Sanna Koskela

GRANULOSA CELL ANTI-MÜLLERIAN HORMONE SECRETION IN OVARIAN DEVELOPMENT AND DISEASE
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Abstract

Anti-Müllerian hormone (AMH) was identified originally in connection with its role in male sexual differentiation. In females, AMH is secreted by ovarian granulosa cells of growing follicles and its serum levels correlate well with the remaining number of follicles, thus reflecting ovarian reserve. The aim of this study was to explore the expression and secretion of AMH in human ovarian development and in various disorders resulting in decreased reproductive function.

In fetal ovaries, AMH expression was found to be initiated at midgestation and it increased gradually towards term. In serum samples from infant girls, transient postnatal activation of the pituitary-ovarian axis was found and it occurred later in premature infants, reflected in lower serum AMH levels and lower numbers of growing follicles. This immaturity resulted in insufficient feedback from ovary to pituitary and may explain the higher levels of follicle-stimulating hormone (FSH) in these girls.

Ovarian follicle reserve is typically diminished in women with primary ovarian insufficiency (POI). Assay of serum AMH may be used to identify women with POI and existing follicles, as women with FSH receptor mutation who were known to have follicles had serum AMH levels in low normal range and girls/women with Turner syndrome mosaicism had higher serum AMH levels compared with those with other karyotypes.

The diagnostics and follow-up of ovarian granulosa cell tumors (GCTs) are challenging as a result of the rarity of the disease and late possible recurrences. Assay of serum AMH combined with assay of the currently used marker inhibin B was shown to improve follow-up of GCTs in individual patients, compared with serum inhibin B measurement alone. The analyses revealed that AMH and inhibin B assays perform similarly in detecting macroscopic tumors.

Continuous use of combined hormonal contraceptives inhibited ovarian activity independently of the administration route, as serum AMH levels decreased significantly and similarly during oral, transdermal or vaginal administration. The decrease of serum AMH levels indicates that AMH is secreted partially from FSH-dependent follicles.

The study provides novel information on AMH secretion in ovarian development, and the use of AMH assay in assessing ovarian reserve and detecting ovarian disorders such as ovarian insufficiency and GCTs.

Keywords: anti-Müllerian hormone, combined contraceptives, fetal development, follicle, FSH, FSH receptor, granulosa cell tumor, infancy, ovary, primary ovarian insufficiency
Koskela, Sanna, Anti-Müllerian hormoni munasarjan toiminnan merkkiaineena munasarjan kehityksessä ja sairauksissa.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Synnytys ja naistentaudit; Kliinisen tutkimuksen keskus; Oulun yliopistollinen sairaala; Valtakunnallinen kliininen tutkijakoulu
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Naisen munasarjassa munarakkuloiden granuloosasolot erittävät anti-Müllerian hormonia (AMH), ja sen pitoisuudet seerumissa heijastavat jäljellä olevien munarakkuloiden määrää. Tässä tutkimuksessa tarkasteltiin AMH:n eritystä ja ilmentymistä munasarjan kehityksen aikana ja erilaisissa munasarjan toiminnan häiriötiloissa.


Munasarjan granuloosasolukasvaimen diagnostiikka ja seuranta ovat haastavia kasvaimen myöhäisen uusituutensaikaun ja harvinaisuuden vuoksi. Tulokset osoittivat, että seerumin AMH-määrityksen yhdistäminen tällä hetkellä käytössä olevaan inhibiinii B -määritykseen voisi parantaa yksittäisten potilaiden seurantaa makroskooppihin kasvaimen toteamisen osalta.

Tutkimus osoitti hormonaalisten yhdistelmäkehäksyvalmistieden jatkuvan käytön vähentävän munasarjan aktiivisuutta merkittävästi annostelureitteistä riippumatta, koska seerumin AMH-pitoisuudet laskivat samalla tavalla ehkäisypilliä, -rengasta ja -laastaria käytävällä naisilla. Tutkimus toi uutta tietoa AMH:n erityksestä munasarjan kehityksen aikana sekä AMH-määrityksen käytön mahdollisuuksista munarakkuloiden määrän arvioinnissa ja eri häiriötilojen tunnistamisessa.

Asiakirjat: anti-Müllerian hormoni, ennenäikainen munasarjan toiminnan hiipuminen, FSH, FSH-reseptori, granuloosasolukasvaimen, munarakkula, munasarja, sikiön kehitys, varhaislapsuus, yhdistelmäkehäsy
In loving memory of my Mother
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Oulu, October 2013

Sanna Koskela
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFC</td>
<td>Antral follicle count</td>
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<tr>
<td>AMH</td>
<td>Anti-Müllerian hormone</td>
</tr>
<tr>
<td>AMHRII</td>
<td>Anti-Müllerian hormone receptor II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CHC</td>
<td>Combined hormonal contraceptive</td>
</tr>
<tr>
<td>CLMA</td>
<td>Chemiluminometric assay</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol</td>
</tr>
<tr>
<td>EE</td>
<td>Ethinyl estradiol</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>FSHR</td>
<td>Follicle-stimulating hormone receptor</td>
</tr>
<tr>
<td>FSHRO</td>
<td>Follicle-stimulating hormone-resistant ovaries</td>
</tr>
<tr>
<td>GA</td>
<td>Gestational age</td>
</tr>
<tr>
<td>GC</td>
<td>Granulosa cell</td>
</tr>
<tr>
<td>GCT</td>
<td>Granulosa cell tumor</td>
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<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MIS</td>
<td>Müllerian inhibitory substance</td>
</tr>
<tr>
<td>PA</td>
<td>Primary amenorrhea</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>POI</td>
<td>Primary ovarian insufficiency</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operator characteristic</td>
</tr>
<tr>
<td>SA</td>
<td>Secondary amenorrhea</td>
</tr>
<tr>
<td>SCA</td>
<td>Structural X chromosome abnormalities</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TR-IFA</td>
<td>Time-resolved immunofluorometric assay</td>
</tr>
<tr>
<td>TS</td>
<td>Turner syndrome</td>
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</tbody>
</table>
List of original publications

The thesis is based on the following articles, which are referred in the text by their Roman numerals:


*= Koskela S née Kallio S
**=equal contribution
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1 Introduction

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β (TGF-β) family (Knight & Glister 2006). It was originally identified in connection with its role in male sexual differentiation, as it causes regression of the Müllerian ducts, which form the major part of the female reproductive system (Josso et al. 1998). However, little is still known about AMH secretion in females during the fetal period and infancy.

Recently, the role of AMH in ovarian follicular development and follicular reserve has attracted great clinical interest and assay of AMH has already been utilized in several different clinical applications, such as predicting the ovarian response in vitro fertilization (IVF) treatment (Broer et al. 2009). In several studies, AMH has been shown to be a good marker of ovarian reserve. Its serum levels decline with age and correlate well with the number of antral follicles and with primordial follicle number as well (de Vet et al. 2002, Hansen et al. 2011, van Rooij et al. 2002). The granulosa cells (GCs) of growing follicles up to small antral stage produce the most AMH and its secretion has been shown to gradually decrease after the follicle becomes dependent on follicle-stimulating hormone (FSH) at the small antral stage (Andersen et al. 2010, Jeppesen et al. 2013, Weenen et al. 2004). It has been suggested that AMH inhibits growth of the primordial follicle pool and reduces follicle sensitivity to FSH (Durlinger et al. 1999, Pellatt et al. 2011, Visser & Themmen 2005). However, this possible role of AMH in the ovary is based mainly on the results of studies in animals and is not well understood.

Normal reproductive aging shows great individual variation and early signs of diminishing ovarian reserve are clinically difficult to recognize (Broekmans et al. 2009). Approximately 1% of women suffer from primary ovarian insufficiency (POI), which is a disorder defined as at least 4 months of amenorrhea with two measurements of serum FSH levels in the menopausal range (>40 IU/l) and decreased serum estradiol (E₂) levels in women under 40 years of age (De Vos et al. 2010). Reasons for POI may be iatrogenic, genetic or autoimmune, for example, but in the majority of cases the etiology remains unknown (Nelson 2009, Welt 2008). Some women with POI have remaining ovarian function (Bidet et al. 2011, Nelson et al. 2005) and previous studies have shown that AMH measurement may be helpful in diagnostics (Knauff et al. 2009).

Serum AMH levels have been shown to be high in women with granulosa cell tumors (GCTs) (Gustafson et al. 1992, Lane et al. 1999, Long et al. 2000,
Petraglia *et al.* 1998, Rey *et al.* 1996). Adult form of GCT is a relatively rare ovarian tumor with low malignant potential (Jamieson & Fuller 2012). However, in cases of relapse, the mortality rate is high (Schumer & Cannistra 2003) and thus a sensitive and specific marker is needed to improve GCT diagnostics. Previous reports on the use of assay of AMH as a marker of GCT have been retrospective, with small sample sizes, and comprehensive studies on its usefulness are lacking.

Combined hormonal contraceptives (CHCs) are widely used and their effects are mainly based on suppression of the hypothalamic-pituitary-ovarian axis, which results in inhibition of follicular development and ovulation (van Heusden & Fauser 2002). The use of different routes of administration results in variability in circulating levels of ethinyl estradiol (EE) (van den Heuvel *et al.* 2005) and this may be reflected in follicular growth. The effect of continuous use of CHCs on serum AMH levels is not well known and there are no clear comparative data concerning different routes of administration and serum AMH levels.

The aim of this study was to investigate granulosa cell AMH secretion and its relationship with the pituitary-ovarian axis in infancy and to explore the ontogeny of AMH in fetal ovarian development. Furthermore, other goals were to evaluate the clinical usefulness of assay of serum AMH in follow-up of GCT and in the identification of women with POI and existing follicles, and to compare the effects of different combined hormonal contraceptives on ovarian function in healthy women.
2 Review of the literature

2.1 Ovarian follicular development

2.1.1 Development of follicular reserve

In humans, ovarian development is initiated during the fourth week of fetal development when the primordial germ cells begin to migrate from the yolk sac to the gonadal ridges of the undifferentiated gonad. Thereafter, the primordial germ cells differentiate into oogonia, and proliferate and transform into primary oocytes at 11–12 weeks of gestation (McGee & Hsueh 2000). After the 24th week of development most of the primary oocytes are enveloped by a single layer of pregranulosa cells, forming the primordial follicles (Sadler 2006). The maximal average population of 300 000 primordial follicles per ovary (with wide individual variation estimated to be from 35 000 to over 2.5 million follicles) is present at the 18th–20th week of gestation and thereafter the number of these cells starts to decrease (Wallace & Kelsey 2010).

At birth, the total number of oocytes will have declined to approximately 295 000 primordial follicles per ovary, with wide individual variation (35 000 to 2.5 million) (Baker 1963, Block 1953, Forabosco et al. 1991, Wallace & Kelsey 2010) and the depletion continues throughout childhood. At the onset of puberty a mean of 180 000 resting follicles remain in the ovary (Wallace & Kelsey 2010), and only around 400 will ovulate during the fertile period of life. Eventually, less than 1000 resting follicles remain when menopause occurs at about 51 years of age (Faddy et al. 1992, Gougeon et al. 1994). However, and in contrast, the results of some studies in animals have indicated that oocytes may also be derived from stem cells after birth and hence the ovarian reserve is not entirely formed during fetal life (Johnson et al. 2004, Johnson et al. 2005, White et al. 2012).

2.1.2 Early follicular development

After the formation of follicles, some of them start to grow as early as during fetal life and occasionally follicles up to antral stage can be observed (Block 1953, Polhemus 1953). However, most follicles remain at a resting stage and form a stock known as the primordial follicle pool, mainly made up of primordial
(oocytes surrounded by a layer of flattened GCs) and transitional follicles (oocytes surrounded by flattened and cuboidal GCs) (Gougeon 2003) (Figure 1).

When follicular growth continues from the transitional stage, GCs turn cuboidal and primary follicles are formed. Subsequently, the GCs proliferate and a secondary follicle is formed, with two or more complete layers of GCs surrounding the oocyte (Figure 1). The surrounding connective tissue differentiates into two parts: theca interna and theca externa. Prior to this stage, follicles lack an independent blood supply, but at this stage follicles become exposed directly to factors circulating in the blood (Gougeon 2003). When the oocyte is surrounded by six or more GC layers, the follicle is defined as a preantral follicle (Gougeon 1996).

The exact time period of follicular growth from resting to preantral stage is not known. It has been estimated that over 120 days are required for a secondary follicle to attain the preantral stage and an even longer period is needed for development of the preceding stages (Gougeon 1986).

![Fig. 1. The stages of follicular development. Modified from Gougeon 1986.](image)

The initial recruitment of resting follicles is a continuous process throughout life until menopause, when the follicular pool is depleted. The mechanism behind the initiation of growth is not fully understood, but some intra-ovarian factors, such as anti-Müllerian hormone (AMH), kit ligand, growth differentiation factor-9, leukemia inhibitory factor and bone morphogenetic proteins have been shown to play a role in this process (Knight & Gliister 2006, Skinner 2005). The growth of early developing follicles is considered to be independent of gonadotropins, as follicles may grow to the preantral/early antral stage without functional FSH.
receptors or with minimal circulating FSH levels (e.g. prepuberty) (Aittomäki et al. 1996, Kumar et al. 1997, Lintern-Moore et al. 1974, McGee & Hsueh 2000). Furthermore, studies on hypophysectomized rodents (Halpin et al. 1986, Wang & Greenwald 1993) and a case study of women with hypogonadotropic hypogonadism (Barnes et al. 2002) have shown that follicular growth is impaired but not abolished, and exogenous administration of FSH has been shown to restore follicular growth (Halpin & Charlton 1988, Schoot et al. 1994). Thus, preantral follicles may be responsive to but not entirely dependent on FSH.

2.1.3 Cyclic recruitment of follicles and ovulation

When preantral follicles are filled with small fluid cavities, they become antral follicles (Figure 1) (Gougeon 2003). After the onset of puberty the increase in cyclic FSH secretion rescues a cohort of small antral follicles from atresia, allowing them to continue growth (McGee & Hsueh 2000). These follicles (2–5 mm in diameter) become dependent on the cyclic changes of circulating FSH, but the levels of E2 in follicular fluid are low. Due to atrophy of the corpus luteum in the late luteal phase of the previous menstrual cycle, the serum levels of E2 decrease, which results in diminished negative feedback to the pituitary and subsequently an increase in FSH levels (le Nestour et al. 1993). To induce follicle growth, circulating FSH concentrations need to surpass a certain level known as the FSH threshold (Brown 1978). It has been shown that the number of follicles recruited to continue growth is dependent more on the time period in which the FSH levels are above the threshold, rather than the level of FSH (Schipper et al. 1998). This critical time period is known as the FSH window (Fauser & Van Heusden 1997). The sensitivity of follicles to FSH is dependent on follicular stage and intra-ovarian factors (e.g. AMH, insulin-like growth factors, activin), which may increase or reduce FSH sensitivity locally (Mason et al. 1993, Miro & Hillier 1992, Visser & Themmen 2005).

FSH increases follicle proliferation and stimulates aromatase activity in GCs of larger (>5 mm) antral follicles, which induces the conversion of androstenedione, derived from theca cells, into E2 (Baerwald et al. 2012, Hillier et al. 1980). According to this two-cell, two-gonadotropin theory, both LH stimulation in theca cells and FSH stimulation in GCs are required for E2 production. Estradiol stimulates the mitotic activity of GCs and enhances the proliferative effect of FSH in the follicles. One of the preovulatory follicles grows faster than the rest of the rescued cohort and it produces the highest levels of
estrogens (Fauser & Van Heusden 1997, Gougeon 1996). This dominant follicle requires less FSH to continue development, i.e. its FSH sensitivity is the highest (McNatty et al. 1983). The increase of E2 and inhibin B production from the GCs of this largest preovulatory follicle in the mid-follicular phase of the menstrual cycle leads to negative feedback at the pituitary and circulating FSH levels decline (Gougeon 1996). The rest of the large non-ovulatory follicles become atretic (via apoptosis) as a result of insufficient FSH stimulation (Macklon & Fauser 2001). The dominant follicle is well vascularized and despite less circulating FSH, its effect in the dominant follicle may be sufficient to enable growth (Fauser & Van Heusden 1997). Furthermore, LH and its receptors may play a role in this survival process as well (Gougeon 1996, Yong et al. 1992).

During the late follicular phase of the menstrual cycle GCs produce high levels of E2, which results in positive feedback at the hypothalamus and consequently LH and FSH levels increase sharply. This midcycle gonadotropin surge induces ovulation and capillaries from the theca cells invade the GC layers (Gougeon 2003). GCs and theca cells form a corpus luteum, which is regulated by LH and the production of progesterone strongly increases (Gougeon 1996). Regression of the corpus luteum in the late luteal phase enables follicle maturation for the next menstrual cycle.

2.1.4 Modulation of follicular development: combined hormonal contraceptives

Combined hormonal contraceptives (CHCs) are widely used (Skouby 2004) and generally have two components: an estrogen (mainly ethinyl estradiol, EE) and a progestin (e.g. desogestrel, norelgestromin, etonogestrel or levonorgestrel).

The effect of CHCs is based primarily on suppression of the hypothalamic-pituitary-ovarian axis. The negative feedback caused by the estrogen component of a CHC decreases the secretion of gonadotropins and consequently inhibits follicular development in the ovary (Cohen & Katz 1979, Mishell et al. 1977). In addition, CHCs inhibit the LH surge and thus prevent ovulation, diminish permeability of cervical mucus, decrease endometrial receptivity for implantation and change uterine and tubal motility, impairing gamete transport (van Heusden & Fauser 2002). The 21/7 administration regimen (21 days of CHC use and a 7-day hormone-free interval) is still the most common (Sulak 2008). However, during the hormone-free interval the pituitary-ovarian axis resumes its activity and this results in transient follicular development, even up to the preovulatory
stage, with increases of serum E₂, inhibin B, LH and FSH levels (van der Spuy et al. 1990, van Heusden & Fauser 1999, Vandever et al. 2008, Willis et al. 2006). When CHC intake is again initiated, follicular development is diminished and no ovulation occurs.

CHCs may be administered via oral (pill), transdermal (patch) or vaginal (ring) routes and this causes fluctuation and variability in circulating levels of steroids (Bitzer & Simon 2011). Overall exposure to EE seems to be lowest among women using vaginal rings compared with pills and patches (van den Heuvel et al. 2005), but whether or not this results in differences in follicular growth is not well known.

2.2 Activation of the pituitary-ovarian axis after birth

During fetal life serum gonadotropin levels increase transiently at midgestation in both sexes and decline thereafter to low levels at birth (Beck-Peccoz et al. 1991, Kaplan & Grumbach 1978, Reyes et al. 1974). The increase in FSH levels is more pronounced in females than in males and it coincides with the timing of follicle formation in the ovary (Debieve et al. 2000, Faiman et al. 1976, Winter et al. 1977).

At birth, removal of the placenta results in decreased concentrations of human chorionic gonadotropin, estrogens and progesterone in the infant’s circulation (Faiman et al. 1976). It has been suggested that this decline initiates a transient rise of circulating gonadotropin concentrations during the first week of life. In girls, serum FSH levels peak at approximately 1–3 months of age and thereafter reach the low prepubertal range at 4 years of age (Schmidt & Schwarz 2000, Shinkawa et al. 1983, Winter et al. 1975). A similar but more rapid surge has also been detected in serum LH levels. In addition, follicle growth increases after birth and antral follicle number has been shown to be highest around 4 months of age, decreasing thereafter, and larger antral follicles are relatively common during the first year of life (Cohen et al. 1993, Polhemus 1953). Furthermore, circulating E₂ levels are more frequently detected during the first 4 months of life than later in the prepubertal period (Winter et al. 1976).

In premature neonates, the serum FSH surge is more pronounced than in full-term infants, but the mechanism behind this difference is not properly understood (Greaves et al. 2008, Massa et al. 1992, Shinkawa et al. 1983, Tapanainen et al. 1981). Possible causes may be immaturity of the hypothalamic-pituitary-ovarian axis or insufficient negative feedback from the ovaries (Faiman et al. 1976,
Greaves et al. 2008, Tapanainen et al. 1981). The negative feedback system at the pituitary level may already be functional during the last trimester, as the administration of exogenous estradiol and progesterone to premature infants suppresses gonadotropin secretion to a level close to normal (Trotter et al. 1999).

In childhood, the secretion of gonadal steroids and gonadotropins is low because of minimal stimulation from hypothalamus caused by central nervous system inhibition. At the onset of puberty, this inhibition decreases and the pulsatile secretion of GnRH increases pituitary LH and FSH secretion, which activates ovarian function and the negative feedback system (Baerwald et al. 2012).

2.3 Disorders of follicular development and ovarian function

2.3.1 Primary ovarian insufficiency

Primary ovarian insufficiency (POI) is a disorder defined as at least 4 months of amenorrhea with two measurements of serum FSH levels in the menopausal range (>40 IU/l) and decreased serum E2 levels in women under 40 years of age (De Vos et al. 2010). The incidence of POI is approximately 1% and the cause may be iatrogenic (e.g. in connection with ovarian surgery, chemotherapy, radiation) or spontaneous (idiopathic [most cases], genetic or caused by autoimmunity) and it has also been associated with smoking (Nelson 2009, Welt 2008). The severity of POI varies greatly and approximately 50% of women with POI present with remaining ovarian function – spontaneous pregnancy rates of 5–10% have been reported (Bidet et al. 2011, Nelson et al. 2005).

In most cases, genetic POI originates from X chromosome defects: Turner syndrome (discussed in more detail below), fragile X premutations or other more uncommon X chromosome deletions, translocations or duplications (Welt 2008). Other genetic causes include e.g. mutations in the FSH receptor gene (discussed in more detail below), the LH receptor gene or in genes encoding steroidogenic enzymes, resulting in dysfunction of follicular development (Nelson 2009).

Autoimmunity is the cause of spontaneous POI in at least 2–10% of all cases (Persani et al. 2009). Approximately 10–20% of female patients with autoimmune adrenal insufficiency develop POI owing to autoimmune oophoritis as a result of the actions of antibodies against steroid-producing cells or steroidogenic enzymes (Bakalov et al. 2005, Welt 2008).

24
**Turner syndrome**

Turner syndrome (TS) is caused by absence or abnormalities of one of the X chromosomes and it affects approximately one in 2500 newborn girls (Stochholm et al. 2006). Fifty percent of TS girls have monosomy (45,X), 5–10% have structural abnormalities in an X chromosome and the rest have mosaicism, in which karyotype 45,X/46,XX is the most common (Sybert & McCauley 2004). Growth failure and POI are general manifestations of TS, affecting 95% of patients (Gravholt 2004). Other common clinical characteristics are congenital lymphedema, heart and kidney failure, endocrine disturbances (e.g. hypothyroidism), particular physical features and psychosocial problems (Gravholt 2004, Sybert & McCauley 2004).

The development of ovarian failure in TS generally begins during the fetal period. The number of primordial follicles has been estimated to be normal up to the 18th gestational week, after which accelerated apoptosis occurs and the follicle pool is depleted, usually prior to adulthood (Carr et al. 1968, Reynaud et al. 2004, Singh & Carr 1966, Weiss 1971). However, 15–30% of TS girls show signs of spontaneous puberty but less than 5% have spontaneous menstruation and thus few can achieve pregnancy without assistance (Hovatta 1999, Pasquino et al. 1997). Turner women with mosaicism have most likely functional ovaries compared to other karyotypes. However, in cases of monosomy, viable follicles have been shown to present in ovarian biopsy samples (Borgström et al. 2009, Hreinsson et al. 2002) and even a few instances of pregnancy have been reported (Mortensen et al. 2010).

Hormone replacement therapy is used in cases of TS to induce pubertal development and to improve bone mineral density, liver function and the quality of life (Pinsker 2012). Growth hormone treatment is needed in most TS cases to improve adult height (Pinsker 2012, Sybert & McCauley 2004). Ovarian tissue cryopreservation may be an option for TS women as regards fertility preservation. However, pregnancies in women with TS show a high miscarriage rate and the risks of aneuploidy and malformation are higher than in the general population (Karnis 2012). In addition, maternal morbidity as a result of aortic dissection or rupture, and the risks of preeclampsia and gestational diabetes are increased among women with TS (Hagman et al. 2013, Karnis 2012).
FSH receptor mutation

Follicle-stimulating hormone is essential for normal ovarian function and fertility. Its stimulatory effect is mediated via FSH receptors (FSHRs), which are located in ovarian GCs (Rannikko et al. 2002, Tilly et al. 1992) and are expressed in growing follicles from primary stage onwards (Oktay et al. 1997). The FSHR is a G protein-coupled receptor and its main signal transduction mechanism is activation of adenylate cyclase, which brings about elevation of intracellular cyclic AMP (cAMP) levels and subsequently activation of intracellular kinases and enzymes (Simoni et al. 2002).

Several single-nucleotide mutations (also known as point mutations) of the FSHR gene have been detected. These point mutations may affect FSH binding, receptor expression or signal transduction, for example (Lussiana et al. 2008). The mutations are classified as activating, inactivating or neutral. Activating mutations bring about constitutive activation of FSHRs and may increase the risk of ovarian hyperstimulation syndrome (Montanelli et al. 2004, Smits et al. 2003, Vasseur et al. 2003). Inactivating mutations block normal FSH action in GCs and consequently result in elevated serum FSH levels (Aittomäki et al. 1995, Allen et al. 2003, Beau et al. 1998, Doherty et al. 2002, Meduri et al. 2003, Touraine et al. 1999). Neutral mutations have no physiological consequences (Lussiana et al. 2008).

The first inactivating mutation of the FSHR gene to be discovered was a C566T transition in exon 7 of the gene (Aittomäki et al. 1995). This single nucleotide mutation results in Ala to Val amino acid substitution at residue 189 of the extracellular domain of the FSHR protein (Aittomäki et al. 1995). The number of FSHRs at the cell surface is decreased, but the binding affinity of remaining receptors is unaltered (Aittomäki et al. 1995, Rannikko et al. 2002). However, the signal transduction response of cAMP and another second messenger, inositol triphosphate, is greatly reduced, causing an insufficient effect of FSH in GCs (Aittomäki et al. 1995, Rannikko et al. 2002). Only women with homozygous mutations show clinical consequences; POI with elevated FSH levels and infertility (Aittomäki et al. 1995, Aittomäki et al. 1996). Ovarian structure in affected women differs from that seen in connection with other types of POI. In ovarian biopsy samples and ultrasonography, most women with FSHR mutations have follicles, even up to small antral stage (Aittomäki et al. 1996), while in other POI types follicles are usually lacking. Men with homozygous FSHR mutations have variable degrees of spermatogenic failure, but not complete infertility.
(Tapanainen et al. 1997). The frequency of FSHR mutation is approximately 1% in Finland, with strong enrichment in the northeastern part of the country, while it is uncommon in other populations (Aittomäki et al. 1996, Jiang et al. 1998).

2.3.2 Granulosa cell tumors

Ovarian tumors may be divided into three major types: epithelial, sex cord-stromal and germ cell tumors (Jamieson & Fuller 2012). Granulosa cell tumors are relatively rare ovarian malignancies which represent the majority of sex cord-stromal tumors and they account for approximately 5% of all ovarian tumors (Jamieson & Fuller 2012). Granulosa cell tumors are hormonally active and secrete E₂, inhibins and AMH, for example (Geerts et al. 2009, Schumer & Cannistra 2003). This endocrine activity results in symptoms such as menstrual irregularity, metrorrhagia, infertility, postmenopausal bleeding, endometrial hyperplasia or even endometrial carcinoma (Stuart & Dawson 2003, Unkila-Kallio et al. 2000). Abdominal pain and distension are common manifestations as a result of a large ovarian mass (Jamieson & Fuller 2012). Granulosa cell tumors can be divided into two subgroups: juvenile and adult forms. Juvenile GCTs occur usually before the age of 30 years (Young et al. 1984), while the more common adult form is diagnosed mainly during the perimenopausal or early postmenopausal period (Schumer & Cannistra 2003).

Low malignant potential, slow growth rate and late recurrences are characteristics of GCTs. However, in 25–50% of cases of GCT there is relapse, even after decades, and then the mortality rate is high (Cronje et al. 1999, Schumer & Cannistra 2003). The most important prognostic factor for survival is the stage of the tumor at the time of diagnosis (Bjorkholm & Silfversward 1981, Miller et al. 1997, Sun et al. 2012). The initial treatment is surgery, which is essential even to define the exact diagnosis and stage (Jamieson & Fuller 2008). Adjuvant therapy may be considered after surgery for women with advanced disease or recurrence, but the impact of systemic chemotherapy or radiotherapy on long-term survival remains uncertain (Jamieson & Fuller 2012, Stuart & Dawson 2003).
Tumor markers

Considering the difficulty of early detection of recurrence, and the high mortality rate, several tumor markers for GCTs (e.g. E\textsubscript{2}, inhibin, AMH and activin) have been identified to improve follow-up of the patients.

Estradiol is a product of GCs and its secretion in GCTs is often increased. However, the lack of correlation between disease progression and serum E\textsubscript{2} levels has indicated that it is not a reliable tumor marker for GCT patients (Lappohn \textit{et al.} 1989, Rey \textit{et al.} 1996).

Inhibin is produced by GCs of developing follicles. It is a member of the TGF-\(\beta\) family and it consists of an \(\alpha\)-subunit linked to either a \(\beta\)A or a \(\beta\)B subunit, forming inhibin A and B (Chand \textit{et al.} 2010). Serum inhibin B levels are high in the early follicular phase of the menstrual cycle (Roberts \textit{et al.} 1993), whereas levels of serum inhibin A are highest in the late follicular and luteal phases, as it is produced by GCs of the dominant follicle and corpus luteum (Roberts \textit{et al.} 1993). After menopause, serum inhibin levels are low or undetectable (Chand \textit{et al.} 2010). Inhibin B is a commonly used tumor marker GCTs (Geerts \textit{et al.} 2009). Inhibin B seems to associate more properly with disease status and its serum levels are more elevated compared with those of inhibin A (Geerts \textit{et al.} 2009). However, serum inhibin levels may also be elevated in cases of epithelial ovarian tumors and thus it is not a specific marker for GCTs (Healy \textit{et al.} 1993).

Activins are inhibin-related peptides, consisting of inhibin \(\beta\) subunits (Chand \textit{et al.} 2010). Elevated activin levels have been reported in GCT patients, but because of the low correlation to the status of the disease, it is not a suitable marker of GCTs (Petraglia \textit{et al.} 1998).

The role of AMH as a tumor marker in cases of GCTs is discussed in section 2.4.5.

2.4 Anti-Müllerian hormone

2.4.1 AMH in gonadal differentiation

Anti-Müllerian hormone (AMH), also known as Müllnerian inhibiting substance (MIS), is a dimeric glycoprotein and a member of the TGF-\(\beta\) superfamily (Knight & Glister 2006, La Marca \textit{et al.} 2009). The main signaling route of AMH is via AMH type II receptor (AMHRII) (Teixeira \textit{et al.} 2001, Visser 2003). Originally, AMH was identified in connection with its role in fetal sexual differentiation. In
early prenatal development both female and male embryos have paired genital ducts named Wolffian (mesonephric) ducts and Müllerian (paramesonephric) ducts (Wilhelm et al. 2007). In males, Sertoli cells of the developing testis produce AMH, which causes the regression of Müllerian ducts (Josso et al. 1998). In females, owing to the absence of a Y chromosome, AMH secretion is lacking, and Müllerian ducts form and maintain the major parts of the female reproductive organs: Fallopian tubes, uterus and the upper part of the vagina (Wilson et al. 1981) (Figure 2). In males, mutations in AMH or AMHRII genes result in persistent Müllerian duct syndrome, characterized by the presence of Müllerian ducts in otherwise normally masculinized males (di Clemente & Belville 2006). Fetal ovaries exposed to high AMH levels, similarly to fetal testis, show a loss of germ cells and subsequent fibrosis in animal and in vitro studies (Behringer et al. 1990, Vigier et al. 1987).

Fig. 2. The role of AMH in sexual differentiation. In males, testicular Sertoli cells secrete AMH, which causes degeneration of the Müllerian ducts, while the androgen secretion of Leydig cells results in the differentiation of Wolffian ducts to male reproductive organs. In females, the Wolffian ducts degenerate and the Müllerian ducts differentiate into Fallopian tubes, uterus and the upper part of the vagina. Modified from Rey 2005.
2.4.2 AMH in ovarian development

In contrast to testicular development, AMH is not expressed in the fetal ovary until the end of the third trimester in humans (Rajpert-De Meyts et al. 1999) and in sheep (Bezard et al. 1987), and postnatally in rodents, when follicular growth is initiated (Hirobe et al. 1992, Munsterberg & Lovell-Badge 1991, Ueno et al. 1989). However, as regards human development, AMH expression has been found already at the end of first trimester (Modi et al. 2006) and it is mainly detected in developing follicles from primary stage onwards (Bezard et al. 1987, Rajpert-De Meyts et al. 1999) (see section 2.4.3). Interestingly, AMHRII is expressed in the gonads of both sexes as early as at the time of sexual differentiation when AMH expression is still absent (Baarends et al. 1994).

Circulating serum AMH levels are low or undetectable during the fetal period (Guibourdenche et al. 2003, Josso et al. 1993, Lutterodt et al. 2009) and in infancy (Bergada et al. 2006, Guibourdenche et al. 2003, Lee et al. 1996) in females but they have also been reported to increase temporarily from birth to 3 months of age (Hagen et al. 2010a). Thereafter, serum AMH levels remain consistently low, and a slight increase is observed in prepubertal and pubertal years, continuing until the age of 25 years (Hagen et al. 2010a, Kelsey et al. 2011, Lee et al. 1996, Lie Fong et al. 2012) (Figure 4).

2.4.3 AMH in follicular development

In both human and animal studies, the expression of AMH has been observed in GCs of growing follicles from primary to antral stage, but not beyond this stage or in the corpus luteum (Baarends et al. 1995, Bezard et al. 1987, Hirobe et al. 1994, Rajpert-De Meyts et al. 1999, Ueno et al. 1989, Weenen et al. 2004) (Figure 3). AMH expression has been shown to be localized in GCs near the oocyte (Baarends et al. 1995, Hirobe et al. 1992, Ueno et al. 1989, Weenen et al. 2004), indicating possible paracrine intrafollicular coordination of follicular development (Salmon et al. 2004). Expression of the AMH gene and protein in adult human ovarian samples is most prominent in preantral and small (≤4 mm) antral follicles, whereafter it declines and is thought to be absent in follicles of > 8 mm in diameter (Jeppesen et al. 2013, Stubbs et al. 2005, Weenen et al. 2004). A similar pattern is observed in follicular fluid, in which the highest concentrations of AMH are found in small antral follicles and low levels in larger more advanced follicles (Andersen & Byskov 2006, Andersen et al. 2010,
Fanchin et al. 2005). In a recent study it was estimated that follicles of 5–8 mm produce approximately 60% of AMH in the serum (Jeppesen et al. 2013). However, in contrast to GCs, AMH expression remains high in the cumulus cells surrounding the oocyte in larger antral follicles (10–14 mm), but only low levels of expression are found in preovulatory follicles (Grondahl et al. 2011). The pattern of AMH secretion in individual follicles has been shown to be independent of age and to remain unchanged throughout fertile life (Andersen et al. 2010).

Fig. 3. The secretion and possible role of AMH in the ovary. AMH is secreted by granulosa cells of growing follicles up to small antral stage (≤ 8 mm). It has been suggested that AMH inhibits the growth of resting follicles (1) and inhibits the effect of FSH on follicular growth (2). Modified from Visser & Themmen 2005.

The role of AMH in the ovary

The exact role of AMH in the ovary is still unclear. AMH has been shown to inhibit initiation of primordial follicle growth in mouse neonatal ovarian culture, but the results concerning human ovarian tissue are contradictory (Carlsson et al. 2006, Durlinger et al. 2002, Nilsson et al. 2011, Schmidt et al. 2005). AMH-deficient mice have been shown to have more small growing and atretic follicles compared with wild-type mice and also their primordial follicle pool is depleted earlier (Durlinger et al. 1999, Visser et al. 2007). Furthermore, it has been suggested that AMH inhibits the stimulatory effect of FSH on the growth of
preantral and small antral follicles (Figure 3), which increases the FSH threshold of the follicle. AMH-deficient mice have more growing follicles than wild-type mice regardless of low FSH levels, and when exogenous FSH has been administered folliculogenesis increased significantly compared with that in wild-type females (Durlinger et al. 2001). It has also been shown that AMH decreases FSHR expression in GCs (Pellatt et al. 2011). Thus, in the absence of AMH, follicles may become more sensitive to FSH (Visser & Themmen 2005). In addition, AMH has been shown to inhibit and correlate negatively to FSH-induced aromatase expression in GCs (Eilso Nielsen et al. 2010, Grossman et al. 2008, Pellatt et al. 2011). Hence, AMH reduces E_2 levels via FSH, but no direct effect of AMH on E_2 production has been observed (Grossman et al. 2008, Pellatt et al. 2011). However, the results of in vitro studies concerning the role of AMH on FSH-stimulated follicular growth are inconsistent (Durlinger et al. 2001, McGee et al. 2001).

2.4.4 Ovarian reserve and aging

The number of primordial follicles decreases with age, as does oocyte quality, but the early signs of diminishing ovarian reserve are clinically difficult to recognize (Broekmans et al. 2009). Fertility gradually decreases from the age of 30 years onward, with wide individual variation (te Velde & Pearson 2002). The first clinical sign of a decrease in the follicle pool is shortened and irregular menstrual cycles. The size of the antral follicle cohort (secreting inhibin B) is reduced, which causes a gradual rise of serum FSH levels owing to insufficient ovarian negative feedback at the pituitary (Knauff et al. 2009, te Velde & Pearson 2002). The levels of E_2 and inhibin A remain relatively unaltered until the later stages of reproductive life (Broekmans et al. 2009). The mean proportion of small antral follicles (<8 mm) has been shown to decrease with increasing age, whereas the proportion of larger follicles (8–10 mm in diameter) increases as a result of increased FSH-stimulated follicular recruitment in the late luteal phase (Bentzen et al. 2013).

As normal reproductive aging shows great individual variation and the early signs of ovarian aging are mainly undetectable, several clinical tests have been developed to provide additional information on ovarian reserve. Ovarian AFC (total number of 2- to 10-mm antral follicles present in both ovaries as assessed by ultrasonography) and measurements of serum FSH, inhibin B, E_2 and AMH levels have been used to test ovarian reserve, and AFC and AMH measurements
are currently considered the most useful (Hansen et al. 2011). Only AMH is discussed in more detail in this thesis.

**AMH as a marker of ovarian reserve**

Anti-Müllerian hormone has been shown to be a useful marker of ovarian reserve, as its serum levels decline linearly with age and become undetectable prior to menopause (de Vet et al. 2002, Sowers et al. 2008, van Rooij et al. 2005) (Figure 4). Furthermore, serum AMH levels correlate well with AFC, which in turn reflects the size of the primordial follicle pool (de Vet et al. 2002, van Rooij et al. 2002). In addition, a direct correlation between serum AMH levels and primordial follicle number counted in ovarian tissue samples has been demonstrated previously (Hansen et al. 2011, Kevenaar et al. 2006). Previous studies have shown that AMH assay can be used for prediction of age at menopause (Broer et al. 2011b, Freeman et al. 2012b, Tehrani et al. 2013, van Disseldorp et al. 2008, van Rooij et al. 2004) and the individual rate of change of serum AMH levels has a significant impact on menopause timing as well (Freeman et al. 2012a).

During the menstrual cycle, serum AMH levels remain unchanged (Hehenkamp et al. 2006, La Marca et al. 2004) or fluctuate slightly, as some studies have shown higher serum AMH concentrations in the follicular phase (Streuli et al. 2009, Wunder et al. 2008) or midcycle (Cook et al. 2000) compared with the luteal phase. However, this variation probably has no clinical relevance (Streuli et al. 2009).

Serum AMH measurement is currently clinically used as a predictor of the response to ovarian stimulation in assisted reproduction as its levels are highly correlated with the number of oocytes retrieved (Broer et al. 2009, Broer et al. 2011a, van Rooij et al. 2002). Serum AMH levels may be used to individualize IVF treatment (Nelson et al. 2009), but caution must be taken when interpreting the results, especially in women with low serum AMH concentrations, as these women have been shown to have normal fecundity despite low serum AMH (Fraisse et al. 2008, Hagen et al. 2012b). Recently, serum AMH levels have been shown to be positively correlated to live-birth rates as well (Brodin et al. 2013, Broer et al. 2009). Age-specific nomograms have been constructed, mainly using data from infertility patients (Nelson et al. 2011, Seifer et al. 2011), and sample sizes in studies concerning healthy women have been relatively small (La Marca et al. 2012, Lie Fong et al. 2012, Masse et al. 2011). The lack of exact threshold
levels in normal populations has delayed the wider use of AMH measurement in other clinical applications.

![Fig. 4. Mean serum AMH levels according to age. Modified from Kelsey et al. 2011.](image)

**AMH and ovarian insufficiency**

In women with POI, ovarian function may vary from temporary amenorrhea to consistent infertility and thus an accurate marker to estimate the size of the remaining ovarian follicle pool is needed. Serum AMH levels are low in most women with POI of unknown etiology (La Marca et al. 2006b, Meduri et al. 2007). However, previous studies have indicated that some women with POI have growing follicles and measurable AMH levels. Indeed, most women with POI due to steroidogenic cell autoimmunity have normal serum AMH levels if the time since diagnosis is less than five years (La Marca et al. 2009). Furthermore, functional hypothalamic amenorrhea patients have normal or even higher AMH levels compared with healthy women (Jonard et al. 2005, La Marca et al. 2006b). When serum AMH levels were investigated in relation to ovarian histology in women with POI, significantly higher AMH concentrations were found in women with over 15 follicles in biopsy samples (Meduri et al. 2007). In the same study, AMH expression was found to be normal in preantral follicles, but in antral stage follicles expression was decreased as a result of atresia (Meduri et al. 2007). Serum AMH assessment provides more accurate information on the size of the follicle pool than assay of FSH or inhibin B alone in women with elevated FSH
levels and regular menstrual cycles or oligomenorrhea (Knauff et al. 2009). In addition, measurement of AMH is a useful tool in fertility counseling of women surviving cancer who are at risk of developing POI as a result of chemotherapy or irradiation treatment (Anderson & Cameron 2011, Bath et al. 2003, Brougham et al. 2012, Lie Fong et al. 2009, van Beek et al. 2007). In TS, girls with spontaneous menarche, normal FSH levels and follicles found in ovarian biopsy samples have higher serum AMH levels than those with opposite findings (Borgström et al. 2009, Hagen et al. 2010b). These features are most common in cases of mosaicism, but some girls with structural chromosomal abnormalities or monosomy have measureable AMH levels as well (Borgström et al. 2009, Hagen et al. 2010b, Visser et al. 2013).

2.4.5 AMH in combined hormonal contraceptive users

The results of previous studies concerning serum AMH levels during CHC use have been controversial. In large or population-based studies, serum AMH levels have been found to be lower among CHC users compared with non-users (Bentzen et al. 2012, Kristensen et al. 2012, Shaw et al. 2011), but in a smaller study no difference was observed (Deb et al. 2011). An increase of serum AMH levels was observed when AMH measurements were performed during the hormone-free interval and in subsequent natural cycles (van den Berg et al. 2010). However, in other studies in healthy women, no differences have been found prior to treatment and during CHC use (Arbo et al. 2007, Li et al. 2011, Somunkiran et al. 2007, Streuli et al. 2008). There are no clear comparative data concerning different administration routes and serum AMH levels.

2.4.6 AMH as a marker of granulosa cell tumors

Previous studies have shown that serum AMH concentrations are elevated in women with GCTs but not in women with other ovarian tumors or extraovarian cancers (Gustafson et al. 1992, Rey et al. 1996). The sensitivity of assay of serum AMH in previous retrospective studies with small sample sizes has varied between 76% and 100% (Gustafson et al. 1992, Lane et al. 1999, Long et al. 2000, Petraglia et al. 1998, Rey et al. 1996). The results of immunohistochemical studies support these reports, as the expression of AMH is localized only in granulosa and Sertoli cell tumors (Matias-Guiu et al. 1998, Rey et al. 2000).
However, in larger sized GCTs, AMH expression has been shown to be reduced (Anttonen et al. 2005, Dutertre et al. 2001).

Serum AMH measurement may be used to estimate the effectiveness of surgery or other treatment, as its levels decrease within a few days after surgery, and AMH assay can be used to predict recurrence even years prior to the clinical manifestation of relapse (Gustafson et al. 1992, Lane et al. 1999, Long et al. 2000, Rey et al. 1996). Serum AMH levels are relatively stable throughout the menstrual cycle (Hehenkamp et al. 2006, La Marca et al. 2006, Wunder et al. 2008) and consequently AMH may be a more reliable marker in premenopausal women compared with inhibins. However, follow-up studies with large numbers of patients are lacking because of the rarity of GCTs and thus the utility of serum AMH measurements in women with these tumors needs clarification.
3 Aims of the study

The clinical utility of serum AMH assay in connection with different disorders of ovarian function has been studied intensively during the last decade. However, little is still known of AMH secretion during the fetal period and infancy, and its role in follicle development has been studied mainly in animals. Combined hormonal contraceptives are widely used among women, but the inhibitory effects on follicular development as regards different administration routes have not been compared previously, in particular by using serum AMH measurement. Moreover, serum AMH levels have been shown to be high in women with GCTs, but no large clinical studies have been carried out to examine this issue further.

Thus, the aims of this study were to explore human GC AMH secretion in early ovarian development and in disorders of ovarian function. The specific aims of the study were:

1. To explore how AMH secretion in infancy is related to function of the pituitary-ovarian axis and ovarian follicular development, and to investigate the ontogeny of AMH during the fetal period.
2. To evaluate the utility of serum AMH
   a) in identifying women with POI and existing follicles
   b) as a marker in GCT follow-up compared with the currently used marker inhibin B.
3. To compare the effects of combined contraceptives administered via different routes on the pituitary-ovarian axis and ovarian function.
4. To study in more detail stage-dependent AMH secretion in follicular development.
4 Materials and methods

4.1 Subjects and materials

This study was approved by the Ethics Committee of Oulu University Hospital and the University of Oulu, the Ethics Committee of the Pohjois-Savo Health Care District (Study I), the Ethics Committee of Helsinki University Central Hospital (Study III) and the National Supervisory Authority for Welfare and Health in Finland.

4.1.1 Infant girls (Study I)

Sixty-three healthy infant girls were recruited for Study I at Kuopio University Hospital. The subjects were divided into three groups according to gestational age (GA) at birth: full term (n = 29; GA 37–41.7 wk), near term (n = 17; GA 34–36.7 wk) and preterm (n = 17; GA 24.7–33.9 wk). There was one set of triplets and one set of twins in the preterm group, three sets of twins in the near-term group and one set of twins in the full-term group.

4.1.2 Girls and women with POI (Study II)

Forty-three women with ovarian insufficiency (aged 18–43 years) were recruited originally for a previous study (Aittomäki et al. 1996) and were divided into three groups in Study II: women with FSH-resistant ovaries (FSHRO; n = 12), women with POI and primary amenorrhea of unknown etiology (PA; n = 11) and women with POI and secondary amenorrhea of unknown etiology (SA; n = 20). All subjects had normal female karyotype (46,XX), high serum gonadotropin levels (FSH >40 IU/l) and no known cause of hypergonadotropic ovarian insufficiency (e.g. autoimmune ovarian diseases). Twenty-three healthy normal-weight women (aged 18–44 years) with regular menstrual cycles served as controls (Piltonen et al. 2003, Puurunen et al. 2009).

Girls/women with TS (n = 35, aged 6–43 years) were recruited by contacting members of the Finnish TS Association or during clinical follow-up visits to specialist care units. The subjects were divided in three groups according to karyotype: 45,X (n = 18), mosaicism (n = 7) and structural X chromosome abnormalities (SCAs, n = 10). Five subjects with mosaicism and three subjects
with SCAs had had spontaneous menarche. One woman with mosaicism had had two spontaneous pregnancies. Thirty-four healthy girls, or women with regular menstrual cycles (aged 7–44 years) served as controls (Piltonen et al. 2003, Puurunen et al. 2009, Siljander et al. 2009).

4.1.3 Women with GCTs (Study III)

A total of 123 adult-type GCT patients (107 women from Helsinki University Central Hospital and 16 women from Oulu University Hospital) were included in Study III. The diagnoses were histologically re-confirmed by an expert pathologist and stage classification was performed according to FIGO 2009 criteria. The mean follow-up time was 12.4 years (range 0.3–49.9). Thirty-five patients (28.5%) had at least one recurrence during follow-up.

4.1.4 Healthy women (Study IV)

A total of 54 healthy normal-weight women (aged 20–33 years) with regular menstrual cycles and no medication participated in Study IV, and 42 subjects completed the study. Subjects with previous use of contraceptives had a wash-out period (minimum of two months) before entering the study. Exclusion criteria were cigarette smoking, abuse of alcohol, lactation and general contraindications for the use of hormonal contraceptives.

4.1.5 Tissue samples (Studies I and II)

Tissue samples were collected from autopsy or biopsy samples at the Department of Pathology, Oulu University Hospital. The samples were fixed in 10% phosphate-buffered neutral formalin for 24 h and embedded in paraffin. Histological sections were cut and processed for immunohistochemistry (IHC). A permit to study human autopsy tissues and resection material was obtained from the National Supervisory Authority for Welfare and Health in Finland.

Fetal ovarian tissues

A total of 37 fetal ovarian tissue samples with a normal (46,XX) karyotype were collected from 13 fetuses (GA 13–24 wk) after spontaneous or therapeutic abortions and from 18 fetuses (GA 22–41 wk) after intrauterine death. In addition,
ovaries from six neonates who died because of perinatal asphyxia or infection within 30 min to 4 d after birth were studied (Study I).

In Study II, ovarian tissue from 7 fetuses (GA 13–22 wk) with 45,X karyotype were studied. Of these, six fetal ovaries were obtained after therapeutic abortions and one ovarian sample after autopsy because of intrauterine death. Human fetal testis tissue was used as a positive control.

**Adult ovarian tissues**

Adult ovarian tissue samples were obtained from 14 women (aged 22–45 years) undergoing ovariectomy because of endometriosis. There were two ovarian biopsy samples available from women with FSHRO, which were taken for clinical purposes at the time of diagnosis of POI.

### 4.2 Methods

#### 4.2.1 Serum and urine sampling and hormone measurements

Serum samples were collected from infant girls during the first week of life, at three months of age and at the corrected age of 14 months (14 months from the expected date of delivery). Among the women with regular menstrual cycles, the samples were taken at the follicular phase of the menstrual cycle (in Study II on cycle days 1–7 and in Study IV on cycle days 2–4 before treatment). In women with GCTs, serum samples were collected retrospectively at Helsinki University Central Hospital and Oulu University Hospital (n=237) and prospectively at Helsinki University Central Hospital (n=323). The samples were taken prior to treatment (surgery or chemotherapy) and/or during follow-up and were classified into two groups (with disease [WD] and disease-free [DF]). In the WD group (n = 117) macroscopic tumor growth was confirmed by way of surgical operation, CT, MRI or ultrasonography at the time of blood sampling, whereas in the DF group (n = 443) no macroscopic tumor was detected. Serum samples in all studies were kept at -80 °C until analyses were carried out.

Spot urine samples from infant girls were collected in plastic collection bags or by clean catch during the first week of life and thereafter monthly until 6 months of age and at the corrected age of 14 months.
Details of the hormone assays used are given in Table 1. All assays were performed by following the instructions of the manufacturers.

Table 1. Characteristics of the assays used.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Method</th>
<th>Sensitivity</th>
<th>Intra-assay coefficient of variation (%)</th>
<th>Interassay coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-AMH</td>
<td>ELISA</td>
<td>0.05 ng/ml</td>
<td>4.6 &amp;</td>
<td>8.0 &amp;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.16 ng/ml</td>
<td>6.1</td>
<td>7.3</td>
</tr>
<tr>
<td>U-FSH</td>
<td>TR-IFA</td>
<td>0.05 IU/l</td>
<td>-</td>
<td>&lt;5</td>
</tr>
<tr>
<td>S-FSH</td>
<td>CLMA</td>
<td>0.3 IU/l</td>
<td>2.9</td>
<td>3.9</td>
</tr>
<tr>
<td>S-LH</td>
<td>CLMA</td>
<td>0.07 IU/l</td>
<td>2.3</td>
<td>2.7</td>
</tr>
<tr>
<td>S-E₂</td>
<td>RIA</td>
<td>5 pmol/l</td>
<td>2.8</td>
<td>5.8</td>
</tr>
<tr>
<td>S-inhibin B</td>
<td>ELISA</td>
<td>2.6 ng/l</td>
<td>2.4</td>
<td>4.4</td>
</tr>
</tbody>
</table>

1The lowest standards used in the assays. ELISA = enzyme-linked immunosorbent assay, TR-IFA = time-resolved immunofluorometric assay, CLMA = chemiluminometric assay, RIA = radioimmunoassay

AMH immunoassay

Serum AMH levels were measured using two-site immunoassay kits, DSL-II MIS/AMH ELISA (Studies I-II) and Beckman-Coulter AMH Gen II ELISA (Studies III-IV). The AMH Gen II assay was developed more recently (Kumar 2010). The same antibodies are used in Gen II assay as in DSL-II assay, but the calibrators are different. However, the assay procedure is similar in both kits.

First, the calibrators/standards, controls and samples were added to anti-AMH antibody coated microtitration wells. The wells were incubated for one hour in room temperature and washed with solution. Thereafter, anti-AMH detection antibody labeled with biotin was added into the wells. After the second step of incubation and washing, the streptavidin-labeled horseradish peroxidase was added into each well, followed by a third incubation and washing step. Thereafter, the tetramethylbenzidine substrate solution was added, and the color reaction was stopped by using stopping solution. The degree of enzymatic turnover of the substrate was determined by wavelength absorbance measurement. The measured absorbance was directly proportional to the AMH concentration in the samples. The calibrators were used to plot a cubic regression standard curve of absorbance versus serum AMH concentration.
4.2.2 Ultrasonographic measurements

Transabdominal ovarian ultrasonography was performed in infant girls during the first week of life, and thereafter monthly until 6 months of age and at the corrected age of 14 months by three pediatric radiologists. The number of visible antral follicles was counted, and the diameter of follicles of ≥ 6mm was registered. Ovarian volume was measured by using the formula for an ellipsoid (length × width² × π/6) and all cysts were included.

Transvaginal ultrasonography was performed in Study IV before treatment in all subjects to verify normal ovarian morphology.

4.2.3 Immunohistochemistry

Immunohistochemistry was performed to detect protein expression in paraffin-embedded tissue samples. Tissue sections were deparaffinized in xylene, rehydrated gradually through a series of graded alcohol solutions and washed in water. The tissues were pretreated in 10 mM sodium citrate in a microwave oven to enhance their permeability. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Nonspecific staining was prevented by using normal blocking serum incubation. Tissue samples were incubated overnight with primary goat anti-human MIS/AMH antibody (R&D Systems, MN, USA), dilution 1:50 in phosphate-buffered saline (PBS). An avidin-biotin immunoperoxidase system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) was used to visualize the bound antibody. The color reaction was produced with diaminobenzidine tetrahydrochloride (DAB, DakoCytomation Ltd., Ely, UK) and samples were counterstained with hematoxylin, dehydrated through a series of graded alcohol solutions and cleared in xylene. For negative controls, PBS was applied instead of primary antibody.

All samples were analyzed by using light microscopy in whole ovary or ovarian biopsy sections, evaluating staining intensity and extent.

4.2.4 Treatment with combined contraceptives (Study IV)

The subjects in Study IV were randomized to use continuously one of the following preparations for 9 weeks: a combined oral contraceptive (ethinyl estradiol [EE], 20 μg and desogestrel, 150 μg [Mercilon®; Organon, Oss, Holland]), a transdermal contraceptive patch (EE, 20 μg/day and norelgestromin,
150 μg/day [Ortho Evra®; Janssen Ortho, LLC, Manati, Puerto Rico]) or a contraceptive vaginal ring (EE, 15 μg/day and etonogestrel [active metabolite of desogestrel], 120 μg/day [NuvaRing®; Schering-Plough Corp., Kenilworth, NJ, USA]). Serum samples were collected before the treatment and at 5 and 9 weeks of treatment.

4.2.5 Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics or PASW Statistics software and with JMP Pro (Study III). In Study I, comparisons of FSH and AMH levels and antral follicle numbers between and within groups were analyzed by mixed models analysis.

To compare serum AMH levels between healthy women and subjects with POI of different etiologies (Study II), an independent samples $t$-test (with more statistical power) was used and the results were confirmed by using the Mann–Whitney test. In Study III, receiver operator curves (ROCs) were constructed and area under the curve (AUC) values were analyzed to investigate the cut-off values and the accuracy of serum markers in diagnostics. Sensitivity, specificity and positive and negative predictive values were calculated with contingency tables. Changes of hormone concentrations during follow-up in individual patients were studied by way of Friedman test and Wilcoxon’s test was used for matched-pairs/paired samples analysis. Correlations of hormone levels to variables were analyzed by using Wilcoxon test (categorical variables) and linear regression (continuous variables). In Study IV, analysis of variance (ANOVA) for repeated measures was performed to analyze changes in serum hormone levels within the groups of women using oral, transdermal or vaginal CHCs. To assess the changes in hormone levels before treatment and after 5 or 9 weeks, the paired samples $t$-test was used. One way ANOVA was used to explore differences between the study groups. The limit of statistical significance was set at $p < 0.05$ in all studies.
### Table 2. Summary of materials, methods and main results of the studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects &amp; materials</th>
<th>Methods</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Serum and urine samples from healthy infant girls 46,XX fetal ovarian tissue samples</td>
<td>S-AMH: ELISA U-FSH: TR-IFA Ultrasonography IHC: AMH antibody</td>
<td>Postnatal pituitary activation results in transient ovarian maturation. In preterm girls folliculogenesis was delayed. AMH expression was detected from midgestation onwards in fetal ovaries.</td>
</tr>
<tr>
<td>II</td>
<td>Serum samples from girls/women with FSHRO, TS and POI of unknown etiology FSHRO biopsy samples 45,X fetal ovarian tissue samples</td>
<td>S-AMH: ELISA IHC: AMH antibody</td>
<td>Serum AMH measurement can be used to identify patients with POI and existing follicles. AMH is secreted mainly by small non-selected follicles. Women with FSHRO have no follicles beyond small antral stage and their serum AMH levels were close to normal.</td>
</tr>
<tr>
<td>III</td>
<td>Serum samples from women with GCTs</td>
<td>S-AMH: ELISA S-Inhibin B: ELISA</td>
<td>Serum AMH performed equally as a tumor marker for GCTs as the currently used serum marker inhibin B. Combination of S-AMH and S-inhibin B measurements improves follow-up of GCT in individual patients.</td>
</tr>
<tr>
<td>IV</td>
<td>Serum samples from healthy, normal-weight women with regular menstrual cycles</td>
<td>S-AMH: ELISA S-FSH &amp; S-LH: CLMA S-E₂: RIA S-Inhibin B: ELISA</td>
<td>Follicular development is inhibited by continuous use of CHCs independently of administration route. It was reflected in reduced serum AMH and inhibin B levels.</td>
</tr>
</tbody>
</table>

TR-IFA = time-resolved immunofluorometric assay, IHC = immunohistochemistry, CLMA = chemiluminometric assay, RIA = radioimmunoassay.
5 Results and discussion

5.1 AMH expression in the human ovary

Expression of AMH in the ovary has been mainly studied in animals and in adult human ovarian tissue samples (Baarends et al. 1995, Bezard et al. 1987, Hirobe et al. 1994, Ueno et al. 1989, Weenen et al. 2004). Data concerning AMH expression in fetal ovaries is scarce (Modi et al. 2006, Rajpert-De Meyts et al. 1999) and there are only a few studies available concerning ovarian AMH expression in cases of POI of different etiologies (Massin et al. 2008, Meduri et al. 2007). To clarify these issues further, AMH expression was analyzed in fetal and adult ovaries in Studies I & II.

5.1.1 Fetal ovaries

It has been thought, based on the animal studies, that AMH is expressed in fetal ovaries only when follicular growth is initiated. The results of previous studies concerning AMH expression in human fetal ovaries have been contradictory (Modi et al. 2006, Rajpert-De Meyts et al. 1999). The results of Study I demonstrated that AMH expression is initiated at midgestation, approximately at the time of follicular formation, and it increases gradually towards term. As early as at the 23rd week of gestation, a few GCs of transitional follicles expressed AMH. From the 25th gestational week to term the staining of AMH was more intensive and localized in GCs of growing follicles from transitional stage onwards (Figure 5). Antral follicles were stained positively but detected only near term. Our results partially support the results of a previous study, in which the expression of AMH was observed first at 36 weeks of gestation (Rajpert-De Meyts et al. 1999), but were not in line with those of another study where AMH mRNA was already observed at the end of first trimester (Modi et al. 2006). The differences may be explained by different methods and antibodies used and in addition, the sample sizes in all studies were relative small owing to limited availability of tissue samples. Furthermore, there may be individual variation in ovarian development and maturation which may affect the results as well.
Fig. 5. AMH expression in human fetal ovaries. A. At the 23rd gestational week AMH expression was observed in a few transitional follicles. B. Thereafter, AMH expression increased gradually towards term. At 31 weeks of gestation AMH expression was detected in primary, secondary and preantral follicles. The expression was focused in the center of the follicle. C. At term (40th weeks of gestation) AMH staining was observed in primary, secondary, preantral and small antral follicles. The primordial follicles were stained negatively. D. No clear follicle formation or immunostaining was detected in a 45,X fetal ovarian sample at 21 weeks of gestation. E. A fetal ovarian sample from the 40th week of gestation served as a negative control (PBS used instead of primary antibody), showing no AMH expression. Scale bar 50 µm.

In fetal 45,X ovarian samples (aged 13–22 gestational weeks) in Study II, no clear follicle formation was found and all the samples were AMH-negative (Figure 5), which was expected and in line with the results of Study I. Indeed, previous studies have shown that follicle loss occurs from the 18th gestational week in most TS girls with monosomy (Reynaud et al. 2004). Unfortunately, owing to limited
data available, there was no opportunity to study AMH expression in TS subjects with mosaicism, who more often have follicles up to adolescence.

### 5.1.2 Adult ovaries

Ovarian biopsy samples from women with FSHRO were investigated in Study II. There were no secondary or more advanced follicles observed in the samples. AMH expression was detected mainly in GCs of primary follicles, supporting the results of previous studies (Baarends et al. 1995, Weenen et al. 2004), but some transitional and primordial follicles stained positively as well (Figure 6).

Ovarian tissue samples from healthy women served as positive controls in Studies I & II. AMH expression was observed from primary to antral stage and most of the primordial and transitional follicles were AMH-negative (Figure 6). Atretic follicles and stroma showed no staining. The results were partly in line with those of previous studies (Baarends et al. 1995, Stubbs et al. 2005, Weenen et al. 2004), and the differences concerning staining of primordial and transitional follicles may be explained by different methodologies and antibodies used. The observed staining of AMH in primordial and transitional-stage follicles suggests that the expression of AMH precedes or coincides with the initiation of follicular growth. The results of a previous study suggest that the alteration of AMH secretion at these stages may be related to disorders of follicular development, such as polycystic ovary syndrome, in which the number of growing follicles is abnormally high (Stubbs et al. 2005).
5.2 AMH secretion and postnatal function of the pituitary-ovarian axis

Serum AMH levels in fetal and neonatal girls have been reported to be low or undetectable (Bergada et al. 2006, Guibourdenche et al. 2003, Josso et al. 1993, Lee et al. 1996, Lutterodt et al. 2009), but a slight increase has been detected at 1–3 months of age (Bergada et al. 2006, Hagen et al. 2010b). Only one longitudinal study of full-term infant girls has been performed (Hagen et al. 2010a) and the relationship between AMH secretion during fetal life and infancy and the function of the pituitary-ovarian axis and ovarian folliculogenesis has not been studied longitudinally. A transient postnatal increase of serum FSH levels has been reported in previous studies in both sexes, as well as a temporary increase of follicular growth in girls (Polhemus 1953, Schmidt & Schwarz 2000, Shinkawa et al. 1983, Winter et al. 1975). Preterm infants show more pronounced FSH levels compared with infants born at term, but the underlying mechanism behind this is not known (Greaves et al. 2008, Shinkawa et al. 1983, Tapanainen et al. 1981).

Serum AMH levels were measureable in fetal girls from the age of 26 postmenstrual weeks in Study I and increased gradually towards term, which is in line with AMH protein expression in fetal ovaries (Figure 5, and see section 5.1.1).
The levels were lowest in the first week of life and increased significantly to three months of age in preterm, near-term and full-term infants, supporting the results of a previous study (Hagen et al. 2010b) (Table 3). Thereafter, levels were decreased at the (corrected) age of 14 months in full-term and near-term girls, but in girls born prematurely, AMH levels remained elevated. A similar temporary increase in the number of antral follicles was observed in all infants. More prolonged and pronounced U-FSH levels were found in near-term and preterm girls compared with full-term infants, confirming the findings in earlier reports (Greaves et al. 2008, Tapanainen et al. 1981) (Table 3).

Table 3. Urinary FSH and serum AMH levels, and the numbers of antral follicles observed per ovary in full-term, near-term and preterm infants during the first week of life, at three months of age and at the (corrected) age of 14 months.

<table>
<thead>
<tr>
<th>Variable</th>
<th>First week of life</th>
<th>Three months of age</th>
<th>Corrected age of 14 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (miU/mg Cr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-term</td>
<td>12.8 (7.0–33.4)*,**</td>
<td>8.9 (5.5–15.7)**</td>
<td>12.7 (5.6–20.0)</td>
</tr>
<tr>
<td>Near-term</td>
<td>113 (85.0–250)</td>
<td>6.8 (4.5–15.6)**</td>
<td>12.6 (8.1–17.9)</td>
</tr>
<tr>
<td>Preterm</td>
<td>121 (91.2–411)</td>
<td>26.7 (7.9–245)</td>
<td>15.6 (12.1–18.9)</td>
</tr>
<tr>
<td>AMH (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-term</td>
<td>0.33 (0.20–0.76)*,**</td>
<td>2.78 (1.35–3.37)**</td>
<td>1.58 (0.77–2.24)</td>
</tr>
<tr>
<td>Near-term</td>
<td>0.22 (0.16–0.27)</td>
<td>3.80 (2.22–5.40)**</td>
<td>1.30 (0.87–1.87)</td>
</tr>
<tr>
<td>Preterm</td>
<td>0.16 (0.10–0.20)</td>
<td>1.13 (0.35–3.82)</td>
<td>1.28 (0.46–1.60)</td>
</tr>
<tr>
<td>Number of AFs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-term</td>
<td>1 (0–4)</td>
<td>3 (1–7)</td>
<td>1 (0–4)</td>
</tr>
<tr>
<td>Near-term</td>
<td>1 (0–2)</td>
<td>4 (1–13)**</td>
<td>2 (1–3)</td>
</tr>
<tr>
<td>Preterm</td>
<td>-</td>
<td>1 (0–1)</td>
<td>2 (1–2)</td>
</tr>
</tbody>
</table>

Data are expressed as median (quartile) for FSH and AMH and as median (range) for number of AFs.

* p ≤ 0.01 vs. near-term group, ** p < 0.05 vs. preterm group.

The results of Study I demonstrated for the first time a relationship between the FSH surge, changes in follicular growth and serum AMH levels. The results showed that the more prolonged FSH surge in near-term and preterm girls compared with full-term girls was associated with the delayed increase of serum AMH levels and the number of antral follicles. Thus, immature ovarian folliculogenesis may result in insufficient negative feedback from ovary to pituitary and thereby explain the high FSH levels in preterm girls, as in previous studies the negative feedback system has been shown to be already operational during the third trimester (Trotter et al. 1999).
When the results were analyzed according to postmenstrual age (Figure 7), U-FSH levels were high prior to 40 weeks of age and decreased thereafter rapidly. The first antral follicles were observed at 36 weeks of postmenstrual age and the number of antral follicles was highly positively correlated with serum AMH levels. The results suggest that maturation of ovarian follicular growth occurs during fetal life, with a specific time window, and this maturation continues postnatally in preterm girls. Even though lower reproduction rates have been reported previously in premature born adults (Swamy et al. 2008), the possible later effects of the altered function of the pituitary-ovarian axis in preterm girls are not clear and need further investigation.
Fig. 7. Relationship between urinary FSH and serum AMH levels, and the numbers of antral follicles in infant girls according to postmenstrual age. Published by permission of The Endocrine Society.
5.3 AMH as a clinical serum marker

During the last decade knowledge of AMH as a clinical serum marker of ovarian reserve and different disorders of ovarian function has increased significantly. However, more research data is needed prior to wider clinical use of AMH measurement. In the present study, useful information on serum AMH measurement in women with POI and in GCT patients was gained.

5.3.1 AMH and POI of different etiologies

The severity of POI varies widely and approximately half of the cases have some remaining ovarian function, but there are only a few possibilities to clinically estimate individual ovarian reserve reliably. The serum AMH level has been shown to be a good marker of ovarian reserve, but data on serum AMH concentrations in POI of different etiologies is limited.

In Study II, serum AMH levels were measured in women with POI (unknown reason), women with FSHRO and TS subjects. The results demonstrated slightly but not significantly lower AMH levels in women with FSHRO compared with controls (Figure 8). In women with POI of unknown etiology, circulating AMH levels were low or undetectable, supporting results in previous reports (Knauff et al. 2009, La Marca et al. 2006a, Meduri et al. 2007). The low-normal serum AMH levels in FSHRO subjects support the concept that AMH is secreted by small non-selected follicles, which are not yet dependent on FSH, as women with FSHR mutations have follicles only up to small antral stage as a result of the lack of a stimulating effect of FSH on follicular growth (Aittomäki et al. 1996). However, the constitutional activity of FSHR may have some effect on follicular growth, but as most of the mutated FSHRs have been shown to be sequestered intracellularly, the possible effect is mainly theoretical (Rannikko et al. 2002). The slightly lower AMH levels may also be explained by the smaller amount of follicles in larger phases and thus a lower number of AMH-secreting GCs. Approximately 75% of the FSHRO subjects studied had follicles in previously performed ultrasonography, but follicles (mainly primordial and primary stage) were detected in all ovarian biopsy samples studied (Aittomäki et al. 1996). Thus, the ovarian reserve may be unaffected in FSHRO subjects, as they had low-normal serum AMH levels, but the number of antral follicles in cases of FSHRO is decreased.
Fig. 8. Median (solid line) serum AMH levels in women with POI of unknown etiology (primary amenorrhea [PA] and secondary amenorrhea [SA]), women with FSHRO and control subjects. Dotted line represents mean. *p < 0.001 vs. controls.

Turner syndrome girls/women with mosaicism had significantly higher serum AMH levels than subjects with monosomy or SCAs, supporting the results of previous studies (Borgström et al. 2009, Hagen et al. 2010b) (Figure 9). The measureable concentrations in some girls/women of the two latter groups indicate that remaining ovarian function may be detected regardless of karyotype. It has been hypothesized previously that most of 45,X women may have undiagnosed partial mosaicism in some of their cell lines (Zhong & Layman 2012), which may theoretically explain the detectable serum AMH levels in some of 45,X women in this study. Great individual variation was observed in serum AMH levels among girls/women with mosaicism and most of those with spontaneous menarche had high AMH levels. Variability of serum AMH levels in TS women with mosaicism reflects the fact that some of them have already POI and some do not. However, the number of subjects in this study was relatively low, which has to be taken into consideration.
The results of Study II demonstrate that serum AMH measurement may be used as a tool to identify girls/women with POI and remaining ovarian function, as women with FSHRO who had follicles up to small antral stage had significantly higher AMH levels compared with other subjects with POI. This concept is supported by the results of previous studies of patients with POI due to steroidogenic cell autoimmunity and patients with functional hypothalamic amenorrhea, who had measureable serum AMH levels despite a diagnosis of POI (Falorni et al. 2012, Jonard et al. 2005, La Marca et al. 2006a, La Marca et al. 2009). However, in some cases of hypogonadotropic hypogonadism, serum AMH levels have been found to be undetectable or low (Sonntag et al. 2012, Tran et al. 2011). In the future, after validation of AMH assay in larger clinical studies carried out for the purpose, AMH data may be used as a tool for counseling women (or parents of younger girls) who may be at risk of developing POI. Ovarian tissue cryopreservation may provide an option for TS girls to preserve their fertility in the future, but the risks of pregnancies have to be carefully taken into account (Hagman et al. 2013, Karnis 2012).
5.3.2 AMH as a marker of GCTs

The utility of serum AMH measurement in GCT patients was investigated in Study III. Both serum AMH and inhibin B levels were significantly elevated in patients with macroscopic disease compared with patients with no evidence of disease – in subjects without ovarian function as well as in subjects with one or two functional ovaries (Figure 10). This finding was confirmed in longitudinal follow-up of individual patients. However, in some subjects with no evidence of macroscopic disease, serum AMH and/or inhibin B levels were elevated (Figure 10) and the majority of these patients suffered relapse after a median time of 3.4 years (range 0.7–8.2) in connection with AMH and 2.8 years (range 0.3–8.3) as regards inhibin B. Nevertheless, the utility of an early rise of marker levels needs to be confirmed in future studies. It has to be noted, however, that in epithelial ovarian cancer, early treatment of patients with elevated levels of tumor markers but no macroscopic disease does not improve survival (Rustin et al. 2010).
Fig. 10. Serum AMH and inhibin B levels in women with GCTs according to the presence of macroscopic disease. WD = with macroscopic disease, DF = no macroscopic disease. Serum AMH levels (A) and inhibin B levels (C) in patients with no ovarian function at sample retrieval (Ov-). Dashed oval in DF boxplot indicates elevated values with no macroscopic tumor growth. Serum AMH levels (B) and inhibin B levels (D) in patients with one or two functional ovaries at sample retrieval (Ov+). Data is presented in quartile box plots and a logarithmic scale. Dashed line indicates mean. **p < 0.001.

The diagnostic accuracy of serum AMH and inhibin B measurements in the detection of macroscopic disease was investigated and the data are presented in Table 4 and Figure 11. The cut off-values for pre- and postmenopausal women given by the manufacturers and used in study were <13 µg/l and <0.1 µg/l for AMH, and <200 ng/l and <10 ng/l for inhibin B. There were no differences in sensitivity, specificity or positive and negative predictive values of AMH and inhibin B. However, the best diagnostic accuracy was reached when the data concerning these two markers were combined (Table 4). It has to be noted that a few of patients with GCTs (6%) were negative as regards both markers.
Table 4. Sensitivity, specificity, and positive and negative predictive values of S-AMH and S-inhibin B concentrations based on the cut-off-values given by the manufacturers.

<table>
<thead>
<tr>
<th>Value</th>
<th>AMH</th>
<th>Inhibin B</th>
<th>AMH+Inhibin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>83%</td>
<td>88%</td>
<td>93%</td>
</tr>
<tr>
<td>Specificity</td>
<td>88%</td>
<td>90%</td>
<td>92%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>54%</td>
<td>59%</td>
<td>64%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>97%</td>
<td>98%</td>
<td>99%</td>
</tr>
</tbody>
</table>

ROC curves and AUC values were analyzed to confirm the accuracy of the measurements in detecting the macroscopic tumors in individual patients (Figure 11). No statistically significant difference was observed between AMH and inhibin B in either of the groups concerned: subjects without ovarian function and subjects with one or two functional ovaries. In subjects with one or two functional ovaries, the cut-off value of AMH given by the manufacturer (13 μg/l) may be too high, leading to several false-negative diagnoses. The establishment of validated age-specific reference values for healthy premenopausal women and the adjustment of optimal AMH cut-off values by studying a larger number of GCT patients with one or two functional ovaries would improve the diagnostics in the future.
Fig. 11. Receiver Operator Characteristic (ROC) curves for S-AMH (solid line) and inhibin B (dashed line) in all subjects (A), in patients with no ovarian function (B) and in patients with one or two functional ovaries (C). Ov− = patients with no functional ovaries at sample retrieval, Ov+ = patients with at least one functional ovary at sample retrieval, AUC = area under the curve, CI = confidence interval, Sens. = sensitivity, Spec. = specificity.
Serum AMH levels were correlated positively to tumor size, supporting the results of a previous study (Chang et al. 2009). In large-sized GCTs AMH expression has been shown to be decreased (Anttonen et al. 2005). This finding was not supported in the current study. However, the form in which AMH is secreted into the circulation in women with GCTs is not known.

There was no fluctuation in serum AMH or inhibin B levels during longitudinal follow-up of the patients with no macroscopic disease, regardless of ovarian status. Serum AMH levels have been shown to fluctuate only slightly during the menstrual cycle, compared with the wider fluctuation of inhibin B levels in healthy premenopausal women (Groome et al. 1996, Streuli et al. 2009), but this difference was not observed in the current study. However, the sample size of the premenopausal subjects during follow-up in the present study was relatively small, which may have affected the results.

5.4 The effect of different CHCs on ovarian function

The effects of continuous use of oral, transdermal and vaginal CHCs on ovarian function were compared in Study IV. Serum AMH levels decreased gradually in all three study groups, but the decline was significant only after 9 weeks of treatment (Figure 12, Table 5).

![Fig. 12. Serum AMH levels at baseline and after 5 and 9 weeks of use of CHCs via three different administration routes (oral [Pill], transdermal [Patch] and vaginal [Ring]). *p = 0.001 vs. baseline. Published by permission of Elsevier.](image-url)
Table 5. Mean (SD) hormone levels at baseline and at 5 and 9 weeks of treatment in the oral pill, transdermal patch and vaginal ring groups. Published by permission of Elsevier.

<table>
<thead>
<tr>
<th>Hormone (ng/ml)</th>
<th>wk</th>
<th>Pill</th>
<th>p</th>
<th>Patch</th>
<th>p</th>
<th>Ring</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>0</td>
<td>3.88 (3.0)</td>
<td></td>
<td>3.86 (3.6)</td>
<td></td>
<td>4.27 (3.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.34 (2.8)</td>
<td></td>
<td>2.89 (1.9)</td>
<td></td>
<td>3.75 (2.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.91 (1.5)</td>
<td>0.001</td>
<td>1.96 (1.3)</td>
<td>0.001</td>
<td>2.25 (1.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Inhibin B (ng/l)</td>
<td>0</td>
<td>75.56 (29.4)</td>
<td></td>
<td>53.47 (34.1)</td>
<td></td>
<td>80.37 (4.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.58 (5.9)</td>
<td></td>
<td>5.37 (3.5)</td>
<td></td>
<td>10.60 (10.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4.14 (3.2)</td>
<td>&lt;0.001</td>
<td>3.03 (2.8)</td>
<td>&lt;0.001</td>
<td>8.00 (12.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>0</td>
<td>5.91 (1.9)</td>
<td></td>
<td>5.64 (2.7)</td>
<td></td>
<td>5.74 (1.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.33 (1.8)</td>
<td></td>
<td>0.61 (0.9)</td>
<td></td>
<td>1.14 (1.9)</td>
<td></td>
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<tr>
<td></td>
<td>9</td>
<td>0.86 (1.0)</td>
<td>&lt;0.001</td>
<td>0.36 (0.5)</td>
<td>&lt;0.001</td>
<td>1.65 (1.9)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>0</td>
<td>5.00 (1.9)</td>
<td></td>
<td>4.71 (1.9)</td>
<td></td>
<td>5.18 (2.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.39 (0.5)</td>
<td></td>
<td>0.27 (0.6)</td>
<td></td>
<td>0.36 (0.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.22 (0.4)</td>
<td>&lt;0.001</td>
<td>0.10 (0.2)</td>
<td>&lt;0.001</td>
<td>0.58 (1.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E2 (pmol/l)</td>
<td>0</td>
<td>0.12 (0.07)</td>
<td></td>
<td>0.12 (0.11)</td>
<td></td>
<td>0.10 (0.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.014 (0.01)</td>
<td></td>
<td>0.008 (0.004)</td>
<td></td>
<td>0.015 (0.006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.013 (0.01)</td>
<td>&lt;0.001</td>
<td>0.007 (0.002)</td>
<td>&lt;0.001</td>
<td>0.013 (0.01)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values of p indicate differences in serum hormone levels within a group.

* p = 0.019 vaginal ring vs. transdermal patch group after 9 weeks' use of CHCs.

Significant but more rapid declines were detected in serum levels of inhibin B, E2 and gonadotropins at 5 weeks of treatment (Table 5). Thus, the results demonstrate that CHCs inhibit follicular growth equally, independently of administration route.

The decline of serum AMH levels may be explained by the arrest of follicular growth of small antral follicles, which are already responsive to FSH but not yet selected to the FSH-dependent cohort (Gougeon 1996). Theoretically, the decline of small antral follicles, which have just reached FSH-dependency, may partly contribute to the decline of circulating AMH as well, as follicles of 5–8 mm diameter have been shown to secrete AMH and follicles of >5mm are already dependent on FSH (Andersen et al. 2010, Gougeon 1996, Jeppesen et al. 2013). The granulosa cell mass of these small antral follicles is greater than that of follicles of preceding phases and thus the decrease of circulating FSH levels
caused by CHCs mainly affects these follicles (Figure 13). This hypothesis is supported by the more rapid and intense decrease of serum inhibin B and E2 levels observed in the study, as these hormones originate mainly from larger FSH-dependent follicles (Andersen et al. 2010, Gougeon 2003). However, the decrease of serum AMH levels may at least partially be a result of protracted growth of preantral follicles as well, as in previous studies the absence of FSH caused impairment of early follicular growth, and exogenous administration of FSH restored the growth (Halpin et al. 1986, Tran et al. 2011, Wang & Greenwald 1993). In addition, a direct inhibitory effect of E2 on serum AMH levels cannot be fully excluded, as E2 has been shown to inhibit AMH secretion in vitro in a recent study (Grynberg et al. 2012).
Fig. 13. The effect of FSH on follicle development and AMH secretion in the ovary without (A) and during the continuous use of combined contraceptives (B). Combined contraceptives reduce the stimulatory effect of FSH on follicular growth, mainly at the antral stage, which leads to a significant decrease in AMH secretion. Published by permission of Elsevier.

The decrease of serum AMH levels was more pronounced in the current study than in previous reports in which cyclic oral CHCs were mainly used, and serum AMH concentrations were slightly decreased or remained unchanged after initiation of the CHCs (Li et al. 2011, Somunkiran et al. 2007, Streuli et al. 2008, van den Berg et al. 2010). The continuous administration of CHCs used in this
study may explain this difference, as the results of some previous studies have suggested that a shortened hormone-free interval during the use of oral CHCs suppresses pituitary-ovarian function more effectively than the standard 21/7 administration regimen (Schlaff et al. 2004, Spona et al. 1996, Sullivan et al. 1999, Willis et al. 2006). Furthermore, long-lasting GnRH treatment used in a previous study resulted in a reduction of serum AMH levels by approximately 50% and serum inhibin B, E2 and gonadotropin levels were suppressed nearly completely (Hagen et al. 2012a), supporting the results of the present study. In contrast, during shorter GnRH treatment, serum AMH levels have been found to increase slightly, while the concentrations of other hormones (inhibin B, E2, FSH, LH) were decreased (Jayaprakasan et al. 2008). The role of continuous use of CHCs on ovarian activity needs to be investigated further.

Serum levels of AMH, inhibin B, E2 and LH measured at baseline and after 5 and 9 weeks of treatment were comparable between the groups. However, serum FSH levels were higher in the vaginal ring group than in transdermal patch users after 9 weeks of treatment. Exposure to EE has been demonstrated to be lowest in vaginal ring users when compared with oral pill or transdermal patch users (Vandever et al. 2008). This may induce less effective negative feedback to the pituitary and subsequently higher FSH levels in vaginal CHC users. However, the similar significant declines of other hormones measured suggest that a threshold for follicular growth inhibition was reached with all CHCs studied.

The continuous use of CHCs is increasing (Sulak 2008). There are no previous data available on how this regimen affects serum AMH levels. The present results indicate that the continuous use of CHCs has a strong effect on follicular growth as serum AMH levels were approximately 50% lower compared with baseline in all study groups. This alteration must be taken into account if AMH measurement is used as an indicator of ovarian reserve in healthy women with noncyclic use of CHCs.

5.5 Methodological considerations

There may be several factors affecting the study results. The availability of human tissue samples is limited and thus the sample sizes were relatively low in Studies I & II. The tissues were collected at autopsy and biopsy and were carefully processed to minimize damage. However, it is possible that some protein degradation may have occurred prior to fixation. In immunohistochemical procedures, AMH propeptide antibody was used. Propeptide AMH is not yet
cleaved and thus this may give different results compared with antibodies binding AMH protein after cleavage. There was no blocking peptide available for the AMH antibody used, but negative controls showed no staining and the results were validated with positive control tissue samples as well. However, even with a carefully planned protocol, there is always a possibility of some nonspecific staining, which has to be taken into consideration when interpreting the results.

Circulating concentrations of serum AMH were measured by means of an ELISA. Commercial assays for AMH have not been available for long and thus there are limited data concerning the functionality of the assays. The previously available DSL-II assay was used in Studies I and II and the current AMH Gen II assay was used in Studies III & IV (Kumar et al. 2010) according to the instructions of the manufacturers. There was no comparison between the two assays in the studies. Serum samples containing more AMH than the highest standard were diluted and additional control samples were used to confirm the results.

Recently, problems have been reported in the Gen II assay. According to manufacturer, in the undiluted samples the AMH concentrations may be lower than expected due to interference from complement. This may affect the clinical interpretation of especially low serum AMH levels analyzed by the assay. This problem was noticed by our study group in the study III prior official information from the manufacturer. In study III, we originally diluted all the samples with buffer solution and noticed that the serum AMH levels were significantly higher in diluted than in nondiluted samples. The manufacturer recommended to analyze samples without buffer dilution. Therefore we reanalyzed the samples without dilution, and samples with AMH values higher than the highest standard were diluted with a pool of normal postmenopausal female serum. A correlation of serum AMH levels was observed between buffer-diluted samples and samples which were not diluted with buffer. In study IV, this problem was not yet known but none of the study samples contained especially low serum AMH concentrations. Currently the manufacturer recommends buffer dilution but optimal conditions for Gen II assay need still to be clarified.

Serum samples from women with FSHRO (Study II) and some samples from GCT subjects (Study III) were collected for our earlier studies and it is possible that the long storage period may have affected the AMH levels measured. However, the samples were stored mainly at \(-80\,\degree\text{C}\) and the results of previous studies indicate that long-term storage does not significantly affect serum AMH
levels if the samples are stored properly (Fleming et al. 2013, Mulders et al. 2004).

The sample sizes in all studies except Study III were relatively small, which is explained by the rarity of the diseases studied and partly by the lack of volunteers fulfilling the necessary criteria. However, follow-up was conducted prospectively and longitudinally in all studies, with several samples from the same subjects, apart from in Study III in which some of the samples were collected retrospectively. In Study III serum was not available from all patients for analysis prior to removal of the tumor. However, despite the rarity of the disease, the number of GCT patients was the highest so far reported in a study and they were carefully followed, which makes the study unique.

Study IV was a spin-off study of a prospective, randomized trial, and the study protocol was not designed for the current study. Unfortunately ultrasonography was performed only at baseline and thus possible changes in AFC could not be detected. In addition, in Study II, only retrospective data was available as regards ultrasonographic findings in women with FSHRO and there was no such data concerning TS subjects. Measurement of AFC in Study II may have provided additional support to the results. Furthermore, the transabdominal ultrasonography in infants in Study I was challenging as the ovaries were hard to identify. The most obvious reason for this was the lack of antral follicles in immature ovaries, rather than technical problems.
6 Summary and conclusions

The current study produced novel information concerning AMH secretion in ovarian development and various disorders of ovarian function.

In fetal ovaries, the expression of AMH in follicular GCs was found to be initiated as early as at midgestation, approximately at the time of follicular formation and it increased gradually towards term. In infancy, serum levels of AMH were low during the first week of life and increased with postmenstrual age, indicating that AMH secretion and number of follicles beyond very early stages is still low at birth in preterm girls.

In the neonatal and postnatal period, transient ovarian maturation was observed. This maturation occurred later in prematurely born girls, as reflected in low AMH levels and lower numbers of antral follicles. Thus negative feedback from the ovaries to the pituitary was insufficient, which resulted in higher circulating FSH levels compared with full-term infants. The effect of this delayed maturation as regards later reproductive function is not clear and needs further investigation.

The results indicate that serum AMH assay can be used to identify women with POI and existing follicles, as women with FSHRO, who are known to have follicular growth up to small antral stage, had serum AMH levels in low normal range. Furthermore, girls and women with TS mosaicism had significantly higher serum AMH levels compared with other karyotypes, reflecting higher ovarian reserve.

The diagnostics and follow-up of granulosa cell tumors are challenging as a result of the rarity of the disease and late possible recurrences. The present results indicate that combining serum AMH measurement with assay of the currently used marker inhibin B improves GCT follow-up in individual patients compared with inhibin B assessment alone. ROC analyses revealed that serum AMH and inhibin B measurements perform similarly in detecting macroscopic tumors. Since there is no clinically significant cycle fluctuation in AMH levels in premenopausal women, it can be measured at any time, as also supported by unchanged levels during the follow-up of disease-free GCT subjects in this study. The results demonstrate that serum AMH levels may be elevated several years prior to the detection of macroscopic tumor. Whether this improves survival needs further investigation.

The continuous use of CHCs inhibited ovarian activity significantly and independently of the administration route, as serum AMH levels decreased
similarly during the use of oral, transdermal and vaginal contraceptives. As the inhibition of AMH secretion during continuous use of CHCs seems to be more profound than that seen in traditional cyclic administration, the mode of administration needs to be taken into account when estimating ovarian reserve in CHC users.

The expression of AMH protein was observed in small amounts as early as from the primordial/transitional stage of follicle development. Thus, the secretion of AMH precedes or coincides with the initiation of follicular development and the possible growth-inhibitory role of AMH may already be operational from resting follicular stage.

The hypothesis that AMH is secreted mainly from follicles which have not reached FSH dependency was confirmed in the study, as women with FSHRO had low normal serum AMH levels despite the lack of a stimulatory effect of FSH on follicular growth. Furthermore, the results of Study IV indicate that some small antral follicles, which are sensitive to FSH or which have already reached FSH dependency, may contribute significantly to AMH secretion, as the continuous use of CHCs decreased serum AMH levels by approximately 50% as a result of diminished gonadotropin stimulation. The GC mass of these follicles is higher than that of preantral follicles, leading to higher AMH secretion. This concept is supported by the notion that in GCT patients with large GC masses (Study III) there was a positive correlation between serum AMH levels and tumor size.

The role of AMH as a regulator of ovarian function is still not well understood and needs further investigation. Nevertheless, serum AMH assay has already been shown to be clinically beneficial and the indications for its use are constantly extending. However, the existing assays are not optimal, especially where there are high or low AMH levels, but new assays with better sensitivity and wider detection ranges are under development and should be available in the near future.
References


Original publications


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1204. Arvonen, Miika (2013) Intestinal immune activation in juvenile idiopathic arthritis
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