen APN** 2002 Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076-84
31. **Jones EC, Krohn PL** 1961 The relationships between age, numbers of oocytes and fertility in virgin and multiparous mice. J Endocrin 21:469-495
32. **Gosden RG, Laing SC, Felicio LS, Nelson JF, Finch CE** 1983 Imminent oocyte exhaustion and reduced follicular recruitment mark the transition to acyclicity in aging C57BL/6J mice. Biol Reprod 28:255-60
33. **Hirshfield AN** 1982 Follicular recruitment in long-term hemicastrate rats. Biol Reprod 27:48-53
34. **Gosden RG, Telfer E, Faddy MJ, Brook DJ** 1989 Ovarian cyclicity and follicular recruitment in unilaterally ovariectomized mice. J Reprod Fertil 87:257-64

35. **Brook JD, Gosden RG, Chandley AC** 1984 Maternal ageing and aneuploid embryos--evidence from the mouse that biological and not chronological age is the important influence. Hum Genet 66:41-5
36. **Peppler RD** 1971 Effects of unilateral ovariectomy on follicular development and ovulation in cycling, aged rats. Am J Anat 132:423-7
37. **van Rooij IA, Tonkelaar I, Broekmans FJ, Looman CW, Scheffer GJ, de Jong FH, Themmen APN, te Velde ER** 2004 Anti-mullerian hormone is a promising predictor for the occurrence of the menopausal transition. Menopause 11:601-6
38. **van Rooij IA, Broekmans FJ, Scheffer GJ, Looman CW, Habbema JD, de Jong FH, Fauser BJ, Themmen APN, Te Velde ER** 2005 Serum antimullerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: A longitudinal study. Fertil Steril 83:979-87

Chapter 4

AMH and AMH type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women

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ABSTRACT

Background: In mice, AMH inhibits primordial follicle recruitment and decreases FSH sensitivity. Little is known about the role of AMH in human ovarian physiology. We hypothesize that in women AMH has a similar role in ovarian function as in mice and investigated this using a genetic approach.

Methods: The association of the AMH Ile^{49}Ser and the AMH type II receptor –482 A>G polymorphisms with menstrual cycle characteristics was studied in a Dutch (n=32) and a German cohort (n=21) of normo-ovulatory women.

Results: Carriers of the AMH ^{49}Ser allele had higher estradiol (E_2) levels on menstrual cycle day three compared to non-carriers in the Dutch cohort (P=0.012) and in the combined Dutch and German cohort (P=0.03). Carriers of the AMHR2 –482G allele also had higher follicular phase E_2 levels compared to non-carriers in the Dutch cohort (P=0.028), the German cohort (P=0.048), and hence also the combined cohort (P=0.012). Women carrying both AMH ^{49}Ser and AMHR2 –482G alleles had highest E_2 levels (P=0.001). For both polymorphisms no association with AMH or FSH levels was observed.

Conclusions: Polymorphisms in the AMH and AMHR2 gene are associated with follicular phase E_2 levels, suggesting a role for AMH in the regulation of FSH sensitivity in the human ovary.

INTRODUCTION

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a member of the transforming growth factor-β (TGFβ) family. The ovarian expression pattern of AMH is similar in women and mice. During folliculogenesis AMH expression starts in the granulosa cells of primary follicles, is highest in granulosa cells of preantral and small antral follicles and gradually diminishes in the subsequent stages of follicle development [1]. The expression pattern of AMH is flanked by two major regulatory steps of folliculogenesis, primordial follicle recruitment and cyclic selection [2], suggesting that AMH may influence these steps. Indeed, studies in AMH knockout (AMHKO) mice revealed that in the absence of AMH, follicles are recruited at a faster rate and that follicles display an increased sensitivity to follicle stimulating hormone (FSH) [3, 4].

In mice and women, the increase in circulating FSH at the day of estrus and during the follicular phase of the menstrual cycle, respectively, results in selection of a group of antral follicles from the growing follicle pool. In this selected group, each follicle exerts its own threshold concentration of FSH for further development and the follicle(s) with highest FSH sensitivity will gain dominance and ovulate [2]. Thus, the inhibitory effect of AMH on FSH sensitivity of follicles might play a role in the process of follicle selection. Furthermore, AMH attenuates the FSH-dependent increase in aromatase activity and luteinizing hormone (LH) receptor expression in rat granulosa cell cultures [5]. Therefore, AMH might affect estradiol (E_2) production by granulosa cells.

Studies in women, so far, have focused on the role of serum AMH as a marker for ovarian function. AMH levels strongly correlate with the number of antral follicles detected by ultrasound [6] and reflect the size of the primordial follicle pool, which makes AMH an ideal marker for ovarian reserve, as reviewed by Visser *et al.* [7]. In pathophysiologic conditions, such as Polycystic Ovary Syndrome (PCOS), AMH serum levels also correlate with antral follicle count [8, 9] and may be used as a diagnostic marker for this syndrome [7].

Little attention has been paid to the functional role of AMH in human ovarian physiology. Based on its expression pattern in women, we anticipated that AMH has a similar role in ovarian function as in mice, *i.e.* inhibition of primordial follicle recruitment and of FSH-sensitivity. In this study we explored the function of AMH in normo-ovulatory women using a genetic approach. Individual variation in menstrual cycle dynamics partially results from genetic variation (*i.e.* polymorphisms) in genes encoding proteins involved in this process. For example, a single nucleotide polymorphism (SNP) at position 680 in the FSH receptor, resulting in an asparagine into serine change ($Asn^{680}Ser$; rs6166), has

been shown to be associated with FSH levels and the length of the menstrual cycle [10].

In the present study, we first identified polymorphisms in the AMH gene and its specific type II receptor (AMHR2) gene and subsequently investigated the association of a number of these polymorphisms with hormone levels and ovarian parameters in two population-based cohorts of Caucasian healthy pre-menopausal women. We observed that these polymorphisms are associated with estrogen levels during the follicular phase of the menstrual cycle.

MATERIAL AND METHODS

Identification of polymorphisms in the AMH and AMHR2 genes

To identify novel polymorphisms, we sequenced the complete coding region, including intron/exon boundaries, of the AMH and AMHR2 genes, and 1 kb of promoter region of the AMH gene in 45 randomly selected Dutch Caucasian blood donor samples. In addition, the NCBI (www.ncbi.nlm.nih.gov) and International HapMap Project (www.hapmap.org) [11] SNP databases were used to search for polymorphisms in the AMH and AMHR2 gene. PCR reactions were performed using PCR Master Mix (Promega, Leiden, The Netherlands), with conditions according to the instructions of the manufacturer. To amplify GC rich regions 5% DMSO was added to the PCR reaction. PCR products were purified using a GFX 96 wells kit (Amersham Biosciences, Roosendaal, The Netherlands), according to the instructions of the manufacturer. Sequence reactions were performed using the Big-Dye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) and purified with the Dyex 96 kit (Qiagen, Venlo, The Netherlands). Sequence products were analyzed on a ABI prism 3100 automatic capillary sequencer (Applied Biosystems).

Subjects

We studied a Caucasian subset (n=32) of a previously described Dutch cohort [12] of whom DNA and a complete hormone assessment were available. These normo-ovulatory women had an age of 20-35 years and a body mass index (BMI) of 19-26 kg/m^2. Assessment of serum hormone levels and transvaginal ultrasounds were performed on day 3 of the menstrual cycle. Serum FSH and LH were measured by chemiluminescent immunoassay [Immulite, Diagnostic Products corporation (DPC), Los Angeles, CA, USA]. Serum E_2 concentrations were measured using radioimmunoassay kits provided by DPC, as described

previously [13]. Inhibin B was measured using an immunoenzymometric assay (Serotec, Oxford, UK) and AMH levels were measured using an ELISA assay (Immunotech-Coulter, Marseille, France). Intra- and interassay coefficients of variation (CV) were <3% and 8% for FSH, <5% and 15% for LH, <5% and 7% for E_2, <9% and 15% for inhibin B and <5% and 8% for AMH, respectively [12, 14, 15].

In addition, a German cohort consisting of 21 premenopausal normo-ovulatory women of Caucasian origin, selected by FSH receptor genotype [10] was studied. Women in this study cohort had an age of 18-34 yr and a BMI of 19-28 kg/m^2. In these women the complete menstrual cycle was monitored by performing transvaginal ultrasound and measuring serum hormone levels [10]. FSH and LH were measured by an immunofluorimetric assay and E_2 by fluoroimmunoassay using the Autodelfia system (Perkin-Elmer, Freiburg, Germany) as described previously [16]. Progesterone was measured by RIA using the Coat-a-Count RIA kit by DPC (Bad Nauheim, Germany) according to the instructions of the manufacturer. Inhibin A and inhibin B were measured by highly specific ELISA using the Serotec kits purchased from DSL (Sinsheim, Germany). AMH levels were measured using an in-house AMH ELISA assay [17]. This AMH assay shows close correlation with the AMH ELISA assay of Immunotech (r=0.99). The values from the Immunotech assay were adjusted to the in-house AMH ELISA assay (x 1.73). For both FSH and LH intra- and interassay CV were <3%. Intra- and interassay CV were 2.2% and 2.7% for E_2, 3.7% and 6.1% for progesterone, 6.3% and 7.0 % for inhibin A and 5.3% and 7.0% for inhibin B and <10% and <5% for AMH, respectively.

Genotyping

Genomic DNA was extracted from peripheral blood using standard DNA extraction methods. Genotypes were determined using Taqman allelic discrimination assays. For the AMH Ile^{49}Ser polymorphism an Assay-by-Design with the following probes was used: 5'-CTCCAGGCA**t**CCCACAA-3' and 5'-CCAGGCA**g**CCCACAA-3'. For the AMHR2 -482 A>G promoter SNP we used an Assay-on-Demand, Assay ID C_ 1673084_10. (Applied Biosystems).

Each PCR reaction contained 2 ng of dried genomic DNA, 1 µl of Taqman Universal PCR Master Mix 2x, 0.025 µl of the 80X AMH Ile^{49}Ser Assay Mix or 0.1 µl of the 20X AMHR2 -482 A>G mix in a total volume of 2 µl. The PCR reaction was performed according to the instructions of the manufacturer. The genotyping results were analyzed using an ABI prism 7900HT Sequence Detection System.

Statistical analysis

Using the blood donor samples, haplotypes of the AMH and AMHR2 gene were constructed using the PHASE program [18, 19]. To estimate linkage disequilibrium between SNPs, the pair-wise linkage disequilibrium coefficient (D'), and the correlation coefficient (r^2) were calculated by PHASE and Haploview version 3.2 [11].

Genotype distribution in the Dutch and German population was tested for Hardy-Weinberg equilibrium and the difference in genotype frequencies between the cohorts was tested using a chi-squared test for independence. One way analysis of (co)variance (AN(C)OVA) was used to determine differences between genotype groups in both cohorts. For reasons of statistical power, carriers of the AMH ^{49}Ser allele and carriers of the AMHR2 –482G allele were compared with non-carriers. Differences in AMH, FSH, E_2 and inhibin B levels and follicle number were adjusted for age. E_2 levels were log transformed to normalize their distribution. In the German cohort, a chi-squared test was used to test whether the distribution of the homozygous ^{680}Asn and ^{680}Ser FSHR genotypes over the AMH and AMHR2 genotypes was random. In the latter cohort, hormone levels were compared on two different time scales. The first time scale was based on the day of onset of menstruation (cycle day 0). The second scale was based on the day of the midcycle LH surge (day LH 0). The differences in hormone levels between the genotypes were tested using AN(C)OVA with repeated measures. In addition, results were corrected for multiple testing using the Bonferroni method. Subsequently, to increase statistical power, the Dutch and German cohort were analyzed together. An additive genetic effect of AMH and AMHR2 genotypes on E_2 levels was tested; trend analysis for the combination of the AMH and AMHR2 genotypes was performed for the presence of zero, one or two copies of the carrier genotypes, incorporating the additive variable in a multiple linear regression model. All analyses were performed using Statistical Package for Social Sciences, SPSS, version 11.0.1 (SPSS Inc, Chicago, IL). Data are presented as mean ± SEM. $P \leq 0.05$ was considered to be significant.

RESULTS

Identification of AMH and AMHR2 polymorphisms

Sequence analyses of 45 blood-donor samples revealed fifteen sequence variations, including eight novel SNPs, in the AMH gene and four variations, including one novel SNP in the AMHR2 gene (Figure 4.1). For the association studies polymorphisms with a minor-allele frequency (MAF) > 10% were selected for reasons

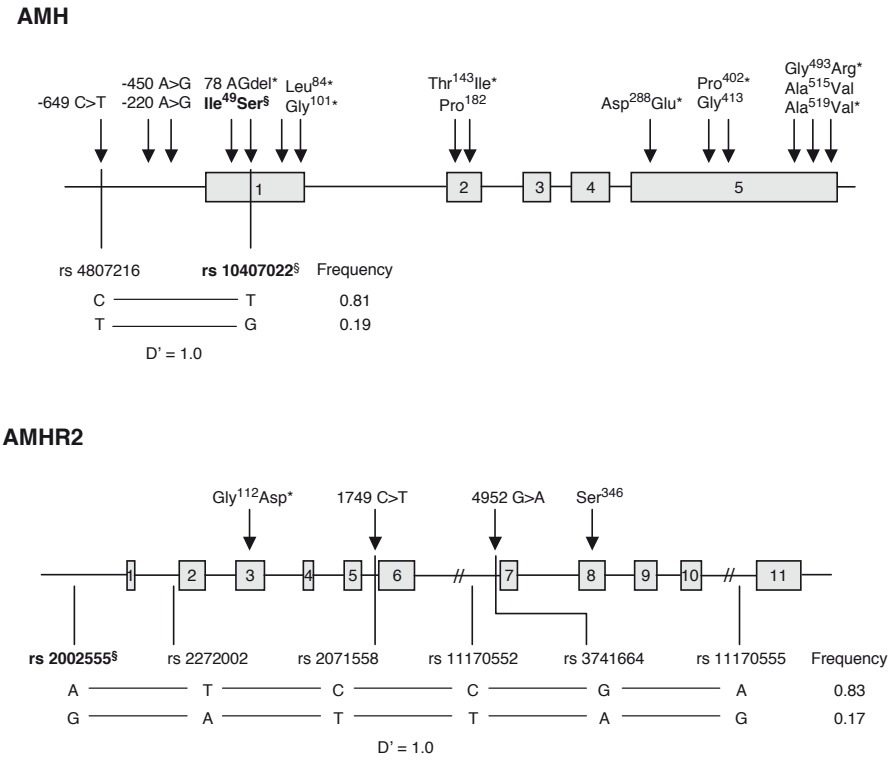

Figure 4.1 Schematic overview of the AMH (Chr. 19p13.3) and AMHR2 genes (Chr. 12q13), depicting the polymorphisms identified by sequencing (arrows). For the polymorphisms used for haplotype reconstruction, the database single nucleotide polymorphism (SNP) rs numbers are shown (including those identified in the HapMap database for AMHR2). D'=1.0 between those SNPs (D' = pair-wise linkage disequilibrium coefficient). Nucleotides are numbered relative to the translation start site. * Novel polymorphisms. §Genotyped for association study.

of statistical power. For the AMH gene two polymorphisms fulfilled this criterion; the –649 C>T promoter polymorphism (rs4807216) and the Ile^{49}Ser polymorphism (rs10407022). Reconstruction of haplotype alleles in the PHASE program revealed that these polymorphisms are in complete linkage disequilibrium (D' =1.0) and therefore we only genotyped the Ile^{49}Ser polymorphism. Similarly, in the AMHR2 gene two polymorphisms were selected: 1749 C>T (rs2071558) and 4952 G>A (rs3741664). Furthermore, four additional polymorphisms with a MAF > 0.10 were identified in the HapMap database, all in the noncoding region of the AMHR2 gene [17]. Analyses in Haploview showed that in Caucasians, these six AMHR2 polymorphisms are in complete linkage disequilibrium (D'=1 and r^2=1) (Figure 4.1). Hence, we only genotyped the -482 A>G promoter polymorphism as a tagging SNP.

Table 4.1 AMH Ile[49]Ser genotypes: hormone levels and ovarian parameters on day 3 of the menstrual cycle

	Dutch cohort			Dutch + German cohort		
AMH Ile49Ser	Ile/Ile	Ile/Ser + Ser/Ser	P	Ile/Ile	Ile/Ser + Ser/Ser	P
N (%)[a]	24 (75.0)	8 (25.0)	0.42	39 (73.6)	14 (26.4)	0.40
Age (year)	30.5 ± 0.8	28.1 ± 1.8	0.17	28.5 ± 0.7	27.3 ± 1.2	0.42
BMI (kg/m^2)	22.4 ± 0.7	20.5 ± 0.5	0.26	21.8 ± 0.4	22.5 ± 0.9	0.44
AMH (ng/ml)[b]	2.9 ± 0.5	3.4 ± 0.9	0.61	3.5 ± 0.4	4.8 ± 0.7	0.10
FSH (IU/L)[b]	7.1 ± 0.5	7.0 ± 0.9	0.93	6.8 ± 0.3	6.4 ± 0.6	0.47
LH (IU/L)	3.4 ± 0.3	3.3 ± 0.5	0.89	4.1 ± 0.3	3.7 ± 0.4	0.44
Estradiol (pmol/L)[b]	151.3 ± 13.2	215.0 ± 23.2	0.012	141.1 ± 10.2	178.7 ± 17.0	0.03
Inhibin B (ng/L)[b]	105.3 ± 11.8	126.7 ± 21.6	0.40	93.5 ± 8.2	109.7 ± 14.2	0.33
Antral follicle count[b,c]	14.7 ± 0.9	15.0 ± 1.6	0.87	15.6 ± 0.9	18.9 ± 2.5	0.13
Cycle length (day)	28.5 ± 0.4	28.3 ± 0.6	0.81	28.2 ± 0.3	28.3 ± 0.7	0.84

Data are presented as mean ± SEM. E_2, estradiol; BMI, body mass index.
[a] P-value for Hardy-Weinberg equilibrium. [b] Adjusted for age. [c] Antral follicles detectable by ultrasound, > 2-3 mm.

Association studies for the AMH Ile[49]Ser polymorphism

The AMH Ile[49]Ser genotype distribution in the Dutch cohort was in Hardy Weinberg equilibrium proportions and did not differ from frequencies observed in blood-donor samples or in Caucasians reported in the NCBI and HapMap database (Table 4.1). Age and body mass index (BMI) were similar between the AMH genotype groups, as were serum AMH, FSH, LH and Inhibin B levels (Table 4.1). However, carriers of the AMH [49]Ser allele had significantly higher E_2 levels on day 3 of the menstrual cycle compared to non-carriers (Table 4.1,

Table 4.2 AMHR2 –482 A>G genotypes: hormone levels and ovarian parameters on day 3 of the menstrual cycle

	Dutch cohort			Dutch + German cohort		
AMHR2 –482 A>G	A/A	A/G + G/G	P	A/A	A/G + G/G	P
N (%)[a]	20 (62.5)	12 (37.5)	0.20	35 (66.0)	18 (34.0)	0.51
Age (year)	29.4 ± 0.9	30.7 ± 1.1	0.38	27.6 ± 0.7	29.3 ± 1.1	0.12
BMI (kg/m^2)	22.3 ± 0.9	21.6 ± 0.9	0.62	22.1 ± 0.5	21.6 ± 0.7	0.52
AMH (ng/ml)[b]	3.6 ± 0.5	2.2 ± 0.7	0.12	4.2 ± 0.4	3.3 ± 0.6	0.21
FSH (IU/L)[b]	7.4 ± 0.5	6.5 ± 0.7	0.29	7.0 ± 0.4	6.1 ± 0.5	0.14
LH (IU/L)	3.5 ± 0.3	3.1 ± 0.4	0.34	4.0 ± 0.3	3.9 ± 0.4	0.89
Estradiol (pmol/L)[b]	145.6 ± 14.3	203.3 ± 18.5	0.028	132.6 ± 10.4	186.9 ± 14.7	0.012
Inhibin B (ng/L)[b]	107.4 ± 13.1	115.6 ± 16.8	0.70	93.9 ± 9.1	104.2 ± 12.2	0.52
Antral follicle count[b,c]	15.6 ± 0.9	13.4 ± 1.2	0.17	17.0 ± 1.0	15.4 ± 1.9	0.41
Cycle length (day)	28.4 ± 0.4	28.6 ± 0.5	0.62	28.3 ± 0.4	28.1 ± 0.5	0.80

Data are presented as mean ± SEM. [a] P-value for Hardy-Weinberg equilibrium. [b] Adjusted for age. [c] Antral follicles detectable by ultrasound, > 2-3 mm.

P=0.012). No association of the AMH genotypes with antral follicle count or cycle length was observed (Table 4.1).

The AMH Ile^{49}Ser genotype distribution in the German cohort was similar to the Dutch cohort (P=0.20). Since the German cohort was selected by FSHR genotype [10], we analyzed the distribution of the AMH genotypes over the FSHR genotypes. The AMH Ile^{49}Ser genotypes (Chr.19p13.3) were not randomly distributed over the genotypes of the FSHR Asn^{680}Ser polymorphism (Chr.2p21-16) (results not shown). Hence, possible associations of the AMH SNP could be driven by the FSHR SNP. Therefore, association analysis using the AMH genotypes was not performed in the German cohort alone. In contrast, in the Dutch cohort, the AMH Ile^{49}Ser genotypes were randomly distributed over the FSHR genotypes (results not shown), indicating that this non-random distribution found in the German cohort is a coincidence, and not due to genetic linkage.

Subsequently, the Dutch and German cohort were analyzed together and the FSHR genotype distribution was found to be randomly distributed over the AMH Ile^{49}Ser genotypes. In this combined cohort carriers of the ^{49}Ser allele again had higher E_2 levels (P=0.03) compared to non-carriers, whereas other hormone levels were not different between the genotype groups (Table 4.1).

Association studies for the AMHR2 –482 A>G polymorphism

The AMHR2 –482 A>G genotype distribution in the Dutch cohort was in Hardy Weinberg equilibrium proportions and did not differ from frequencies observed in blood donor samples or in Caucasians reported in the NCBI and HapMap database (Table 4.2). Carriers of the AMHR2 –482G allele had higher E_2 levels (P=0.028) compared to non-carriers, whereas age, BMI, other hormone levels and ovarian parameters were similar between carriers and non-carriers (Table 4.2).

Also in the German cohort, the AMHR2 –482 A>G genotype distribution was in Hardy Weinberg equilibrium proportions (P=0.51) and did not differ from the Dutch cohort (P= 0.30). The AMHR2 –482 A>G genotypes were equally distributed over the FSHR Asn^{680}Ser genotypes (P=0.62). In this German cohort, the AMHR2 polymorphism was not associated with AMH, FSH (Figures 4.2 and 4.3) and LH levels (results not shown) throughout the menstrual cycle. However, similar to the Dutch cohort, AMHR2 genotypes were significantly associated with E_2 levels during the early follicular phase of the menstrual cycle. AMHR2 -482G allele carriers had higher E_2 levels from cycle day 0 to cycle day 5 (P=0.048) and tended to have higher E_2 levels for the total follicular phase (P=0.077) (Figure 4.2). When aligned relative to the day of the LH peak E_2 levels were not significantly different (P=0.15) (Figure 4.3). Moreover, the preovulatory E_2 peak

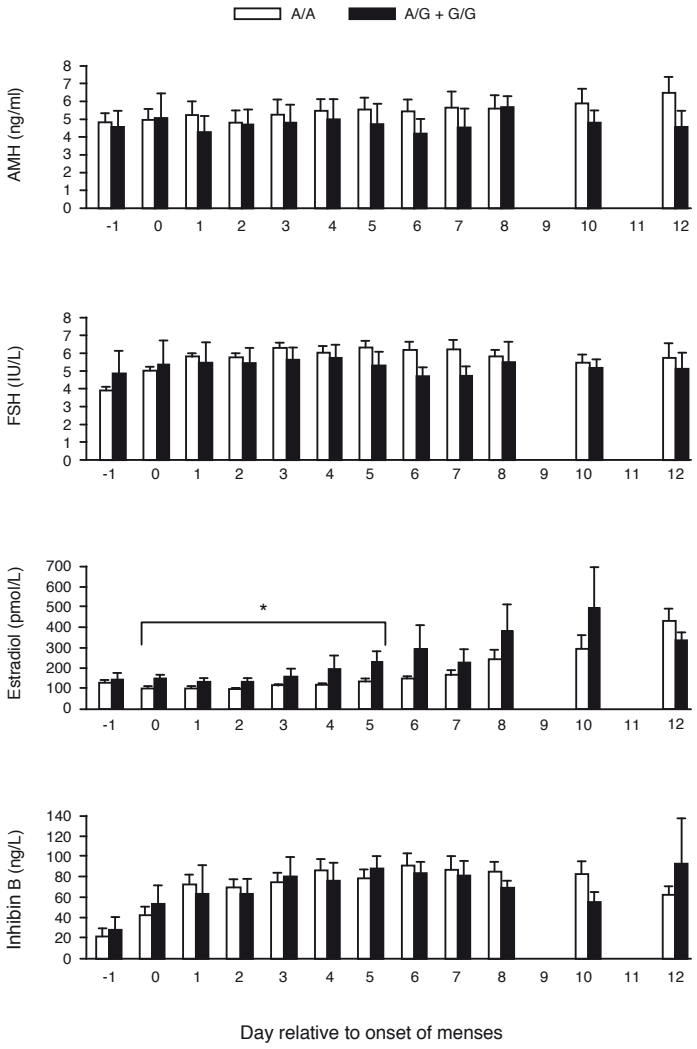

Figure 4.2 Serum levels of AMH, FSH, estradiol (E_2) and inhibin B during the follicular phase referenced to the first day of the menstrual cycle (0) in non-carriers (n=15) and carriers (n=6) of the AMHR2 –482G allele in the German cohort. Data represent the mean ± SEM; *significantly different between carriers and non-carriers, P ≤ 0.05.

was higher in the AMHR2 -482G allele carriers, although this failed to reach significance (P=0.10) (Table 4.3).

To obtain more insight in the difference in estrogen exposure between the carriers and non-carriers, we calculated the area under the curve (AUC) of the follicular phase E_2 levels, defined as the interval from the onset of menses till the preovulatory E_2 peak, and for the total menstrual cycle (Table 4.3). The AUC for follicular phase E_2 was significantly larger in the G-allele carriers (P=0.04).

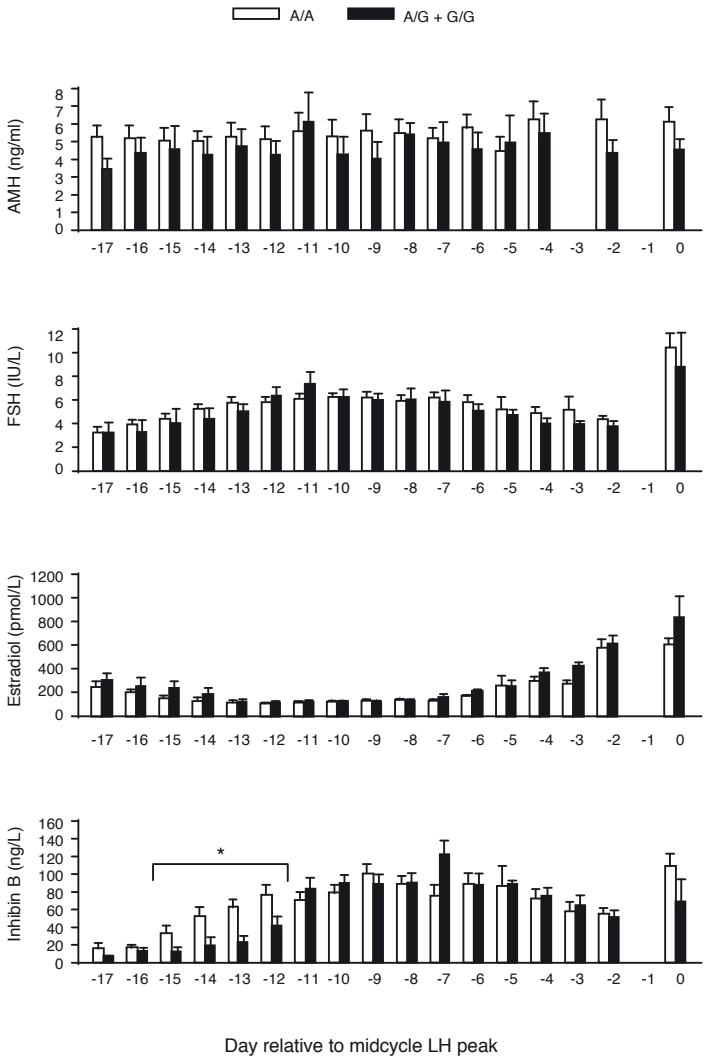

Figure 4.3 Serum levels of AMH, FSH, E_2 and inhibin B during the menstrual cycle referenced to the day of the LH surge (0) in non-carriers (n=15) and carriers (n=6) of the AMHR2 -482G allele in the German cohort. Data represent the mean ± SEM; *significantly different between carriers and non-carriers, $P \leq 0.05$.

However, the AUC for E_2 levels during the complete menstrual cycle was not different between carriers and non-carriers.

Inhibin B levels showed no differences between carriers and non-carriers of the G-allele when aligned relative to the onset of menses (P=0.61, Figure 4.2) or when aligned relative to the LH day (P=0.31). Nevertheless, when aligned relative to the LH day, inhibin B levels appear to rise later in carriers of the G-allele (Figure 4.3). Indeed, inhibin B levels from day LH -15 to day -12 were

Table 4.3 AMHR2 –482 A>G genotypes: ovarian parameters throughout the menstrual cycle.

	German cohort		
AMHR2 –482 A>G	A/A	A/G + G/G	P
N (%)[a]	15 (71.4)	6 (28.6)	0.51
Age (year)	25.1 ± 0.9	26.5 ± 1.9	0.46
BMI (kg/m^2)	22.0 ± 0.5	21.6 ± 1.2	0.70
E2 peak follicular phase[b]	738 ± 52	905 ± 243	0.10
AUC follicular E2[c]	2893 ± 172	3577 ± 225	0.04
AUC E2 total cycle	7613 ± 455	7909 ± 405	0.70
Length of follicular phase (day)[d]	14.1 ± 0.6	12.7 ± 1.4	0.30
Earliest detection of dominant follicle[e]	9.3 ± 0.8	6.8 ± 1.1	0.10
Mean diameter dominant follicle	14.9 ± 0.7	13.8 ± 0.8	0.36
Mean no of antral follicles[f]	19.6 ± 1.8	18.0 ± 3.4	0.72
Mean ovarian volume total (cm3)	13.8 ± 1.3	11.2 ± 0.8	0.24

Data are presented as mean ± SEM. AUC, area under the curve.
[a]P-value for Hardy-Weinberg equilibrium. [b]E_2 in pmol/l. [c]AUC from onset of menstruation until day of E_2 peak. [d]Onset of menstruation until LH surge. [e]Relative to the onset of menstruation. [f]Antral follicles detectable by ultrasound, > 2-3 mm.

significantly lower (P=0.05) in carriers of the G-allele compared to non-carriers (Figure 4.3). No differences in progesterone and inhibin A levels were observed between carriers and non-carriers in both time scales (results not shown). We did not observe any significant associations of AMHR2 genotypes with ovarian

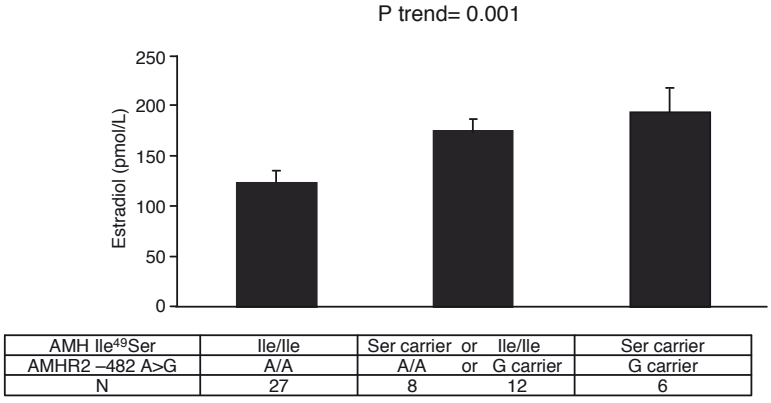

Figure 4.4 Additive effect of AMH and AMHR2 genotypes for E_2 levels on cycle day 3 of the menstrual cycle in the combined Dutch and German cohort. Women are grouped by the presence of zero, one or two carrier alleles (AMH Ile/Ile and AMHR2 A/A genotype, n= 27; AMH Ser^{49}-allele carriers and AMHR2 A/A, n=8 or AMH Ile/Ile and AMHR2 G-allele carriers, n=12; AMH Ser^{49}-allele carriers and AMHR2 G-allele carriers, n=6, respectively). Data represent the mean ± SEM. *P* trend = 0.001.

parameters, and although the dominant follicle tended to be detected 2.5 days earlier in carriers of the G-allele (Table 4.3), this was not significant.

In addition, the analysis of the combined Dutch and German cohort demonstrated once more that carriers of the AMHR2 –482G allele have higher E_2 levels on cycle day 3 compared to non-carriers (P=0.012, Table 4.2).

Combination of AMH Ile[49]Ser and AMHR2 –482 A>G polymorphism

Analysis of the combined Dutch and German cohort for the presence of both the AMH and AMHR2 genotypes revealed that carriers of the AMH [49]Ser and the AMHR2 -482G allele have highest E_2 levels (P trend=0.001), indicating an additive effect of both genotypes (Figure 4.4).

DISCUSSION

The current study was designed to investigate the role of AMH in the human ovary using a genetic approach. To accomplish this, polymorphisms in two genes of the AMH signal transduction pathway, AMH and its specific type II receptor (AMHR2), were identified. Association studies in two cohorts of normo-ovulatory women revealed that the AMH Ile[49]Ser and AMHR2 -482 A>G polymorphisms are associated with E_2 levels during the early follicular phase of the menstrual cycle.

Recently, an inverse correlation of AMH follicular fluid levels and E_2 levels in small antral follicles was demonstrated, suggesting a close interdependent regulation between AMH and E_2 [20]. Our findings of an association of AMH and AMHR2 polymorphisms with E_2 levels suggest that AMH regulates E_2 levels via modulation of FSH sensitivity in the human ovary. During the early follicular phase primary and secondary follicles are FSH-responsive whereas during the mid and late follicular phase follicles become FSH-dependent and subsequently one follicle is selected to become the dominant one [2]. Under influence of FSH a significant increase in aromatase activity occurs in this dominant follicle, resulting in the conversion of theca cell-derived androgens into estrogens [21-25]. Consequently, the development of the dominant follicle coincides with a rise in plasma E_2 levels [21]. Hence, carriers of the AMH [49]Ser or AMHR2 -482G allele, who both had higher E_2 levels, may have a lower threshold for FSH, resulting in more efficient stimulation of E_2 production by granulosa cells and/or an accelerated growth of the selected follicles during the early follicular phase. Moreover, carriers of both the AMH [49]Ser and the AMHR2 -482G allele may exhibit the lowest

threshold since an additive effect on E_2 levels was observed when genotypes were combined.

For the AMHR2 -482 A>G polymorphism, association studies in the German cohort revealed higher E_2 levels in the early follicular phase of the menstrual cycle in carriers of the G-allele compared with non-carriers. These findings again suggest that carriers of the AMHR2 G-allele display an increased sensitivity for FSH. This conclusion is supported by a trend towards an earlier detection of the dominant follicle in carriers of the AMHR2 G-allele. Furthermore, increased FSH sensitivity might reduce follicular phase length. An inverse relationship between follicular E_2 levels and follicular phase length was reported previously by several studies in normo-ovulatory women [26-28]. The latter studies indicated that high (mean, peak and baseline) plasma E_2 levels [26] and high (mean and baseline) urinary estrogen metabolite levels [28] during the follicular phase did correlate with a shorter follicular phase length.

In our study, the follicular phase length was not significantly different between the AMHR2 genotypes, although the small sample size may have not provided sufficient power to detect subtle differences. Nevertheless, differences in inhibin B levels aligned for LH day suggest a difference in follicular phase length between carriers and non-carriers of the AMHR2 -482G allele. During the luteo-follicular transition inhibin B levels rise rapidly [29], and since an increase in the levels of inhibin B reflects early gonadotropin-dependent follicle growth [30], the follicular phase length is reflected by the number of days from the first rise in inhibin B levels to the day of ovulation. Indeed, in carriers of the AMHR2 G-allele the time interval between the initial rise of inhibin B and the LH peak is shorter compared with non-carriers, reflecting a shortened follicular phase length as a result of increased FSH sensitivity. When aligned by menstrual cycle day, inhibin B levels do not directly reflect this increased FSH sensitivity in carriers of the G-allele because inhibin B secretion by preantral and small antral follicles in the early follicular phase is stimulated not only by FSH but also by local growth factors [31]. Moreover, during the late follicular phase, the preovulatory follicle does not contribute to serum inhibin B levels in response to FSH stimulation [32].

In young women with normal ovarian function large inter-individual variation in FSH threshold concentrations are described, with a lack of correlation between maximum follicular phase serum FSH concentrations and menstrual cycle characteristics, such as maximum E_2 levels and follicular phase length [14, 21]. These findings indicate differences in the FSH threshold of the ovary, which might be determined by various intra-ovarian factors, *e.g.* inhibin B, insulin-like growth factor-I and AMH [33]. Furthermore, it has been reported that genetic variation contributes to this individual FSH-setpoint [10, 34]. In our study, no dif-

ferences in the levels of the pituitary gonadotropin hormones, LH and FSH, were observed among the AMH and AMHR2 genotypes, suggesting a direct effect of AMH on the ovary. Therefore, we suggest that the AMH Ile[49]Ser and the AMHR2 -482 A>G polymorphism contribute to the individual variation in the FSH threshold of the ovary. These polymorphisms might constitute valuable clinical markers in determining the individual FSH threshold in patients suffering from normogonadotropic normo-estrogenic anovulatory infertility. In the latter, distinct differences in the amount of exogenous FSH required to elicit an ovarian response (*e.g.* mono-ovulation) may underlie the risk in developing hyper-response and its subsequent complications, *e.g.* ovarian hyperstimulation syndrome and poly-ovulation resulting in multiple pregnancies [35, 36]. Since previous models to predict the individual FSH threshold on basis of clinical parameters (*i.e.* LH serum levels, BMI, age and insulin resistance) suffered from limited specificity and sensitivity the AMH and AMHR2 genotypes might be of additive value in establishing the FSH threshold.

In vivo and *in vitro* studies in mice and *in vitro* studies in human ovarian tissue showed that AMH also regulates primordial follicle recruitment [3, 37]. Therefore, we hypothesized that less active AMH signalling may result in increased recruitment of primordial follicles, and thus more growing follicles. However, in this study, antral follicle count was not different between the AMH and AMHR2 polymorphisms. Also, AMH levels, reflecting the size of the growing follicle pool, did not differ. Therefore, the AMH Ile[49]Ser and AMHR2 -482 A>G polymorphisms do not appear to be related to primordial follicle recruitment, although compensating mechanisms may mask a possible effect.

Although only two small cohorts of women were studied, the AMHR2 –482G allele was associated with higher early follicular phase E_2 levels in both cohorts independently as well as in the combined cohort. The AMH Ile[49]Ser polymorphism was also associated with E_2 levels in the Dutch cohort and in the combined cohort. However, since the AMH Ile[49]Ser polymorphism could not be analyzed in the German cohort alone, an independent validation for the observed association is necessary. In general, the issue of multiple testing requires attention in association studies. However, the strong *a priori* rationale and the consistency in the occurrence and directions of our findings make it unlikely that our results could be explained by chance alone [38, 39]. Nevertheless, replication studies in larger unselected study cohorts of normo-ovulatory women are needed to obtain more definite conclusions. It also remains to be determined whether the higher E_2 levels in carriers of the AMH [49]Ser and the AMHR2 –482G allele are the result of a functional effect of these polymorphisms. Since for both AMH and AMHR2 extensive linkage disequilibrium was observed, it is possible

that the AMH Ile[49]Ser and AMHR2 -482 A>G polymorphisms are merely markers for the truly functional polymorphism elsewhere in these genes.

In conclusion, we have shown for the first time that genetic variants in the AMH and AMHR2 gene are associated with follicular phase E_2 levels, suggesting a role for AMH in the regulation of FSH sensitivity in the ovary. It might be of interest to determine whether the AMH Ile[49]Ser and AMHR2 -482 A>G polymorphism also affect cumulative lifelong estrogen exposure and whether these polymorphism are associated with an altered risk of estrogen-dependent diseases. Last but not least these genotypes might be involved in the pathophysiology of normo-estrogenic anovulatory infertility and polycystic ovary syndrome since in these women the individual FSH threshold seems to be elevated [40].

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REFERENCES

1. **Durlinger ALL, Visser JA, Themmen APN** 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124:601-9
2. **McGee EA, Hsueh AJ** 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-14
3. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-96
4. **Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-9
5. **di Clemente N, Goxe B, Remy JJ, Cate R, Josso N, Vigier B, Salesse R** 1994 Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine 2:553-558
6. **van Rooij IAJ, Broekmans FJM, te Velde ER, Fauser BCJM, Bancsi LF, de Jong FH, Themmen APN** 2002 Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod 17:3065-71
7. **Visser JA, de Jong FH, Laven JSE, Themmen APN** 2006 Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 131:1-9
8. **Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, Dewailly D** 2003 Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. J Clin Endocrinol Metab 88:5957-62
9. **Laven JSE, Mulders AGMGJ, Visser JA, Themmen APN, De Jong FH, Fauser BCJM** 2004 Anti-Mullerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age. J Clin Endocrinol Metab 89:318-23
10. **Greb RR, Grieshaber K, Gromoll J, Sonntag B, Nieschlag E, Kiesel L, Simoni M** 2005 A common single nucleotide polymorphism in exon 10 of the human follicle stimulating hormone receptor is a major determinant of length and hormonal dynamics of the menstrual cycle. J Clin Endocrinol Metab 90:4866-72
11. **The International HapMap Project** 2003 Nature 426:789-96
12. **de Vet A, Laven JSE, de Jong FH, Themmen APN, Fauser BCJM** 2002 Antimullerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril 77:357-62
13. **Fauser BCJM, Pache TD, Lamberts SWJ, Hop WC, de Jong FH, Dahl KD** 1991 Serum bioactive and immunoreactive luteinizing hormone and follicle-stimulating hormone levels in women with cycle abnormalities, with or without polycystic ovarian disease. J Clin Endocrinol Metab 73:811-7
14. **Schipper I, de Jong FH, Fauser BCJM** 1998 Lack of correlation between maximum early follicular phase serum follicle stimulating hormone concentrations and menstrual cycle characteristics in women under the age of 35 years. Hum Reprod 13:1442-8
15. **Hohmann FP, Laven JSE, de Jong FH, Eijkemans MJC, Fauser BCJM** 2001 Low-dose exogenous FSH initiated during the early, mid or late follicular phase can induce multiple dominant follicle development. Hum Reprod 16:846-54
16. **Simoni M, Gromoll J, Hoppner W, Kamischke A, Krafft T, Stahle D, Nieschlag E** 1999 Mutational analysis of the follicle-stimulating hormone (FSH) receptor in normal and infertile men: identification and characterization of two discrete FSH receptor isoforms. J Clin Endocrinol Metab 84:751-5

17. **Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groome NP, Themmen APN, Visser JA** 2006 Serum Anti-Mullerian hormone levels reflect the size of the primordial follicle pool in mice. Endocrinology 147:3228-34
18. **Stephens M, Donnelly P** 2003 A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 73:1162-9
19. **Stephens M, Smith NJ, Donnelly P** 2001 A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978-89
20. **Andersen CY, Byskov AG** 2006 Estradiol and regulation of anti-mullerian hormone, inhibin-a, and inhibin-B secretion: analysis of small antral and preovulatory human follicles' fluid. J Clin Endocrinol Metab 91:4064-9
21. **van Santbrink EJP, Hop WCJ, van Dessel TJ, de Jong FH, Fauser BCJM** 1995 Decremental follicle-stimulating hormone and dominant follicle development during the normal menstrual cycle. Fertil Steril 64:37-43
22. **van Dessel HJ, Schipper I, Pache TD, van Geldorp H, de Jong FH, Fauser BCJM** 1996 Normal human follicle development: an evaluation of correlations with oestradiol, androstenedione and progesterone levels in individual follicles. Clin Endocrinol (Oxf) 44:191-8
23. **Hillier SG, Reichert LE, Jr., Van Hall EV** 1981 Control of preovulatory follicular estrogen biosynthesis in the human ovary. J Clin Endocrinol Metab 52:847-56
24. **Short RV** 1962 Steroids in the follicular fluid and the corpus luteum of the mare. A 'two-cell type' theory of ovarian steroid synthesis. J Endocrinol 24:59-63
25. **Macklon NS, Fauser BCJM** 2001 Follicle-stimulating hormone and advanced follicle development in the human. Arch Med Res 32:595-600
26. **Landgren BM, Unden AL, Diczfalusy E** 1980 Hormonal profile of the cycle in 68 normally menstruating women. Acta Endocrinol (Copenh) 94:89-98
27. **Nagata C, Kaneda N, Kabuto M, Shimizu H** 1997 Factors associated with serum levels of estradiol and sex hormone-binding globulin among premenopausal Japanese women. Environ Health Perspect 105:994-7
28. **Windham GC, Elkin E, Fenster L, Waller K, Anderson M, Mitchell PR, Lasley B, Swan SH** 2002 Ovarian hormones in premenopausal women: variation by demographic, reproductive and menstrual cycle characteristics. Epidemiology 13:675-84
29. **Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather JP, McNeilly AS** 1996 Measurement of dimeric inhibin B throughout the human menstrual cycle. J Clin Endocrinol Metab 81:1401-5
30. **Welt CK, Adams JM, Sluss PM, Hall JE** 1999 Inhibin A and inhibin B responses to gonadotropin withdrawal depends on stage of follicle development. J Clin Endocrinol Metab 84:2163-9
31. **Welt CK, Schneyer AL** 2001 Differential regulation of inhibin B and inhibin a by follicle-stimulating hormone and local growth factors in human granulosa cells from small antral follicles. J Clin Endocrinol Metab 86:330-6
32. **Welt CK, Smith ZA, Pauler DK, Hall JE** 2001 Differential regulation of inhibin A and inhibin B by luteinizing hormone, follicle-stimulating hormone, and stage of follicle development. J Clin Endocrinol Metab 86:2531-7
33. **Laven JSE, Fauser BCJM** 2006 What role of estrogens in ovarian stimulation. Maturitas 54:356-62
34. **de Castro F, Moron FJ, Montoro L, Galan JJ, Hernandez DP, Padilla ES, Ramirez-Lorca R, Real LM, Ruiz A** 2004 Human controlled ovarian hyperstimulation outcome is a polygenic trait. Pharmacogenetics 14:285-93
35. **Fauser BCJM, Van Heusden AM** 1997 Manipulation of human ovarian function: physiological concepts and clinical consequences. Endocr Rev 18:71-106

36. **Mulders AG, Eijkemans MJ, Imani B, Fauser BC** 2003 Prediction of chances for success or complications in gonadotrophin ovulation induction in normogonadotrophic anovulatory infertility. Reprod Biomed Online 7:170-8
37. **Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen APN, Hovatta O** 2006 Anti-Mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. Hum Reprod 21:2223-7
38. **Newton-Cheh C, Hirschhorn JN** 2005 Genetic association studies of complex traits: design and analysis issues. Mutat Res 573:54-69
39. **Rivadeneira F, van Meurs JB, Kant J, Zillikens MC, Stolk L, Beck TJ, Arp P, Schuit SC, Hofman A, Houwing-Duistermaat JJ, van Duijn CM, van Leeuwen JP, Pols HA, Uitterlinden AG** 2006 Estrogen receptor beta (ESR2) polymorphisms in interaction with estrogen receptor alpha (ESR1) and insulin-like growth factor I (IGF1) variants influence the risk of fracture in postmenopausal women. J Bone Miner Res 21:1443-56
40. **Laven JSE, Imani B, Eijkemans MJC, Fauser BCJM** 2002 New approach to polycystic ovary syndrome and other forms of anovulatory infertility. Obstet Gynecol Surv 57:755-67

Chapter 5

A polymorphism in the AMH type II receptor gene is associated with age at menopause in interaction with parity.

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ABSTRACT

Background: Anti-Müllerian hormone (AMH) inhibits primordial follicle recruitment in the mouse ovary. We hypothesize that in women AMH signaling also regulates the usage of the primordial follicle pool and hence, influences the onset of menopause. Since age at menopause has a strong genetic component we investigated the role of AMH signaling using a candidate gene approach.

Methods: In two large population-based cohorts of Dutch postmenopausal women (N= 2381 and N=248) we examined the association between two polymorphisms, one in the AMH gene and one in the AMH type II receptor gene (AMHR2), and natural age at menopause.

Results: The AMH Ile[49]Ser polymorphism (rs10407022) was not associated with age at menopause in either cohort. In the Rotterdam cohort, the AMHR2 –482 A>G polymorphism (rs2002555) was associated with age at menopause in interaction with the number of offspring (P=0.001). Nulliparous women homozygous for the G-allele entered menopause 2.6 years earlier compared with nulliparous women homozygous for the A-allele (P=0.005). In the LASA cohort, women with the G/G genotype tended to enter menopause 2.8 years earlier compared to the A/A genotype (P=0.063).

Conclusions: The observed association of the AMHR2 –482 A>G polymorphism with natural age at menopause suggests a role for AMH signaling in the usage of the primordial follicle pool in women.

INTRODUCTION

Menopause marks a dramatic change in the endocrine and reproductive status of women. In women the onset of menopause is determined by the exhaustion of the ovarian follicle pool [1]. From the establishment of the primordial follicle pool onwards, just before (for primates) or directly after (for mice) birth, dormant primordial follicles are continuously recruited into the growing follicle pool, a process called initial recruitment. After pubertal onset, a cohort of antral follicles is selected from this growing follicle pool as a result of the increase in circulating FSH levels during each reproductive cycle [2]. From this rescued cohort, only one (for primates) or several (for rodents) follicles will ovulate during each cycle, whereas most growing follicles will die as a result of atresia.

Primordial follicle recruitment is predominantly regulated by intra-ovarian factors. One of the factors known to regulate initial recruitment in mice is anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS). AMH, a member of the transforming growth factor-β (TGFβ) family, is expressed in the ovary from the onset of primordial recruitment onwards in a similar pattern in women and mice [3]. AMH expression starts in the granulosa cells of primary follicles, is highest in granulosa cells of preantral and small antral follicles and gradually diminishes in the subsequent stages of follicle development [4]. Studies in the AMH knockout (AMHKO) mice revealed that primordial follicles are recruited at a faster rate in the absence of AMH, illustrating that AMH plays an inhibitory role in the recruitment of primordial follicles. The absence of AMH results in a prematurely exhausted follicle pool and, subsequently, an earlier cessation of the estrus cycle [5]. AMH inhibits mouse, bovine and human primordial follicle growth *in vitro* [6-8], although conflicting results have been reported [9]. In addition to recruitment, AMH attenuates FSH sensitivity in mice [10, 11], albeit also for this role of AMH contrary results have been found [12]. On the basis of the similar expression pattern of AMH in women and in mice, we hypothesize that also in women AMH inhibits primordial follicle recruitment and thus might influence the onset of menopause.

In Western countries the average age at menopause is 50-51 years, but ranges from 40 to 60 years [13]. Environmental factors and personal history (*e.g.* smoking and parity) explain only a minor part of the variety in natural age at menopause, while the main part is explained by genetic factors [14]. This conclusion is mainly based on the strong correlation of age at menopause in monozygotic twins, in whom heritability estimates range from 0.63 up to 0.72 [15, 16]. In addition, a genetic component of age at menopause was suggested by several candidate gene studies. For example, polymorphisms in genes involved in estrogen metabolism

have been associated with age at menopause, *e.g.* ERα [17], CYP 17 [18] and CYP1B1 [19], although these findings have not been replicated [18-20].

In a recent study, we have shown that in premenopausal women genetic variants in AMH and its specific AMH type II receptor (AMHR2) gene are associated with estradiol levels, suggesting modulation of intra-ovarian FSH sensitivity by these variants [21]. In the present study we have evaluated whether the AMH Ile[49]Ser (rs10407022) and the AMHR2 –482 A>G (rs2002555) polymorphisms are associated, independently and in interaction with environmental factors, with age at menopause in two large cohorts of Dutch postmenopausal women.

MATERIAL AND METHODS

Subjects

The first study cohort was derived of women from the Rotterdam Study (n= 7983, 61.6% women), a prospective population-based study of determinants of chronic disabling diseases in the elderly. The design and rationale of this study have been described earlier [22]. Written informed consent was obtained from each participant and the Rotterdam study was approved by the Medical Ethics Review board of Erasmus MC. During the home interview each woman provided information on her reproductive and gynaecological history, including the use of sex steroids at any time [17]. Confounding factors, such as height, weight, smoking and socio-economic status were defined as described previously [17]. For this study, only women with a natural age at menopause were selected (n=3256). Natural age at menopause was defined as the age at the last menstrual period, which can only be defined retrospectively after at least 12 consecutive months of amenorrhoe. This last menstrual period should not be induced by surgery or other obvious causes, such as irradiation or hormone therapy [23]. Women who reported hormone use during the onset of menopause were excluded to avoid uncertainty on menopausal age. DNA was available for 2564 women, of whom 92.9% was successfully genotyped for the AMH and AMHR2 polymorphism, resulting in a final study cohort of 2381 women.

The second study cohort was derived from the Longitudinal Aging Study Amsterdam (LASA) study, an ongoing interdisciplinary cohort study on predictors and consequences of changes in autonomy and well-being in an aging population in The Netherlands [24]. The design of this study has been described previously [25, 26]. Informed consent was obtained from all respondents and the study was approved by the Medical Ethics Review board of the VUMC. Information on oral contraceptive use and age at menarche was provided in the main

interview of the first examination (1992/1993). At the medical interview during the second data collection (1995/1996), other gynecological and reproductive information was provided, including age at menopause, number of children and sex steroid use, along with the confounding factors height, weight, smoking (ever versus never smoking) and socio-economic status. DNA was available in 966 of the 1509 participants of the medical interview (471 men and 495 women) [25]. In 461 of the women, the AMH and AMHR2 polymorphism were successfully genotyped. For the present study, only women with a natural menopause were selected. Furthermore, women who had ever used HRT or oral contraceptives were excluded, resulting in a final study cohort of 248 women.

Genotyping

Genomic DNA was extracted from peripheral blood using standard DNA extraction methods. The AMH Ile[49]Ser and AMHR2 –482 A>G genotypes were determined using Taqman allelic discrimination assays. For the AMH Ile[49]Ser polymorphism an Assay-by-Design with the following probes was used: 5'-CTCCAGGCA**t**CCCACAA-3' and 5'-CCAGGCA**g**CCCACAA-3'. For the AMHR2 -482 A>G promoter SNP, we used an Assay-on-Demand, Assay ID C_ 1673084_10 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Reactions were performed as described previously [21]. A random selection of 5% of samples was independently repeated to confirm genotyping results. In the Rotterdam Study, the disagreement rate for the AMH Ile[49]Ser SNP was 0.4%, whereas it was 0.0% in the LASA study. The disagreement rate for the AMHR2 –482 A>G SNP was 0.0% in both study cohorts.

Statistical analysis

In both populations, genotype frequencies were tested for Hardy-Weinberg equilibrium proportions using the ARLEQUIN package [27]. Differences between the cohorts and differences between genotype groups within each cohort were tested using one-way analysis of variance (ANOVA) for continuous variables and the chi-squared test for categorical variables. Differences in age at menopause between genotype groups were adjusted for potential confounders (age, BMI, smoking, socio-economic status, age at menarche, parity and use of oral contraceptives and hormone replacement therapy) using ANCOVA. Possible interactions between genotypes and covariates were explored in plots and tested using the general linear model procedure of ANCOVA including product terms of main effects. In the Rotterdam cohort stratified analysis for the number of offspring was performed. Because of the relatively small sample size, this stratified analysis was not performed in the LASA cohort. Subsequently, to increase

statistical power, both cohorts were combined and differences in age at menopause between the AMHR2 genotype groups were analyzed using ANCOVA. All analyses were performed using Statistical Package for Social Sciences, SPSS, version 11.0.1 (SPSS Inc, Chicago, IL). P≤0.05 was considered to be significant.

RESULTS

Characteristics of the two study cohorts

Women in the Rotterdam cohort had on average a lower age at the time of the interview and a lower BMI compared with women in the LASA cohort, although these differences were only minor. The mean age at natural menopause was similar in both cohorts. Possible confounding factors for age at menopause, such as smoking and age at menarche were not different between both cohorts, whereas the average number of offspring and socio-economic status were different between the cohorts (Table 5.1). In addition, age at natural menopause (49.6 ± 4.4 yr, mean ± SD) in our study subset of the Rotterdam cohort was nearly identical to the mean age at natural menopause (49.6 ± 4.5 yr) in the total Rotterdam cohort.

Table 5.1 Characteristics of the two study cohorts

	Rotterdam cohort	LASA cohort	P
N	2381	248	
Age at interview (year) (range)	70.1 ± 9.3 (55.0-98.7)	76.9 ± 6.4 (65.6-88.3)	< 0.001
BMI (kg/m^2)	26.8 ± 4.1	27.4 ± 4.5	0.03
Ever smoked (%)	1081 (45.6)[a]	103 (41.5)	0.23
SES education level I-II (%)	1523 (64.2)[a]	175 (70.6)	
education level III-IV (%)	849 (35.8)	73 (29.4)	0.046
Age at menopause	49.6 ± 4.4	49.2 ± 4.8	0.18
Median	50	50	
Age at menarche	13.7 ± 1.8	13.8 ± 1.8	0.48
Median	14	14	
Offspring 0 (%)	511 (21.5)	45 (18.1)	< 0.001
1 or 2 (%)	1023 (43.0)	80 (32.3)	
>2 (%)	847 (35.6)	123 (49.6)	

[a] Information available for 2372 women
Data are presented as mean ± SD

Genotype distributions in the study populations

The allele and genotype frequencies of the AMH Ile[49]Ser and the AMHR2 -482 A>G polymorphism were similar in the Rotterdam study and the LASA study and did not differ from the frequencies in premenopausal women [21] or in Caucasians in the NCBI (www.ncbi.nlm.nig.gov) and in the HapMap database (www.hapmap.org) [28]. In both study cohorts, the genotype frequencies were in Hardy-Weinberg equilibrium proportions (Tables 5.2 and 5.3).

Table 5.2 Characteristics of both cohorts by AMH Ile[49]Ser genotype

	Rotterdam cohort				LASA cohort			
AMH	Ile/Ile	Ile/Ser	Ser/Ser	P	Ile/Ile	Ile/Ser	Ser/Ser	P
N (%)	1631 (68.5)	682 (28.6)	68 (2.9)	0.75[a]	162 (65.3)	75 (30.2)	11 (4.4)	0.54[a]
Age (year)	70.0 ± 0.2	70.1 ± 0.4	71.8 ± 1.1	0.28	76.8 ± 0.5	77.1 ± 0.7	77.0 ± 1.2	0.94
BMI (kg/m^2)	26.7 ± 0.1	27.0 ± 0.2	26.7 ± 0.4	0.30	27.2 ± 0.4	27.7 ± 0.5	28.3 ± 1.3	0.59
Age at menopause	49.6 ± 0.1	49.5 ± 0.2	49.6 ± 0.6	0.66	48.9 ± 0.4	49.6 ± 0.6	49.6 ± 1.0	0.58
Median	50	50	50		50	50	50	
Age at menarche	13.7 ± 0.05	13.6 ± 0.07	13.5 ± 0.20	0.07	13.8 ± 0.2	13.7 ± 0.2	14.0 ± 0.6	0.89
Median	14	13	13		14	14	13.5	
Offspring 0 (%)	352 (21.6)	148 (21.7)	11 (16.2)		33 (20.4)	11 (14.7)	1 (9.1)	
1 or 2 (%)	699 (42.9)	291 (42.7)	33 (48.5)		58 (35.8)	17 (22.7)	5 (45.5)	
>2 (%)	580 (35.6)	243 (35.6)	24 (35.3)	0.84	71 (43.8)	47 (62.7)	5 (45.5)	0.08

[a] P-value for Hardy-Weinberg Equilibrium
Data are presented as mean ± SEM

Table 5.3 Characteristics of both cohorts by AMHR2 –482 A>G genotype

	Rotterdam cohort				LASA cohort			
AMHR2	A/A	A/G	G/G	P	A/A	A/G	G/G	P
N (%)	1562 (65.6)	740 (31.1)	79 (3.3)	0.45[a]	159 (64.1)	79 (31.9)	10 (4.0)	0.96[a]
Age (year)	70.1 ± 0.2	70.0 ± 0.3	70.5 ± 1.0	0.91	76.6 ± 0.5	77.1 ± 0.7	80.4 ± 1.7	0.18
BMI (kg/m^2)	26.8 ± 0.1	26.7 ± 0.1	27.5 ± 0.5	0.28	27.0 ± 0.3	28.1 ± 0.5	27.7 ± 2.1	0.21
Age at menopause	49.7 ± 0.1	49.4 ± 0.2	49.2 ± 0.5	0.26	49.6 ± 0.3	48.7 ± 0.6	46.8 ± 1.5	0.054[b]
Median	50	50	50		50	50	45	
Age at menarche	13.7 ± 0.05	13.7 ± 0.07	13.5 ± 0.22	0.44	13.7 ± 0.2	13.9 ± 0.2	14.3 ± 0.9	0.73
Median	14	14	13		14	14	13.5	
Offspring 0 (%)	351 (22.5)	135 (18.2)	25 (31.6)		29 (18.2)	15 (19.0)	1 (10.0)	
1 or 2 (%)	670 (42.9)	330 (44.6)	23 (29.1)		55 (34.6)	23 (29.1)	2 (20.0)	
>2 (%)	541 (34.6)	275 (37.2)	31 (39.2)	0.01	75 (47.2)	41 (51.9)	7 (70.0)	0.65

[a] P-value for Hardy-Weinberg Equilibrium
[b] A/A genotype tested versus G/G genotype
Data are presented as mean ± SEM

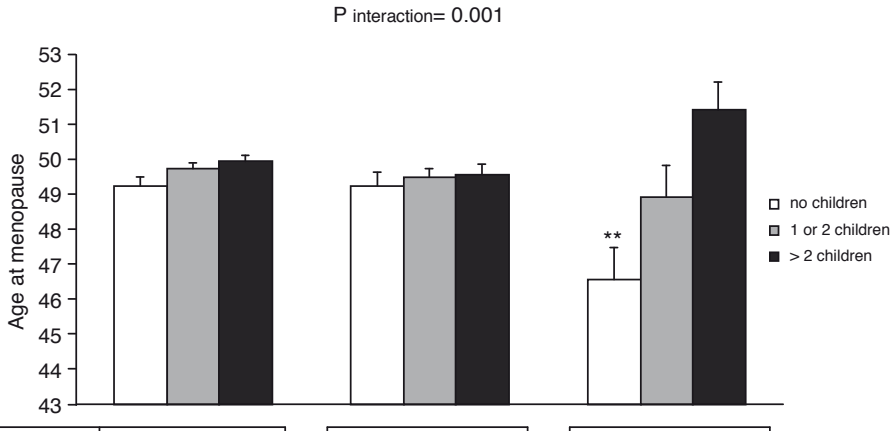
Analysis of the AMH Ile[49]Ser polymorphism

No differences were observed in the basal characteristics between the genotype groups of the AMH Ile[49]Ser polymorphism in both cohorts (Table 5.2). Age at natural menopause was similar between the genotype groups of the AMH Ile[49]Ser polymorphism, as were age at menarche, number of offspring (Table 5.2), smoking, socio-economic status and sex steroid use, including hormone replacement therapy and oral contraceptive use (results not shown). Adjustment of age at menopause for possible confounders did not affect the results.

Analysis of the AMHR2 –482 A>G polymorphism

Basal characteristics were similar between the genotype groups of the AMHR2 -482 A>G polymorphism in both cohorts (Table 5.3). In the Rotterdam cohort, crude age at menopause was not different between the AMHR2 genotypes, as were age at menarche and hormone use, whereas for the number of offspring a significant difference was observed (P=0.01). Homozygous carriers of the -482G allele were more frequently nulliparous (31.6 %) compared with women with the AMHR2 -482 A/A genotype (22.5 %) or AMHR2 -482 A/G genotype (18.2 %) (Table 5.3). Since the number of offspring was different between the AMHR2 genotypes and the number of offspring is associated with age at menopause, we stratified the association analysis of age at menopause for this parameter. We observed a significant influence of the number of children on age at menopause in the AMHR2 –482 G/G homozygous group (n=79). Nulliparous women with the G/G genotype had a 2.6 years earlier onset of menopause (46.6 ± 0.9 yr, mean ± SEM) compared with nulliparous women with the AMHR2 A/A genotype (49.2 ± 0.2 yr, P = 0.005) (Figure 5.1). Women with one or two children and the G/G genotype had a similar onset of menopause compared with the other AMHR2 genotypes (P=0.51), whereas women with the G/G genotype and more than two children tended to have a 1.5 years later onset of menopause (51.4 ± 0.8) compared with the A/A genotype (49.9 ± 0.2), although this does not reach significance (P= 0.072) (Figure 5.1). When differences in age at menopause among genotype groups were tested in an univariate regression model, adjusted for all possible confounders, a strong synergistic interaction (P=0.001) between the AMHR2 G/G genotype and the number of offspring was observed.

In the LASA cohort, women homozygous for the AMHR2 –482G allele tended to enter menopause 2.8 years earlier compared with women homozygous for the –482A allele (P=0.054) (Table 5.3). After adjustment of age at menopause for possible confounders this difference remained borderline significant (P=0.063). In the LASA cohort no differences were observed between the AMHR2 genotype groups



AMHR2	A/A			A/G			G/G		
N	351	670	541	135	330	275	25	23	31
Age at Menopause	49.2	49.7	49.9	49.2	49.5	49.6	46.6	48.9	51.4

Figure 5.1 Interaction between AMHR2 –482 A>G genotypes and parity in the Rotterdam cohort. Age at menopause for women with zero, one or two, and more than two children, by AMHR2 –482 A/A, A/G and G/G genotype groups adjusted for age, BMI, smoking, socio-economic status, age at menarche and hormone use. Data are presented as mean ± SEM. **: G/G genotype significantly different from A/A genotype, P = 0.005.

in age at menarche, number of offspring (Table 5.3), smoking and socio-economic status (results not shown).

When the Rotterdam cohort and the LASA cohort were analyzed together with adjustment for possible confounders, the AMHR2 –482 A>G polymorphism tended to be associated with age at menopause (A/A 49.7 ± 0.1, A/G 49.4 ± 0.2, G/G 48.9 ± 0.5, mean ± SEM, P=0.068). Combined analysis of the AMH Ile^{49}Ser polymorphism and the AMHR2 -482 A>G polymorphism revealed no additional associations with age at menopause (results not shown).

DISCUSSION

In the present study we investigated for the first time whether genetic variants in the AMH signaling pathway influence the onset of natural menopause. In two Dutch cohorts of postmenopausal women, the association of two polymorphisms in the AMH and AMHR2 gene, which both capture the common genetic variation in the gene [21], with age at menopause was studied. In the Rotterdam study, the AMHR2 -482 A>G polymorphism was associated with age at menopause in interaction with parity. Also in the LASA cohort and when both cohorts were

combined, the AMHR2 –482 A>G polymorphism tended to be associated with age at menopause.

Women with the AMHR2 –482 G/G genotype tended to have an earlier onset of menopause compared to women with the A/A genotype, which is indicative of less inhibition of primordial follicle recruitment. Hence, the AMHR2 -482 G/G genotype could result in diminished AMH signaling, which is in concordance with a previous study [21], in which the -482G allele was associated with higher estradiol levels in premenopausal women, correlating with less inhibition of FSH sensitivity by AMH. Indeed, the –482 A>G polymorphism is located at a potential c-Myb and c-Myc transcription factor binding site (www.cbil.upenn.edu/tess)[29], and therefore may modify promoter activity.

Besides the subtle differences in age at menopause between the AMHR2 genotypes in the combined cohort, we observed a strong synergistic interaction between the AMHR2 G/G genotype and the number of children in the Rotterdam cohort. This interaction suggests that the -482 A>G polymorphism influences the relation between age at menopause and parity. The relation between age at menopause and parity has been demonstrated in many epidemiological studies [30-34]. Nulliparous women enter menopause 0.5 [30] to 1.5 years [33] earlier compared with parous women, as is also observed in the Rotterdam study (0.6 years difference) (results not shown). Nevertheless, little is known about the underlying mechanism of this relation between age at menopause and parity. Two possible explanations have been proposed. First, it has been suggested that age at menopause and parity are not causally related but are both reflecting the process of ovarian aging [35]. The second explanation is that during pregnancy less primordial follicles are recruited, resulting in a delayed onset of menopause [2, 30, 33]. The latter explanation is supported by rodent studies. In mice the number of follicles that start growing is reduced during pregnancy [36], and rats allowed to undergo multiple pregnancies show a delay in reproductive aging [37]. Furthermore, prolonged elevation of circulating progesterone in rats suppresses initial follicle recruitment, thus maintaining a larger primordial follicle pool [38, 39]. During pregnancy in women, AMH serum levels, which reflect the size of the growing and, indirectly, the primordial follicle pool [40, 41], apparently do not change [42], suggesting that during pregnancy initial recruitment continues. Alternatively, initial recruitment might halt but growing follicles might be rescued from atresia during pregnancy.

In view of the effects of AMH and possibly also parity on primordial follicle recruitment, it is intriguing that the relation between parity and age at menopause appears to be influenced by the AMHR2 –482 A>G polymorphism. The -482 A>G SNP, located in the promoter region of the gene, is in linkage disequilibrium with several other SNPs [21], and therefore also other variants can drive the observed as-

sociation. However, it is possible that changes in hormone levels during pregnancy, such as progesterone, prolactin and estradiol, alter the expression or function of the receptor. Although signaling of the G-allele derived AMHRII in regularly cycling women is probably less compared to the A-allele derived AMHRII, altered hormone levels during pregnancy might have a stronger effect on the G-allele AMHRII than on the A-allele AMHRII. This may result in increased expression and/or activity of the G-allele derived AMHRII and thereby a stronger inhibition of primordial follicle recruitment during pregnancy. However, functional studies and additional replication studies are necessary to obtain definite conclusions regarding the effect of the AMHR2 -482 A>G polymorphism on age at menopause.

For the AMH Ile[49]Ser polymorphism no association with age at menopause is observed in both cohorts, suggesting that this polymorphism does not affect AMH function in follicle recruitment. In contrast, in our previous study [21] we observed that the AMH Ile[49]Ser polymorphism is associated with altered FSH sensitivity. It is possible that the effect of this polymorphism on primordial follicle recruitment is masked or compensated by other factors.

In the Rotterdam and the LASA cohort, age at menopause was determined retrospectively, which has been shown to be susceptible to bias [43, 44]. Nevertheless, it seems unlikely that misclassification due to recall bias is different across genotypes.

In conclusion, the observed association of genetic variation in the AMHR2 gene with age at menopause suggests a role for AMH signaling in the complex process of human ovarian aging. Although the potential consequences of the AMHR2 -482 A>G polymorphism on receptor function still need to be elucidated, our results suggest that the AMHR2 polymorphism contributes to the wide range in onset of menopause. Furthermore, our results may provide more insight into the mechanism that drives the relationship between age at menopause and parity. It will be interesting to determine whether the AMHR2 polymorphism also influences the risk of menopause-related diseases, such as osteoporosis and breast cancer.

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REFERENCES

1. **te Velde ER, Scheffer GJ, Dorland M, Broekmans FJM, Fauser BCJM** 1998 Developmental and endocrine aspects of normal ovarian aging. Mol Cell Endocrinol 145:67-73
2. **McGee EA, Hsueh AJ** 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-14
3. **Weenen C, Laven JSE, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BCJM, Themmen APN** 2004 Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 10:77-83
4. **Durlinger ALL, Visser JA, Themmen APN** 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124:601-9
5. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-96
6. **Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 2002 Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076-84
7. **Carlsson IB, Scott JE, Visser JA, Ritvos O, Themmen APN, Hovatta O** 2006 Anti-Mullerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. Hum Reprod 21:2223-7
8. **Gigli I, Cushman RA, Wahl CM, Fortune JE** 2005 Evidence for a role for anti-Mullerian hormone in the suppression of follicle activation in mouse ovaries and bovine ovarian cortex grafted beneath the chick chorioallantoic membrane. Mol Reprod Dev 71:480-8
9. **Schmidt KL, Kryger-Baggesen N, Byskov AG, Andersen CY** 2005 Anti-Mullerian hormone initiates growth of human primordial follicles in vitro. Mol Cell Endocrinol 234:87-93
10. **Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-9
11. **Visser JA, Durlinger ALL, Peters IJ, van den Heuvel ER, Rose UM, Kramer P, de Jong FH, Themmen APN** 2007 Increased Oocyte Degeneration and Follicular Atresia during the Estrous Cycle in Anti-Mullerian Hormone Null Mice. Endocrinology 148:2301-8
12. **McGee EA, Smith R, Spears N, Nachtigal MW, Ingraham H, Hsueh AJ** 2001 Mullerian inhibitory substance induces growth of rat preantral ovarian follicles. Biol Reprod 64:293-8
13. **te Velde ER, Dorland M, Broekmans FJ** 1998 Age at menopause as a marker of reproductive ageing. Maturitas 30:119-25
14. **Kok HS, van Asselt KM, van der Schouw YT, Peeters PHM, Wijmenga C** 2005 Genetic studies to identify genes underlying menopausal age. Hum Reprod Update 11:483-93
15. **Snieder H, MacGregor AJ, Spector TD** 1998 Genes control the cessation of a woman's reproductive life: a twin study of hysterectomy and age at menopause. J Clin Endocrinol Metab 83:1875-80

16. **de Bruin JP, Bovenhuis H, van Noord PA, Pearson PL, van Arendonk JA, te Velde ER, Kuurman WW, Dorland M** 2001 The role of genetic factors in age at natural menopause. Hum Reprod 16:2014-8
17. **Weel AEAM, Uitterlinden AG, Westendorp ICD, Burger H, Schuit SCE, Hofman A, Helmerhorst TJM, van Leeuwen JPTM, Pols HAP** 1999 Estrogen receptor polymorphism predicts the onset of natural and surgical menopause. J Clin Endocrinol Metab 84:3146-50
18. **Gorai I, Tanaka K, Inada M, Morinaga H, Uchiyama Y, Kikuchi R, Chaki O, Hirahara F** 2003 Estrogen-metabolizing gene polymorphisms, but not estrogen receptor-alpha gene polymorphisms, are associated with the onset of menarche in healthy postmenopausal Japanese women. J Clin Endocrinol Metab 88:799-803
19. **Hefler LA, Grimm C, Heinze G, Schneeberger C, Mueller MW, Muendlein A, Huber JC, Leodolter S, Tempfer CB** 2005 Estrogen-metabolizing gene polymorphisms and age at natural menopause in Caucasian women. Hum Reprod 20:1422-7
20. **Kok HS, Onland-Moret NC, van Asselt KM, van Gils CH, van der Schouw YT, Grobbee DE, Peeters PHM** 2005 No association of estrogen receptor alpha and cytochrome P450c17alpha polymorphisms with age at menopause in a Dutch cohort. Hum Reprod 20:536-42
21. **Kevenaar ME, Themmen APN, Laven JSE, Sonntag B, Lie Fong S, Uitterlinden AG, de Jong FH, Pols HAP, Simoni M, Visser JA** 2007 Anti-Mullerian hormone and anti-Mullerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women. Hum Reprod 22:1547-54
22. **Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA** 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 7:403-22
23. **WHO Scientific Group** 1996 Research on the menopause in the 1990s. World Health Organ Tech Rep Ser 866:1-107
24. **Deeg D, Knipscheer C, van Tilburg W** 1993 Autonomy and well-being in the aging population: concepts and design of the longitudinal Aging Study Amsterdam. Bunnili: Netherlands Institute of Gerontology.
25. **Pluijm SM, van Essen HW, Bravenboer N, Uitterlinden AG, Smit JH, Pols HA, Lips P** 2004 Collagen type I alpha1 Sp1 polymorphism, osteoporosis, and intervertebral disc degeneration in older men and women. Ann Rheum Dis 63:71-7
26. **Schaap LA, Pluijm SM, Smit JH, van Schoor NM, Visser M, Gooren LJ, Lips P** 2005 The association of sex hormone levels with poor mobility, low muscle strength and incidence of falls among older men and women. Clin Endocrinol (Oxf) 63:152-60
27. **Schneider S, Roessli D, Excoffier L** 2000 Arlequin version 2.000: a software for population genetics data analysis, 2.000 ed. Genetics and Biometry Laboratory, University of Geneva, Geneva
28. **The International HapMap Consortium** 2003 The International HapMap Project. Nature 426:789-96
29. **Schug J, Overton GC** 1997 TESS: Transcription Element Search Software. Computational Biology and Informatics Laboratory, University of Pennsylvania, pp Technical report CBIL-TR-1997-1001-v0.0
30. **Whelan EA, Sandler DP, McConnaughey DR, Weinberg CR** 1990 Menstrual and reproductive characteristics and age at natural menopause. Am J Epidemiol 131:625-32
31. **van Noord PAH, Dubas JS, Dorland M, Boersma H, te Velde E** 1997 Age at natural menopause in a population-based screening cohort: the role of menarche, fecundity, and lifestyle factors. Fertil Steril 68:95-102

32. **Cramer DW, Xu H, Harlow BL** 1995 Does "incessant" ovulation increase risk for early menopause? Am J Obstet Gynecol 172:568-73
33. **Stanford JL, Hartge P, Brinton LA, Hoover RN, Brookmeyer R** 1987 Factors influencing the age at natural menopause. J Chronic Dis 40:995-1002
34. **Cassou B, Derriennic F, Monfort C, Dell'Accio P, Touranchet A** 1997 Risk factors of early menopause in two generations of gainfully employed French women. Maturitas 26:165-74
35. **Kok HS, van Asselt KM, van der Schouw YT, Grobbee DE, te Velde ER, Pearson PL, Peeters PHM** 2003 Subfertility reflects accelerated ovarian ageing. Hum Reprod 18:644-8
36. **Pedersen T, Peters H** 1971 Follicle growth and cell dynamics in the mouse ovary during pregnancy. Fertil Steril 22:42-52
37. **Matt DW, Sarver PL, Lu JK** 1987 Relation of parity and estrous cyclicity to the biology of pregnancy in aging female rats. Biol Reprod 37:421-30
38. **Lapolt PS, Yu SM, Lu JK** 1988 Early treatment of young female rats with progesterone delays the aging-associated reproductive decline: a counteraction by estradiol. Biol Reprod 38:987-95
39. **Lapolt PS, Matt DW, Lu JK** 1998 Progesterone implants delay age-related declines in regular estrous cyclicity and the ovarian follicular reserve in Long-Evans rats. Biol Reprod 59:197-201
40. **van Rooij IAJ, Broekmans FJM, te Velde ER, Fauser BCJM, Bancsi LF, de Jong FH, Themmen APN** 2002 Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod 17:3065-71
41. **Visser JA, de Jong FH, Laven JSE, Themmen APN** 2006 Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 131:1-9
42. **La Marca A, Giulini S, Orvieto R, De Leo V, Volpe A** 2005 Anti-Mullerian hormone concentrations in maternal serum during pregnancy. Hum Reprod 20:1569-72
43. **Hahn RA, Eaker E, Rolka H** 1997 Reliability of reported age at menopause. Am J Epidemiol 146:771-5
44. **den Tonkelaar I** 1997 Validity and reproducibility of self-reported age at menopause in women participating in the DOM-project. Maturitas 27:117-23

Chapter 6

A functional AMH polymorphism is associated with follicle number and androgen levels in Polycystic Ovary Syndrome patients

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Submitted

ABSTRACT

Background: The common characteristic of Polycystic Ovary syndrome (PCOS) is a disturbance in the selection of the dominant follicle resulting in anovulation. In PCOS women serum Anti-Müllerian Hormone (AMH) levels are elevated. As AMH decreases FSH sensitivity in mice, the elevated AMH levels may contribute to the disturbed follicle selection in PCOS women. The aim of this study was to investigate the role of the AMH signaling pathway in the pathophysiology of PCOS using a genetic approach.

Methods: The association of the AMH Ile^{49}Ser (rs10407022) and the AMH type II receptor -482 A>G (rs2002555) polymorphism with PCOS susceptibility and phenotype was studied in large a cohort of PCOS women (n=331). The control group consisted of 32 normo-ovulatory women and 3635 population based controls.

Results: Genotype and allele frequencies for the AMH Ile^{49}Ser and AMHR2 –482 A>G polymorphism were similar in PCOS women and controls. However, within the group of PCOS women, carriers of the AMH ^{49}Ser allele had less often polycystic ovaries (92.7% versus 99.5%, P=0.0004), lower follicle numbers (P=0.03) and lower androgen levels compared to non-carriers (P=0.04). In addition, *in vitro* studies demonstrated that the bioactivity of the AMH ^{49}Ser protein is diminished compared to the AMH ^{49}Ile protein (P<0.0001).

Conclusions: Genetic variants in the AMH and AMHR2 gene do not influence PCOS susceptibility. However, our results suggest that the AMH Ile^{49}Ser polymorphism contributes to the severity of the PCOS phenotype.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is the most frequent endocrine disorder and most common cause of anovulation in women of reproductive age [1]. According to the Rotterdam 2003 consensus [2], PCOS is characterized by (at least) two out of the following three criteria: oligo- or anovulation, clinical or biochemical hyperandrogenism and polycystic ovaries on ultrasound. The etiology of this very heterogeneous syndrome is still poorly understood. However, a major genetic component has been demonstrated, *i.e.* the heritability estimate in monozygotic twin sisters is 0.72 [3], and a candidate gene approach has been widely used to identify the molecular genetic mechanisms and the metabolic and/or biochemical pathways that are implicated in the etiology of PCOS [4]. In particular, candidate genes involved in pathways that regulate gonadotropin secretion, affect androgen production and action, and influence insulin signaling have been considered, as reviewed in [4, 5]. However, little is known about factors involved in early follicle development [6]. An important regulator of folliculogenesis that may play a role in the pathophysiology of PCOS is anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance [7]. AMH is produced by the granulosa cells of growing follicles in the ovary and serum AMH levels correlate with the number of antral follicles as observed by transvaginal ultrasound [8]. PCOS women display a two to threefold increase in serum AMH levels compared to normo-ovulatory women, reflecting the increased number of small antral follicles [9, 10].

Although little is known about the role of AMH in the human ovary, studies in mice showed that AMH inhibits initial recruitment [11] and reduces FSH sensitivity of growing follicles [12]. Given its comparable expression pattern in women and mice [13, 14], AMH may have similar roles in human ovarian folliculogenesis. Hence, the high AMH levels in women with PCOS may contribute to their aberrant follicle selection. Since AMH inhibits FSH-induced aromatase activity in *in vitro* cultured mouse [15] and human granulosa cells [16], AMH may also be responsible for the reduced aromatase activity in PCOS granulosa cells [7, 17], and contribute to the elevated androgen levels in PCOS women. Indeed, AMH serum levels are positively correlated with androgen levels in PCOS patients [9, 10], supporting the latter hypothesis.

Recently, we showed that two genetic variants of AMH (Ile^{49}Ser; rs 10407022) and its specific type II receptor (AMHR2 –482 A>G; rs 2002555) genes are associated with estradiol levels in normo-ovulatory women, suggesting that these polymorphisms modulate intra-ovarian FSH sensitivity and thereby aromatase activity [18]. In the present study, we investigated for the first time whether these genetic variants of the AMH signaling pathway are associated with the suscepti-

bility or phenotype of PCOS in a large Dutch Caucasian cohort of PCOS women (n=331). In addition, *in vitro* studies were performed to analyze the functional aspects of the AMH Ile[49]Ser polymorphism.

MATERIAL AND METHODS

Subjects

Dutch Caucasian patients attending our fertility clinic between 1993 and 2004, who fulfilled the definition of PCOS according to the Rotterdam criteria [2] were enrolled in this study. Hyperandrogenism was defined as elevated (> 4.5) free androgen index (testosterone x 100/ SHBG). Polycystic ovaries were defined as 12 or more follicles (measuring 2-9 mm) per ovary, and the ovarian volume was considered to be increased above 10 ml [19]. Standardized initial screening (clinical investigation, transvaginal ultrasound, and fasting blood withdrawal) was performed on a random cycle day between 09.00 and 11.00h, irrespective of the interval between blood sampling and the preceding menses.

For the present study, 331 PCOS women, of whom DNA was available and genotyping for the AMH and AMHR2 polymorphisms was successful, were included.

For sonographic imaging, we used a 6.5 MHz vaginal transducer (model EUB-415, Hitachi Medical Corp., Tokyo, Japan). Ovarian volume and follicle number were assessed as described earlier [20]. Serum hormone levels were assessed using the following assays: Serum FSH, LH and SHBG were measured by luminescence-based immunometric assays (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA). Serum estradiol and testosterone were measured using radioimmunoassays (Diagnostic Products Corporation). Serum androstenedione was measured using the Immulite 2000. Inhibin B was measured using an enzyme-immunometric assay (Oxford BioInnovation, Oxford, UK) and AMH levels were measured using an in-house AMH ELISA assay [21], commercially available through Diagnostic Systems Laboratories (Webster, Texas, USA). Intra- and interassay coefficients of variation (CV) were less than 3% and 5.8% for FSH, less than 3.5% and 7.1% for LH, less than 10.9% and 10.7% for androstenedione, less than 6.0% and 4.8% for SHBG, less than 10.2% and 8.8% for estradiol, less than 5.7% and 8.4% for testosterone, less than 7.0% and 15% for inhibin B and less than 3.5% and 4% for AMH. Free testosterone levels were calculated using the equation according to Sodergard [22, 23].

The control group consisted of 32 Dutch Caucasian normo-ovulatory women in whom the AMH and AMHR2 polymorphism were genotyped, as described

previously [18]. Inclusion criteria were a regular menstrual cycle (26–30 d), age between 20–36 yr and normal body mass index (18–25 kg/m^2). In this cohort, serum testosterone, serum AMH and follicle number were assessed on day 3 of the menstrual cycle using the methods described above.

In addition, a large population-based cohort, the Rotterdam study, was used to determine the allele and genotype frequency of the AMH and AMHR2 polymorphisms in the general population. The design and rationale of this study have been described earlier [24]. For the present study, only women were included (n=3635).

Furthermore, allele frequencies of the AMH and AMHR2 polymorphisms in the PCOS group were compared to the frequencies reported for Caucasians in the HapMap database (www.hapmap.org) [25].

Genotyping

Genomic DNA was extracted from peripheral blood using standard DNA extraction methods. Previously we have shown that the AMH Ile^{49}Ser and the AMHR2 –482 A>G polymorphism are both in complete linkage disequilibrium (D'=1 and r^2=1) with the additional polymorphisms with an allele frequency > 10% located in the coding and non-coding regions of each gene, including 1 kb of the promoter region [18]. The AMH Ile^{49}Ser and AMHR2 –482 A>G genotypes were determined using Taqman allelic discrimination assays. For the AMH Ile^{49}Ser polymorphism an Assay-by-Design with the following probes was used: 5'-CTCCAGGCA**t**CCCACAA-3' and 5'-CCAGGCA**g**CCCACAA-3'. For the AMHR2 -482 A>G promoter SNP we used an Assay-on-Demand, Assay ID C_ 1673084_10 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Reactions were performed as described previously [18].

Recombinant human AMH production

The full-length human AMH (hAMH) cDNA was isolated from human testis and subcloned into the pcDNA3.1 expression vector (Invitrogen, Breda, The Netherlands) as described previously [14]. Quick change site-directed mutagenesis was performed according to the manufacturer (Stratagene, Amsterdam, The Netherlands) to introduce the Ile^{49}Ser polymorphism. HEK293 cells were transfected with the hAMH-^{49}Ile and hAMH-^{49}Ser expression vectors. Cells transfected with the empty pcDNA3.1 vector served as control. Supernatants were collected under serum free culture conditions, and were concentrated approximately 40-fold using a Centriprep system (Millipore Corp., Amsterdam, The Netherlands) and the amount of AMH was measured by the in-house AMH ELISA assay as described previously [21].

Western blot

Western blot analysis was performed using the mouse monoclonal antibodies 5/6A and 9/6A [14, 26]. Proteins from conditioned medium were separated using 12% polyacrylamide gel electrophoresis under reducing conditions, transferred to nitrocellulose membranes and incubated with the 5/6A or 9/6A antibody at a 1:1000 dilution, followed by a secondary Alexa Fluor-680 goat antimouse antibody (Molecular Probes, Invitrogen, Breda, The Netherlands) at a 1:15000 dilution. Proteins were visualized using the Licor Odyssey imaging system and blots were analyzed with the Odyssey software version 2.1 (LI-COR Biosciences, Westburg, Leusden, The Netherlands).

Cell culture and transfections

The mouse granulosa cell line KK-1 [27] (a kind gift of Dr. I. Huhtaniemi), and the human granulosa cell line COV434, derived from a human granulosa cell tumor but possessing many characteristics of normal granulosa cells [28, 29], were cultured in DMEM/F12 (Gibco, Invitrogen, Breda, The Netherlands) containing 10% FCS and penicillin (400 IU/ml) and streptomycin (0.4 mg/ml), and stably transfected with an AMHRII expression vector [30]. For AMH-induced luciferase assays, KK-1 and COV434 cells were seeded at 20% confluency in 24 wells plates and transfected with the BRE-Luc reporter plasmid (150 ng/well) [31] (kind gift of Dr. P. ten Dijke) using Fugene 6 transfection reagent (Roche Diagnostics Nederland B.V., Almere, The Netherlands) The pRL-TK plasmid (Promega, Leiden, The Netherlands) served as an internal control to normalize for transfection efficiency. Twenty-four hours after transfection, cells were cultured for 2 hours in medium containing 0.2% FCS followed by 16 hours treatment with increasing concentrations of the hAMH variants. Luciferase activity was determined using the Dual-Glo™ Luciferase Assay (Promega, Leiden, The Netherlands) in the TOPCOUNT luminometer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Statistical analysis

AMH levels were compared between normo-ovulatory controls and PCOS women using one-way analysis of (co)variance (AN(C)OVA), with adjustment for age and BMI. Within the PCOS cohort, Spearman's correlation coefficient was used to correlate AMH serum levels with additional hormone levels and total follicle number.

In each group of women, genotype frequencies of the AMH and AMHR2 polymorphisms were tested for Hardy-Weinberg equilibrium proportions using the ARLEQUIN package [32]. Differences in allele and genotype frequencies

between cases and controls were tested using a Chi-squared test. For reasons of statistical power, carriers of the AMH ^{49}Ser allele and carriers of the AMHR2 –482G allele were compared to non-carriers. If appropriate, hormone levels were log transformed to normalize their distribution. Within the PCOS group, one-way analysis of (co)variance (AN(C)OVA) was used to determine differences in continuous variables between genotype groups. Androgen related traits and ovarian parameters were adjusted for age and BMI. Categorical parameters were analyzed using Fisher's exact test. Subsequently, to correct for multiple testing, we obtained an empirical P-value by permutation analysis using Haploview version 3.32 [33]. The phenotypic status of each individual was permuted 10,000 times and association analysis was performed to obtain the test statistic under the null hypothesis of no association. The empirical P-value was obtained as the proportion of the 10,000 replicates that had a P-value less than or equal to the one obtained from the actual (unshuffled) data.

Prism software was used to fit the sigmoidal dose-response curves of the *in vitro* studies and to calculate the EC50 and maximal response values. To test differences in EC50 and maximal response between the AMH variants the F-test comparison method was used (GraphPad Prism 4.0 Software, Inc. San Diego, CA, USA). Unless stated otherwise, analyses were performed using Statistical Package for Social Sciences, SPSS, version 11.0.1 (SPSS Inc, Chicago, IL). $P \leq 0.05$ was considered to be significant.

RESULTS

AMH serum levels in PCOS women

Clinical characteristics of the normo-ovulatory women and the women with PCOS are shown in Table 6.1. As shown previously, PCOS patients had elevated AMH levels compared with the normo-ovulatory controls (Table 6.1). To provide insight into the relationship of AMH serum levels with androgen levels and follicle number in the PCOS cohort, correlation coefficients between AMH levels and these parameters were determined. In the PCOS cohort, serum AMH levels were positively correlated with total testosterone levels ($r=0.44$, $P<0.001$), free testosterone levels ($r=0.36$, $P<0.001$), androstenedione levels ($r=0.44$, $P<0.001$), LH levels ($r=0.31$, $P<0.001$) and total follicle number ($r=0.54$, $P<0.001$), but not with estradiol levels ($r=0.01$, $P=0.80$).

Table 6.1 Clinical characteristics of normo-ovulatory and PCOS women

	Normo-ovulatory women	PCOS patients
Number	32	331
Age (year) (range)	29.9 ± 4.1 (20-36)	28.7 ± 4.7 (15-44)
BMI (kg/m^2)	22.0 ± 2.7	27.1 ± 6.2*
PCO (%)	0.0	97.0*
Total follicle number	14.8 ± 4.6	41.1 ± 21.9*
AMH (ng/ml)	3.1 ± 2.7	12.2 ± 7.7*
Hyperandrogenism (%)	0.0	52.0*
Testosterone (nmol/L)	1.3 ± 0.5	2.0 ± 0.9*
Oligo- or Anovulation	0.0	100.0*

* P< 0.001 compared to control group.
Data represent mean ± SD.

PCOS risk by AMH and AMHR2 genotypes

Genotype and allele frequencies of the AMH Ile^{49}Ser and the AMHR2 –482 A>G polymorphism in the 331 PCOS women did not differ from the frequencies in the normo-ovulatory controls and in the Rotterdam study (Table 6.2). In addition, the allele frequencies for both polymorphisms in PCOS women were similar to allele frequencies of Caucasians in the HapMap database (www.hapmap.org) [25].

Table 6.2 Genotype distributions of AMH and AMHR2 polymorphisms in PCOS cases and controls

		AMH Ile^{49}Ser					AMHR2 –482 A>G				
	N	Ile/Ile n (%)	Ile/Ser n (%)	Ser/Ser n (%)	MAF	P[a]	A/A n (%)	A/G n (%)	G/G n (%)	MAF	P[a]
PCOS	331	208 (62.9)	110 (33.2)	13 (3.9)	0.21		224 (67.7)	94 (28.4)	13 (3.9)	0.18	
Normo-ovulatory controls	32	24 (75)	8 (25)	0 (0.0)	0.13	0.28	20 (62.5)	12 (37.5)	0 (0.0)	0.19	0.33
Rotterdam study	3635	2457 (67.6)	1075 (29.6)	103 (2.8)	0.18	0.16	2386 (65.6)	1125 (31.0)	124 (3.4)	0.19	0.59

PCOS= Polycystic Ovary Syndrome, MAF=Minor Allele Frequency
[a] P-value of X^2 test for genotype frequencies in PCOS cases versus control group

The genotype distributions of the AMH Ile^{49}Ser polymorphism and the AMHR2 –482 A>G polymorphism were in Hardy Weinberg equilibrium proportions in both cases and controls (results not shown).

PCOS phenotype by AMH Ile^{49}Ser genotype

Within the group of PCOS women, genotypes of the AMH Ile^{49}Ser polymorphism were not associated with general characteristics, such as age, BMI and Waist Hip Ratio (Table 6.3). However, carriers of the AMH ^{49}Ser allele had less often polycystic ovaries compared to non-carriers (92.7% versus 99.5%, P=0.0008),

Table 6.3 General characteristics and ovarian parameters of PCOS patients by AMH Ile[49]Ser and AMHR2 –482 A>G genotypes

	AMH			AMHR2		
	Ile/Ile	Ile/Ser + Ser/Ser	P	A/A	A/G + G/G	P
N	208	123		224	107	
Age (yr)	28.8 ± 0.3	28.5 ± 0.5	0.65	28.5 ± 0.3	29.1 ± 0.5	0.29
BMI (kg/m^2)	27.0 ± 0.4	27.3 ± 0.5	0.73	27.1 ± 0.4	27.2 ± 0.6	0.90
WHR	0.83 ± 0.01	0.85 ± 0.01	0.07	0.83 ± 0.01	0.84 ± 0.01	0.13
PCO n (%)	207 (99.5)	114 (92.7)	0.0004[a]	219 (97.8)	102 (95.3)	0.23
Total Follicle Count[b]	43.1 ± 1.5	37.7 ± 2.0	0.03	41.6 ± 1.5	40.0 ± 2.1	0.84
Mean Ovarian volume[b]	9.6 ± 0.3	9.1 ± 0.4	0.21	9.5 ± 0.3	9.2 ± 0.4	0.46
Amenorrhea n (%)	59 (26.5)	45 (34.9)	0.10	69 (28.6)	35 (31.5)	0.58

Data represent the mean ± SEM, PCO = polycystic ovaries, defined as described in material and methods

[a]P-value obtained using permutation analysis

[b]corrected for age and BMI

which remained significant after permutation analysis (P=0.0004) (Table 6.3). Hence, [49]Ser allele carriers also had a lower total follicle number, on average 5.4 follicles less (=12%), compared to non-carriers (P=0.03) (Table 6.3). The mean ovarian volume and the percentages of women with amenorrhea were not different between both genotype groups (Table 6.3).

AMH levels were similar between carriers and non-carriers of the AMH [49]Ser allele but carriers of the AMH [49]Ser allele had almost 10% lower total testosterone (P=0.05) and androstenedione levels (P=0.04) compared with non-carriers (Table 6.4). However, SHBG levels were also lower in carriers of the AMH [49]Ser allele compared with non-carriers (P=0.04), and hence, free testosterone levels were

Table 6.4 Hormone levels in PCOS patients by AMH Ile[49]Ser and AMHR2 –482 A>G genotypes:

	AMH			AMHR2		
	Ile/Ile	Ile/Ser + Ser/Ser	P	A/A	A/G + G/G	P
N	208	123		224	107	
AMH (ng/ml)[a]	12.4 ± 0.5	11.9 ± 0.7	0.32	12.3 ± 0.5	12.1 ± 0.7	0.48
LH (IU/L)[a]	8.2 ± 0.3	8.0 ± 0.4	0.28	8.2 ± 0.3	7.9 ± 0.5	0.86
FSH (IU/L)[a]	4.6 ± 0.1	4.7 ± 0.2	0.73	4.6 ± 0.1	4.9 ± 02	0.17
Inhibin B (ng/L)[a]	103.9 ± 5.5	104.5 ± 7.1	0.95	107.6 ± 5.2	96.8 ± 7.5	0.24
Total testosterone (nmol/L)[a]	2.03 ± 0.06	1.84 ± 0.08	0.05	1.99 ± 0.06	1.90 ± 0.08	0.38
Free testosterone (pmol/L)[a]	43.1 ± 1.4	40.1 ± 1.8	0.17	42.8 ± 1.3	40.3 ± 1.9	0.27
Androstenedione (nmol/L)[a]	13.0 ± 0.3	11.9 ± 0.4	0.04	12.7 ± 0.3	12.2 ± 0.5	0.34
E2 (pmol/L)[a]	294.3 ± 11.8	263.8 ± 15.3	0.08	283.0 ± 11.4	282.7 ± 16.5	0.98
SHBG (nmol/L)[a]	47.3 ± 2.0	42.8 ± 2.7	0.04	45.6 ± 2.0	45.8 ± 2.9	0.53

[a]corrected for age and BMI. E_2 = Estradiol

Data represent the mean ± SEM

not significantly different between the AMH genotype groups (P=0.17). FSH, LH, estradiol and inhibin B levels were similar in both groups (Table 6.4).

Functional analysis of the AMH Ile[49]Ser polymorphism

The results described above suggest that the AMH Ile[49]Ser polymorphism modulates AMH function. To determine whether this polymorphism had an effect on the secretion and/or processing of AMH, Western blot analysis of supernatants from hAMH-[49]Ile and hAMH-[49]Ser expressing cells was performed using proregion-specific (Mab 9/6A) and mature region-specific (Mab 5/6A) antibodies. For both AMH variants the N-terminal proregion, the C-terminal mature region and an additional cleavage band as a result of a potential second cleavage site, were detected with comparable intensities (Figure 6.1), suggesting similar processing. The observed incomplete processing of recombinant hAMH-[49]Ile and hAMH-[49]Ser is consistent with previous reports [34, 35]. Introduction of an optimized cleavage

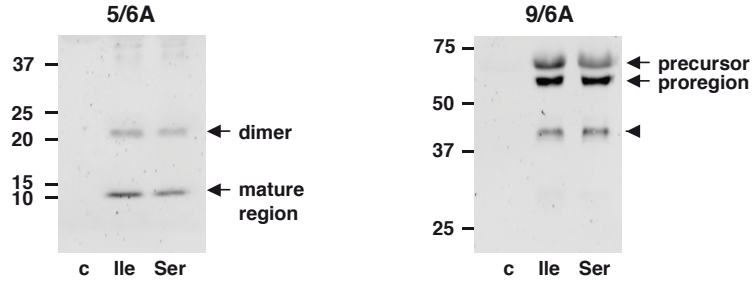

Figure 6.1 Western blot analysis of human recombinant AMH variants, AMH-[49]Ile (Ile) and AMH-[49]Ser (Ser), using mouse monoclonal AMH antibodies. The antibody 5/6A recognized the C-terminal ~12 kDa mature region of AMH, including the stable dimer. The antibody 9/6A recognizes the full-length N-terminal ~57 kDa pro-region and a second subunit due to a possible second cleavage site (~40 kDa, indicated by arrowhead). In addition, these antibodies recognize the AMH precursor protein. No AMH protein is detected in the control medium (c). Relative molecular mass (kDA) of the standards are indicated on the left. No difference was observed in the cleavage of the AMH variants.

site (RARR) resulted in fully cleaved AMH. Again, no differences in processing between hAMH-RARR-[49]Ile and hAMH-RARR-[49]Ser were observed (results not shown).

To determine the effect of the Ile[49]Ser polymorphism on AMH bioactivity, a mouse granulosa cell line (KK1) and a human granulosa cell line (COV434) were transiently transfected with the AMH-responsive luciferase reporter BRE-Luc, and stimulated with hAMH-[49]Ile and hAMH-[49]Ser. Dose-response experiments in both cell lines revealed that stimulation with equal amounts of hAMH-[49]Ser resulted in a similar EC50 but a significantly lower maximal induction of luciferase activity

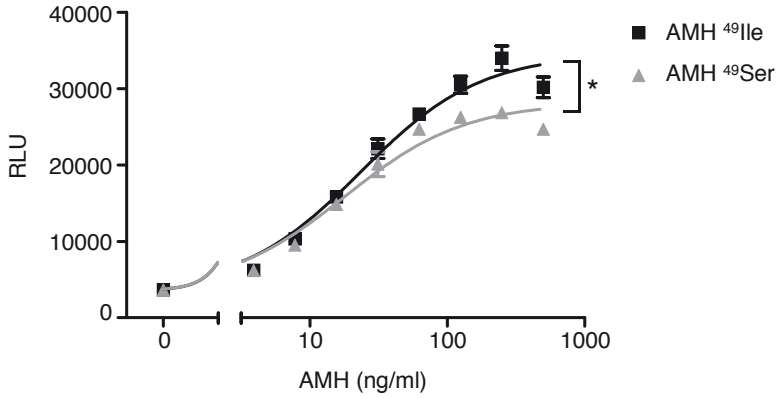

Figure 6.2 Dose response analysis of the recombinant human AMH variants in mouse KK-1/AMHRII cells. KK-1/AMHRII cells were transiently transfected with a luciferase reporter plasmid and incubated with equal concentration ranges of rhAMH-^{49}Ile (black line) or rhAMH-^{49}Ser (grey line). Stimulation with rhAMH-^{49}Ser protein resulted in a similar EC50 but a lower maximum response compared to stimulation with rhAMH-^{49}Ile (*P<0.0001). Data are expressed as relative luciferase units (RLU) and are the mean ± SEM of triplicates from a representative experiment that was performed at least three times with two independent batches of the recombinant AMH variants from independent cultures. Some *error bars* are too small to be visible in the graph.

compared to stimulation with hAMH-^{49}Ile (P<0.0001) (Figure 6.2, and results not shown). These observations suggest that the hAMH-^{49}Ser constitutes an AMH protein with lower bioactivity.

PCOS phenotype by AMHR2 –482 A>G genotype

The AMHR2 –482 A>G polymorphism was not associated with age, BMI and Waist Hip Ratio (Table 6.3). Furthermore, no association of the AMHR2 genotypes with polycystic ovaries, follicle number or ovarian volume was observed (Table 6.3). AMH serum levels, androgen levels and other hormones also did not differ between the genotype groups (Table 6.4).

DISCUSSION

The present study was designed to investigate the functional role of AMH in PCOS using a genetic approach. The association of polymorphisms in genes of the AMH signaling pathway with PCOS susceptibility and phenotype was studied in a large cohort of PCOS women. We observed that polymorphisms in the AMH and AMHR2 gene did not contribute to the risk for development of PCOS. However, within the PCOS group, the AMH Ile^{49}Ser polymorphism was

associated with follicle number, androgen levels and the percentage of women exhibiting polycystic ovaries.

The main feature of follicular dysfunction in PCOS is the disturbed selection of the dominant follicle resulting in anovulation. Studies in mice have shown that AMH decreases FSH sensitivity *in vivo* and *in vitro* [12]. Hence, the elevated AMH levels in PCOS women may contribute to the refractoriness to FSH-induced follicle differentiation, leading to the aberrant selection process. The observed association of the AMH Ile[49]Ser variant with follicular parameters supports this hypothesis. The [49]Ser variant is associated with a lower follicle number and a lower PCO frequency compared to the [49]Ile variant, suggesting that the AMH [49]Ser variant is less effective in reducing the individual FSH-sensitivity of antral follicles. Indeed, our *in vitro* studies demonstrated that the bioactivity of the AMH [49]Ser protein is diminished compared to the AMH [49]Ile protein.

The Ile[49]Ser polymorphism is located in the proregion of the AMH protein. It has been suggested that this domain is involved in protein stability and folding and mutations within this proregion could affect biosynthesis or bioactivity of AMH. Indeed, mutations in the proregion of AMH can render the protein inactive as has been demonstrated in patients with persistent Müllerian duct syndrome (PMDS) [36]. The AMH Ile[49]Ser polymorphism did not affect the processing of AMH, but did affect its bioactivity. The presence of a serine at amino acid position 49 possibly alters the folding of the protein rendering the protein slightly less bioactive compared to the protein with an isoleucine at this position. However, EC50 values were not changed, suggesting that the binding/interaction of both variants with the AMHRII/type I receptor complex is not different, but that the [49]Ser variant induces weaker or altered conformational changes in the receptor complex that lead to a lower maximal transduction efficacy compared to the [49]Ile variant.

Although the etiology of PCOS is not clearly established, accumulating evidence suggests that PCOS results primarily from exposure of the fetal ovary to high androgen levels [37]. Subsequently, secondary genetic and environmental factors may interact with this underlying process and lead to the heterogeneity in the phenotype of PCOS [38]. The absence of an association of the functional AMH Ile[49]Ser polymorphism with PCOS risk indicates that the AMH signaling pathway is not directly involved in the pathophysiology of PCOS. Nevertheless, the association of the AMH Ile[49]Ser polymorphism with follicle number suggests that AMH may be one of the factors modifying the final PCOS phenotype.

In addition, AMH may be responsible for the diminished induction of aromatase activity in PCOS granulosa cells [7]. In a previous study, we observed that normo-ovulatory women carrying the AMH [49]Ser allele had higher follicular

phase estradiol levels compared to women carrying the AMH ^{49}Ile allele, also suggesting that the AMH ^{49}Ser variant results in less inhibition of FSH-induced aromatase activity in normal granulosa cells [18].

In the present study in PCOS women, carriers of the ^{49}Ser allele had lower androstenedione and testosterone levels compared to non-carriers, also suggesting less inhibition of FSH-induced aromatase activity. Nevertheless, the AMH Ile^{49}Ser polymorphism was not associated with estradiol levels, but this may be explained by the peripheral conversion of androgens, also contributing to final serum estradiol levels and thereby masking the subtle differences in follicular fluid estradiol levels. Indeed, serum AMH levels were also not correlated with serum estradiol levels in the PCOS cohort.

Interestingly, AMH is located in the same chromosomal region (19p13) as a promising locus for genetic susceptibility for PCOS (STS marker D19S884, chr 19p13.2 [39, 40]). However, the AMH Ile^{49}Ser polymorphism is not associated with PCOS risk and given the large distance and the lack of linkage disequilibrium between the AMH Ile^{49}Ser polymorphism and this marker (about 5850 Kb) or any SNPs in its region (based on HapMap database [25]), it is very unlikely that our findings with the AMH Ile^{49}Ser polymorphism are related to the proposed candidate gene region on chromosome 19p13.2.

The AMHR2 -482 A>G polymorphism is neither associated with PCOS susceptibility nor with the final phenotype. Although we observed in our previous study that also the AMHR2 -482G allele was associated with higher follicular phase estradiol levels in premenopausal women, suggesting less inhibition of FSH-sensitivity by this AMHR2 variant [18], in PCOS women this effect may be masked by the elevated AMH levels.

Candidate gene studies in PCOS suffer from a lack of reproducibility between cohorts, which may be attributed to the different criteria used to define PCOS, but also to several additional factors [4]. First, most PCOS studies reported so far have been based on very small sample sizes, therefore lacking sufficient statistical power. In contrast, our cohort, which consists of 331 PCOS women, is among the largest studied in PCOS genetics. Second, in many studies only one or a few variants per gene have been tested, whereas it is critical to characterize the genetic variation of the entire candidate gene to unravel the etiology of genetically complex diseases such as PCOS [4]. In our study, the analyzed polymorphisms in the AMH and AMHR2 gene both capture the common genetic variation in the gene, including 1 kb of the promoter region [18]. Last but not least, the issue of multiple testing requires attention in association studies. Nevertheless, the association of the AMH Ile^{49}Ser polymorphism with the PCO phenotype withstands correction for multiple testing using permutation analysis. In addition, the strong

a priori rationale in combination with the functional evidence makes it very unlikely that our results could be explained by chance alone [41].

In conclusion, our results provide new insight into the role of AMH in the pathophysiology of PCOS. The observed association between the AMH Ile[49]Ser polymorphism and follicle number and androgen levels, together with the *in vitro* evidence of the functional effect of this polymorphism, strongly suggests that AMH contributes to the severity of the PCOS phenotype. Although the findings of the association study need to be replicated in additional cohorts, our results imply that the AMH Ile[49]Ser polymorphism is one of the genetic factors contributing to the complex etiology of PCOS.

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REFERENCES

1. **Franks S** 1995 Polycystic ovary syndrome. N Engl J Med 333:853-61
2. **The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group** 2004 Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 19:41-7
3. **Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI** 2006 Heritability of polycystic ovary syndrome in a Dutch twin-family study. J Clin Endocrinol Metab 91:2100-4
4. **Urbanek M** 2007 The genetics of the polycystic ovary syndrome. Nat Clin Pract Endocrinol Metab 3:103-11
5. **Escobar-Morreale HF, Luque-Ramirez M, San Millan JL** 2005 The molecular-genetic basis of functional hyperandrogenism and the polycystic ovary syndrome. Endocr Rev 26:251-82
6. **Franks S** 2006 Candidate genes in women with polycystic ovary syndrome. Fertil Steril 86 Suppl 1:S15
7. **Visser JA, de Jong FH, Laven JSE, Themmen APN** 2006 Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 131:1-9
8. **de Vet A, Laven JSE, de Jong FH, Themmen APN, Fauser BCJM** 2002 Antimullerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril 77:357-62
9. **Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, Dewailly D** 2003 Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. J Clin Endocrinol Metab 88:5957-62
10. **Laven JSE, Mulders AGMGJ, Visser JA, Themmen APN, De Jong FH, Fauser BCJM** 2004 Anti-Mullerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age. J Clin Endocrinol Metab 89:318-23
11. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-96
12. **Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-9
13. **Durlinger ALL, Visser JA, Themmen APN** 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124:601-9
14. **Weenen C, Laven JSE, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BCJM, Themmen APN** 2004 Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 10:77-83
15. **di Clemente N, Goxe B, Remy JJ, Cate R, Josso N, Vigier B, Salesse R** 1994 Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine 2:553-558
16. **Grossman MP, Nakajima ST, Fallat ME, Siow Y** 2007 Mullerian-inhibiting substance inhibits cytochrome P450 aromatase activity in human granulosa lutein cell culture. Fertil Steril
17. **Laven JSE, Imani B, Eijkemans MJC, Fauser BCJM** 2002 New approach to polycystic ovary syndrome and other forms of anovulatory infertility. Obstet Gynecol Surv 57:755-67
18. **Kevenaar ME, Themmen APN, Laven JSE, Sonntag B, Lie Fong S, Uitterlinden AG, de Jong FH, Pols HAP, Simoni M, Visser JA** 2007 Anti-Mullerian hormone and anti-

Mullerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women. Hum Reprod 22:1547-54

19. **Balen AH, Laven JSE, Tan SL, Dewailly D** 2003 Ultrasound assessment of the polycystic ovary: international consensus definitions. Hum Reprod Update 9:505-14
20. **van Santbrink EJP, Hop WCJ, Fauser BCJM** 1997 Classification of normogonadotropic infertility: polycystic ovaries diagnosed by ultrasound versus endocrine characteristics of polycystic ovary syndrome. Fertil Steril 67:452-8
21. **Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groome NP, Themmen APN, Visser JA** 2006 Serum Anti-Mullerian hormone levels reflect the size of the primordial follicle pool in mice. Endocrinology 147:3228-34
22. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H** 1982 Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801-10
23. **de Ronde W, van der Schouw YT, Pols HA, Gooren LJ, Muller M, Grobbee DE, de Jong FH** 2006 Calculation of bioavailable and free testosterone in men: a comparison of 5 published algorithms. Clin Chem 52:1777-84
24. **Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA** 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 7:403-22
25. **The International HapMap Consortium** 2003 The International HapMap Project. Nature 426:789-96
26. **Al-Qahtani A, Muttukrishna S, Appasamy M, Johns J, Cranfield M, Visser JA, Themmen APN, Groome NP** 2005 Development of a sensitive enzyme immunoassay for anti-Mullerian hormone and the evaluation of potential clinical applications in males and females. Clin Endocrinol (Oxf) 63:267-73
27. **Kananen K, Markkula M, Rainio E, Su JG, Hsueh AJ, Huhtaniemi IT** 1995 Gonadal tumorigenesis in transgenic mice bearing the mouse inhibin alpha-subunit promoter/simian virus T-antigen fusion gene: characterization of ovarian tumors and establishment of gonadotropin-responsive granulosa cell lines. Mol Endocrinol 9:616-27
28. **Zhang H, Vollmer M, De Geyter M, Litzistorf Y, Ladewig A, Durrenberger M, Guggenheim R, Miny P, Holzgreve W, De Geyter C** 2000 Characterization of an immortalized human granulosa cell line (COV434). Mol Hum Reprod 6:146-53
29. **van den Berg-Bakker CA, Hagemeijer A, Franken-Postma EM, Smit VT, Kuppen PJ, van Ravenswaay Claasen HH, Cornelisse CJ, Schrier PI** 1993 Establishment and characterization of 7 ovarian carcinoma cell lines and one granulosa tumor cell line: growth features and cytogenetics. Int J Cancer 53:613-20
30. **Visser JA, Olaso R, Verhoef-Post M, Kramer P, Themmen APN, Ingraham HA** 2001 The serine/threonine transmembrane receptor ALK2 mediates Mullerian inhibiting substance signaling. Mol Endocrinol 15:936-45
31. **Korchynskyi O, ten Dijke P** 2002 Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883-91
32. **Schneider S, Roessli D, Excoffier L** 2000 Arlequin version 2.000: a software for population genetics data analysis, 2.000 ed. Genetics and Biometry Laboratory, University of Geneva, Geneva
33. **Barrett JC, Fry B, Maller J, Daly MJ** 2005 Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263-5

34. **di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard JY, Vigier B, Josso N, Cate R** 1994 Cloning, expression, and alternative splicing of the receptor for anti-Mullerian hormone. Mol Endocrinol 8:1006-20
35. **Nachtigal MW, Ingraham HA** 1996 Bioactivation of Mullerian inhibiting substance during gonadal development by a kex2/subtilisin-like endoprotease. Proc Natl Acad Sci U S A 93:7711-6
36. **Belville C, Van Vlijmen H, Ehrenfels C, Pepinsky B, Rezaie AR, Picard JY, Josso N, di Clemente N, Cate RL** 2004 Mutations of the anti-mullerian hormone gene in patients with persistent mullerian duct syndrome: biosynthesis, secretion, and processing of the abnormal proteins and analysis using a three-dimensional model. Mol Endocrinol 18:708-21
37. **Franks S, McCarthy MI, Hardy K** 2006 Development of polycystic ovary syndrome: involvement of genetic and environmental factors. Int J Androl 29:278-85; discussion 286-90
38. **Abbott DH, Dumesic DA, Franks S** 2002 Developmental origin of polycystic ovary syndrome - a hypothesis. J Endocrinol 174:1-5
39. **Stewart DR, Dombroski BA, Urbanek M, Ankener W, Ewens KG, Wood JR, Legro RS, Strauss JF, 3rd, Dunaif A, Spielman RS** 2006 Fine mapping of genetic susceptibility to polycystic ovary syndrome on chromosome 19p13.2 and tests for regulatory activity. J Clin Endocrinol Metab 91:4112-7
40. **Urbanek M, Woodroffe A, Ewens KG, Diamanti-Kandarakis E, Legro RS, Strauss JF, 3rd, Dunaif A, Spielman RS** 2005 Candidate gene region for polycystic ovary syndrome on chromosome 19p13.2. J Clin Endocrinol Metab 90:6623-9
41. **Wacholder S, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N** 2004 Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. J Natl Cancer Inst 96:434-42

Chapter 7

Variants in the ACVR1 gene are associated with AMH levels in women with Polycystic Ovary Syndrome.

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ABSTRACT

Background: Polycystic ovaries display an increased number of preantral and antral follicles compared to normal ovaries, suggesting that early and late follicle development are disturbed. Although the pathophysiology of this process is poorly understood, TGFβ family members, such as anti-Müllerian hormone (AMH) and bone morphogenetic proteins (BMPs), may be involved. Since AMH and several BMPs inhibit FSH sensitivity, AMH and/or BMP signaling may contribute to the aberrant follicle development in these women. The aim of this study was to investigate the role of ALK2, a type I receptor for BMP and AMH signaling, in PCOS using a genetic approach.

Methods: Seven single nucleotide polymorphisms in the ACVR1 gene, encoding ALK2, were genotyped in PCOS patients (n=359), normo-ovulatory control women (n=30) and population based control women (3543) and haplotypes were determined. Subsequently, the association of ACVR1 variants with ovarian parameters was investigated.

Results: The polymorphisms rs1220134, rs10497189 and rs2033962 and their corresponding haplotypes within ACVR1 did not have different frequencies from controls but were associated with AMH levels in PCOS women (P=0.001, P=0.002 and P=0.007, respectively). Adjustment for follicle number revealed that the association with AMH levels was, in part, independent from follicle number, suggesting that variants in ACVR1 also influence AMH production per follicle.

Conclusion: Genetic variation within ACVR1 is associated with AMH levels and follicle number in PCOS women, suggesting that ALK2 signaling contributes to the disturbed folliculogenesis in PCOS patients.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is the most frequent endocrine disorder and most common cause of anovulation in women of reproductive age [1, 2]. PCOS is a complex genetic disorder, in which multiple susceptibility genes interact with lifestyle and environmental factors [3]. These genetic factors not only contribute to PCOS risk, but also appear to modulate component traits of PCOS, such as insulin resistance, androgen levels and follicle development [4, 5].

The disturbance of folliculogenesis, resulting in anovulation and infertility, is a major characteristic of PCOS. In polycystic ovaries, the selection of a dominant follicle is disturbed, suggesting aberrant FSH sensitivity of follicles at the antral stage. In addition, polycystic ovaries display an increased density of small preantral follicles compared to normal ovaries, suggesting that early follicle development is also abnormal [6, 7].

Intraovarian growth factors that play an important role in early and late follicle development are members of the Transforming Growth Factor-β (TGFβ) superfamily, such as Bone Morphogenetic Proteins (BMPs) and anti-Müllerian hormone (AMH) [5, 8]. BMPs and AMH signal via a heteromeric receptor complex consisting of a ligand-specific type II receptor and shared type I receptors (ALK2, ALK3, and ALK6) [9-12]. The BMP/AMH signaling system is present in somatic cells and/or oocytes [13, 14], and can exert autocrine and/or paracrine actions. This signaling pathway has been implicated as a negative (AMH) or positive regulator (BMP4 and BMP7) of primordial follicle recruitment [15-17]. Furthermore, AMH and BMPs contribute to the FSH-dependent follicle selection by suppressing FSH actions [17-20]. Interestingly, serum AMH levels are elevated in PCOS women compared to normo-ovulatory women [21, 22], and therefore may contribute or further aggravate the disturbed follicle development and selection in PCOS patients [23]. Indeed, in a recent study we observed that the AMH Ile^{49}Ser polymorphism contributes to the frequency of polycystic ovaries, number of follicles, and level of androgens in PCOS patients (Chapter 6).

In this study we investigated whether ALK2, one of the type I receptors shared by the AMH/BMP signaling pathway, contributes to PCOS susceptibility and/or phenotype using a genetic approach. The common genetic variation across the ACVR1 gene, encoding for ALK2, was captured by selecting tagging single nucleotide polymorphisms (SNPs). Subsequently these SNPs and the corresponding haplotypes were analyzed in a large cohort of PCOS women. We observed that variations in ACVR1 were associated with AMH levels and follicle number in PCOS patients.

MATERIAL AND METHODS

Subjects

The local Medical Ethics Review Committee approved this study, and informed consent was obtained from all participants. Dutch Caucasian patients attending our fertility clinic between 1993 and 2004, who fulfilled the definition of PCOS by the Rotterdam criteria [24] were enrolled in this study (n=409). Hyperandrogenism was defined as an elevated (> 4.5) free androgen index (testosterone x 100/ SHBG) (with testosterone and SHBG both expressed in nmol/L). Polycystic ovaries were defined as 12 or more follicles (measuring 2-9 mm) per ovary and the ovarian volume was considered to be increased above 10 ml [25]. Standardized initial screening (clinical investigation, transvaginal ultrasound, and fasting blood withdrawal) was performed on a random day between 09.00 and 11.00h, irrespective of the interval between blood sampling and the preceding menses.

For sonographic imaging, we used a 6.5 MHz vaginal transducer (model EUB-415, Hitachi Medical Corp., Tokyo, Japan). Ovarian volume, and the mean follicle number were assessed as described earlier [26]. Serum hormone levels were assessed using the following assays: Serum FSH and LH were measured by luminescence-based immunometric assays (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA). Serum estradiol and testosterone were measured using radioimmunoassays (Diagnostic Products Corporation). Serum androstenedione was measured using the Immulite 2000. Inhibin B was measured using an enzyme-immunometric assay (Oxford BioInnovation, Oxford, UK) and AMH levels were measured using an in-house AMH ELISA assay [27], commercially available through Diagnostic Systems Laboratories (Webster, Texas, USA). Intra- and interassay coefficients of variation (CV) were less than 3% and 5.8% for FSH, less than 3.5% and 7.1% for LH, less than 10.9% and 10.7% for androstenedione, less than 10.2% and 8.8% for estradiol, less than 5.7% and 8.4% for testosterone, less than 7.0% and 15% for inhibin B and less than 3.5% and 4% for AMH.

For the present study, DNA was available for 394 PCOS women, and genotyping for all selected ACVR1 polymorphisms was successful in 359 PCOS women. In a subset of 295 women, AMH serum levels were measured.

The control group consisted of a previously described cohort of Dutch Caucasian normo-ovulatory women (n=32) [28, 29], for which genotyping of the seven tagging SNPs in ACVR1 was successful in 30 women. Inclusion criteria were a regular menstrual cycle (26–30 d), age of 20–36 yr and normal body mass index (18–25 kg/m^2). In this cohort, assessment of serum hormone levels (FSH, LH, testosterone, estradiol, inhibin B, AMH) and transvaginal ultrasound were

performed on day 3 of the menstrual cycle, using the methods as described above.

In addition, a large population based cohort, the Rotterdam study, was used to determine the genotype and allele frequencies of the seven tagging SNPs in the ACVR1 gene in the general population. The design and rationale of this study have been described earlier [30]. For the present study, only women in whom all seven ACVR1 SNPs were successfully genotyped were included (n=3543). The mean age of these women was 70.4 years (range 55.0-99.2).

Genotyping and haplotype determination

We selected seven SNPs, rs1220134, rs10497189, rs2033962, rs10933441, rs17798043, rs10497192, rs1372115, which span the genomic length of ACVR1. These seven SNPs were selected because they are predicted to tag the haplotypes across the entire gene including 40 kb of the promoter region (upstream of the first translated exon) and 20 kb of the 3'UTR region, and occurred at >5% frequency in the Caucasian population of the HapMap database [31]. These SNPs were genotyped using Taqman allelic discrimination assays. For four SNPs Assays-on-Demand (*i.e.* Pre-Designed Assay) with the following assay numbers were used; rs1220134, C_7544932_10; rs10933441, C_31158472_10; rs17798043, C_33166336_10; rs10497192, C_8503188_10. (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). For the additional three SNPs Assays-by-Design (*i.e.* Custom Assay) with the following probes were used; rs10497189, 5'-ACTAATGTCC**a**AGAACAC-3' and 5'-AATGTCC**g**AGAACAC-3'; rs2033962, 5'-TCAGCTTTC-**c**GAGCTC-3' and 5'-AGCTTTC**a**GAGCTC-3'; rs1372115, 5'-TTCAGTCC**a**TGGTTTAT-3' and 5'-CAGTCC**g**TGGTTTAT-3'. Each PCR reaction contained 2 ng of dried genomic DNA, 1 µl of Taqman Universal PCR Master Mix 2x, 0.025 µl of the 80X Assay-on–Demand mix or 0.05 µl of the 40X Assay-by-Design mix in a total volume of 2 µl. The PCR reaction was performed according to the instructions of the manufacturer. The genotyping results were analyzed using an ABI prism 7900HT Sequence Detection System. In the Rotterdam study, a random selection of 5% of samples was independently repeated to confirm genotyping results. The disagreement rate for each SNP in ACVR1 was less than 0.4%.

Statistical analysis

The PHASE program [32] and Haploview version 3.32 [33] were used to construct haplotypes and haplotype blocks. To estimate linkage disequilibrium (LD) between SNPs, the pair-wise linkage disequilibrium coefficient (D') and the correlation coefficient (r^2) were calculated using Haploview. The solid spine of LD method was used to define haplotype blocks.

In the 359 PCOS patients, the 30 normo-ovulatory controls, and the 3543 women of the Rotterdam study, genotype frequencies of each ACVR1 SNP were tested for Hardy-Weinberg equilibrium proportions using Haploview. Differences in single marker or haplotype frequencies were compared between cases and controls using the chi-squared test in Haploview. Furthermore, an empirical P-value by permutation analysis was obtained using Haploview. The phenotypic status of each individual was permuted 10,000 times and association analysis was performed to obtain the test statistic under the null hypothesis of no association. The empirical P-value was obtained as the proportion of the 10,000 replicates that had a P-value less than or equal to the one obtained from the actual (unshuffled) data [33, 34].

If appropriate, hormone levels were log transformed to normalize their distribution. Correlations between hormone levels were determined using Spearman's rank correlation coefficient. Within the subset of PCOS women in whom AMH levels were measured (n=295), differences in hormone levels and ovarian parameters were tested between the genotype and haplotype groups using one-way analysis of (co)variance (AN(C)OVA) with adjustment for age and BMI. Because of statistical power, only haplotypes with an allele frequency of more than 5% were included in this analysis. Trend analysis assuming an additive genetic model was performed for the presence of zero, one or two copies of the associated allele, incorporating the genotype or haplotype variable as a continuous term in a linear regression model [35]. Correction for multiple testing was performed by applying a Bonferroni correction to the level of significance, which was reset from $P<0.05$ to $P<0.0072$ considering the number of SNPs (n=7) or haplotypes (n=7) analyzed in this study. All statistical analyses were performed using Statistical Package for Social Sciences, SPSS, version 11.0.1 (SPSS Inc, Chicago, IL).

RESULTS

Clinical characteristics of the PCOS cohort

Characteristics of the 359 PCOS women, the 30 normo-ovulatory controls, and the 3543 women of the Rotterdam study, are shown in Table 7.1. As previously shown [22], AMH serum levels were elevated in the PCOS women compared to the normo-ovulatory controls (Table 7.1). AMH levels were strongly correlated with total follicle number ($r=0.51$, $P<0.001$) and total testosterone levels ($r=0.47$, $P<0.001$). Total testosterone levels were also positively correlated with total follicle number ($r=0.30$, $P<0.001$).

Table 7.1 Clinical characteristics of the study population

	Rotterdam Study	Normo-ovulatory women	PCOS women
Number	3543	30	359
Age (year) (range)	70.4 ± 4.1 (55-99)	30.1 ± 4.1 (20-36)	28.2 ± 4.9 (15-44)
BMI (kg/m^2)	26.7 ± 4.1	22.4 ± 2.6	27.5 ± 6.3*
PCO (%)	n.a.	0.0	97.3*
Total follicle number	n.a.	14.8 ± 4.7	41.2 ± 22.2*
AMH (ng/ml)[a]	n.a.	2.8 ± 2.6	12.3 ± 7.7*
Hyperandrogenism (%)	n.a.	0.0	55.6*
Testosterone (nmol/L)	n.a.	1.3 ± 0.5	2.0 ± 0.9*
FAI	n.a.	n.d.	6.1 ± 5.6

[a] measured in 295 PCOS women.

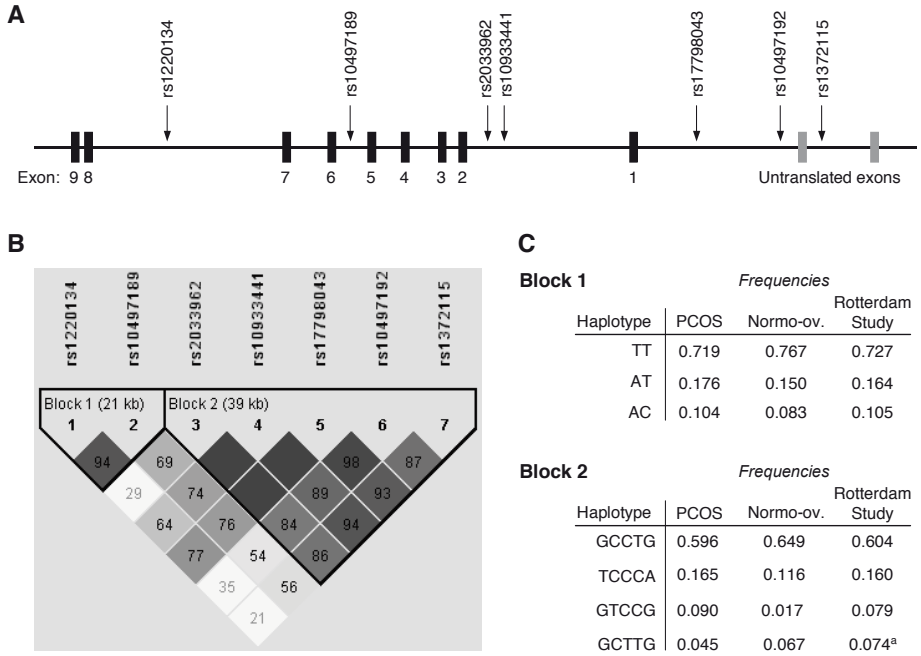
* P< 0.001 compared to normo-ovulatory control group.

Data represent mean ± SD. n.a.: not applicable; n.d: not determined.

PCOS risk by ACVR1 gene variants

The seven selected polymorphisms in ACVR1 were all located within non-coding regions of the gene (Figure 7.1A and Table 7.2). Genotype frequencies of the seven ACVR1 polymorphisms were in Hardy-Weinberg equilibrium proportions in the cohort of PCOS patients, the normo-ovulatory controls and the women of the Rotterdam Study (results not shown). The minor allele frequencies (MAF) of the seven SNPs in the different populations are shown in Table 7.2. The T-allele of rs17798043 was less common in PCOS patients compared to the women of the Rotterdam Study (OR=0.58, 95% CI 0.40-0.84, P=0.003, P after permutation analysis=0.02), although the allele frequencies of rs17798043 were not significantly different between the PCOS patients and the normo-ovulatory controls (OR=0.65, 95% CI 0.22-1.91, P=0.43, Table 7.2). Allele frequencies of the other six SNPs in ACVR1 were similar between the PCOS patients, the normo-ovulatory women and the women of the Rotterdam Study (Table 7.2). In addition, the allele frequencies of the ACVR1 SNPs in the two control groups were similar to the Caucasian allele frequencies of these SNPs in the HapMap database (www.hapmap.org) [31].

Linkage Disequilibrium (D') among the seven ACVR1 SNPs in our subjects ranged from 0.21-1.00 (Figure 7.1B). The patterns of linkage disequilibrium among the SNPs determined two haplotype blocks within the gene, the first block comprised the first and second SNP at the 3' part of the gene; the second block comprised the five SNPs remaining at the 5' part of the gene (Figure 7.1B). Frequencies of the haplotypes of block 1 and 2 did not differ between PCOS patients and both control groups (Figure 7.1C, and results not shown). Only the GCTTG haplotype in block 2 had a lower allele frequency in PCOS patients



C

Block 1

Haplotype	PCOS	Normo-ov.	Rotterdam Study
	Frequencies		
TT	0.719	0.767	0.727
AT	0.176	0.150	0.164
AC	0.104	0.083	0.105

Block 2

Haplotype	PCOS	Normo-ov.	Rotterdam Study
	Frequencies		
GCCTG	0.596	0.649	0.604
TCCCA	0.165	0.116	0.160
GTCCG	0.090	0.017	0.079
GCTTG	0.045	0.067	0.074[a]

Figure 7.1 Gene structure, linkage disequilibrium plot, and haplotype frequencies for *ACVR1*. (A) The *ACVR1* gene, encoding ALK2, has nine exons (black boxes) and two untranslated exons (grey boxes) and is located on the reverse strand of chromosome 2 (2q23-q24). Arrows indicate the locations of the genotyped SNPs. (B) The linkage disequilibrium plot displays D' values (%) for each pair of SNPs in the box at the intersection of the diagonals from each SNP. The dark solid boxes indicate D'=1 (100%) for the corresponding pair of variants. The SNPs were considered in two haplotype blocks as indicated. Based on the HapMap database, these haplotype blocks cover the common genetic variation across 40 kb of the 5'UTR (upstream of the first translated exon) and 20 kb of the 3'UTR of the *ACVR1* gene. (C) Common *ACVR1* haplotypes and their frequencies in PCOS women, normo-ovulatory women, and women of the Rotterdam study. [a], significantly different from allele frequencies of PCOS women (P-value from X^2 test = 0.004, P-value obtained using permutation analysis = 0.05).

compared to the Rotterdam Study, which is in line with the individual marker rs17798043 that fully drives this haplotype (results not shown).

Ovarian phenotype in PCOS women by ACVR1 gene variants

Within the group of 295 PCOS women, the different ACVR1 genotypes and haplotypes were not associated with general characteristics, such as age, BMI and Waist Hip Ratio (results not shown). Three ACVR1 polymorphisms, rs1220134, rs10497189 and rs2033962, were associated with serum AMH levels in PCOS patients, in a manner that suggests an allele-dose effect (P-trend=0.001, 0.002 and 0.007 respectively, Table 7.3). For each of these three polymorphisms, women homozygous for the minor allele (rs1220134 A/A, n=23; rs10497189 C/C,

Table 7.2 Frequencies and locations of ACVR1 polymorphisms

Variant	Alleles	Location	PCOS	Normo-ovulatory women	Rotterdam Study	P[a]	P[b]
N			359	30	3543		
			MAF	MAF	MAF		
rs1220134	T/A	Intron 7	0.280	0.233	0.269	0.44	0.51
rs10497189	T/C	Intron 5	0.104	0.083	0.110	0.61	0.68
rs2033962	G/T	Intron 1	0.191	0.133	0.180	0.27	0.45
rs10933441	C/T	Intron 1	0.100	0.033	0.086	0.09	0.21
rs17798043	C/T	5'UTR	0.045	0.067	0.074	0.43	0.003 (0.02[c])
rs10497192	T/C	5'UTR	0.305	0.233	0.283	0.24	0.20
rs1372115	G/A	5'UTR	0.208	0.167	0.190	0.45	0.25

MAF= minor allele frequency
[a]P-value from X^2 test of allele frequencies PCOS women versus normo-ovulatory women
[b]P-value from X^2 test of allele frequencies PCOS women versus women of the Rotterdam Study
[c]P-value obtained using permutation analysis.

Table 7.3 AMH levels and follicle count in PCOS women by ACVR1 genotypes

Polymorphism	Genotype	N	AMH[a]	P-trend[b]	Follicle number[a]	P-trend[b]	AMH[c]	P-trend[b]
rs1220134	T/T	149	10.9 ± 0.6		38.1 ± 1.8		11.5 ± 0.5	
	T/A	123	13.6 ± 0.7		45.1 ± 2.0		13.0 ± 0.6	
	A/A	23	14.2 ± 1.6	0.001	40.3 ± 4.6	0.01[d]	14.3 ± 1.4	0.007
rs10497189	T/T	237	11.5 ± 0.5		39.1 ± 1.4		11.9 ± 0.4	
	T/C	54	15.1 ± 1.0		49.5 ± 3.0		13.7 ± 0.9	
	C/C	4	19.5 ± 3.8	0.002	54.0 ± 11.0	0.001	17.3 ± 3.3	0.10
rs2033962	G/G	197	11.5 ± 0.5		39.9 ± 1.6		11.7 ± 0.5	
	G/T	82	13.7 ± 0.8		42.4 ± 2.5		13.5 ± 0.7	
	T/T	16	15.4 ± 1.9	0.007	50.8 ± 5.6	0.04	13.7 ± 1.7	0.06
rs10933441	C/C	237	12.3 ± 0.5		41.4 ± 1.5		12.2 ± 0.4	
	C/T	57	12.4 ± 1.0		39.3 ± 3.0		12.7 ± 0.9	
	T/T	1	18.1 ± 7.7	0.32	84.4 ± 22.3	0.72	10.5 ± 6.7	0.17
rs17798043	C/C	273	12.5 ± 0.5		41.1 ± 1.4		12.6 ± 0.4	
	C/T	22	9.4 ± 1.6		42.3 ± 4.8		9.2 ± 1.4	
	T/T	0	-	0.09	-	0.98	-	0.05
rs10497192	T/T	150	11.6 ± 0.6		39.7 ± 1.8		11.9 ± 0.5	
	T/C	110	12.5 ± 0.7		42.0 ± 2.1		12.4 ± 0.6	
	C/C	35	14.5 ± 1.3	0.03	44.7 ± 3.8	0.31	13.9 ± 1.1	0.05
rs1372115	G/G	190	11.4 ± 0.5		39.7 ± 1.6		11.7 ± 0.5	
	G/A	85	14.2 ± 0.8		43.7 ± 2.4		13.7 ± 0.7	
	A/A	20	13.0 ± 1.7	0.02[d]	44.7 ± 5.0	0.17	12.3 ± 1.5	0.08

Data represent mean ± SEM. Bonferroni corrected significance level, P<0.0072.
[a]Means are adjusted for age and bmi. [b]P-trend obtained using linear regression analysis.
[c]Means are adjusted for age, bmi and follicle number. [d]P-value obtained using ANCOVA.

n=4; or rs2033962 T/T, n=16) had respectively 30, 70 and 34% higher AMH levels compared with women homozygous for the major allele. Polymorphism rs10497189 was also associated with follicle number. Women with the rs10497189 C/C genotype had on average 14.9 more follicles (1.4 fold) compared to women with the T/T genotype (P-trend=0.001, Table 7.3). Moreover, the other two polymorphisms tended to be associated with follicle number, although these associations did not reach the Bonferroni corrected significance level (P<0.0072, Table 7.3). Since serum AMH levels are correlated with follicle number, we adjusted the AMH levels of the different genotypes for follicle number, using ANCOVA. After adjustment, the rs1220134 polymorphism remained significantly associated with AMH levels (P-trend=0.007, Table 7.3). Furthermore, the effect size of the difference in AMH levels did not differ substantially before or after adjustment (Δ3.3 vs Δ2.8, respectively), indicating that the observed association

Table 7.4 AMH levels and follicle count in PCOS women by ACVR1 haplotypes

Haplotypes	Number of copies	N	AMH[a]	P-trend[b]	Follicle number[a]	P-trend[b]	AMH[c]	P-trend
Block 1								
TT	0	23	14.2 ± 1.6		40.3 ± 4.6		14.3 ± 1.4	
	1	123	13.6 ± 0.7		45.1 ± 2.0		13.0 ± 0.6	
	2	149	10.9 ± 0.6	0.001	38.1 ± 1.8	0.01[d]	11.5 ± 0.5	0.007
AT	0	197	12.1 ± 0.5		41.2 ± 1.6		12.1 ± 0.5	
	1	89	13.0 ± 0.8		42.2 ± 2.4		12.8 ± 0.7	
	2	9	11.1 ± 2.6	0.18	30.2 ± 7.5	0.04[d]	13.0 ± 2.2	0.07
AC	0	237	11.5 ± 0.5		39.1 ± 1.4		11.9 ± 0.4	
	1	54	15.1 ± 1.0		49.4 ± 3.0		13.7 ± 0.9	
	2	4	19.5 ± 3.7	0.002	53.9 ± 11.0	0.001	17.3 ± 3.3	0.10
Block 2								
GCCTG	0	49	13.9 ± 1.1		41.6 ± 3.2		13.8 ± 0.9	
	1	134	12.7 ± 0.7		42.8 ± 1.9		12.5 ± 0.6	
	2	112	11.1 ± 0.7	0.02	39.1 ± 2.1	0.66	11.5 ± 0.6	0.01
TCCCA	0	207	11.7 ± 0.5		39.9 ± 1.5		11.9 ± 0.5	
	1	75	13.4 ± 0.9		42.3 ± 2.6		13.2 ± 0.8	
	2	13	14.9 ± 2.1	0.02	53.9 ± 6.2	0.03	12.7 ± 1.8	0.19
GTCCG	0	243	12.2 ± 0.5		41.3 ± 1.4		12.2 ± 0.4	
	1	51	12.5 ± 1.1		39.8 ± 3.1		12.7 ± 0.9	
	2	1	18.1 ± 7.7	0.33	84.4 ± 22.3	0.81	10.5 ± 6.7	0.20
GCTTG	0	273	12.5 ± 0.5		41.1 ± 1.4		12.6 ± 0.4	
	1	22	9.4 ± 1.6		42.3 ± 4.8		9.2 ± 1.4	
	2	0	-	0.09	-	0.98	-	0.05

Data represent mean ± SEM. Bonferroni corrected significance level, P<0.0072. [a]Means are adjusted for age and bmi. [b]P-trend obtained using linear regression analysis. [c]Means are adjusted for age, bmi and follicle number. [d]P-value obtained using ANCOVA.

was (in part) independent of follicle number. For the rs10497189 and rs2033962 polymorphisms the effect size also remained present after adjustment (Δ5.4 and Δ2.0, respectively), but because of power, failed to reach significance. Polymorphisms rs10497192 and rs1372115 also tended to be associated with serum AMH levels (P=0.03 and P=0.02, respectively), but these associations did not reach the Bonferroni corrected significance level (Table 7.3).

Inhibin B levels were not significantly associated with the different ACVR1 genotypes, but for the rs1220134, rs10497189 and rs10497192 genotypes trends in inhibin B levels in the same direction as the AMH levels were observed (results not shown). Furthermore, no associations were observed between the different ACVR1 genotypes and LH, FSH, androgen (testosterone and androstenedione) and estradiol levels in the PCOS cohort. In the normo-ovulatory women (n=30), the ACVR1 genotypes or haplotypes were not associated with follicle number or serum AMH levels, which may be due to lack of power (results not shown).

Subsequently, the association of the ACVR1 haplotypes with AMH levels and follicle number in PCOS women was analyzed (Table 7.4). Consistent with the results of the individual markers in haplotype block 1 (rs1220134 and rs10497189), the haplotypes TT and AC of this block were associated with serum

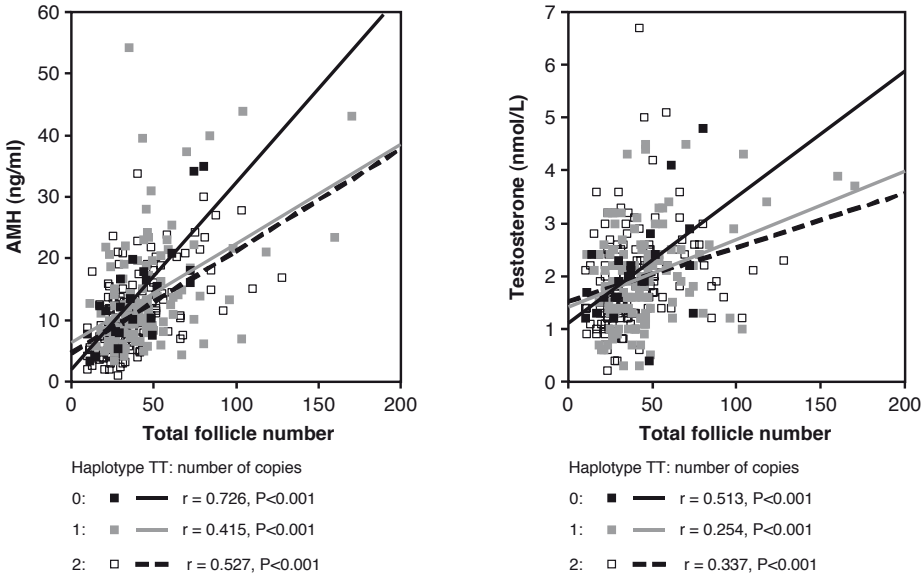


Figure 7.2 Scatter plots depicting the correlations between the individual total follicle number and AMH or Testosterone serum levels in 295 PCOS women, carrying 0 (black squares, black solid line, n=23), 1 (grey squares, grey solid line, n=123), or 2 (open squares, black dotted line, n=149) copies of the *ACVR1* TT haplotype. Spearman's rank correlation coefficient (r) and its significance level (P) are depicted.

AMH levels in PCOS women (P-trend=0.001 and P-trend=0.002, respectively). Carriers of the TT haplotype had lower AMH levels, whereas carriers of the AC haplotype had higher AMH levels compared to non-carriers. After adjustment for follicle number, the TT haplotype remained significantly associated with AMH levels (similar to the rs1220134 genotypes, P-trend=0.007). Indeed, scatter plots showing the correlation between AMH and total follicle number for the TT haplotypes revealed a lower AMH production per follicle number in carriers of the TT haplotype compared to non-carriers (Figure 7.2). In line, also testosterone production per follicle number appeared lower in carriers of the TT haplotype compared to non-carriers (Figure 7.2), although the association of the TT haplotype with testosterone levels was not significant (p=0.25). The AC haplotype failed to reach significance after adjustment for follicle number, although the effect size remained present (Δ5.4) (similar to the rs10497189 genotypes), indicating that the association of this haplotype with AMH levels is in part driven by the observed association with follicle number. The haplotypes GCCTG and TCCCA of block 2 also tended to be associated with AMH levels but this association did not reach significance (P-trend=0.02 and 0.02). After adjustment for follicle number, the association of the GCCTG haplotype with AMH levels nearly reached significance (P-trend=0.01, effect size Δ2.8).

DISCUSSION

Polycystic ovaries contain an increased number of preantral and antral follicles compared to normal ovaries, suggesting that early and late follicle development are disturbed [2, 6, 7, 36, 37]. Intra-ovarian factors, in particular members of the TGFβ family, such as GDF9, follistatin and AMH, may play a role in this pathophysiologic process [7, 23, 38, 39]. In this study, we observed an association of genetic variants in one of the type I receptors for members of the TGFβ superfamily, ALK2, with follicle number and AMH levels in PCOS women, supporting the above mentioned hypothesis.

ALK2 is a type I receptor for AMH and BMP ligands. Interestingly, both AMH and BMPs inhibit FSH sensitivity of growing follicles [17-20, 40], and therefore may contribute to the disturbed follicle selection and the accumulation of growing follicles in PCOS patients. Indeed, AMH serum levels are strongly elevated in PCOS women [21, 22]. Furthermore, in a previous study, we found an association of the AMH Ile[49]Ser polymorphism with follicle number and androgen levels in PCOS women, suggesting that this functional polymorphism in the AMH ligand may influence FSH sensitivity and FSH-induced aromatase activity (Chapter 6).

In the present study, genetic variations in ACVR1 were associated with AMH levels and follicle number but not with androgen levels.

The associations observed for the AMH variant and ACVR1 variants do not fully overlap. A simple explanation is that ACVR1 variants not only affect the actions of AMH but also of other BMP ligands. In addition, BMPs and AMH may use different type I receptors for signaling, depending on cell-type and function [8, 12]. Furthermore, it cannot be excluded that, compared to genetic variation in AMH, genetic variation in ACVR1 may result in a different effect on the AMH receptor complex, and thus on AMH mediated function. The association of ACVR1 variants with AMH levels but not with androgen levels may suggest that ALK2 signaling affects predominantly granulosa cell function and not theca cell function in PCOS women. Alternatively, the lack of an association with androgen levels could be explained by the stronger correlation between follicle number and AMH levels than between follicle number and androgen levels. Indeed, in agreement with decreased AMH levels in carriers of the ACVR1 TT haplotype, also androgen levels tended to be lower in TT haplotype carriers.

The polymorphisms rs1220134, rs10497189 and rs2033962 and haplotypes TT and AC (block 1) of the ACVR1 gene were associated with AMH levels, and these associations were in part independent of follicle number. This suggests that these variants not only influence the number of follicles but also the amount of AMH produced per follicle. Indeed, it was recently suggested that granulosa cells of PCOS women produce more AMH per follicle than granulosa cells of normo-ovulatory women [41]. So far, little is known about the factors that regulate AMH production in normal or polycystic ovaries. FSH and LH do not regulate AMH production in granulosa cells of normal ovaries but in polycystic ovaries FSH may suppress and LH may stimulate AMH production [41]. Our findings suggest that ALK2 is one of the factors regulating AMH expression in polycystic ovaries. Studies in AMHRII null mice suggest that AMH expression is not under strong control of its own signaling pathway (Kevenaar, Themmen and Visser, unpublished results), hence, AMH expression may be regulated by other BMP ligands using ALK2. Taken together, in PCOS women ALK2 may not only inhibit follicle selection via AMH or BMP signaling, leading to the accumulation of follicles, but it may also enhance AMH production by these follicles, thereby exaggerating the disturbance of folliculogenesis even more. In the 30 normo-ovulatory women the ACVR1 genotypes or haplotypes were not observed to be associated with AMH levels or follicle growth. Although power is limited to draw firm conclusions, this suggests that in PCOS women the effect of ACVRI variants on folliculogenesis is established by other altered (intra-ovarian) factors.

One of the strengths of our study is that we investigated the genetic variation within the entire ACVR1 gene, including 40 kb of the 5'UTR and 20 kb of the 3'UTR of the gene. Using a conservative Bonferroni correction for multiple testing we observed that three SNPs and the corresponding haplotypes were associated with AMH levels. For two other polymorphisms and haplotypes we observed associations that nearly reached significance. Since the tested markers in ACVR1 are not independent of each other, it is possible that the Bonferroni correction results in an overly stringent correction of our results [42], implying that in fact several more ACVR1 variants may be involved in folliculogenesis in PCOS women. Because of the haplotype/SNP tagging approach used in this genetic study, it is difficult to elucidate which of the polymorphisms in the ACVR1 gene are causative. Nevertheless, the most significant associations were observed for the polymorphisms located in haplotype block 1, which mainly comprises the 3'UTR region of the gene. Since this region is known to regulate mRNA stability, it may be worthwhile to investigate whether genetic variants in the 3'UTR region influence ALK2 expression levels in polycystic ovaries.

Although our PCOS cohort is among the largest studied in PCOS genetics, our results need to be replicated in other cohorts before definite conclusions can be obtained. Nevertheless, the observed associations of polymorphisms in ACVR1 with AMH levels are highly significant, show consistent effects of higher AMH levels associated with the less frequent allele, and correspond to an allele-dose model, making it unlikely that our results can be explained by chance alone.

In conclusion, our study demonstrates for the first time that genetic variants of the ACVR1 gene are associated with follicle number and AMH levels in PCOS women, suggesting that ALK2 contributes to the disturbed folliculogenesis and the production of AMH per follicle. These results provide new insight into the pathophysiology of PCOS and may be important for the interpretation of AMH levels as a marker for PCOS in the clinic. Furthermore, our results indicate that members of the TGFβ superfamily contribute to the complex pathogenesis of PCOS. Hence, it will be of interest to investigate the contribution of other ligands and receptors in this signaling pathway in the future.

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REFERENCES

1. **Franks S** 1995 Polycystic ovary syndrome. N Engl J Med 333:853-61
2. **Laven JSE, Imani B, Eijkemans MJC, Fauser BCJM** 2002 New approach to polycystic ovary syndrome and other forms of anovulatory infertility. Obstet Gynecol Surv 57:755-67
3. **Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI** 2006 Heritability of polycystic ovary syndrome in a Dutch twin-family study. J Clin Endocrinol Metab 91:2100-4
4. **Urbanek M** 2007 The genetics of the polycystic ovary syndrome. Nat Clin Pract Endocrinol Metab 3:103-11
5. **Franks S, Gharani N, McCarthy M** 2001 Candidate genes in polycystic ovary syndrome. Hum Reprod Update 7:405-10
6. **Webber LJ, Stubbs S, Stark J, Trew GH, Margara R, Hardy K, Franks S** 2003 Formation and early development of follicles in the polycystic ovary. Lancet 362:1017-21
7. **Franks S, McCarthy MI, Hardy K** 2006 Development of polycystic ovary syndrome: involvement of genetic and environmental factors. Int J Androl 29:278-85; discussion 286-90
8. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72-101
9. **Massague J, Chen YG** 2000 Controlling TGF-beta signaling. Genes Dev 14:627-44
10. **di Clemente N, Josso N, Gouedard L, Belville C** 2003 Components of the anti-Mullerian hormone signaling pathway in gonads. Mol Cell Endocrinol 211:9-14
11. **Jamin SP, Arango NA, Mishina Y, Hanks MC, Behringer RR** 2003 Genetic studies of the AMH/MIS signaling pathway for Mullerian duct regression. Mol Cell Endocrinol 211:15-9
12. **Visser JA** 2003 AMH signaling: from receptor to target gene. Mol Cell Endocrinol 211:65-73
13. **Erickson GF, Shimasaki S** 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. Reprod Biol Endocrinol 1:9
14. **Visser JA, Themmen APN** 2005 Anti-Mullerian hormone and folliculogenesis. Mol Cell Endocrinol 234:81-6
15. **Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen APN** 1999 Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140:5789-96
16. **Nilsson EE, Skinner MK** 2003 Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 69:1265-72
17. **Lee WS, Otsuka F, Moore RK, Shimasaki S** 2001 Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. Biol Reprod 65:994-9
18. **Durlinger ALL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen APN** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-9
19. **Otsuka F, Yamamoto S, Erickson GF, Shimasaki S** 2001 Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem 276:11387-92
20. **Otsuka F, Moore RK, Shimasaki S** 2001 Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. J Biol Chem 276:32889-95

21. **Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, Dewailly D** 2003 Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. J Clin Endocrinol Metab 88:5957-62
22. **Laven JSE, Mulders AGMGJ, Visser JA, Themmen APN, De Jong FH, Fauser BCJM** 2004 Anti-Mullerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age. J Clin Endocrinol Metab 89:318-23
23. **Visser JA, de Jong FH, Laven JSE, Themmen APN** 2006 Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 131:1-9
24. **The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group** 2004 Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 19:41-7
25. **Balen AH, Laven JSE, Tan SL, Dewailly D** 2003 Ultrasound assessment of the polycystic ovary: international consensus definitions. Hum Reprod Update 9:505-14
26. **van Santbrink EJP, Hop WCJ, Fauser BCJM** 1997 Classification of normogonadotropic infertility: polycystic ovaries diagnosed by ultrasound versus endocrine characteristics of polycystic ovary syndrome. Fertil Steril 67:452-8
27. **Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groome NP, Themmen APN, Visser JA** 2006 Serum Anti-Mullerian hormone levels reflect the size of the primordial follicle pool in mice. Endocrinology 147:3228-34
28. **de Vet A, Laven JSE, de Jong FH, Themmen APN, Fauser BCJM** 2002 Antimullerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril 77:357-62
29. **Kevenaar ME, Themmen APN, Laven JSE, Sonntag B, Lie Fong S, Uitterlinden AG, de Jong FH, Pols HAP, Simoni M, Visser JA** 2007 Anti-Mullerian hormone and anti-Mullerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women. Hum Reprod 22:1547-54
30. **Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA** 1991 Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol 7:403-22
31. **The International HapMap Consortium** 2003 The International HapMap Project. Nature 426:789-96
32. **Stephens M, Smith NJ, Donnelly P** 2001 A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978-89
33. **Barrett JC, Fry B, Maller J, Daly MJ** 2005 Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263-5
34. **Goodarzi MO, Shah NA, Antoine HJ, Pall M, Guo X, Azziz R** 2006 Variants in the 5alpha-reductase type 1 and type 2 genes are associated with polycystic ovary syndrome and the severity of hirsutism in affected women. J Clin Endocrinol Metab 91:4085-91
35. **Sasieni PD** 1997 From genotypes to genes: doubling the sample size. Biometrics 53:1253-61
36. **Franks S, Mason H, Willis D** 2000 Follicular dynamics in the polycystic ovary syndrome. Mol Cell Endocrinol 163:49-52
37. **Hughesdon PE** 1982 Morphology and morphogenesis of the Stein-Leventhal ovary and of so-called "hyperthecosis". Obstet Gynecol Surv 37:59-77
38. **Teixeira Filho FL, Baracat EC, Lee TH, Suh CS, Matsui M, Chang RJ, Shimasaki S, Erickson GF** 2002 Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome. J Clin Endocrinol Metab 87:1337-44

39. **Roberts VJ, Barth S, el-Roeiy A, Yen SS** 1994 Expression of inhibin/activin system messenger ribonucleic acids and proteins in ovarian follicles from women with polycystic ovarian syndrome. J Clin Endocrinol Metab 79:1434-9
40. **Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S, Lee KA** 2004 Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. Mol Reprod Dev 69:159-63
41. **Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S, Mason H** 2006 Granulosa cell production of anti-Mullerian hormone is increased in polycystic ovaries. J Clin Endocrinol Metab
42. **Cardon LR, Bell JI** 2001 Association study designs for complex diseases. Nat Rev Genet 2:91-9

Chapter 8
General Discussion

8.1 INTRODUCTION

The ovary is of major importance for both reproduction and the endocrine status of women. The objective of this thesis was to study the role of anti-Müllerian hormone (AMH) in the ovary. In mice, AMH is known to inhibit initial and cyclic recruitment, two important steps of follicle development. We hypothesized that also in women AMH attenuates these two steps and therefore, AMH may be involved in the regulation of sex steroid hormone production, in the pathophysiology of ovarian dysfunction, resulting in infertility and in the process of ovarian aging, leading to the onset of menopause. In this discussion, the main findings of this thesis are taken together and placed in a broader perspective. The similarities and differences in the role of AMH between the mouse and human ovary are considered. Furthermore, possible clinical applications for AMH as a marker are discussed. Finally, suggestions for future research are made and potential clinical applications are considered, not only for polymorphisms in the AMH signaling pathway, but also for genetic research in general.

8.2 THE ROLE OF AMH IN THE OVARY

We hypothesized that similar to mice, AMH inhibits initial follicle recruitment in the human ovary and investigated this using a genetic approach in a large population-based cohort of postmenopausal women, the Rotterdam Study. In this cohort, we observed an association of the -482 A>G polymorphism in the AMHR2 gene with age at menopause (see Chapter 5). Indeed, these results suggest a role for, in particular, the AMHRII in the usage of the primordial follicle pool in women. However, this association of the AMHR2 polymorphism with age at menopause was only seen in interaction with the number of offspring, whereas in the AMHRII null mice a direct effect on ovarian aging is observed, independent of the number of litters (Chapter 2). How can we explain these differences in AMH function among species? To answer this question, another TGFβ family member, BMP15, for which also species-dependent variation in function has been observed can be taken as an example.

Naturally occurring point mutations of the BMP15 gene in sheep result in impaired follicular development beyond the primary stage and affect fertility profoundly [1, 2], whereas mice lacking BMP15 have only minimal defects in folliculogenesis (but are subfertile due to reduced ovulation and fertilization) [3]. Interestingly, in contrast to the infertile ewes with a homozygous inactivating mutation in BMP15, ewes with a single inactive BMP15 gene are fertile and dis-

play an increased ovulation rate and a higher incidence of twin or triplet births [1, 2, 4]. To explain these phenotypical differences between species several molecular mechanisms affecting the biological effects of BMP15 have been proposed.

One explanation would be the difference in the nature of the mutations in the BMP15 gene, *i.e.* single point mutations in sheep versus deletion of the entire second exon in mice [5, 6]. *In vitro* studies have shown that the mutant BMP15, as observed in sheep, has a dominant negative effect on the secretion of GDF9, a closely resembled TGFβ family member, thereby possibly causing the aberrant phenotype of the BMP15 mutant sheep [7]. In BMP15 knockout mice, the lack of the BMP15 protein, caused by the deletion of a large part of the gene, may prevent the dominant negative effect of the mutant BMP15 protein, possibly explaining its relatively normal folliculogenesis [6]. Hence, the absence of a protein may not have similar physiological consequences compared with the presence of a mutated protein.

Comparable differences in the nature of genetic variation, *i.e.* mutations versus polymorphisms, may play a role in our findings. In the AMHRII null mice exon 5 of the AMHR2 gene was deleted, leading to complete inactivation of the protein [8]. Since the AMHRII is essential for AMH signaling [9], this completely hampers AMH mediated function in these mice (Chapter 2) (Figure 8.1). In contrast, in women a polymorphism was studied, which most likely leads to only a subtle difference in the expression level or in the structure of the AMHRII protein. Subsequently, factors related to parity and pregnancy, such as the hormones progesterone, prolactin and estradiol, may interact with the AMH type II receptor, depending on its genotype, and alter AMH-mediated functions (Figure 8.1). In AMHRII null mice, the complete absence of the AMHRII makes this interaction impossible. Currently, functional studies to investigate the molecular mechanism of the AMHR2 polymorphisms are ongoing.

Another explanation for the discrepancies between the reproductive phenotypes of BMP15 knockout mice and BMP15 sheep is the difference in the relative importance of the two factors in these species [3, 5]. BMP15 may have a prominent role in folliculogenesis in sheep, whereas it may have a supportive role in mice, with GDF9 being the necessary and dominant factor [3]. With regard to our studies, it cannot be excluded that the relative contribution of AMH also differs between mice and women, partially explaining our results.

Interestingly, for one of the type I receptors that is involved in AMH signaling, ALK6, the pronounced differences between sheep that are carriers of mutations in this receptor (the Booroola strain) [10-12] and mice with targeted deletions of the gene encoding ALK6 [13] resemble the species-specific differences between BMP15 knockout mice and BMP15 mutant ewes [5]. These findings indicate that

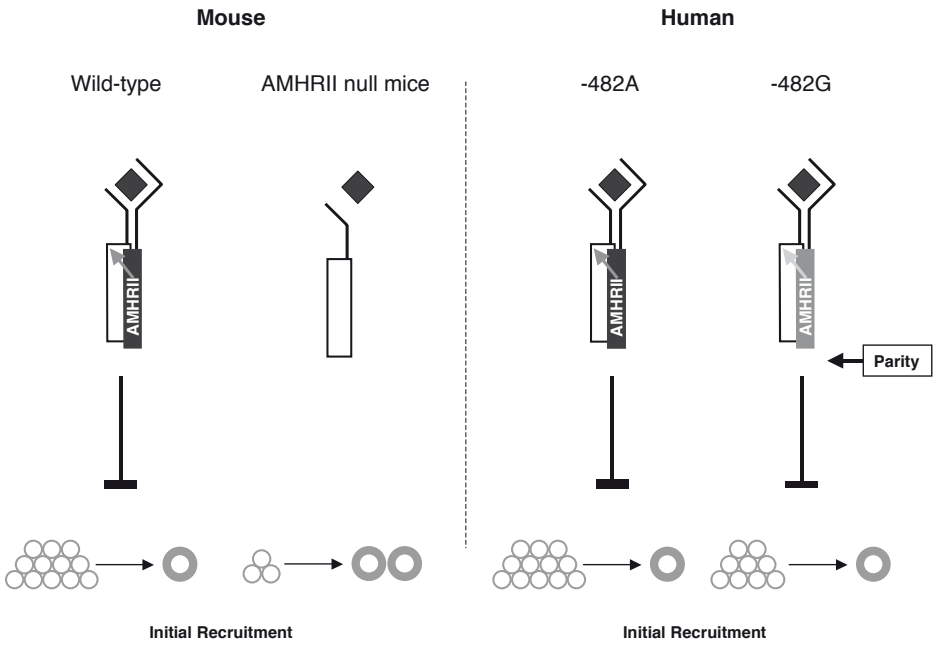

Figure 8.1 Schematic overview of the effects of AMH signaling on initial recruitment in female mice and women. In mice, wild-type and AMHRII null mice are compared. In AMHRII null mice, the AMHRII protein is completely deficient leading to the absence of AMH signaling, and hence, enhanced recruitment. In women, the AMHR2 -482A allele and the -482G allele are compared. The -482 A>G polymorphism may result in subtle differences in expression level or structure of the AMHRII protein. An association with age at menopause in interaction with parity was observed for the AMHR2 -482G allele, suggesting an effect of parity on AMHRII expression and/or function. The black squares represent the AMH ligand. The receptors with open boxes represent the AMH type I receptors. The small circles represent primordial follicles and the larger circles represent growing follicles.

these phenotypical differences, either due to species differences or to the nature of the mutation, are not unique to the homologues oocyte factors BMP15 and GDF9 but indeed, may also play a role in the signaling pathway of other TGFβ family members, such as AMH.

Last but not least, the mono-ovulatory nature of sheep and women versus the poly-ovulatory nature of mice may explain the species variation in the function of TGFβ family members [6, 14]. The number of ovulatory follicles is determined by the FSH sensitivity of antral follicles to be selected for dominance [15]. Therefore, this species difference is unlikely to involve the role of AMH signaling in initial recruitment but rather the role of AMH in the modulation of FSH sensitivity. In mice, AMH inhibits FSH sensitivity in small antral follicles but not in large antral follicles, thereby attenuating the FSH threshold for cyclic recruitment but not for selection of dominance [16, 17]. In normo-ovulatory women we observed an

association of polymorphisms in the AMH and AMHR2 gene with estradiol levels during the early follicular phase of the cycle (day 0-5) (Chapter 4), suggesting that AMH signaling modulates FSH sensivity and FSH-induced aromatase activity in the small antral follicles. In women with Polycystic Ovary syndrome (PCOS), the AMH Ile[49]Ser polymorphism is associated with follicle number, again suggesting that AMH modulates FSH sensitivity of growing follicles (Chapter 6). In addition, *in vitro* studies in both mouse and human granulosa cells have shown that AMH inhibits FSH-induced aromatase activity [18, 19]. Hence, these results suggest that the role of AMH in modulating FSH sensitivity does not vary among species.

In general, we conclude that the role of AMH in the mouse and human ovary is similar. The observed differences with regard to the function of AMH in initial recruitment may be explained by several species-independent factors. Hence, in mice and women, AMH influences the recruitment of primordial follicles into the growing follicle pool and attenuates the FSH sensitivity of growing follicles.

8.3 GENETIC ASSOCIATION STUDIES

Since the role of the AMH signaling pathway in the human ovary was studied using a genetic approach, some general comments on genetic association studies should be made. In recent years many positive genetic association studies have been published but only few of them have been replicated in other study cohorts, suggesting that many of these studies are false positive [20, 21]. One way to reduce the chance of finding false positive results is a strong a priori rationale for the association study, *i.e.* an evident biological mechanism explaining the relation between the candidate gene and the phenotypic trait [22, 23]. With respect to the studies described in this thesis, our a priori rationale was based on the results of studies in mice, obtaining a clear hypothesis about the role of the AMH signaling pathway in the ovary. Another way to avoid spurious findings is the validation of the association in independent study cohorts. For example, despite the small numbers, the association of the AMHR2 –482 A>G polymorphism with estradiol levels was observed in two independent cohorts of women (Chapter 4). Unfortunately, the association analysis of the AMH Ile[49]Ser polymorphism could not be performed in a second cohort of normo-ovulatory women. In this respect it is important to realize that it is very difficult and laborious to obtain cohorts of normo-ovulatory women of which both hormone levels and DNA for genotyping is available. Nevertheless, for this polymorphism we demonstrated that it results in a functional difference on protein level (Chapter 6), thereby also decreasing the change of finding a spurious association (under the condition

that the effects found in the functional and association studies are consistent) [24]. The AMH [49]Ser allele has a lower maximal bio-activity compared to the [49]Ile allele, as shown by *in vitro* studies using two different cell lines (Chapter 6). Indeed, this is consistent with less inhibition of FSH-induced aromatase activity by the [49]Ser allele, as suggested by the association study in normo-ovulatory women. Also in PCOS women, the AMH [49]Ser allele appears to result in lower AMH bioactivity compared to the AMH [49]Ile allele, as the [49]Ser allele is associated with a lower percentage of women exhibiting polycystic ovaries, again suggesting less inhibition of FSH sensitivity by AMH.

Functional studies on the –482 A>G polymorphism in the AMHR2 gene promoter are currently ongoing. This polymorphism is located at a potential c-Myb and c-Myc transcription factor binding site (www.cbil.upenn.edu/tess) [25] and therefore may modify promoter activity, resulting in differences in expression level. However, this polymorphism is in complete linkage disequilibrium with several other polymorphisms in the AMHR2 gene (Chapter 4), which may also drive the observed associations. Hence, although our approach may appear to be a single SNP approach, the complete genetic variation of the AMHR2 gene was captured (Chapter 4). This also accounts for the AMH Ile[49]Ser polymorphism, which was in complete linkage disequilibrium with all other polymorphisms in the gene, including 1 kb of the promoter region, with a minor allele frequency of more than 10% (Chapter 4). Previously, many genetic studies used a single SNP approach, but since multiple variants within one gene may have subtle effects on complex diseases, this may have contributed to the conflicting results of genetic association studies, in particular when different SNPs within the same gene were investigated.

To capture the complete genetic variation of the ACVR1 gene, encoding ALK2 (one of the candidate type I receptors for AMH), genotyping of seven tagging polymorphisms was required. Hence, multiple genotypes and haplotypes within the ACVR1 gene were tested, increasing the chance of obtaining false positive results. To avoid this, we corrected for multiple testing using the conservative Bonferroni method (Chapter 7). In addition, the observed associations in this study all point into the same direction and nicely fit an allele-dose model, also increasing the chance that our results are truly positive. Nevertheless, because of the tagging SNP approach, it is difficult to elucidate which of the polymorphism(s) in the ACVR1 gene is truly functional.

Although AMH, AMHR2 and ALK2 all exert their effect via the AMH signaling pathway, the observed associations of genetic variants within these genes with endpoints related to ovarian function were not completely similar, *e.g.* the AMH and ALK2 polymorphisms were associated with follicle number in PCOS

women whereas the AMHR2 polymorphism was not. This could be explained by compensating effects of other factors but also by a differential effect of AMH signaling. AMH may use different type I receptors for different targets of AMH signaling, depending on cell type and function [6, 26]. Therefore, the effect of genetic variation on the AMH receptor complex, and hence, on its function, may differ between the AMH ligand, the AMH type II receptor and the various type I receptors.

In conclusion, since the chance of obtaining false positive results was reduced in various ways, it is very likely that genetic variation in the AMH, AMHR2 and ACVR1 genes truly contributes to ovarian function related endpoints as described in Chapters 4, 5, 6 and 7. Nevertheless, to obtain definite conclusions, our findings should be replicated in large cohorts of premenopausal, postmenopausal and PCOS women. In addition, it should be noted that all our studies are performed in cohorts of Caucasian women. Since racial descent may lead to differences in the frequency and biological effect of genetic variants [27], our results cannot be directly extrapolated to women of other ethnicities.

8.4 CLINICAL APPLICATIONS AND FUTURE RESEARCH

8.4.1 AMH as a marker for ovarian function

Since AMH serum levels decline with age in premenopausal women and correlate with the number of antral follicles as detected by transvaginal ultrasound, AMH has been proposed as a marker for ovarian aging [28]. In this thesis we have shown in mice that AMH levels are correlated with the number of growing follicles and indirectly with the number of primordial follicles (Chapter 3). Therefore, we assume that also in women AMH levels reflect the quantity of the primordial follicle pool, supporting the use of AMH as a marker for ovarian reserve. AMH serum levels are not regulated by the hypothalamus-pituitary-gonadal (HPG) axis [29-31], resulting in stable AMH serum levels during the menstrual cycle [32-35]. Therefore, AMH levels can be determined at any day of the cycle, which facilitates the use of AMH as a marker in the clinic. This is in particular advantageous because serum markers that are currently used to assess ovarian reserve, such as FSH, inhibin B and estradiol, are regulated by the HPG axis and vary during the cycle. In addition, with increasing age serum AMH concentrations appear to change ahead of these other hormonal markers, making it the earliest marker for ovarian aging [28, 30, 36]. In this paragraph I will give a short overview of the current and future clinical applications for AMH as a marker for ovarian function and reserve (overview Box 8.1).

Assessment of ovarian reserve by measurement of AMH may be of use to identify women with premature ovarian failure (POF), which is characterized by a diminished ovarian follicle pool resulting in the onset of menopause before the age of 40 years [37]. Indeed, in patients with idiopathic POF AMH levels are low compared to women without POF and are below the detection threshold in about 60-80% of the patients [38, 39]. Interestingly, in the small group of POF patients in whom AMH levels are detectable, an association was found between the number of small follicles in ovarian biopsy and AMH levels [38]. Despite their amenorrhea, women with POF have a 5-10% chance of conceiving at some time after the diagnosis [40]. Hence, AMH levels could help identifying these patients more likely of possessing follicles available for development and ovulation and thus, having a higher chance of becoming pregnant [38]. Furthermore, AMH may be of use in predicting ovarian reserve in relatives of women with familiar POF. A small percentage of POF cases (varying from 4-30%) is caused by genetic factors, such as X chromosome abnormalities (*e.g.* Turner's Syndrome) and a premutation in the FMR1 gene (the full FMR1 mutation is associated with fragile X syndrome but not with POF) [41, 42]. At present, only screening tests for these genetic abnormalities are routinely available for women with familiar POF and their relatives [43]. Many other mutations causing POF, such as in those in the BMP15 [44, 45] and Inhibin α gene [46, 47], are not considered in this screening, resulting in a low specificity of these screening tests. Hence, the prediction of ovarian reserve by AMH levels may be of additional use in the counseling offered to these relatives at risk.

Another group of women at risk for diminished ovarian reserve and thus for POF, comprises of cancer survivors who have been treated with radiotherapy or chemotherapy in the past [42]. Both these treatments have adverse effects on the ovary and cause depletion of the primordial follicle pool in a dose- and drug-dependent manner [42, 48-50]. Hence, this loss in ovarian reserve may be reflected by AMH levels. Indeed, in young women treated for childhood cancer, AMH levels were lower compared to controls, whereas other direct products of the ovary, estradiol and inhibin B were not different, indicating that AMH is the most accurate reflection of the primordial follicle pool [51]. In addition, in a group of premenopausal women with breast cancer, AMH levels showed a marked fall during chemotherapy treatment. Differences between chemotherapeutic regimens were observed, with the most gonadotoxic regimen known resulting in the lowest AMH levels, indicating that AMH levels are also useful for comparison of ovarian toxicity of different regimens [52]. The decline in AMH levels was also observed in a cross-sectional study of breast cancer patients, in which basal AMH levels were lower in patients treated with chemotherapy compared to patients who did not

yet receive chemotherapy [53]. Hence, AMH may be useful as a predictor of ovarian reserve and fertility in cancer survivors, which is particularly important because reproductive issues are of major concern for these women [54, 55].

A second future application of AMH in the field of oncology may be concerning the choice of therapy for breast cancer. Since estrogen has a pivotal role in the etiology and development of breast cancer, for over 30 years, tamoxifen, a selective estrogen receptor modulator (SERM), has been the cornerstone in endocrine therapy for breast cancer treatment [56]. However at present, also aromatase inhibitors are widely used to manipulate estradiol action and these are now the optimum adjuvant therapy in postmenopausal women with hormone receptor-positive disease (meaning estradiol and/or progesterone receptor positive) [57, 58]. Clearly, in postmenopausal women, the systemic estrogen is mainly produced by the peripheral aromatization of androgens in bone, breast, adipose and muscle tissue, whereas in premenopausal women the ovaries are the major source for estradiol production [59]. In premenopausal women aromatase inhibitor monotherapy does not block estrogen synthesis completely due to high levels of androstenedione in the ovary. In addition, incomplete suppression of estrogen may activate the negative feedback control of FSH and LH, leading to a reflex rise in gonadotrophins and ultimately overcoming the initial block in estrogen synthesis [57, 58]. Hence, aromatase inhibitors are not suitable as monotherapy in pre- or perimenopausal women, and therefore, an accurate assessment of the menopausal status of each individual is essential to ensure the most appropriate choices are made [58, 60]. However, especially when cancer therapy is either recently completed or ongoing, determining menopausal status can be difficult in these patients, in particular because amenorrhea is a common and well-described side effect of chemotherapy, but not necessarily implying ovarian failure [57, 58]. In addition, chemotherapy and endocrine therapy, such as tamoxifen and LH-releasing hormone analogues (GnRHa), interfere with the HPG axis, making other markers of ovarian reserve, such as FSH and inhibin B uninformative in these patients. Since AMH directly reflects ovarian reserve, AMH may be an excellent marker to determine menopausal status in these breast cancer patients, prior to treatment, but also after primary treatment with chemotherapy or endocrine therapy.

Assessment of the ovarian reserve may also be important in the IVF clinic. In women undergoing treatment for infertility, ovarian aging is characterized by decreased ovarian responsiveness to exogenous gonadotropin administration and poor pregnancy outcome. On the one hand, it is important to correctly identify poor responders by assessment of their ovarian reserve before entering an IVF program, whereas on the other hand, assessment of the ovarian reserve may benefit patients that would generally be excluded because of advanced age [30].

Several studies have shown that serum AMH levels are highly correlated with the number of antral follicles before treatment and the number of oocytes retrieved upon ovarian stimulation, indicating that AMH is an excellent predictor of poor ovarian response and thus, may be very useful in the IVF clinic [29, 61-64] (reviewed in [30, 65]). In addition, basal AMH levels may be associated with an exaggerated response to ovulation induction, suggesting that AMH could be a marker for identifying women at risk for developing ovarian hyperstimulation syndrome [65, 66]. However, the application of AMH to predict (ongoing) pregnancy appears to be limited [29, 30, 63, 67], and also, the value of AMH as a predictor of embryo quality is controversial [68-71].

Furthermore, AMH may be a useful marker for ovarian pathophysiology, such as in polycystic ovary syndrome (PCOS), which is extensively described in Chapters 6 and 7 of this thesis and also in a review by Visser et al. [30]. Interestingly, in situations where accurate ultrasonographic data are not available, elevated AMH levels could be used instead of increased follicle count as a diagnostic criterion and incorporated as such in the Rotterdam criteria of PCOS [72]. Also, AMH levels are elevated in 76% [73] to 93% [74] of women with a much less common disorder of the ovary, ovarian granulosa cell cancer. In these women AMH may be used as a marker for detection and surveillance of the AMH producing granulosa cell tumors [73-75].

Last but not least, measurement of AMH levels to assess ovarian reserve may be of interest in women in general. In Western-style societies, such as the Netherlands, increasing numbers of women deliberately postpone childbearing, resulting in an increased risk of subfertility [76]. Assessment of the size of the ovarian follicle pool may provide insight into the number of fertile years a woman has left, thereby assisting in the timing of having children and reducing the risk of subfertility.

In general, I predict that AMH will make its way into daily clinical practice in the coming years. However, before AMH can be used as a marker for the various applications described above, more prospective studies in normal and infertile women are necessary and age-related reference ranges need to be defined.

8.4.2 Polymorphisms in the AMH signaling pathway

An interesting direction for future research relates to the possible extragonadal effects of AMH. As discussed before, we have shown in this thesis that polymorphisms in the AMH signaling pathway are associated with age at menopause and estradiol levels. Estradiol has widespread biological effects on tissues beyond the reproductive system, such as the skeleton, the cardiovascular system and the brain. Therefore, the withdrawal of the effects of estradiol at the menopausal

transition has a major impact on healthy aging of women and their quality of life. An early onset of menopause results in an increased risk for osteoporosis [77], cardiovascular disease [78], stroke and perhaps Alzheimer's disease [79], whereas a late onset of menopause is associated with an increased breast cancer risk [80]. Since the AMHR2 polymorphism appears to influence age at menopause, it may be related to the risk of these estrogen-dependent diseases.

In addition, the risk of these diseases is influenced by estradiol levels during the reproductive lifespan. For example, higher endogenous estradiol levels in premenopausal women, in particular in the follicular phase of the menstrual cycle, are associated with increased premenopausal breast cancer risk [81]. Since polymorphisms in the AMH signaling pathway are associated with follicular phase estradiol levels (Chapter 4), AMH may also influence breast cancer risk by regulating estradiol exposure. Also, since a woman experiences a large number of menstrual cycles during her total reproductive life, small differences in estradiol levels per cycle may lead to a large difference in cumulative estradiol exposure, influencing estrogen-dependent disease risk in postmenopausal women. In the German normo-ovulatory women we observed that the AMHR2 -482G allele tends to be associated with earlier development of a dominant follicle, probably resulting in a shorter follicular phase length (Chapter 4). This shorter follicular phase may result in a shorter menstrual cycle [82], and therefore a higher number of menstrual cycles during the reproductive lifespan, thereby elevating the cumulative estradiol exposure even more [83]. Hence, for subsequent studies it would be of interest to investigate whether polymorphisms in the AMH signaling pathway are associated with estrogen-dependent diseases, such as osteoporosis and breast cancer. In the future, these polymorphisms may be useful for predicting the onset of menopause and maybe, the risk of estrogen-dependent diseases in individual women. However, as the effect size of these single (tagging) SNPs is only modest, assays including many gene variants related to these diseases are needed to make a substantial contribution to the prediction of disease risk. Furthermore, it is important to keep in mind that predictive testing will only have value in cases in which effective preventative interventions exist, and when modest changes in risk improve clinical decision-making [84].

Another interesting question for future research relates to the modulation of the FSH threshold by the polymorphisms in the AMH and AMHR2 gene. In women with anovulatory infertility, such as in PCOS, follicle selection is disturbed despite normal FSH levels. The first line treatment of normogonadotrophic anovulatory infertility consists of clomiphene citrate administration, but since a substantial number of these women do not ovulate and/or conceive as a result of this therapy, ovulation induction with exogenous FSH is required [85,

[86]. This treatment is, particularly in PCOS patients, associated with an increased risk in developing hyper-response and its subsequent complications, *e.g.* ovarian hyperstimulation syndrome and poly-ovulation resulting in multiple pregnancies [87, 88]. This high risk of complications could be explained by the variation in amount of exogenous FSH required to elicit an ovarian response. Patient characteristics, in particular the individual FSH threshold, rather than the treatment protocol contribute to this wide variation. Therefore, it will be worthwhile to determine whether the identified polymorphisms in the AMH and AMHR2 gene are associated with the amount of FSH required and with outcome parameters of ovulation induction therapy, such as ovulation rate, ovarian hyperstimulation and pregnancy rate. Although this is highly speculative, these polymorphisms may be of additive value in establishing FSH sensitivity to predict ovulation induction outcome in the future.

Last but not least, a direction for future research, worthwhile investigating concerns the role of the candidate AMH type I receptors, ALK2, ALK3 and ALK6. We have shown that ALK2 contributes to the disturbed folliculogenesis in PCOS women (Chapter 7). Obviously, also ALK3 and ALK6 may play a role in normal and/or disturbed follicle development. In addition, each of the AMH type I receptors may be involved in the AMH-mediated effect on ovarian aging, thereby influencing the onset of menopause and possibly the risk of estrogen-dependent diseases, as described above. Nevertheless, in contrast to the AMHRII, the type I receptors are not specific for AMH signaling, hence, they may also mediate the effect of other TGFβ family members (see also Chapter 7) [89]. Furthermore, the type I receptors have a much broader expression pattern than AMH and AMHRII and, subsequently, are involved in growth, differentiation and apoptosis

Box 8.1 Potential clinical applications for serum AMH as a marker of ovarian function

Potential clinical applications for serum AMH as a marker of ovarian function	
Population/ patients	Use
	Assessment of ovarian reserve
General population	Predict fertility and menopausal transition
Women with/ at risk for POF	Predict fertility and menopausal transition
Before and after cancer therapy	Predict fertility, menopausal transition and gonadal toxicity of chemotherapeutics
Breast cancer patients	Determine menopausal status for choice of therapy
Infertile patients undergoing ovulation induction therapy	Predict ovarian response and risk OHSS
	Assessment of ovarian dysfunction
PCOS patients	Diagnosis and surveillance of PCOS therapy
Ovarian granulosa cell carcinoma patients	Detection and surveillance of tumor

POF= Premature ovarian failure, OHSS= Ovarian hyperstimulation syndrome, PCOS= Polycystic ovary syndrome.

in a wide variety of tissues [6, 90, 91]. For example, ALK2, ALK3 and ALK6 are all expressed in the developing skeletal system [92] and play an important role in embryonic chondrocyte and osteoblast differentiation and postnatal bone formation (reviewed in [93]). Hence, AMH type I receptors are important within and beyond the reproductive system, making it of interest to investigate whether genetic variation in these receptors contributes to various complex traits, such as age at menopause, PCOS and osteoporosis.

8.4.3 Genetic research in complex diseases

In this thesis a candidate gene approach was used to unravel the role of the AMH signaling pathway in age at menopause and PCOS, both examples of complex genetic traits. Currently, because of major advances in technology for high-throughput genotyping, genome wide association (GWA) studies are becoming increasingly popular, allowing identification of genetic risk alleles without prior knowledge of function [94, 95]. Since loci associated with complex disease risk are generally assumed to be of modest effect size, GWA studies require multistage analyses in large sample sizes, typically comprising of at least several thousand cases and controls [95]. In compliance with these factors, a recent GWA study was successful in identifying genetic variants associated with bipolar disorder, rheumatoid arthritis, coronary artery disease, Crohn's disease and type 1 and type 2 diabetes [96]. Also, using the GWA approach, progress has been made in the identification of novel breast cancer loci [94].

However, to obtain the large sample sizes needed for these GWA studies, multicenter collaborations and substantial funding are required, complicating the performance of these studies. Furthermore, similar to the candidate gene approach, some problems with GWA studies exist, such as non-replication, genetic heterogeneity among different populations and the difficulty to identify gene-gene and gene-environment interactions [97-99]. Also, the association signals detected in these studies can help to define regions of interest, but they cannot provide unambiguous identification of the causal genes. Therefore, extensive resequencing and fine-mapping work, followed by functional studies will be required [96]. Despite these pitfalls, the GWA approach provides new opportunities to unravel previously unknown biological pathways involved in complex genetic traits and diseases, such as age at menopause and PCOS. In the future, the results of these studies along with the knowledge obtained using candidate gene analysis may provide possibilities to predict disease risk and improve treatment in the individual patient. Eventually, the integration with knowledge obtained at the post-genomic level, such as mRNA expression profiles and proteomics, will aid to optimize this individual patient approach [100].

REFERENCES

1. **Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O** 2000 Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 25:279-83
2. **Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM** 2004 Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). Biol Reprod 70:900-9
3. **Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM** 2001 Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol 15:854-66
4. **Juengel JL, McNatty KP** 2005 The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum Reprod Update 11:143-60
5. **Moore RK, Erickson GF, Shimasaki S** 2004 Are BMP-15 and GDF-9 primary determinants of ovulation quota in mammals? Trends Endocrinol Metab 15:356-61
6. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72-101
7. **Liao WX, Moore RK, Otsuka F, Shimasaki S** 2003 Effect of intracellular interactions on the processing and secretion of bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. Implication of the aberrant ovarian phenotype of BMP-15 mutant sheep. J Biol Chem 278:3713-9
8. **Arango NA, Lovell-Badge R, Behringer RR** 1999 Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 99:409-19
9. **Mishina Y, Rey R, Finegold MJ, Matzuk MM, Josso N, Cate RL, Behringer RR** 1996 Genetic analysis of the Mullerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. Genes Dev 10:2577-87
10. **Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N, Elsen JM** 2001 Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. Proc Natl Acad Sci U S A 98:5104-9
11. **Souza CJ, MacDougall C, Campbell BK, McNeilly AS, Baird DT** 2001 The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. J Endocrinol 169:R1-6
12. **Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP, Montgomery GW** 2001 Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. Biol Reprod 64:1225-35
13. **Yi SE, LaPolt PS, Yoon BS, Chen JY, Lu JK, Lyons KM** 2001 The type I BMP receptor BmprIB is essential for female reproductive function. Proc Natl Acad Sci U S A 98:7994-9
14. **Galloway SM, Gregan SM, Wilson T, McNatty KP, Juengel JL, Ritvos O, Davis GH** 2002 Bmp15 mutations and ovarian function. Mol Cell Endocrinol 191:15-8

15. **McGee EA, Hsueh AJ** 2000 Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-14
16. **Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN** 2001 Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142:4891-9
17. **Visser JA, Durlinger ALL, Peters IJ, van den Heuvel ER, Rose UM, Kramer P, de Jong FH, Themmen APN** 2007 Increased Oocyte Degeneration and Follicular Atresia during the Estrous Cycle in Anti-Mullerian Hormone Null Mice. Endocrinology 148:2301-8
18. **di Clemente N, Goxe B, Remy JJ, Cate R, Josso N, Vigier B, Salesse R** 1994 Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. Endocrine 2:553-558
19. **Grossman MP, Nakajima ST, Fallat ME, Siow Y** 2007 Mullerian-inhibiting substance inhibits cytochrome P450 aromatase activity in human granulosa lutein cell culture. Fertil Steril
20. **Wacholder S, Chanock S, Garcia-Closas M, El Ghormli L, Rothman N** 2004 Assessing the probability that a positive report is false: an approach for molecular epidemiology studies. J Natl Cancer Inst 96:434-42
21. **Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG** 2001 Replication validity of genetic association studies. Nat Genet 29:306-9
22. **Hattersley AT, McCarthy MI** 2005 What makes a good genetic association study? Lancet 366:1315-23
23. **van Rossum EF, Russcher H, Lamberts SW** 2005 Genetic polymorphisms and multifactorial diseases: facts and fallacies revealed by the glucocorticoid receptor gene. Trends Endocrinol Metab 16:445-50
24. **Colhoun HM, McKeigue PM, Davey Smith G** 2003 Problems of reporting genetic associations with complex outcomes. Lancet 361:865-72
25. **Schug J, Overton GC** 1997 TESS: Transcription Element Search Software. Computational Biology and Informatics Laboratory, University of Pennsylvania, pp Technical report CBIL-TR-1997-1001-v0.0
26. **Visser JA** 2003 AMH signaling: from receptor to target gene. Mol Cell Endocrinol 211:65-73
27. **Ioannidis JP, Ntzani EE, Trikalinos TA** 2004 'Racial' differences in genetic effects for complex diseases. Nat Genet 36:1312-8
28. **de Vet A, Laven JSE, de Jong FH, Themmen APN, Fauser BCJM** 2002 Antimullerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril 77:357-62
29. **van Rooij IAJ, Broekmans FJM, te Velde ER, Fauser BCJM, Bancsi LF, de Jong FH, Themmen APN** 2002 Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod 17:3065-71
30. **Visser JA, de Jong FH, Laven JSE, Themmen APN** 2006 Anti-Mullerian hormone: a new marker for ovarian function. Reproduction 131:1-9
31. **Wachs DS, Coffler MS, Malcom PJ, Chang RJ** 2007 Serum anti-mullerian hormone concentrations are not altered by acute administration of follicle stimulating hormone in polycystic ovary syndrome and normal women. J Clin Endocrinol Metab 92:1871-4
32. **Hehenkamp WJ, Looman CW, Themmen APN, de Jong FH, Te Velde ER, Broekmans FJ** 2006 Anti-Mullerian hormone levels in the spontaneous menstrual cycle do not show substantial fluctuation. J Clin Endocrinol Metab 91:4057-63

33. **Cook CL, Siow Y, Taylor S, Fallat ME** 2000 Serum mullerian-inhibiting substance levels during normal menstrual cycles. Fertil Steril 73:859-61
34. **La Marca A, Stabile G, Artenisio AC, Volpe A** 2006 Serum anti-Mullerian hormone throughout the human menstrual cycle. Hum Reprod 21:3103-7
35. **Tsepelidis S, Devreker F, Demeestere I, Flahaut A, Gervy C, Englert Y** 2007 Stable serum levels of anti-Mullerian hormone during the menstrual cycle: a prospective study in normo-ovulatory women. Hum Reprod 22:1837-40
36. **van Rooij IA, Tonkelaar I, Broekmans FJ, Looman CW, Scheffer GJ, de Jong FH, Themmen APN, te Velde ER** 2004 Anti-mullerian hormone is a promising predictor for the occurrence of the menopausal transition. Menopause 11:601-6
37. **Coulam CB, Adamson SC, Annegers JF** 1986 Incidence of premature ovarian failure. Obstet Gynecol 67:604-6
38. **Meduri G, Massin N, Guibourdenche J, Bachelot A, Fiori O, Kuttenn F, Misrahi M, Touraine P** 2007 Serum anti-Mullerian hormone expression in women with premature ovarian failure. Hum Reprod 22:117-23
39. **La Marca A, Pati M, Orvieto R, Stabile G, Carducci Artenisio A, Volpe A** 2006 Serum anti-mullerian hormone levels in women with secondary amenorrhea. Fertil Steril 85:1547-9
40. **van Kasteren YM, Schoemaker J** 1999 Premature ovarian failure: a systematic review on therapeutic interventions to restore ovarian function and achieve pregnancy. Hum Reprod Update 5:483-92
41. **Conway GS, Payne NN, Webb J, Murray A, Jacobs PA** 1998 Fragile X premutation screening in women with premature ovarian failure. Hum Reprod 13:1184-7
42. **Goswami D, Conway GS** 2005 Premature ovarian failure. Hum Reprod Update 11:391-410
43. **Goswami D, Conway GS** 2007 Premature Ovarian Failure. Horm Res 68:196-202
44. **Di Pasquale E, Beck-Peccoz P, Persani L** 2004 Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet 75:106-11
45. **Di Pasquale E, Rossetti R, Marozzi A, Bodega B, Borgato S, Cavallo L, Einaudi S, Radetti G, Russo G, Sacco M, Wasniewska M, Cole T, Beck-Peccoz P, Nelson LM, Persani L** 2006 Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. J Clin Endocrinol Metab 91:1976-9
46. **Shelling AN, Burton KA, Chand AL, van Ee CC, France JT, Farquhar CM, Milsom SR, Love DR, Gersak K, Aittomaki K, Winship IM** 2000 Inhibin: a candidate gene for premature ovarian failure. Hum Reprod 15:2644-9
47. **Dixit H, Deendayal M, Singh L** 2004 Mutational analysis of the mature peptide region of inhibin genes in Indian women with ovarian failure. Hum Reprod 19:1760-4
48. **Himelstein-Braw R, Peters H, Faber M** 1978 Morphological study of the ovaries of leukaemic children. Br J Cancer 38:82-7
49. **Chapman RM, Sutcliffe SB, Malpas JS** 1979 Cytotoxic-induced ovarian failure in women with Hodgkin's disease. I. Hormone function. Jama 242:1877-81
50. **Lutchman Singh K, Davies M, Chatterjee R** 2005 Fertility in female cancer survivors: pathophysiology, preservation and the role of ovarian reserve testing. Hum Reprod Update 11:69-89
51. **Bath LE, Wallace WH, Shaw MP, Fitzpatrick C, Anderson RA** 2003 Depletion of ovarian reserve in young women after treatment for cancer in childhood: detection by anti-Mullerian hormone, inhibin B and ovarian ultrasound. Hum Reprod 18:2368-74

52. **Anderson RA, Themmen APN, Al-Qahtani A, Groome NP, Cameron DA** 2006 The effects of chemotherapy and long-term gonadotrophin suppression on the ovarian reserve in premenopausal women with breast cancer. Hum Reprod 21:2583-92
53. **Lutchman Singh K, Muttukrishna S, Stein RC, McGarrigle HH, Patel A, Parikh B, Groome NP, Davies MC, Chatterjee R** 2007 Predictors of ovarian reserve in young women with breast cancer. Br J Cancer 96:1808-16
54. **Partridge AH, Gelber S, Peppercorn J, Sampson E, Knudsen K, Laufer M, Rosenberg R, Przypyszny M, Rein A, Winer EP** 2004 Web-based survey of fertility issues in young women with breast cancer. J Clin Oncol 22:4174-83
55. **Lee SJ, Schover LR, Partridge AH, Patrizio P, Wallace WH, Hagerty K, Beck LN, Brennan LV, Oktay K** 2006 American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. J Clin Oncol 24:2917-31
56. **Anonymous** 1998 Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 351:1451-67
57. **Winer EP, Hudis C, Burstein HJ, Wolff AC, Pritchard KI, Ingle JN, Chlebowski RT, Gelber R, Edge SB, Gralow J, Cobleigh MA, Mamounas EP, Goldstein LJ, Whelan TJ, Powles TJ, Bryant J, Perkins C, Perotti J, Braun S, Langer AS, Browman GP, Somerfield MR** 2005 American Society of Clinical Oncology technology assessment on the use of aromatase inhibitors as adjuvant therapy for postmenopausal women with hormone receptor-positive breast cancer: status report 2004. J Clin Oncol 23:619-29
58. **Clemons M, Simmons C** 2006 Identifying menopause in breast cancer patients: considerations and implications. Breast Cancer Res Treat
59. **Clemons M, Goss P** 2001 Estrogen and the risk of breast cancer. N Engl J Med 344:276-85
60. **Gainford MC, McCready D, Clemons M** 2006 The latest is the greatest? Results of a structured lecture about aromatase inhibitor use for breast cancer. Breast Cancer Res Treat 96:203-6
61. **Seifer DB, MacLaughlin DT, Christian BP, Feng B, Shelden RM** 2002 Early follicular serum mullerian-inhibiting substance levels are associated with ovarian response during assisted reproductive technology cycles. Fertil Steril 77:468-71
62. **La Marca A, Giulini S, Tirelli A, Bertucci E, Marsella T, Xella S, Volpe A** 2007 Anti-Mullerian hormone measurement on any day of the menstrual cycle strongly predicts ovarian response in assisted reproductive technology. Hum Reprod 22:766-71
63. **Ficicioglu C, Kutlu T, Baglam E, Bakacak Z** 2006 Early follicular antimullerian hormone as an indicator of ovarian reserve. Fertil Steril 85:592-6
64. **Muttukrishna S, Suharjono H, McGarrigle H, Sathanandan M** 2004 Inhibin B and anti-Mullerian hormone: markers of ovarian response in IVF/ICSI patients? Bjog 111:1248-53
65. **Seifer DB, Maclaughlin DT** 2007 Mullerian Inhibiting Substance is an ovarian growth factor of emerging clinical significance. Fertil Steril
66. **Nakhuda GS, Chu MC, Wang JG, Sauer MV, Lobo RA** 2006 Elevated serum mullerian-inhibiting substance may be a marker for ovarian hyperstimulation syndrome in normal women undergoing in vitro fertilization. Fertil Steril 85:1541-3
67. **Penarrubia J, Fabregues F, Manau D, Creus M, Casals G, Casamitjana R, Carmona F, Vanrell JA, Balasch J** 2005 Basal and stimulation day 5 anti-Mullerian hormone serum concentrations as predictors of ovarian response and pregnancy in assisted reproductive technology cycles stimulated with gonadotropin-releasing hormone agonist--gonadotropin treatment. Hum Reprod 20:915-22

68. **Ebner T, Sommergruber M, Moser M, Shebl O, Schreier-Lechner E, Tews G** 2006 Basal level of anti-Mullerian hormone is associated with oocyte quality in stimulated cycles. Hum Reprod 21:2022-6
69. **Smeenk JM, Sweep FC, Zielhuis GA, Kremer JA, Thomas CM, Braat DD** 2007 Anti-mullerian hormone predicts ovarian responsiveness, but not embryo quality or pregnancy, after in vitro fertilization or intracyoplasmic sperm injection. Fertil Steril 87:223-6
70. **Fanchin R, Mendez Lozano DH, Frydman N, Gougeon A, di Clemente N, Frydman R, Taieb J** 2007 Anti-Mullerian hormone concentrations in the follicular fluid of the preovulatory follicle are predictive of the implantation potential of the ensuing embryo obtained by in vitro fertilization. J Clin Endocrinol Metab 92:1796-802
71. **Silberstein T, Maclaughlin DT, Shai I, Trimarchi JR, Lambert-Messerlian G, Seifer DB, Keefe DL, Blazar AS** 2006 Mullerian inhibiting substance levels at the time of HCG administration in IVF cycles predict both ovarian reserve and embryo morphology. Hum Reprod 21:159-163
72. **Pigny P, Jonard S, Robert Y, Dewailly D** 2006 Serum anti-Mullerian hormone as a surrogate for antral follicle count for definition of the polycystic ovary syndrome. J Clin Endocrinol Metab 91:941-5
73. **Lane AH, Lee MM, Fuller AF, Jr., Kehas DJ, Donahoe PK, MacLaughlin DT** 1999 Diagnostic utility of Mullerian inhibiting substance determination in patients with primary and recurrent granulosa cell tumors. Gynecol Oncol 73:51-5
74. **Long WQ, Ranchin V, Pautier P, Belville C, Denizot P, Cailla H, Lhomme C, Picard JY, Bidart JM, Rey R** 2000 Detection of minimal levels of serum anti-Mullerian hormone during follow-up of patients with ovarian granulosa cell tumor by means of a highly sensitive enzyme-linked immunosorbent assay. J Clin Endocrinol Metab 85:540-4
75. **Gustafson ML, Lee MM, Scully RE, Moncure AC, Hirakawa T, Goodman A, Muntz HG, Donahoe PK, MacLaughlin DT, Fuller AF, Jr.** 1992 Mullerian inhibiting substance as a marker for ovarian sex-cord tumor. N Engl J Med 326:466-71
76. **te Velde ER, Pearson PL** 2002 The variability of female reproductive ageing. Hum Reprod Update 8:141-54
77. **Kritz-Silverstein D, Barrett-Connor E** 1993 Early menopause, number of reproductive years, and bone mineral density in postmenopausal women. Am J Public Health 83:983-8
78. **van der Schouw YT, van der Graaf Y, Steyerberg EW, Eijkemans JC, Banga JD** 1996 Age at menopause as a risk factor for cardiovascular mortality. Lancet 347:714-8
79. **Cholerton B, Gleason CE, Baker LD, Asthana S** 2002 Estrogen and Alzheimer's disease: the story so far. Drugs Aging 19:405-27
80. **Collaborative Group on Hormonal Factors in Breast Cancer** 1997 Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Lancet 350:1047-59
81. **Eliassen AH, Missmer SA, Tworoger SS, Spiegelman D, Barbieri RL, Dowsett M, Hankinson SE** 2006 Endogenous steroid hormone concentrations and risk of breast cancer among premenopausal women. J Natl Cancer Inst 98:1406-15
82. **Waller K, Swan SH, Windham GC, Fenster L, Elkin EP, Lasley BL** 1998 Use of urine biomarkers to evaluate menstrual function in healthy premenopausal women. Am J Epidemiol 147:1071-80

83. **Windham GC, Elkin E, Fenster L, Waller K, Anderson M, Mitchell PR, Lasley B, Swan SH** 2002 Ovarian hormones in premenopausal women: variation by demographic, reproductive and menstrual cycle characteristics. Epidemiology 13:675-84
84. **Altshuler D, Daly M** 2007 Guilt beyond a reasonable doubt. Nat Genet 39:813-5
85. **Imani B, Eijkemans MJ, te Velde ER, Habbema JD, Fauser BC** 1998 Predictors of patients remaining anovulatory during clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility. J Clin Endocrinol Metab 83:2361-5
86. **Imani B, Eijkemans MJ, Faessen GH, Bouchard P, Giudice LC, Fauser BC** 2002 Prediction of the individual follicle-stimulating hormone threshold for gonadotropin induction of ovulation in normogonadotropic anovulatory infertility: an approach to increase safety and efficiency. Fertil Steril 77:83-90
87. **Aboulghar MA, Mansour RT** 2003 Ovarian hyperstimulation syndrome: classifications and critical analysis of preventive measures. Hum Reprod Update 9:275-89
88. **Fauser BC, Devroey P, Macklon NS** 2005 Multiple birth resulting from ovarian stimulation for subfertility treatment. Lancet 365:1807-16
89. **Shi Y, Massagué J** 2003 Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113:685-700
90. **ten Dijke P, Ichijo H, Franzen P, Schulz P, Saras J, Toyoshima H, Heldin CH, Miyazono K** 1993 Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene 8:2879-87
91. **Nohe A, Keating E, Knaus P, Petersen NO** 2004 Signal transduction of bone morphogenetic protein receptors. Cell Signal 16:291-9
92. **Dewulf N, Verschueren K, Lonnoy O, Moren A, Grimsby S, Vande Spiegle K, Miyazono K, Huylebroeck D, Ten Dijke P** 1995 Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. Endocrinology 136:2652-63
93. **Cao X, Chen D** 2005 The BMP signaling and in vivo bone formation. Gene 357:1-8
94. **Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, Ballinger DG, Struewing JP, Morrison J, Field H, Luben R, Wareham N, Ahmed S, Healey CS, Bowman R, Meyer KB, Haiman CA, Kolonel LK, Henderson BE, Le Marchand L, Brennan P, Sangrajrang S, Gaborieau V, Odefrey F, Shen CY, Wu PE, Wang HC, Eccles D, Evans DG, Peto J, Fletcher O, Johnson N, Seal S, Stratton MR, Rahman N, Chenevix-Trench G, Bojesen SE, Nordestgaard BG, Axelsson CK, Garcia-Closas M, Brinton L, Chanock S, Lissowska J, Peplonska B, Nevanlinna H, Fagerholm R, Eerola H, Kang D, Yoo KY, Noh DY, Ahn SH, Hunter DJ, Hankinson SE, Cox DG, Hall P, Wedren S, Liu J, Low YL, Bogdanova N, Schurmann P, Dork T, Tollenaar RA, Jacobi CE, Devilee P, Klijn JG, Sigurdson AJ, Doody MM, Alexander BH, Zhang J, Cox A, Brock IW, MacPherson G, Reed MW, Couch FJ, Goode EL, Olson JE, Meijers-Heijboer H, van den Ouweland A, Uitterlinden A, Rivadeneira F, Milne RL, Ribas G, Gonzalez-Neira A, Benitez J, Hopper JL, McCredie M, Southey M, Giles GG, Schroen C, Justenhoven C, Brauch H, Hamann U, Ko YD, Spurdle AB, Beesley J, Chen X, Mannermaa A, Kosma VM, Kataja V, Hartikainen J, Day NE, Cox DR, Ponder BA** 2007 Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 447:1087-93
95. **Amos CI** 2007 Successful Design and Conduct of Genome-Wide Association Studies. Hum Mol Genet
96. **The Wellcome Trust Case Control Consortium** 2007 Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447:661-78
97. **Ioannidis JP** 2007 Non-Replication and Inconsistency in the Genome-Wide Association Setting. Hum Hered 64:203-213

98. **Williams SM, Canter JA, Crawford DC, Moore JH, Ritchie MD, Haines JL** 2007 Problems with genome-wide association studies. Science 316:1840-2
99. **Shriner D, Vaughan LK, Padilla MA, Tiwari HK** 2007 Problems with genome-wide association studies. Science 316:1840-2
100. **Hughes C, Elgasim M, Layfield R, Atiomo W** 2006 Genomic and post-genomic approaches to polycystic ovary syndrome--progress so far: Mini Review. Hum Reprod 21:2766-75

Chapter 9
Summary/Samenvatting

SUMMARY

The female gonad, the ovary, is of major importance for both reproduction and the endocrine status of women. The ovary ensures the differentiation and release of the mature oocyte for fertilization and the production of sex steroid hormones. During reproductive life, ovarian aging results in a gradual decrease of fertility and eventually leads to cessation of ovarian function, which is marked by menopause and causes an almost complete absence of female sex steroid production by the ovaries. This decline in sex steroid levels has major impact on healthy ovarian aging and results in an increased risk for several health problems in women, such as osteoporosis and cardiovascular disease.

Follicle development in the ovary is regulated by the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), but also by intra-ovarian factors, such as anti-Müllerian hormone (AMH). In mice, AMH regulates the recruitment of primordial follicles into the growing follicle pool, thereby influencing the rate of follicle depletion and hence ovarian aging, and the FSH sensitivity of growing follicles. Studies in this thesis reveal the role of AMH in ovarian physiology and aging in mice and women. In addition, the contribution of AMH to the most common cause of ovarian dysfunction in women, polycystic ovary syndrome (PCOS), is investigated. The background of ovarian function, dysfunction and aging along with the aims of this thesis are described in **Chapter 1**.

In **Chapter 2** the role of the AMH type II receptor (AMHRII) in follicle development and ovarian aging was investigated using AMHRII null mice. AMHRII null mice display, similar to AMH null mice, enhanced primordial follicle recruitment. Interestingly, the primordial follicle pool was already decreased in AMHRII null mice at 25 days of age, whereas the size of the pool did not differ between AMH null and wild-type mice at this age. Hence, AMHRII null mice display ovarian aging at an even earlier age than AMH null mice, suggesting that AMHRII has an AMH-independent role in the ovary.

In **Chapter 3** we investigated whether AMH is a useful marker for the ovarian reserve, reflecting the quantity of the primordial follicle pool. As the number of primordial follicles cannot be determined in women, mice were used as a model. We observed that, similar to women, serum AMH levels decline with increasing age and AMH correlated directly with the declining number of growing follicles. AMH expression in individual follicles did not change with age. More interestingly, AMH levels correlated (indirectly) with the declining number of primordial follicles in aging mice, indicating that indeed, AMH is an excellent marker for ovarian reserve.

In **Chapter 4** we studied whether AMH has a similar role in the ovary in women as it has in mice using a genetic approach. Polymorphisms in the AMH and AMHR2 gene were identified by sequence analysis of 45 blood-bank donors. Two tagging polymorphisms were selected, Ile^{49}Ser in the AMH gene and –482 A>G in the AMHR2 gene, and the association of these polymorphism with menstrual cycle characteristics was studied in a Dutch and a German cohort of normo-ovulatory women. For both polymorphisms no association with serum AMH or FSH levels was observed. However, in both cohorts the AMH and AMHR2 polymorphisms were associated with follicular phase estradiol levels, suggesting a role for AMH in the regulation of FSH sensitivity and FSH-induced aromatase activity in the human ovary.

The role of AMH in the usage of the primordial follicle pool, and thus in ovarian aging, in women was studied in **Chapter 5**. In two Dutch cohorts of postmenopausal women, the association of polymorphisms in the AMH and AMHR2 gene with natural age at menopause was examined. The AMH Ile^{49}Ser polymorphism was not associated with age at menopause. However, the AMHR2 -482 A>G polymorphism was associated with age at menopause in interaction with the number of offspring. Nulliparous women homozygous for the G-allele entered menopause 2.6 years earlier compared to nulliparous women homozygous for the A-allele, suggesting a role for AMH signaling in the usage of the primordial follicle pool in women.

In **Chapter 6** we investigated the role of AMH in the most common cause of ovarian dysfunction, *i.e.* PCOS. Serum AMH levels are elevated in PCOS women and since AMH inhibits FSH sensitivity, the elevated AMH levels may contribute to the disturbed follicle development in these women. Indeed, in a cohort of PCOS women, the AMH ^{49}Ser polymorphism was associated with a lower follicle number and lower androgen levels. In addition, *in vitro* studies showed that the bioactivity of the AMH ^{49}Ser protein is diminished compared to the AMH ^{49}Ile protein. Hence, these results suggest that AMH contributes to the FSH refractoriness of growing follicles and, therefore the disturbed follicle selection in PCOS women.

The role of AMH signaling in PCOS was further studied in **Chapter 7**, by investigating the contribution of common genetic variation within ALK2, one of the AMH type I receptors. Several polymorphisms and haplotypes in the ACVR1 gene (encoding ALK2) were associated with AMH serum levels in PCOS women. Adjustment for follicle number revealed that the association with AMH levels was, in part, independent from follicle number, suggesting that variants in ACVR1 influence the number of follicles but also AMH production per follicle.

These results indicate that ALK2 signaling contributes to the disturbed folliculogenesis in PCOS patients.

In the general discussion, **Chapter 8**, the similarities and differences in the role of AMH between the mouse and human ovary are considered. Furthermore, possible clinical applications for AMH as a marker are discussed. Finally, suggestions for future research are made and potential clinical applications are considered, not only for polymorphisms in the AMH signaling pathway, but also for genetic research in general.

SAMENVATTING

Het vrouwelijke geslachtsorgaan, ofwel het ovarium (de eierstok) is heel belangrijk voor de voortplanting en de hormoonhuishouding van de vrouw. Het ovarium zorgt voor de differentiatie en het vrijkomen van een mature eicel en voor de productie van geslachts steroiden, met name oestrogenen. Gedurende het reproductieve leven leidt veroudering van het ovarium tot een geleidelijke afname van vruchtbaarheid en uiteindelijk tot uitval van de ovariële functie. Vanaf het moment van uitval, ofwel de menopauze, produceert het ovarium geen steroid hormonen meer. De daling in oestrogeen spiegels die hiermee gepaard gaat heeft veel invloed op de gezondheid van de vrouw en leidt tot een verhoogd risico op onder andere osteoporose en hart- en vaatziekten.

Follikelgroei en ontwikkeling in het ovarium worden gereguleerd door de gonadotropinen, luteiniserend hormoon (LH) en follikel stimulerend hormoon (FSH), maar ook door intra-ovariële factoren, zoals anti-Müllers hormoon (AMH). In muizen reguleert AMH de rekrutering (initiatie van groei en ontwikkeling) van primordiale follikels naar groeiende follikels. Op deze manier beïnvloedt AMH de snelheid waarmee de follikel voorraad wordt verbruikt en dus ook het proces van ovariële veroudering. Verder reguleert AMH in muizen de gevoeligheid van follikels voor FSH. In de experimenten beschreven in dit proefschrift wordt de rol van AMH in de fysiologie en de veroudering van het ovarium in muizen en mensen onderzocht. Ook wordt de bijdrage van AMH aan polycysteus ovarium syndroom, de meest voorkomende oorzaak van ovariële disfunctie, bestudeerd. In **hoofdstuk 1** wordt de achtergrond van ovariële functie, disfunctie en veroudering beschreven, samen met het doel van het onderzoek.

In **hoofdstuk 2** wordt de rol van de AMH type II receptor (AMHRII) in follikel ontwikkeling en ovariële veroudering onderzocht met behulp van muizen die geen AMHRII tot expressie brengen. In deze muizen zonder AMHRII, bleek er, net zoals in muizen zonder AMH, een toename in de rekrutering van primordiale follikels te zijn. Het was opmerkelijk dat in muizen zonder AMHRII van 25 dagen oud de primordiale follikel voorraad al was afgenomen terwijl in muizen zonder AMH de grootte van de voorraad niet verschilde ten opzichte van de wild-type muizen van deze leeftijd. Bij muizen zonder AMHRII treedt ovariële veroudering dus op een nog jongere leeftijd op dan in muizen zonder AMH. Dit suggereert dat de AMHRII een AMH onafhankelijke rol heeft in het ovarium.

In **hoofdstuk 3** hebben we onderzocht of AMH een bruikbare marker is voor de ovariële reserve, ofwel, of het de kwantiteit van de primordiale follikel voorraad reflecteert. Aangezien het aantal primordiale follikels niet in mensen onderzocht kan worden, hebben we muizen als model gebruikt. We vonden dat, net als in

mensen, serum AMH spiegels afnemen bij oplopende leeftijd en dat AMH direct correleert met het afnemende aantal groeiende follikels. AMH expressie in de individuele follikels veranderde niet met de leeftijd. AMH spiegels correleren ook (indirect) met het afnemende aantal primordiale follikels in verouderende muizen, hetgeen laat zien dat AMH inderdaad een uitstekende marker is voor de ovariële reserve.

In **hoofdstuk 4** hebben we met behulp van genetische studies onderzocht of AMH in vrouwen dezelfde ovariële functie heeft als in muizen. In het DNA van 45 bloed bank donoren werden polymorfismen (algemene variaties in de DNA sequentie) in het AMH en het AMHRII gen geïdentificeerd met behulp van sequencen. Vervolgens werden twee 'tagging' polymorfismen geselecteerd, Ile^{49}Ser in het AMH gen en –482 A>G in het AMHRII gen, en de associatie van deze polymorfismen met karakteristieken van de menstruele cyclus werd bestudeerd in een Nederlands en een Duits cohort van normo-ovulatoire vrouwen. Beide polymorfismen waren niet geassocieerd met serum AMH of FSH levels. Echter, in beide cohorten waren het AMH en het AMHRII polymorfisme geassocieerd met oestrogeen spiegels in de folliculaire fase van de cyclus. Dit suggereert dat AMH betrokken is bij de regulatie van FSH gevoeligheid en FSH-geïnduceerde aromatase activiteit in het ovarium van de vrouw.

De rol van AMH in het verbruik van de primordiale follikel voorraad en dus in ovariële veroudering in vrouwen wordt bestudeerd in **hoofdstuk 5**. In twee Nederlandse cohorten van postmenopauzale vrouwen is onderzocht of de polymorfismen in het AMH en het AMHR2 gen geassocieerd zijn met de natuurlijke leeftijd van menopauze, het moment van uitputting van de primordiale follikel voorraad. Het AMH Ile^{49}Ser polymorfisme was niet geassocieerd met leeftijd van menopauze. Echter, het AMHR2 –482 A>G polymorfisme was geassocieerd met leeftijd van menopauze in interactie met het aantal kinderen. Kinderloze vrouwen, die homozygoot zijn voor het G-allel ondergaan de menopauze 2.6 jaar eerder dan kinderloze vrouwen, die homozygoot zijn voor het A-allel. Dit suggereert een rol voor de AMH signaal transductie in het verbruik van de primordiale follikel voorraad in vrouwen.

In **hoofdstuk 6** hebben we onderzocht of AMH een rol speelt in de meest voorkomende oorzaak van ovariële disfunctie in vrouwen, het polycysteus ovarium syndroom (PCOS). Serum AMH spiegels zijn verhoogd in vrouwen met PCOS. Aangezien AMH de FSH gevoeligheid van groeiende follikels remt, kunnen deze hoge AMH spiegels bijdragen aan de verstoorde follikelgroei en ontwikkeling in PCOS vrouwen. Het AMH ^{49}Ser allel is inderdaad geassocieerd met een lager follikel aantal en lagere androgeen spiegels in deze vrouwen. *In vitro* studies laten ook zien dat de bio-activiteit van het AMH ^{49}Ser eiwit verminderd is ten opzichte

van het AMH ^{49}Ile eiwit. Deze resulaten suggereren dat AMH bijdraagt aan de FSH ongevoeligheid van groeiende follikels en daarmee aan de verstoorde follikel selectie in PCOS vrouwen.

De rol van AMH signaal transductie in PCOS werd verder onderzocht in **hoofdstuk 7**. In dit hoofdstuk werd de bijdrage van veel voorkomende genetische variatie in ALK2, één van de type I receptoren voor AMH, bestudeerd. Verschillende polymorfismen en haplotypen (een combinatie van polymorfismen) in het ACVR1 gen (dat ALK2 codeert) waren geassocieerd met serum AMH spiegels in vrouwen met PCOS. Correctie voor het aantal follikels laat zien dat de associatie met AMH spiegels gedeeltelijk onafhankelijk is van het follikel aantal, hetgeen suggereert dat deze polymorfismen in het ACVR1 gen zowel het aantal follikels als ook de AMH productie per follikel beïnvloeden. Deze resultaten tonen dat signaal transductie via ALK2 waarschijnlijk bijdraagt aan de verstoorde follikel ontwikkeling in PCOS patiënten.

In de algemene discussie, **hoofdstuk 8**, worden de overeenkomsten en verschillen met betrekking tot de rol van AMH in het ovarium van muizen en mensen in beschouwing genomen. Bovendien worden potentiële klinische toepassingen van AMH als marker beschreven. Tenslotte worden aanbevelingen gedaan voor toekomstig onderzoek met betrekking tot de mogelijke klinische toepasbaarheid van polymorfismen in de AMH signaal transductie route en voor genetisch onderzoek in het algemeen.

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CURRICULUM VITAE

Maria Elisabeth Kevenaar, forename Marlies, was born on June 1st, 1979 in Rotterdam, The Netherlands. In 1997 she completed secondary school at the G.S.G. Helinium in Hellevoetsluis, and started her medical study at the Erasmus University in Rotterdam. Her graduation research in 2001 was performed at the Department of Internal Oncology, Erasmus MC, at the laboratory of dr. P.M.J.J. Berns. During this research project the role of genetic variation in luteinizing hormone and estrogen signaling pathways in breast cancer was studied. In March 2004 she obtained her medical degree cum laude. In April 2004 she started the work presented in this thesis at the Department of Internal Medicine, under supervision of dr.ir. J.A. Visser, dr.ir. A.P.N. Themmen and Prof.dr. H.A.P. Pols. During her graduation project she attended several courses at the Netherlands Institute for Health Sciences (NIHES) and the Molecular Medicine postgraduate school. In September 2005 and May 2007 she received a travel grant of the Dutch Endocrine Society. In June 2006 a travel grant from the Endocrine Society, USA was obtained. In May 2007 she acquired the "Neena Schwartz Award for Excellence in Basic Science" of "Women in Endocrinology, USA". In September 2007 she started her residency in internal medicine at the IJsselland Hospital, Capelle aan den IJssel, under supervision of dr. H.E. van der Wiel.

PUBLICATIONS

Powell BL, Piersma D, **Kevenaar ME**, van Staveren IL, Themmen APN, Iacopetta BJ, Berns EMJJ. Luteinizing hormone signaling and breast cancer: polymorphisms and age of onset. *J Clin Endocrinol Metab 2003; 88:1653-7.*

Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groome NP, Themmen APN, Visser JA. Serum Anti-Mullerian hormone levels reflect the size of the primordial follicle pool in mice. *Endocrinology 2006; 147:3228-34.*

Kevenaar ME, Themmen APN, Laven JSE, Sonntag B, Lie Fong S, Uitterlinden AG, de Jong FH, Pols HAP, Simoni M, Visser JA. Anti-Mullerian hormone and anti-Mullerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women. *Human Reproduction 2007; 22:1547-54.*

Kevenaar ME, Themmen AP, Rivadeneira F, Uitterlinden AG, Laven JS, van Schoor NM, Lips P, Pols HA, Visser JA. A polymorphism in the AMH type II receptor gene is associated with age at menopause in interaction with parity. *Human Reproduction 2007; 22:2382-8.*

Kevenaar ME, Laven JSE, Lie Fong S, Uitterlinden AG, de Jong FH, Themmen APN, Visser JA. A functional AMH polymorphism is associated with follicle number and androgen levels in polycystic ovary syndrome patients. *Submitted.*

Kevenaar ME, Themmen APN, van Kerkwijk AJ, Valkenburg O, Uitterlinden AG, de Jong FH, Laven JSE, Visser JA.Variants in the ACVR1 gene are associated with AMH levels in women with polycystic ovary syndrome. *Submitted.*

Kevenaar ME, Kramer P, McLuskey A, Behringer RR, Themmen APN, Visser JA. AMH type II receptor null mice display a more severe ovarian phenotype than AMH null mice. *To be submitted.*

LIST OF ABBREVIATIONS

AFC	antral follicle count
ALK	activin-receptor like kinase
AMH	anti-Müllerian hormone
AMHRII	anti-Müllerian hormone type II receptor
AN(C)OVA	analysis of (co)variance
AUC	area under the curve
BMI	body mass index
BMP	bone morphogenetic protein
BMPRII	bone morphogenetic protein type II receptor
bp	base pairs
BSA	bovine serum albumine
CV	coefficient of variation
CYP1B1	Cytochrome P450 1B1
CYP17	Cytochrome P450 17α-hydroxylase/ c17-20 lyase
CYP19	aromatase
D'	linkage disequilibrium coefficient
DMEM	Dulbecco's minimal essential medium
DNA	deoxyribonucleic acid
e.g.	for example (exempli gratia)
E_2	estradiol
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FCS	fetal calf serum
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GDF	growth and differentiation factor
GnRH	gonadotropin-releasing hormone
GnRHa	gonadotropin-releasing hormone agonist
GWA	genome wide association
HEK	human embryonic kidney
HPE	high-performance ELISA
HPG axis	hypothalamus-pituitary-gonadal axis
HRT	hormone replacement therapy
HWE	Hardy-Weinberg equilibrium
i.e.	in other words (id est)
IU	international unit
IVF	in vitro fertilisation
Kb	kilo base pairs
KDa	kilo Dalton
KO	knockout
LASA	longitudinal aging study Amsterdam
LD	linkage disequilibrium
LH	luteinizing hormone
LHR	luteinizing hormone receptor
Mab	monoclonal antibody
MAF	minor allele frequency
MIS	Müllerian-inhibiting substance
MMP2	matrix metalloproteinase 2
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline
PCO	polycystic ovaries
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PMDS	persistant Müllerian duct syndrome
POF	premature ovarian failure
RIA	radioimmunoassay
r^2	correlation coefficient
SD	standard deviation
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
SHBG	sex hormone binding globulin
SNP	single nucleotide polymorphism
TGF	transforming growth factor
wt	wild-type