CLINICAL APPLICATIONS OF SERUM ANTI-MÜLLERIAN HORMONE

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CLINICAL APPLICATIONS OF SERUM ANTI-MÜLLERIAN HORMONE

Klinische toepassingen
van het anti-Müllers hormoon in serum

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PROMOTIECOMMISSIE

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GENERAL INTRODUCTION

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1.1 OVARIAN PHYSIOLOGY

Embryology

During the fifth week of fetal life, formation of the genital ridges starts in the posterior abdominal wall, in response to colonization by primordial germ cells, migrating from the yolk sac. By the end of the sixth week, the male and female gonads are indifferent. Differentiation of the gonads is triggered by the sex-determining-region on the Y-chromosome. The first step in male gonadal development is differentiation of Sertoli cells, which produce anti-Müllerian hormone (AMH). Subsequently, between the eighth and tenth week, AMH induces regression of the Müllerian ducts. Thereafter, mesenchymal cells differentiate into Leydig cells, which produce testosterone. Further differentiation of the urogenital system into male derivatives is then ensured by testosterone. In absence of the sex-determining-region on the Y-chromosome, producing the testis determining factor, the gonad differentiates into a female genital system. In addition, production of AMH is lacking and Müllerian ducts, also called the paramesonephric ducts, continue to differentiate into fallopian tubes, uterus and upper part of the vagina. From the seventh week of fetal life onwards, germ cells differentiate into oogonia. Through mitotic cell divisions, the number of oogonia increases steadily and during the third month, the first oogonia begin to transform into oocytes. From the fifth month onwards, the oogonia enter meiosis. In addition, the oocytes are encapsuled by a single layer of pregranulosa cells, forming the primordial follicles (1). Meiosis is arrested during the first prophase of the first meiotic division, when the homologous chromosomes pair and the primordial follicles enter a dormant state.

Menstrual cycle

Menarche is the onset of the menstrual cycle and thus the start of the reproductive lifespan, which begins after full establishment of the hypothalamic-pituitary-gonadal axis. Normal reproductive function involves monthly follicle development, ovulation and preparation of the endometrium for implantation. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play a central role in the regulation of ovarian function. These hormones are produced by the gonadotrophes of the anterior pituitary gland. Secretion of the gonadotrophins is regulated by gonadotrophin-releasing hormone (GnRH), a decapeptide secreted by the neurons of one of the basal nuclei in the hypothalamus. Both gonadotrophins are required for follicular development and steroid hormone production in the ovary. These steroid hormones regulate gonadotrophin secretion and GnRH pulsatility by positive and negative feedback during the menstrual cycle (2).

During the luteo-follicular transition of a normal menstrual cycle, a certain concentration of FSH is needed to recruit follicles. More specifically, only those antral follicles responding to the FSH surge during the luteo-follicular transition continue to grow. One of the factors, exerting a negative feedback on FSH secretion is serum inhibin B, a member of the TGF-β family (3). It is produced by granulosa cells of small antral follicles and serum levels increase with increasing diameters of follicles during

the early follicular phase. Subsequently, high inhibin B levels decrease FSH levels via a negative feedback, thereby restricting the window of FSH sensitivity and allowing only a limited number of small antral follicles to be recruited for further growth and development. Follicles that are not responsive to the FSH surge will degenerate and become atretic. This process is also referred to as the FSH threshold concept (4). Each month a few of these follicles will surpass the 'FSH threshold' in order to develop until the antral stage. At the end of the follicular phase, only one of these follicles, due to differences in size and biopotency will gain dominance and resume gametogenesis by completing the first meiotic division. Eventually this follicle will ovulate and upon fertilization this oocyte will complete the second meiotic division (Figure 1.1) (1, 4).

The number of follicles that will ultimately reach the pre-ovulatory stage will be limited by the magnitude and duration of FSH stimulation (5). In the end, only 400 follicles will fully mature and ovulate during a woman's reproductive lifespan resulting in about 400 menstrual cycles. At any point during the menstrual cycle, follicles of different developmental stages are present and can become atretic. Indeed, the majority of follicles leaving the primordial follicle pool will eventually undergo atresia (1). Hence, a follicle seems to be destined to deteriorate rather than to develop until the pre-ovulatory stage and ovulate. Therefore, this process of follicle growth, development, and selection appears to be quite wasteful. This suggests the presence
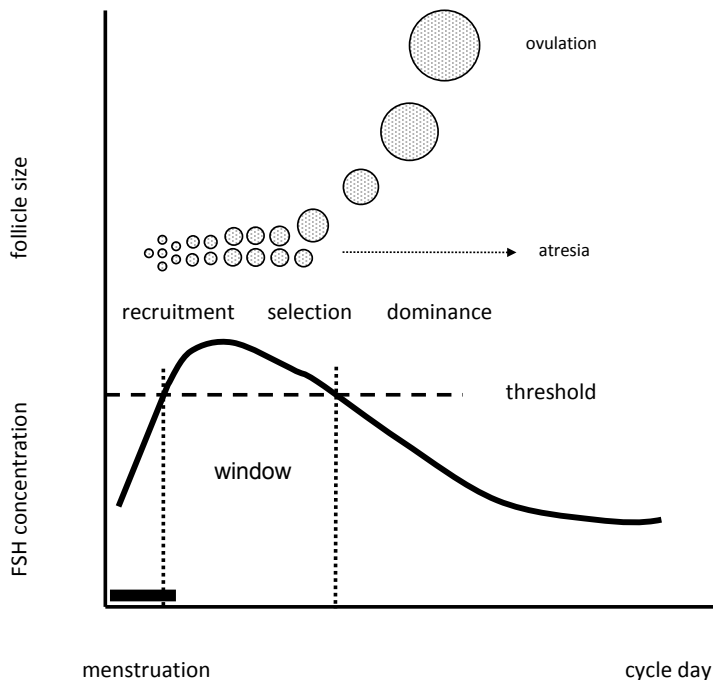

Figure 1.1. FSH dependent recruitment of antral follicles in the FSH window.

of some regulatory mechanism at various points in folliculogenesis that distinguishes healthy follicles, destined to ovulate, from the less viable ones, those destined to become atretic. FSH is one of these regulating factors, because of its important role in follicle selection, i.e. FSH-dependent selection of pre-antral follicles and subsequent selection for dominance during the antral stage (5). Functional FSH receptors are not expressed in primordial follicles (1, 6, 7). Indeed, recruitment from the primordial follicle pool is thought to be independent from FSH (Figure 1.1). Besides FSH itself, the FSH sensitivity of antral follicles seems to be regulated by intra-ovarian growth factors (1, 4). Apparently, these intra-ovarian factors are also key factors of early folliculogenesis (8, 9). The transforming growth factor β (TGF-β) superfamily, which includes activins, inhibins, growth and differentiation factors, bone morphogenetic proteins and several other members such as AMH, has been suggested to affect different steps of folliculogenesis, in a paracrine or autocrine fashion (9, 10) (Figure 1.2). The role of AMH in the ovary is discussed in paragraph 3.4.

Ovarian reserve throughout life

Duration of fertility is determined by the number of primordial follicles, the ovarian reserve (11). Around the fifth month, a maximum number of approximately 7 million germ cells is reached (12-14). Since at this stage, the first oocytes will enter meiosis, their number will not increase any further (1, 12). In addition, the oocytes are encapsuled by a single layer of pregranulosa cells, forming the primordial follicles. Those oocytes that do not become surrounded by pregranulosa cells are lost through apoptosis (1). As a result, an exponential decrease in the number of primordial follicles is observed until birth and continues during childhood, so that at menarche approximately 500.000
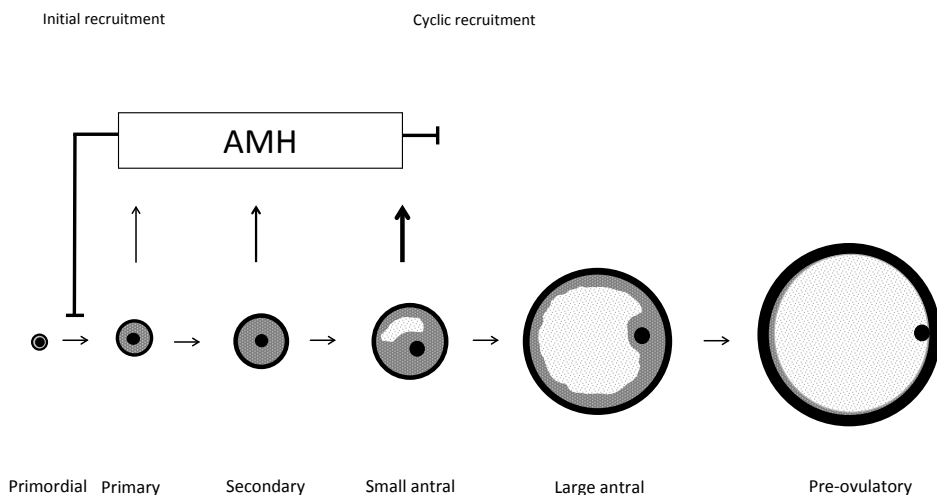
Figure 1.2. Initial recruitment and cyclic recruitment during stages of folliculogenesis, with AMH expression and AMH function.

primordial follicles remain (13-15). During reproductive lifespan, the number of follicles decreases gradually at a fixed rate, until the age of 35 years. From this point onwards, follicle loss is accelerated and finally, the primordial follicle pool is exhausted and menopause is reached.

In natural fertility populations, the mean maternal age at the birth of their last child is at 40-41 years. This age can be regarded at the mean age at which female fertility comes to an end and sterility starts. At that age, most women have seemingly normal regular cycles, with a biphasic basal body temperature, reflecting progesterone production during the luteal phase. Surprisingly, although the menopause is traditionally regarded as the age at which sterility ensues, it occurs about ten years later, at a mean age of 51 years with a range from 40 to 60 years (16-18). The age at which women become sterile shows the same degree of variation as observed for the age of menopause (19). Oligomenorrhoea and cycle shortening, which are distinct clinical signs of ovarian ageing, do not seem to be indicative of the onset of decreasing fertility. Since the end of fertility may be reached about 10 years prior to menopause, the decline of fertility may start as early as mid-twenties (20). So, whereas menopause is a clear clinical feature, the onset of accelerated follicle loss, and thus decreased fertility are far less distinct.

Along with this decrease in the number of oocytes, there is also a noticeable decrease in oocyte quality (21-25). The primordial follicles leaving the pool later in life have been exposed to more environmental disruptors which might damage DNA integrity and functionality. Accordingly, more chromosomal abnormalities, such as trisomy 21 due to non-disjunction of chromosomes in older oocytes, are observed in embryos of women of advanced age as compared with those of younger women (21, 26-28). Similarly, advanced maternal age is associated with increased rates of miscarriage, congenital malformations and stillbirth (11, 24, 29, 30). Hence, both oocyte quantity and oocyte quality decrease with increasing age.

1.2. OVARIAN PATHOPHYSIOLOGY

Anovulation

Ovarian dysfunction results in subfertility and endocrine disturbances. A woman is proven fertile after a spontaneous pregnancy has been established. In females, this requires at least the development of a competent oocyte and ovulation. About 80% of normal fertile couples will conceive within one year of unprotected intercourse, while the remaining 20% is subfertile. Besides physiological ageing, one of the most common disorders in female subfertility is anovulation (25, 31). Oligo-ovulation is defined as less than 8 ovulations per year and anovulation is defined as no ovulations at all (32). Several disorders may coincide with ovarian dysfunction, resulting in anovulation and consequently infertility. Classification of anovulatory infertility should serve multiple goals (33). First, a correct diagnosis of disease states related to ovarian dysfunction might establish short-term (i.e., adrenal tumours, prolactinomas, or autoimmune

disease) and long-term health risks, such as type 2 diabetes mellitus, cardiovascular disease, and endometrial cancer. Secondly, the classification also provides guidance for therapy; it should be established whether ovulation induction may be useful. Finally, classification may discriminate between patients with favourable or unfavourable chances to respond to different ovulation induction strategies, and may help to identify the best and most cost effective strategy in a particular patient (33). The current classification for anovulatory infertility, supported by the World Health Organization (WHO), is based on the measurement of three distinctive hormones i.e., FSH, LH and oestradiol. There are three distinct classes to be distinguished: the first class, WHO I, includes patients with low gonadotrophin and low oestradiol levels. If these women remain anovulatory and thus stay in a hypo-oestrogenic state, they are at risk for osteoporosis and probably bone fractures later in life (34, 35). When gonadotrophin and oestradiol levels are normal, patients are classified into the second class, designated as WHO II. Depending on the definition used, this class also includes a smaller or larger number of women suffering from polycystic ovary syndrome (PCOS), the most common endocrinopathy in women of reproductive age. Women with PCOS may present with oligo-anovulation, hyperandrogenism and/or polycystic ovarian morphology on ultrasound (33, 36). There is clear evidence that women with PCOS are at increased risk for developing type 2 diabetes mellitus (37-39). Those women suffering from oligomenorrhea or amenorrhea also have a life time increased risk to develop endometrial cancer. In addition, PCOS seems to be associated with unfavourable lipid profiles and other cardiovascular disease markers. It remains to be established whether hard endpoints, such as hypertension, ischaemic heart disease and cerebrovascular accidents are also increased in these women (40-42). Finally, patients with hypergonadotrophic, hypo-oestrogenic anovulation constitute the WHO III class. In fact this is the end point of every woman's reproductive life. In some women this phase might ensue prematurely, leading to premature ovarian insufficiency (POI). Apparently, the ovary is not responsive, despite extremely elevated gonadotrophin levels, because the primordial follicle pool has become exhausted. These women may have postmenopausal symptoms and besides the risk of osteoporosis, they might also develop cardiovascular disease and possibly Alzheimer's disease (43).

Accordingly, in WHO I and II classes, ovulation induction, by increasing endogenous FSH levels, will be the first treatment of choice. In WHO III patients, endogenous gonadotrophins levels are already increased and due to the exhaustion of the primordial follicle pool, *in vitro* fertilization treatment (IVF) with oocyte donation remains the only therapeutic option (33).

Iatrogenic ovarian pathology

Survival rates of childhood and adult cancer have improved dramatically during the last few decades (44). Consequently, long-term effects of chemotherapy and/or radiotherapy are becoming more apparent. One of the adverse long-term effects of cancer treatment is gonadotoxicity and consequently impaired fertility (45-47). Although some chemotherapeutic approaches do not affect the number of primordial

follicles, most cancer treatments seem to reduce the number of primordial follicles and subsequently give rise to subfertility or even sterility. Currently, options for preservation of fertility, such as cryopreservation of ovarian tissue or oocytes, are available. However, due to considerable differences in numbers of primordial follicles between women at any given age, markers measuring the actual primordial follicle pool are needed. These markers of residual fertility and reproductive potential are needed, in order to guide the clinician in appropriately counseling patients and their parents on the possibilities to preserve fertility prior to treatment.

1.3. MARKERS OF OVARIAN DYSFUNCTION

FSH

In women with PCOS, FSH levels are within the normal range. It seems that in women with PCOS, follicles are less sensitive to FSH so that selection of a dominant follicle fails (48). Indeed, after administration of low doses of exogenous FSH, the FSH threshold can be easily surpassed and ovulation usually occurs. FSH levels did not seem to be correlated with the extent of PCOS or predictive for chances of successful treatment outcome (49-51).

In the ageing ovary, the number of growing follicles has decreased and consequently, increasing amounts of FSH are necessary to ensure follicle growth. In addition, due to the decreased number of follicles serum inhibin B levels are low and do not exert any negative feedback, resulting in a further increase of serum FSH levels (52). However, FSH is an indirect marker of ovarian function, since it is produced in the pituitary gland at an increased rate due to the release of the negative feedback of inhibins and oestrogens which are not produced anymore by the insufficient ovary. Hence, the rise in FSH serum concentrations constitute a rather late sign of ovarian ageing (53). In addition, for appropriate interpretation of ovarian reserve, FSH levels should be assessed during the early follicular phase.

Inhibins

During the late follicular phase, inhibin B production decreases and inhibin A levels increase, which is the predominant inhibin during the peri-ovulatory and early luteal phase (54). Inhibin B is produced by granulosa cells of developing early antral follicles and constitutes a reflection of the number of recruited antral follicles, cyclicly recruited by FSH. Hence, in order to reflect the size of ovarian reserve, assessment of inhibin B levels is needed during the early follicular phase when small antral follicles are present (2, 22). Although inhibin B is a direct product of the ovary and its serum levels correlate well with the number of small antral follicles, it is not a direct product of the primordial follicle pool (55, 56). Hence, although it constitutes a better marker for ovarian reserve than basal FSH levels, it lacks sufficient power to predict the size of the primordial follicle pool accurately (22, 57-63).

Anti-Müllerian hormone

Recently, serum AMH levels have been described as novel marker of ovarian reserve. In rodents, AMH expression starts immediately after birth in granulosa cells of primary follicles and increases until follicles reach the small antral stage. From the antral stage onwards, AMH expression decreases and is absent in the pre-ovulatory stage (64). This specific expression pattern between initial recruitment and cyclic recruitment indicates that AMH may have a role in these two important steps during folliculogenesis (1, 65).

In AMH null mice, an increased number of growing follicles was observed concomitant with a smaller primordial follicle pool at the age of 4 months, as compared to wild type mice. It was concluded that AMH inhibits initial recruitment, since this process was accelerated in absence of AMH and led to earlier depletion of the primordial follicle pool (66) (Figure 1.2). In addition, a larger number of small antral follicles was observed in adult AMH null mice, despite lower FSH levels. Furthermore, the number of large pre-antral follicles was increased at estrous, whereas in wild type mice these follicles are not sensitive to FSH. Nevertheless, AMH null mice did not have more pre-ovulatory follicles, suggesting that the final selection for dominance was not influenced by AMH. It was suggested that the sensitivity for FSH during monthly follicle selection was increased in absence of AMH. Hence, AMH seems to reduce FSH sensitivity of individual antral follicles (67, 68) (Figure 1.2).

In humans, AMH expression was observed in female fetuses from 36 weeks onwards and continued until menopause (69-72). Similar to the pattern in mice, AMH is expressed in granulosa cells of primary follicles and was highest in small antral follicles, suggesting that AMH may have a comparable role in human folliculogenesis (65, 73, 74) (Figure 1.2). This was indeed supported by genetic studies showing that a polymorphism in the gene encoding for the AMH type II receptor (AMHRII) was associated with the age of menopause. Although this association was only observed in interaction with the number of offspring, this finding confirmed that AMH has a role in the recruitment of primordial follicles in the human ovary (75). Furthermore, polymorphisms in the genes encoding for AMH and AMHRII were associated with oestradiol levels during the follicular phase, suggesting that also in human AMH may affect FSH sensitivity (76-78).

Based on the specific expression pattern solely in small growing follicles, between initial recruitment and cyclic recruitment, it was suggested that serum AMH levels might indirectly reflect the size of the primordial follicle pool, i.e. the ovarian reserve. Indeed, in mice, serum AMH levels decreased with increasing age and were correlated with the decreasing number of growing follicles. As expected, a strong positive correlation was also observed between serum AMH levels and the number of primordial follicles. Importantly, AMH expression in the individual growing follicles did not change with increasing age, indicating that decreasing serum AMH levels in mice reflect the decrease in size of the primordial follicle pool (79).

Clinical studies confirmed that concentrations AMH levels correlated well with antral follicle count. Accordingly, in women of advanced reproductive age, serum AMH

levels were already low, whereas FSH and inhibin B levels were still within normal ranges (80). Moreover, serum AMH concentrations were more predictive of ovarian response to ovarian stimulation during IVF treatment cycles than the traditional markers of ovarian reserve (63, 81). Finally, recent studies have shown that several years prior to menopausal transition, serum AMH levels were low or undetectable and were more predictive of the onset of menopause compared to estimates based on chronological age (82-84). Therefore, serum AMH concentrations are the earliest marker of ovarian ageing and best reflect the reproductive decline.

Since serum AMH levels are a direct marker of the number of small growing follicles, AMH concentrations may also be used as marker in ovarian pathophysiology, such as PCOS. Although the syndrome includes a variety of clinical features, most women with PCOS present with polycystic ovaries, in which the number of pre-antral and small antral follicles is increased. As expected, also in women with PCOS, the antral follicle count was well correlated with serum AMH levels, reflected by the two- to threefold increase of both markers in women with PCOS (51). In addition, serum AMH levels were also correlated with other PCOS features such as cycle duration, ovarian volume and androgen levels (51, 85). Therefore, it was suggested that AMH may be a marker of the severity of PCOS and thus of ovarian dysfunction.

Antral follicle count

The number of primordial follicles decreases with increasing age and consequently, the number of antral follicles decreases as well. Indeed, the number of primordial follicles is indirectly reflected by the number of growing follicles (86). Therefore, the antral follicle count (AFC), defined as all follicles between 2 to 10 mm in diameter, is a good marker for ovarian ageing. Indeed, AFC was predictive for the number of oocytes retrieved after ovarian stimulation (86, 87). Transvaginal ultrasound provides the most direct way to assess the antral follicle count. However, reproducibility of antral follicle count seemed to be rather limited, both within and between observers (88, 89).

One of the phenotypical characteristics of PCOS is an increased number of antral follicles (36). It has been suggested that the failure of follicle selection by FSH results in accumulation of follicles. However, recent studies showed that not only early follicular growth is enhanced in ovaries of PCOS women but there is also a reduced rate of follicle loss as compared to that in normo-ovulatory women (90, 91). Although the etiology of PCOS remains unclear, the cohort of primary and secondary follicles is increased in PCOS (48). Indeed, the extent of anovulation seems to be correlated with a higher number of follicles (92). In addition, an appearance of ovaries on ultrasound was a predictive factor for the outcome of ovulation induction treatment with gonadotrophins (93).

1.4. OUTLINE OF THE THESIS

The aim of this thesis was to study clinical applications of serum AMH measurements. First, normal ranges of serum AMH values in healthy subjects from childhood to menopause are described in the first chapter. In the second part, the use of AMH as marker of ovarian reserve was described in women suspected of impaired fertility. These women have been treated with chemotherapy and/or radiotherapy at adult age (chapter 3.1) or during childhood (chapter 3.2). In addition, pregnancy outcome was described in female cancer survivors (chapter 3.3). In the third part, the role of AMH as a marker of ovarian dysfunction is studied. In chapter 4.1 the role of AMH in the diagnosis of anovulatory infertility was studied. In addition, the diagnostic value of AMH as predictor of ovarian response during ovulation induction treatment in women with PCOS was studied (chapter 4.2). Chapter 4.3 focuses on the predictive value of AMH for embryo quality. Finally, the last chapter provides a general discussion on the application of serum AMH levels in clinical practice and recommendations for future research.

SERUM ANTI-MÜLLERIAN HORMONE LEVELS IN HEALTHY FEMALES: A NOMOGRAM RANGING FROM INFANCY TO ADULTHOOD

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ABSTRACT

Background: Anti-Müllerian hormone (AMH) is a novel marker of ovarian reserve. However, sufficiently large sets of normative data from infancy to the end of reproductive life are still lacking.

Objective: assessment of serum AMH levels in healthy females in relation with follicle stimulating hormone and antral follicle count.

Subjects: In 804 healthy females ranging from infancy until the end of the reproductive period serum AMH levels were measured with an ultrasensitive enzyme-linked immunometric assay. All adults had regular menstrual cycles. The majority was proven fertile and none of them had used oral contraceptive pills three months prior to assessment.

Results: In the total cohort, AMH was inversely correlated with age (r = -0.24; P < 0.001). The age at which the maximum AMH value was attained was at 15.8 years. In girls younger than 15.8 years, serum AMH and age were positively correlated (r = +0.18; P = 0.007). Thereafter, AMH levels remained stable (r = -0.33; P = 0.66). From the age of 25.0 years, there was an inverse correlation between AMH and age (r = -0.47; P < 0.001). At any given age, considerable inter-individual differences in serum AMH levels were observed.

Conclusions: Follicular dynamics during childhood seem to differ from that at adult age. During infancy and puberty, AMH levels increase, whereas during adolescence, levels stabilize and adult follicular dynamics are established. Hence, serum AMH levels seem to constitute a valuable marker of ovarian reserve only from the age of 25 years onwards. Our nomogram may facilitate counseling women on their reproductive potential.

INTRODUCTION

Serum anti-Müllerian hormone (AMH) is solely produced in the human ovary by granulosa cells (73). AMH expression starts in primary follicles as soon as recruitment from the primordial follicle pool is initiated, until the early antral stage during which expression is strongest (64, 73, 77). Expression ceases in follicles with a diameter between 8×10 mm (73). Based on this expression pattern, serum AMH was suggested to reflect the number of early growing follicles (73). Indeed, in mice serum AMH levels correlated strongly with the number of small growing follicles, and more importantly with the number of primordial follicles (79). Although direct assessment of the primordial follicle pool in women is difficult, one study using oophorectomy for benign gynecological reasons confirmed the correlation between serum AMH and primordial follicle number in women (94). In addition, several clinical studies have confirmed the strong correlation between serum AMH and antral follicle count (AFC) (70, 80, 92), and AMH seems the most accurate proxy for not only the cohort of small growing follicles, but also for the actual size of the primordial follicle pool (80). Furthermore, it was shown that AMH can be used as a marker of diminished ovarian reserve (70, 80, 95). Similar to AFC, AMH has been shown to be a better predictor of poor response to controlled ovarian hyperstimulation than age, serum follicle stimulating hormone (FSH) or serum inhibin B concentrations (81, 96, 97).

Besides being a marker of ovarian reserve, AMH was also described as marker for an excess of growing follicles in normogonadotropic normoestrogenic anovulatory infertility and polycystic ovary syndrome (PCOS). As compared with women with regular menstrual cycles, two to three fold higher AMH levels have been observed in women with PCOS (51, 85). Although, serum AMH is used as a marker for ovarian function in clinical practice, data on the range of AMH levels in the normal population have not been determined and thus, information on cut-off levels of the normal range is still lacking. Recently, several studies have aimed to fill this gap in knowledge (97-102). Although large cohorts were studied, the vast majority of women included in these studies were recruited at fertility clinics (97, 98, 100, 101) and may therefore not represent normo-ovulatory women. The present study is unique, by measuring serum AMH levels in a large cohort of healthy females, ranging from infancy to the end of reproductive lifespan, in a single laboratory. In addition, most of the described women of reproductive age were proven fertile.

MATERIALS AND METHODS

Subjects

Healthy children, adolescents and adult women were included. Most subjects had participated in earlier studies as healthy control subjects. For detailed information on recruitment strategy and study populations, we refer to the original papers (70, 80, 86, 103-106). All studies had been previously approved by the different local ethical review

boards. All subjects had given written informed consent. Healthy girls aged up to 18 years were recruited at local primary and secondary schools or at the Sophia Children's hospital prior to minor surgery. Adults (18 years and older) had regular menstrual cycles between 25 and 35 days (70, 80, 86, 103-105) or were proven fertile (106). Adults did not use any hormones or oral contraceptive pills during the last three months prior to participation. Proven fertile women at had given birth at least six months earlier (106). Serum was drawn randomly during the menstrual cycle. In those adult women screened during the early follicular phase, also serum FSH was measured (70, 80, 86, 103). In adults, transvaginal ultrasound was performed on cycle day 3, 4 or 5 to assess AFC (80, 86, 88, 103, 105).

Hormone assays

All serum measurements were performed within one laboratory at the Erasmus MC, Rotterdam, the Netherlands (FdJ). All samples had been stored at − 20˚C until assayed. AMH immunoreactivity in serum samples was stable after repeated freeze–thaw cycles (79). In the older cohorts (70, 80, 86), AMH was measured with an ultrasensitive enzyme-linked immunosorbent assay (ELISA) (Immunotech-Coulter, Marseilles, France) (80) and an in-house AMH-ELISA (commercially available as the GenII Beckman Coulter, Beckman Coulter, Inc., Webster, Texas) was used in study subjects, in whom serum was drawn more recently (76). The values obtained with the Immunotech assay were adjusted with a factor 1.564 for comparison with the currently used ELISA. Intra-assay and interassay coefficients of variation in the Immunotech-Coulter assay were <5% and 8%, and <5% and <10% in the in-house ELISA. The detection limit of the currently used ELISA, defined as the mean of the absorbance of the blank replicates + 2 SDs, was 6.3 pg/ml. For analysis, serum AMH levels below 0.1 microg/L were valued at 0.0 microg/L. FSH was measured with a fluorescence-based immunoassay (Immulite, Diagnostic Products Corp., Los Angeles, CA, USA), with intra- and interassay coefficients of variance <3% and 8%.

Data analysis

Because samples were obtained from subjects that had participated in different studies, AMH levels were compared between the included study cohorts with a univariate analysis of covariance with adjustment for age. Since serum was drawn across the follicular and luteal phase of the menstrual cycle, a similar test was used to compare AMH levels drawn during the follicular phase with those drawn during the luteal phase.

Reference curves for serum AMH levels as function of age were calculated from a linear regression model using a natural cubic spline fitted on log-transformed AMH values. From the fitted curve, a piecewise linear function was calculated to describe variation in AMH levels with increasing age. In addition, we determined the age at which AMH values attained their maximum value, and the 95% CI interval of this age was determined by bootstrapping 5000 times. Consequently, two subgroups could be distinguished: one with increasing AMH levels and a subgroup with decreasing AMH levels. In the total cohort as well as in subgroups, bivariate correlations were performed

between serum AMH and age. In addition, a bivariate correlation was performed between serum AMH levels and the number of follicles. This correlation was also performed between serum AMH levels and FSH levels in samples drawn during the early follicular phase.

RESULTS

A total of 804 healthy infants, children, adolescents and adult women were included. The median age was 24.6 years. The youngest subject was 3.1 months old and the oldest was 46.8 years old. Measurement of AMH concentrations using the two AMH ELISAs did not result in significant differences (median 1.6 vs. 3.3 µg/L; $P = 0.11$) (80). There were no differences in AMH levels drawn during the follicular phase and those measured during the luteal phase after adjustment for age differences (median 1.9 vs. 3.3 µg/L, respectively; $P = 0.08$).

In the total cohort, serum AMH levels were inversely correlated with age ($r = -0.24$; $P < 0.001$). The natural cubic spline function explained 41% of the variance in log(AMH), indicating that variance in AMH was for 41% determined by age. The fitted curve showed that, from birth onwards, serum AMH levels increased slightly until the end of puberty and seemed to remain rather constant until levels decreased from the age of 25.0 onwards (Figures 2.1 and 2. 2). The piecewise linear function failed to show a significant rise in AMH levels during childhood or an attenuation in AMH during adolescence (data not shown). The age at which the maximum value of AMH was attained was estimated as 15.8 years (95% CI 13.5 to 20.3). Prior to the age of 15.8 years, serum AMH and age were positively correlated ($r = +0.18$; $P = 0.007$). Thereafter, AMH levels remained stable, and consequently, the correlation between AMH and age
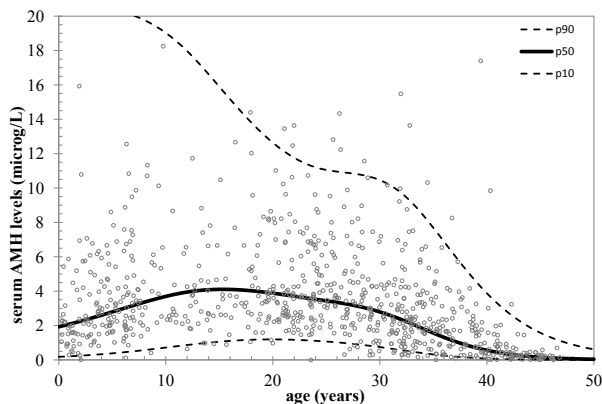
Figure 2.1. Anti-Müllerian hormone (AMH) nomogram based on natural linear spline interpolation. Reference lines of serum AMH for the 10th, 50th and 90th percentile of predicted AMH values versus age.
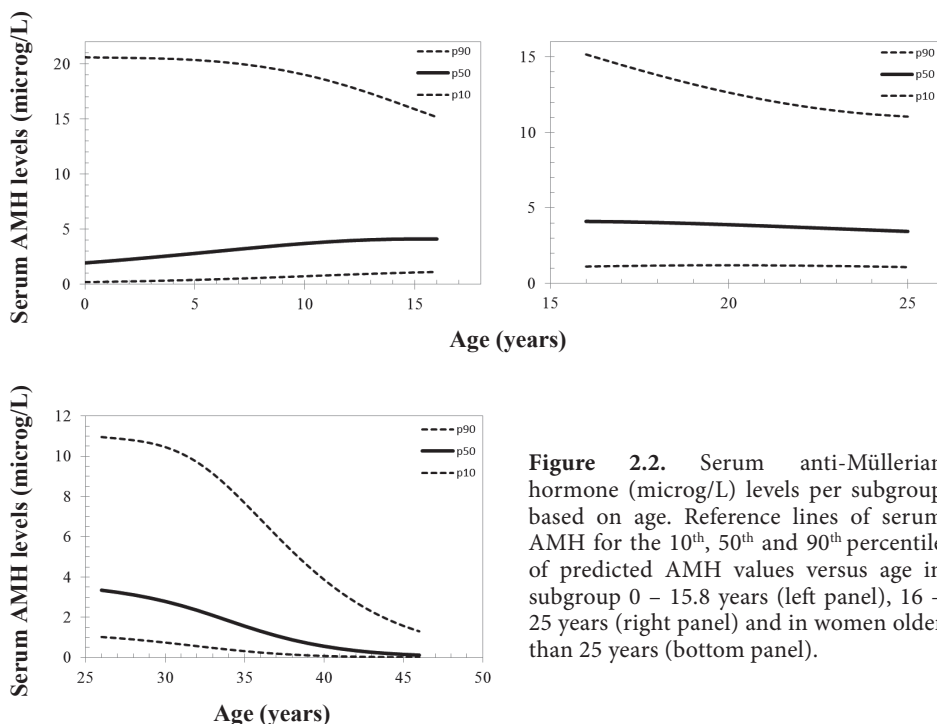
Figure 2.2. Serum anti-Müllerian hormone (microg/L) levels per subgroup based on age. Reference lines of serum AMH for the 10th, 50th and 90th percentile of predicted AMH values versus age in subgroup 0 – 15.8 years (left panel), 16 – 25 years (right panel) and in women older than 25 years (bottom panel).

did not reach statistical significance (r = -0.33; P = 0.66). From the age of 25.0 years, there was an inverse correlation between AMH and age (r = -0.47; P < 0.001).

Antral follicle counts were not available in girls younger than 18 years. In 465 women, aged 18 years and older, antral follicle counts were available. The median antral follicle count was 13 (range 0 - 48). Serum AMH and antral follicle count were positively correlated (r = +0.67; P < 0.001). In 253 adults serum was drawn during the early follicular phase and the median FSH was 6.4 IU/L (range 2.0 – 35.9 IU/L) . Serum AMH and FSH levels were negatively correlated (r = - 0.29; P < 0.001).

DISCUSSION

In this study, serum AMH levels were assessed in healthy females from birth to the end of reproductive life. Our data clearly indicate that the correlation between serum AMH levels and age throughout childhood is different from that during the reproductive lifespan. Serum AMH levels slightly increased during childhood until the mid-teens, and reached a maximum at the age of 15.8 years. Thereafter, AMH levels remained stable until early twenties, and from the age of 25 years decreased with increasing age. Recently, a study in healthy children has described increasing AMH levels during early childhood and thereafter stable AMH concentrations until early adulthood (99). These

findings were confirmed in a nomogram based on previously described AMH levels in children and adults (102). In addition, the authors conclude that the variation in AMH concentrations was mainly determined by factors other than age (102). Indeed, in our data the variance AMH levels was for 41% determined by age, and hence, variation in AMH concentrations may also be determined by variation in the number of AMH producing follicles or by genetic predisposition.

The positive correlation between AMH and age in girls up to 15.8 years is in contrast with the negative correlation between AMH levels and age in women older of 25 years. Therefore, our data suggest that follicular dynamics during childhood differ from that at adult age. Until now, scant data are available on follicular dynamics during childhood. Data on follicle counts were unfortunately not available in our cohort of girls younger than 18 years. The rising AMH levels during childhood suggest that the number of AMH producing granulosa cells increases or that the number of small growing follicles, in which AMH expression is strongest, increases (73). It may be postulated that prior to menarche, an increasing number of follicles is recruited into the growing follicle cohort. Since gonadotropins are absent, these follicles do not reach the antral stage and will eventually atrophy. At the age of 15.8 years a peak level in serum AMH values is reached, suggesting that until the first years after menarche, the size of the growing follicle cohort increases. Possibly, because of irregular FSH pulsatility during the first years after menarche (107, 108), cyclic recruitment of small antral follicles is not yet very efficient, resulting in irregular ovulations. In addition, adrenarche induces an increase in androgen levels prior to the onset of puberty. This rise in androgen concentrations may induce atresia of AMH producing follicles (109), and consequently, attenuating the increasing size of the AMH producing cohort. In the end, these changes during childhood and puberty may inhibit a further increase of AMH levels and thereafter, a steady state in AMH levels between the age of 15.8 and 25.0 years is established. Apparently, cyclic recruitment into the antral stage is more organized, as reflected by regular menstrual cycles.

In contrast to childhood and adolescence, a strong inverse correlation between AMH and age was observed from the age of 25 years onwards, confirming results of previous studies (100, 110). In one study, the authors showed that a quadratic equation was the best model to describe declining serum AMH values with increasing age (100). In our data, a quadratic model could not fit the results of our total cohort. However, when the quadratic equation was applied to AMH levels in women of 25 years and older, a similar curve was obtained as with the natural linear spline interpolation model (data not shown). Our data support the earlier described inverse correlation between serum AMH levels and the decreasing number of antral follicles in women and in rodents (79, 80) and thus, confirm the role of AMH as a proxy of ovarian reserve (70, 80). However, our results also suggest that AMH is only applicable as marker of ovarian reserve in women of 25 years and older.

As described in the previously mentioned studies (99, 102), we observed a large inter-individual variation in AMH levels. Most likely, this variation in AMH levels

may reflect the range in reproductive aging and age of menopause, i.e. from 40 to 60 years (16). Indeed, it has been shown that the age of menopause was more accurately predicted by serum AMH concentrations than by chronological age (83). A recent longitudinal cohort study showed that serum AMH levels drawn approximately 10 years earlier, were highly predictive of the age of menopausal transition (109). The authors of this latter study suggested that AMH is capable of predicting future age at menopause for a given woman. Hence, it may be proposed that at any age, women with AMH at the upper limit of the normal range will enter menopause at a later age as compared to women with AMH levels at the lower limit of the normal range. However, the mean age of women included in the latter cohort study was 35 years (109), at which the inter-individual variation in AMH is smaller than at younger ages, as shown in our data. Consequently, a prediction of age of menopause based on AMH levels measured at young age has to be interpreted with caution, since such a prediction may be less accurate. Furthermore, it remains unknown whether follicle loss follows a similar rate in all women and whether the initial number of primordial follicles, endowed during early fetal life, is similar in all women. Therefore, additional studies with long term follow-up of frequently measured AMH levels in young women are needed to confirm whether AMH can predict age of menopausal transition.

Recent studies in regularly cycling women have remained inconclusive on the relation between serum AMH and FSH levels (80, 111-113). In our results, serum AMH and FSH levels in women older than 25 years were negatively correlated, despite a large inter-individual variation of both hormones. In addition, the inverse correlation between AMH and age was stronger than that between FSH and age, suggesting that AMH is a more accurate marker of ovarian ageing than FSH (80).

In conclusion, our results confirm and expand earlier findings of AMH levels across the reproductive lifespan. We demonstrated increasing AMH levels during childhood until a maximum is reached at the age of 15.8 years. Thereafter, a plateau until the age of 25 years was observed. From the age of 25 years onwards, serum AMH correlate inversely with age, implying that AMH is only applicable as marker of ovarian reserve in women of 25 years and older. Our data further suggest that follicular dynamics during childhood are different than at adult age. Our nomogram may facilitate counseling of women on their reproductive potential.

ANTI-MÜLLERIAN HORMONE AS MARKER OF OVARIAN RESERVE IN CANCER SURVIVORS.

3

ANTI-MÜLLERIAN HORMONE AS A MARKER OF OVARIAN FUNCTION IN WOMEN AFTER CHEMOTHERAPY AND RADIOTHERAPY FOR HAEMATOLOGICAL MALIGNANCIES

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3.1

ABSTRACT

Background: In female cancer survivors, the accelerated loss of primordial follicles as a result of gonadal damage may lead to premature ovarian insufficiency (POI). However, the extent of the damage is unpredictable. Anti-Müllerian hormone (AMH) constitutes a sensitive marker of ovarian reserve. Serum AMH levels were measured to assess sub-clinical ovarian damage in patients treated with gonadotoxic therapy.

Methods: In 25 patients with haematological malignancies serum AMH concentrations were measured prior to and after cancer therapy and were compared with normo-ovulatory controls.

Results: In all patients, AMH concentrations were lower than controls prior to treatment. Thirteen patients were treated with multi-drug chemotherapy. Although in most patients treated with chemotherapy menstrual cyclicity was restored, median serum AMH levels were lower than in controls. Twelve patients had stem cell transplantation (SCT) after total body irradiation. They all developed POI and their serum AMH concentrations were undetectable.

Conclusion: Female cancer survivors treated with SCT all developed POI. Hence, in these patients fertility preservation should be considered. In patients treated with chemotherapy, ovarian reserve seems to be compromised as well.

INTRODUCTION

The number of patients surviving cancer is increasing, hence long-term effects of chemotherapy and/or radiotherapy are becoming more apparent (44, 114). The extent of gonadal damage depends on the treatment modality, the dose received and patients' age. Alkylating agents, such as cyclophosphamide (CY) and procarbazine, as well as radiotherapy are highly gonadotoxic. However, non-gonadotoxic drugs in multi-drug regimens may have a cumulative toxic effect on reproductive function (115). So far, long-term follow-up studies have assessed ovarian function after cancer treatment using parameters such as follicle stimulating hormone (FSH) serum levels or clinical end-points such as cycle length or pregnancy rates (116-122). However, a recent report on gonadal function in childhood cancer survivors showed that, notwithstanding regular menstrual cycles, in patients treated with chemotherapy and cranial radiotherapy, ovarian reserve was compromised as assessed by serum anti-Müllerian hormone (AMH) concentrations (116). Moreover, other studies showed that female cancer survivors with regular menses and normal serum FSH levels had significantly smaller ovaries along with a lower antral follicle count as compared to age-matched controls (123, 124). These observations indicate that parameters such as cycle length and FSH serum levels are not accurate markers of ovarian function. Serum AMH levels, antral follicle count and ovarian volume, appear to be more reliable predictors (116, 123).

Recently, it was shown that serum AMH levels may constitute a valuable marker of ovarian reserve (125). Clinical trials indicated that serum AMH concentrations correlated well with antral follicle count and age. With increasing age, the number of primordial, preantral and small antral follicles declines and concomitantly serum AMH levels decrease. Moreover, in ageing females, AMH levels were decreased, while other factors associated with perimenopausal status, such as early follicular phase FSH, inhibin B or oestradiol serum levels did not change significantly (68, 80, 96). Finally, AMH levels are unaffected by cyclic variations or the use of oral contraceptive pills (OCP) (126-129). Currently, serum AMH levels seem to represent the most reliable marker to determine ovarian reserve.

In the current study, serum AMH concentrations were measured in order to assess the ovarian reserve in women previously treated with gonadotoxic agents for haematological malignancies.

MATERIALS AND METHODS

Subjects

Approval for the study was obtained from the local medical ethics review board. Written informed consent was obtained from each participant.

Premenopausal women from whom serum samples were taken before and after treatment for haematological malignancy at the Erasmus MC University Hospital between January 1995 and December 2004 were eligible.

Women without a history of cancer and with a regular menstrual cycle were recruited as controls. Inclusion criteria were age between 20 and 35 years, body mass index (BMI) between 19 and 26 kg/m2 and normal menstrual cycle length between 26 and 31 days. These women had participated in previous studies (80, 130). In addition, they were healthy and all proven fertile. They did not have any actual or previous endocrine disease, they used neither medication, nor oral contraceptive pills (OCPs), nor any hormonal treatment.

Study design
Prior to treatment as well as during follow-up, blood samples were drawn and serum was stored at   -20°C. Clinical screening at follow-up included recording of obstetric and cycle history, medication use and OCP use (50). Ultrasound scanning included measurement of ovarian volume and total number of follicles and was performed by a single investigator (SLF). In patients with regular menstrual cycles, screening was performed in the early follicular phase (between cycle day 2 and 5). In patients using OCPs, screening was planned at the last day of the pill-free interval (131). Endocrine screening included assays of serum gonadotrophins, oestradiol, inhibin B and AMH. Luteinizing hormone (LH) and FSH levels were measured using a luminescence based immunoassay (Immulite, Diagnostic Products Corp., Los Angeles, CA, USA). A radioimmunoassay was used to assess oestradiol serum levels (Diagnostic Products Corp., Los Angeles, CA, USA). Intra- and interassay coefficients of variation were less than 5 % and 15 % for LH, less than 3% and 8% for FSH and less than 5% and 7% for oestradiol. Serum Inhibin B levels were determined using an enzyme-linked immunoassay as described earlier (Oxford Bio Innovation, Oxford, United Kingdom) (132). Intra- and interassay coefficients of variation were less than 9% and 15%. Serum AMH levels were measured by double-antibody enzyme-linked immunosorbent assay (ELISA) in serum samples taken before and after treatment as described before (133). Intra- and interassay coefficients of variation were 3.6% and 4.0%, respectively. AMH immunoreactivity in serum samples was stable for several days at room temperature and after repeated freeze-thaw cycles (79). Values were adjusted to allow comparison with AMH levels in controls, which were assayed with an ELISA (Immunotech-Coulter, Marseille, France) (80).

Statistics
To assess selection bias, general patient characteristics were compared between enrolled patients and drop-outs, using non-parametric tests. Similarly, non-parametric tests were performed to compare demographic, endocrine, cycle and treatment characteristics in patient subgroups. Serum AMH levels were compared using univariate analysis of covariance, adjusting for age differences. The decline in AMH levels in controls was calculated according to standard formulas for prediction intervals in linear regression analysis, reflecting 5th, 50th and 95th percentiles. The formula: AMH*exp(3.47-0.102*age) reflects the mean decline in AMH levels in controls, adjusting for increasing age. The formula: $AMH*exp(3.47-0.102*age) \pm 1.48*\sqrt{(1.013 + ((age-30.48)/38.45)^2)}$

reflects the 5th and 95th prediction intervals for decline of AMH levels, with adjustment for increasing age (80). The interval between the first and last visit was calculated using the formula: (date of first visit – date of last visit)/365 days. The remaining fraction of ovarian function after cancer therapy was calculated according to a formula comparing the remaining AMH in serum to the AMH serum level at the first visit, controlling for the interval between first and last visit: (AMH at last visit/AMH at first visit)*exp(1/interval). Statistical analysis was performed using Statistical Package for Social Sciences 12.0 (SPSS Inc, Chicago, IL). A P value < 0.05 indicates statistical significance.

RESULTS

For 37 patients pretreatment serum samples were available. All patients were treated with chemotherapy and/or radiotherapy according to HOVON (Dutch haemato-oncology association) protocols (http://www.hovon.nl). Diagnosis, treatment protocol and cumulative doses of chemotherapy and irradiation were recorded for each patient (Table 3.1.1). From 12 women serum was not obtained post-treatment. Two of these twelve had died from their disease. Participation was refused in 4 patients with severe complaints attributable to their treatment. The remaining 6 patients refused for unknown reasons. Amongst the group of drop-outs, a significantly higher number of OCP users was observed. In those enrolled, an increased incidence of oligomenorrhea before treatment was observed ($P < 0.001$). Finally, 25 women were eligible for analysis.

	Lost in follow up n = 12	Participants n= 25
Age at diagnosis (years) (median; range)	31.5 (20.1 – 35.0)	27.5 (16.0 – 36.7)
Age at follow-up (years) (median; range)	34.4 (24.3 – 41.9)	31.4 (22.2 – 40.6)
BMI at diagnosis (kg/m²) (median; range)	22.7 (18.3 – 33.3)	23.7 (16.9 – 33.1)
Cycle at diagnosis		
regular	2 (16.7%)	18 (72.0%)[a]
oligomenorrhoea	0	5 (20.0%)[a]
OCP	4 (33.3%)	0[a]
unknown	6 (50.0%)	2 (8.0%)
Treatment		
Non AA	1 (8.3%)	3 (12.0%)
AA	5 (41.7%)	10 (40.0%)
SCT	6 (50.0%)	12 (48.0%)

Table 3.1.1. Patient characteristics in groups of patients lost in follow-up and participating patients.
[a] = $P < 0,001$ lost in follow-up group versus participants by Chi-square test; BMI = body mass index; OCP = oral contraceptive pills; Non AA= non alkylating chemotherapeutics; AA = alkylating agents; SCT = stem cell transplantation.

These patients were divided into subgroups according to their treatment, i.e. multi-drug chemotherapy (group A, n=13) or stem cell transplantation (SCT) after total body irradiation (TBI) and high dose cyclophosphamide (CY) (group B, n=12). Group C consisted of 42 control women. Patient numbers within group A were too small to allow separate analysis between patients treated with 'non-alkylating agents' and those treated with 'alkylating agents'.

Treatment

In group A, 10 patients received alkylating agents, i.e. cyclophosphamide, procarbazine and/or ifosfamide. The remaining three were treated with non-alkylating chemotherapy, such as methotrexate or idarubicine. In group B, all 12 patients received autologous

	A N = 13	B N = 12	C N= 42
Age at first visit or at treatment (years)	29.4 (16.0 – 36.2)	25.3 (17.9 – 36.6)[b]*	29.9 (19.6 – 35.6)
Cycle before	10 (77%)		
regular	3 (23%)	8 (67%)	42 (100%) [c]***
irregular	0	2 (17%)	
amenorrhoea	0	0	
OCP		0	
unknown		2 (17%)	
AMH before (µg/L)	1.0 (0.01 – 2.9)[a]**	0.9 (0.12 – 2.2)[b]*	2.1 (0.1 – 7.4)
Interval (years)	3.4 (1.2 – 10.6)[a]*	2.8 (1.5 – 11.1)[b]*	1.6 (1 – 7.3)
Age at last visit (years)	33.1 (25.7 – 40.6)	28.9 (22.2 – 40.6)	32.9 (21.3 – 37.9)
Cycle after	3 (23%)	0	42 (100%)[c]**
regular	5 (38%)	0	
irregular	2 (15%)	11 (92%)	
amenorrhoea	3 (23%)	1 (8%)	
OCP			
unknown			
AMH after (µg/L)	0.3 (0 – 1.9)[a]*	0 (0 – 0)[b]***	1.3 (0 – 5)
FSH after (IU/L)	8.5 (3.8 – 154)[a]*	64.4 (7.3 – 313)[b]***	5.8 (2.4 – 13.4)
Inhibin B after (ng/L)	35 (9 – 127)[a]*	n.d. (n.d.)[b]***	113 (4 – 206)
Oestradiol (pmol/L)	239 (27 – 516)	37 (10 – 83)[b]***	163 (70 – 620)
Total number of follicles	8 (1 – 15)[a]**	1 (0 – 3)[b]***	14 (2 – 24)
Median remaining fraction of AMH	73%	13%[b]***	84%

Table 3.1.2. Hormonal and sonographic characteristics before and after treatment in group A, B and C; patients treated with multi drug-chemotherapy, patients treated with stem cell transplant and controls, respectively.
*= P < 0,05; ** = P < 0,01; *** = P < 0,001; [a]= comparison between A and C by Mann-Whitney-U test; [b] = comparison between B and C by Mann-Whitney-U test; [c]= comparison between A and C, B and C by Chi-square test; n.d. = non-detectable; OCP = oral contraceptive pills.

or allogenic SCT after a standardized conditioning regimen including TBI (2 Gray [Gy] or 2x6 Gy) combined with high dose alkylating chemotherapy (CY 60 mg/kg). Serum AMH concentrations were lower in patients as compared to controls (group A versus group C: $P = 0.007$, group B versus group C: $P = 0.02$) (Table 3.1.2). Moreover, patients in group B were younger than controls ($P < 0.05$) and 20% of the patients was oligomenorrhoeic before treatment was started.

After treatment

At follow-up, 24 patients were in complete remission. Three of them had relapsed, but were re-treated successfully. Only one patient was in partial remission. The median interval between the pre- and post-treatment visit was 5.0 years (range 1.2 –11.1 years) (Table 3.1.2).

In subgroup A, serum AMH, inhibin B levels and antral follicle count were significantly lower than in controls ($P = 0.01$; P = 0.02; $P = 0.006$, respectively). In addition, FSH levels were significantly increased ($P = 0.03$). Three (23%) women had continued OCPs immediately after finishing chemotherapy. In the group of patients with regular menstrual cycles at the start of therapy (n = 10), four (31%) had become oligomenorrhoeic and two (15%) amenorrhoeic. Three patients (23%) had regular cycles after treatment, of which one was oligomenorrhoeic prior to chemotherapy. The cycle pattern did not change in one patient (8%) who suffered from oligomenorrhea prior to therapy (Table 3.1.2).

In group B, serum AMH concentrations were significantly decreased, as were inhibin B levels, oestradiol levels and follicle count as compared to group C ($P <0.001$), and FSH serum levels were increased ($P <0.001$). In addition, all patients in group B were amenorrhoeic (Table 3.1.2).

Prior to therapy, patients were younger than controls and had lower AMH levels than controls. Moreover, intervals between the first and last visit were different in the three subgroups. Therefore, the remaining fraction in AMH was calculated. In subgroup C, the remaining fraction of AMH reflected the normal decline in AMH serum levels during one year, independent from baseline AMH levels. Within one year, AMH serum levels decreased by 15% (median remaining fraction 85%; range 0 – 120%) in healthy normo-ovulatory women. The remaining fraction in subgroup A (median 73%; range 20% – 110%) was not different from that in controls ($P = 0.42$). In subgroup B, the remaining fraction (median 13%; range 0 – 50%) was significantly smaller than in controls ($P <0.001$) (Table 3.1.2) (Figure 3.1.1).

In group A, 3 patients conceived spontaneously within 5 years after finishing chemotherapy.

DISCUSSION

Regardless of their age or ovarian function prior to treatment, all patients treated with SCT developed premature ovarian insufficiency and their serum AMH concentrations
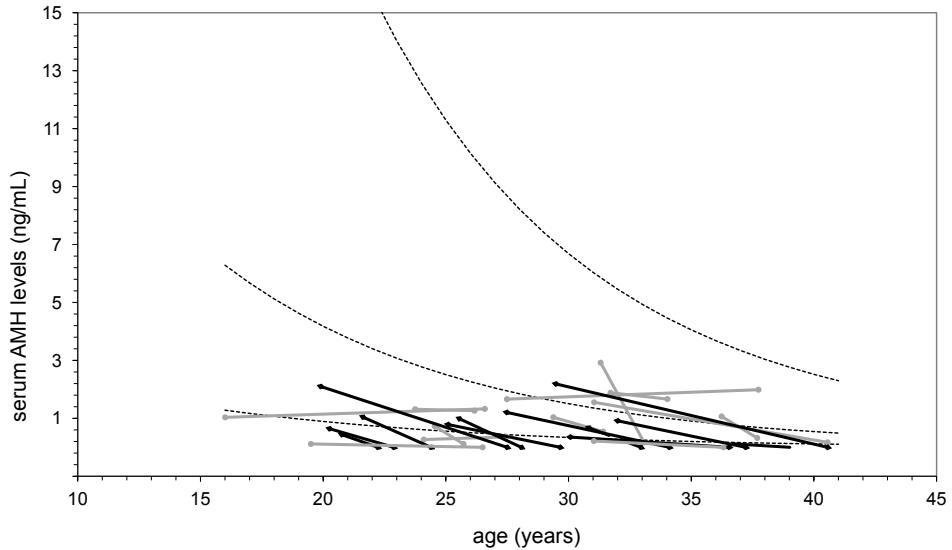
Figure 3.1.1. Decrease in serum anti-Müllerian hormone (AMH) levels in relation to age in patients treated with multi-drug chemotherapy (black squares and lines) and patients treated with stem cell transplant (grey dots and lines) as compared with the decrease in AMH levels in healthy controls (dashed lines; upper line = 95th percentile, middle line = 50th percentile and lower line = 5th percentile).

were undetectable. In accordance to data from literature, the conditioning regimen for SCT is a high risk factor for developing ovarian insufficiency (114). Moreover, TBI causes irreversible damage to the ovaries (134). Hence, all women treated with SCT, especially those receiving TBI, are candidates for fertility preservation techniques, such as ovarian cryopreservation or vitrification of oocytes.

Serum AMH levels as well as antral follicle counts were significantly decreased in patients treated with chemotherapy alone. Although most of these patients had menstrual cycles and some of them conceived spontaneously after chemotherapy, sub-clinical ovarian damage had occurred. Hence, they might be at risk for premature ovarian insufficiency as well and long-term follow up of the reproductive function in these patients is necessary.

Three recent studies have described predictors for ovarian reserve in breast cancer survivors. In premenopausal patients treated with gonadotoxic chemotherapy, serum AMH and inhibin B levels were decreased after treatment, although the difference was statistically not significant. Antral follicle count was identified as the best predictor for ovarian function after chemotherapy (135). It may be hypothesized that directly after chemotherapy, the number of small growing follicles and eventually the number of antral follicles is decreased. Indeed, immediately after chemotherapy AMH levels fall rapidly, suggesting that most of the small growing follicles are lost during the first 3 months after therapy (136). In most of their patients, AMH levels were undetectable

and recovery of ovarian function did not occur, most likely because their ovarian reserve prior to therapy was reduced due to advanced age at start of chemotherapy. Hypothetically, in younger patients ovarian reserve is also diminished, but menstrual cycles will be restored, as observed in the current study. Until now, data are insufficient to determine the time-interval needed for recovery (114). In another study, a seemingly intact ovarian function was observed in women treated for breast cancer. These patients had regular menstrual cycles and normal antral follicle counts. However, most of these women had decreased inhibin B levels and 50% of them had increased FSH levels as well. Moreover, all these women had had an entire ovary removed for cryopreservation prior to treatment. Hence, these women had an additional risk factor for compromised ovarian function after cancer therapy (137).

Prior to therapy, AMH levels in patients were lower than in controls, even though the latter group was older. This indicates that ovarian reserve in patients was already compromised before any therapy was initiated. Remarkably, all patients who developed premature ovarian insufficiency after gonadotoxic chemotherapy had pretreatment AMH levels lower than 10th percentile of AMH levels in age-matched controls. However, not all patients with pretreatment AMH below this threshold point developed premature ovarian insufficiency. Hence, in our study sample, AMH levels prior to treatment were not predictive for ovarian reserve after treatment.

The remaining fraction of AMH in patients treated with chemotherapy alone, reflecting the decrease in AMH levels per year, was not significantly different from controls. This suggests that ovarian damage following gonadotoxic therapy is due to a diminished primordial follicle pool rather than to an increased rate of follicle loss, as described earlier (136).

In conclusion, the present data show that patients receiving TBI developed premature ovarian insufficiency. Hence, to these women fertility preservation, either through unilateral ovariectomy and subsequent cryopreservation or vitrification of collected oocytes, should be offered. In patients treated with multi-drug chemotherapy, the ovarian reserve is compromised as well. Although to a lesser extent, these patients are also at risk for premature menopause.

ASSESSMENT OF OVARIAN RESERVE IN ADULT CHILDHOOD CANCER SURVIVORS USING ANTI-MÜLLERIAN HORMONE

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3.2

ABSTRACT

Background: The aim was to assess possible treatment-induced gonadal damage in a cohort of adult female childhood cancer survivors (CCS) using anti-Müllerian hormone (AMH), the most sensitive marker of ovarian reserve.

Methods: A cohort of 185 survivors was compared with 42 control subjects. The median follow-up time was 18.1 years (range 4.1 – 43.2 years).

Results: Median AMH concentrations in the analyzed cohort were not different from controls (median 1.7 vs. 2.1 µg/L; P = 0.57). However, AMH levels were lower than the 10th percentile of normal values in 27% (49/182) of the survivors. In addition, 43% (79/182) had AMH levels lower than 1.4 µg/L, a previously established cut-off value which predicts ongoing pregnancy after assisted reproduction. There were no differences in AMH levels in subgroups classified according to disease. However, survivors treated with three or more procarbazine containing chemotherapy cycles had significantly lower AMH levels than controls (median 0.5 µg/L; P = 0.004). Also survivors treated with abdominal or total body irradiation had significantly lower AMH levels than controls (median <0.1 µg/L; P <0.001).

Conclusions: AMH can be used to identify subgroups of CCS at risk for decreased fertility or premature ovarian failure. In these survivors options for fertility preservation should be considered prior to starting treatment since they may be at risk for poor chances of pregnancy after assisted reproductive treatment.

INTRODUCTION

Gonadotoxicity is a well-known side effect of cancer therapy in adult survivors. Alkylating agents, such as cyclophosphamide and procarbazine, are known to be highly gonadotoxic. In addition, multi-drug regimens may have a cumulative toxic effect on reproductive function (138). Damage to the ovaries causes follicle loss and may result in premature ovarian insufficiency (POI). Indeed, impaired fertility in childhood cancer survivors (CCS) has been described (139). In several studies, survivors of childhood haematological and solid tumours had high follicle stimulating hormone (FSH) levels and amenorrhoea, indicative of ovarian failure (140-143). However, most studies have described ovarian function in small groups, in which the extent of damage to the ovarian reserve was not predictable (140-142). Despite the presence of high FSH levels and amenorrhoea after chemotherapy, pregnancies in CCS have been reported as well (144, 145). Hence, FSH levels or menstrual cyclicity after cancer treatment do not seem to be predictive of the chance to conceive (140, 144).

Recently, serum anti-Müllerian hormone (AMH) was established as a novel marker of ovarian reserve (80). Results from pre-clinical studies confirmed the role of AMH in human folliculogenesis, i.e. inhibition of initial recruitment and attenuation of FSH sensitivity (146). Clinical studies showed that the number of antral follicles and age correlate well with serum AMH levels. Moreover, serum AMH levels seem to be related to the onset of menopause (83). Furthermore, decrease in AMH levels seems to precede changes in conventional parameters associated with peri-menopausal status, such as FSH, inhibin B and oestradiol (70, 80). Therefore, serum AMH levels are the most reliable marker of ovarian ageing. Furthermore, several studies have described AMH as a predictor of ongoing pregnancy after assisted reproductive treatment (60, 147-149). Finally, in contrast to gonadotrophins, AMH levels were unaffected by cyclic variations or the use of oral contraceptive pills (OCPs) (128, 129).

Although AMH has been described as a useful marker of ovarian reserve in survivors of cancer diagnosed at adult age, data on the value of AMH in adult survivors of childhood cancer are scarce (135, 136, 150). Recently, in two studies AMH was measured in survivors of childhood Hodgkin lymphoma (n = 32), haematological malignancies and solid tumours (n = 21) (116, 151). These studies confirmed that, in CCS with regular menstrual cycles, serum AMH levels were indicative of limited ovarian reserve, whereas FSH, oestradiol and inhibin B levels were not different from healthy controls (151). So far, no studies are available on AMH levels in large cohorts of CCS. In the current study, ovarian reserve was assessed in a large single centre cohort of 185 CCS by measuring serum AMH levels and comparing them with controls.

MATERIALS AND METHODS

Subjects

The study was approved by the local medical ethical review board. Written informed consent was obtained from all participants. These adult female CCS had been treated at the Erasmus MC – Sophia Children's Hospital from October 1958 until December 2000. Participants were 18 years and younger at time of diagnosis and had finished treatment at least 5 years prior to study inclusion. At the time of follow-up they were 17 to 50 years old and in complete remission. Participants were recruited at the outpatient clinic during follow-up of long-term effects of cancer treatment. Because of possible hypothalamic-pituitary-axis dysfunction, survivors of brain tumours were excluded from this study. Details on cancer treatment were recorded, including type and cumulative doses of chemotherapy, site, field, cumulative dose of radiotherapy, type of surgery and/or conditioning regimen prior to stem-cell transplantation, as well as complications and relapse. Subsequently, a general health screening, including extensive history taking and physical examination, was performed. Serum samples were taken randomly during the menstrual cycle, in pregnant survivors as well as in survivors taking OCPs.

A cohort of 42 women without a history of cancer and with a regular menstrual cycle was recruited as controls, as described previously (80). Briefly, inclusion criteria were age between 20 and 35 years, normal body mass index (BMI) (between 19 and 26 kg/m2) and regular menstrual cycle length between 26 and 31 days. In addition, these women were healthy and were all proven fertile. They did not have any endocrine disease and did not use any medication, OCPs or hormonal treatment. Blood samples were drawn during the early follicular phase.

Hormone assays

AMH was measured in controls using the Immunotech-Coulter assay and in survivors using an in-house double-antibody enzyme-linked immunosorbent assay (ELISA) (76, 80). The values from the in-house ELISA assay were adjusted (÷ 2.145) for comparison with the Immunotech-Coulter assay. Intra- and interassay coefficients of variance were <10% and <5% in the in-house ELISA assay and <5% and 8% in the Immunotech-Coulter assay (79, 80). Fluorescence–based immunoassays were used to measure FSH levels (Immulite, Diagnostic Products Corp., Los Angeles, CA, USA). A radioimmunoassay was used to assess oestradiol serum levels (Diagnostic Products Corp., Los Angeles, CA, USA). Intra- and interassay coefficients of variance were <3% and 8% for FSH, <5% and 7% for oestradiol. Serum Inhibin B levels were determined using an enzyme-linked immunoassay (Oxford Bio Innovation, Oxford, United Kingdom). Intra- and interassay coefficients of variation were less than 9% and 15%.

Data analysis

Non-parametric tests were used to compare general characteristics between survivors and controls. Chi-square tests and non-parametric tests were used to compare

participants and non-participants. Univariate analysis of covariance (ANCOVA) was performed to compare AMH in survivors and controls. Likewise, subgroup analysis was performed. Survivors were grouped according to their diagnosis or treatment received. Multi-drug chemotherapy regimens containing cyclophosphamide, procarbazine or ifosfamide were referred to as "chemotherapy with alkylating properties". Correlations between AMH and total cumulative dose of chemotherapy were determined using ANCOVA for categorical parameters (more or less than 3 mechlorethamine, vincristine, procarbazine and prednisone [MOPP] cycles) and partial correlation analysis for continuous variables (total cumulative dose of cyclophosphamide or ifosfamide). Similarly, the correlation between AMH and the total dose of radiation (Gray [Gy]) was assessed using partial correlation analysis. In addition, subanalysis was performed using ANCOVA in subgroups according to treatment before or after menarche, cyclicity and pregnancy rate. Oligomenorrhoea was defined as a cycle interval longer than 35 days, but shorter than 6 months. Amenorrhoea was defined as an interval between cycles of longer than 6 months. All outcome parameters were adjusted for age differences. Survivors with AMH <0.1µg/L were analysed separately and their characteristics were compared with survivors with AMH >0.1µg/L. Data were presented as the median and range.

For the 42 control women, linear regression of age on log-transformed AMH values was performed and 90% prediction limits were calculated. The formula: $AMH*\exp(3.47-0.102*age)$ was used to calculate the mean decline in AMH levels in controls, adjusting for increasing age. The 10th and 90th prediction intervals for decline of AMH levels were calculated using the formula: $AMH*\exp(3.47-0.102*age) \pm 1.23*\sqrt{(1.013 + ((age-30.48)/38.45)^2))}$, with adjustment for increasing age. For graphical display, results of the linear regression were back-transformed. Statistical analysis was performed using Statistical Package for Social Sciences 12.0 (SPSS Inc, Chicago, IL). A P value < 0.05 indicates statistical significance.

RESULTS

Survivors

The total cohort of CCS included 238 women, of whom 25 withdrew from participation. Out of 213 eligible subjects, serum AMH levels were measured in 185 (185/238; 78%) survivors (Figure 3.2.1). In 21% (n = 38) of the 185 CCS samples for measurement of FSH, oestradiol and inhibin B levels were drawn during the early follicular phase of the menstrual cycle and thus were useful for analysis. Median age at diagnosis was 8.2 years (range 0.3 - 15.8 years) in survivors who discontinued follow-up and those in whom serum AMH levels were not available, and was similar in participants (median 5.8 years; range 0.1 – 16.8 years; P = 0.82). Neither the type of malignancies (P = 0.14), nor the frequency of relapse (13% vs. 12%; P = 0.54), the number of survivors receiving chemotherapy (93% vs. 87%; P = 0.22) or radiotherapy (36% vs. 32%; P = 0.73) were different between participants and non-participants.
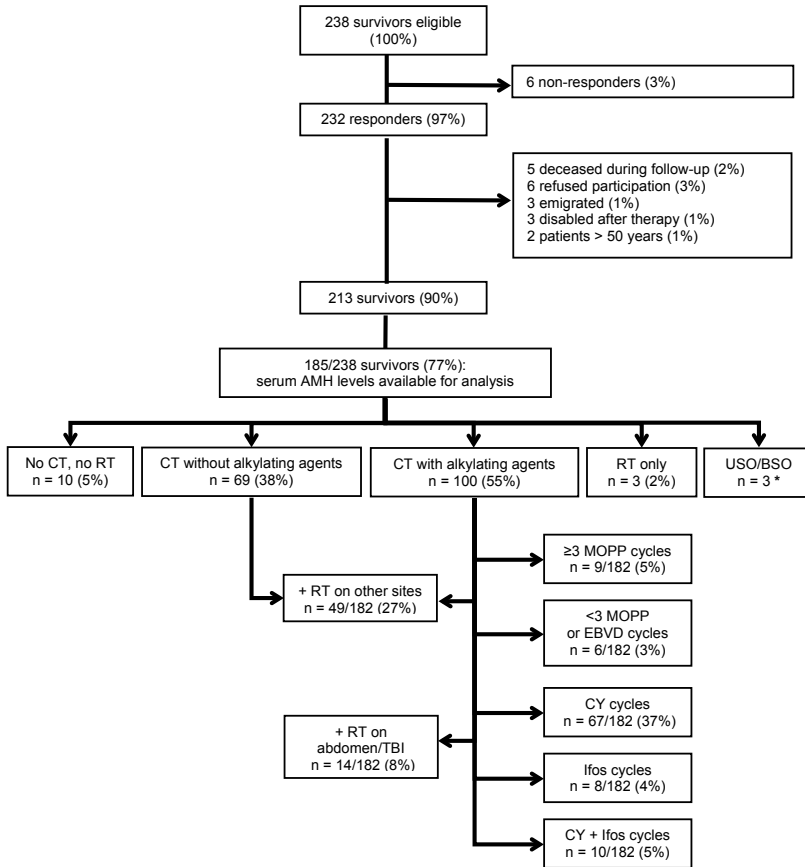
Figure 3.2.1. Flow diagram of subjects.
CT = chemotherapy; RT = radiotherapy; USO = unilateral salpingoophorectomy; BSO = bilateral salpingoophorectomy; MOPP = mechlorethamine, vincristine, procarbazine and prednisone; EBVD = epirubicin, bleomycin, vinblastine and dacarbazine; TBI = total body irradiation; CY = cyclofosfamide; Ifos = Ifosfamide
 * = not included in the analysis.

Diagnosis and treatment

In participants, median age at time of treatment was 5.8 years (range 0.1 – 16.8 years) and median age at time of AMH assessment was 25.5 years (range 17.0 – 47.4 years). Twenty-four (13%) survivors were in second complete remission after relapse. Seventy-seven survivors had been treated for acute lymphoblastic leukaemia (ALL) or non-Hodgkin lymphoma (NHL). These survivors were assigned to the "leukaemia group". Eight survivors were diagnosed with acute myeloid leukaemia (AML). Fifteen survivors with Hodgkin lymphoma (HL) were included, of whom 14 have been described earlier as part of a multi-centre study from our group (151). Since our objective was to analyse our total cohort of female survivors, these women were also included in the present

study. The remaining subgroups consisted of 17 survivors of neuroblastoma (NBL), 25 survivors of sarcoma, 28 with a nephroblastoma, 9 with Langerhans cell histiocytosis (LCH), 3 with a germ cell tumour, 1 with a hepatoblastoma and 1 with a carcinoid tumour. Three survivors diagnosed with Burkitt lymfoma (n = 1) and germ cell tumours (n = 2) were treated with chemotherapy and an unilateral salpingoophorectomy or bilateral salpingoophorectomy. Since their ovarian reserve was reduced by surgery, these three survivors were analysed separately. Finally, excluding these three subjects, 182 survivors were analysed (Table 3.2.1).

	CCS	Serum AMH (µg/L)	P
All subjects included in analysis	N = 182 (%)	1.7 (<0.1 – 19.9)	0.57
Diagnosis			
Leukemia group (ALL, NHL)	77 (42%)	2.2 (<0.1 – 14.1)	0.50
AML	8 (4%)	0.5 (<0.1 – 5.1)	0.17
Hodgkin's Lymphoma	15 (8%)	0.8 (<0.1 – 12.1)	0.32
Neuroblastoma group	17 (9%)	1.9 (0.1 – 6.2)	0.28
Sarcoma group	25 (14%)	1.5 (<0.1 – 19.9)	0.15
Wilms tumour	28 (15%)	1.5 (<0.1 – 13.1)	0.38
LCH	9 (5%)	4.2 (1.6 – 11.5)	0.011
Other tumours	3 (2%)	3.7 (2.0 – 6.4)[*]	-
Time of treatment			
Treated prior to menarche	114 (63%)	1.7 (<0.1 – 19.9)	0.72
Treated after menarche	31 (17%)	1.4 (<0.1 – 12.1)	0.94
Age of menarche unknown	37 (20%)	1.9 (<0.1 – 12.6)[†]	-
Menstrual cycles			
Regular cycles	33 (18%)	2.3 (<0.1 – 13.1)	0.37
Oligo- or amenorrhoea	28 (15%)	1.2 (<0.1 – 14.1)	0.13
OCPs	98 (54%)	1.8 (<0.1 – 19.9)	0.72
HRT	5 (3%)	<0.1 (<0.1 – 1.0)[*]	-
Gravida	4 (2%)	0.9 (0.4 – 3.7)[*]	-
Data on menstrual cycles missing	14 (8%)	1.8 (<0.1 – 6.7)[†]	-

Table 3.2.1. Serum AMH levels in childhood cancer survivors (CCS) as compared with 42 control subjects.
P values indicate differences in serum AMH levels (in µg/L) between survivors and controls by ANCOVA, adjusting for age differences. Median AMH levels in controls was 2.1 µg/L, range (0.1 – 7.4µg/L).
Values are absolute numbers (proportions %) or medians (range).
ALL = acute lymphoblastic leukemia; NHL = non-Hodgkin lymphoma; AML = acute myeloid leukemia; LCH = Langerhans cell histiocytosis; OCPs = oral contraceptive pills; HRT = hormonal replacement therapy.
* = Analysis was not performed because of small number of patients. † = Analysis was not performed because data were missing.

Sixty-three survivors (63/182; 35%) received chemotherapy and radiotherapy. Fourteen of these 63 women (22%) received alkylating chemotherapy as well as radiotherapy on their whole body or abdomen (Figure 3.2.1). Two of the nine survivors of LCH had received radiotherapy on the neck or face and eight were treated with chemotherapy. Survivors of AML were analysed separately from the 'leukemia' group, since their treatment is radically different: five of the eight AML survivors received stem cell transplantation after a conditioning regimen of alkylating chemotherapy and total body irradiation (TBI). Most survivors (63%) had been treated before they had reached menarche, 17% were treated after menarche and in 20% of cases the age of menarche was unknown. At the time of inclusion, 4/182 survivors (2%) were pregnant. Thirty-three survivors (33/182; 18%) had regular menstrual cycles, whereas 28/182 (15%) survivors suffered from oligomenorrhoea or amenorrhoea. In 14/182 survivors (8%) data on menstrual cycle at the time of screening were not available. All other survivors used OCPs (98/182; 54%) or hormonal replacement therapy (5/182; 3%) at the time of follow-up (Table 3.2.1). Median time of follow-up was 18.1 years (4.1 – 43.2 years).

Endocrine profiles
Median serum AMH levels in the 182 survivors were within the normal range after adjustment for age ($P = 0.57$). There were no differences in AMH levels between subgroups classified according to malignancy and controls, except for survivors of LCH in whom serum AMH was higher than in controls ($P = 0.011$) (Table 3.2.1). AMH concentrations in survivors treated before menarche were not different from those in controls ($P = 0.72$). Similarly, AMH levels in survivors treated after menarche were within the normal range ($P = 0.94$) (Figure 3.2.2A). In addition, after excluding survivors treated with TBI or abdominal radiotherapy and survivors treated with three or more procarbazine containing cycles, serum AMH concentrations were not different in survivors treated prior to or after menarche (2.0 vs. 2.1 µg/L; $P = 0.88$). AMH levels were not different in survivors with regular cycles, oligomenorrhoea or amenorrhoea, or in those taking OCPs and controls (2.3 µg/L, 1.2 µg/L, 1.8 vs. 2.1 µg/L, respectively). In the 5 survivors taking hormonal replacement therapy, which was prescribed because of secondary amenorrhoea, median AMH level was <0.1 (<0.1 – 1.0 µg/L). Moreover, no differences were observed in AMH concentrations between survivors with regular cycles and oligomenorrhoea or amenorrhoea (2.3 vs. 1.2 µg/L; $P = 0.43$) (Figure 3.2.2B) (Table 3.2.1). Finally, no differences were observed in AMH levels in multipara survivors or nullipara survivors versus controls (1.5; 1.7 vs 2.1 µg/L; $P = 0.51$; $P = 0.78$). Unfortunately, in survivors who were pregnant at time of inclusion, cycle information or gonadotrophin levels prior to their pregnancy were not available. No correlation was observed between the duration of follow-up and serum AMH concentrations ($P = 0.69$).

Serum AMH levels were significantly lower in survivors who received radiotherapy on the abdomen, pelvis, sacrum or their total body (<0.1 vs. 2.1 µg/L; $P < 0.001$) than in controls, whereas survivors irradiated on other parts of their body had normal
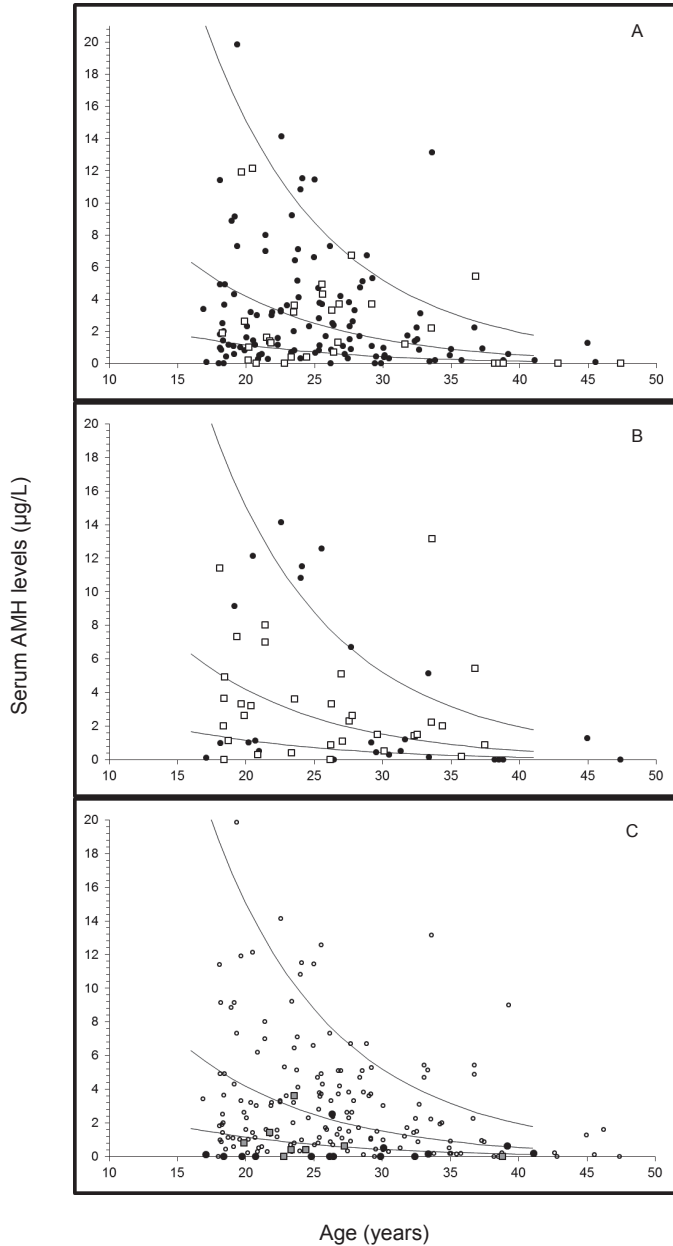
Figure 3.2.2. Serum AMH levels in subgroups of survivors versus 10th, 50th and 90th percentile of AMH levels in controls (solid lines). A. Survivors treated prior to (black dots) or after (open squares) menarche. B. Survivors with regular menstrual cycles (black dots) or oligo- or amenorrhoea (open squares). C. Survivors treated total body irradiation or abdominal radiotherapy (black dots), more than 3 MOPP cycles (grey squares) or other treatment regimen (open dots).

AMH levels (1.5 μg/L(<0.1 – 11.4); $P = 0.52$) (Figure 3.2.2C). In 25 survivors of ALL, irradiated on the cranium, AMH levels were similar to controls ($P = 0.96$) and to ALL-survivors without cranial radiotherapy (2.3 vs. 1.5 μg/L; $P = 0.87$) (Table 3.2.2). The cumulative radiation dose administered on the cranium varied between 18 and 49 Gy. It varied from 10 to 70 Gy when administered on the abdomen and was not correlated with serum AMH concentrations ($P = 0.54$).

In survivors treated with chemotherapy AMH concentrations were comparable with those in controls (1.8 vs. 2.2 μg/L; $P = 0.38$). Although median AMH levels in the whole group of HL-survivors were normal (median 0.8 μg/L; $P = 0.32$), they were significantly lower after three or more MOPP cycles as compared with controls (0.5

	CCS	Serum AMH (μg/L)	P
All subjects included in analysis	N = 182 (%)	1.7 (<0.1 – 19.9)	0.57
Treatment			
Patient with USO[*]	1[*]	8.9[*]	-
Patient with BSO[*]	2[*]	<0.1 [*]	-
Surgery (no USO/BSO), no CT, no RT	10 (5%)	3.3 (0.1 – 11.9)	0.029
RT	3 (2%)	0.2 (<0.1 – 9.0)[†]	-
CT without alkylating agents	69 (38%)	1.7 (<0.1 – 14.1)	0.30
CT containing alkylating agents	100 (55%)	1.8 (<0.1 – 19.9)	0.95
Type of alkylating CT and/or RT			
≥3 MOPP cycles	9 (5%)	0.5 (<0.1 – 3.6)[‡]	0.004
<3 MOPP cycles or EBVD cycles	6 (3%)	3.2 (<0.1 – 12.1)	0.30
Cyclophosphamide cycles	67 (37%)	1.5 (<0.1 – 12.7)	0.63
(Cyclophosphamide+)Ifosfamide cycles	18 (10%)	3.2 (<0.1 – 19.9)	0.08
CT + RT on abdomen or TBI	14 (8%)	<0.1 (<0.1 – 2.5)	<0.001
CT + RT other sites	49 (27%)	1.5 (<0.1 – 11.4)	0.52
ALL	64		0.29
CT with cranial RT	25	2.3 (<0.1 – 7.3)	0.96
CT without cranial RT	39	1.5 (<0.1 – 14.2)	0.62

Table 3.2.2. Serum AMH levels in childhood cancer survivors according to treatment as compared with 42 control subjects.
P values indicate differences in serum AMH levels (in μg/L) in survivors and controls, by ANCOVA, adjusting for age differences. Median AMH levels in controls was 2.1 μg/L, range (0.1 – 7.4μg/L).
Values are absolute numbers (%) or medians (range). USO = unilateral salpingoophorectomy; BSO = bilateral salpingoophorectomy; CT = chemotherapy; RT = radiotherapy; MOPP = mechlorethamine, vincristine, procarbazine and prednisone; EBVD = epirubicin, bleomycin, vinblastine and dacarbazine; TBI = total body irradiation.
* = Patients were not included in analysis because ovarian reserve was compromised by ovarian surgery. † = Analysis was not performed because of small number of patients. ‡ = ANCOVA between CCS treated with ≥3 MOPP cycles vs. CCS treated with <3 MOPP cycles or EBVD cycles: *P* = 0.04

vs. 2.1 µg/L; P = 0.004) and compared with HL-survivors treated with epirubicin, bleomycin, vinblastine and dacarbazine (EBVD) (0.5 vs. 3.2 µg/L; P = 0.04) (Figure 3.2.2C). No significant correlations were observed between serum AMH levels and total cumulative dose cyclophosphamide or ifosfamide (P = 0.63; P = 0.08, respectively). In the only patient treated with an unilateral salpingoophorectomy, serum AMH was 8.9 µg/L. In both survivors who had a bilateral salpingoophorectomy, serum AMH levels were undetectable. Survivors treated with non-gynaecological surgery without any chemotherapy or radiotherapy (n = 10) had higher AMH levels than controls (P = 0.029) (Table 3.2.2).

In 27% (49/182) of our survivors, AMH levels were lower than the 10th percentile of normal values. In only 11 of these 49 survivors, FSH, oestradiol and inhibin B levels were evaluable: FSH levels were within normal ranges (5.0 vs. 6.2 U/L; P = 0.17), whereas inhibin B levels were lower than in controls (9 vs. 113 ng/L; P <0.001). Even after excluding survivors older than 35 years, 41 (23%) survivors had AMH levels below the 10th percentile. Recently, serum AMH levels ≥1.4 µg/L were described as predictive for ongoing pregnancy after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment cycles in healthy, but infertile women (148). Since this cut-off value was assessed with an assay comparable to the Immunotech-Coulter assay used in our study, the cut-off value was applied to our cohort of survivors. In 79/182 (44%) women serum AMH was lower than this threshold value. These 79 survivors were significantly older than the group survivors with AMH levels ≥1.4 µg/L (median 27 vs. 25 years; P = 0.02). However, their age ranged between 17 and 47 years, which was similar to the range in the younger group (16 – 46 years).

The group of women with AMH levels <0.1µg/L was compared with the remaining group of survivors (Table 3.2.3). Women with undetectable AMH levels were significantly older at diagnosis (P = 0.03). At follow-up, they had significantly more irregular menstrual cycles and received more hormonal replacement therapy because of secondary amenorrhea (P <0.001). In addition, in this group, more women had received radiotherapy, especially on their whole body or abdomen, than survivors with AMH levels >0.1µg/L (P <0.001) (Table 3.2.3).

DISCUSSION

Due to increased survival rates, more CCS may tend to delay childbearing, as observed in the general population (152). In healthy women older than 35 years, depletion of the primordial follicle pool is accelerated and the chances of conceiving spontaneously are decreased (22). In CCS, follicle loss may be even more accelerated due to gonadal damage caused by cancer treatment. Consequently, in some cancer survivors, reproductive function will be more compromised as compared with controls. Although AMH concentrations in the whole cohort of survivors were similar to those in controls, in 27% of our survivors AMH levels were lower than the 10th percentile of controls. Serum AMH levels are the most sensitive marker of ovarian reserve. Low AMH levels

	All CCS n = 182	AMH <0.1 µg/L n = 17	AMH >0.1 µg/L n = 165	P
Age at treatment (years)	5.8 (0.1 – 16.8)	10.4 (1.1 – 16.1)	5.3 (0.14 – 16.8)	<0.05
Age at follow-up (years)	25.5 (16.9 – 47.4)	28.0 (18.0 – 47.4)	25.9 (16.9 – 46.2)	NS
Menstrual cycle (n; %)				
Regular	33	2 (6%)	31 (94%)	<0.001
Oligomenorrhea/amenorrhea	28	4 (14%)	24 (86%)	
Oral contraceptive pills	98	5 (5%)	93 (95%)	
Hormonal replacement therapy	5	4 (80%)	1 (20%)	
Data not available	15	2 (13%)	13 (87%)	
Time of treatment (n; %)				
Prior to menarche	114	5 (4%)	109 (96%)	<0.01
After menarche	31	7 (23%)	24 (77%)	
Diagnosis (n; %)				
Leukemia group (ALL, NHL)	77	4 (5%)	73 (95%)	<0.05
AML	8	4 (50%)	4 (50%)	
M Hodgkin	15	3 (20%)	12 (80%)	
Sarcoma	25	3 (12%)	22 (88%)	
Wilms tumour	28	3 (11%)	25 (89%)	
Treatment (n; %)				
Chemotherapy alone or radiotherapy alone	109	2 (2%)	107 (98%)	NS
Chemotherapy and radiotherapy	63	15 (24%)	48 (76%)	<0.001
≥3 MOPP	9	2/15 (13%)	11 (87%)	NS
Radiotherapy on abdomen or total body	14	9 (64%)	5 (36%)	<0.001

Table 3.2.3. Patient characteristics in survivors with serum AMH < 0.1µg/L as compared with survivors with serum AMH >0.1µg/L. *P* values indicate differences in characteristics in survivors with serum AMH < 0.1µg/L as compared with the remaining group of survivors with serum AMH >0.1µg/L by Chi-square test (categorical variables) or Mann Whitney-U test (continuous variables). For survivors with AMH serum <0.1µg/L, numbers per subgroup and percentage of the total number of CCS (n = 182) are given.
ALL = acute lymphoblastic leukemia; NHL = non-Hodgkin lymphoma; AML = acute myeloid leukemia.
MOPP = mechlorethamine, vincristine, procarbazine and prednisone.

are predictive for poor ovarian response in IVF treatment cycles (70). Thus it may be concluded that low AMH levels and low ovarian response to ovarian stimulation are correlated with ovarian ageing and, hence, with decreased fertility (59, 80, 96). Therefore, we postulated that women are at risk of decreased fertility, based on low ovarian reserve, and consequently, these women may have low chances of pregnancy after assisted reproductive techniques. Accordingly, their chances of spontaneous pregnancy may be even lower. Moreover, taking into account the previously described

threshold value, indicating poor outcome of IVF treatment, only 56% of our survivors would be likely to have an ongoing pregnancy after IVF or ICSI (148). Furthermore, not only the elder, but also survivors younger than 20 years had AMH levels below this threshold. Hence, despite normal AMH levels, chances of both future spontaneous pregnancy and pregnancy after assisted reproductive treatment may be impaired in the majority of our CCS. It may be premature to apply a cut off value, since this has not been validated in the population under study. However, counseling on fertility is an important topic, especially for the youngest survivors with low AMH levels. Although these young women may not yet think about their future, in terms of reproduction, they should be discouraged from postponing their first pregnancy until an age at which their ovarian reserve is depleted. Until now, cancer survivors can only be counseled based on a limited amount of data from currently available reports which include cut-off values (60, 147-149, 153). Most studies used a discriminating cut-off value twice as high than the one applied to our cohort (60, 147, 149). A higher cut-off value would result in an even worse outcome for our survivors. However, results of different studies should be interpreted with care, since international standards for an AMH assay are lacking. In addition, these cut-off levels have been described in women with infertility and the number of normal fertile women, who may have AMH levels lower than this cut-off level is unknown. Unfortunately, the number of our control subjects was limited to 42 and 11 of them had AMH levels lower than 1.4 ng/mL. Nevertheless, there seems to be a large variation in serum AMH levels in normal women, reflecting the large variation in their reproductive status and age of menopause (83). Therefore, it can be hypothesized that normal women, including our controls, will have AMH values within the normal range for their age.

To our knowledge, this is the first study to describe ovarian reserve in a large cohort of adult CCS using AMH. Until now, the few available studies on ovarian reserve in large cohorts of CCS have defined ovarian failure as amenorrhoea or failure to conceive, while endocrine profiles were not assessed (47, 139, 143, 154, 155). Moreover, studies using gonadotrophin levels as a parameter of gonadal reserve after cancer therapy were performed in small groups of survivors (140, 142, 156). A further limitation of these studies is that increased FSH levels, amenorrhoea and subfertility are present at the very end of the reproductive life span (22). In the present study, the majority of survivors used OCPs and consequently, gonadotrophin levels or menstrual cyclicity were not informative concerning ovarian function. Nevertheless, in clinical practice, survivors should be counseled regarding their future chances of conception. Therefore, survivors with reduced reproductive potential should be identified at a stage in their fertile life span when interventions such as fertility preservation are still feasible. AMH provides an opportunity for survivors to be counseled on their level of fertility. Indeed, a recent study demonstrated that a healthy woman with low serum AMH for her age is likely to reach menopause before the median age of 51 years (83). Hence, serum AMH levels may be related to the age of menopause in healthy women (83). In another study, healthy pre- and perimenopausal women were followed for six years. Their serum AMH levels were undetectable five years prior to their final menstrual period (82). It

was concluded that low or non-detectable AMH levels were predictive of menopausal transition, whereas serum inhibin B and FSH levels were less predictive (82). Hence, these studies indicate that our survivors with AMH levels lower than their age-matched controls will probably reach menopause before the average 51 years.

Until recently, the extent of gonadal damage was thought to depend on the age of the patient at the start of therapy, the type of treatment and the dose (136). In this study no differences were observed in the number of women treated with chemotherapy and radiotherapy between survivors with AMH<0.1μg/L and those with AMH>0.1μg/L. However, in the first group significantly more women had been treated with TBI or abdominal radiotherapy. Indeed, only AML survivors who had received TBI had undetectable AMH levels. Likewise, only those Hodgkin's lymphoma-survivors who had been treated with more than three MOPP cycles had low AMH levels, as described previously (151). These results indicate that type of treatment is the most important predictive factor for residual ovarian function after treatment, rather than age at treatment. Neither the menarcheal state, nor the total cumulative dose of cyclophosphamide seemed to influence the extent of damage to the ovaries found in our study. Many studies have shown that administration of alkylating agents was an independent risk factor for POI (143, 144), but a direct correlation between the dose of chemotherapy and the endocrine parameters of ovarian reserve was not observed (124, 143, 154). Indeed, in our study, the total cumulative dose of cyclophosphamide did not correlate with AMH levels. Presumably, the gonadotoxic effect of cancer treatment is determined by different agents (138). Furthermore, it could be hypothesized that other factors, such as genetic predisposition may be involved. The presence of a single nucleotide polymorphism in the AMH gene or the AMH type II receptor gene may result in a slightly faster depletion of the ovarian reserve (75). Cancer treatment may be an additional risk factor for POI in these women. Therefore, long-term follow-up and sequential AMH measurements will be needed to confirm the early age of menopause in CCS.

In conclusion, this is the first study in which AMH was used to identify subgroups of CCS highly at risk for POI, i.e. survivors treated with abdominal irradiation, total body irradiation or three or more MOPP cycles. Especially in these survivors, options for fertility preservation should be considered before starting therapy. A substantial part of our survivors had AMH levels below the 10th percentile and may have POI. In addition, a high proportion of CCS is at risk for poor chances of pregnancy after assisted reproductive treatment. Obviously, chances of spontaneous pregnancy will be even lower, especially if survivors postpone their first pregnancy until the third decade.

PREGNANCY OUTCOME IN FEMALE CHILDHOOD CANCER SURVIVORS

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3.3

ABSTRACT

Background: The number of childhood cancer survivors has dramatically increased and consequently, an increasing number of survivors may now wish to conceive. Recently, several studies have described that previous treatment with abdominal radiotherapy may increase the risk of adverse pregnancy outcome.

Methods: We conducted a retrospective single centre cohort study of childhood cancer survivors with a singleton live birth between January 2000 and December 2005. Pregnancy outcome was compared with data from the *Netherlands* Perinatal Registry, a nationwide database of pregnancy outcome parameters of all births in the Netherlands registered by midwives, obstetricians and paediatricians.

Results: Data were available on 40 survivors and 9.031 controls. Median age at diagnosis was 6.9 years (range 0.1 – 16.8 years). The median interval between diagnosis and date of delivery was 21.6 years (range 7.4 – 36.1 years). In the whole cohort, pregnancy outcome was not different between survivors and controls. However, survivors treated with abdominal radiotherapy delivered preterm and had post-partum haemorrhage (mean gestational age in survivors = 34.9 weeks versus 39.2 weeks in controls, $P = 0.001$; 33% in survivors versus 5% in controls, $P = 0.007$, respectively). The offspring of survivors had normal birth weight after adjustment for gestational age (mean birth weight in offspring of survivors 2,503 g versus 1,985 g; $P = 0.22$).

Conclusion: Childhood cancer survivors irradiated to the abdomen have an earlier delivery and higher incidence of postpartum haemorrhage. This stresses the need for close monitoring of the delivery, including inpatient perinatal care, in this group of childhood cancer survivors.

INTRODUCTION

As a result of dramatic improvements in survival of childhood cancer, the number of adult women that have received cancer therapy during their childhood or adolescence will increase (157). Consequently, an increasing number of survivors will reach an age at which they wish to have children. Many uneventful pregnancies have been reported in childhood cancer survivors (158-161). However, previous studies by our group suggested that ovarian reserve was impaired in cancer survivors (150, 151, 162). In accordance, recent studies have proposed that these women may encounter problems while trying to conceive, which has recently been illustrated in a large report of the Childhood Cancer Survivor Study (163, 164). Moreover, an increased risk of miscarriage, preterm delivery and offspring with low birthweight has been recognized as a serious problem in at least subsets of cancer survivors. Hence, studies on pregnancy outcome have shown conflicting results (161, 165-174). The availability of information on pregnancy outcome in childhood cancer survivors may be biased; reporting on adverse pregnancy outcome may be more challenging than describing successful pregnancies. In addition, so far, results were based on small series of survivors and never compared with population-based control settings (159).

Recent studies on pregnancy outcome in childhood cancer survivors have recruited healthy siblings of these survivors as controls (167, 168, 170, 171, 173). In the control group of healthy siblings, the number of unsuccessful pregnancy outcomes may be lower due to recall bias. Consequently, the risk of unfavourable outcome in survivors may seem higher than in their healthy sibling controls. In addition, most studies on pregnancy outcome in female cancer survivors have focused mainly on neonatal outcome, such as stillbirth, birthweight and congenital malformations (145, 154, 170, 171).

To our knowledge, the current study is the first to describe pregnancy outcome in a single centre cohort of female childhood cancer survivors compared to outcome parameters of a nationwide perinatal database.

MATERIALS AND METHODS

Subjects

Childhood cancer survivors from the Erasmus MC - Sophia Children's Hospital in Rotterdam were included, if they had a singleton live birth between January 2000 and December 2005. At inclusion, they were at least 18 years old and at least 5 years after discontinuation of cancer treatment. Participants were recruited at the out-patient clinic for follow-up of long-term effects of childhood cancer treatment. Thirty-five women had participated in a previous study by our group (162). Details on disease and cancer treatment were recorded, including type and cumulative doses of chemotherapy, as well as site, field and cumulative dose of radiotherapy, and, if applicable, type of surgery. In addition, data on pregnancy outcome, delivery and possible complications during pregnancy or during delivery were collected by extensive history taking. Moreover,

these self-reported data were completed by and checked with data retrieved from the national registry of perinatal data, the *Netherlands* Perinatal Registry (http://www. perinatreg.nl/home_english). This registry contained data on all pregnancies, deliveries and neonatal outcome in the Netherlands between January 2000 and December 2005 and had been provided by medical professionals, i.e. midwives, obstetricians and paediatricians, who were responsible for the women during pregnancy, delivery, birth and the post-natal period.

Control subjects were selected from the *Netherlands* Perinatal Registry, after matching with survivors by parity, year of birth and month and year of delivery. Hence, all women in the Netherlands who had a singleton live birth between January 2000 and December 2005 were possible control subjects.

This study was approved by the local medical ethics review board and written informed consent was obtained from each participant.

Outcome parameters

Maternal outcome parameters were the highest diastolic blood pressure measured during pregnancy and the prevalence of pre-eclampsia. In offspring, gestational age, birthweight, Apgar score, gender and the percentage of congenital malformations were analysed. Outcome parameters after delivery were post-partum haemorrhage and the prevalence of a manual removal of placenta. Pregnancy-induced hypertension was defined by diastolic blood pressure >90 mmHg. Preterm birth was defined as duration of pregnancy less than 37 weeks. Postpartum haemorrhage was defined as more than 1 litre blood loss within 24 hours after the delivery. In the national registry, birth weight was reported in grams and length of gestation in weeks. Apgar scores were only available for the assessment carried out 5 minutes after birth.

Statistical analysis

Outcome parameters were compared between controls and survivors using Students' T test for continuous variables or Chi square test for categorical variables. A univariate analysis of covariance (ANOVA) was performed to calculate differences between subgroups and controls. We expected that besides diagnosis, the type of treatment might also be predictive of pregnancy outcome. Therefore, outcome parameters were compared between groups allocated according to diagnosis and controls, and between groups based on type of treatment and controls. Consequently, in the analysis, parameters in the following groups were compared: childhood acute lymphoblastic leukaemia/non-Hodgkin lymphoma (ALL/NHL) versus controls (other groups allocated according to diagnosis were too small for comparison, see Results), survivors treated with radiotherapy to the abdomen versus controls, and survivors treated with chemotherapy only versus controls (survivors treated with surgery only were omitted from the analysis, since this group was too small, see Results). In the ANOVA calculating differences in birth weight, data were adjusted for gestational age.

Correlations were calculated between the duration of pregnancy and the total dose of radiation with a bivariate correlation. Age at diagnosis was correlated with the length

of gestation, using a partial correlation after adjustment for parity. Characteristics in survivors were presented as median and range. Since the group of controls was very large, results of the comparisons between survivors and controls were presented as mean and SD. A P value for two-sided tests < 0.05 indicates statistical significance. Because of the large sample size of the perinatal registry, these data were provided in SAS/Stat software version 9.0 (SAS Institute Inc., Cary, NC, USA). Statistical analysis on differences between survivors and controls was therefore performed in SAS/Stat. Analyses within the group of childhood cancer survivors only were performed with Statistical package for Social Sciences version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Subjects

From the total cohort of 238 female survivors, six women were lost to follow-up. Of the 232 eligible women, five had died during follow-up and six refused participation. In addition, three women had emigrated and three women were severely disabled, and were not able to visit the out-patient clinic. Finally, in the studied cohort of 215 childhood cancer survivors, 64 women had been pregnant, of whom 40 (n = 40/215; 18.6%) had a singleton live birth between 1 January 2000 and 31 December 2005. At study inclusion, the survivors included in the analysis (n = 40) were significantly older than childhood cancer survivors that had not been pregnant (median age 29.9 versus 23.8 years, respectively; P < 0.001). However, diagnoses and treatment were similar in the 40 women included and the excluded survivors (P = 0.44).

At diagnosis, the 40 study subjects were 6.9 years old (median; range 0.1 – 16.8 years) and their median age at time of delivery was 29.7 years (range 21.4 – 36.8 years). The median interval between diagnosis and date of delivery was 21.6 years (range 7.4 – 36.1 years) and the median interval between end of therapy and date of delivery was 20.2 years (range 6.9 – 32.5 years). Most women were primiparous (n = 28/40; 70%); 20% (n = 8/40) had been pregnant for the second time, three women (7.5%) had been pregnant three times and one survivor (2.5%) had 5 pregnancies before study inclusion.

The 40 women were treated for different malignancies; the largest group had survived ALL or NHL (the 'ALL&NHL group') (Table 3.3.1). Survivors had been treated according to standard national and international treatment protocols, containing a variety of multiple drug combinations. Therefore, it was not feasible to evaluate the effect of each type of chemotherapy on pregnancy outcome parameters. Sixteen women had received radiotherapy, six of whom had received radiotherapy to the abdomen (n = 1) or flank (n = 4) and in one patient data on the exact size of the radiation field could not be retrieved. The six survivors who had received radiotherapy to the abdomen were referred to as 'group treated with abdominal radiotherapy' and at diagnosis their median age was 1.1 years (range 0.3 – 5.2 years). The median total cumulative dose of radiation given to the 16 irradiated survivors was 25.0 Gray (Gy) (2.0 – 40.0 Gy). This dose was 19.8 Gy (2.0 – 26.5 Gy) in the group treated with abdominal radiotherapy. The

Diagnosis	Total n (%)	chemotherapy (CT) and/or radiotherapy (RT)				
		CT only	CT+RT to abdomen	RT to abdomen only	CT + RT to CNS	surgery only
ALL&NHL	17 (42.5%)	9	-	-	8	-
Wilms' tumour	7 (17.5%)	3	3	1	-	-
Neuroblastoma	5 (12.5%)	2	2	-	-	1
Hodgkin's lymphoma	4 (10.0%)	2	-	-	2	-
Sarcoma	3 (7.5%)	3	-	-	-	-
Germ cell tumour	1 (2.5%)	1	-	-	-	-
LCH	1 (2.5%)	1	-	-	-	-
Other (teratoma, carcinoid)	2 (5.0%)	-	-	-	-	2
Total	40 (100%)	21	5	1	10	3

Table 3.3.1. Diagnosis and treatment characteristics. ALL = acute lymphoblastic leukaemia; NHL = non Hodgkin lymphoma; LCH = Langerhans cell Histiocytosis; CNS = central nervous system.

three women who had been treated with surgery only, were excluded from analysis. Table 3.3.1 shows details of diagnosis and treatment. Data on 9.031 control subjects was retrieved from the nationwide perinatal database.

Pregnancy outcome

There were no differences in maternal outcome parameters between the total cohort of survivors and controls after matching by parity, year of birth, month and year of delivery. Neonatal outcome parameters, such as gender, gestational age, Apgar score and the percentage of congenital abnormalities, were not different in children born to survivors compared to those born to healthy controls (Table 3.3.2).

In survivors of the 'ALL&NHL group' (n = 17), neither neonatal nor maternal outcome parameters were different from those in controls (data shown in the Supplementary Table 3.3.3). Other groups based on diagnosis were considered too small for separate analysis (Table 3.3.1). In the group treated with abdominal radiotherapy (n = 6), the highest diastolic pressure and the percentage of spontaneous vaginal deliveries were similar to that of controls (*P* = 0.80; *P* = 0.63) (Table 3.3.2). However, the number of preterm deliveries was higher in survivors who received abdominal radiotherapy than in controls (mean term of delivery in survivors 34.9 weeks versus 39.2 weeks in controls, *P* = 0.001 (Table 3.3.2). Consequently, birthweight in offspring of survivors was lower than children of control subjects (mean birth weight 2,503 g versus 3,271 g, respectively; *P* = 0.02). After adjustment for gestational age, birthweight was similar in children born to survivors and those born to control subjects (mean birthweight 2,503 versus 1,985 g, respectively; *P* = 0.22) (Figure 3.3.1) (175). Birthweight was lower than the 50th percentile in five out of six children born to women who received abdominal

	Controls (n = 9.031)	Whole cohort CCS (n = 40)	CCS with RT to abdomen (n = 6/40)
Highest diastolic tension (mmHg)	81.4 (11.7)	81.9 (12.9)	80.8 (14.6)
Preeclampsia			
No	8.991 (98.9%)	40 (100%)	6 (100%)
Yes	40	0	0
Delivery			
Vaginal: spontaneous	4.928 (55%)	21 (55%)	5 (83%)
Vaginal: assisted delivery	2.746 (31%)	12 (32%)	1 (17%)
Secondary caesarean section	1.296 (14%)	5 (13%)	0
Missing	61	2	0
Gestational age (weeks)	39.2 (3.0)	38.9 (2.8)	34.9 (4.3)*a*
Birth weight (grams)	3.271 (714)	3.266 (705)	2.503 (1.026)
Apgar score			
<8	458 (5%)	0	0
8	526 (6%)	3 (8%)	1 (17%)
9	2.165 (24%)	10 (25.0%)	2 (33%)
10	5.856 (65%)	27 (68%)	3 (50 %)
Congenital malformations			
No	8.834 (98%)	38 (100%)	6 (100%)
Yes	145 (2%)	0	0
Missing	52	2	0
Gender			
Male	4.727 (52%)	19 (48%)	3 (50.0%)
Female	4.301 (48%)	21 (53%)	3 (50.0%)
Unknown	3	0	0
Postpartum hemorrhage			
No	8.232 (95%)	35 (92%)	4 (67%)
Yes	449 (5%)	3 (8%)	2 (33%)*a*
Missing	350	2	0
Manual removal of the placenta			
No	8.430 (97%)	37 (97%)	5 (83%)
Yes	251 (3%)	1 (3%)	1 (17%)*b*
Missing	350	2	0

Table 3.3.2. Parameters in controls, the whole cohort childhood cancer survivors and childhood cancer survivors treated with radiotherapy to the abdomen. Data are presented as number (n [%]) or mean (SD). CCS = childhood cancer survivors; RT = radiotherapy.
[a] P <0.01
[b] P = 0.08

radiotherapy (Figure 3.3.1). Apgar score five minutes after birth was similar in offspring of survivors treated with abdominal radiotherapy and offspring of controls (*P* = 0.26) (Table 3.3.2). A significantly larger proportion of survivors had a post-partum haemorrhage than controls (33% versus 5%, respectively; *P* = 0.007). The comparison of the number of these 6 survivors and controls needing a manual removal of the placenta did not reach statistical significance (17% versus 3%, respectively; *P* = 0.08). In all women treated with radiotherapy, the total dose of radiation was correlated with the
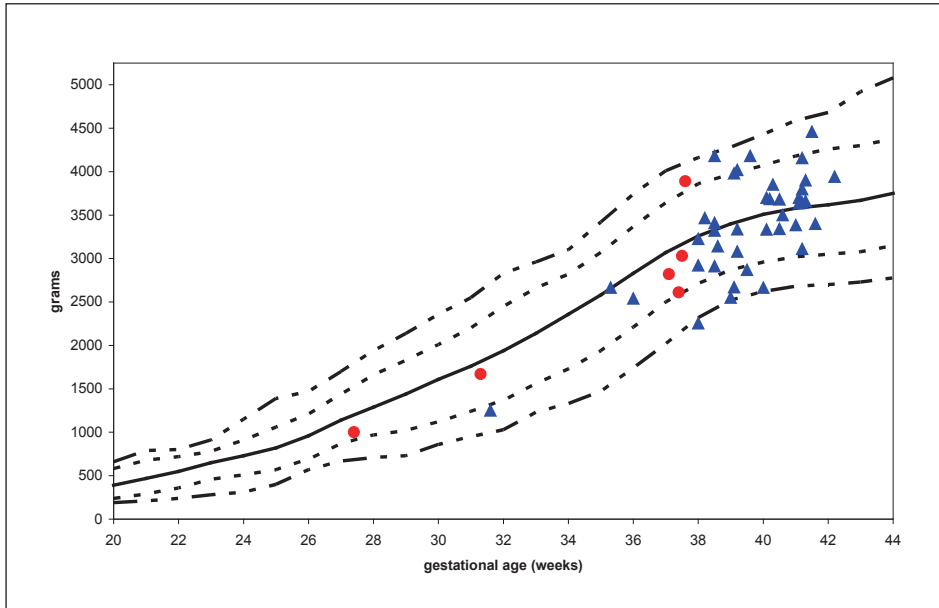
Figure 3.3.1. Birth weight (in grams) in offspring of childhood cancer survivors in population-based birth weight curves per gestational age (in weeks).

– · – · –   2.3th and 97.7th percentile

·············   10th and 90th percentile

————   50th percentile

▲   Offspring of survivors not treated with abdominal radiotherapy (n = 34)

•   Offspring of survivors treated with abdominal radiotherapy (n = 6)

length of gestation ($r = 0.67$; $P = 0.004$). However, the dose of radiation administered to the abdomen was not correlated with the term of birth ($r = 0.46$; $P = 0.35$) (data not shown).

Twenty-one women had been treated with chemotherapy only. Their median age at diagnosis was 9.9 years (range 0.1 – 15.6 years). No significant differences in maternal or neonatal variables were observed between these survivors and controls (data shown in the Supplementary Table 3.3.3).

Age at diagnosis was positively correlated with the duration of pregnancy ($r = 0.48$, $P = 0.006$) in the whole cohort. However, this correlation no longer reached statistical significance after exclusion of the 6 survivors treated with abdominal radiotherapy ($r = - 0.20$; $P = 0.34$). Since these 6 women had been treated at the median age of 1.1 years (range 0.3 – 5.2 years), age at diagnosis and pregnancy duration were not significantly correlated ($r = 0.59$, $P = 0.30$) in the group treated with abdominal radiotherapy.

DISCUSSION

To our knowledge, this is the first retrospective analysis on pregnancy outcome in a single centre cohort of childhood cancer survivors, which compares these data with healthy age-matched controls recruited from a nationwide database. In the total cohort of childhood cancer survivors, pregnancy outcome was not different from that in the general population. However, the current study shows that in the group of survivors who had undergone abdominal irradiation, the frequency of preterm deliveries was higher than in controls, as previously reported (167, 169-171, 176). Besides that, the frequency of post-partum haemorrhage was also higher than in controls: although our findings were based on data of only six survivors, this has not been reported so far in childhood cancer survivors. Most of these women received radiation to the flank and none of them was irradiated to the pelvis. In addition, these 6 women had been treated at a very young age, when the uterus is small. Hence, it may not be expected that radiotherapy to the abdomen had caused the adverse pregnancy outcome. Indeed, earlier studies showed that abdominal irradiation of at least 20 Gy caused irreversible damage to the uterine musculature and blood flow (176). Although scatter radiation to the uterus could not be prevented, it is not plausible that the different radiotherapy regimens in our 6 survivors have resulted in compromised uterine musculature. Possibly, chemotherapy might have damaged uterine structures, resulting in smaller uterine volume and hence, restricting uterine distensibility (166). This finding will need to be confirmed in a larger prospective study sample.

Impaired vascular adaptation to placentation has been associated with pregnancy-induced hypertension, pre-eclampsia and intra-uterine growth restriction (177, 178). Although the severity of pre-eclampsia and its maternal complications may vary considerably, women with pre-eclampsia are at risk of preterm birth and children small for gestational age (179). We therefore speculated that women previously treated with abdominal radiotherapy may also encounter vascular-related problems during pregnancy. However, surprisingly, there were no significant differences in diastolic blood pressure between survivors and controls. To date, no studies have addressed possible hypertension related problems during pregnancy in long-term childhood cancer survivors (169).

It has been assumed that prior to menarche, blood flow through the internal genital system is lower than during puberty and the reproductive lifespan (180). Consequently, it could be hypothesized that cancer treatment may cause less damage when administered prior to menarche. However, in our results, age at diagnosis did not influence the term of delivery. Since none of our six survivors had reached menarche at time of abdominal irradiation, their pre-menarcheal stage did not seem protective against adverse effects of cancer treatment regarding uterine distensibility and contractility. These findings confirm that chemotherapy and radiotherapy administered in children may cause permanent damage to the uterine musculature, independent of menarcheal stage at treatment.

In contrast, in girls previously treated with total body irradiation it was shown that young age at treatment was correlated with smaller uterine volume, assessed at young adulthood (165). In that study, controls were childhood cancer survivors treated with chemotherapy, but without radiotherapy to the uterus (165). Another recent study showed that adult childhood cancer survivors with regular menstrual cycles had lower uterine blood flow and smaller uterine volume than age-matched healthy controls (166). This illustrates the importance of the design of the current study, in which healthy age-matched women were used as controls. In addition, findings of the aforementioned studies suggest that a smaller uterine volume may be expected in our survivors previously treated with radiotherapy to the uterus. Unfortunately, ultrasound data on uterine volume were not available, neither in our survivors nor in controls, but the higher frequency of preterm deliveries may reflect the restricted uterine volume in irradiated women in our study group.

In the current study, data from the nationwide registry, which were recorded by medical professionals, were used as control data. Consequently, recall bias that is characteristic of self-report studies was avoided (164, 181, 182). However, in the *Netherlands* Perinatal Registry, only births between 2000 and 2005 were available for analysis and hence, matching for parity, year of birth, month and year of delivery resulted in a study group of only 40 survivors. So, despite the solid control cohort, only a small sample of survivors was included. In addition, the national registry did not document ethnicity. Therefore, our cases and controls could not be matched for this parameter, in contrast to previous siblings-control studies (145, 164, 171, 173, 181, 183, 184). Similarly, neither BMI nor smoking habits were registered in the Dutch nationwide database.

Offspring of our survivors treated with abdominal radiotherapy had a birthweight appropriate for gestational age after adjustment for gestational age. Similarly, in another recent study, women treated with flank irradiation delivered prematurely and consequently, their children had lower birth weight (164, 167). It was concluded that childhood cancer survivors were at risk of children with low birthweight although that it was not clear whether birthweight had been adjusted for gestational age in that study (164, 167). Another study reported an increased risk of having children small for gestational age in female survivors previously treated with abdominal irradiation. However, they compared pregnancy outcome in survivors treated with abdominal radiotherapy to those treated without abdominal radiotherapy, without comparison to offspring of healthy controls (164, 171). In our study, low birthweight seems to be attributable to preterm birth. Nevertheless, the majority of these children had a birthweight lower than the 50th percentile of offspring of healthy women. Hence, these findings will need to be confirmed in a larger cohort and should be compared with a large cohort of age-matched healthy controls.

Based on our results, health care-providers should be aware of the increased frequency of possible complications during delivery and the post-partum period in survivors treated with abdominal radiotherapy during childhood. Therefore, perinatal

care should be optimized, specifically in female childhood cancer survivors who received abdominal radiotherapy. Consequently, home birth should be discouraged in this particular group of childhood cancer survivors.

In conclusion, in our whole cohort of survivors, pregnancy outcome was similar to that in healthy controls. As described in earlier studies, women treated with abdominal radiotherapy delivered preterm. In addition, we were the first to describe an increased frequency of post-partum haemorrhage in female childhood cancer survivors irradiated to the abdomen, which stresses the importance of delivery in an inpatient setting in these women.

3.3

	Controls (n = 9.031)	'ALL/NHL' group (n = 17/40)	CCS treated with chemotherapy only (n =21/40)
Highest diastolic tension (mmHg)	81.4 (11.7)	81.5 (12.7)	84.7 (14.1)
Preeclampsia			
No	8.991 (98.9%)	17 (100%)	21 (100%)
Yes	40	0	0
Delivery			
Vaginal: spontaneous	4.928 (54.9%)	6 (46.2%)	9 (60.0%)
Vaginal: assisted delivery	2.746 (30.6%)	5 (38.5%)	4 (26.6%)
secondary caesarean section	1.296 (14.4%)	2 (15.4%)	2 (13.3%)
Missing	61	4	6
Gestational age (weeks)	39.2 (3.0)	40.3 (1.3)	39.8 (1.3)
Birth weight (grams)	3.271 (714)	3.493 (516)	3.382 (509)
Apgar score			
<8	458 (5.1%)	0	0
8	526 (5.8%)	2 (11.6%)	0
9	2.165 (24.0%)	3 (17.7%)	6 (28.5%)
10	5.856 (65.0%)	12 (70.6%)	15 (71.4%)
Missing	26	0	0
Congenital malformations			
No	8.834 (98.4%)	13 (100%)	13 (100%)
Yes	145 (1.6%)	0	0
Missing	52	4	8
Gender			
Male	4.727 (52.4%)	8 (47.1%)	11 (52.4%)
Female	4.301 (47.6%)	9 (52.8%)	10 (47.6%)
unknown	3	0	0
Postpartum hemorrhage			
No	8.232 (94.8%)	12 (100%)	20 (95.2%)
Yes	449 (5.2%)	0	1 (4.8%)
missing	350	0	0
Manual removal of the placenta			
No	8.430 (97.1%)	12 (100%)	21 (100%)
Yes	251 (2.9%)	0	0
Missing	350	0	0

Supplementary table 3.3.3 Parameters in controls, the childhood cancer survivors with ALL or NHL and childhood cancer survivors treated with chemotherapy only. Data are presented as number (n [%]) or mean (SD). CCS = childhood cancer survivors; ALL – acute lymphoblastic leukemia; NHL = non-Hodgkin lymphoma.

ANTI-MÜLLERIAN HORMONE AS MARKER OF OVARIAN DYSFUNCTION

4

THE ROLE OF ANTI-MÜLLERIAN HORMONE IN THE CLASSIFICATION OF ANOVULATION AND THE DIAGNOSIS OF POLYCYSTIC OVARY SYNDROME

Sharon Lie Fong, Izaäk Schipper, Olivier Valkenburg, Frank H. de Jong, Jenny A. Visser, Joop S.E. Laven

4.1

SERUM ANTI-MÜLLERIAN HORMONE AND INHIBIN B CONCENTRATIONS ARE NOT USEFUL PREDICTORS OF OVARIAN RESPONSE DURING OVULATION INDUCTION TREATMENT WITH RECOMBINANT FOLLICLE STIMULATING HORMONE IN WOMEN WITH POLYCYSTIC OVARY SYNDROME

Sharon Lie Fong, Izaäk Schipper, Frank H. de Jong,
Axel P.N. Themmen, Jenny A. Visser, Joop S.E. Laven

4.2

ABSTRACT

Objective: To describe changes of anti-Müllerian hormone (AMH) and inhibin B during low-dose gonadotrophin ovulation induction (OI) treatment in women with polycystic ovary syndrome (PCOS), and thus disturbed selection of the dominant follicle.

Design: observational study .

Setting: A referral fertility clinic.

Patients: PCOS women (n=48) and normo-ovulatory women (n=23)

Intervention and main outcome measures: Serum AMH, inhibin B, follicle stimulating hormone and oestradiol concentrations were measured at start of stimulation, on the day of follicle selection, and at administration of human chorionic gonadotrophin during OI cycles and were compared with concentrations measured during the normal menstrual cycle.

Results: Development of a single dominant follicle was observed in 92% of all OI cycles, reflected by similar oestradiol concentrations compared with those in spontaneous cycles. AMH concentrations were constant during low dose ovarian stimulation. Inhibin B concentrations remained elevated in PCOS patients, suggesting prolonged survival of small antral follicles, whereas in controls inhibin B concentrations declined during the late follicular phase.

Conclusions: The lack of change in AMH and inhibin B suggest that follicle dynamics during low dose stimulation seem different from those during controlled ovarian hyperstimulation. In addition, constant AMH and inhibin B suggest that neither AMH nor inhibin B is an accurate marker of ovarian response after low dose gonadotrophin OI in PCOS patients.

INTRODUCTION

Chronic anovulation is a major cause of subfertility (31). The great majority of chronic anovulatory patients (80%) presents with serum follicle stimulating hormone (FSH) and oestradiol concentrations within the normal range and are classified as World Health Organization class II for anovulatory infertility (WHO II) (211). Typically, a considerable proportion of these women also suffers from polycystic ovary syndrome (PCOS) (33). This syndrome is defined by the presence of at least two of three Rotterdam consensus criteria: anovulation, hyperandrogenism and/or polycystic ovaries (PCO) (212). Although the aetiology of PCOS still remains unclear, this syndrome is characterized by failure in dominant follicle selection (48). The selection of antral follicles is disturbed and follicles accumulate, resulting in anovulation and PCO (48, 185).

Fertility treatment in women with WHO II anovulation aims at restoring ovulation, by inducing maturation and ovulation of a single dominant follicle (185). If first line treatment with clomifene citrate does not result in ovulation or pregnancy, second-line treatment consisting of exogenous gonadotrophins is adopted (213). In women with PCOS, the increased follicle number may facilitate multifollicular growth during low-dose stimulation and so these women may be at risk of multiple pregnancies (93). In addition, inhibin B and AMH are considered as accurate predictors of ovarian response during ovarian stimulation cycles for *in vitro* fertilization (70, 81). However, results are inconclusive regarding the prediction of ovarian response upon stimulation with low dose recombinant follicle stimulating hormone (recFSH) (214, 215).

The aim of this study was to compare changes in serum AMH, inhibin B, FSH and oestradiol concentrations in PCOS women during low-dose ovarian stimulation with those during normal menstrual cycles in healthy regularly cycling women, in order to gain insight in follicle dynamics in PCOS women during low-dose gonadotrophin ovarian stimulation.

4.2

MATERIALS AND METHODS

Subjects

This study was approved by the local medical ethics review board. Written informed consent was obtained from each participant.

General patients' characteristics and phenotypical characteristics to diagnose PCOS were obtained at initial clinical workup, as described earlier (50). Briefly, clinical, biochemical and sonographic screening was performed, including history taking, anthropometric measurements, pelvic ultra-sonography, and endocrine measurements. The median age of PCOS women was 28.7 years (range 23.8 – 39.9 years). The median body mass index (BMI) was at the upper limit of normal (median 25.1 kg/m2; range 16.6 – 38.9 kg/m2). Most women suffered from primary infertility (63%; n = 30/48) and at start of OI treatment, the median duration of infertility was 2.8 years (range 0.5 – 17.5 years) (Table 4.2.1).

Characteristic	Women with PCOS	Normo-ovulatory women
Number (n)	48	23
Age (years) (median, range)	28.7 (23.8 – 39.9)*	27.5 (20.0 – 30.0)
Menstrual cycle (n)		
Regular	0	23
Oligomenorrhoea	27	0
amenorrhoea	21	0
Fertility (n)		
Spontaneous pregnancy	0	14/23
Primary subfertility	30	0
Secundary subfertility	18	0
Total follicle count in both ovaries (mean, range)	22 (4.5 – 51)	11 (4 – 21)
Hyperandrogenism (n)	27	0

Table 4.2.1. General patients' characteristics in women with polycystic ovary syndrome (PCOS) and normo-ovulatory women (*: $P = 0.09$).

Inclusion criteria for the current study were: a diagnosis of PCOS according to the Rotterdam consensus criteria, with the wish to conceive and ovulation induction (OI) treatment with recFSH (Gonal-F® [follitropine α], Merck-Serono B.V., the Netherlands) between July 2006 and August 2009. Hyperandrogenism was defined as free androgen index (FAI) >4.5 (calculated using the formula [serum testosterone concentrations (nmol/L) x 100]/serum sex binding globulin hormone concentrations (nmol/L)), testosterone concentrations >3.5 nmol/L and/or serum androstenedione concentrations >15.0 nmol/L. Polycystic ovaries (PCO) were defined as 12 or more follicles between 2-9 mm diameter (216).

Normo-ovulatory subjects had participated in an earlier study (4). They had regular menstrual cycles and did not use any hormonal treatment or oral contraceptive pills. In addition, they had not received treatment for infertility. At inclusion in the earlier study, the median age was 27.5 years (range 20.0 – 33.0 years) and the BMI ranged between 19 and 25 kg/m2. Natural menstrual cycles were monitored with daily blood sampling and pelvic ultra-sonography, starting 10 to 12 days after a positive urinary LH test during a first cycle until normal ovulation in the next cycle. The latter was confirmed sonographically and 7 days later by measuring serum progesterone concentrations (4). For the current study, only data from the natural follicular phase were included.

Treatment protocol

Ovulation induction treatment was started on the third to fifth day after spontaneous menstrual bleeding or after progestagen-induced bleeding. Treatment protocols and assessment of ovarian response have been described previously (93). In summary: the first treatment cycle was started with daily subcutaneous injection of 37.5 IU recFSH. If ovarian response was present upon the starting dose during the first cycle, a second cycle was performed using the same fixed dose protocol. If not, the second treatment

cycle was started at a dose 37.5 IU/day above the response dose during the first cycle, according to the step-down regimen. Then, the recFSH dose was decreased, with 37.5 IU/day during 3 days in case of response (217). Human chorionic gonadotrophin (hCG; [Pregnyl®; Schering-Plough, The Netherlands]) was administered intramuscularly as a single dose of 5000 IU on the day upon which one or two follicles of 18 mm could be visualized. The presence of three or more follicles larger than 16 mm was considered as hyperresponse and consequently, the cycle was cancelled. OI treatment was continued until pregnancy was achieved, with a maximum of six cycles.

Endocrine measurements

Serum was drawn on three sequential time points for assessment of AMH, inhibin B, FSH and oestradiol. In study subjects, samples were drawn at the start of exogenous FSH administration (T1), at selection of the dominant follicle (T2) and on the day of hCG administration, at the end of ovarian stimulation (T3). The day of selection of the dominant follicle was defined as the day on which a follicle reached a diameter of 10 mm or larger and enlargement of this follicle during subsequent days until ovulation. In normo-ovulatory women, samples were drawn on cycle day 3, 4 or 5, on the day of follicle selection and on the day prior to the LH surge and were used for comparison with samples from study subjects. Samples were stored at -20 ˚C until assessment of AMH. Inhibin B, FSH and oestradiol were measured on the same day of withdrawal.

Serum AMH concentrations were measured with an in-house double-antibody enzyme-linked immunosorbent assay (79). The assay is available through DSL Inc. (DSL-10-14400; Diagnostic Systems Laboratories Inc. Webster, TX, USA) (79). Serum inhibin B concentrations were determined using an enzyme-linked immunoassay (Oxford Bio Innovation, Oxford, UK). Luminescence-based immunoassays were used to measure FSH concentrations (Immulite, Siemens DPC, Los Angeles, CA, USA). A coated tube radioimmunoassay was used to assess oestradiol serum concentrations (Siemens DPC). Intra- and interassay coefficients of variance were <10 and <5% for AMH, <9 and 15% for inhibin B, <3 and 8% for FSH, <5 and 7% for oestradiol, respectively.

Data analysis

General characteristics of eligible patients and normo-ovulatory women were compared using non-parametric tests, because of skewed distribution of the data (not shown) and limited sample size. ANOVA for repeated measurements was used to compare the effect of time on endocrine parameters in PCOS women and normo-ovulatory women and to compare changes in endocrine parameters between PCOS women and normo-ovulatory women, followed by an independent samples t-test in case of the presence of significant differences. Data are presented as medians with ranges. AMH and oestradiol concentrations were logarithmically transformed to achieve normal distribution. Serum AMH concentrations are presented in μg/L (1 μg/L = 7.14 pmol/L). Statistical analysis was performed using Statistical Package for Social Sciences 15.0 (SPSS Inc., Chicago, IL, USA). A P value of <0.05 indicates statistical significance.

RESULTS

Subjects' characteristics

In accordance with the inclusion criteria, 48 women with PCOS were eligible for analysis. All 48 women had irregular cycles. All but 2 women had PCO and 27 women (27/48; 56%) were hyperandrogenic (Table 4.2.1). The median age in PCOS women was not significantly different from that in 23 normo-ovulatory women (median 27.5 years, range 20.0 – 33.0 years) (P = 0.09). Fourteen of the 23 normo-ovulatory women had been pregnant previously (4).

Characteristics of ovarian stimulation

One treatment cycle per woman was included. In 18 patients, the first day on which the dominant follicle was observed, coincided with the day on which hCG was administered. Consequently, in these patients serum was drawn on only two occasions. In the remaining 30 patients, serum sampling was performed on 3 independent days. In 5 patients OI cycles were cancelled: in 3 patients, at least 2 follicles larger than 16 mm were observed at hCG administration, which indicated hyperresponse and an increased risk of multiple pregnancy. In another patient, surplus follicles were punctured prior to hCG administration and finally, in one patient a postovulatory follicle was observed during monitoring of ovarian response, indicative for premature LH surge. In 42 of the 43 ongoing OI cycles, monofollicular growth was observed, whereas one ongoing cycle had resulted in 2 follicles of 16 mm. The median duration of stimulation was 15 days (range 5 – 38 days). Eventually, analysis was performed in 43 women with ongoing OI cycles.

Endocrine parameters

Serum AMH concentrations remained constant during FSH-stimulated cycles and normal cycles (P = 0.136), although AMH concentrations in PCOS women were significantly higher than in normo-ovulatory women (P = 0.007) (Figure 4.2.1). Indeed, post-hoc analysis showed that at all time points PCOS women had higher serum AMH concentrations than normo-ovulatory women (P = 0.004 at start; P = 0.003 at selection; P = 0.02 at end of stimulation) (Table 4.2.2).

Inhibin B concentrations within OI or natural cycles did not change significantly over time (P = 0.343). However, the repeated measures analysis revealed that there was a significant interaction between time and group (P = 0.039), suggesting that there is a change in inhibin B concentrations during OI and natural cycles and that this change differs between both groups. In OI cycles inhibin B concentrations remained relatively constant, whereas in natural cycles inhibin B concentrations increased from T1 to T2 and declined from T2 to T3. As a consequence, inhibin B concentrations were nearly twice as high in PCOS women at the end of an OI cycle than in normo-ovulatory women on the day prior to the LH surge (P < 0.0001) (Table 4.2.2) (Figure 4.2.1).

FSH concentrations were significantly different between PCOS and normo-ovulatory women (P < 0.0001). At all time points FSH concentrations were higher in
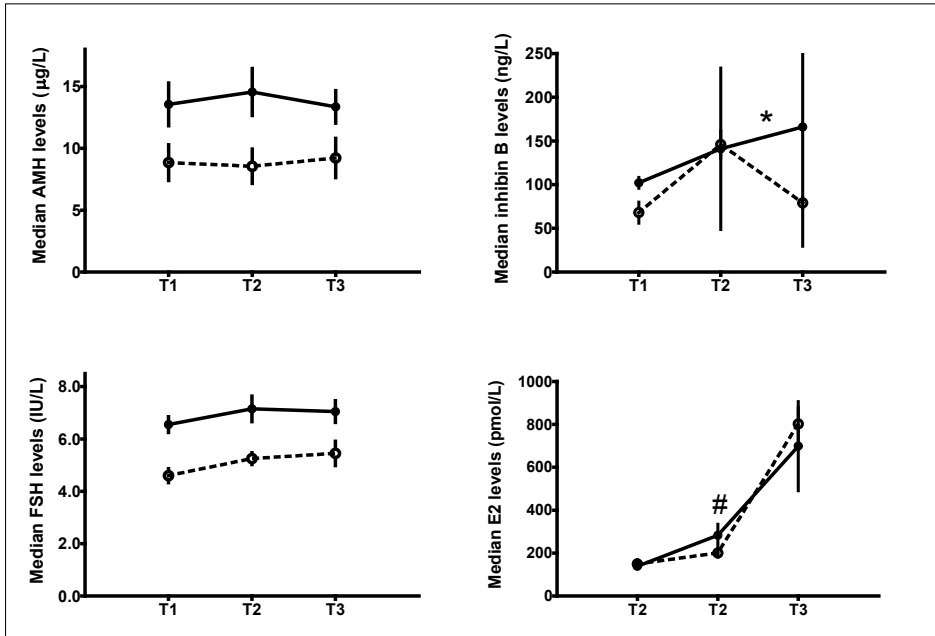
Figure 4.2.1. Changes in median serum anti-Müllerian hormone (AMH), inhibin B, follicle stimulating hormone (FSH) and oestradiol (E2) (and ranges) concentrations in PCOS patients during ovulation induction cycles (filled dots and solid lines) and in normo-ovulatory controls during normal menstrual cycles (open squares and dashed lines) at start of ovarian stimulation (T1), at selection of the dominant follicle (T2) and at the end of stimulation (T3).
* P <0.05: significant change over time, which was different between PCOS and normo-ovulatory women
# P <0.05: significant change over time, which was similar in both PCOS and normo-ovulatory women

PCOS women compared with normo-ovulatory women (P < 0.0001 at T1 and T2; P = 0.01 at T3) (Table 4.2.2) (Figure 4.2.1). However, concentrations remained constant during both OI and natural cycles (P = 0.557) (Figure 3.2.1).

Oestradiol concentrations changed significantly within OI cycles and natural cycles (P = 0.032), however the change over time was similar in both groups (P = 0.396). Both cycles showed an increase in oestradiol concentrations at T3 (Figure 4.2.1). Overall, oestradiol concentrations in OI cycles were not significantly different from concentrations in natural cycles (P = 0.883)

DISCUSSION

To our knowledge, our study is the first to describe the dynamics of AMH, inhibin B, FSH and oestradiol concentrations during low-dose FSH ovarian stimulation in normogonadotropic anovulatory women. This treatment regimen has been shown to

	T1	*P*	T2	*P*	T3	*P*
AMH (µg/L)		0.004		0.003		0.02
PCOS	13.6 (3.4 – 56.4)		14.6 (2.1 – 37.7)		13.4 (2.8 – 42.9)	
controls	8.8 (2.1 – 27.8)		8.5 (1.8 – 25.8)		9.2 (2.0 – 30.8)	
Inhibin B (ng/L)		0.15		0.98		<0.001
PCOS	102 (9 – 212)		141 (67 – 2,554)		166 (39 – 4,757)	
controls	68 (11 – 164)		146 (48 – 310)		79 (31 – 131)	
FSH (IU/L)		<0.001		<0.001		0.01
PCOS	6.6 (0.1 – 11.9)		7.2 (4.3 – 19.1)		7.1 (3.7 – 15.1)	
controls	4.6 (3.1 – 7.7)		5.3 (3.1 – 7.3)		5.2 (2.6 – 10.2)	
oestradiol (pmol/L)		-		-		-
PCOS	139 (57 – 387)		283 (93-1,404)		699 (180 – 6,886)	
controls	149 (98 – 404)		201 (123 – 432)		788 (387 – 1,600)	

Table 4.2.2. Median serum anti-Müllerian hormone (AMH), inhibin B, follicle stimulating hormone (FSH) and oestradiol (E2) concentrations (and ranges) in PCOS patients during ovulation induction cycles compared with concentrations in normo-ovulatory controls during normal menstrual cycles at start of ovarian stimulation (T1), at selection of the dominant follicle (T2) and at the end of stimulation (T3). *P*-values are results of the independent samples *t*-test, performed following a significant difference in the repeated measurement analysis.

be successful and cost-effective in several studies (185, 218, 219). In the present study mono-ovulation was achieved in the majority of women with PCOS, as confirmed by the development of a single dominant follicle and a similar pattern and degree of increasing oestradiol concentrations in both OI cycles and spontaneous cycles.

Serum AMH concentrations were constant in both women with PCOS and normo-ovulatory women, in accordance with previous studies (128, 220). This is in contrast with the decline in serum AMH concentrations observed during controlled ovarian hyperstimulation for *in vitro* fertilization (147, 221). It has been suggested that the decline in AMH concentrations reflects the shift of AMH-producing, small antral follicles to the subsequent stage upon supraphysiological doses of recFSH, since AMH expression gradually decreases in antral follicles sized 6 mm and larger (73). The constant AMH concentrations in PCOS women suggest, however, that upon low-dose recFSH stimulation, the cohort of small antral follicles was not significantly reduced, despite the presence of an increased number of follicles, reflected by high serum AMH concentrations at start of stimulation. Thus, ovarian stimulation with low-dose recFSH mimics a physiological cycle, as reflected by mono-ovulation in most PCOS women.

In contrast with AMH concentrations, inhibin B concentrations did show different dynamics between PCOS and control women. At the end of low-dose ovarian stimulation, inhibin B concentrations remained elevated, whereas in natural cycles, inhibin B declined. This prolonged elevation of inhibin B concentrations has been described previously (222) and would be suggestive of sustained multiple follicular development during the late follicular phase. In spontaneous cycles, multiple antral

follicles are recruited by FSH. Because of decreasing FSH concentrations, most of them will become atretic, resulting in the development of a single dominant follicle. Apparently, upon continuous low-dose FSH administration, the physiological decrease in FSH was overruled and the FSH-recruited follicles survived longer during OI cycles, explaining the significantly elevated inhibin B concentrations at the end of stimulation in our treated subjects.

Prior to stimulation, FSH concentrations were higher in PCOS women than in normo-ovulatory women. Since age was similar in PCOS women and normo-ovulatory women, these high FSH concentrations may reflect impaired FSH sensitivity in our PCOS cohort. Indeed, it is widely considered that selection of the dominant follicle is impaired in women with PCOS, due to failure of FSH-dependent follicle recruitment (223). Apparently, by administration of a small amount of exogenous FSH, the 'blocked' cyclic recruitment in PCOS women can be overruled and multiple follicles surpass the FSH threshold (224). Despite this prolonged survival of follicles with continuous low-dose FSH administration after cyclic recruitment, development of a single dominant follicle was unaffected in the majority of treated PCOS subjects, as reflected by mono-ovulation. This implies that upon stimulation with low-dose exogenous FSH, multiple antral follicles are present, but they seem to be less responsive to FSH and possibly less viable.

We hypothesized that in the studied population of women with PCOS, several treatment cycles would result in hyperresponse to low-dose stimulation. Then, multifollicular growth would be expected and AMH concentrations would decrease during low-dose stimulation. It might be expected that this decrease is predictive for ovarian hyperresponse, and thus cycle cancellation. Ovulation induction cycles were cancelled in five patients, of whom four had multiple follicular growth. This may indicate that the studied cohort of PCOS women had rather favourable factors of successful OI treatment outcome (93). Consequently, in a more obese population with a higher follicle number or a higher percentage of more severe hyperandrogenism, multifollicular growth might occur more often than in the currently studied subjects. In addition, in our study, a starting dose of 37.5 IU recFSH was applied and seemed quite efficient and save, with respect to hyperstimulation. Moreover, the low cancellation rate may also suggest that follicle growth was well monitored.

In conclusion, serum AMH concentrations were constant during low-dose ovulation induction. Although inhibin B concentrations at the end of stimulation were indicative of prolonged survival of antral follicles, mono-ovulation was not affected. Unfortunately, in the current study, data on the number of growing antral follicles during stimulation was not available. However, the lack of change of serum AMH and inhibin B concentrations during low-dose stimulation and mono-follicular development in PCOS women seem distinctly different from that during controlled ovarian hyperstimulation. This suggests that follicle dynamics during ovulation induction are different from that during controlled ovarian stimulation with pharmacological doses of recFSH. In addition, neither AMH nor inhibin B seems useful as a marker of ovarian response after low-dose ovarian stimulation in women with PCOS.

ANTI-MÜLLERIAN HORMONE: A MARKER FOR OOCYTE QUANTITY, OOCYTE QUALITY AND EMBRYO QUALITY?

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4.3

ABSTRACT

Background: Serum anti-Müllerian hormone (AMH) levels decline with increasing age and constitute a sensitive marker for ovarian ageing. In addition, basal serum AMH concentrations predict ovarian response during *in vitro* fertilization (IVF) cycles. Concomitantly, oocyte quantity and embryo quality decrease with advancing age. Hence, it was hypothesized that AMH in serum constitutes a marker for embryo quality.

Methods: Women aged 37 and younger with regular menstrual cycles, normal body mass index and partners with normal semen parameters were randomly assigned to either a standard or mild stimulation protocol for IVF treatment. Blood samples were drawn at cycle day 3 and at the day of hCG administration. Embryo quality was assessed using embryo morphology score and preimplantation genetic screening.

Results: Serum AMH levels on cycle day 3 were correlated with the number of oocytes retrieved in both groups. AMH and embryo morphology were correlated after mild stimulation, but not after conventional ovarian stimulation. AMH and the chromosomal competence of embryos were not correlated.

Conclusions: Serum AMH is predictive for ovarian response to stimulation. However, the lack of a consistent correlation with embryo morphology and embryo aneuploidy rate is not in favour of a direct relationship between oocyte quantity and embryo quality.

INTRODUCTION

Anti-Müllerian Hormone (AMH) is a dimeric glycoprotein and member of the transforming growth factor-β superfamily (225). Members of this family play a role in follicle maturation and development. In rodent studies, accelerated depletion of the primordial follicle pool was observed in AMH knock-out (AMH-KO) mice. In addition, follicles in AMH-KO mice were more sensitive to follicle stimulating hormone (FSH). Despite the presence of low serum FSH concentrations, a higher number of small growing follicles was observed in AMH-KO mice as compared with wild-type mice (67). AMH is expressed in ovarian granulosa cells, especially in granulosa cells of those follicles at the stages of follicular development between initial recruitment and cyclic recruitment by FSH, i.e. preantral and small antral follicles. Hence, AMH seems to be an important regulator at two crucial points in folliculogenesis, i.e. initial follicle recruitment and cyclic selection for dominance (125).

In the human ovary, AMH is expressed in granulosa cells of secondary, preantral and small antral follicles ≤ 4 mm in diameter and expression is absent in follicles > 8 mm (73). This expression pattern in small growing follicles is similar to that in mice, suggesting a similar role for AMH in humans. Consequently, serum AMH concentrations correlate well with the number of antral follicles. Indeed, the decline of antral follicles with age is reflected by a decline in serum AMH levels (70, 80, 84). In addition, different reports have demonstrated that serum AMH levels correlate with outcome of ovarian hyperstimulation. Poor response in in-vitro fertilization (IVF), indicative of a diminished ovarian reserve, is associated with reduced baseline serum AMH concentrations (63, 70, 96, 226, 227). Therefore, AMH serum levels constitute a sensitive marker of ovarian reserve.

With increasing age, the primordial follicle pool diminishes, resulting in a decrease in oocyte quantity. At the same time, the incidence of numeric chromosomal abnormalities is increased in oocytes obtained after ovarian stimulation in women older than 37 years (28). This suggests that with increasing age, a concomitant decrease in oocyte quantity as well as quality is observed. In assisted reproductive techniques, the best embryos are selected for transfer. Until now, these embryos are selected based on rather subjective criteria, according to a widely used morphology score (228). While embryos rated as high quality by this morphological assessment are associated with higher implantation and pregnancy rates, successful implantation and ongoing pregnancy cannot be predicted with certainty. In addition, over 50% of the embryos transferred appears to be chromosomally abnormal (229, 230). In this study, embryo quality was assessed by performing preimplantation genetic screening (PGS) for numeric chromosomal abnormalities as well as by traditional embryo morphology score.

AMH has previously been shown to be a better predictor of ovarian reserve than age (96). Since oocyte quantity as well as oocyte quality affect embryo quality and seem to be associated with ageing, it was hypothesized that AMH might also correlate with embryo quality. Accordingly, in patients receiving IVF treatment cycles, AMH might predict their response to ovarian stimulation as well as their chances of conceiving.

4.3

The aim of this study was to determine the correlation between serum AMH levels and embryo quality, employing PGS.

MATERIALS AND METHODS

Subjects
This study was approved by the local medical ethics review board of the participating hospitals and by the Dutch Central Committee on research involving human subjects (CCMO). Written informed consent was obtained from all participants. Patients participated in a randomized controlled trial (RCT), during one IVF treatment cycle, as described earlier (229).

Study design
Patients were randomly assigned to a conventional treatment group (group 1) or to a mild stimulation group (group 2) as described previously (229, 231). Embryo score was defined as the mean score of all embryos obtained per patient, rated on the third day after oocyte retrieval. Preimplantation genetic screening was performed on embryos with more than five blastomeres. Two cells were removed by biopsy, unless the embryo consisted of only six blastomeres. Fluorescent in-situ hybridization (FISH) analysis was performed to determine the copy number of nine pairs of chromosomes (1, 7, 13, 15, 16, 18, 21, 22, X and Y) (232). Embryos were classified as either euploid or aneuploid according to FISH results obtained from the first biopsied blastomere.

In both groups, baseline blood samples were obtained on cycle day 3, before ovarian stimulation was started. Subsequently, on the day of human chorionic gonadotrophin (hCG) administration, a second blood sample was obtained. Serum samples were stored at -20°C and assayed for AMH, luteinizing hormone (LH), follicle stimulating hormone (FSH) and inhibin B. AMH levels were assayed using an in-house double-antibody enzyme-linked immunosorbent assay (ELISA) kit, with intra- and interassay coefficient of variation of <5% and <10%, respectively (79). Serum levels of LH and FSH were measured using luminescence based immunoassays (Immulite 2000, Diagnostic Products Corp., Los Angeles, CA, USA). Intra- and interassay coefficients of variation were less than 4% and 7% for LH and less than 3% and 6% for FSH, respectively. Dimeric inhibin B levels were assessed using an immunoenzymometric assay obtained from Serotec (Oxford, UK). Intra- and interassay coefficients of variance were less than 7% and 14% respectively.

Data analysis
Data obtained from the blood samples drawn on the day of hCG administration were used to calculate the change in serum AMH concentrations during ovarian stimulation: [AMH on the day of hCG administration] – [AMH before start of ovarian stimulation]. Primary outcome measures were serum AMH concentrations before and after standard or mild ovarian stimulation, embryo morphology assessed by embryo morphology score and aneuploidy rate assessed by PGS.

Non-parametric Mann-Whitney-U and Chi square tests were used to compare data in patient subgroups. Correlations between data were assessed using Pearson or Spearman correlation tests and were expressed as correlation coefficient r. Correlations between AMH on the day of hCG administration and outcome parameters were assessed by analysis of covariance (ANCOVA), after adjustment for baseline AMH values. A P value < 0.05 was considered to be statistically significant. Statistical analysis was performed using a commercially available software package (SPSS, SPSS Inc., Chicago, IL, USA).

RESULTS

In the present study, 125 patients were included, of whom 51 (40.8%) were assigned to the standard protocol (group 1) and 74 (59.2%) to the mild protocol (group 2). On average, participants were 33.2 (range 22.5 – 37.9) years old and their body mass index (BMI) was 22.7 (range 18.1 – 30.5) kg/m2. No differences were observed in demographic patient characteristics between the two groups. Patients in group 1 were stimulated significantly longer as compared with patients in group 2 (median 11 and 9 days, respectively) ($P < 0.001$). Figure 4.3.1 shows details concerning patients who withdrew or were excluded from analysis, as described earlier (229). In the additional patients included in the present study, two patients withdrew prior to ovarian stimulation, two further patients were excluded due to ovarian hyperstimulation syndrome and in another two, fertilization failed after standard ovarian stimulation. Furthermore, another patient dropped out because of a spontaneous pregnancy, one more cycle was cancelled due to low response and in 18 patients embryos were not available for analysis after mild stimulation. Eventually, in 33% of the patients assigned to group 1 (conventional ovarian stimulation), embryos were not suited for transfer. Of the patients in group 2 (mild ovarian stimulation), 42% underwent embryo transfer after biopsy for PGS.

Serum AMH levels before the start of IVF cycle were comparable in both groups ($P = 0.71$) (Figure 4.3.2). During stimulation in group 1, AMH levels decreased significantly ($P = 0.002$). In group 2, baseline AMH levels and AMH levels on the day of hCG administration were not different ($P = 0.193$) (Figure 4.3.2). Serum AMH concentrations on the day of hCG administration were significantly lower in group 1 as compared with group 2 ($P < 0.001$). This difference remained statistically significant after adjustment for the number of oocytes retrieved and basal AMH concentrations ($P < 0.001$). As described in the previous study, more oocytes were retrieved in group 1 as compared with group 2 ($P = 0.001$). In addition, more fertilized oocytes were obtained in group 1 ($P = 0.014$). However, fertilization rate ($P = 0.227$) and the number of ongoing pregnancies ($P = 0.842$) were comparable in both groups (229).

In group 1, serum AMH concentrations on cycle day 3 and the number of oocytes retrieved were positively correlated ($r = + 0.47$; $P = 0.002$) (Figure 4.3.3A). However, baseline serum AMH levels were not correlated with the number of fertilized oocytes ($r = + 0.17$; $P = 0.29$), nor with fertilization rate ($r = - 0.21$; $P = 0.21$). In addition, no
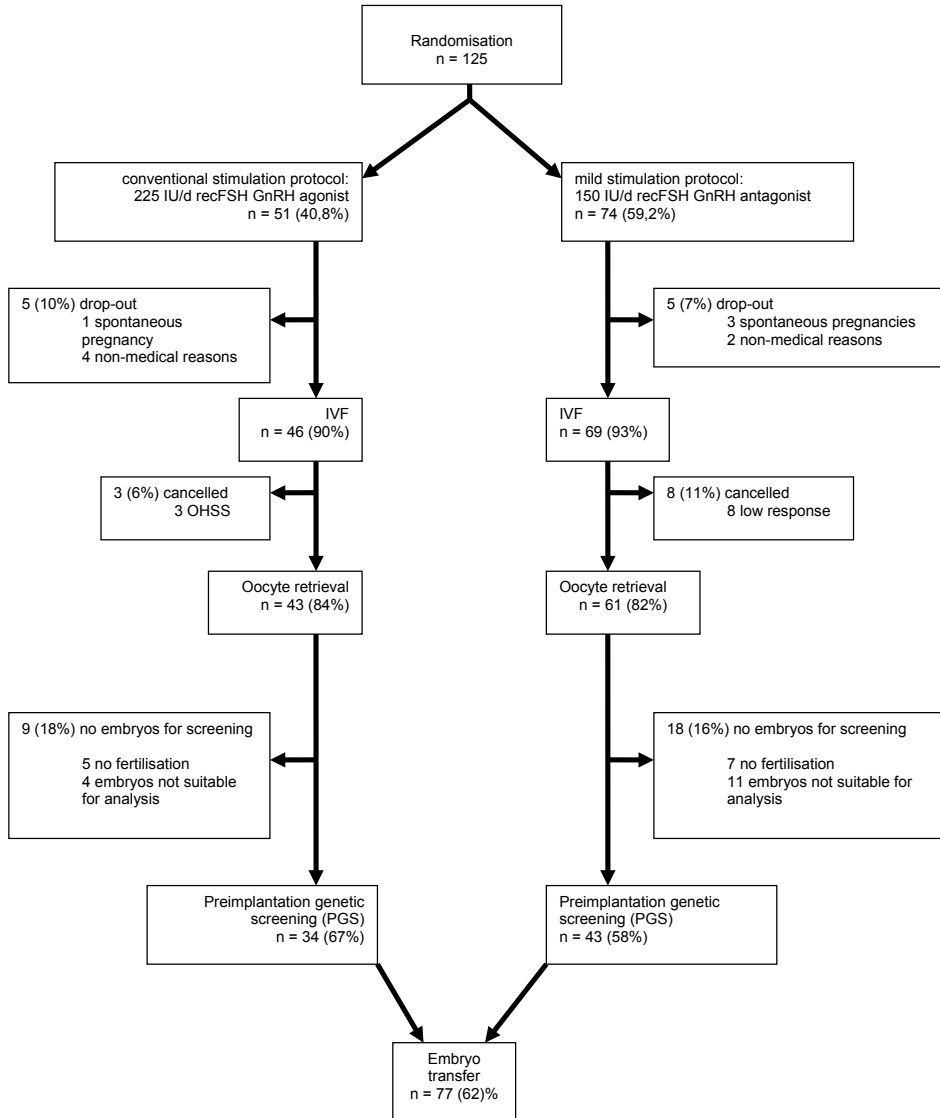
Figure 4.3.1. Flow diagram of patients through phases of trial.

correlation was observed with embryo morphology, defined by morphology score as well as with aneuploidy rate ($r = -0.15$, $P = 0.37$; $r = +0.05$, $P = 0.77$, respectively) (Figure 4.3.3B). The decrease in AMH concentrations during standard ovarian stimulation was not correlated with the number of oocytes retrieved, fertilization rate, embryo morphology or aneuploidy rate ($r = +0.25$, $P = 0.14$; $r = +0.22$, $P = 0.20$; $r = -0.24$, $P = 0.15$; $r = -0.03$, $P = 0.88$, respectively).
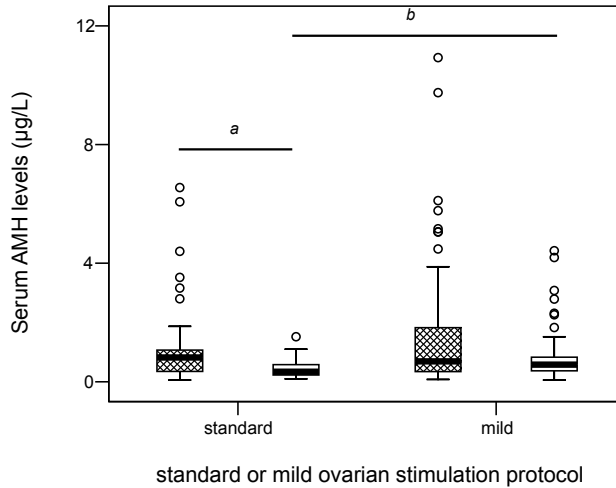
Figure 4.3.2. Box Whisker-plot of serum AMH levels in standard stimulation group versus mild stimulation group.
Serum AMH levels in patients in standard versus mild stimulation group at the start of stimulation cycle (hatched boxes) and on the day of hCG administration (open boxes). A statistically significant decrease in serum AMH levels was only observed in the standard protocol group (a: $P = 0.002$). A statistically significant difference between serum AMH levels at day of hCG administration was observed between the groups (b: $P < 0.001$).

In group 2, baseline serum AMH concentrations were positively correlated with the number of oocytes ($r = + 0.29$; $P = 0.03$) and with the embryo score on day of embryo biopsy expressed by morphology score ($r = + 0.41$; $P = 0.005$) (Figure 4.3.3A and 4.3.3B). However, no correlation was found between baseline AMH and number of fertilized oocytes or fertilization rate, nor between AMH and results of aneuploidy screening ($r = - 0.05$, $P = 0.74$; $r = + 0.22$, $P = 0.12$; $r = - 0.10$, $P = 0.54$, respectively). The decrease in serum AMH concentrations during mild stimulation was correlated with the number of oocytes retrieved ($r = + 0.34$, $P = 0.016$). No correlation was observed with morphology score, aneuploidy rate or fertilization rate ($r = 0.17$, $P = 0.25$; $r = -0.03$, $P = 0.88$; $r = -0.26$, $P = 0.07$ respectively).

In both stimulation protocol groups, serum AMH concentrations on the day of hCG administration were positively correlated with the number of oocytes ($P = 0.023$). No correlations were observed with embryo quality expressed by embryo score or aneuploidy rate, nor with fertilization rate.

DISCUSSION

Ongoing pregnancy and live birth are the most important endpoints in assisted reproductive treatment. These outcome measures are mainly affected by embryo quality and hence, oocyte quality. Therefore, in the current study embryo morphology
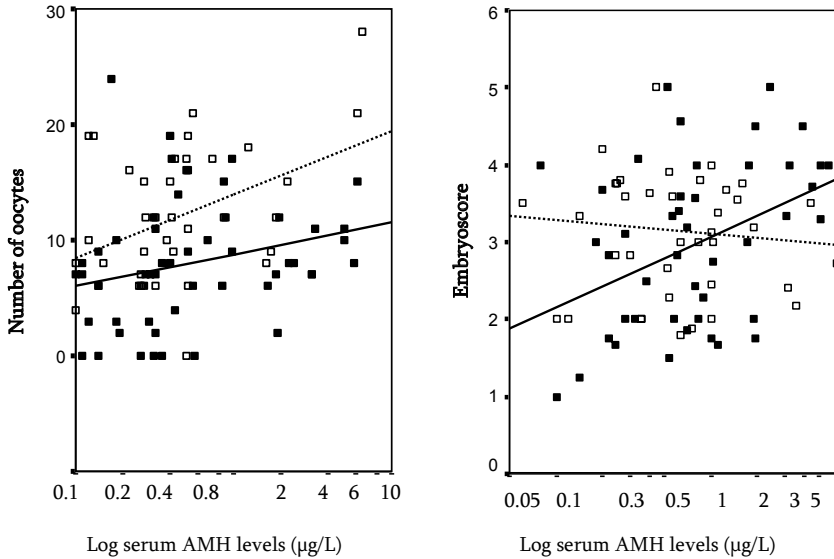
Figure 4.3.3. Scatter plots of baseline AMH serum levels (logarithmically transformed) and Number of oocytes retrieved at oocyte pick. Pearson's correlation coefficient $r = 0,47$ ($P = 0,002$) in the standard (dotted line and open boxes) and $r = 0,29$ ($P = 0,03$) in the mild stimulation group (solid line and filled boxes).

Mean embryo score per number of rated embryos; lowest score represents best embryo morphology. Pearson's correlation coefficient $r = -0,15$ ($P = 0,37$) in the standard (dotted line and open boxes) and $r = 0,41$ ($P = 0,005$) in the mild stimulation group (solid line and filled boxes).

score as well as aneuploidy rate were used to assess embryo quality. Recently, a positive correlation was observed between the number of oocytes retrieved and the percentage abnormal embryos after mild stimulation (229). In the current study, AMH at start of stimulation was correlated with the number of oocytes after mild as well as standard stimulation and therefore, similar findings to the previously mentioned study were expected. Indeed, the current results showed a negative correlation between the number of oocytes retrieved and the percentage of normal oocytes (data not shown). Apparently, among the few oocytes retrieved after mild stimulation, the majority must have been chromosomally normal. However, the lack of correlation between AMH in serum and aneuploidy rate suggests that AMH cannot be used as a marker for the rate or incidence of aneuploidy.

After standard stimulation, AMH serum levels decreased significantly. This decrease in AMH levels between start of stimulation and the day of hCG administration reflects the decline of the cohort of small growing follicles recruited during ovarian stimulation (221, 226). Studies in mice revealed that the FSH sensitivity of follicles is reduced by AMH (67, 68). Therefore, it might be hypothesized that in women, in the presence of low serum AMH levels, FSH sensitivity is increased, and consequently the most FSH-sensitive follicles will be recruited. Because in the present study, basal serum

AMH levels were comparable between the two stimulation protocols, the difference in the number of oocytes retrieved was solely dependent on the stimulation regimen. For this reason, more follicles were recruited during conventional stimulation, due to an extended window of recombinant FSH availability. On the other hand, during mild stimulation, only the most FSH-sensitive follicles were recruited, due to their decreased FSH sensitivity. Consequently, AMH serum concentrations during mild stimulation were correlated with the number of follicles as well as with embryo morphology. Since pregnancy rates were similar between the two protocols, oocyte quality in the mild stimulation group must have been better. In contrast, during conventional stimulation the recruited cohort consisted of a mixed population of follicles with different individual FSH sensitivities and consequently different developmental stages, harbouring apparently good quality and poor quality oocytes. Indeed, in such a protocol the correlation between basal serum AMH levels and follicle number was still present. However, the correlation between AMH and embryo quality was lost, as is apparent from the current study. Similar findings have been reported in two recent studies (130, 231). In patients undergoing IVF, AMH levels on the day of hCG administration exceeding a predefined threshold were positively correlated with embryo quality, higher implantation rates and higher ongoing pregnancy rates as compared with patients with serum AMH concentrations below this threshold value (149). Higher AMH concentrations on the day of hCG administration may have resulted from more physiological ovarian stimulation protocols, during which lower numbers of oocytes are recruited. It may be assumed, that during the physiological process of single follicle selection, the one follicle containing the best oocyte will ovulate. Hence, it seems that the mild stimulation protocol mimics the physiological situation more closely. Although it induces less pre-ovulatory oocytes, they constitute the better quality oocytes since implantation rates were higher, explaining the similar pregnancy rates observed in this study.

AMH levels in follicular fluid might be indicative for oocyte quality. Recently, it has been reported that intra-follicular AMH concentrations from patients with fertilized oocytes were higher than those from patients with non-fertilized eggs (233). In addition, clinical pregnancy rates and embryo implantation rates were significantly higher in patients with follicles containing high AMH concentrations (234). Similar to the present findings, this almost "physiological" stimulation protocol resulted in one follicle containing a qualitatively good oocyte. However, a negative correlation between AMH in serum and patients age or between serum AMH and failure to retrieve oocytes was absent. Apparently, differences in follicular fluid AMH concentrations are not necessarily reflected in serum AMH concentrations. This might be due to the relatively low number of follicles recruited during mild stimulation exhibiting a similar FSH sensitivity and therefore containing similar amounts of AMH. Hence, these follicles all contribute in an equal proportion to the AMH serum level. In contrast, in a conventional stimulation protocol, the recruited follicles exhibit different FSH sensitivities and might also contain different levels of AMH. Hence, the correlation between the serum

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AMH concentration and the number of follicles is lost after conventional ovarian stimulation. Indeed, in the present study AMH serum levels were only positively correlated with embryo morphology in the mild stimulation protocol. This might also be an explanation why data on correlations between serum AMH concentrations and implantation or pregnancy rates are contradictory (63, 149, 226, 235, 236).

In conclusion, baseline serum AMH concentrations are well correlated with ovarian response during conventional as well as during mild ovarian stimulation. Although serum AMH levels were correlated with embryo morphology scores in the mild stimulation group, a similar correlation could not be demonstrated in the conventional stimulation protocol. A consistent correlation between serum AMH concentrations and embryo quality, as assessed by PGS, was not observed. It seems, therefore, that basal serum AMH concentrations are not an adequate marker for embryo quality. However, AMH seems to play an important role during folliculogenesis, by modulating FSH sensitivity of individual follicles. The exact role of AMH in the prediction of oocyte quality during assisted reproductive treatment remains to be established.

5

Postponement of childbearing has led to increased rates of age-related female infertility. In clinical practice, those women with reduced reproductive capacity need to be identified in order to counsel them on their chances of pregnancy. Consequently, increased attention has been given to the identification of markers of ovarian reserve. Serum anti-Müllerian hormone (AMH) levels have been described as an accurate marker of ovarian reserve. AMH is expressed in granulosa cells of primary follicles and continues until the early antral stage during which expression is strongest (73). Many clinical studies have confirmed that serum AMH concentrations correlate well with the antral follicle count (AFC) (51, 80, 85, 96). Consequently, AMH levels not only constitute a direct marker of the AFC but also seem to indirectly reflect the size of the primordial follicle pool (80). In addition, serum AMH has also been described as a marker of polycystic ovary syndrome (PCOS), since it reflects the excessive number of antral follicles (51, 85). Hence, serum AMH levels seem to constitute a promising marker of ovarian reserve and ovarian dysfunction. The aim of this thesis was to study the value of serum AMH levels as a marker of ovarian reserve and ovarian dysfunction in a clinical setting.

5

5.1. AMH AS A MARKER OF OOCYTE QUANTITY

Ideally, a marker of ovarian reserve should be able to predict the onset of decreased fertility and the age of menopause. In addition, such a marker should also be able to reliably detect an early decline in ovarian reserve in the individual woman, and consequently, these women should be advised to not further postpone their childbearing, in order to avoid future assistance in reproduction. Moreover, such an ovarian reserve marker requires a clear cut-off value that discriminates individuals with normal ovarian reserve from those with diminished ovarian reserve. Until now, no clear cut-off levels have been defined for FSH and AFC, the more traditional ovarian reserve markers (237, 238). Despite the lack of well-defined normal values of AMH, assessment of AMH levels is widely applied in the prediction of ovarian response during assisted reproductive techniques (ART) (60, 63, 70, 81, 95, 226, 227, 235, 239-243). Numerous studies have attempted to define a cut-off level for serum AMH that might be predictive of poor ovarian response (148, 149, 235, 242, 244-246). A cut-off level that might discriminate women with expected poor response from those with normal ovarian response and successful outcome of ART, may also serve as a cut-off level of diminished ovarian reserve. However, the discriminative levels that have been proposed for serum AMH so far show a considerable variation, that seems to depend on the etiology of infertility and age of the study subjects included. Indeed, some studies have included infertile women older than 38 years, in whom poor response might be expected (242, 244), whereas other studies have measured AMH levels in a more random infertile population (148, 149). Furthermore, in some of these studies the enzyme-linked immunosorbent assay (Beckman-Coulter) had been used to measure AMH levels (148, 149), whereas others used the ultrasensitive immunoassay available

through Diagnostic System Laboratories (DSL) (242, 244). Fortunately, nowadays a new assay is available (Gen II Beckman-Coulter assay, Beckman Coulter, Inc., Webster, Texas), and its general use would reduce inter-assay variability in future studies. Finally, this large variation in AMH serum concentrations in infertile women indicates the urgent need to investigate variation of AMH levels in the normal population. In our study on normative data in a large cohort of healthy females, cut-off levels of the normal values were assessed using the 10th and 90th percentile of normative data (chapter 2.1). At any age, there was a wide range in AMH levels. A similar range in AMH levels has also been described in large cohort studies in healthy girls (99, 247) and regularly cycling, but infertile women (100). Furthermore, during early childhood and from the age of 25 years onwards, the lower limit of normal values seemed to approximate the limit of detection of AMH levels. As a consequence, it will be extremely difficult to distinguish women with a compromised ovarian reserve from those with normal ovarian reserve based on a single AMH measurement. Furthermore, the large variation in AMH levels in a normal, proven fertile population, indicates that defining a cut-off value for poor response with a sufficient predictive power may be difficult.

Our cross sectional data (chapter 2.1) only provide information on the current status of ovarian reserve, but not on the progression towards menopause. Consequently, from these data it cannot be concluded whether regularly cycling females with an AMH level below the 10th percentile indeed have a diminished ovarian reserve. Although two longitudinal cohort studies have suggested that AMH might be predictive of age at menopause (83, 248), long term follow-up of ovarian function and reproductive status, with frequent serial analysis, is needed in females with normal ovarian function to determine whether women younger than 35 years with AMH below the 10th percentile, have a compromised ovarian reserve. In some women, serial AMH measurements might follow the 10th percentile, since they have been on that level ever since, whereas other women might progress into menopause much faster and hence, their longitudinally assessed AMH concentrations will intersect different percentiles in a similar time span. However, serial measurements of ovarian reserve and follow-up of reproductive events might as well reveal that in some women AMH levels are low, without any consequences for their reproductive outcome.

Consistent with recent studies (99, 102), a variable correlation between AMH and age was observed in our normative data (chapter 2.1). A previous study in rodents may provide a possible explanation for these findings (79). It was shown that during early reproductive life, AMH concentrations remained constant in mice, whereas the number of primordial follicles decreased steadily. Initially, the number of growing follicles was constant, suggesting that the rate of primordial follicle recruitment was increased during early reproductive life. Once the size of the primordial follicle pool fell below a certain threshold, the number of growing follicles did decline and concomitantly, serum AMH levels declined (79). In women, there is limited information on regulation of recruitment of primordial follicles. However, it may be possible that it follows a similar course as in mice. Hence, during adolescence and early reproductive life, the

growing follicle cohort is constant and AMH concentrations are constant, whereas the number of primordial follicles decrease steadily. From the age of 25 years onwards, the number of remaining primordial follicles has reached a certain threshold and as a consequence the size of the growing follicle cohort and AMH levels decline. Because of differences in the rate of primordial follicle recruitment and differences in the size of the primordial follicle pool, the onset of declining AMH levels may vary, reflecting the large variation in AMH concentrations and thus, variation in reproductive ageing. In other words, prior to the age of 25, the variation in AMH levels may reflect several different stages of reproductive ageing, i.e. a stage at which the number of growing follicles remains constant or a stage at which the cohort of growing follicles is declining.

A specific group of women in whom assessment of the ovarian reserve is relevant are survivors of cancer (chapter 3.1 and 3.2). Gonadotoxicity is a known side-effect of cancer treatment and may damage primordial follicles as well as growing follicles (249, 250). Depending on the extent of ovarian damage, accelerated follicle loss might result in a transient reduction in the size of the growing follicle cohort and consequently, a temporary ovarian dysfunction, whereas loss of primordial follicles might result in premature ovarian insufficiency (POI) (119, 136, 143, 154, 251-253). Hence, female cancer survivors are suspected of diminished ovarian reserve and its early detection is especially important in those who still might want to conceive. In chapter 3.1, it was shown that in premenopausal women who had received chemotherapy and total body irradiation for hematological cancer at adult age, serum AMH levels after treatment were undetectable and all women had developed acute POI. In addition, in premenopausal women treated with chemotherapy, serum AMH levels were lower than AMH levels in age-matched controls, despite restoration of menstrual cyclicity after cancer therapy. These results indicate that in female cancer survivors, AMH constitutes a useful marker of ovarian reserve. Moreover, assessment of AMH may be valuable to detect early decrease of ovarian reserve in women at risk to lose their reproductive capacity early in life. In young adult survivors of childhood cancer that had been treated with procarbazine-containing chemotherapy, in those who received irradiation to the abdomen and in survivors treated with total body irradiation, serum AMH levels were significantly lower than in age-matched healthy controls, indicating that these females may be at risk of POI. Indeed, in 27% (49/182) of our survivors, AMH levels were lower than the 10th percentile of normal values, suggesting that follicle loss in cancer survivors was accelerated as compared to that in controls (chapter 3.2). However, these results should be interpreted with caution, since they were based on a single assessment of ovarian reserve. Since a single AMH measurement only reflects the current status of the ovarian reserve and not the progression of follicle loss. Therefore, longitudinally designed studies with repeated assessments of AMH levels are recommended, which may provide more insight in the rate of follicle loss after cancer treatment. Moreover, the progression of follicle loss in cancer survivors should be compared with that in age-matched healthy control women over a similar period of follow-up, to determine whether in cancer survivors AMH levels within the normal range are predictive of

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normal age at menopause or whether gonadotoxicity has accelerated primordial follicles loss, resulting in POI. In addition, it would be useful to be able to predict the extent of ovarian damage and consequence of gonadotoxicity in each individual patient prior to therapy. A recent long term follow-up study showed that pretreatment AMH levels were predictive of restoration of menstrual cycles in women after treatment for breast cancer, independent of age at start of treatment (254). Although data on reproductive potential in survivors with restoration of menstrual cycles was lacking in that study and follow-up of AMH levels was not performed in a control cohort, these data do indicate that pretreatment assessment of the ovarian reserve may provide information on residual ovarian function after therapy. Consequently, cancer patients with low AMH levels at diagnosis seem to be at increased risk of POI after treatment. These females should be counseled on the possibilities of fertility preservation prior to initiation of cancer treatment.

5.2. AMH AS MARKER OF NORMOGONADOTROPIC ANOVULATORY DYSFUNCTION AND POLYCYSTIC OVARIES

Because of the strong correlation between AMH and the number of small antral follicles, AMH has been described as a marker of ovarian dysfunction i.e. anovulatory infertility (146). Most women presenting with anovulatory infertility are diagnosed with normogonadotropic anovulatory dysfunction, class II according to the World Health Organization classification for anovulatory dysfunction (class WHO II). Among normogonadotropic anovulatory women, the polycystic ovary syndrome (PCOS) is a common endocrine disorder. PCOS is based on its clinical presentation, which encompasses a broad spectrum of symptoms (255). Typically, women with normogonadotropic anovulation and PCOS present with high AMH levels as compared with normo-ovulatory women (51, 85), which seems to be attributable to an increased number of pre-antral and antral follicles (48). Therefore, in chapter 4.1 we have tested the diagnostic value of AMH levels the presence of an excessive number of follicles, i.e. polycystic ovaries (PCO). Surprisingly, the largest proportion of women with PCO studied had AMH levels within the normal range. This finding may be explained by the wide the range in AMH levels and concomitant number of follicles in the regularly cycling, age-matched control women. More specifically, in normo-ovulatory control women between 18 and 30 years old, the median AFC was 18, and thus would be diagnosed as having PCO. Hence, the presence of 12 or more follicles per ovary in regularly cycling women is far more common than previously described (208, 216). In agreement, a recent study has suggested that the cut off level of 12 or more follicles may no longer be valid for the diagnosis of PCO. Because of more accurate detection of small follicles, the threshold for PCO should be elevated to 19 follicles or more. In addition, the authors propose that the presence of 19 or more follicles per ovary in

women with regular menstrual cycles, thus, comparable to women described in our control cohort, may be a silent precursor form of PCOS (191). Indeed, several studies have described endocrine abnormalities, such as increased androgen and insulin levels in non-hirsute, normo-ovulatory women with PCO (103, 203, 256), indicating that PCOS may be more common and far more heterogeneous than recognized until so far. Other studies have proposed that PCO is an age-dependent, common finding among ovulatory women (209, 210, 257). The presence of PCO in almost two-third of our control subjects supports the latter studies and suggests that the threshold of 12 or more follicles as cut-off level for PCO is not very specific for the diagnosis of PCOS (216). Consequently, the cut-off level for PCO may need to be revisited (191). Because of the age-related decline of the AFC, we propose that this cut-off level should be age-dependent. Possibly, an age-adjusted and higher threshold for PCO may yield a more accurate diagnosis of PCO and thus, of PCOS in adolescents, with an increased follicle number (42). Longitudinal follow-up studies of menstrual cycles, follicle counts, endocrine and metabolic profiles in a random cohort of adolescents may provide more insights on the clinical significance of PCO in the diagnosis of PCOS.

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5.3. AMH AS A MARKER OF OOCYTE QUALITY

Concomitant with the gradual decline of follicle numbers, an increased miscarriage rate and a higher incidence of children with chromosomal abnormalities have been observed at advanced maternal age, and are believed to be due to a decrease in oocyte quality (258, 259). Hence, the process of ovarian ageing is not only accompanied by a steady decrease in the number of ovarian follicles but is also associated with a concomitant decrease in oocyte quality (16). Studies on the two aspects of ovarian ageing in infertile women showed increased miscarriage rates and low chances of ongoing pregnancy in women with poor response to ART treatment cycles (260-262). Since poor response seems to reflect a decrease in the number of follicles (59), these findings suggest a possible relation between oocyte quantity and quality. However, other studies showed that markers predictive of poor response such as AFC, serum FSH and inhibin B were not predictive of chances of ongoing pregnancy (16, 57, 101, 238, 263, 264). In agreement, in chapter 4.3, we describe that serum AMH levels were predictive of the number of oocytes retrieved after ovarian stimulation in infertile women aged up to 37 years. However, there was no consistent correlation between AMH and embryo quality, expressed by aneuploidy rate and embryo morphology score or with fertilization rate. These results suggest that oocyte quantity and numerical chromosomal abnormalities are not tightly linked. Indeed, aneuploidic embryos have been observed after *in vitro* fertilization cycles in infertile women younger than 38 years, in whom FSH and inhibin B levels were within normal ranges (265). In addition, our data confirm that markers of the quantitative aspect of ovarian reserve, such as FSH, AFC and AMH, do predict the number of oocytes retrieved after ovarian stimulation, but do not constitute markers of oocyte quality. This suggests that, besides chromosomal segregation defects, multiple

factors may contribute to the developmental capacity of an oocyte, and that an accurate marker has yet to be identified. Although the number of good quality oocytes is decreasing with increasing age, apparently a few normal oocytes are still present which can be recruited from time to time and especially during ART cycles.

In conclusion, the most important clinical application of serum AMH levels is assessment of ovarian reserve. Additional longitudinal follow-up studies are needed to determine a cut-off level at the lower limit of the normal range of serum AMH concentrations, that can be used to discriminate women at risk of infertility or early menopause (Table 5.1). Such studies may provide information on the rate of follicle loss and are required not only in healthy, normo-ovulatory women, but also in women at risk of POI, i.e. after gonadotoxic treatment. In addition, serum AMH concentrations at diagnosis may predict residual ovarian reserve after cancer treatment. Longitudinal follow-up studies of ovarian reserve in cancer survivors may provide insight into the progression of follicle loss after gonadotoxic treatment and the consequences for reproductive potential (Table 5.1). In the diagnosis of PCOS, the role of AMH seems to be limited; most studied PCOS women had AMH levels within the normal range and consequently, the upper limit of the normal range of AMH levels in normo-ovulatory women needs further investigation (Table 5.1). Concomitantly, normo-ovulatory adolescents and young adults women should be screened on the presence of PCO, since our data indicate that this may be a rather physiological phenomenon. Furthermore, these adolescents and women might have an occult form of PCOS, and therefore, screening of endocrine profiles, especially serum androgen levels, should also be included. Confirmation of the current findings may require the assessment of a new threshold for the presence of PCO and thus, for the diagnosis of PCOS.

Population	Research topic
General population	Prediction of decreased fertility and age of menopause
	Variation in the age of menopause
	Follicular dynamics during childhood and adolescence
	Prevalence of polycystic ovaries and polycystic ovary syndrome
Cancer survivors	Prediction of residual ovarian function after cancer treatment
	Follicular dynamics after cancer treatment

Table 5.1. Recommendations for future research

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SUMMARY

The ovary is of major importance in the process of reproduction and hormone production. The age-related decrease in ovarian follicles and a concomitant decline in oocyte quality dictate the occurrence of natural loss of fertility and, ultimately, menopause. Over the past few decades, postponement of childbearing has led to a shift of the age at which women deliver their first child towards the age at which fertility starts to decrease. Consequently, the incidence of age-related female subfertility has increased and as a consequence the need for assisted reproductive treatment. Since ovarian reserve is predictive of ovarian response during assisted reproductive treatment, increased attention has been given to the identification of markers of ovarian reserve. **Chapter 1**, the general introduction, provides the background of ovarian physiology, ovarian reserve and ovarian pathophysiology. In addition, the most currently used markers of ovarian reserve are described. Pre-clinical and clinical studies, discussed in detail in this first chapter, confirmed that serum AMH concentrations constitute a marker of ovarian reserve and ovarian pathophysiology, such as polycystic ovary syndrome (PCOS). The aim of the thesis was to study clinical applications of serum AMH as marker of ovarian reserve and ovarian dysfunction.

Chapter 2 describes normal values of AMH in a large cohort of healthy females, ranging from childhood to the end of the reproductive lifespan. During childhood, serum AMH increased with advancing age, whereas in adults from the age of 25 years onwards, serum AMH levels and age were inversely correlated. This suggests that follicular dynamics during childhood might differ from that at adult age and implies that AMH is only applicable as marker of ovarian reserve in women of 25 years and older. In addition, the inter-individual range in serum AMH levels was very large at any given age. Most likely, this reflects the large variation in age of menopause.

The second part of the thesis addresses ovarian reserve in cancer survivors. Gonadotoxicity is a well-known side effect of cancer therapy, which may lead to premature ovarian insufficiency (POI). However, the extent of the damage is unpredictable. **Chapter 3.1** describes the predictive value of serum AMH levels for the residual ovarian reserve in women with haematological malignancies. In all patients, AMH concentrations were lower than in age-matched controls prior to treatment, suggesting that in cancer patients the ovarian reserve might already be compromised prior to any therapy. Patients who had received total body irradiation all developed POI and in this subgroup of cancer patients fertility preservation should be considered. In the studied cohort of cancer survivors, serum AMH levels prior to therapy were not predictive of the residual ovarian reserve after cancer therapy.

Ovarian reserve in survivors of childhood cancer was investigated in **chapter 3.2**. Serum AMH concentrations in the total cohort were not different from controls. However, significantly lower AMH levels were observed in survivors treated with procarbazine-containing chemotherapy cycles, with abdominal or total body irradiation. Consequently, also in adults who had been treated for cancer during childhood or adolescence, serum AMH can be used to identify subgroups at risk for

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decreased fertility or POI. Some of these childhood cancer survivors had become pregnant. Pregnancy outcome in a cohort of childhood cancer survivors was described in **chapter 3.3.** Pregnancy outcome was not different between survivors and controls in the total cohort. However, survivors treated with abdominal radiotherapy delivered preterm and had post-partum hemorrhage. Therefore, we recommend that this group of childhood cancer survivors needs inpatient perinatal care and close monitoring of the delivery.

In chapter 4 the role of serum AMH as marker a of ovarian pathophysiology was investigated. First, in **chapter 4.1**, the diagnostic value of AMH in the classification of anovulatory dysfunction and in the prediction of polycystic ovaries was assessed in a large cohort of anovulatory women. Based on AMH levels, hypogonadotropic women could not be discriminated from normogonadotropic women and the value of serum AMH levels as a diagnostic marker of anovulatory dysfunction seemed to be limited to the prediction of hypergonadotropic anovulation. Serum AMH levels were predictive of PCO and might therefore replace PCO as a criterion in the diagnosis of PCOS. However, especially in young control subjects also PCO was observed. This finding suggests that the threshold for PCO is age-dependent and stresses the need to revise the Rotterdam consensus criteria for PCOS.

AMH is considered as accurate predictor of ovarian response in controlled ovarian stimulation for *in vitro* fertilization treatment cycles. It was hypothesized in **chapter 4.2** that serum AMH concentrations might also be a marker of ovarian response during ovulation induction treatment with low dose exogenous gonadotrophins in women with PCOS, since these women are at risk of multiple follicular growth. Serum AMH concentrations remained stable during ovulation induction in PCOS women, which was similar to the findings in natural cycles. Apparently, follicular dynamics in ovulation induction treatment cycles with low dose exogenous gonadotrophins are different from those during controlled ovarian hyperstimulation. Therefore, in these PCOS women, serum AMH concentrations did not seem to be useful as marker of ovarian response in low dose ovarian stimulation cycles.

Ovarian ageing encompasses not only decrease in the number of oocytes, but also oocyte quality decreases with increasing age. In **chapter 4.3** the predictive value of AMH as marker of oocyte quantity and embryo quality was studied in women with regular menstrual cycles and younger than 37 years. Serum AMH concentrations determined on cycle day three were predictive for ovarian response to stimulation. However, the lack of a consistent correlation between AMH and embryo morphology and embryo aneuploidy rate suggests that oocyte quantity and embryo quality are not directly related.

In **chapter 5**, the general discussion, the different applications of serum AMH concentrations are considered. Based on the current studies, the most important role for AMH in clinical practice is assessment of ovarian reserve, in healthy women as well as in women treated with gonadotoxic agents in the past. In addition, suggestions are made for future research to further establish the role of AMH as marker of ovarian reserve and ovarian dysfunction.

SAMENVATTING

De eierstok (het ovarium) is een belangrijk orgaan voor de voortplanting en hormonenproductie. Met toenemende leeftijd neemt het aantal en de kwaliteit van de eiblaasjes (follikels) in het ovarium, ofwel de ovariële reserve, geleidelijk af en neemt ook de vruchtbaarheid af. Uiteindelijk zijn er zo weinig follikels over, dat de menstruaties stoppen en de menopauze is bereikt. Door de groeiende welvaart van de afgelopen decennia, hebben vrouwen hun kinderwens steeds verder uitgesteld, zodat de leeftijd waarop vrouwen hun eerste kind krijgen steeds verder opschuift richting de leeftijd waarop de vruchtbaarheid begint te verminderen. Als gevolg hiervan is er, om zwanger te kunnen worden, een toenemende vraag naar vruchtbaarheidsbehandelingen, ofwel geassisteerde voortplanting. De uitkomst van deze behandelingen wordt sterk bepaald door het aantal follikels in de eierstokken. Daarom is het van belang om dit aantal zo nauwkeurig mogelijk te kunnen inschatten. In **hoofdstuk 1** wordt een overzicht gegeven van de normale follikelgroei en ontwikkeling, de ovariële reserve en verstoorde follikelgroei. Daarnaast worden verschillende markers van ovariële reserve besproken. Recent is het anti-Müllers hormoon (AMH) beschreven als nieuwe en accurate marker van de ovariële reserve. Klinische studies worden beschreven, die aantonen dat AMH waarden in serum een goede weergave zijn van het aantal groeiende follikels. Derhalve kan AMH als marker van de ovariële reserve worden gebruikt en als marker voor disfunctioneren van de eierstok, zoals in het polycysteus ovarium syndroom (PCOS), waarbij er een overschot aan antrale follikels is. In dit proefschrift wordt beschreven in hoeverre AMH kan worden toegepast als marker van ovariële reserve en ovarieel disfunctioneren.

In **hoofdstuk 2** worden normaalwaarden van AMH beschreven in een groot cohort vrouwen, van kinderleeftijd tot het eind van de vruchtbare levensfase. Op kinderleeftijd was er een lichte stijging in AMH waarden. Vanaf de leeftijd van 25 jaar, daalden AMH waarden met toenemende leeftijd. Dit lijkt te duiden op een verschil in follikeldynamiek op kinderleeftijd ten opzichte van volwassenleeftijd en dat AMH pas als marker voor ovariële reserve te gebruiken is vanaf de leeftijd van 25 jaar. Daarnaast was er op elke leeftijd een grote inter-individuele range in AMH waarden. Zeer waarschijnlijk is dit een weergave van de aanzienlijke variatie in menopauzeleeftijd.

Hoofdstuk 3 is gewijd aan ovariële functie en reserve in overlevenden van kanker. Gonadotoxiciteit is een bekende bijwerking van verschillende kankerbehandelingen, die schade aan de eierstokken veroorzaakt, waardoor versnelde afbraak van primordiale follikels kan optreden. Vrouwen die een gonadotoxische behandeling hebben ondergaan lopen daarom het risico op jonge leeftijd al verminderd vruchtbaar te zijn en vervroegd in de overgang te komen. De mate van ovariële schade na behandeling is echter niet te voorspellen. In **hoofdstuk 3.1** wordt onderzocht of AMH vóór behandeling van kanker voorspellend is voor de ovariële reserve na de behandeling. AMH werd gemeten voor en na therapie in vrouwen die werden behandeld voor hematologische nieuwvormingen en werd vergeleken met AMH in vrouwen van vergelijkbare leeftijd, met normale ovariële functie, de controle groep. In alle patiënten was AMH voor de behandeling

lager dan in de controles, wat erop kan wijzen dat de ovariële reserve vóór enige kankerbehandeling al gecompromitteerd kan zijn. Vrouwen die werden behandeld met bestraling over het gehele lichaam waren na deze behandeling allen vervroegd in de overgang. Deze vrouwen moeten daarom worden gecounseld voor de mogelijkheid tot fertiliteitspreservatie. In dit cohort vrouwen, was AMH vóór kankerbehandeling niet voorspellend voor de ovariële reserve na behandeling.

Hoofdstuk 3.2 beschrijft serum AMH concentraties van vrouwen die op kinderleeftijd zijn behandeld voor kanker. In de gehele groep vrouwen was AMH niet verschillend van de controle vrouwen. Echter, vrouwen die waren behandeld met minimaal 3 chemotherapiebehandelingen met procarbazine hadden lagere AMH waarden dan controles, evenals vrouwen die bestraling op het gehele lichaam of op de buik hadden gehad. Ook in volwassen vrouwen die op kinderleeftijd zijn behandeld voor kanker, kan op basis van AMH een verhoogd risico op verminderde vruchtbaarheid of vervroegde menopauze worden opgespoord. Zwangerschapsuitkomsten in een deel van de vrouwen die behandeld werden voor kinder- en jeugdkanker werden vergeleken met data uit een groot nationaal cohort en beschreven in hoofdstuk 3.3. Over het algemeen waren er geen verschillen in zwangerschapsuitkomst tussen de vrouwen die kanker hadden gehad en controles. Echter, vrouwen die waren behandeld met buikbestraling bevielen bij een vroegere zwangerschapsduur en hadden vaker een fluxus postpartum (bloedverlies >1 liter). Daarom adviseren wij dat vrouwen die behandeld zijn voor kanker een medische indicatie krijgen voor de zwangerschap en de partus.

In hoofdstuk 4 wordt de rol van AMH als marker van ovariële disfunctie onderzocht. Hoofdstuk 4.1 beschrijft de voorspellende waarde van AMH bij het diagnosticeren van de drie belangrijkste oorzaken van het uitblijven van de eisprong (anovulatie) en bij de diagnose van PCOS. Op basis van alleen een AMH waarde, was het niet mogelijk om vrouwen met hypogonadotrope anovulatie te onderscheiden van vrouwen met normogonadotrope anovulatie, omdat beide groepen normale AMH waarden hadden. AMH in serum heeft met name een voorspellende waarde bij de diagnose van hypergonadotrope anovulatie, het voortijdig verouderen van de eierstokken. Hoge AMH waarden waren zeer specifiek was voor de aanwezigheid van polycysteuze ovaria (PCO). Omdat een groot deel van de jonge gezonde controle vrouwen ook PCO hadden, lijkt het dat de afkapwaarde voor de diagnose van PCO moet worden aangepast op basis van de leeftijd, hetgeen gevolgen heeft voor de diagnose van PCOS.

In hoofdstuk 4.2 wordt verondersteld dat tijdens ovulatie inductie behandeling in vrouwen met PCOS eenzelfde daling in AMH kan worden waargenomen zoals tijdens een in vitro fertilisatie (IVF) behandeling. De aanmaak van AMH neemt af naarmate follikels groter worden en tijdens stimulatie voor een IVF behandeling zal de serum AMH concentratie afnemen. Bij ovulatie inductie behandelingen wordt de eisprong van een eiblaasje op gang gebracht. AMH waarden bleven stabiel tijdens de ovulatie-inductie behandeling in bijna alle PCOS vrouwen en er was geen verschil met de verandering van AMH tijdens een normale menstruele cyclus. Blijkbaar lijkt de follikeldynamiek tijdens een ovulatie inductie cyclus meer op die van een normale

menstruatie cyclus, dan de follikeldynamiek tijdens ovariële stimulatie voor IVF. Serum AMH waarden lijken daarom niet bruikbaar als marker van respons tijdens ovulatie inductie behandeling in PCOS vrouwen.

Naarmate de eierstokken verouderen is er niet alleen sprake van afname van het aantal follikels, maar er is ook een verminderde kwaliteit van de oocyten. In **hoofdstuk 4.3** wordt onderzocht of serum AMH waarden voorspellend zijn voor zowel het aantal follikels als de kwaliteit van embryo's na ovariële stimulatie bij IVF behandeling. Vrouwen jonger dan 37 jaar met regelmatige menstruaties werden gerandomiseerd voor ovariële stimulatie volgens het standaard IVF protocol dan wel volgens een mild stimulatie schema. Serum AMH correleerde met het aantal verkregen eicellen (oocyten). AMH was gecorreleerd met embryo morfologie, in embryo's verkregen na milde stimulatie, maar niet na standaard stimulatie. Concluderend is AMH voorspellend voor het aantal verkregen oocyten. De inconsistente correlatie tussen AMH en embryo morfologie pleit tegen het bestaan van een directe relatie tussen oocyt kwantiteit en embryo kwaliteit.

Hoofdstuk 5, het laatste hoofdstuk, is een algemene beschouwing van de relevantie van de klinische toepassingen van AMH. Ook de noodzaak aan en mogelijkheden voor toekomstig onderzoek naar de rol van AMH worden besproken.

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LIST OF ABBREVIATIONS

AFC	antral follicle count
ALL	acute lymphoblastic leukemia
AMH	anti-müllerian hormone
AMH-KO	amh knock out
AMH-RII	amh type ii receptor
AML	acute myeloid leukemia
ANCOVA	univariate analysis of covariance
ANOVA	univariate analysis of variance
ART	assisted reproductive techniques
BMI	body mass index
CCMO	centrale commissie mensgebonden onderzoek
CCS	childhood cancer survivors
CI	confidence interval
CY	cyclophosphamide
DNA	deoxyrubonucleic acid
E2	oestradiol
ELISA	enzyme-linked immunosorbent assay
ESHRE	european society of human reproduction and embryology
FISH	fluorescent in-situ hybridization
FSH	follicle stimulating hormone
Gy	gray
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HL	hodgkin lymphoma
Hovon	hemato-oncologie voor volwassenen nederland
IVF	*in vitro* fertilization
ICSI	intracytoplasmic sperm injection
IU/L	international units per liter
LCH	langerhans cell histiocytosis
LH	luteinizing hormone
MOPP	mechlorethamine, vincristine, procarbazine, prednisone
NBL	neuroblastoma
NHL	non-hodgkin lymphoma
OCP	oral contraceptive pills
OI	ovulation induction
PCO	polycystic ovaries
PCOS	polycystic ovary syndrome
PGS	preimplantation genetic screening
POI	premature ovarian insufficiency
RCT	randomized controlled trial
recFSH	recombinant fsh
ROC	receiver operating characteristic
SCT	stem cell transplantation
TBI	total body irradiation
TGF-β	transforming growth factor β
WHO	world health organization

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PHD PORTFOLIO

Name PhD student: Sharon Lie Fong
Erasmus MC Department: Obstetrics and Gynaecology, Division of Reproductive Medicine
Research School: Molecular Medicine Postgraduate school (MolMed)
PhD period: 2005-2011
Promotors: prof. J.S.E. Laven and prof. A.P.N. Themmen
Supervisors: J.A. Visser and I. Schipper

1. PHD TRAINING

	Year	Workload (Hours/ECTS)
General courses		
Biomedical English Writing and Communication	2009	2
Oral presentations		
Fertility in childhood cancer survivors. 2nd National SKION Late effects of childhood cancer meeting, Rotterdam 2010	2010	2
Pregnancy outcome in long term childhood cancer survivors. 25th Annual Meeting of European Society of Human Reproduction and Embryology (ESHRE), Amsterdam 2009	2009	2
Pregnancy outcome in long term childhood cancer survivors. Voorjaarsvergadering 2009 Vereniging voor Fertiliteitsstudies, Antwerpen 2009	2009	2
Ovarian reserve in long term survivors of childhood cancer using anti-Müllerian hormone. 40th Annual Meeting of the International Society of Pediatric Oncology, Berlijn 2008	2008	2
Anti-Müllerian Hormone, een nieuw marker voor PCOS. Refereeravond Rotterdamse Gynaecologen Opleidingscluster (RGOC), Rotterdam 2008	2008	2
Anti-Müllerian Hormone in serum replaces transvaginal ultrasound in the diagnosis of PCOS. Jaarlijkse wetenschapsdag afdeling Verloskunde en Vrouwenziekten Erasmus MC en Rotterdamse Gynaecologen Opleidingscluster (RGOC), Rotterdam 2007	2007	2
Komt een vrouw bij de dokter……met een cyclusstoornis Voorjaarsvergadering 2007 Vereniging voor Fertiliteitsstudies, Gent 2007	2007	1
Protocol voor weeënremming in het Rotterdamse cluster Refereeravond Rotterdamse Gynaecologen Opleidingscluster (RGOC), Dordrecht 2002	2002	1
Poster presentations		
The role of anti-Müllerian hormone in the classification of anovulation and the diagnosis of polycystic ovary syndrome 67th Annual Meeting of the American Society of Reproductive Medicine (ASRM), Orlando 2011	2011	1
Normale serum Anti-Müllers hormoon waarden in gezonde vrouwenvan kinderleeftijd tot menopauze 38e Gynaecongres november 2010 Arnhem	2010	1

	Year	Workload (Hours/ECTS)
Is anti-Müllers hormoon nuttig bij het diagnosticeren van anovulatie en PCOS? 38ᵉ Gynaecongres november 2010 Arnhem	2010	1
Postpartum haemorrhage in female childhood cancer survivors previously treated with abdominal radiotherapy. 11th International Conference on Long-term Complications of Treatment of Children and Adolescents for Cancer (LCTCC 2010), Williamsburg 2010	2010	1
Postpartum haemorrhage in female childhood cancer survivors previously treated with abdominal radiotherapy. 2nd European Symposium on Late Complications after Childhood Cancer 2009 (ESLCCC09), Edinburgh 2009	2009	1
Serum anti-Müllerian Hormone levels in healthy girls. 91st Annual Meeting of the Endocrine Society, Washington D.C. 2009	2009	1
Gonadal damage in female adult childhood cancer survivors after total body irradiation, assessed by anti-Müllerian Hormone. 5th International congress of the European Working group of Myelodysplastic Syndrome in childhood and Juvenile Myelomonocytic Leukemia (EWOG-MDS/JMML), Rotterdam 2009	2009	1
Serum anti-Müllerian Hormone levels as marker of ovarian reserve in adult survivors of childhood cancer. 90th Annual Meeting of the Endocrine Society, San Francisco 2008	2008	1
Serum anti-Müllerian Hormone levels as marker of ovarian reserve in adult survivors of childhood cancer. 10th International Conference on Long term complications of treatment of children and adolescents for cancer, Niagara on the Lake 2008	2008	1
The role of anti-Müllerian hormone in the classification of anovulation. 55th Annual Meeting of the Society of Gynecologic Investigation (SGI), San Diego 2008	2008	1
Long term follow-up of clinical characteristics of Polycystic ovary syndrome. 22nd Annual Meeting of European Society of Human Reproduction and Embryology (ESHRE), Praag 2006	2006	1

International conferences

	Year	Workload (Hours/ECTS)
89th Annual Meeting of the Endocrine Society, Toronto 2007	2007	1

2. TEACHING

Lecturing

	Year	Workload (Hours/ECTS)
Curriculum Bachelor Medicine, Faculty Medicine and Health Sciences, Erasmus University Medical Center Rotterdam	2006-2009	2

Supervising Master's theses

	Year	Workload (Hours/ECTS)
mw. S. Stevens (2007-2008)	2007	2
mw. A.E. Combé (2008-2009)	2008	2
mw. Z.A. Brown (2009)	2009	2
mw. J.A. Burgers (2009)	2009	2

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CURRICULUM VITAE

Sharon Lie Fong was born on November 11th, in Purmerend, the Netherlands. She grew up in Purmerend and Saint Germain en Laye, France. In 1995 she started medical school at the Catholic University of Leuven, Belgium. After her *cum laude* graduation in July 2002, she worked as resident at the department of Obstetrics and Gynaecoloy at the Ikazia Ziekenhuis Rotterdam and almost a year later, at the department of Obstetrics and Gynaecoloy at the Erasmus MC Rotterdam. From July 2005, she worked on her PhD project at the division of Reproductive Medicine until March 2010, when she started her residency in Obstetrics and Gynaecology at the Maasstad Ziekenhuis Rotterdam. Since January 2012 she is in training at the Erasmus MC Rotterdam.

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