AMH mutations with reduced in vitro bioactivity are related to premature ovarian insufficiency

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STUDY QUESTION: Could anti-Müllerian hormone (AMH) mutations be implicated in the development of idiopathic premature ovarian insufficiency (POI)?

SUMMARY ANSWER: Three rare or unknown missense variants of the AMH gene were identified in a cohort of 55 POI patients; all three variants showed a drastically reduced in vitro bioactivity.

WHAT IS KNOWN ALREADY: Genetic factors are implicated in 5–15% of cases of POI. However, only a few genes have been shown to be involved in its development. AMH inhibits the recruitment of primordial follicles in the ovary and defective or absent AMH leads to premature depletion of the primordial follicle pool in AMH null mice.

STUDY DESIGN, SIZE, DURATION: The whole coding sequence and the exon–intron junction of the AMH gene was sequenced in a cohort of 55 POI patients recruited over a period of 8 years. The studied variants were also sequenced in 197 ethnically matched controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: POI was defined as amenorrhea of more than 4 months with increased FSH before the age of 40. Patients with POI resulting from radio- or chemotherapy, surgery, chromosomal anomalies or FMR1 gene pre-mutation were excluded from the study. Recombinant human wild-type (wt) and mutated AMH proteins were produced in HEK293 T cells. KK-1 cells transfected with the AMH receptor type 2 (AMHR2) and a BMP responsive element coupled to a luciferase reporter vector were stimulated with different concentrations of wt AMH and the three tested variants.

MAIN RESULTS AND THE ROLE OF CHANCE: The whole coding sequence of the AMH gene could be performed and analyzed for 50 POI patients: 16 variants were found, including 6 missense variants from which 1 was unknown (R444H) and 2 were very rare (G264R and D288E). The variant D288E was also found in one of the patient’s mother who also underwent POI at 32 years old. The stimulation of the AMHR2 assessed by the luciferase activity was drastically reduced for the three variants when compared with the wt AMH.

LIMITATIONS, REASONS FOR CAUTION: The study is limited by a relatively small number of patients in the POI cohort.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first time that the bioactivity of AMH variants related to POI patients is tested in vitro. The functional study showed a drastic reduction of the protein activity for the three variants, supporting their contribution to the development of the ovarian insufficiency. The familial segregation further supports the implication of AMH in the development of POI.

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Key words: premature ovarian insufficiency / POI / AMH / AMH mutations / AMH bioactivity
**Introduction**

Premature ovarian insufficiency (POI) is characterized by amenorrhea of more than 4 months with increased FSH before the age of 40 (Nelson, 2009). The cause of POI is undetermined in most cases, but the incidence of family-related cases can achieve a percentage from 4 to 31% (Van Kasteren et al., 1999), suggesting a genetic etiology in a large proportion of patients. In contrast with the number of genes implicated in the development of the ovary and the ovarian folliculogenesis (Matzuk and Lamb, 2008), until now, only a few genes have been associated with POI (Persani et al., 2010).

The development of POI might be explained by either a diminished ovarian reserve at birth, an accelerated rate of primordial follicle recruitment, an increased atresia of the follicles or a follicular dysfunction (Christin-Maitre and Braham, 2008). Exhaustion of the pool of primordial follicles is the cause of POI in most women. Evidence is accumulating that factors synthesized by the oocyte or surrounding granulosa cells play a key role in early follicular development (Matsuda et al., 2012). Members of the transforming growth factor-beta (TGF-β) family are crucial for the transition of resting primordial follicles to primary growing follicles (Knight and Glister, 2006). Among these, BMP15 and GDF9 have been suggested to play a role in POI (Di Pasquale et al., 2006; Driix et al., 2006; Laisse et al., 2006; Tiotiu et al., 2010; Otsuka et al., 2011). Anti-Müllian hormone (AMH) is another dimeric glycoprotein belonging to the TGF-β superfamily. It was first named Mullerian inhibitor by Alfred Jost who postulated that a testicular factor was responsible for the regression of the Mullerian ducts during sexual differentiation (Jost, 1947). In females, AMH secretion has been detected as early as 32 gestational weeks (Rapport-De Meyts et al., 1999) and is produced by the granulosa cells of the pre-antral and small antral follicles of the ovary. The AMH level is correlated with the pool of primordial follicles and the number of antral follicles (de Vet et al., 2002) and is considered today as a useful marker of the ovarian reserve (Anderson et al., 2012; Broer et al., 2014). Studies in AMH null female mice show that AMH inhibits the recruitment of primordial follicles in the ovary (Durlinger et al., 1999), probably by regulating the sensitivity of the ovarian follicles to FSH (Visser and Themmen, 2014). AMH null mice at 4 months of life showed a decreased number of primordial follicles and an increased number of non-atretic small follicles when compared with wild-type (wt) AMH mice. At 13 months, there were still no primordial follicles left (Durlinger et al., 1999). Thus, a defective or absent AMH could lead to an earlier than expected depletion of the primordial follicle pool.

In this article, we identified and tested the bioactivity of three missense mutations of the AMH gene found in three patients from a cohort of 55 patients with POI. One of the mutations had never been described previously and the two others appeared to be very rare variants.

**Materials and Methods**

**Patients**

A total of 55 POI patients were included in the study. Inclusion criteria were primary or secondary amenorrhea occurring before the age of 40 years and an FSH serum level above 40 IU/l. Patients with POI resulting from radio- or chemotherapy, surgery, chromosomal anomalies or FMR1 gene pre-mutation were excluded from the study.

Patients were recruited in the outpatient clinic of the Fertility Clinic of the Erasme Hospital (Brussels, Belgium); most of them were referred for infertility treatment. The Ethics Committee of the hospital approved the study and all participants gave their written informed consent for blood sampling and genetic investigations. Patients were asked to complete a questionnaire about their ethnic origin, up to their four grandparents.

Of the patients included in this study, 44 were of Caucasian origin, 4 originated from North Africa, 5 were from Sub-Saharan Africa, 1 was from West Indies and 1 was from Asia. There were 8 patients with primary amenorrhea and 47 patients with secondary amenorrhea. Among these, 6 were diagnosed with POI after stopping the contraceptive pill. For the others, the mean age of onset of POI was of 28.9 ± 6.5 years (mean ± SD) (range from 15 to 39 years old).

**Controls**

The control population was recruited in collaboration with the occupational medicine center of the hospital among hospital employees. The collection of a DNA database in a control population was part of a transdisciplinary project of genetic susceptibility to diseases and treatments. The project was approved by the Ethics Committee of the hospital. All controls received written information about the project. After having signed an informed consent, they were asked to complete a questionnaire about their ethnic origins up to their four grandparents. The control population was composed of 113 women of Caucasian origin and 84 women originating from Sub-Saharan Africa.

**DNA extraction and sequencing**

Genomic DNA was extracted from peripheral blood leukocytes. The entire coding sequence and the intron—exon junctions of the human AMH were analyzed for all patients. PCR products were treated with shrimp alkaline phosphatase and exonuclease I as described by the manufacturer (USB, Cleveland, OH, USA) and directly sequenced, using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). Only the exon containing the variant was tested in the control group.

**Sequence analyses**

The resulting sequences were analyzed using the SeqScape v2.5 software (Applied Biosystems) and confirmed by manual analysis.

To determine the potentially deleterious effect of the amino acid changes, two software programs were exploited: PolyPhen (http://genetics.bwh.harvard.edu/ php/index.html) and SIFT (http://sift.jcvi.org/www/SIFT_BLink_submit.html). The PolyPhen software predicts the possible impact of an amino acid substitution on the structure and function of a human protein by considering structural parameters and three-dimensional protein structures (Sunyaev et al., 2001). The SIFT software predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (Kumar et al., 2009). The score is calculated according to the current available human AMH sequence (P03971 UniProt/SwissProt). For multiple alignments and allele frequency of the found variants, Ensembl database was exploited (http://www.ensembl.org/index.html).

**Recombinant human AMH production**

The human AMH cDNA (GenBank Accession BC049194, from PlasmID, DF/HCC DNA Resource Core, Harvard Medical School) was cloned into pcDNA3 vector plasmid and used as a template for site-directed mutagenesis using PfuTurbo® DNA Polymerase (Stratagene) and specific primers containing the identified AMH mutations (available upon request). After verification of the presence of the mutation in the obtained cDNAs by sequencing analysis, the cDNAs were cloned into the expression vector pcDNA3. HEK293 T cells were transfected with expression vectors containing the wt AMH or the very rare or not previously described missense AMH variants.
An empty pcDNA3 vector was transfected in some HEK293 T cells for use as a control. Serum-free supernatant was collected and AMH was concentrated with an ultracentrifugation filter system (Amicon Ultra, Millipore, Cork, Ireland).

**AMH dosage**

AMH levels in the serum-free supernatant were measured by enzyme immunoassay using commercially available kits (AMH Gen II Elisa; Beckman Coulter). The AMH functional detection limits were 0.1 ng/ml and the intra-assay and inter-assay coefficients of variation were 5 and 8%, respectively.

**Cell culture, DNA transfection and bioactivity measurements**

KK-1 cells (Kananen et al., 1995), a kind gift of Professor Huhtaniemi (Institute of Reproduction and Developmental Biology, Imperial College of London, London, UK), were cultured in Dulbecco’s medium with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% amphotericin B, 1% penicillin streptomycin. This mouse ovarian granulosa cell line is characterized by the maintained responsiveness to gonadotrophins and steroidogenesis in culture. They constitute, thus, a good cellular model to study ovarian molecular function.

KK-1 cells were transiently transfected with AMHR2 and a BMP responsive element (Bre) coupled to a luciferase reporter (Bre-Luc) plasmid (kind gift of Peter van Dijke, The Netherlands Cancer Institute, The Netherlands) using polyethylenimine (PEI) and cultured to 20% confluence. Plasmid containing firefly luciferase plasmid was co-transfected as an internal control. One day after transfection, they were incubated for 2 h with DMEM medium without serum followed by 16 h incubation with increasing concentrations of wt AMH and the different studied variants. Luciferase activity was measured using Dual Glo luciferase assay (Promega), following the manufacturer instructions, in an EG&G Berthold LB 96 V luminometer.

**Western blot analysis**

Serum-free supernatant of the cells transfected with different AMH variants was boiled at 100°C for 10 min with Laemmli buffer. The separation of proteins by electrophoresis was done with a NuPAGE 10% Bis-Tris Gel (Novex). The day after, after washing the membrane four times with PBS-Tween, it was incubated with the secondary antibody (anti-mouse Ig horseradish peroxidase linked whole antibody; Amersham Biosciences, Buckinghamshire, UK) for 2 h at room temperature. The membrane was washed four times with PBS-Tween and then incubated with WesternLigthning Plus-ECL, Enhanced Luminol and oxidizing reagents Plus for 1 min before autoradiography.

**Results**

**Sequencing and analysis**

The whole coding sequence of the AMH gene could be analyzed for 50 POI patients. Five patients were excluded because some exons or part of them could not be completely sequenced.

Sixteen variants of the AMH gene were found in the heterozygous status, including six missense variants, of which two were very rare variants (G264R, D288E) and another one (R444H) had never been described. None of these three missense variants were found in any of the ethnically matched controls.

These three variants, on account of their rarity, were selected for further investigation (in silico and in vitro). The variants G264R and D288E have been found in a high-throughput sequencing database (Exome Sequencing Project, ESP). Their allele frequency is shown in Table I.

The in silico analysis of the missense variants predicted an alteration of the protein function for the variants G264R and R444H. The variant D288E was predicted to be benign and tolerated.

**Clinical characteristics of the patients with a missense variant of the AMH gene**

**G264R**

The patient with the heterozygous mutation G264R (amino acid change from glycine to arginine) had a secondary amenorrhea that started at 30 years of age. She comes from Sub-Saharan Africa and had a family case of severe sterility that was not further investigated (Table II).

**D288E**

The patient with the heterozygous mutation D288E (amino acid change from aspartic acid to arginine) presented with a secondary amenorrhea at the age of 26. She is Caucasian and her mother was also diagnosed with POI when she was 32. Analysis of her mother’s AMH gene showed that she is heterozygous for the same variant.

<table>
<thead>
<tr>
<th>Table I. Missense variants found in AMH gene of POI patients.</th>
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<td>Missense variant</td>
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<tr>
<td>S481I &gt;G</td>
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<tr>
<td>G264R &gt;C</td>
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<tr>
<td>D288E &gt;C</td>
</tr>
<tr>
<td>Q325R &gt;G</td>
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<tr>
<td>R444H &gt;A</td>
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<td>A515V &gt;G</td>
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*Allele count from ESP (Exome Sequencing Project) database, except for the variant Q325R, from 1 000 Genomes Project, via Ensembl database (http://www.ensembl.org/index.html).
The patient with the heterozygous mutation R444H (amino acid change from arginine to histidine) presented with secondary amenorrhea after stopping the birth control pill at 26 years old. She is Caucasian and had a family case of induction of puberty (no more information was available).

Intracellular biosynthesis and secretion of the AMH variants

The recombinant human wt AMH and the other three variants were obtained from the culture medium from transfected HEK293T cells, as it is a secreted hormone. The concentration of wt AMH was about 150 ng/ml, which was ultracentrifugated with 50 kDa filters (Amicon Ultra) to reach a median concentration of 500 ng/ml. The concentration ranges were similar for both variants G264R and R444H and the wt, in contrast with the variant D288E that was never detected by this method. The ELISA used for the detection is a commercial kit under confidential conditions, therefore the information about the specific epitopes was not available for the investigators. No AMH protein was found in the control medium or in the medium of the cells transfected with the empty vector.

A western blot was performed in order to discriminate a real absence of the protein, an absence of secretion or the production of a defective protein. The variants G264R and R444H showed, as the wt AMH, a 110 kDa band corresponding to the dimerized form of the protein, a 70 kDa band corresponding to the full length N-terminal pro-region and a 12 kDa band corresponding to the C-terminal mature domain. Meanwhile, the variant D288E was weakly detected as a dimerized protein, and no bands were visible at 70 or 12 kDa or at other sizes (Fig. 1).

Bioactivity of the AMH variants

The recombinant human wt AMH stimulated the AMHR2 in a dose-dependent manner, which validates the production method and the functional system.

A western blot did not show any luciferase activity when stimulated by the variants G264R and R444H. The luciferase activity was strongly reduced when AMHR2 was stimulated with the variant D288E, but low residual activity could still be measured in the best experiments (Fig. 2).
Discussion

We report in this article three missense variants of the AMH gene found in three different patients of a cohort of POI patients. The mother of one of the three patients had also undergone POI and appeared to be carrier of the same mutation as her daughter. We analyzed the three AMH variant proteins to determine how they impact the secretion and function of the protein and if they could be implicated in the development of the ovarian failure.

None of the controls sequenced for these three variants were carriers. Furthermore, high-throughput sequencing databases found that the variants G264R and D288E are extremely rare in a general population. The G264R was found in 1 allele among 1,895 American-African subjects, and the D288E was found in 5 alleles among 4,050 European-American subjects. The variant R444H had never been described. The extremely rare frequency of the variants G264R, D288E and R444H in the general population (see Table I for details) and the software prediction of a possible influence of these variants on the protein function drove us to continue with an in vitro investigation to elucidate their possible implication in the development of POI.

The ELISA kit AMH GenII used on the medium culture of cells transfected with the different variants allowed detection at the same level as the wt form for the variants G264R and R444H, confirming their normal production and secretion. The western blot of the culture medium confirmed the secretion of the two domains with a similar intensity as the wt AMH. The two variants G264R and R444H did not show any activity when tested with the luciferase reporter system, despite the fact that their production, secretion and concentration appeared to be normal. Accordingly, we can consider that when any of the variants G264R and R444H occurs, the function of the protein appears to be, at least, reduced when compared with the same concentration level of wt AMH, or that much higher amounts than the wt form are needed to produce any activation of the receptor.

In contrast, the variant D288E was not detected by the ELISA assay. The western blot showed a weak secretion of this variant. Nevertheless, this was the only variant that slightly activated the AMHR2 in the best experiments. The problem with the detection of the variant protein in the ELISA assay could be explained by the specificity of the antibodies, which are confidential information of the commercial company (Beckman Coulter), with the mutation interfering at the epitope recognition.
However, as the western blot showed only a weak secretion of the variant, it is also possible that the secretion of the variant protein is diminished but the activity is conserved or even slightly increased. That would explain the weak remaining activity and would be in accordance with the *in silico* prediction as a tolerated variant. This variant was found in the heterozygous state in the mother of the patient, who also had POI at 32 years old. This familial segregation supports a relationship between the variant and the development of the disease.

The three patients with a rare or novel AMH variant presented secondary amenorrhea at the age of 30, 26 and after stopping birth control pill at 26 years old, respectively. This is in accordance with the role of AMH in the ovary, as this hormone does not act as a blocker of the follicle development but as an inhibitor of the follicular depletion (Durlinger et al., 1999; Visser et al., 2007). Thereafter the expected clinical presentation is compatible with a secondary amenorrhea: the ovaries can accomplish their function but during a shorter period of time, even if the number of primordial follicles was normal at birth.

Similar to other members of the TGF-β superfamily growth factors, AMH needs dimerization to accomplish its function. The dimeric precursor peptide is cleaved in a pro-region of 70 kDa and a much smaller bioactive domain of 25 kDa in the C-terminal region. After cleavage, the pro-region remains non-covalently associated with its active domain (Pepinsky et al., 1988; Wilson et al., 1993). AMH signals through a transmembrane serine/threonine kinase receptor complex of type I and II components that activate the cascade of the Smads 1, 5 and 8 which, in turn, will activate other transcription factors to regulate the responsive genes (Josso and Clemente, 2003). The dimerization process could explain the deleterious effect of a variant in a heterozygous patient. Theoretically, if the secretion of the two alleles is comparable and the dimerization occurs equivalently, only 25% of the dimers will be formed by two normal proteins, and therefore the function of the protein could be altered by the non/less functional dimers. If the secretion is altered for one of the alleles, the activity could be diminished because of a reduced concentration. Supporting the clinical implication of a heterozygous mutation of the AMH, female mice heterozygous for AMH null mutation, had a faster depletion of the primordial follicle pool when compared with the wt mice, with numbers of follicles per class falling in the heterozygous state, which could also contribute to a diminution of the bioactivity of the protein. Nevertheless, the sequence used for the functional study in our experience contains the Serine in position 49 of the gene in a homozygous status for both the wt AMH and the variants. Therefore, we can exclude a contribution of the Ser49Ile variant to the diminished bioactivity of the tested variants (D264R and R444H) *in vitro*.

Several mutations in AMH have been described in males with Persistent Mullerian Duct Syndrome (PMDS) (for a review, see Josso et al., 2012). They are inherited in an autosomal recessive manner, and the carriers may be homozygous or compound heterozygous for the different types of mutations (deletions, insertions, nonsense or missense). Most of them have an altered secretion or produce truncated proteins, but no functional studies testing them have been published. Furthermore, a potential female phenotype has not been described in their families; nothing is described about the age at the menopause of the mothers or sisters of males having a PMDS with a mutation in the AMH gene inherited from their mother.

In this study, three AMH missense variants, of which one has never previously described, were identified among POI patients. Two out of three variants (G264R and R444H) showed a loss of function when functionally tested *in vitro*, while the third one (D288E) showed a reduced secretion or a decreased.

In summary, our study reports unknown or very rare AMH variants in POI patients. When functionally tested *in vitro*, AMH variants showed a drastic reduction of protein activity. Furthermore, the mother of one of the patients, who also developed POI at age 32, appeared to be carrier of the same variant as her daughter, strongly supporting its implication in the development of POI.

The studied AMH variants are very rare in the general population. This could be explained by the lower chances of reproduction of the carriers due to a reduction in their fertility by many years. In our cohort, eight patients had a family history of reproductive troubles (Table II). It is worth noting that the three missense variants of the AMH gene were identified in this specific subgroup, suggesting that AMH mutations could be implicated in a non-negligible proportion of POI patients with a family history of ovarian insufficiency. Searching for AMH mutations in larger cohorts of POI patients, particularly with a family history, would permit us to draw firmer conclusions about the implication of the AMH gene in the pathogenesis of POI.

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### Authors’ roles


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### Conflict of interest

The authors declare no conflicts of interest.