Anti-Müllerian hormone and anti-Müllerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women

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BACKGROUND: In mice, anti-Müllerian hormone (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 Ile49Ser and the AMH type II receptor (AMHR2) 2482 A>G polymorphisms with menstrual cycle characteristics was studied in a Dutch (n = 32) and a German (n = 21) cohort of normo-ovulatory women. RESULTS: Carriers of the AMH Ser49 allele had higher serum estradiol (E2) levels on menstrual cycle day 3 when compared with non-carriers in the Dutch cohort (P = 0.012) and in the combined Dutch and German cohort (P = 0.03). Carriers of the AMHR2 2482G allele also had higher follicular phase E2 levels when compared with 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 Ser49 and AMHR2 2482G alleles had highest E2 levels (P = 0.001). For both polymorphisms no association with serum AMH or FSH levels was observed. CONCLUSIONS: Polymorphisms in the AMH and AMHR2 genes are associated with follicular phase E2 levels, suggesting a role for AMH in the regulation of FSH sensitivity in the human ovary.

Keywords: anti-Müllerian hormone/estrogen/menstrual cycle/ovarian function/polymorphism

Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance, is a member of the transforming growth factor-β 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 pre-antral and small antral follicles and gradually diminishes in the subsequent stages of follicle development (Durlinger et al., 2002). The expression pattern of AMH is flanked by two major regulatory steps of folliculogenesis, primordial follicle recruitment and cyclic selection (McGee and Hsueh, 2000), suggesting that AMH may influence these steps. Indeed, studies in AMH knockout mice revealed that in the absence of AMH, follicles are recruited at a faster rate and that follicles display an increased sensitivity to FSH (Durlinger et al., 1999, 2001).

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 the 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 (McGee and Hsueh, 2000). 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 LH receptor expression in rat granulosa cell cultures (di Clemente et al.,...
1994). Therefore, AMH might affect estradiol (E2) 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 (van Rooij et al., 2002) and reflect the size of the primordial follicle pool, which makes AMH an ideal marker for ovarian reserve, as reviewed by Visser et al. (2006). In pathophysiologic conditions, such as polycystic ovary syndrome (PCOS), AMH serum levels also associate with antral follicle count (Pigny et al., 2003; Laven et al., 2004) and may be used as a diagnostic marker for this syndrome (Visser et al., 2006).

Little attention has been paid to the functional role of AMH in human ovarian physiology. On the basis of 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<sup>680</sup>Ser; rs6166), has been shown to be associated with FSH levels and the length of the menstrual cycle (Greb et al., 2005).

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 premenopausal 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 promoter region of the AMH gene in 45 randomly selected Dutch Caucasian blood donor samples. 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% dimethyl-sulphoxide 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 analysed on an ABI prism 3100 automatic capillary sequencer (Applied Biosystems). In addition, the NCBI (www.ncbi.nlm.nih.gov) and International HapMap Project (www.hapmap.org) (The International HapMap Project, 2003) SNP databases were used to search polymorphisms in the AMH and AMHR2 genes.

**Subjects**

We studied a Caucasian subset (n = 32) of a previously described Dutch cohort (de Vet et al., 2002) for whom DNA and a complete hormone assessment were available. These normo-ovulatory women had an age range of 20–35 years and a body mass index (BMI) of 19–26 kg m<sup>-2</sup>. 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 immunoenooassay [Immulite, Diagnostic Products corporation (DPC), Los Angeles, CA, USA]. Serum E<sub>2</sub> concentrations were measured using radioimmunoassay kits provided by DPC, as described previously (Fauser et al., 1991). Inhibin B was measured using an immunoenzymometric assay (Serotec, Oxford, UK) and AMH levels were measured using an enzyme-linked immunosorbent assay (ELISA) (Immunotech-Coulter, Marseille, France). Intra- and inter-assay coefficients of variation (CV) were <3% and 8% for FSH, <5% and 15% for LH, <5% and 7% for E<sub>2</sub>, <9% and 15% for inhibin B and <5% and 8% for AMH, respectively (Schipper et al., 1998; Hohmann et al., 2001; de Vet et al., 2002).

In addition, a German cohort consisting of 21 premenopausal normo-ovulatory women of Caucasian origin, selected by FSH receptor (FSHR) genotype (Greb et al., 2005), was studied. Women in this study cohort had an age range of 18–34 years and a BMI of 19–28 kg m<sup>-2</sup>. In these women, the complete menstrual cycle was monitored by performing transvaginal ultrasound and measuring serum hormone levels (Greb et al., 2005). FSH and LH were measured by an immunofluorimetric assay and E<sub>2</sub> by fluoroiimunoassay using the Autodelfia system (Perkin-Elmer, Freiburg, Germany) as described previously (Simoni et al., 1999). Progesterone was measured by radioimmunoassay 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 DSI (Sinsheim, Germany). AMH levels were measured using an in-house AMH ELISA assay (Kevenaar et al., 2006). This AMH assay shows close correlation with the AMH ELISA assay of Immunootech (r = 0.99). The values from the Immunootech assay were adjusted to the in-house AMH ELISA assay (x 1.73). For both FSH and LH, intra- and inter-assay CV were <3%. Intra- and inter-assay CV were 2.2% and 2.7% for E<sub>2</sub>, 3.7% and 6.1% for progesterone, 6.3% and 7.0% for inhibin A, 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<sup>49</sup>Ser polymorphism, an Assay-by-Design with the following probes was used: 5′-CTCCAGGCATCCACA-3′ and 5′-CCAGGCCgGCCCAAC-3′. For the AMH2<sup>–482</sup> 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 2 x, 0.025 μl of the 80 x AMH Ile<sup>49</sup>Ser Assay Mix or 0.1 μl of the 20 x AMH2<sup>–482</sup> A>G 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 analysed using an ABI prism 7900HT Sequence Detection System.

**Statistical analysis**

Using the blood donor samples, haplotypes of the AMH and AMHR2 genes were constructed using the PHASE program (Stephens et al., 2001; Stephens and Donnelly, 2003). 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 (The International HapMap Project, 2003).

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 $\chi^2$-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 Ser$^{49}$ allele and carriers of the AMHR2 $\sim$482G allele were compared with non-carriers. Differences in AMH, FSH, E2 and inhibin B levels and follicle number were adjusted for age. E2 levels were log-transformed to normalize their distribution. In the German cohort, a $\chi^2$-test was used to test whether the distribution of the homozygous Asn$^{680}$ and Ser$^{680}$ 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 the midcycle LH surge (day LH 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 cohorts were analysed together. An additive genetic effect of AMH and AMHR2 genotypes on E2 levels was tested; trend analysis for the combination of the AMH and AMHR2 genotypes was performed for the presence of one, zero 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, USA). 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 15 sequence variations, including eight novel SNPs, in the AMH gene and four variations, including one novel SNP, in the AMHR2 gene (Figure 1 and Supplementary data, Table 1). For the association studies, polymorphisms with a minor-allele frequency (MAF) $> 0.10$ were selected for reasons of statistical power. For the AMH gene, two polymorphisms fulfilled this criterion; the $\sim$482 C>T promoter polymorphism (rs 4807216) and the Ile$^{49}$Ser polymorphism (rs 10407022). Reconstruction of haplotype alleles in the PHASE program revealed that these polymorphisms are in complete linkage disequilibrium ($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 non-coding region of the AMHR2 gene [$\sim$482 A>G promoter (rs 2002555), T>A intron 1 (rs 2272002), C>T intron 6 (rs 11170552) and A>G intron 10 (rs 11170555)]. Analyses in Haploview showed that in Caucasians, these six AMHR2 polymorphisms are in complete linkage disequilibrium ($D' = 1.0$ and $r^2 = 1$) (Fig. 1). Hence, we only genotyped the $\sim$482 A>G promoter polymorphism as a tagging SNP.

Figure 1. Schematic overview of the anti-Müllerian hormone (AMH) (Chr. 19p13.3) and AMH type II receptor (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.

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 1). Age and BMI were similar between the AMH genotype groups, as were serum AMH, FSH, LH and Inhibin B levels (Table 1). However, carriers of the AMH Ser$^{49}$ allele had significantly higher E2 levels on day 3 of the menstrual cycle when compared with non-carriers (Table 1, $P = 0.012$). No association of the AMH genotypes with antral follicle count or cycle length was observed (Table 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 (Greb et al., 2005), we analysed 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) (Supplementary data, Table 2A). 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 (Supplementary data, Table IIA), 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 cohorts were analysed 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 Ser$^{49}$ allele again had higher E$_2$ levels ($P = 0.03$) compared with non-carriers, whereas other hormone levels were not different between the genotype groups (Table 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 2). Carriers of the AMHR2 $-482$G allele had higher E$_2$ levels ($P = 0.028$) compared with non-carriers, whereas age, BMI, other hormone levels and ovarian parameters were similar between carriers and non-carriers (Table 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$^{660}$Ser genotypes ($P = 0.62$, Supplementary data, Table 2B). In this German cohort, the AMHR2 polymorphism was not associated with AMH, FSH (Figs 2 and 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 $-482$G allele carriers had higher E$_2$ levels from cycle day 0 to cycle day 5 ($P = 0.048$) and there was a trend towards higher E$_2$ levels for the total follicular phase ($P = 0.077$) (Fig. 2). When aligned relative to the day of the LH peak, E$_2$ levels were not significantly different ($P = 0.15$) (Fig. 3). Moreover, the pre-ovulatory E$_2$ peak tended to be higher in the AMHR2 $-482$G allele carriers, although this failed to reach significance ($P = 0.10$) (Table 3).

To obtain more insight into 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 pre-ovulatory E$_2$ peak and for the total menstrual cycle (Table 3). The AUC for follicular phase E$_2$ was significantly larger in the G-allele carriers ($P = 0.04$). However, the AUC

### Table 1. Anti-Müllerian hormone (AMH) Ile$^{49}$Ser genotypes: hormone levels and ovarian parameters on day 3 of the menstrual cycle

<table>
<thead>
<tr>
<th>AMH Ile$^{49}$Ser</th>
<th>Dutch cohort</th>
<th></th>
<th></th>
<th>Dutch cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ile/Ile</td>
<td>Ile/Ser + Ser/Ser</td>
<td>$P$</td>
<td>Ile/Ile</td>
</tr>
<tr>
<td>$N$ (%)$^a$</td>
<td>24 (75.0)</td>
<td>8 (25.0)</td>
<td>0.42</td>
<td>39 (73.6)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>30.5 ± 0.8</td>
<td>28.1 ± 1.8</td>
<td>0.17</td>
<td>28.5 ± 0.7</td>
</tr>
<tr>
<td>BMI (kg m$^{-2}$)</td>
<td>22.4 ± 0.7</td>
<td>20.5 ± 0.5</td>
<td>0.26</td>
<td>21.8 ± 0.4</td>
</tr>
<tr>
<td>AMH (ng ml$^{-1}$)$^b$</td>
<td>2.9 ± 0.5</td>
<td>3.4 ± 0.9</td>
<td>0.61</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>FSH (IU l$^{-1}$)$^b$</td>
<td>7.1 ± 0.5</td>
<td>7.0 ± 0.9</td>
<td>0.93</td>
<td>6.8 ± 0.3</td>
</tr>
<tr>
<td>LH (IU l$^{-1}$)</td>
<td>3.4 ± 0.3</td>
<td>3.3 ± 0.5</td>
<td>0.89</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>E$_2$ (pmol l$^{-1}$)$^b$</td>
<td>151.3 ± 13.2</td>
<td>215.0 ± 23.2</td>
<td>0.012</td>
<td>141.1 ± 10.2</td>
</tr>
<tr>
<td>Inhibin B (ng l$^{-1}$)$^b$</td>
<td>105.3 ± 11.8</td>
<td>126.7 ± 21.6</td>
<td>0.40</td>
<td>93.5 ± 8.2</td>
</tr>
<tr>
<td>Antral follicle count$^c$</td>
<td>14.7 ± 0.9</td>
<td>15.0 ± 1.6</td>
<td>0.87</td>
<td>15.6 ± 0.9</td>
</tr>
<tr>
<td>Cycle length (day)</td>
<td>28.5 ± 0.4</td>
<td>28.3 ± 0.6</td>
<td>0.81</td>
<td>28.2 ± 0.3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.

$E_2$, estradiol; BMI, body mass index.

*P*-value for Hardy–Weinberg equilibrium.

$^a$Adjusted for age.

$^c$Antral follicles detectable by ultrasound, 2–3 mm.

### Table 2. Anti-Müllerian hormone type II receptor (AMHR2) $-482$ A$>$G genotypes: hormone levels and ovarian parameters on day 3 of the menstrual cycle

<table>
<thead>
<tr>
<th>AMHR2 $-482$A$&gt;$G</th>
<th>Dutch cohort</th>
<th></th>
<th></th>
<th>Dutch cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/A</td>
<td>A/G + G/G</td>
<td>$P$</td>
<td>A/A</td>
</tr>
<tr>
<td>$N$ (%)$^a$</td>
<td>20 (62.5)</td>
<td>12 (37.5)</td>
<td>0.20</td>
<td>35 (66.0)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>29.4 ± 0.9</td>
<td>30.7 ± 1.1</td>
<td>0.38</td>
<td>27.6 ± 0.7</td>
</tr>
<tr>
<td>BMI (kg m$^{-2}$)</td>
<td>22.3 ± 0.9</td>
<td>21.6 ± 0.9</td>
<td>0.62</td>
<td>22.1 ± 0.5</td>
</tr>
<tr>
<td>AMH (ng ml$^{-1}$)$^b$</td>
<td>3.6 ± 0.5</td>
<td>2.2 ± 0.7</td>
<td>0.12</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>FSH (IU l$^{-1}$)$^b$</td>
<td>7.4 ± 0.5</td>
<td>6.5 ± 0.7</td>
<td>0.29</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>LH (IU l$^{-1}$)</td>
<td>3.5 ± 0.3</td>
<td>3.1 ± 0.4</td>
<td>0.34</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>E$_2$ (pmol l$^{-1}$)$^b$</td>
<td>145.6 ± 14.3</td>
<td>203.3 ± 18.5</td>
<td>0.028</td>
<td>132.6 ± 10.4</td>
</tr>
<tr>
<td>Inhibin B (ng l$^{-1}$)$^b$</td>
<td>107.4 ± 13.1</td>
<td>115.6 ± 16.8</td>
<td>0.70</td>
<td>93.9 ± 9.1</td>
</tr>
<tr>
<td>Antral follicle count$^c$</td>
<td>15.6 ± 0.9</td>
<td>13.4 ± 1.2</td>
<td>0.17</td>
<td>17.0 ± 1.0</td>
</tr>
<tr>
<td>Cycle length (day)</td>
<td>28.4 ± 0.4</td>
<td>28.6 ± 0.5</td>
<td>0.62</td>
<td>28.3 ± 0.4</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.

*P*-value for Hardy–Weinberg equilibrium.

$^a$Adjusted for age.

$^c$Antral follicles detectable by ultrasound, 2–3 mm.

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for E2 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 ($P = 0.61$, Fig. 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 (Fig. 3). Indeed, inhibin B levels from day LH $-15$ to day $-12$ were significantly lower ($P = 0.05$) in carriers of the G-allele compared with non-carriers (Fig. 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 parameters, and although the dominant follicle tended to be detected 2.5 days earlier in carriers of the G-allele, this was not significant (Table III).

In addition, the analysis of the combined Dutch and German cohort demonstrated once more that carriers of the AMHR2 $−482$G allele have higher E2 levels on cycle day 3 compared with non-carriers ($P = 0.012$, Table II).
suggest that AMH regulates E2 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 (McGee and Hsueh, 1999), the follicular phase length is reflected by the number of fAntral follicles detectable by ultrasound. 

Consequently, the development of the dominant follicle coincides with a rise in plasma E2 levels (van Santbrink et al., 1995; van Dessel et al., 1995). Hence, 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 E2 levels and follicular phase length was reported previously by several studies in normo-ovulatory women (Landgren et al., 1980; Nagata et al., 1997; Windham et al., 2002). The latter studies indicated that high (mean, peak and baseline) plasma E2 levels, who both had higher E2 levels, may have a lower threshold for FSH, resulting in more efficient stimulation of E2 production by granulosa cells and/or an accelerated growth of the selected follicles during the early follicular phase. Moreover, carriers of both the AMH Ser49 and the AMHR2 −482G alleles may exhibit the lowest threshold since an additive effect on E2 levels was observed when genotypes were combined.

For the AMHR2 −482 A>G polymorphism, association studies in the German cohort revealed higher E2 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 E2 levels and follicular phase length was reported previously by several studies in normo-ovulatory women (Landgren et al., 1980; Nagata et al., 1997; Windham et al., 2002). The latter studies indicated that high (mean, peak and baseline) plasma E2 levels (Landgren et al., 1980) and high (mean and baseline) urinary estrogen metabolite levels (Windham et al., 2002) 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 (Groome et al., 1996), and since an increase in the levels of inhibin B reflects early gonadotrophin-dependent follicle growth (Welt et al., 1999), the follicular phase length is reflected by the number.

![Figure 4. Additive effect of AMH and AMHR2 genotypes on E2 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 genotypes, N = 27; AMH Ser49-allele carriers and AMHR2 A/A, n = 8 or AMH Ile/Ile and AMHR2 G-carrier, n = 12; AMH Ser49-allele carriers and AMHR2 G-allele carriers, n = 6, respectively). Data represent the mean ± SEM. P-trend = 0.001.](image-url)
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 pre-antral and small antral follicles in the early follicular phase is stimulated not only by FSH but also by local growth factors (Welt and Schneyer, 2001). Moreover, during the late follicular phase, the pre-ovulatory follicle does not contribute to serum inhibin B levels in response to FSH stimulation (Welt et al., 2001).

In young women with normal ovarian function, large 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 E2 levels and follicular phase length (van Santbrink et al., 1995; Schipper et al., 1998). 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 (Laven and Fauser, 2006). Furthermore, it has been reported that genetic variation contributes to this individual FSH-setpoint (de Castro et al., 2004; Greb et al., 2005).

In our study, no differences in the levels of the pituitary gonadotrophin 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<sup>49</sup>Ser and the AMHR2 −482 A>G polymorphisms 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 normogonadotrophic 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 stimulation syndrome and poly-ovulation resulting in multiple pregnancies (Fauser and Van Heusden, 1997; Mulders et al., 2003). Since previous models to predict the individual FSH threshold on the 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 (Durlinger et al., 1999; Carlsson et al., 2006). 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<sup>49</sup>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 E2 levels in both cohorts independently as well as in the combined cohort. The AMH Ile<sup>482</sup>Ser polymorphism was also associated with E2 levels in the Dutch cohort and in the combined cohort. However, since the AMH Ile<sup>482</sup>Ser polymorphism could not be analysed 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 (Newton-Cheh and Hirschhorn, 2005; Rivadeneira et al., 2006). 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 E2 levels in carriers of the AMH Ser<sup>49</sup> and the AMHR2 −482G alleles 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<sup>482</sup>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 genes are associated with follicular phase E2 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<sup>482</sup>Ser and AMHR2 −482 A>G polymorphisms also affect cumulative lifelong estrogen exposure and whether these polymorphisms 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 PCOS since in these women, the individual FSH threshold seems to be elevated (Laven et al., 2002).

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Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

References


Fauser BC, Pache TD, Lamberts SW, Hop WC, de Jong FH and 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 73,811–817.


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