Anti-Müllerian hormone as a predictor of follicular reserve in ovarian insufficiency: special emphasis on FSH-resistant ovaries

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BACKGROUND: Anti-Müllerian hormone (AMH) is secreted by ovarian granulosa cells and its serum levels reflect ovarian follicle reserve. The main objective of this study was to test the use of AMH assay in identifying women with primary amenorrhea (PA) and existing follicles and to study follicle phase dependent AMH secretion.

METHODS: Serum levels of AMH were measured in subjects with FSH-resistant ovaries (FSHRO, n = 12), primary ovarian insufficiency (POI) with PA (n = 11) or secondary amenorrhea (SA n = 20) of unknown etiology, and controls (n = 23), and in Turner syndrome (TS) [45,X (n = 18), mosaicism (n = 7), structural X chromosome abnormalities (SCA, n = 10)], and healthy controls (n = 34).

RESULTS: Serum levels of AMH in women with FSHRO were comparable with those in control women (2.76 ± 2.37 versus 3.77 ± 2.36 ng/ml) and significantly higher than in women with PA (0.05 ± 0.04 ng/ml; P < 0.001) or SA of unknown origin (0.12 ± 0.20 ng/ml; P < 0.001). TS girls/women with 45,X or SCA had low serum AMH levels (0.13 ± 0.09 and 0.27 ± 0.19 ng/ml) compared with their controls (3.34 ± 2.23 ng/ml) or subjects with mosaicism (2.33 ± 2.81 ng/ml). AMH expression was detected in granulosa cells of women with FSHRO but not in any of the 45,X fetal ovarian specimens.

CONCLUSIONS: A serum AMH assay could be used to identify patients with decreasing ovarian reserves and POI. Moreover, our results support the notion that AMH is secreted mainly by small non-selected follicles, since follicular granulosa cells were AMH-positive and serum AMH levels were normal/low normal in women with FSHRO, who lack follicle development beyond the small antral stage.

Key words: AMH / ovarian reserve / FSH receptor mutation / turner syndrome / ovarian insufficiency

Introduction

Ovarian insufficiency is a heterogeneous syndrome causing primary amenorrhea (PA) and abnormal pubertal development or secondary amenorrhea (SA) before the age of 40 years as a result of follicle depletion (Nelson, 2009; Rebar, 2009). It may result from several different underlying causes, including sex chromosome abnormalities, gene mutations and autoimmune diseases, or it can be induced by chemotherapeutic agents or irradiation (Nelson, 2009; Persani et al., 2009; Rebar, 2009). However, in most women with a normal (46,XX) karyotype, the etiology is unknown. As a result of a diminished or absent ovarian response, serum concentrations of gonadotrophins are increased to menopausal levels.

Most women with Turner syndrome (TS) exhibit primary ovarian insufficiency (POI) with streak gonads and infertility (Gravholt, 2004). In TS, germ cell loss may start as early as during fetal life, although the exact timing of the process is unclear and may vary (Speed, 1986; Singh and Carr, 1966; Reynaud et al., 2004). Some girls with TS have follicles in adolescence (Hreinsson et al., 2002; Borgström et al., 2009), and even spontaneous pregnancies have been reported, mainly in women with sex chromosome mosaicism (Hovatta, 1999).
We have previously identified a recessively inherited inactivating mutation (a missense mutation c.566C>T causing an Ala189Val substitution) in the FSH receptor gene (FSHR), which results in a dramatic reduction in signal transduction and FSH-resistant ovaries (FSHRO). As a consequence, the women concerned have PA and infertility (Aittomäki et al., 1995). In contrast to most other forms of POI, women with FSHRO have early growing follicles, but their development is arrested at the small antral stage (Aittomäki et al., 1996).

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance (MIS), is a member of the transforming growth factor-β family. It is mainly produced by granulosa cells of growing ovarian follicles, from primary up to the small antral stage (Baarends et al., 1995; Weenen et al., 2004). In girls and women, serum AMH levels increase slightly from childhood to puberty, decreasing thereafter with age, and they become undetectable before menopause (Lee et al., 1996; de Vet et al., 2002; Piltonen et al., 2005). Several investigators have reported a positive correlation between serum AMH levels and antral follicle count, and therefore there is a consensus of opinion that levels of AMH reflect ovarian follicle reserve well (de Vet et al., 2002; Seifer et al., 2002; van Rooij et al., 2002; Fanchin et al., 2003). The results of previous studies in mice also suggest that AMH may have a significant role in early follicular development by inhibiting initial follicle recruitment and FSH-stimulated follicular growth (Durlinger et al., 1999, 2002), but results of human in vitro studies are inconsistent (Schmidt et al., 2005; Carlsson et al., 2006).

In earlier reports, serum AMH levels have been found to be low or undetectable in women with POI (La Marca et al., 2006; Meduri et al., 2007; Knauff et al., 2009) and to correlate with the number of AMH-positive follicles in immunohistochemical analyses (Meduri et al., 2007). However, women with functional hypothalamic amenorrhea and with normal follicular reserves have normal serum AMH levels as well as some women with POI due to steroidogenic autoimmunity, suggesting that AMH could be used clinically as a marker of ovarian reserve in women with amenorrhea (La Marca et al., 2006, 2009).

Our objective was to study serum AMH levels in patients with FSHRO, POI of unknown etiology with PA or SA, and TS and especially to identify women with PA and existing follicles. Furthermore, the unique patient material, particularly women with FSHRO, allowed us to explore in more detail phases of follicular development in which AMH is produced.

## Materials and Methods

### Subjects

Three groups of patients with ovarian insufficiency were recruited. Patients with FSHRO (n = 12) and POI with PA of unknown etiology (n = 11) were recruited at five University Hospitals and patients with POI with SA of unknown etiology (n = 20) at Helsinki University Central Hospital. All patients in these three groups had normal female chromosome constitution (46,XX), repeatedly high serum FSH concentrations of >40 IU/l and no known cause of hypergonadotropic ovarian insufficiency (e.g. autoimmune ovarian diseases) (Aittomäki et al., 1996). At the time of sampling, the ages of the patients with FSHRO varied from 22 to 43 years and those with PA from 18 to 42 years. All patients with SA had had amenorrhea between the ages of 16–35 years. At the time of sampling, their ages varied from 19 to 41 years. Twenty-three healthy women with regular menstrual cycles (aged 16–44 years) served as controls (Piltonen et al., 2003; Puurunen et al., 2009). The samples were frozen at −80°C until AMH analysis.

Thirty-five girls/women with TS (aged 6–43 years) participated in the study and were divided in three groups according to the karyotype: 45,X (n = 18), mosaicism (n = 7) and structural X chromosome abnormalities (SCA, n = 10; Table I). Thirty-four healthy girls or subjects with regular menstrual cycles (aged 7–44 years) served as controls (Piltonen et al., 2003; Puurunen et al., 2009; Siljander et al., 2009). Twenty-five serum samples from girls and women with TS were obtained by contacting members of the Finnish TS Association and ten samples from girls with TS were collected during clinical follow-up visits to specialist care units. Informed consent was obtained from the participants or their parents. Among the women with menstrual cycles, the samples were taken on cycle Days 1–7. The serum samples were kept at −80°C until analyses were carried out. One of the subjects with 45,X/46,XX had two spontaneous pregnancies.

### Tissue samples

Fetal and adult ovarian tissue samples were collected from the files of the Department of Pathology, Oulu University Hospital. Ovaries from 7 fetuses (gestational age 13–22 weeks) with 45,X karyotype were studied. Of these, six fetal ovaries were obtained after therapeutic abortions and one fetal ovarian sample after autopsy because of intrauterine death. Samples did not contain any detectable autolysis. In addition, one adult ovarian tissue sample as a control was obtained from a 37-year-old woman with normal karyotype undergoing ovariectomy because of endometriosis. Furthermore, there were two ovarian biopsies samples available from patients with FSHRO (Aittomäki et al., 1996). The samples had been taken for clinical purposes at the time of diagnosis of ovarian insufficiency. Human fetal testis tissue was used as a positive control as it is known to express AMH (Tran et al., 1987; Rajpert-De Meyts et al., 1999).

All samples were fixed in 10% phosphate-buffered neutral formalin for 24 h and embedded in paraffin. Histological sections (4 μm) were cut and processed for immunohistochemistry. The study was approved by the Ethics Committees of Oulu University Hospital. A permit to study human autopsy tissues and resection material was obtained from the Finnish National Authority for Medicolegal Affairs.

### Table I Clinical data of subjects with TS.

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>n</th>
<th>Age (year, mean, range)</th>
<th>Spontaneous menarche</th>
<th>HRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>45,X</td>
<td>18</td>
<td>16 (6–43)</td>
<td>0/18 (0%)</td>
<td>10/18 (56%)</td>
</tr>
<tr>
<td>Mosaicism</td>
<td>7</td>
<td>24 (11–35)</td>
<td>5/7 (71%)</td>
<td>2/7 (29%)</td>
</tr>
<tr>
<td>SCA</td>
<td>10</td>
<td>24 (12–42)</td>
<td>3/10 (30%)</td>
<td>6/10 (60%)</td>
</tr>
</tbody>
</table>

SCA, Structural X chromosome abnormalities; HRT, hormone replacement therapy.
**Immunohistochemistry**

Tissue sections were deparaffinized in xylene and rehydrated gradually through graded alcohols and then washed in water. The tissues were pretreated in 10 mM sodium citrate in a microwave oven (800 W for 2 min and 300 W for 15 min). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Tissues were incubated overnight with primary goat anti-human MIS/AMH propeptide antibody (AF2748, R&D Systems, MN, USA), working concentration 1:50 in phosphate-buffered saline (PBS). For negative controls, PBS was applied instead of the primary antibody. No blocking peptide was available for the antibody. Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA, USA) were used to visualize the bound antibody and commercial diaminobenzidine tetrahydrochloride (DAB, DakoCytomation Ltd., Ely, UK) was used as a chromogen at a concentration of 15 μl/ml. Samples were counterstained with hematoxylin, dehydrated through graded alcohols and cleared in xylene.

**ELISAs**

Serum AMH concentrations were determined by using two-site AMH enzyme immunoassay kits (Kevenaar et al., 2006), following the instructions of the manufacturer (Diagnostic Systems Laboratories, Webster, TX, USA). The range of the AMH standards used in this assay was 0.05–15 ng/ml. Intra- and inter-assay coefficients of variation were 4.6 and 8.0%. To convert the results into SI units the following conversion factor should be used: AMH 1 ng/ml = 7.14 pmol/l.

**Microscopy and statistics**

All tissue samples were analyzed by two independent observers using light microscopy. Data were analyzed by means of the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). To test normality of AMH values, the Shapiro–Wilk test was used. The distributions of AMH levels were close to normal, only in two groups (45,X and SA) the distribution was skewed. Parametric independent samples t-test with more statistical power was performed and the results were confirmed with non-parametric test (Mann–Whitney test). The cut-off value for FSHRO was calculated using an ROC-curve. The results are presented as mean ± SD. The limit of statistical significance was set at \( P < 0.05 \).

**Results**

**AMH expression and serum AMH levels in women with FSHRO**

Mean serum level of AMH in women with FSHRO was slightly lower (2.76 ± 2.37 ng/ml) than in control subjects (3.77 ± 2.36 ng/ml), but the difference was not statistically significant. In women aged over 45, serum AMH levels were low in both groups (0.12 ± 0.96 and 0.34 ± 0.24 ng/ml). In subjects with PA (0.05 ± 0.04 ng/ml) or SA (0.12 ± 0.20 ng/ml) serum AMH levels were low or undetectable (Fig. 1A). Individual serum AMH levels in women with FSHRO are shown in Table II.

In the two FSHRO ovarian biopsy samples, AMH expression was detected in granulosa cells of primary follicles and in some primordial and transitional follicles. Most of the primordial follicles were AMH-negative (Fig. 2A). No secondary or more advanced follicles were found in the ovarian biopsy samples studied. The control adult ovarian tissue sample showed intensive staining in granulosa cells of primary and more developed follicles, and as in FSHRO, in some primordial as well as in transitional follicles. No immunostaining was observed in stromal tissue (Fig. 2B). Human fetal testis was used as a positive control and showed specific staining in Sertoli cells (Fig. 2C). AMH expression was not observed in an adult ovarian tissue sample in which PBS was used instead of primary antibody (Fig. 2D).

**AMH expression in 45,X fetal ovaries and serum AMH levels in girls/women with TS**

In girls/women with TS and mosaicism, serum AMH levels showed wide individual variation (Table II). One subject, with the highest serum AMH level, had two spontaneous pregnancies.
Subjects with SCA had lower serum AMH levels (0.27 ± 0.19 ng/ml) than the control group (3.34 ± 2.23 ng/ml) or subjects with mosaicism (2.33 ± 2.81 ng/ml; Table II). All women/girls with 45,X karyotype had low serum AMH levels (0.13 ± 0.09 ng/ml) and no spontaneous menarche (Fig. 1B, Table II). Regardless of karyotype, women aged over 45 years had low serum AMH levels, similarly to control women at the same age.

Immunostaining for AMH was not detected in any of the seven 45,X fetal ovarian specimens (gestational age 13–22 weeks) (data not shown).

**Discussion**

The present results demonstrate that assay of serum AMH can be used as a clinical tool to identify patients with PA and existing follicles. We report here, for the first time, that serum AMH levels in women with FSHRO mutation causing FSHRO were normal or low normal. Moreover, in most of the subjects with FSHRO, serum AMH levels were higher than in those with other types of ovarian insufficiency studied, including TS and POI with PA or SA of unknown etiology. Furthermore, these observations in women support and strengthen the hypothesis based mainly on animal studies that AMH is secreted by small growing follicles.

Expression of AMH in biopsy samples from women with FSHRO was detected in granulosa cells of primary follicles as observed previously in the normal ovarian tissue (Baarends et al., 1995; Weenen et al., 2004). In addition, granulosa cells in some primordial and transitional follicles expressed AMH, although most of primordial follicles were AMH-negative. The same expression pattern was detected in adult ovarian control samples as observed earlier (Stubb et al., 2005). The expression in primordial/transitional stage suggests that AMH expression, at least to some extent, precedes or coincides with the initiation of follicle recruitment.

An interesting finding was that serum AMH levels in women with FSHRO were measurable and significantly higher in comparison with levels in other types of ovarian insufficiency. The cut-off value to distinguish serum AMH concentrations between women with FSHRO and POI of unknown etiology was 0.64 ng/ml with a 91% sensitivity and 97% specificity for women with FSHRO. The mean AMH level in women with FSHRO was ≏ 25% lower than in the control group with normal ovarian function. This tendency may be explained by the higher number of follicles in the growing phase and consequently a higher number of granulosa cells in control women. It is supported by the finding that only half of the women with FSHRO had visible follicles in transvaginal ultrasonography (Aittomäki et al., 1996). The smaller volume of the ovaries in women with FSHRO most probably reflects a lower number of follicles beyond the antral stage and not decreased total follicle count. Hence, it is

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<th>Age (year)</th>
<th>AMH (ng/ml)</th>
<th>Age (year)</th>
<th>AMH (ng/ml)</th>
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<th>AMH (ng/ml)</th>
<th>Age (year)</th>
<th>AMH (ng/ml)</th>
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SCA, Structural X chromosome abnormalities.

*Prepubertal.

*Karyotype 45,X/46,X,r(Xp).

*Lower than the lowest standard.
possible that the lower levels of AMH in women with FSHR mutation are not necessarily a result of decreased follicle reserve, but a different phase of follicular development.

The concept that AMH is secreted mainly by small growing follicles is supported by the present observations. Follicular development in FSHR-O is arrested at the pre-antral/small antral stage, and despite that these women had normal/low normal AMH levels compared with healthy women. As the blood samples from the women with FSHR-O were collected for our earlier study (Aittomäki et al., 1996), it is possible that the long period of preservation of the samples may have had an effect on the apparent serum levels of AMH. However, the results of previous studies indicate that AMH is relatively stable and long-term storage does not significantly influence AMH levels (de Vet et al., 2002).

The observation that serum AMH levels in women with POI of unknown origin were low or undetectable strengthens the findings of previous studies (La Marca et al., 2006; Meduri et al., 2007; Knauff et al., 2009). No women with POI due to steroidogenic cell autoimmunity were investigated in the present study, but they have been reported to have measureable or normal serum AMH concentrations indicating a preserved follicle pool in these women (La Marca et al., 2009). The results of a recent reports suggest that AMH may be a good predictor of POI patients with remaining follicles but with symptoms preceding POI diagnosis (elevated FSH levels with regular menstruation or oligomenorrhea) (Knauff et al., 2009) or in women treated for cancer in childhood (Bath et al., 2003; van Beek et al., 2007). Our patients with SA were diagnosed at least 1 year before blood sampling (range 1–20 years, mean 8.2 years) and the AMH levels were already low. This means that AMH measurement could probably be used to predict diminishing ovarian reserve as early as years before actual ovarian insufficiency, as it would be reflected by low AMH levels. The time interval, however, cannot be determined on the basis of the present results. The results of one earlier longitudinal study suggest that serum AMH levels become very low or undetectable in the majority of women ~5 years before the final menstrual period, but the exact time of menopause is difficult to predict (Sowers et al., 2008).

In cases of chromosomal mosaicism in women with TS, serum AMH levels were significantly higher than in those with the 45,X karyotype or structural abnormalities in one of the X chromosomes, supporting the findings of a recent study (Hagen et al., 2010). In these two latter groups, serum AMH levels were low. The measurable concentrations in some of the women can be explained by the fact that follicles have been found in ovarian biopsy samples in girls with TS, regardless of karyotype (Hreinsson et al., 2002; Borgström et al., 2009). Nearly all TS girls with mosaicism have been shown to have

**Figure 2.** AMH expression in the ovary. Scale bar 50 μm. (A) In FSHR-O ovarian samples positive staining was detected in primary follicles and some primordial follicles. (B) A similar expression pattern was observed in the adult ovarian control sample, including positive staining in secondary follicles. (C) Fetal testis tissue as a positive control showed staining in Sertoli cells. (D) In ovarian sections used as negative controls no immunostaining was detected.
follies compared with girls with 45,X, of whom ~10% had follicles in ovarian biopsy samples, and normal serum FSH and AMH levels were more common in girls with follicles (Borgström et al., 2009). Moreover, TS women with mosaicism are more likely to show spontaneous menarche and fertility (Sybert and McCauley, 2004). In the present study, it was not possible to obtain ovarian biopsy material for research purposes only. We found great individual variation in serum AMH levels in women with mosaicism and most of them with spontaneous menarche had AMH levels close to normal.

Data concerning AMH expression in human fetal ovaries are to some extent controversial since the expression of AMH mRNA in normal 46,XX fetal ovaries has been shown as early as at the 13th week (Modi et al., 2006), but another study has demonstrated immunostaining of AMH only during the third trimester (Rajpert-De Meyts et al., 1999). No significant AMH expression was detected in samples of fetal 45,X ovaries at 13–22 weeks of gestation. This was in line with the results of our recent study showing AMH expression in normal fetal ovary from midgestation onwards (Kuiri-Hänninen et al., 2011).

The ovarian tissue and serum samples from women with FSRHO provided a unique possibility to study the origin of AMH secretion as these women have follicles mainly up to the small antral stage demonstrated earlier by ovarian biopsy samples and ultrasonography (Aittomäki et al., 1996). The normal serum levels of AMH in these women support the concept that AMH is secreted from small growing follicles. Furthermore, the present results show that the measurement of serum AMH helps, especially in POI with PA, to identify subjects with existing follicles.

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Authors’ roles

S.K., K.A., T.E.V. L.D. and J.S.T. designed the study. S.K., K.A., R.V., T.P., A.L. and J.S.T. contributed to data collection. S.K., T.E.V. and J.S.T. executed the study and analysed the results. S.K. wrote the first draft of the paper. All authors participated to critical discussion and revising the manuscript. All authors approved the final version of the manuscript. J.S.T. is chairman of the publication subcommittee and Chairman-Elect of ESHRE.

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Conflict of interest

None declared.

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