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Female Aging

Quantification of ovarian reserve and its association with cardiovascular health

Vrouwelijke Veroudering

Meten van ovariële reserve en de relatie met cardiovasculaire gezondheid

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedgen op donderdag 11 mei 2017 des middags te 4.15 uur

door

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To my parents

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Chapter 1

Chapter 1

General Introduction

Ovarian reserve decline

Women are born with a finite number of oocytes, which gradually decreases as they age (1). The size of the pool of remaining oocytes is known as ovarian reserve. The decline of ovarian reserve continues until the ovaries are 'depleted' at an estimated number of 1000 remaining oocytes (2), which heralds the onset of menopause. The median age at which this occurs is 51 years, with a normal variation between 40-60 years (3-5). The distribution of age at menopause is slightly skewed towards earlier ages at menopause, of which 1% are thought to occur before the age of 40, and 5% before the age of 45 (6). The distribution of age at menopause furthermore appears to have been conserved over time and between ethnicities (7-11).

Data from a 19th century cohort in Québec, giving a rare insight into female reproduction unaffected by the use contraceptive methods, suggest that the ability to achieve a spontaneous pregnancy ceases at an interval of ~10 years before the onset of menopause (12, 13). With the current societal tendency to delay the age of childbearing, it has become relevant to identify women who will cease to be able to reproduce naturally at an early age. This is one of the reasons that markers representing the remainder of ovarian reserve have received ample attention over the past decades. These quantitative markers include inhibin B (14), follicle-stimulating hormone (FSH) (15), degree of response to ovarian stimulation (16, 17), antral follicle count measured by ultrasonography (18, 19), and anti-Müllerian hormone (AMH).

Anti-Müllerian hormone is produced by the granulosa cells of developing ovarian follicles and is correlated to the size of the primordial follicle pool. The AMH molecule is a dimeric protein that belongs to the transforming growth factor-beta (TGFβ) superfamily (20). After being produced by the granulosa cells of developing ovarian follicles (21, 22), the precursor protein proAMH is cleaved to the $AMH_{N,C}$ complex, which covalently binds a N- and mature C-terminal domain (23). Both proAMH and $AMH_{N,C}$ are then released into circulation, where they together represent the concentration of circulating AMH (24).

Peripherally circulating AMH levels are correlated to the size of the primordial follicle pool (25, 26). Along with the decline of the primordial follicle pool with age, peripherally circulating levels of AMH thus also decrease (Figure 1). Levels of AMH are thereby suggested to provide an estimation of 'reproductive age', irrespective of chronological age (18, 27-34). In other words, a woman of a certain (chronological) age with a low AMH level is believed to have a lower ovarian reserve, and thus a shorter time to menopause than a woman with a higher AMH level of the same (chronological) age. Currently, there are multiple available assays that can measure circulating AMH concentrations, with differing detection ranges. The association between two different AMH assays differs per study (35) and it is still unknown to which extent the advent of highly

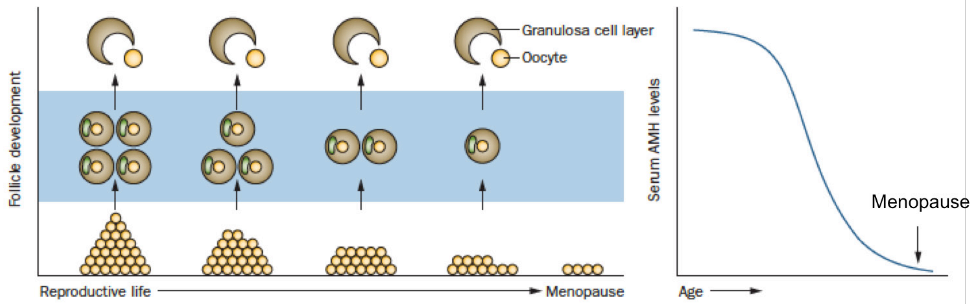


Figure 1. *Schematic overview of the depletion of the primordial follicle pool and decrease in AMH levels of the aging woman. Adapted from Visser et al. (36).*

sensitive AMH assays can increase the number of predicted AMH levels in a low range.

Although the level of premenopausal AMH is associated with age at menopause (18, 30, 37-39), this does not necessarily mean that AMH can give an individual prediction of age at menopause that is reliable enough to be used in clinical practice. A recent study demonstrated that due to wide prediction intervals and large overlap of predicted ranges, it is not possible to generate a precise age at menopause estimation with AMH for an individual (Figure 2) (39). Moreover, the prediction of age at menopause is most relevant for young women faced with the decisions of family planning, and women with an early age at menopause (≤45 years). The relative underrepresentation of young women and high or low extremes of age at menopause in the cohorts of current studies (18, 30, 37-39) hinders the translation of the current prediction models to clinical practice. Lastly, most current prediction models are based on the use of a single AMH measurement (18, 30, 38, 39). Predicting an individual's time to menopause with the use of a single AMH measurement assumes that AMH levels decline in similar fashion between women, with no available long-term data to support this. Thus, if a 30-year-old woman wishes to know her estimated age at menopause, it is currently unknown whether her AMH levels will decline as quickly as that of other women of the same age; and if her AMH level was on the 95th percentile, whether her AMH levels will remain in the top 5% throughout her reproductive lifespan. These gaps in the knowledge of individual AMH decline need to be addressed in order to first be able to better interpret the value of a single AMH measurement, and then to evaluate whether knowledge of the AMH decline trajectory can improve the prediction of age at menopause.

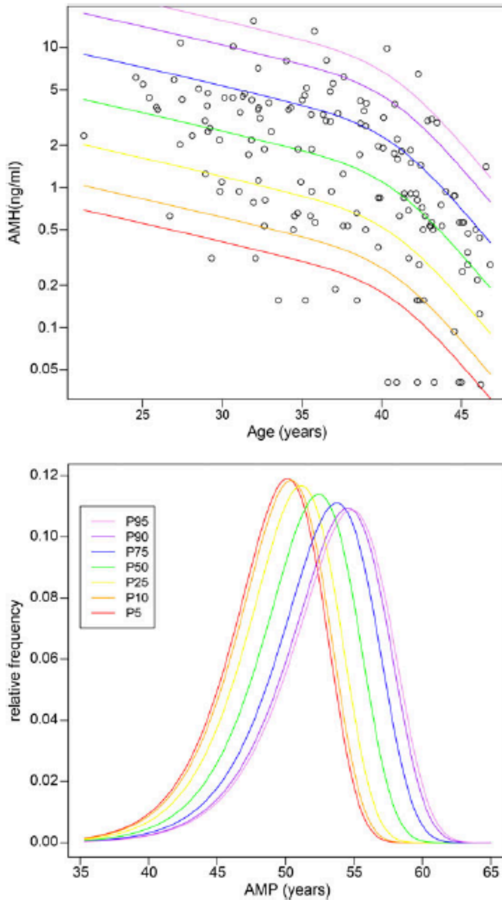


Figure 2. *Estimated age at menopause distribution of women in age-specific AMH percentiles. Upper panel: colored lines indicate the age-specific AMH percentile. Lower panel: estimated age at menopause distribution for women in each age-specific AMH percentile band (39).*

Ovarian reserve decline in relation to cardiovascular health

Globally, cardiovascular disease (CVD) is the main cause of morbidity and mortality in women (40). It is widely accepted that an earlier age at menopause, in particular surgical menopause, is associated with an increased risk of CVD occurrence and resulting mortality (41, 42) in later years. To date, it is not yet fully clear which mechanisms govern this relationship.

There are indications that the menopausal transition and onset of menopause are associated or coincide with a deterioration of cholesterol and blood pressure levels (43-50), which are well-known risk factors of CVD. This could be due to the role of estrogen, which sharply decreases with the onset of menopause (49), and is thought to have protective effects on vascular remodeling and lipid metabolism (51). However, inconsistencies in the benefits of estrogen supplementation in postmenopausal women (52-54) at the very least suggest that there are other factors at play. With increasing chronological age, CVD risk factors are known to become more unfavorable (55, 56). As postmenopausal women are generally older than premenopausal women and the menopausal transition occurs over several years (57), it is challenging to separate the independent effects of menopause or ovarian aging from general or vascular aging. To date, a small number of studies have tried to overcome this barrier, either by longitudinally following women throughout the menopausal transition (45), or studying the effects of menopause in women of the same age (44, 58). These studies found an independent effect of meno-

pause on CVD risk factor levels, but are limited by the fact that the simultaneous effects of general aging and menopause cannot be disentangled within the same study participant (45), or that only a small age range was taken into account (44, 58). The independent effects of aging and menopause thus require additional investigation in order to get a better indication of menopause-related changes in the involvement of CVD occurrence.

The notion that an adverse cardiovascular environment may influence the pacing of ovarian aging, rather than the other way around, comes from a longitudinal study in 695 premenopausal women, where women with a more unfavorable lipid profile had an earlier age at menopause (59). This hypothesis is supported by indications that premenopausal ovarian AMH levels are inversely associated with cholesterol levels (60-62), but this is contested by other reports (63, 64). To make matters more complicated, the discovery of the AMH receptor on fetal heart tissue (65) suggests that circulating AMH could have a direct effect on cardiovascular function, rather than only representing a proxy of ovarian reserve. These hypotheses deserve merit, but current literature on the association between ovarian and cardiovascular aging is still scarce and heterogeneous. The existence of this association should thus be confirmed before drawing conclusions on its causative mechanisms. Further exploration of this topic could potentially help identify prevention and treatment strategies for ovarian or cardiovascular health, or both.

Aims of this thesis

The research presented in this thesis is driven by two main questions:

1. Can knowledge of individual AMH decline improve the prediction of time to menopause?
2. Is there a relationship between ovarian and cardiovascular aging?

To help answer the main research questions, the following sub-questions were addressed:

1a. Is the measurement of AMH comparable between different assays?

1b. Do individual AMH trajectories differ from one another?

2a. Through which mechanisms can the relationship between ovarian and cardiovascular aging be mediated?

Part I: Quantification of ovarian reserve decline

In *Chapter 2*, current literature on the physiology of female reproductive aging and ovarian reserve markers is discussed in more detail as an introduction to ovarian reserve decline.

In *Chapter 3*, the Gen II (Beckman Coulter) and picoAMH (AnshLabs) assays are quantitatively compared to one another, providing a basis for the use and interpretation of AMH measurements in this thesis.

In *Chapter 4*, individual longitudinal decline trajectories of AMH are characterized, in order to determine how AMH declines with age and whether AMH decline trajectories differ between women.

In *Chapter 5*, prediction of time to menopause is studied with the use of multiple AMH measurements. This chapter aims to investigate whether the knowledge of individual AMH decline trajectories can improve the prediction of menopause occurrence.

Part II: The relationship between ovarian and cardiovascular aging

In *Chapter 6*, levels of cardiovascular risk factors are simultaneously related to female age and menopausal status, in order to determine to which extent chronological and reproductive age are independently associated with cardiovascular risk.

In *Chapter 7*, an introduction to the relationship of ovarian reserve with cardiovascular risk is provided, by summarizing current literature on the association between premenopausal AMH levels and cardiovascular risk factors in women.

In *Chapter 8*, premenopausal AMH levels are studied in relation to cardiovascular risk factors in a cross-sectional study, which aims to provide further insight into whether there is a relationship between ovarian reserve decline and cardiovascular health in women from the general population.

In *Chapter 9*, cardiovascular disease outcomes are related to individual decline trajectories of AMH. This chapter assesses whether there is an association between ovarian and cardiovascular aging over time and reflects on potential mechanisms of this relationship.

In *Chapter 10*, the meaning and implications of the combined results of this thesis are discussed and placed in perspective of other relevant research.

Chapter 2

Chapter 2

Female age and reproductive chances

A.C. de Kat, F.J.M. Broekmans

Chapter 1 from 'The Prevention of Age Related Fertility Loss', Springer Publishing

Currently in press

Introduction

We currently live in an era of family planning and female work-force emancipation, while experiencing an ever-increasing lifespan. With this has come the freedom and ability to delay the age of childbearing and facilitate conception. However, for some women this delay may result in having to undergo assisted reproductive treatment (ART) to achieve pregnancy or even in the inability to conceive at all. While calendar, or 'chronological age' is very much related to biological or 'reproductive age', they can also represent separate entities. This means that while some women will be able to achieve a spontaneous pregnancy at age 35 without any problems, others may then have already missed their window of optimal opportunity. This chapter will cover the basic aspects of the reproductive physiology of the aging woman.

Physiology of reproductive aging

Oocyte quantity

During the intra-uterine development of a female she is endowed with a supply of egg cells, or oocytes, which is not able to multiply and will thus decrease throughout her reproductive lifespan. The oocytes are surrounded by a layer of granulosa and theca cells, together constituting a follicle. In its earliest stage of development, while in the resting and non-developing pool, the follicle is considered to be primordial. Starting at birth, follicle numbers in the resting pool decline through apoptosis of the resting follicles. After puberty, primordial follicles are either recruited to undergo development during the menstrual cycle, or go into apoptosis at any stage from resting through development (Figure 1). The vast majority of all ovarian follicles will ultimately be lost through apoptosis (66, 67). Through these pathways, the follicle pool declines with time, and thus with age.

At around 20 weeks of gestation, the pool of oocytes is fully developed, reaching a number of about 7 million (1, 67, 68). At birth, the number of follicles will have already decreased to approximately 1-2 million (1, 69). With time, the oocyte pool declines further. At menarche, the number of remaining oocytes is thought to be 300,000-400,000 (12), with an estimated 1000 oocytes remaining at the time of menopause (2), marking the end of the female reproductive lifespan. The onset of menopause coincides with the final menstrual period and occurs at an average age of 51 years, with ages between 40-60 years considered as the normal variation (1, 3-5).

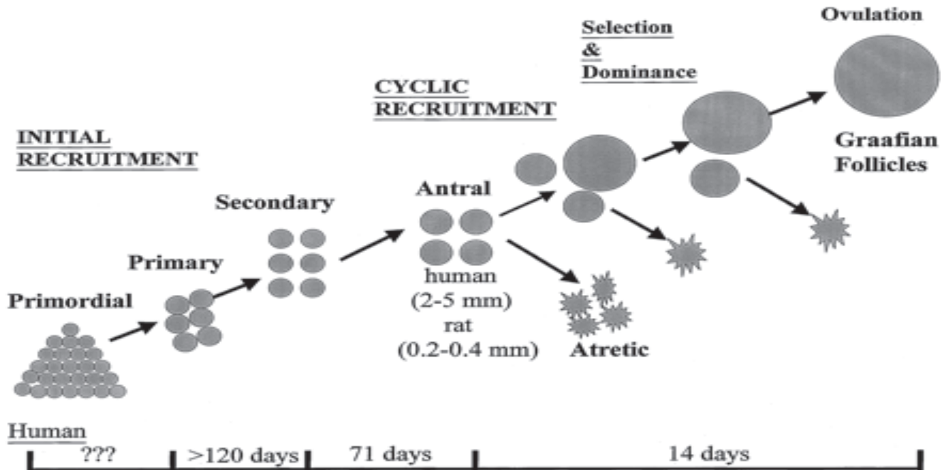


Figure 1. *Schematic overview of follicular recruitment and apoptosis (70).*

Oocyte quality

In addition to a decrease in absolute follicle numbers with time, the aging ovary is also affected by a deterioration of oocyte quality. In the development from a primordial follicle to fertilization, it is necessary for the oocyte to undergo two stages of meiosis in order to reach a haploid state. With advancing female age, the ability of the oocyte to undergo successful and high quality meiosis decreases, as reviewed by Handyside et al. (71). Experimental mice studies demonstrated increased aneuploidy rates resulting from impaired meiotic divisions of oocytes in long-living females, also known as meiotic non-disjunction, with various causative mechanisms related to errors in the cohesion and division of chromatids (72-75). In humans, embryonic and fetal aneuploidies due to failures in both the meiosis I and II stages more often stem from aneuploidies in oocytes than spermatozoa (71, 76-78). In assisted reproduction embryos, the number of aneuploidies exponentially increased with increasing maternal age (79, 80). The significantly lower aneuploidy rate in older women with donor oocyte pregnancies confirms the aging oocyte to be the most important contributor to aneuploidic pregnancies (81).

Another consequence of female aging for the ovary is the effect on mitochondrial DNA, which is maternally inherited. Mitochondrial DNA functions as a reactive oxygen species (ROS) scavenger and is involved in cell metabolism by generating ATP for several functions (82). As a woman ages, mitochondrial DNA sustains more damage and an increase in the number of mutations. After meiosis, the oocytes of aging women therefore increasingly contain damaged mitochondrial DNA, with a concomitant decline of total mitochondrial DNA content (82-84).

Besides the intrinsic aspects of aging described above, oocyte quality is also suggested to be influenced by extrinsic factors related to aging such as lifestyle (e.g. smoking (85)), disease and environmental factors and oxidative stress exposure (71, 86). These factors may cause damage to the oocyte directly, but are also known to influence the epigenetic cell milieu. In animal studies, aging was associated with changes in DNA methylation and histone modification in oocytes, which resulted in an increased aneuploidy rate (87). Epigenetic modifications are thought to lead to disturbances in the RNA expression necessary for follicular development, changes in the expression of DNA governing the meiosis process, and post-ovulatory DNA modifications (87), which in turn can cause aneuploidy.

The reproductive consequences of biological aging

Oocyte quantity decline

During the development of follicles recruited from the primordial follicle pool, a constant interplay consists between hormones produced by the follicular granulosa cells and those secreted from the hypothalamus and pituitary. This enables the regular cyclic pattern of ovulation and menstruation. When the primordial follicle pool, and thus the number of developing follicles selected from this pool, decrease to a certain threshold, the endocrine balance is altered (88). Briefly, the relative lack of released gonadal hormones, such as estradiol and inhibin B initiates a mitigated negative feedback signal to the hypothalamus and pituitary, leading to an increase in gonadotrophin-releasing hormone (GnRH). The ensuing higher levels of FSH in combination with the decrease of FSH-sensitive follicles (1) cause dysregulation of follicle development and –release. Initially, the increase of FSH-levels gives the development of antral, and selection of dominant, follicles an impulse. This results in an uninhibited dominant follicle selection during the menstrual cycle, which therefore remains regular. With increasing FSH levels, the chance of early, or 'advanced' dominant follicle growth increases (89). This leads to the shortening of the menstrual cycle, which is the first noticeable sign of decreasing ovarian reserve. Eventually, the relative lack of available antral follicles inhibits the regular selection of a dominant follicle and ovulation, thus leading to an irregular length of the menstrual cycle, marking the beginning of the perimenopausal transition (57, 88).

The irregularity of menstrual cycles becomes more pronounced in the late stage of the perimenopausal transition, which continues until the final menstrual period, heralding the onset of menopause (57). The estimated time of 2-3 years between the onset of irregular cycles and the onset of menopause is thought to be similar for all women, irrespective of their age at menopause (albeit studied in a population of women in which age at menopause ranged between 44 and 55 years) (90).

Oocyte quality decline

Although changes in the menstrual cycle pattern are indicative of having reached the later stages of reproductive aging, they will already have been preceded by a decline in fertility. Figure 2 summarizes the putative stages of fertility decline with age, from optimal fertility to menopause. At a mean age of 30-31 years, the per-cycle chance of achieving an ongoing pregnancy starts to decrease, due to either impaired fertilization or implantation (91, 92). Data from a contained, religious community not applying any form of reproductive constriction, in the Québec region in the early 19th century suggest that natural sterility subsequently occurs at an average age of 41 years, with a putative fixed interval of 10 years before the onset of menopause (13). The occurrence of natural sterility a whole decade before depletion of the follicle pool is can be problematic for women delaying their age of conception. It was recently estimated that women who wish to have respectively two or three children through natural conception should start as early as age 27 or 23 in order to have a 90% chance of realizing this objective (93).

The impact of deteriorating oocyte quality on fertility extends further than just fertilization. Miscarriage rates start increasing in women trying to conceive after 30 years of age, and exponentially so after age 35 (94). The observed miscarriage rates are likely only the tip of the iceberg, as a large number of pregnancies may have miscarried before they could become clinically apparent (12). Chromosomal

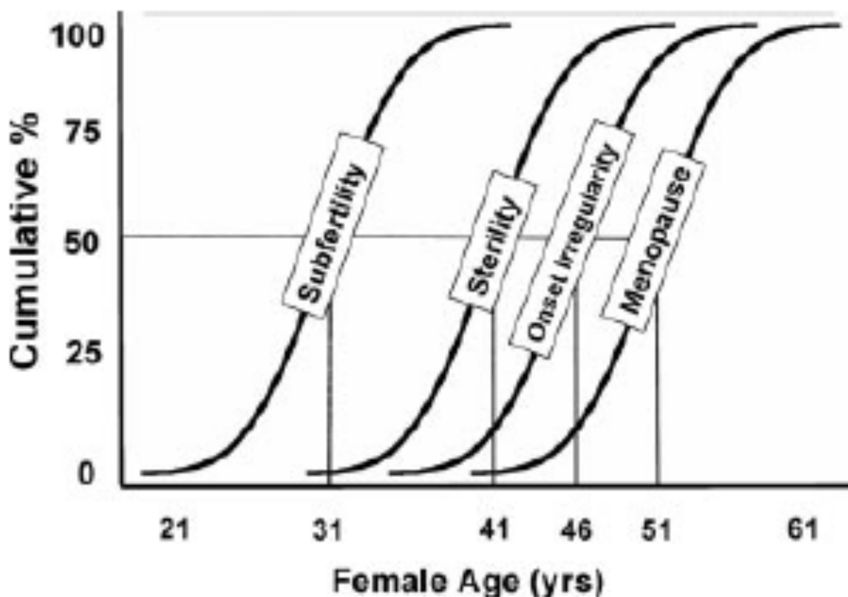


Figure 2. *Stages of fertility decline and menstrual cycle changes with increasing female age (12).*

abnormalities lie at the heart of at least 50% of all known miscarriages in the general population (95), but even in the event of an ongoing pregnancy, chromosomal abnormalities can still occur. The incidence of trisomy 13, 18 and in particular 21 sharply increases with advancing maternal age (74, 96, 97). Between the age of 15 and 45, the incidence of a fetus with Down syndrome (trisomy 21) increases from 0.6/1,000 to 4.1/1,000 (98, 99). In a large epidemiological study from the United Kingdom, at least 95% of all Down syndrome cases was associated with meiotic non-disjunction (100).

The importance of oocyte quality in addition to quantity is exemplified by a group of women with the same extent of diminished ovarian reserve, wherein younger women had significantly better pregnancy rates (101). Furthermore, oocyte quantity decline cannot independently predict implantation and pregnancy rates (102). Male and uterine factors are also an important aspect of fertilization and implantation, but the high pregnancy rates in older women with donated oocytes suggest that the aging oocyte plays a primary role (103).

Measuring reproductive aging

Chronological aging does not necessarily follow the same pace as reproductive aging. It can thus be the case that two women of the same chronological age have a very different 'reproductive age' and, as a result, different reproductive chances. A method of quantifying ovarian age was searched for in the field of ART, in order to individualize ovulation induction dosages and estimate the feasibility of fertility treatment.

Oocyte quantity markers

The past decade saw the emergence of several markers that represent the size of the remaining primordial follicle pool. Here, we briefly present two such markers frequently used in clinical practice, also known as ovarian reserve tests (ORT): antral follicle count (AFC) and anti-Müllerian hormone (AMH). From the non-growing pool, follicles are continuously selected to develop under the influence of FSH. Although the primordial follicle pool cannot be directly measured, the number of antral follicles, which can be determined by ultrasound, is directly correlated to the number of primordial follicles (26, 104). Another proxy of the size of the primordial follicle pool is AMH (25), which is produced by the granulosa cells of small developing follicles (21, 22) and can be measured in the peripheral circulation. Both AFC and AMH give an indication of the expected response to ovarian stimulation (105-107), but are also used as markers of the reproductive lifespan. Antral follicle count and AMH concentrations can better predict age at menopause than chronological aging or family history alone, in which respect AMH appears superior to AFC (108). Predicting the age at which a woman will reach

menopause could potentially guide women and clinicians in decisions regarding family planning (when to start having children) and fertility treatment. However, despite initially hopeful results of age at menopause prediction with AMH, it is still not possible to pinpoint an exact age at menopause for an individual woman (108).

Oocyte quality markers

In order to provide an estimation of the reproductive chances of women with advanced age, it would be desirable to have a marker of oocyte quality in addition to oocyte quantity. In theory, if the aging processes that influence both oocyte quantity and quality run in parallel, the decline in oocyte quantity could also be a measure of deteriorating oocyte quality. There is some dispute as to whether markers of oocyte quantity are indeed representative oocyte quality. This can be divided into two categories: fecundability, or the per-cycle chance of achieving a pregnancy, and fetal or embryonic aneuploidy. Levels of AMH below 0.7 ng/mL were associated with a 62% reduced chance of achieving pregnancy in an ovulatory cycle (109), but others found no association between ovarian reserve markers and pregnancy rate (110-112) or time to pregnancy (113). With regard to fetal aneuploidy, there is evidence suggesting that trisomy occurrence is related to reduced AMH levels (114), or decreased ovarian reserve due to congenital ovarian absence or unilateral surgery (115, 116), whereas no association between oocyte quantity and fetal aneuploidy is reported elsewhere (117, 118). The latter is supported by a study in which embryonic aneuploidy rates were strongly related to maternal age, but not to the number of available embryos per stimulated cycle (79). In other words, oocyte quantity does not appear to be unequivocally related to oocyte quality. The use of oocyte quantity markers for fertility work-up and counseling may therefore be limited. To date, there are still no known markers that are solely indicative of oocyte quality.

Strategies for reproduction at advanced age

In practice, the majority of women will be able to successfully achieve an ongoing pregnancy. Those who do not and have clear reasons for their sub- or infertility, such as tubal factor, azoospermia, or anovulation may benefit from targeted treatment strategies to their problem. However, when a reduced oocyte quantity or quality lies at the heart of involuntary childlessness, the solution is less simple. To date, there are no known ways to increase oocyte quantity or improve oocyte quality. Treatment for ovarian aging therefore currently has a more preventative nature: women are advised not to postpone a pregnancy for too long and should consider lifestyle habits such as smoking to be a constant threat factor for their (future) fertility. At a late stage of ovarian aging, oocyte donation, using eggs from young or at least previously fertile women, may be

the only remaining treatment option for a viable euploidic pregnancy.

Chapter 3

Chapter 3

A quantitative comparison of AMH measurement and its shifting boundaries between two assays

A.C. de Kat, F.J.M. Broekmans, A.C. van Westing, E. Lentjes, W.M.M. Verschuren, Y.T. van der Schouw

Submitted for publication

Abstract

Objective Over the past decades, numerous anti-Müllerian hormone (AMH) assays have been developed. Among other factors, the lack of large-scale comparisons between various assays hinders the universal interpretation of AMH levels. Moreover, little is known of the practical performance of high-sensitive compared to conventional assays with regard to AMH levels in a very low range.

Design Cross-sectional study.

Setting General population.

Participants 1,985 premenopausal women who completed the second visit of the Doetinchem Cohort Study, with a mean age of 42 ± 7 years.

Main Outcome Measure(s) AMH levels were measured with the Gen II assay (Beckman Coulter) and picoAMH assay (AnshLabs). Passing-Bablok and Bland Altman analyses were performed and differences in the proportion of detectable samples were assessed.

Results The Gen II and picoAMH assays were highly correlated, with a Spearman correlation coefficient of 0.92. The Passing-Bablok regression formula was *picoAMH = 0.01 + 1.69*Gen II*, meaning that on average picoAMH levels were 69% higher than Gen II levels. Of the 670 samples with an undetectable value with the Gen II assay, 78% could be detected with the picoAMH assay, with a median [interquartile range] of 0.05 [0.01-0.14] ng/mL.

Conclusion These results indicate that, despite a high correlation, there is a large relative difference between results of the Gen II and picoAMH assays. The use of a high-sensitive AMH assay is furthermore likely to result in a large increase of samples with detectable levels, but the clinical relevance of AMH levels in a very low range remains to be studied.

Introduction

Over the past decade, anti-Müllerian hormone (AMH) has received much attention. Its emergence held the promise of the improvement of non-invasive techniques for quantifying ovarian reserve. The assessment of ovarian reserve is used for several clinical purposes (1), including the prediction of the response to ovarian stimulation for infertility treatments, assessing the extent of ovarian damage caused by agents chemotherapy, aiding the diagnosis of polycystic ovary syndrome and as a potential marker for the duration of the reproductive lifespan. The process of measuring AMH has evolved during this time, resulting in multiple AMH assays currently on the market. As others have previously demonstrated (35, 119-129), none of the currently available AMH assays are directly comparable to one another. Moreover, due to the heterogeneity of available comparative studies and small, selected study populations, it is not known whether a certain proportional difference between two AMH assays found in one center is generalizable to others. These two factors combined form an important impediment to the creation and implementation of international standards of AMH measurement and interpretation.

Amongst the recently developed AMH assays, there has been a focus on the detection of very low AMH levels (127). The detection of low AMH levels could potentially be beneficial to observe subtle differences in ovarian reserve between women nearing the menopausal transition, potentially before the advent of menstrual cycle irregularities. Little is currently known of AMH levels in this phase of the reproductive lifespan. One such sensitive assay is the picoAMH assay marketed by AnshLabs (Webster, Texas, USA), directed at measuring AMH levels in a very low range (124). One study with 22 undetectable samples measured by Beckman Coulter's Gen II assay, the standard assay at that time, found 15 samples to yield a detectable level with the picoAMH assay (124). Other studies, with 68 (130) and 12 (131) samples of undetectable AMH levels with the Gen II assay, additionally detected AMH levels in more samples with the picoAMH compared to the Gen II assay. The implication of the use of very sensitive assays remains elusive.

In this study, we aimed to assess how well the measurements with the relatively new picoAMH assay and widely used Gen II assay correspond to one another, thereby possibly enabling translation of AMH data from one assay system to the other. Furthermore, we sought to evaluate to which extent the more sensitive picoAMH assay was able to detect AMH levels that the Gen II did not. We therefore made a quantitative comparison of the AMH measurements of the Gen II and picoAMH assays in a large population-based sample of women.

Materials and methods

Anti-Müllerian hormone levels were measured in premenopausal women who com-

pleted the second visit of the population-based Doetinchem Cohort Study (132). This second visit occurred on a random day of the menstrual cycle between 1999-2002, at which time peripheral blood was withdrawn and stored at -80°C (132). Prior to the AMH measurements, each sample underwent one freeze-thaw cycle (133). In 2011, frozen serum samples were retrieved from storage, after which AMH was measured with the Gen II ELISA (Beckman-Coulter, Sinsheim, Germany) at the in-house laboratory of the National Institute of Public Health and the Environment, by a single operator (133). In 2015, frozen plasma samples were retrieved from storage, after which AMH was transported on dry ice to Webster, Texas, USA. The samples were then temporarily stored at -20°C until AMH was measured with the picoAMH ELISA (AnshLabs, Webster, TX, USA), by two operators. The plasma samples of each individual were measured in a single assay run by a single laboratory operator. Due to the limited remaining volume of the stored plasma samples, less samples were retrieved than had previously been measured with the Gen II assay. Of the 2,034 women in the ongoing Doetinchem Cohort Study with an available Gen II assessment, AMH levels were additionally measured by the picoAMH assay in 1,985 women. All participants gave written informed consent. Ethical approval for AMH measurement was granted by the Medical Ethics Committee of the Netherlands Organization of Applied Scientific Research for the Gen II measurements (133) and the Ethical Committee for Biobank Studies of the University Medical Center Utrecht for the picoAMH measurements.

The precision of the Gen II assay measurements was validated with a linearity-of-dilution assessment (133). The limit of detection was 0.08 ng/mL and the limit of quantification 0.16 ng/mL. The respective inter- and intra-assay coefficients of variation (CV) were 3.4 and 4.0% (133). Because the Gen II measurements took place prior to the implementation of the revised protocol in 2013 (134), measurements were performed with a non-dilution protocol. For the picoAMH assay, the limit of detection was 1.8 pg/mL and the limit of quantification was 3.0 pg/mL. The inter- and intra-assay CV of the picoAMH assay were 4.4 and 3.9%, respectively. There was no indication of plate drift, as all CVs within plate columns and rows of the picoAMH assay were below 5%. For the purposes of comparison, all AMH levels are henceforth presented in ng/mL.

Levels of AMH, measured by the Gen II and picoAMH assays, were directly compared with a Spearman's correlation coefficient, in order to minimize the effect of outliers. A Passing-Bablok regression analysis was performed to quantify the relationship between levels of both assays. The R package 'mcr' was used for this purpose, with the number of bindings set at $1E^{7}$ due to the large number of samples. The Passing-Bablok analysis was performed with all available measurements, including undetectable levels (which were assigned a value of 0.000 ng/mL). As the assumption of normality does not apply to Passing-Bablok analyses (135), AMH levels were not log-transformed prior to

analysis. The differences between values of both assays at various mean levels were assessed with a Bland Altman plot. All statistical analyses were performed in R (http://www.R-project.org).

Results

The mean age ± standard deviation (SD) was 41.5 ± 7.3 years, with a range between 26 and 58 years. There were 661 (33%) current smokers and 546 (28%) current oral contraceptive (OC) users. Table 1 summarizes the AMH level characteristics of both assays. The medians [interquartile range, IQR] of the Gen II and picoAMH assays were 0.43 [0.00-1.34] ng/mL and 0.71 [0.08-2.09] ng/mL, respectively.

A correlation plot of the Gen II and picoAMH measurements is provided in Figure 1. For both assays, the majority of the measurements accumulated around the lower limit of detection. There was one exceptionally high value, detected as such by both the Gen II and picoAMH assays (with a value of 22.8 and 31.2 ng/mL, respectively). The corresponding Spearman's correlation coefficient was 0.92. When only including AMH measurements above the limit of detection for both assays, the Spearman's correlation coefficient was 0.88.

Table 1. *Characteristics of AMH measurement by the Gen II and picoAMH assays*

	Gen II assay (Beckman Coulter)	picoAMH assay (AnshLabs)
Median [IQR] (ng/mL)	0.43 [0.00-1.34]	0.71 [0.08-2.09]
Mean ± SD (ng/mL)	0.99 ± 1.55	1.59 ± 2.41
Range (ng/mL)	0.00-22.75	0.00-31.23
Levels below the LoD n (%)	670 (33.8)	159 (8.0)

IQR = interquartile range; SD = standard deviation; LoD = limit of detection

The difference in detected AMH levels between the Gen II and picoAMH assays at various mean levels of both assays is depicted in a Bland Altman plot in Figures 2A and 2B. As can be seen in Figure 2A, the absolute difference between the estimates of both assays increases with increasing AMH levels. The differences between log-transformed detectable AMH levels is portrayed by mean logAMH level in Figure 2B, from which can be seen that the majority of the proportional differences remained consistent with increasing AMH levels.

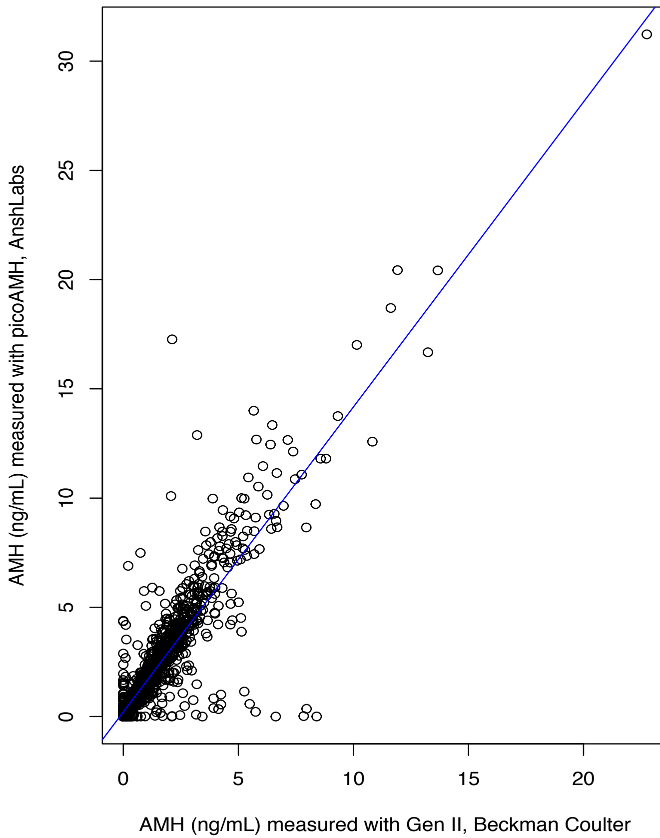


Figure 1. *Scatter plot and regression line of AMH measured by the Gen II and picoAMH assays.*

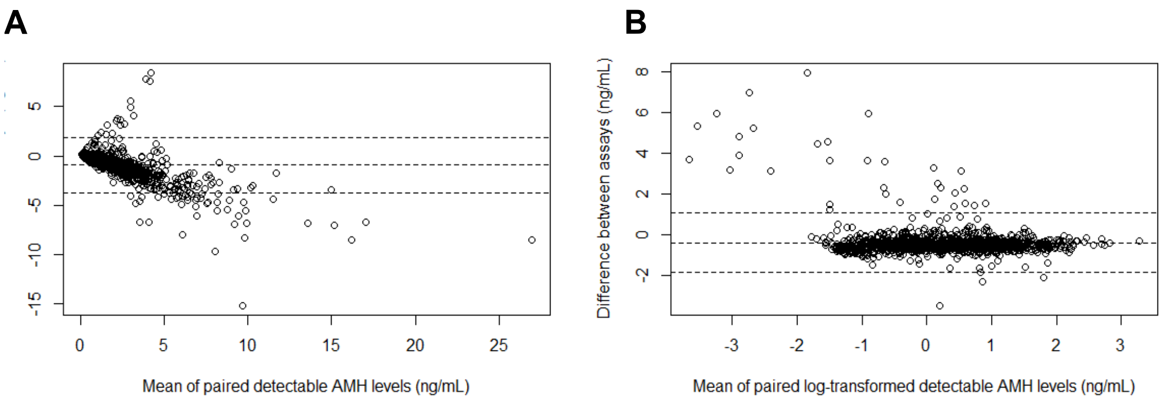


Figure 2. *Bland-Altman plot of absolute (A) and log-transformed (B) AMH levels. The difference between the detectable AMH levels as measured by the Gen II and picoAMH assays is portrayed relative to each mean estimated value of both assays.*

A Passing-Bablok analysis was performed in order to quantify the absolute and proportional differences between the Gen II and picoAMH measurements. The resulting formula for all AMH measurements was *picoAMH = 0.01 + 1.69*Gen II*, with a 95% confidence interval (CI) of the slope of 1.66 to 1.72. Supplementary Figure 1 shows the Passing-Bablok regression plot. The regression formula remained the same after exclusion of the highest 1% of the Gen II and picoAMH measurements. When only including the measurements above the limit of detection of both assays, the regression formula was *picoAMH= 0.03 + 1.63*Gen II*, with a 95% CI of the slope of 1.60 to 1.67. Thus, all picoAMH measurements were 0.01 ng/mL + 69% higher than Gen II measurements, and for measurements above the limit of detection there was a similar proportional difference of 63%, with an added absolute increase of 0.03 ng/mL.

Six hundred seventy (34%) women had AMH levels below the limit of detection of the Gen II assay, compared to 159 (8%) of the picoAMH assay. Of the 670 women with AMH levels below the detection of the Gen II assay, 149 (22%) also had levels below the detection of the picoAMH assay (Table 2), and 521 (78%) had quantifiable levels. The median [IQR] AMH level measured by the picoAMH assay in these 521 women was 0.05 [0.01-0.14] ng/mL. Vice versa, there were 10 women with AMH levels above the level of detection for Gen II and below the level of detection for picoAMH (Table 2). The median [IQR] range of AMH levels of these women was 0.30 [0.13-0.48] ng/mL.

Table 2. *Comparison of proportion of detectable and undetectable AMH levels between Gen II and picoAMH assays*

			Gen II assay		Total
			≥0.08 ng/mL	<0.08 ng/mL	
			n (%)	n (%)	n (%)
picoAMH assay	**≥0.0018 ng/mL**	**n (%)**	1305 (65.7)	521 (26.2)	1826 (92.0)
	<0.0018 ng/mL	**n (%)**	10 (0.5)	149 (7.5)	159 (8.0)
Total		**n (%)**	1315 (66.2)	670 (33.8)	1985 (100.0)

Discussion

In the largest quantitative comparison of two AMH assays to date, we found that AMH levels measured with the picoAMH assay were highly correlated to measurements with the unmodified Gen II assay, but were 69% higher overall. Moreover, 78% of the undetected samples with the Gen II assay yielded a detectable value with the picoAMH assay. Use of the picoAMH assay will therefore allow for the identification of women with a very low ovarian reserve status, before complete depletion of the follicle pool.

Two studies, with 143 (125) and 90 (123) participants, previously compared the results of Gen II to picoAMH assay measurement. The former study (125) divided the participants into categories, based on their Gen II measurement, of <0.1 ng/mL (n=30) and ≥0.1 ng/mL (n=113). In the low AMH group, the slope parameter of the Passing-Bablok regression was 0.94, compared to 1.14 in the high group. This does not correspond to our results, as we found the slope to be slightly lower in the group of women with only detectable AMH levels for both assays (0.16 and 0.0018 ng/mL respectively for the Gen II and picoAMH assays). The origin and age of the study participants was not reported (125), therefore a potential explanation for this discrepancy can only be limited to the difference in study size and lack of measurements below the limit of detection. The comparison of both assays in 90 breast cancer patients (123) was more similar to the current study, with a slope estimate of 1.45 and a Spearman's correlation coefficient of 0.92. However, these values were derived from linear regression analyses with untransformed AMH levels, rather than a Passing-Bablok analysis, which could in part explain the difference in slope estimates (1.45 vs. 1.69).

The ability to measure AMH concentrations in very low ranges is relatively novel. Robertson et al. (122, 136) previously reported an increased intra-cycle variability of AMH levels in a late stage of reproductive life, which was better detected with the AnshLabs UltraSensitive assays than the Gen II assay (122). Use of the picoAMH assay led to a 68% increase in detected samples in a prior small study (124). Our results support this observation, as 78% of the undetectable levels with the Gen II assay were detectable with the picoAMH assay. While this gain of information may be advantageous, the question remains whether it is clinically relevant to make a distinction between AMH levels in a very low range. Potentially, it could help identify women with low ovarian reserve who may still have some room for ovarian stimulation, or on the other hand those with very low ovarian reserve who may have a more unfavorable cardiovascular risk profile (137). However, oocyte quality plays an important role in female fertility, meaning that the mere presence of oocytes is by itself not sufficient for reproduction. Future studies should therefore focus on whether the reproductive lifespan and pregnancy chances of women with very low, de-

tectable AMH levels differ from those with undetectable levels on the current assays.

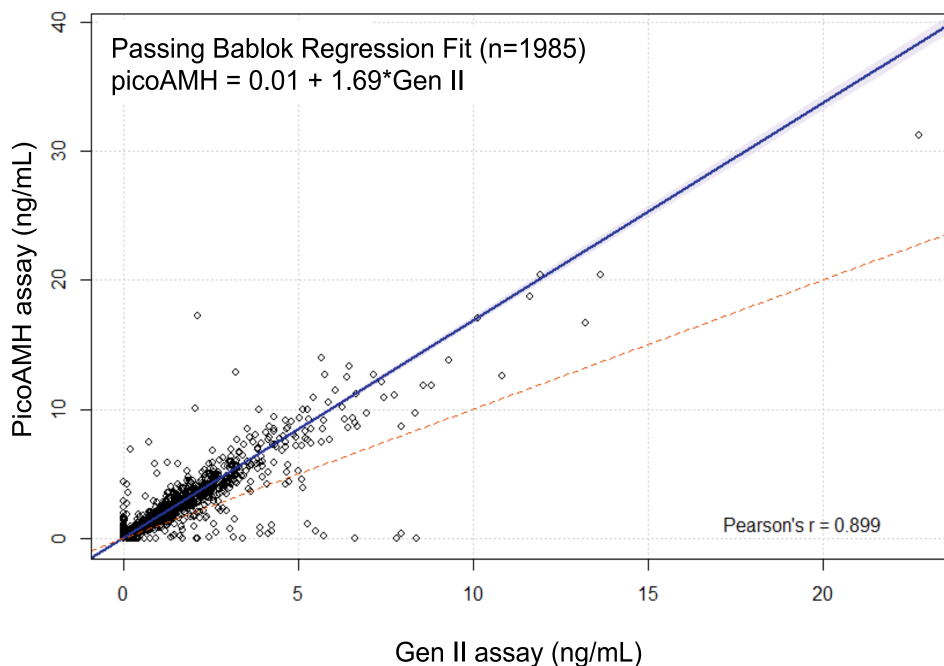
Surprisingly, the current study included 10 women (0.5%) with a detectable level measured by the Gen II assay, but undetectable level with the picoAMH assay. As all samples were treated similarly in the time preceding and during the study, this is unlikely to be caused by batch effects. The other studies in which Gen II assay results were compared to those of the picoAMH assay (122, 123, 131) did not report whether such a situation occurred. This is not surprising considering the small likelihood and small prior studies, but it is therefore not possible to place these results in an appropriate context. The antibodies of the Gen II ELISA are directed at the mature regions of the AMH molecule (119, 123) and those of the picoAMH ELISA are directed at the linear epitope of the mid-pro and mature regions of the AMH molecule (119, 123, 124). It could potentially be the case that mutations or differences in the makeup of the AMH molecule between women explain these differences in detection.

Altogether, storage effects are not assumed to play an important role in the current study, as all samples only underwent one prior freeze-thaw cycle and were simultaneously stored at -80°C. Two studies (138, 139) have systematically assessed the effects of storage and freeze-thaw cycles on AMH measurements with the Gen II assay. Concentrations of AMH remained unchanged after two freeze-thaw cycles and storage at -80°C during 5 days (138), while the second study observed more variability of AMH concentrations measured with the unmodified Gen II protocol after 12 hours (139). A study reporting on the development of an AMH assay reported <1% variation in samples stored at -20°C for 7 days, measured with the Gen II assay (119). A comparative study of the Access AMH assay (Beckman Coulter), Elecsys assay (Roche) and Ultrasensitive assay (AnshLabs) assessed the effects of two-week storage at -20°C and -80°C (129). Compared to fresh samples, the Access and Elecsys assays measured lower AMH levels in the stored samples, whereas the Ultrasensitive assay measured higher AMH levels (129). The reasons for these differences remain unknown, but at the least stress the importance of handling all samples in similar conditions for the sake of comparability. Little is still known of the stability of the AMH molecule during long-term storage, but the location of the binding site of both assays theoretically helps prevent the measurement of proteolytic elements and contributes to assay stability over time.

The great advantage of this comparative study of Gen II and picoAMH assays is its size. Previous studies with a similar objective (123, 125) had a considerably smaller study size, thereby potentially putting more emphasis on chance findings. Moreover, the use of a sample of the general population, rather than a selected study population, enables the generalization of the current assay comparisons to other populations (but not specific patient subgroups). A downside to the current study is the use

of serum and plasma samples for the measurement of AMH. A comparative study of the assessment of AMH in plasma or serum is lacking, but considering the high correlation coefficient this may only play a role in the relative differences in AMH levels over the detectable range. A second pitfall to the generalizability of our results is the use of the original Gen II rather than modified protocol, which could cause an overestimation of AMH levels (138, 139). To assess the extent of this effect in our laboratory, 150 samples that were measured with the original Gen II protocol were re-measured with the modified protocol. The differences in detected levels fell within the coefficients of variation of 3-5% (data not shown, available upon request), thereby suggesting a lack of a systematic difference between AMH measured with both protocols.

The results of this study are in agreement with a previous report of systematic higher AMH levels measured with the picoAMH compared to Gen II assay. Variability potentially caused by differences in sample storage and assay protocol can still hinder a universal conversion factor of levels of one assay to another. Recently, a new nomogram with age was established for the modified Gen II measurements (140). Use of such nomograms per site, which should additionally appear for the newest AMH assays, in addition to knowledge of the site-specific differences between assays, can help aid the construction of AMH measurement and interpretation guidelines. This study therefore does not solve all currently present issues, but does bring this reality one step closer. The capability of new sensitive assays to provide a more nuanced view of the state of low ovarian reserve is furthermore confirmed by this study, but requires additional investigation to fully estimate the clinical consequences.



Supplementary Figure 1. *Passing-Bablok regression analysis of all AMH levels measured by the Gen II and picoAMH assays.*

Chapter 4

Chapter 4

Back to the basics of ovarian aging: a population-based study on longitudinal AMH decline

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Abstract

Background Anti-Müllerian hormone (AMH) is currently used as an ovarian reserve marker for individualized fertility counseling, but very little is known of individual AMH decline in women. This study assessed whether the decline trajectory of AMH is uniform for all women, and whether baseline age-specific AMH levels remain consistently high or low during this trajectory.

Methods 3,326 female participants from the population-based Doetinchem Cohort Study were followed with 5 visits over a 20-year period. Baseline age was 40 ± 10 years with a range of 20-59 years.AMH was measured in 12,929 stored plasma samples using the picoAMH assay (AnshLabs). Decline trajectories of AMH were studied with both chronological age and reproductive age, i.e. time to menopause (TTM). Multivariable linear mixed effects models characterized the individual AMH decline trajectories.

Results The overall rate of AMH decline accelerated after 40 years of age. Mixed models with varying age-specific AMH levels and decline rates provided the significantly best fit to the data, indicating that the fall in AMH levels over time does not follow a fixed pattern for individual women. AMH levels remained consistent along individual trajectories of age, with an intraclass correlation coefficient (ICC) of 0.87. The ICC of 0.32 for AMH trajectories with TTM expressed the large variation in AMH levels at a given time before the menopause. The differences between low and high age-specific AMH levels remained distinguishable, but became increasingly smaller with increasing chronological and reproductive age.

Conclusions This is the first study to characterize individual AMH decline over a long time period and broad age range. The varying AMH decline rates do not support the premise of a uniform AMH decline trajectory. Although age-specific AMH levels remain consistently high or low with increasing age, the converging trajectories and variance of AMH levels at a given time before menopause shed doubt on the added value of AMH to represent individualized reproductive age.

Introduction

Women are born with an endowment of oocytes, which decreases as they age. The decline in oocyte quantity eventually leads to menopause, marking the end of the reproductive lifespan (1). The ability to achieve spontaneous pregnancies ceases several years before the onset of menopause (12), and is thought to be related to the quality of remaining oocytes. With an ever-expanding societal tendency to delay childbearing to a later age, more women may thus unknowingly surpass their window of fertile years due to a decline in oocyte quality and quantity.

Over the past decades, many research efforts have aimed at quantifying the remaining pool of oocytes, otherwise known as the ovarian reserve. Anti-Müllerian hormone (AMH), produced by follicular granulosa cells, emerged as a promising biomarker representing the number of remaining follicles in the ovaries (1). Herein, AMH levels are suggested to provide an estimation of 'reproductive age', irrespective of chronological age (18, 27-34). In other words, a woman with a low AMH level would have a lower ovarian reserve, and thus a shorter time to menopause than a woman with a higher AMH level of the same (chronological) age. This concept has found its way into clinical practice, as women are currently receiving personalized family planning or fertility treatment counseling based on their AMH levels. Although this may seem like an advancement in reproductive health care, evidence to support this practice is lacking. Current knowledge of AMH is scarce, and limited by the use of a single measurement (141-147); small study populations (29, 31, 148); selected study groups rather than a population-based approach (29, 31, 141-147); theoretical rather than empiric models (149, 150) and restricted age ranges (141, 144). The individualized use of AMH as an indicator of the reproductive lifespan is therefore still hampered by two main questions.

First, little is known about whether the rate by which AMH declines is the same for all women. In other words: are individual AMH decline trajectories parallel to one another, or not? Secondly, the value of a single AMH measurement remains elusive; can a woman with a high age-specific AMH level at 20 years be expected to also have a high age-specific value at age 35, and what does this mean for her trajectory with reproductive age, i.e. time to menopause? We aimed to answer these two questions by characterizing the longitudinal decline trajectories of AMH in relation to both chronological age and time to menopause in a large population-based study.

Methods

Study population

Our study population consisted of the female participants of the Doetinchem Cohort Study. The Doetinchem Cohort is a population-based cohort, whose participants were randomly recruited from the Doetinchem area of the Netherlands in 1987 (132). The objective of the Doetinchem Cohort Study is to observe the impact of lifestyle and biological factors on chronic disease occurrence and quality of life (132). At the time of recruitment, participants were aged between 20-59 years. After the baseline visit (round 1), participants were invited for follow-up every 5 years. At the time of the study, round 1 through 5 had been completed, leading to an approximate follow-up time of 20 years.

At each visit, lifestyle, general health and reproductive history were assessed through extensive questionnaires, and biometric and laboratory measurements were performed. In addition to the laboratory measurements that were performed directly after each consecutive blood withdrawal, aliquots with additional plasma samples of each participant were immediately stored for future use. All participants provided written informed consent and ethical approval was granted by the Medical Ethics Committee of the Netherlands Organization of Applied Scientific Research. The use of stored samples specimens was ethically approved by the Ethical Committee for Biobank Studies of the University Medical Center Utrecht.

Only female participants from the Doetinchem Cohort with at least one available stored plasma sample, regardless of their age or menopausal status, were eligible for the current study. Of the total number of 4,128 participating women, 3,326 had an available plasma sample of at least one of the follow-up rounds. Round 1, the baseline visit, comprised plasma samples of 3,133 women; round 2 comprised plasma samples of 2,914 women; round 3 comprised plasma samples of 2,507 women; round 4 comprised plasma samples of 2,324 women; and round 5 comprised plasma samples of 2,051 women.

AMH measurements

The plasma samples from round 1 were stored in EDTA aliquots at -30°C. The samples derived from round 2-5 were stored in EDTA aliquots at -80°C. Prior to the current study, the samples were thawed once for additional measurements and immediately refrozen. For the current study, stored plasma samples of round 1-5 were utilized. In March 2015, all the available samples of each participant had been retrieved from storage and were shipped on dry ice to AnshLabs (Webster, Texas, USA), where they were temporarily stored at -20°C until the analyses were performed. AMH levels were

measured with the picoAMH assay (AnshLabs), because of its low limit of detection and the small aliquot size necessary, which is crucial for cohort studies with a limited pool of biological samples. The plasma samples of each individual were measured in a single assay run, by a single laboratory operator. In total, two laboratory operators performed all measurements. At a mean level of 91.2 pg/mL, the coefficient of variation was 4.0%. At 290.3 pg/mL the coefficient of variation was 4.8%. The limit of quantification was 3.0 pg/mL and the limit of detection 1.8 pg/mL. There were no indications of plate drift, with all coefficients of variation within plate columns and rows under 5%.

Time to menopause

Age at the time of the final menstrual period (FMP) was assessed by taking into account questionnaire information of cycle regularity, number of menstrual periods in the prior 12 months, OC use, pregnancy, reproductive surgery and self-reported age at menopause. Due to slightly differing questionnaires throughout the follow-up rounds, the assessment of the timing of the FMP differed per round. The earliest estimation of the timing of the FMP was considered to be the most accurate, being the most proximate to the event. Time to menopause was calculated by subtracting a participant's age at the FMP from her age at follow-up. Women who ever underwent a bilateral oophorectomy were excluded from this calculation, in order to obtain the time to natural menopause at each follow-up round. Women who underwent a hysterectomy before the onset of natural menopause were considered to have an unknown age at menopause.

Missing data

For information on smoking, oral contraceptive (OC) use, menstrual cycle regularity in rounds 1 and 4-5, age at menarche and body mass index (BMI), the percentage of missing information was below 2%. In rounds 2 and 3, missing information for cycle status was 6.8 and 14.6%, due to missing information of the date of the last menstrual period. Missing information for hormone replacement therapy (HRT) use increased with each round, and varied between 7.1 and 59.8%. Multiple imputation through predictive mean matching with 10 iterations was performed for these variables, including participant ID, age and AMH levels solely as predictor variables, and all remaining variables both as predictors and outcomes. Multiple imputation was performed with R (http://www.R-project.org), using the 'mice' library (http://www.jstatsoft.org/v45/i03/).

Assessment of individual decline rate: parallel or non-parallel trajectories?

To assess whether the decline rate of AMH differed for individuals, AMH trajectories in relation to age and time to menopause were fitted with a mixed model approach, using

the 'lme4' package in R. Mixed models enable the evaluation of multilevel longitudinal data, and thus are able to take into account multiple measurements over time for each participant, with varying AMH levels (i.e., random intercept) and decline rates (i.e., random slope) for each individual. As AMH had a skewed distribution, AMH levels were logarithmically transformed. Levels below the detection limit of 0.0018 ng/mL were set at this level for the purpose of logarithmic transformation. $_{Log}$AMH was used as the outcome of the mixed models, with chronological age or time to menopause as the time variable and participant ID as the group indicator variable. We modeled age and time to menopause with non-linear natural splines and checked the significance of non-linearity (a p-value of <0.05 indicated significant non-linearity). Models were adjusted for current OC use and OC use 5 years prior, HRT use, current smoking and smoking 5 years prior, as these determinants were associated with the longitudinal AMH levels (data not shown). To decide whether women had differing age-specific AMH levels, differing decline rates, or both, the multivariable-adjusted models with a random intercept, slope or both, were compared to models with only fixed terms for these two parameters. The Akaike's Information Criterion (AIC) of the models was used for this purpose. A lower AIC by at least 2 points represented a significantly better fit of the data.

Assessment of the consistency of age-specific AMH levels: does high remain high and vice versa?

To get an indication of whether individual AMH levels that were relatively low or high based on age, remained comparatively low or high as time progressed, women were divided into age-standardized AMH quartiles in round 1. The CG-LMS method, previously described in detail by Dólleman et al. (133), was used for age standardization. The AMH decline trajectory of women in these four quartile groups was then plotted against chronological age and time to menopause.

In order to measure whether the AMH levels of the individual participants remained on a single trajectory, and did not vary between the 95th and 5th percentile over time for example, the variance of AMH measurements within and between individuals was assessed for the final mixed models. By dividing the between-individual variance by the total variance (between-individual + within-individual variance), the intraclass correlation coefficient (ICC) was calculated. The ICC gives an indication of the correlation of AMH measurements on each individual's trajectory, which is directly relative to the amount of variation between individuals. For AMH decline with age, we hypothesized that women would follow a consistent high or low trajectory, i.e. that the variance of their AMH levels around a trajectory would be low. We therefore postulated that most of the variance would arise from differences in AMH levels between individuals,

in which case the ICC would approach 1. For AMH decline with time to menopause, we hypothesized that there would be little variance of AMH levels between individuals; for example, we expected that the AMH level at 10 years before menopause would be roughly similar across the whole group. In this case, the ICC would approach 0.

Participant involvement

Participants of the Doetinchem Cohort were not directly involved in the formulation of the study question or realization of the study design. As this was a population-based study design, patients were not involved.

Results

Population characteristics

On average, women in the study population completed 3.1 visits, and 79% of the participants completed two or more visits. The longest follow-up time was 21 years. In Table 1, the participant characteristics per follow-up round are presented. The number of women and their characteristics at each visit are listed per 5-year age groups in Supplementary Tables 1-6. The youngest age at baseline was 20 and the highest age at the end of follow-up was 81 years. At baseline, 18% of the women were aged between 20 and 30 years, and 32% were between 30 and 40 years. At each visit, the percentage of OC users and smokers decreased with increasing age (Table 1, Supplementary Tables 1-3). The percentage of smokers within the same age categories (thus comprising different women at each visit) decreased over time. BMI levels increased both with age and over time within the same age categories, meaning that on average, a 40-year-old woman had a lower BMI in 1987 than in 2007 (Table 1, Supplementary Table 4). Within the entire study population, the median [interquartile range (IQR)] ages at menarche and FMP were 13 [12-15] and 50 [48-53], respectively.

By the end of follow-up (visit 5), there were 1,882 women with a known age at menopause. In total, 440 women (13.2%) underwent a hysterectomy, 139 women (4.2%) had a unilateral oophorectomy, and 77 women (2.3%) had a bilateral oophorectomy. The women with no available blood samples had similar baseline characteristics to the study population with regard to age, age at menarche, age at FMP and OC use. They smoked more frequently at baseline (41.9% vs. 33.6%, $p<0.001$) and had a higher average BMI (25.9 vs. 24.6 kg/m^2, $p<0.001$).

Table 1. *Population characteristics per follow-up round.*

	Round 1	Round 2	Round 3	Round 4	Round 5
	n=3133	*n=2914*	*n=2507*	*n=2324*	*n=2051*
Age (years)	40 ± 10	46 ± 10	50 ± 10	55 ± 10	59 ± 10
OC use (%)	32	25	21	12	8
HRT use (%)	-	4	4	2	2
Smoker (%)	34	31	26	22	18
BMI (kg/m²)	25 ± 4	26 ± 4	26 ± 4	27 ± 5	27 ± 5
Regular cycle*	60	54	45	20	10
Premenopausal (%)	86	66	54	48	36
Pregnant (%)	2	1	1	0	0

*Numbers are given in % or mean ± SD. *OC users excluded. OC=oral contraceptive; HRT=hormone replacement therapy; BMI=body mass index.*

General AMH decline

Anti-Müllerian hormone levels decreased with age and remained relatively stable over time within the visit-specific age categories (Supplementary Table 6). The percentage of AMH levels below the lower limit of detection reached a maximum of 88.3% between the ages 61-65 (Supplementary Table 7). In Table 2, the observed AMH levels and number of measurements below the level of detection are listed for fixed chronological age and time to menopause points. Each individual AMH trajectory over age and time to menopause is depicted in Figure 1. The decline patterns were not linear, but appeared to follow a sigmoid-shaped trajectory, indicating that the overall rate of AMH change differed depending on age and time to menopause. Generally, the decline rate appeared to be relatively low until 40 years, after which there was an apparent acceleration until a plateau at the lower limit of detection around the age of 55 was reached. There also appeared to be an acceleration of the overall decline rate with time to menopause at 10 years before the FMP, although this acceleration seemed more gradual than with age.

Table 2. *Observed AMH levels and proportion of undetectable levels at specified ages and time points before and after menopause.*

	AMH (ng/mL)	< 0.0018 ng/mL
Age (years)		
20	3.86 [2.66-5.28]	0 (0)
30	2.84 [1.88-4.86]	0 (1)
40	0.93 [0.40-2.00]	1 (3)
50	0.01 [0.00-0.08]	28 (61)
60	0.00 [0.00-0.00]	82 (111)
Time to menopause (years)		
20	3.61 [2.25-6.28]	0 (0)
15	2.06 [0.94-3.28]	0 (0)
10	0.82 [0.50-1.51]	1 (1)
5	0.18 [0.08-0.34]	4 (7)
0	0.00 [0.00-0.01]	37 (76)
+5 *	0.00 [0.00-0.00]	73 (139)

*Numbers indicate median [IQR] and % (n), respectively. *5 years after the final menstrual period.*

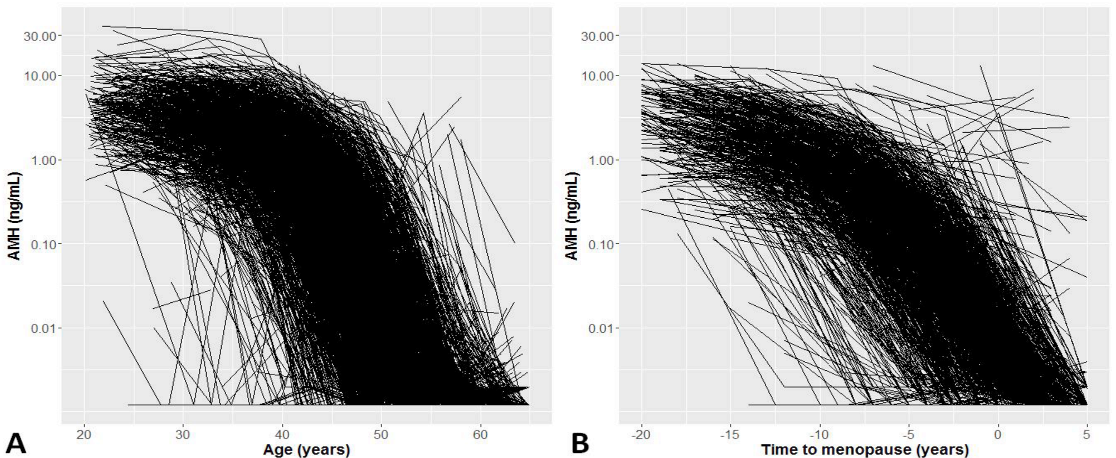
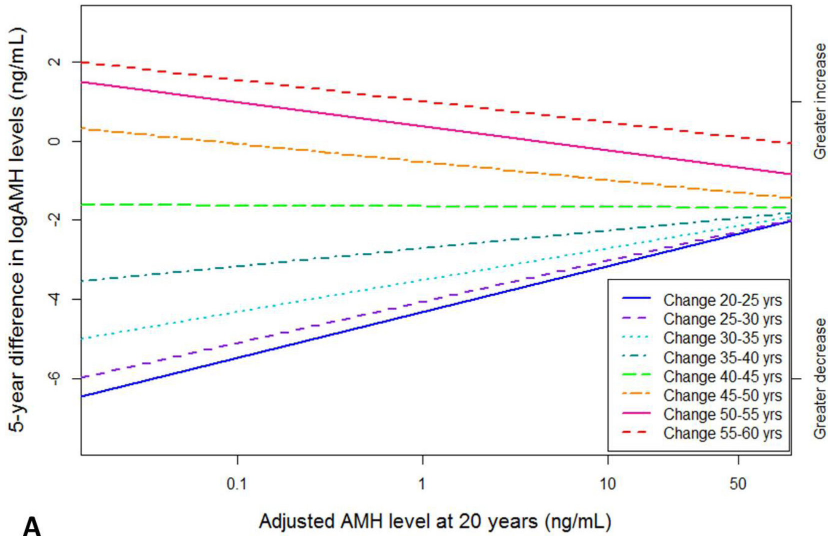


Figure 1. *Decline of AMH with chronological age (A) and time to menopause (B). Each line represents an individual trajectory based on observed AMH levels. Abbreviations: AMH anti-Müllerian hormone.*

Table 3. *Estimated rate of AMH change per time interval for age and per time interval to menopause, based on multivariable-adjusted mixed models.*

	5-yr rate of change	**1-yr rate of change**
Age period (years)		
20-25	0.05 ± 0.86 (-3.1;3.3)	0.01 ± 0.17 (-0.63;0.66)
25-30	-0.12 ± 0.78 (-2.9;2.8)	-0.02 ± 0.16 (-0.59;0.56)
30-35	-0.46 ± 0.60 (-2.6;1.9)	-0.09 ± 0.12 (-0.51;0.37)
35-40	-0.97 ± 0.35 (-2.0;0.42)	-0.19 ± 0.07 (-0.40;0.08)
40-45	-1.65 ± 0.13 (-2.3;-0.3)	-0.33 ± 0.03 (-0.46;-0.05)
45-50	-2.2 ± 0.37 (-3.5;0.3)	-0.43 ± 0.08 (-0.71;0.05)
50-55	-1.9 ± 0.46 (-3.6;0.3)	-0.38 ± 0.09 (-0.71;0.06)
55-60	-0.95 ± 0.37 (-2.4;0.4)	-0.19 ± 0.07 (-0.47;0.07)
Time to menopause period (years)		
20-15	-2.18 ± 0.65 (-3.75;-0.30)	-0.44 ± 0.13 (-0.75;-0.06)
15-10	-2.11 ± 0.48 (-3.25;-0.70)	-0.42 ± 0.10 (-0.65;-0.14)
10-5	-1.98 ± 0.17 (-2.58;-1.39)	-0.39 ± 0.03 (-0.52;-0.28)
5-0	-1.78 ± 0.32 (-2.87;-0.80)	-0.36 ± 0.06 (-0.57;-0.16)



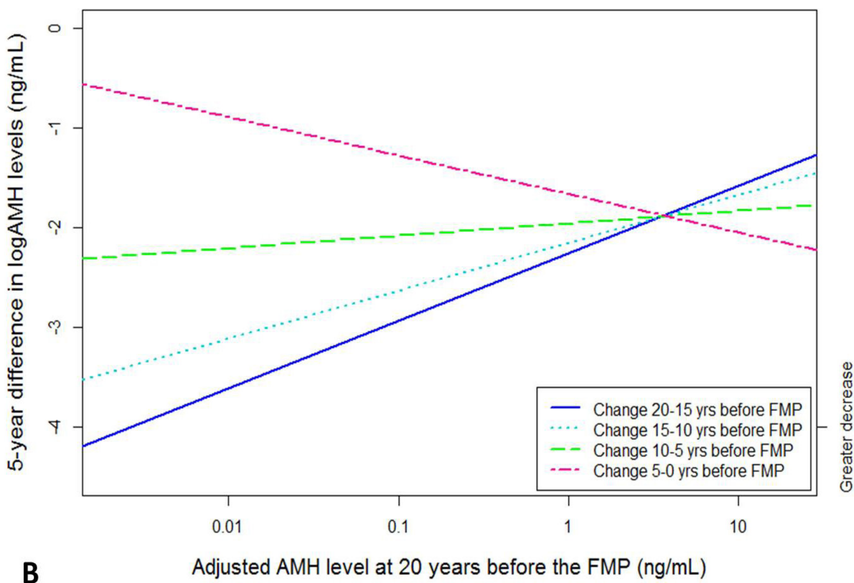


***Figure 2**. Association of multivariable-adjusted baseline AMH level with rate of change in different time intervals. Colored lines indicate varying time intervals. A: A higher AMH level at age 20 was associated with a slower decline rate between the ages 20-25 (blue line), and a higher decline rate between the ages 55-60 (orange line). B: A higher AMH level at 20 years before the final menstrual period was associated with a slower decline rate between 20-15 years before the final menstrual period (blue line) and a higher decline rate in the 5 years before the final menstrual period (pink line). Abbreviations: AMH anti-Müllerian hormone; FMP final menstrual period.*

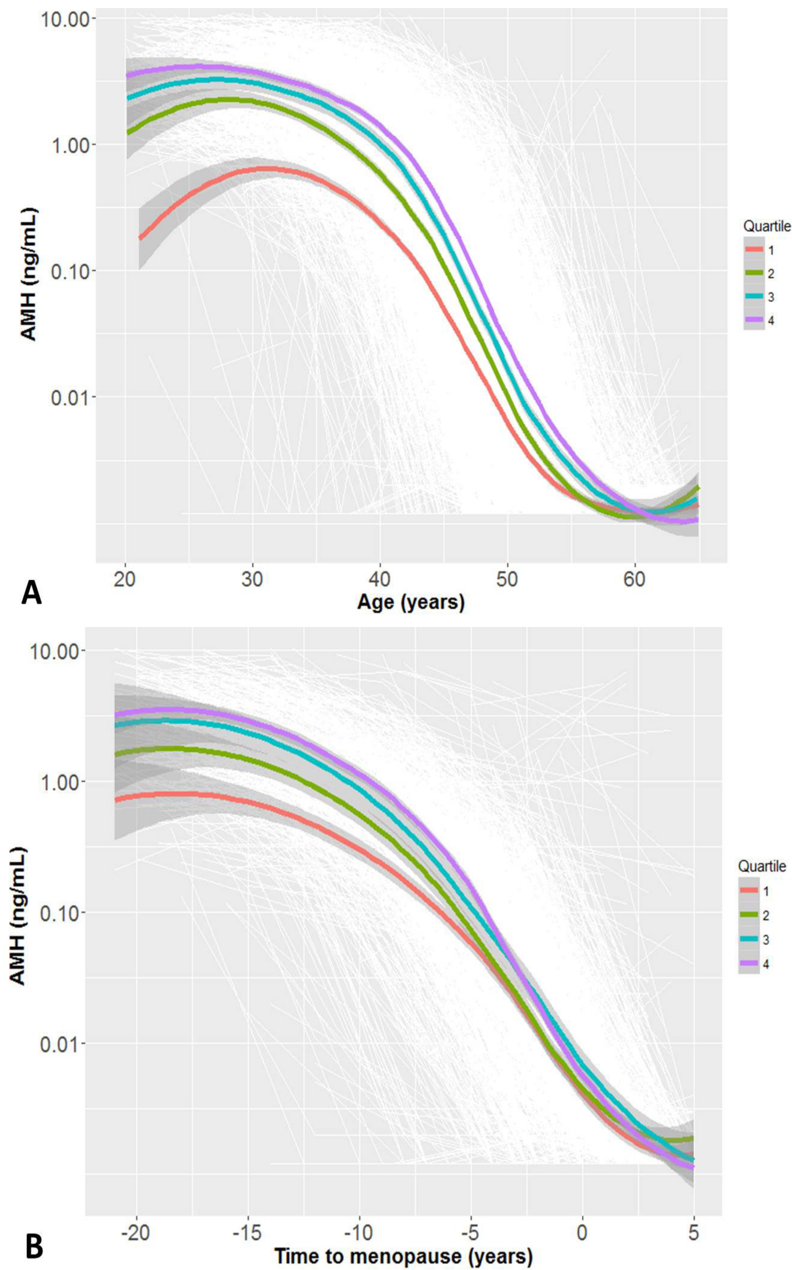


***Figure 3.** AMH decline with age and time to menopause. White lines represent individual trajectories based on observed AMH levels. Colored lines represent the group trajectories of women based on baseline age-specific AMH quartiles and gray areas indicate the standard error of a group trajectory. A: The trajectories of women in the baseline age-specific AMH quartiles are distinguishable until they overlap between age 55-60. The difference between women in low and high age-specific quartiles is largest at age 20. B: The trajectories of women in the baseline age-specific AMH quartiles are distinguishable until they overlap around 5 years before the final menstrual period. The difference between women in low and high age-specific quartiles is largest at 20 years before the final menstrual period. Abbreviations: AMH anti-Müllerian hormone.*

Assessment of individual decline rate: parallel or non-parallel trajectories?

i. Chronological age

For AMH decline with age, the best fitting mixed models incorporated a random slope and random intercept and were significantly non-linear. The random slopes indicate that individual women had differing decline rates, and the non-linearity indicates that the overall rate of decline varied with age. Based on the multivariable-adjusted AMH levels, the rate of change in various age intervals was assessed per participant, as shown in Table 3. The average rate of AMH decline was greatest between 45 and 50 years of age.

Because the AMH decline rates with age differed between participants, we plotted the adjusted AMH levels at age 20 against the decline rate at different age intervals, in order to estimate whether there was a relationship between AMH levels and rate of decline. As seen in Figure 2A, women who had a higher adjusted AMH level at age 20 had a slower decline rate between ages 20-25. This was also true between the ages 25-40 years, whereas between the ages 40-45 all women had approximately equal decline rates, regardless of their adjusted AMH levels at age 20. In contrast, after age 45, women with higher AMH levels at age 20 had a faster decline rate.

ii. Time to menopause

For time to menopause, the best fitting mixed models incorporated a random slope and random intercept and were significantly non-linear. Thus, individual women had differing AMH levels and decline rates, and the rate of decline varied with time to menopause. In Table 3, the multivariable-adjusted rate of change per time to menopause interval is displayed, indicating that the average decline rate gradually decreased closer to the menopause.

A higher AMH level at 20 years before the FMP was associated with a slower decline rate of AMH between 20-15 years before the FMP (Figure 2B). In the last 5 years before the FMP, this relationship reversed, such that a high AMH level at 20 years before the FMP was associated with a faster AMH decline rate.

Assessment of the consistency of age-specific AMH levels: does high remain high and vice versa?

i. Chronological age

According to the distribution of AMH with age at baseline, women were divided into age-specific AMH quartiles. The overall decline trajectory of each quartile group with chronological age is depicted in Figure 3A. The difference between low and high age-specific AMH levels was maintained with increasing age, but the absolute differ-

ence became smaller. The ICC of the mixed model for AMH decline with age was 0.87, indicating that 13% of the total variance could be accounted for by variability *within* individual AMH decline trajectories with age. Including only regularly cycling women at baseline (n=2,070), the ICC was 0.93, leaving 7% of the total variance to be explained by variability within individual trajectories.

ii. Time to menopause

The overall decline trajectory of each age-specific AMH quartile group with time to menopause is depicted in Figure 3B. The difference between low and high age-specific AMH levels was distinguishable from 20 years before menopause to the FMP, but the absolute differences became smaller as women neared their FMP. The ICC for time to menopause was 0.32 for the whole group (n=1,882) and 0.31 for only the baseline regular cyclers (n=1,046), meaning that approximately one-third of the total variance arose from *between*-individual differences in AMH levels. Thus, differences between high and low age-specific AMH levels were also distinguishable before menopause, but there was more overlap of AMH trajectories with time to menopause than with chronological aging.

Discussion

With this longitudinal study, we are able to shed light on the individual decline of AMH. We found that AMH trajectories with age and time to menopause were not identical, as the rate of decline differed between individuals. The rate of AMH decline was dependent on initial AMH levels, and this relationship differed with age and time to menopause. The evident differences between women with relatively high and low age-specific AMH levels at baseline became increasingly smaller as time progressed. The AMH levels of an individual woman correlated well with one another and thus did not deviate far from her trajectory. Contrary to our expectations, there was considerable variation of AMH levels between individuals with time to menopause. Taken together, these results indicate that women do not all follow the same decline trajectory of AMH, and that the largest differences in AMH levels between women can be found in early adult and reproductive life, after which they become increasingly smaller.

In a study of 81 fertile women, the variation of 2 AMH levels measured over an interval of 4 years was compared per study participant (151). AMH levels were strongly associated with chronological age, and the change of individual AMH levels was comparable with the overall group decline (with a within-individual residual correlation of 0.66) (151). Our results indicate that there is indeed a high within-individual correlation of AMH levels over a long trajectory with age, which appeals to

the reliability of multiple AMH measures to estimate the trajectory of an individual.

Building an overall model for AMH decline with age previously proved to be a complex matter. In some cross-sectional studies, a quadratic decline function of AMH with age best fit the data (141, 143, 144, 147), while others built models with polynomials or flexible splines (18, 142, 145, 146). Consequently, the proposed relationship of AMH with age took on various forms. Some models estimated the decline of age-specific AMH levels with age to be parallel (18, 143, 144), where others reported converging AMH levels with higher age (141, 142, 145-147). With our longitudinal data, we show that the individual decline of AMH levels is more approximate to the latter observation, as the differences between low and high AMH levels decreased with age. The reality of the physiology of the decline of ovarian reserve furthermore appears more complex than could previously be studied, as the individual decline rates depended on both initial AMH levels and age or time to menopause.

The notion of differing decline rates of AMH was previously put forward by Faddy et al. (2), who suggested that the decline rate of follicles accelerates after reaching a certain numeric threshold. The number of follicles at this threshold was primarily estimated to be around 25,000 (152), and later hypothesized to differ per individual (2). Thilagam recently extended this mathematical model to include the influence of other hormones involved in ovarian aging (150). Indeed, our results indicate that there are more factors at play. We observed an average acceleration of AMH decline with chronological age, while the average decline rate gradually decreased with time to menopause. This could mean that follicle quantity changes are more dependent on chronological rather than reproductive aging, or could be an indication of increased AMH production per follicle due to increasing FSH levels nearer to menopause (49). Moreover, higher initial AMH levels were first associated with a slower AMH decline rate, but later with a quicker decline. This could potentially mean that the suggested inhibitory effect of AMH on follicle recruitment (153-155) is effective up to a certain age or ovarian reserve threshold. This concept was previously brought up in a mouse study, in which the decline of growing follicles and AMH levels accelerated only later in reproductive life (25), leading to the hypothesis that compensatory mechanisms are present earlier on. In any case, this observation may prove detrimental for the hopes of improved reproductive age estimation with repeated AMH measurements.

Current time to menopause estimations with single AMH measurements are based on the concept that comparatively high (or low) AMH levels for age will remain high (or low) with age. Following this principle, a lower age-specific AMH level at any age should be associated with a shorter time to menopause (18, 28, 30, 32-34, 39). While we did indeed observe differences between women in different baseline age-specific quartiles, it was surprising that there was such variability of AMH levels between women at

a given time before menopause. This may in part explain the currently limited discriminatory capacity of AMH for time to menopause (39). A related finding in this study is the 62.9% observed AMH levels above the limit of detection within a year of the FMP. While this may partly be attributed to the high sensitivity of the assay, measurement error or recall bias for the timing of the FMP, earlier research has also suggested that the follicle pool is not entirely depleted at the time of menopause (12, 152, 156). It may well be that this critical threshold differs between women, or that in the minority of the cases other causes such as hypothalamic dysregulation are at the root of the cessation of menses (12).

Prior longitudinal studies of AMH decline with age at menopause are in disagreement on whether AMH decline rate is associated with time to menopause (29, 31). Contrary to our current findings, these studies assumed a linear decline of $_{log}$AMH with time to menopause. The rate of decline was assessed over a maximum period of 14 years in 293 women (31), and over 6 annual intervals in 50 women (29). A striking difference with our current study is the difference in age; Freeman *et al.* included women aged between 35-48 (mean 41) years (31) and Sowers *et al.* included women with a mean age of 42 ± 2.7 years (no range provided) (29). This age difference may explain the perceived linear decline, as we found the decline rate of AMH to be highest between the ages 40 and 55, at which time the overall decline trajectory did appear more or less linear. Importantly, if AMH is ever to be used for individual estimations of the remaining number of fertile years in the light of family planning, the AMH measurements should occur at a far earlier age than in these studies. Our results indicate that the currently available prediction models with AMH decline rate cannot be extrapolated to the ages at which the measurement of AMH would be most useful. The added value of multiple AMH measurements for the prediction of time to menopause therefore requires further investigation.

This study is the first to characterize the longitudinal decline of AMH with regard to age and time to menopause, in the largest study population to date. Further strengths include the random selection of study participants, large age range, standardized data assessment and storage of the samples from round 2 onwards at -80 °C. There are no data on the effects of long-term storage on sample degeneration, but the selectivity of the picoAMH antibodies to a single binding site on the N-terminal domain of the AMH molecule minimizes the risk of measuring degradation products. The women with no available sample for AMH measurement smoked more often and had a higher BMI at baseline, suggesting that our results may represent a slightly more healthy part of the female general population. We were adequately able to correct for smoking status and BMI had no influence on AMH levels, and therefore do not believe this to have substantial consequences for the results presented here. As there were no women below the age of 20 in the cohort, with the majority of women above the age of 30 at baseline, our study is limited by the fact that we have relatively lit-

tle information of ovarian reserve decline in the early years of female reproduction.

This longitudinal study reveals that there is no fixed decline trajectory of AMH. The decline rate of AMH differs between women and although age-specific AMH levels remain distinct, these differences decrease as age progresses. Moreover, the finding of varying AMH levels between women at the same time before menopause highlights the potential limitation of AMH for estimating time to menopause. Future studies of AMH as a predictor of the reproductive lifespan should consider the non-linear decline with age and time to menopause, the differences in rate of decline, and the converging, rather than parallel, trajectories.

In the light of reproductive aging and family planning, it would also be interesting to focus on the association of AMH decline and pregnancies. Levels of AMH were not associated with time to pregnancy in a prior study (113), and the wide range of AMH levels in proven fertile women (157) indicates that there is more to fertility than ovarian reserve. In a contemporary population-based cohort such as the Doetinchem Cohort Study, it is difficult to address this question due to the widespread use of contraceptives and the burden of collecting reliable information regarding the menstrual cycle, spontaneous pregnancies, infertility treatment, time to pregnancy and age at last childbirth. Future studies, with a younger study population, may be better suited to answer this question.

In conclusion, this study gives an insight into the physiology of ovarian reserve decline and paves the way for studies measuring the true feasibility of the individualized clinical use of single and multiple AMH measurements. Until then, AMH levels in regard to the reproductive lifespan should be interpreted with caution.

Supplementary Table 1. *Number of women in each age category per follow-up round*

Age category	Round 1 *n=3133*	Round 2 *n=2914*	Round 3 *n=2507*	Round 4 *n=2324*	Round 5 *n=2051*
20-25	228	-	-	-	-
26-30	341	170	-	-	-
31-35	490	294	164	-	-
36-40	515	459	269	161	-
41-45	523	488	423	260	155
46-50	364	520	402	383	239
51-55	366	338	448	401	362
56-60	300	327	290	415	364
61-65	-	280	282	271	369
66-70	-	35	202	246	229
71-75	-	-	26	168	202
76-80	-	-	-	18	117
81-85	-	-	-	-	12

Supplementary Table 2. *Current oral contraceptive users per age category and follow-up round (n (%))*

Age category	Round 1 *n=3133*	Round 2 *n=2914*	Round 3 *n=2507*	Round 4 *n=2324*	Round 5 *n=2051*
20-25	180 (89.1)	-	-	-	-
26-30	194 (61.7)	112 (70.9)	-	-	-
31-35	175 (39.9)	130 (47.3)	81 (51.3)	-	-
36-40	120 (25.7)	144 (34.3)	93 (36.9)	104 (32.9)	-
41-45	62 (13.9)	116 (25.5)	112 (28.4)	67 (27.0)	43 (28.7)
46-50	34 (12.7)	75 (16.4)	91 (24.3)	72 (20.7)	49 (21.6)
51-55	23 (10.1)	19 (7.6)	44 (11.5)	40 (10.8)	26 (7.9)
56-60	1 (0.8)	2 (1.0)	2 (0.9)	1 (0.3)	4 (1.2)
61-65	-	2 (1.5)	2 (1.1)	0 (0.0)	0 (0.0)
66-70	-	0 (0.0)	2 (1.9)	0 (0.0)	0 (0.0)
71-75	-	-	0 (0.0)	0 (0.0)	3 (2.7)
76-80	-	-	-	0 (0.0)	1 (1.9)
81-85	-	-	-	-	0 (0.0)

Supplementary Table 3. *Current smokers per age category and follow-up round (n (%))*

Age category	Round 1 *n=3133*	Round 2 *n=2914*	Round 3 *n=2507*	Round 4 *n=2324*	Round 5 *n=2051*
20-25	75 (72.8)	-	-	-	-
26-30	142 (65.4)	53 (31.2)	-	-	-
31-35	196 (57.6)	101 (34.4)	49 (29.9)	-	-
36-40	184 (52.7)	148 (34.4)	86 (32.0)	36 (22.5)	-
41-45	193 (57.1)	175 (35.9)	130 (30.7)	71 (27.3)	30 (19.4)
46-50	103 (53.6)	178 (34.2)	124 (30.6)	109 (28.5)	58 (24.3)
51-55	88 (53.7)	97 (28.7)	119 (26.6)	104 (26.1)	89 (24.7)
56-60	69 (53.9)	73 (22.3)	63 (21.7)	88 (21.3)	66 (18.2)
61-65	-	55 (19.6)	51 (18.1)	46 (17.0)	54 (14.6)
66-70	-	6 (17.1)	33 (16.4)	28 (11.7)	28 (12.4)
71-75	-	-	4 (15.4)	21 (12.6)	22 (10.9)
76-80	-	-	-	6 (33.3)	10 (8.8)
81-85	-	-	-	-	2 (16.7)

Supplementary Table 4. *BMI levels per age category and follow-up round (mean ± SD)*

Age category	Round 1 *n=3133*	Round 2 *n=2914*	Round 3 *n=2507*	Round 4 *n=2324*	Round 5 *n=2051*
20-25	22.7 ± 3.2	-	-	-	-
26-30	23.2 ± 3.3	24.1 ± 4.3	-	-	-
31-35	23.6 ± 3.5	24.3 ± 3.8	25.2 ± 4.1	-	-
36-40	24.1 ± 3.5	24.6 ± 3.9	25.3 ± 4.1	26.1 ± 5.1	-
41-45	24.5 ± 3.4	25.1 ± 4.1	25.2 ± 4.0	25.7 ± 4.5	26.3 ± 5.0
46-50	25.5 ± 3.8	25.6 ± 3.7	25.9 ± 4.2	26.1 ± 4.3	26.3 ± 4.6
51-55	26.5 ± 4.1	26.4 ± 4.2	26.4 ± 4.0	26.3 ± 4.5	26.5 ± 4.5
56-60	26.7 ± 3.6	27.4 ± 4.4	26.8 ± 4.4	26.5 ± 4.2	26.5 ± 4.5
61-65	-	27.3 ± 4.0	27.6 ± 4.6	27.1 ± 4.5	26.7 ± 4.1
66-70	-	27.6 ± 4.2	28.0 ± 4.5	28.3 ± 5.2	27.7 ± 4.8
71-75	-	-	27.7 ± 3.9	27.8 ± 4.7	28.3 ± 5.1
76-80	-	-	-	28.3 ± 4.0	29.2 ± 5.9
81-85	-	-	-	-	29.2 ± 4.4

Supplementary Table 5. *Current estrogen users for climacterial complaints per age category and follow-up round (n (%))*

Age category	Round 1 *n=3133*	Round 2 *n=2914*	Round 3 *n=2507*	Round 4 *n=2324*	Round 5 *n=2051*
20-25	-	-	-	-	-
26-30	-	0 (0.0)	-	-	-
31-35	-	1 (0.3)	1 (0.6)	-	-
36-40	-	2 (0.4)	1 (0.4)	2 (1.3)	-
41-45	-	7 (1.4)	2 (0.5)	4 (1.6)	1 (0.6)
46-50	-	35 (6.9)	12 (3.0)	8 (2.1)	8 (3.3)
51-55	-	38 (11.8)	50 (11.6)	16 (4.0)	7 (1.9)
56-60	-	25 (7.8)	23 (8.6)	12 (2.9)	8 (2.2)
61-65	-	10 (3.6)	11 (4.0)	5 (1.9)	2 (0.5)
66-70	-	1 (2.9)	3 (1.6)	4 (1.6)	3 (1.3)
71-75	-	-	0 (0.0)	3 (1.8)	2 (1.0)
76-80	-	-	-	0 (0.0)	1 (0.8)
81-85	-	-	-	-	0 (0.0)

Supplementary Table 6. *AMH levels per age group and follow-up round in ng/mL (median [IQR])*

Age category	Round 1 *n=3127*	Round 2 *n=2911*	Round 3 *n=2506*	Round 4 *n=2323*	Round 5 *n=2049*
20-25	3.7 [2.2-5.8]	-	-	-	-
26-30	3.6 [2.0-5.9]	3.4 [1.8-5.5]	-	-	-
31-35	2.8 [1.5-5.0]	2.3 [1.2-4.0]	2.2 [1.0-4.4]	-	-
36-40	1.9 [0.9-3.8]	1.6 [0.8-3.3]	1.4 [0.7-2.7]	1.4 [0.7-3.1]	-
41-45	0.7 [0.3-1.7]	0.6 [0.2-1.4]	0.5 [0.2-1.4]	0.5 [0.1-1.2]	0.5 [0.1-1.5]
46-50	0.1 [0.0-0.5]	0.1 [0.0-0.3]	0.0 [0.0-0.3]	0.0 [0.0-0.3]	0.0 [0.0-0.2]
51-55	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]
56-60	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]
61-65	-	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]
66-70	-	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]
71-75	-	-	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]
76-80	-	-	-	0.0 [0.0-0.0]	0.0 [0.0-0.0]
81-85	-	-	-	-	0.0 [0.0-0.0]

Supplementary Table 7. *Number of women with undetectable AMH levels (<1.8 pg/mL) per age category and follow-up round (n (%))*

Age category	**Round 1** *n=3127*	**Round 2** *n=2911*	**Round 3** *n=2506*	**Round 4** *n=2323*	**Round 5** *n=2049*
20-25	1 (0.4)	-	-	-	-
26-30	2 (0.6)	1 (5.9)	-	-	-
31-35	1 (0.2)	4 (1.4)	3 (1.8)	-	-
36-40	6 (1.2)	2 (0.4)	3 (1.1)	7 (4.3)	-
41-45	11 (2.1)	13 (2.7)	9 (2.1)	11 (4.2)	13 (8.3)
46-50	55 (15.1)	89 (17.1)	63 (15.7)	75 (19.6)	46 (19.2)
51-55	198 (54.1)	197 (58.3)	262 (58.4)	216 (53.9)	202 (55.8)
56-60	245 (81.7)	274 (83.8)	233 (80.3)	331 (79.8)	294 (80.8)
61-65	-	240 (85.7)	249 (88.3)	221 (81.5)	298 (80.8)
66-70	-	11 (31.4)	172 (85.1)	204 (82.9)	181 (79.0)
71-75	-	-	5 (19.2)	133 (79.2)	170 (84.2)
76-80	-	-	-	6 (33.3)	96 (82.1)
81-85	-	-	-	-	3 (25.0)

Chapter 5

Chapter 5

Can menopause prediction be improved with multiple AMH measurements? Results from the prospective Doetinchem Cohort Study

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Manuscript in preparation

Abstract

Objectives Prediction of age at menopause could potentially help to advise women on decisions regarding family planning. Anti-Müllerian hormone (AMH) was previously identified as a potential promising predictor of age at menopause, but led to inaccurate individual age at menopause estimations. We investigated whether an individual's AMH decline rate could improve the prediction of age at menopause.

Methods This study included 2,434 premenopausal women from the population-based Doetinchem Cohort Study. Participants were followed up every 5 years for a total of 20 years, and AMH was measured in stored plasma samples from each follow-up round with the picoAMH assay. Adjusted AMH levels and decline rates were assessed for each individual with the use of a linear mixed regression analysis. These values were related to age at menopause and early menopause in a time-varying Cox proportional hazards analysis. (Added) predictive value of the models was evaluated using C-statistics and Somers' D statistic.

Results At baseline, the mean age was 36.1 ± 8.1 years. There were 96 (4%) women who became postmenopausal by the age of 45 years. Overall, every unit ng/mL lower AMH level was associated with a 23% higher risk of menopause. For a 25-year-old woman, adding AMH decline rate between the ages 20-25 to a model with AMH level at age 25 increased the C-statistic with 5%, while for a 30-year-old, models with the AMH decline rate did not improve. The prediction of early menopause (≤45 years) did not benefit from the use of multiple AMH measurements.

Conclusion These results suggest that knowledge of the AMH decline rate can slightly improve the prediction of age at menopause, but not early menopause. The relatively low discriminative ability furthermore argues against the use of AMH decline trajectories as a screening method for an early age at menopause.

Introduction

Women reach menopause as a result of a depletion of the primordial follicle pool at an average age of 51 years, with a normal range between 40-60 years (3-5). At an estimated 10 years before the onset of menopause women lose the ability to conceive naturally (12). Although uncertainty remains on a possible variation in this time relation between individual women, it may imply that in some 20-25% of the female population this inability may already be present in their mid- to late thirties. In an era where motherhood is continually being postponed (158), this means that women may unknowingly surpass their window of childbearing opportunity. In order to help guide women in decisions involving their family planning, it would be helpful to identify those with an early menopause well before the onset of natural sterility. Measuring the current state of ovarian reserve could be beneficial to estimate the duration of the remaining reproductive lifespan, or age at menopause (22).

The level of circulating anti-Müllerian hormone (AMH), produced by developing follicles in the female ovary, is related to the size of the remaining primordial follicle pool and is thus considered a reliable quantitative marker of ovarian reserve (1). Prior studies indicate that AMH level is significantly associated with time to or age at menopause (18, 29, 30, 32, 33, 38, 108, 148, 159). However, predicting an individualized age at menopause is still in need of considerable improvement, illustrated by the lack of ability to predict very early ages at menopause (108). Current prediction models are further limited by the selection of fertile, regularly cycling women (18, 30, 32, 39) and small study sizes (27, 29), with a possible underrepresentation of extreme ages at menopause on either side of the spectrum.

It has been suggested that the use of multiple rather than single measures of AMH, taking into account the individual decline rate of AMH, could improve menopause prediction (31). To date, one study has studied longitudinal AMH trajectories leading up to menopause (148). It reported on short-term predictions from age 43 onwards, but did not report the added value of the AMH trajectory to a single measurement. We previously observed that the decline rate of AMH with age differed between women (160), suggesting that the combination of AMH level and decline rate may better characterize an individual trajectory of ovarian reserve decline. In this study, we therefore sought to investigate whether the use of multiple AMH measurements can improve the individualized prediction of menopause, and in particular early menopause, in the general population.

Methods

Study population

For this study, female participants from the population-based Doetinchem Cohort Study were selected. The Doetinchem Cohort Study originated in 1987 with an age- and sex-stratified random sample of inhabitants of the town of Doetinchem in the Netherlands. The study objective and design are described in more detail elsewhere (132). After the baseline visit, participants were invited for follow-up every 5 years. Each visit included a questionnaire, anthropometric measurements and non-fasting blood withdrawal. Plasma samples were immediately stored for future use after each blood withdrawal. At the time of the current study, 5 visits had been completed, resulting in an approximate follow-up time of 20 years. All participants provided written informed consent and the Medical Ethics Committee of the Netherlands Organization of Applied Scientific Research granted ethical approval.

Anti-Müllerian hormone was measured in stored plasma samples of 3,326 women. Women who experienced surgical menopause, i.e. a bilateral oophorectomy (n=52) were excluded. Next, women with an age at menopause or hysterectomy before baseline were excluded (n=810), as well as women with a missing menopausal status (n=30). This left 2,434 women for inclusion with one or more AMH measurements preceding the occurrence of menopause or censoring for a protocolled reason.

AMH measurements

Anti-Müllerian hormone concentration was measured in all the available stored plasma samples with the picoAMH assay (AnshLabs, Webster, Texas, USA). We previously provided a detailed description of these measurements (160). The inter- and intra-assay coefficient of variation (CV) were 4.4 and 3.9%, respectively. There was no indication of plate drift, with all CVs within plate columns and rows below 5%. Levels of AMH below the limit of detection of 1.8 pg/mL were set at this level to avoid the use of null-values. The use of the stored sample specimens for AMH measurements was approved by The Committee for Biobank Studies of the University Medical Center Utrecht.

Covariates

Questions regarding both current smoking and current oral contraceptive (OC) use were included in the questionnaires at every follow-up round and were assessed in the same manner at each round. Current smoking was defined as having smoked one or more cigarettes in the month prior to the visit.

Age at menopause estimation

Age at the time of the final menstrual period (FMP) was assessed with the use of the questionnaires. The questionnaires included information regarding current and previous cycle status, date of the last menstrual period, number of menstrual periods in the prior 12 months, use of hormonal contraception, reproductive surgery and self-reported age at menopause. Menopausal status and the timing of the FMP was assessed at each individual round. The earliest estimation of the timing of the FMP was considered to be the most accurate, being the most proximate to the event.

Statistical analysis

The primary study outcome was natural menopause, which was considered an event. The follow-up time of each women ended after this event, or by censoring at hysterectomy, at the time of lost to follow-up, or at the last follow-up round. Over all follow-up rounds, missing information of current smoking was 0.04% and missing information of current OC use was 0.4%. The missing data was imputed through multiple imputation, using 10 iterations and predictive mean matching. Multiple imputation was performed in SPSS Statistics (IBM), version 21. All further analyses were performed with R (http://www.R-project.org). Three separate analytical steps were designed and carried out in order to arrive at an answer to the research questions as summarized in Figure 1.

i. Menopausal status prediction with baseline AMH and overall 20-year AMH decline

First, the predictive capacity of the baseline AMH measurement and the overall AMH decline rate in addition to age was assessed, without taking into account differences between individual AMH decline trajectories. This provided a crude view of the predictive capacity of a single AMH measurement and decline rate over a 20-year period, without taking into account the pattern of individual AMH decline. Conform the methods used by Freeman et al. (31), the last AMH measurement or first undetectable AMH measurement was identified. The difference in $_{log}$AMH between the baseline measurement and last measurement was then divided by the corresponding time interval to calculate the overall annual decline ($_{log}$AMH/year). Baseline age, the baseline AMH level and overall annual decline were consecutively added to a Cox proportional hazards model with timing of reaching the postmenopausal status as the outcome and observation time as the time variable. Natural cubic smoothing splines with 2 knots were included in the baseline age covariate, in order to account for a non-linear decline of AMH with age (160). The models were additionally adjusted for baseline smoking status and OC use. The predictive capacity of baseline age, baseline AMH and overall AMH decline were

compared with the use of C-statistics of the corresponding prediction models. To assess whether the time to menopause prediction of AMH differed with age, the results were compared in subgroups of women aged below 40 and from 40 onwards at baseline. The Cox models were constructed using the R package 'survival'.

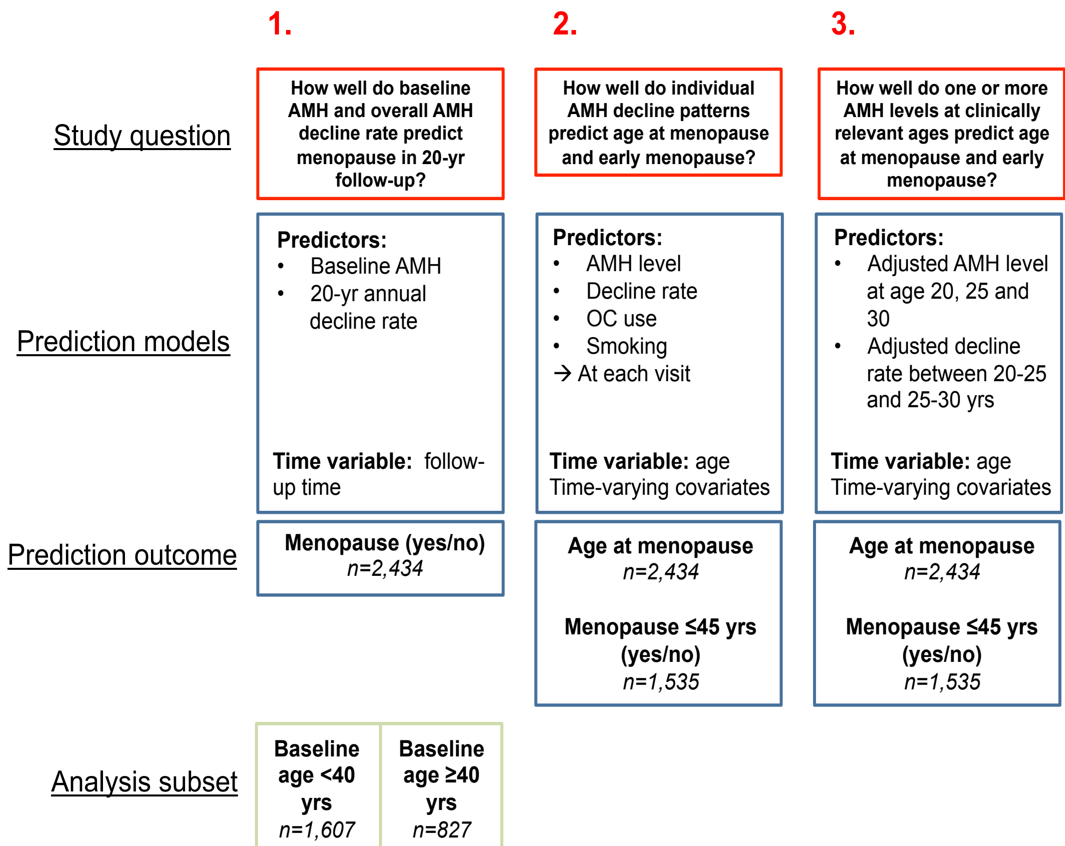

Figure 1. *Schematic representation of the three main analyses performed in the study. 1 indicates menopause prediction with baseline AMH and overall 20-year AMH-decline; 2 indicates age at menopause prediction with individual AMH trajectories; 3 indicates age at menopause prediction with single or repeated AMH measurements.*

ii. Age at menopause prediction with individual AMH trajectories

Next, we sought to take into account the shape of the individual AMH trajectories for the prediction of age at menopause. To this end, a Cox proportional hazards model with time-varying covariates was used in order to make use of the multiple measurements of AMH over time within one individual. In order to assess the true predictive capacity of AMH independently of age, participants' age was used as the time variable and divided into intervals, known as the landmarking method (161). Each interval began with the age at baseline or follow-up round and either ended at the age of menopause, censoring or the next visit round, whichever came first. There is an important distinction to be made between the resulting Cox proportional hazards models from this method as opposed to the models described under *i*. In the first models, follow-up time was the time indicator and age was included as a potential predictor of time to menopause. In contrast, in the time-varying models age was used as the time indicator, thereby no longer including age as a predictor. This was expected to lead to lower C-statistics, as the variance in time to menopause explained by age was incorporated in these models, thereby providing a more accurate estimation of the predictive effect of AMH independently from age.

In order to assess the predictive effect of all observed AMH *levels* throughout the study, a participant's observed $_{log}$AMH level and smoking and OC status at the start of each interval were related to the outcome (menopause yes or no) at the end of each interval. Additionally, we aimed to assess the individual and additive predictive value of the AMH *decline rate* at the starting age of each interval. To achieve this, a linear mixed effects model was used to quantify the individual AMH decline trajectories of women in the study population, giving an estimation of their $_{log}$AMH level throughout the entire age range up to an event or censoring. The linear mixed effects model included $_{log}$AMH as the outcome, a random intercept and slope per individual, and age (using natural splines with 3 knots, to encompass a triphasic decline trajectory) (160), smoking and OC use as time-varying covariates. The R package 'lme4' was used for the linear mixed effects model. The adjusted observed $_{log}$AMH level and predicted annual $_{log}$AMH decline at each study visit were extracted from this model for each participant. The 'momentary' slope was calculated by estimating the rate of change of between the current and consecutive year. The adjusted AMH level and decline rate at each visit were included in the time-varying Cox model, as well as an interaction term between annual decline rate and age, in order to account for a differing slope with age (160).

The predictive capacity of the models was assessed with the resulting C-statistics (estimated percentage of correct predictions of menopause occurrence in the same age interval). The correlation of AMH levels and decline rates with correct at age menopause estimations were assessed using the Somers' D statistic, where a

value of -1 indicates no concordance between an AMH level and/or slope with age at menopause, a value of 0 indicates 50% agreement of AMH level and/or slope with age at menopause (equal to tossing a coin) and a value of 1 indicates 100% agreement.

The time-varying Cox models predicted age at menopause. However, it may be clinically more useful to predict the risk of becoming postmenopausal *early on*, i.e. before the age of 45, rather than distinguishing between an arbitrary menopausal age such as 52 or 54. We therefore chose to repeat the analyses with right-censoring of the follow-up time at age 45. Right-censoring at 45 years excluded women who entered the study when they were over 40 years old, leaving 1,535 women for analysis. Henceforth we will refer to these separate analyses as 'age at menopause' and 'early menopause' prediction.

iii. Comparison of age at menopause prediction with single or repeated AMH measurements

In the last analytical step, we sought to assess the predictive capacity of one or two AMH measurements taken at three different ages for the prediction of age at menopause. These ages were considered to be relevant from the perspective of preventive management of infertility. From the linear mixed effects model, the adjusted $_{log}$AMH levels at the ages 20, 25 and 30 were determined, and the annual rate of AMH change between these ages was estimated for each participant, based on her individual trajectory. The prediction of age at menopause was compared between one or two AMH measurements at these ages, by comparing the C-statistics and Somers' D-statistics of the corresponding models. Again, this analysis was performed both for the prediction of age at menopause (including all ages at follow-up) and the prediction of the occurrence of early menopause (with right-censoring at age 45).

Results

The population characteristics for each follow-up round are listed in Table 1. The average number of measurements per participant was 2.8. There were 369 (15%) women with 5 AMH measurements, 367 women (15%) with 4 measurements, 476 women (19%) with 3 measurements, 1,172 women (48%) with 2 measurements and 50 women (2%) with 1 measurement. The median follow-up time was 11.6 years and the maximum follow-up time was 21.9 years. The median age at menopause [interquartile range] was 51 [49-53] years. The total number of women who reached menopause during the entire follow-up period was 1,298 (53%), and for early menopause this was 96 (4%).

Table 1. *Population characteristics at each follow-up round*

	Baseline n=2,434	Round 2 n=2,338	Round 3 n=1,947	Round 4 n=1,818	Round 5 n=1,663
Age (years)	36.1 ± 8.1	42 ± 8.0	46.9 ± 8.0	51.9 ± 7.9	56.5 ± 7.9
OC use (%)	33	27	22	12	6
Smoker (%)	35	32	27	23	18
AMH (ng/mL)	1.82 [0.55-3.82]	0.64 [0.04-2.04]	0.10 [0.00-0.94]	0.00 [0.00-0.17]	0.00 [0.00-0.26]
Postmenopausal (%)	0	12	27	60	77

Values given in %, mean ± SD or median [IQR]

Table 2. *Time to menopause prediction with baseline AMH and overall decline rate*

	All baseline ages (n=2,434)		<40 years at baseline (n=1,607)		≥40 years at baseline (n=827)	
	HR (95% CI)	**C-statistic**	**HR (95% CI)**	**C-statistic**	**HR (95% CI)**	**C-statistic**
Age, OC, smoking	-	0.89	-	0.83	-	0.75
+ AMH		0.90		0.85		0.78
$_{log}$AMH Round 1 ↓	1.24 (1.20-1.28)		1.42 (1.35-1.50)		1.21 (1.16-1.25)	
+ decline rate		0.90		0.86		0.78
$_{log}$AMH Round 1↓	1.37 (1.31-1.42)		1.60 (1.50-1.71)		1.30 (1.24-1.36)	
$_{log}$AMH/year↓	3.72 (2.84-4.88)		26.8 (13.2-54.3)		2.53 (1.77-3.62)	

Results indicate the hazard of menopause in the 20-year follow-up period in models adjusted for 1) baseline age, OC use and smoking; 2) baseline age, OC use, smoking and AMH level; 3) baseline age, OC use, smoking, AMH level and overall 20-year decline rate. Analyses were performed in three groups: 1) all women at baseline; 2) women aged below 40 years at baseline; 3) women aged 40 years and older at baseline. Interpretation example: each ng/mL lower logAMH level at baseline was associated with a 24% higher risk of menopause by the end of follow-up.

i. Menopausal status prediction with baseline AMH and overall 20-year AMH decline

The results of the prediction of time to menopause with baseline AMH and overall slope are presented in Table 2. For the whole study population, each lower ng/mL unit of $_{log}$-AMH was associated with a 37% higher risk of menopause during follow-up, and each additional 10% decrease/year was associated with an almost quadrupled risk of becoming postmenopausal during the follow-up period. The C-statistic of the model including baseline age, smoking and OC use was 0.89, i.e. the chance of correctly predicting who would become postmenopausal between two women in the period of 20 years would be 89%. Adding baseline AMH and overall annual AMH decline to this model with baseline age, smoking and OC use only led to an increase of 0.1 in the C-statistic for the whole study population. The C-statistics in both subgroups for baseline age were lower, theoretically meaning that there was more room for small increases of predictive capability with the addition of AMH and/or AMH decline rate. Indeed, in both groups, addition of baseline AMH and the overall rate of AMH decline led to an increased C-statistic of 0.3.

ii. Age at menopause prediction with individual AMH trajectories

The results of the time-varying Cox models with the AMH observed and adjusted measurements at all visits are presented in Table 3. Taking smoking and OC use at each visit into account, every unit ng/mL lower observed AMH level was associated with a 23% higher risk of menopause occurring within the same follow-up interval, with a C-statistic of 70%. For the prediction of early menopause the predictive capacity of the observed AMH measurements was larger, with a C-statistic of 0.78.

The addition of the momentary AMH decline slope and slope*age interaction to the adjusted AMH levels at each interval led to an increased C-statistic from 0.68 to 0.70 for age at menopause prediction, where there was a slight decrease from 0.75 to 0.74 for early menopause prediction. The Somers' D statistic for prediction of age at menopause and early menopause was 0.40 and 0.45, respectively, indicating a fair but not good concordance of AMH level, slope and predicted age at menopause.

iii. Comparison of age at menopause prediction with single or repeated AMH measurements

The results of the time-varying Cox models with the adjusted AMH levels and decline rates at the set ages are listed in Table 4. Here, the results can be interpreted from the perspective of a 20-, 25- or 30-year-old woman. For a 20-year-old, each ng/mL *lower* adjusted AMH level was associated with a *later* age at menopause and *lower* risk of early menopause. The corresponding C-statistics for age at meno-

Table 3. *Age at menopause prediction with time-varying Cox models.*

	Age at menopause prediction (n=2,434)		Early menopause prediction (n=1,535)	
	HR (95% CI)	**C-statistic**	**HR (95% CI)**	**C-statistic**
Observed AMH		0.70		0.76
$_{log}$AMH ↓	1.20 (1.18-1.24)		1.50 (1.39-1.63)	
Observed AMH + OC + smoking		0.70		0.78
OC	0.72 (0.62-0.84)		0.52 (0.30-0.89)	
Smoking	1.14 (1.01-1.28)		0.97 (0.63-1.49)	
$_{log}$AMH ↓	1.23 (1.20-1.26)		1.53 (1.42-1.66)	
Adjusted AMH		0.68		0.75
$_{log}$AMH ↓	1.28 (1.24-1.32)		1.86 (1.64-2.09)	
Momentary slope		0.65		0.72
Slope (10% ↓AMH/year)	1.36 (1.30-1.42)		2.01 (1.73-2.32)	
Adjusted AMH + slope		0.69		0.75
$_{log}$AMH ↓	1.50 (1.33-1.69)		1.73 (1.22-2.44)	
Slope (10% ↓AMH/year)	0.80 (0.68-0.94)		1.09 (0.73-1.65)	
Adjusted AMH + slope + slope:age		0.70		0.74
$_{log}$AMH ↓	1.02 (0.88-1.18)		1.84 (1.29-2.62)	
Slope (10%↓ AMH/year)	3.36 (2.48-4.55)		0.73 (0.30-1.76)	
Slope*age	0.99 (0.98-0.99)		1.01 (0.99-1.02)	

Models indicate prediction of menopause in the entire range of follow-up ages ("menopause prediction") and prediction of menopause by the age of 45 ("early menopause prediction"). 'Observed AMH' indicates the AMH levels measured at each follow-up round. 'Adjusted AMH' indicates the individual AMH levels adjusted for OC use and smoking at each follow-up round, based on the linear mixed effects model. 'Momentary slope' indicates the difference in adjusted logAMH levels within one year. Example of interpretation: at any age during follow-up, each ng/mL lower adjusted logAMH level was associated with a 28% higher risk of menopause during follow-up.

Table 4. *Age at menopause prediction with time-varying Cox models.*

	Age at menopause prediction (n=2,434)		Early menopause prediction (n=1,535)	
	HR (95% CI)	**C-statistic**	**HR (95% CI)**	**C-statistic**
Perspective from 20 years				
$_{log}$AMH age 20 ↓	0.32 (0.26-0.38)	0.62	0.75 (0.41-1.36)	0.47
Perspective from 25 years				
$_{log}$AMH age 25 ↓	2.06 (1.74-2.43)	0.64	2.48 (1.88-3.26)	0.73
Slope (10%↓AMH/ year) 20-25	1.03 (1.03-1.04)	0.70	1.03 (1.02-1.04)	0.72
$_{log}$AMH age 20 ↓ +	0.79 (0.64-0.98)	0.69	1.66 (1.12-2.46)	0.73
Slope (10%↓AMH/ year)	1.14 (1.12-1.16)		1.17 (1.13-1.21)	
Perspective from 30 years				
$_{log}$AMH age 30 ↓	2.37 (2.16-2.59)	0.70	2.49 (2.04-3.05)	0.75
Slope (10%↓ AMH/ year) 20-25	1.13 (1.12-1.14)	0.70	1.13 (1.09-1.16)	0.72
$_{log}$AMH age 25 ↓ +	1.01 (0.80-1.27)	0.70	1.84 (1.30-2.61)	0.73
Slope (10%↓ AMH/ year)	1.13 (1.11-1.14)		1.11 (1.07-1.14)	

Models indicate prediction of menopause in the entire range of follow-up ages ("menopause prediction") and prediction of menopause by the age of 45 ("early menopause prediction"). The adjusted AMH levels at age 20, 25 and 30 and corresponding slopes were calculated based on the linear mixed effects model. Example of interpretation: at age 25, each ng/mL lower logAMH level was associated with a 2.1 times higher risk of menopause during the follow-up period and a 2.5 times higher risk of early menopause. Each additional 10%/year increase between the ages 20-25 was associated with a 3% higher risk of menopause during follow-up and early menopause.

pause and early menopause predictions were 0.62 and 0.47 respectively, indicating that the probability of correctly discriminating the age at menopause or occurrence of early menopause was low, i.e. near the threshold of 50% (equal to tossing a coin). The Somers' D statistics were 0.24 and -0.06 respectively, indicating poor concordance between AMH levels and age at menopause/early menopause.

For a 25-year-old woman, knowledge of the decline rate between the ages 20-25 to an AMH measurement at age 25 would increase the number of correct age at menopause predictions by 5% to 0.69. Each 10% additional relative AMH decrease between 20-25 years was associated with an *earlier* age at menopause. The prediction of early menopause for a 25-year-old woman did not improve with the addition of her AMH decline rate between 20-25 years, with a C-statistic of 0.73. Prediction of both age at menopause and early menopause was better for the 25- versus the 20-year-old. The Somers' D statistics of the age at menopause and early menopause predictions were 0.38 and 0.46, respectively.

For a 30-year-old woman, it would not make a difference for her prediction of age at menopause if she had previously had an AMH measurement at age 25, or a single measurement at age 30 (with C-statistics of 0.70 in either case). For the prediction of *early* menopause, the discriminatory ability of a single AMH measurement even appeared better than a combination with AMH decline rate between the ages 25-30 (with a C-statistic of 0.75 versus 0.73). At age 30, the Somers' D statistics for age at menopause and early menopause prediction with the full model were 0.40 and 0.46 respectively.

In order to visualize these results, we compared the effect of extremely low and high AMH levels and decline rates from the perspective of a 30-year-old woman. Figure 2 shows a predicted Kaplan-Meier curve of women with high (>p95) and low (<p5) AMH levels at age 25 and a swift (>p95) or slow (<p5) annual AMH decline between the ages 25 and 30 years, for the whole follow-up period (A) and for follow-up until 45 years (B). In these most extreme examples of a 30-year old, the difference in median age at menopause for the entire follow-up is 3 years, dictated solely by the difference in AMH decline rate between the ages 25 and 30 (Figure 2). Moreover, for a woman with a very low AMH level at age 25 with a very fast speed of decline to age 30, her risk of becoming postmenopausal by age 45 would be larger than 45% (Figure 3). The number of women with an AMH level at age 25 in the lowest 5% and among the 5% fastest decline rates was 34, 2.2% of the population with right-censoring at age 45.

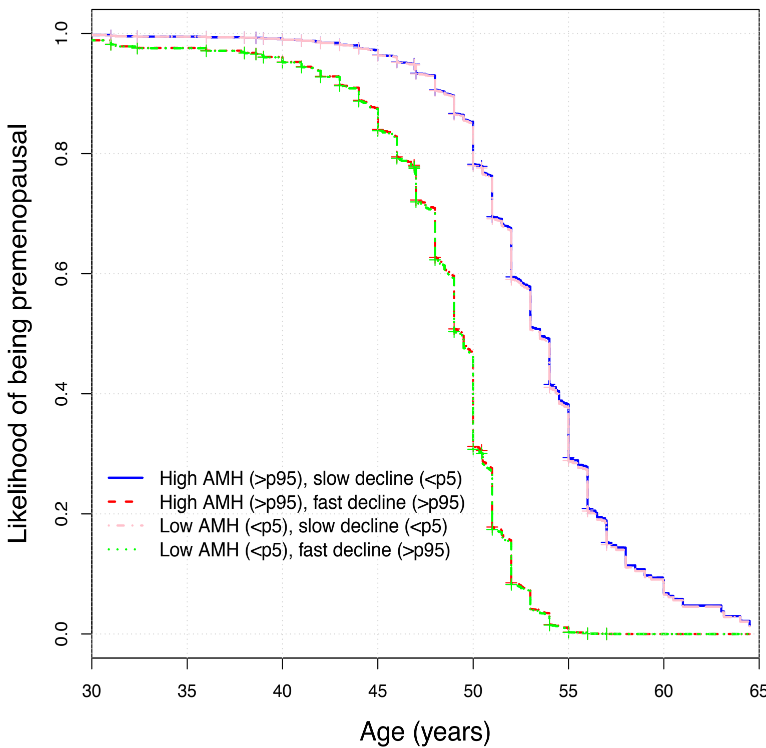

Figure 2. *Predicted likelihood of premenopausal status by age, based on the 5% highest and lowest AMH levels at age 25, and 5% fastest or slowest decline rates between 25-30 years.*

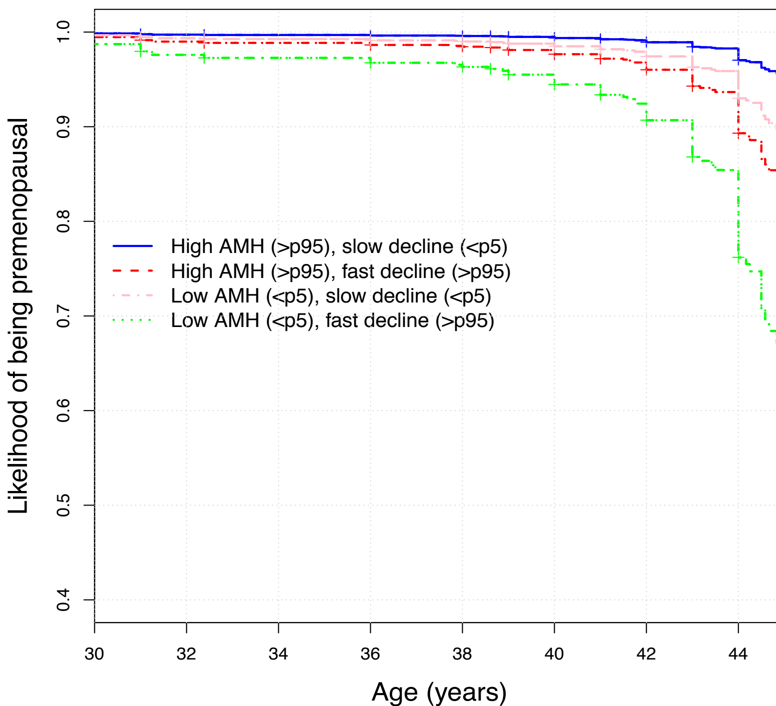

Figure 3. *Predicted likelihood of premenopausal status until age 45, based on the 5% highest and lowest AMH levels at age 25, and 5% fastest or slowest decline rates between 25-30 years.*

Discussion

This study aimed to address whether knowledge of AMH decline, through the use of multiple AMH measurements, could improve prediction of age at menopause. The use of multiple AMH measurements before the age of 25 resulted in a slight improvement in age at menopause prediction, but the prediction of occurrence of early menopause was not improved by the use of multiple AMH measurements.

Our findings that both a lower baseline AMH and a swifter overall annual decline were associated with a shorter time to menopause are in line with the findings from the Penn Ovarian Aging Study (31), which studied the predictive capacity of AMH change over 14 years in women aged between 35-48 years at baseline. Our finding that adding a baseline AMH measurement to a model only containing age increased the predictive capacity of time to menopause in the entire population from 89% to 90% corresponds to the findings of C-statistics of previous long-term prediction models with a single AMH measurement plus age (18, 33, 38, 39, 159). There was no apparent added value of overall rate of annual decline, although a slight improvement could theoretically be less noticeable at a high C-statistic of 0.90. When restricting our analysis to the women aged below 40 years at baseline, addition of both the overall annual AMH decline to the model with baseline AMH led to an increase in predictive capacity of 3%, whereas the rate of AMH decline did not make a difference in the subgroup above 40 years. In a longitudinal study of 6 years among 50 women, the rate of AMH decline was not associated with time to menopause, after adjustment for AMH (29). Given that the women in this study had a mean age of 42 ± 3 years at baseline, it is possible that their higher age difference explains the lack of significance of the rate of AMH decline, in accordance with our findings.

We previously reported that individual AMH trajectories with time are non-linear, that the rate of AMH decline differs between individuals, and that the individual decline rate changes both with AMH level and chronological age (160). It is therefore unsurprising that the age*slope interaction was a significant predictor of age at menopause. This was not the case for the prediction of the occurrence of early menopause, where the changes in slope after age 45 were not taken into account in the analysis. Interestingly, the current slope had a small or negligible effect on the respective age at menopause and early menopause prediction models with current AMH levels, suggesting that short-term AMH changes may not be of added relevance to menopause prediction with multiple AMH measurements.

A prior study from Iran (148) related individual AMH trajectories to time to menopause and concluded that time to menopause prediction with multiple AMH measurements allows for "individualized prediction tailored to each woman" (148). However, there may be several limitations to this study that do not warrant this strong

conclusion. Firstly, the predictive capacity of a single AMH measurement was not compared to that of the AMH trajectories, thereby not providing any information of the additive effect of an individual trajectory. Moreover, the authors reported predictions for 3, 4 or 5 years from age 43 onwards, when the management of age at childbearing, which the authors allude to, is no longer relevant. For this reason, we chose to study the predictive capacity of 1 or 2 AMH measurements at clinically relevant ages. For age at menopause prediction, the use of 2 measurements before the age of 30 increased the prognostic value. On the other hand, a 25-year-old woman wanting to know her risk of becoming postmenopausal by age 45 may not increase the accuracy of this prediction with the addition of an AMH measurement 5 years later.

Although the subpopulation that was followed up until 45 years was smaller, the C-statistics of the prediction models of the occurrence early menopause were higher overall. This could have its origin in the fact that the hazards corresponding to AMH levels are not proportional with age (39, 159). The risk of menopause automatically increases with increasing age, most notably in the years surrounding the average age at menopause (39), thereby decreasing the room for added prognostic value of AMH.

It is a novel, and counter-intuitive, observation that lower AMH levels at age 20 were associated with a *later* age at menopause, and a *lower* risk of early menopause. This observation could potentially be due to the variability of increasing AMH levels after adolescence, which are thought to peak and plateau between the ages of 20-25 years (142, 162). AMH is thought to play an inhibiting role in the rate of follicle recruitment from the primordial follicle pool and subsequent development (153-155). It is therefore conceivable that insensitivity towards the effect of AMH, for example through an AMH receptor polymorphism, could lead to increased follicle development at a young age, leading to higher AMH levels and an eventual swifter depletion of the follicle pool (163). This is supported by our previous observation that high AMH levels at age 20 were associated with a slower initial AMH decline and a swifter decline at a later age (160). If this is indeed the case, it could potentially be one of the mechanisms of the heritability of age at menopause between mothers and daughters (164). It may be speculated that higher per-follicle AMH production could be caused by auto-antibody stimulation, as women with auto-immune disorders are thought to have a higher risk of early menopause (165).

There are several strengths and limitations of the current study that should be discussed. Its strengths include the population-based participant selection, enabling generalization of the results to women of the general population. The longitudinal design with 5 visits in a 20-year follow-up period is well suited for inferences of AMH decline rate and long-term age at menopause prediction. The high sensitivity of the picoAMH assay (with a detection limit of 1.8 pg/mL) allows for a nuanced view of AMH levels and rate of change in the low AMH ranges, which could potentially be rel-

evant for age at menopause prediction. The response rate of >75% in each follow-up round furthermore limited the effect of loss to follow-up. This study is the largest to date to study AMH changes in relation to age at menopause. An important advantage of this study size was the inclusion of 96 women with a very low age at menopause.

A potential practical limitation of the current study is the long time interval of 5 years between two visits and AMH measurements, which could be a long time to wait for someone with a wish to know her future age at menopause. AMH is thought to remain stable over short-term periods and the menstrual cycle (166, 167), or differ up to 0.5 ng/mL throughout the menstrual cycle after the age of 30 (168). This would then plead against spacing the AMH measurements too close to one another. In the Doetinchem Cohort Study, the ages at each follow-up round were normally distributed, which meant that the AMH measurements were available throughout the entire age range, enabling us to estimate an individual trajectory for each participant across this range. Another potential limitation is the assessment of age at menopause, which was entirely done through questionnaires and is thus subject to recall bias, which could affect those with a longer time since menopause, i.e. women with an early menopause, most (169). As the time difference between menopause and the questionnaire was 5 years at most and any irregularities or inconsistencies were filtered out by the use multiple questionnaires for the age at menopause estimation, we do not think misclassification of age at menopause will have had a large impact on our results.

In the aforementioned, we highlighted the limitations of existing models for the prediction of AMH based on a single sample. Refinement of the models with the AMH decline rate, i.e. measuring AMH on multiple occasions, only slightly increased the predictive capacity of overall age at menopause. Knowledge of AMH decline rate overall furthermore did not improve the prediction of early menopause. It is therefore questionable whether knowledge of an individual decline trajectory of AMH will enable women to make better-informed decisions of (postponement of) family planning. We did observe that the median ages at menopause and risks of early menopause were quite different in individuals with extremely high or low AMH level and rates of decline. Women seen in clinical practice with such values may thus benefit from knowing they are at risk. However, as only 2.2% of the women in the group followed until age 45 had this combination of AMH levels and slope, the number of women that this may benefit is likely very limited.

In summary, the results of this study suggest that knowledge of the AMH decline rate can slightly improve age at menopause. Due to the relatively low discriminative ability of the prediction models, our results do not provide a solid basis for AMH screening for long-term menopause prediction.

Chapter 6

Chapter 6

Unraveling the associations of age and menopause with cardiovascular risk factors in a large population-based study

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Abstract

Background Although the association between menopause and cardiovascular disease (CVD) risk has been studied extensively, the simultaneous role of chronological aging herein remains underexposed. This study aimed to disentangle the relationships of menopausal status and chronological aging with CVD risk factors in the largest study population to date.

Methods In this cross-sectional study, CVD risk factors were compared between women with a different menopausal status within the same yearly age strata. The study population comprised female participants of the baseline visit of the population-based Lifelines Cohort Study. A total of 63,466 women, aged between 18-65 years, was included. Of them, 39,379 women were considered to be premenopausal, 8,669 were perimenopausal, 14,514 were naturally postmenopausal, and 904 women were surgically postmenopausal.

Results Compared to postmenopausal women aged 45 years, average total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) were respectively 0.5 and 0.4 mmol/L higher in postmenopausal women aged 50. Systolic and diastolic blood pressure (SBP and DBP) levels were 4 and 1 mm Hg higher, respectively. At all ages between 46 and 55 and after adjustment for confounders, naturally postmenopausal women had 0.2 to 0.4 mmol/L higher TC and 0.1 to 0.3 mmol/L higher LDL-c levels compared to premenopausal women in the same age range. SBP levels were up to 4 mmHg lower in naturally post- compared to premenopausal women at all ages between 29 and 52 years. Body mass index levels were up to 3.2 kg/m^2 higher in women with surgical menopause compared to all other women between the ages 32 and 52 years. All aforementioned results were statistically significant.

Conclusions Chronological age and menopausal status are both independently associated with CVD risk factors. Based on the comparatively smaller observed differences associated with menopausal status than with chronological aging, the significance of a more unfavorable lipid profile in a later reproductive stage may be less obvious than previously thought.

Introduction

Menopause is the final result of the continuous decline of ovarian reserve, marking the end of a woman's reproductive lifespan. An earlier age of reaching menopause is considered to be associated with an increased risk of cardiovascular disease (CVD) (41, 170), but the mechanisms through which menopause is associated with CVD are still unclear. The menopausal transition and postmenopausal status have been associated with adverse CVD risk factor levels (43-50), but others recently contended that chronological aging or prior CVD risk play a more important role (171-173).

As postmenopausal women are by definition older than premenopausal women, it is challenging to separate the effects of biological aging from the various phases of the reproductive aging process (174). This problem was previously circumvented by exclusively studying 53-year-old women born within the same week (44), longitudinally estimating the rate of change of CVD risk factors in the time surrounding the final menstrual period (45, 175), or comparing blood pressure levels between women in biannual age strata (58). However, as the menopausal transition occurs over several years, its longitudinal effects can be ascribed to both aging and menopausal status in the same participant. The currently available studies were furthermore not able to assess the individual effects of chronological and reproductive aging over a large age interval.

In this study, we aimed to disentangle the associations of menopausal status and chronological aging with CVD risk factors over a wide age range. To this end, we compared levels of CVD risk factors with menopausal status, within and between yearly age strata, in the largest study population to date.

Methods

Cohort profile

For our study population, there were 80,853 potentially eligible women between 18-65 years old who participated in the baseline examination of the Lifelines Cohort Study. Lifelines is a multi-disciplinary prospective population-based cohort study examining, in a unique three-generation design, the health and health-related behaviors of 167,729 persons living in the north of The Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioral, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on multi-morbidity and complex genetics (176, 177). The cohort participants were recruited through general practitioner registrations between 2006 and 2013. Cohort members are examined at baseline and will be prospectively followed up with visits in five-year intervals and questionnaires every 1,5 years. The cur-

rent study was based on information from the baseline examination, which includes a questionnaire, anthropometric measurements and blood withdrawal. All participants gave written informed consent (31) and ethical approval was granted by the medical ethics committee of University Medical Center Groningen (177). Lifelines is a facility that is open for all researchers. Information on application and data access procedure is summarized on www.lifelines.net.

Menopausal status assessment

Women with an intra-uterine contraceptive device (n=2,445, 3.0%), who previously underwent a hysterectomy (n=4,937, 6.2%) and/or who reported never having had a regular menstrual cycle (n=4,780, 5.9%) were excluded, leaving 73,662 women. Participants were then divided into groups based on menopausal status, which were defined as premenopausal, perimenopausal, naturally postmenopausal or surgically menopausal. Group allocation was based on baseline questionnaire information and followed the Stages of Reproductive Aging Workshop (STRAW) criteria (57). Women with a currently regular menstrual cycle (n=39,379, 53.4%) were classified as premenopausal. Women with an irregular menstrual cycle since several months (n=7,661) or years (n=1,260; total n=8,669, 11.8%) were considered to be perimenopausal. Women who answered that they were postmenopausal when asked about cycle regularity, and from whom the date of their last menstruation was more than one year before the visit (n=14,514, 19.7%) were considered to be naturally postmenopausal. Women who reported having had a bilateral oophorectomy (n=904, 6.7%) were classified as surgically postmenopausal. The reproductive status of 5,293 (7.2%) women could not be determined. This left 63,466 women in the study population.

Cardiovascular risk factor assessment

At the baseline examination, height and weight were measured by trained staff, from which body mass index (BMI) (in kg/m^2) was calculated. Systolic and diastolic blood pressure (SBP and DBP) were measured 10 times during 10 minutes using a Dynamap PRO (GE Healthcare, Freiburg, Germany) (177), from which the average values were used. The baseline examination furthermore included fasting venous blood withdrawal. Directly after blood withdrawal, prespecified biomarkers in each fasting blood sample were routinely assessed at the in-house laboratory of the University Medical Center Groningen. Serum levels of total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-c) were assessed with an enzymatic colorimetric method, low-density lipoprotein cholesterol (LDL-c) was assessed with a colorimetric method and triglyceride (TG) levels were measured with a colorimetric ultraviolet method, with a Roche Mod-

ular P chemistry analyzer (Roche, Basel, Switzerland). Fasting blood glucose was assessed with a hexokinase method (178).

Other variables

The questionnaires additionally contained questions about hormonal contraception or postmenopausal hormone therapy (HT) use and smoking status. Participants were asked whether they had ever or were currently using oral contraceptives, a hormonal intrauterine device, contraceptive injection (henceforth altogether referred as hormonal contraception) or HT. Current use included any use in the prior month. Smoking status was assessed by asking participants whether they were current smokers or had smoked the previous months. Current and ever smokers were furthermore asked about the total duration and daily frequency of smoking. For this study, smoking status was defined as current smoker (yes or no), including women who had smoked up until the prior month.

Women who were pregnant at the time of examination (n=109, 0.1%) were asked to fill out the questionnaire about the period preceding their pregnancy. They completed their baseline visit at least six months after their pregnancy and three months after ceasing to breastfeed, at which point the questionnaire was handed in and blood withdrawal occurred.

Data analysis

For all variables of interest, the number of complete cases was 60,811 (96%) and missing information per variable did not exceed 1%. Missing values were imputed by conditional multiple imputation with 10 iterations, through predictive mean matching for continuous variables and proportional odds for categorical variables. All CVD risk factor variables, with the exception of TG, were normally distributed. As the distribution of TG levels was right-skewed, TG levels were log-transformed. Baseline characteristics were presented across menopausal status groups as mean ± SD or n (%), unless stated otherwise.

To gain a first insight in differences in CVD risk factor levels between the menopausal status categories independently of age, a linear regression analysis was performed within each one-year age stratum for each outcome, with premenopausal women as the reference category. Women below the age of 34 were all included in a 34-years and younger group, due to the relative lack of postmenopausal women before this age. In similar fashion, women above age 56 were all included in a 56-years and older age stratum. The regression analyses were adjusted for smoking status, current hormonal contraception and BMI, due to their potential association with both menopausal status and CVD risk factors. Because BMI was considered to be both a potential confounder and CVD risk factor, a model with BMI as an outcome

was also fit, which adjusted for smoking and hormonal contraception use only. Models were furthermore adjusted for antihypertensive and lipid-lowering medication.

The objective of investigating an independent association of both calendar age and menopausal status with CVD risk factor levels was addressed by creating a linear regression model for each CVD risk factor as an outcome, with menopausal status and age as independent covariables. In order to adjust for smoking status, hormonal contraception use, antihypertensive or lipid-lowering medication and BMI (except in the case of BMI as a CVD risk factor outcome), these parameters were additionally added to the model. To test whether the association with age differed between the menopausal status groups, we included an interaction term of menopausal status with age in the model and tested its significance with an analysis of variance (ANOVA). Furthermore, in order to take into account a potential non-linear relationship of age with CVD risk factors, restricted cubic splines for age were added to the model (179, 180). The model was then tested for non-linearity with an ANOVA analysis. Using the resulting best fitting model (excluding the interaction term or splines if the interaction term or test for non-linearity were non-significant), the adjusted values for each outcome were plotted against age for each menopausal status group.

All statistical analyses were performed with R (www.r-project.org), version 3.1.3. Multiple imputation was done using the 'mice' library, using a prediction matrix with all determinants, outcomes and confounders (181). The regression models were fitted with the fit.mult.impute function from the 'Hmisc' library.

Sensitivity analyses

We performed four sensitivity analyses. First, the analyses described above were repeated after including women with missing reproductive status information, by assigning them to menopausal groups based on their age, similar to the methods by Clavel-Chapelon *et al.* (182). Secondly, the analyses were repeated after excluding women who reported current use of cholesterol- or blood pressure-lowering medication. Thirdly, the analyses were performed with only inclusion of women who reported an irregular cycle 'since several months' as the perimenopausal group. Lastly, as the classification of the STRAW criteria for the whole study population was based on the answers to the question of cycle regularity and menopause, hormonal contraception and HT use were not taken into account for this determination. To assess the differences between the menopausal status groups independently from exogenous hormone use, women who had ever used HT or currently used hormonal contraception were excluded from analysis.

Patient involvement

The development of the research question and study design occurred without the involvement of patients. The research question fits within the scope of healthy aging in the general population, an objective set by Lifelines.

Results

In Table 1, the number of women in each age stratum and menopausal status group is listed. Characteristics for women in each reproductive category are presented in Table 2. Mean age increased over the pre-, peri-, and postmenopausal groups, and so did the mean levels of all of CVD risk factors. Hormonal contraception usage decreased over the pre-, peri- and postmenopausal groups, with the lowest percentage of users in the surgically postmenopausal group. The vast majority of women who reported ever using HT (3% of the study population) were postmenopausal (77%), with the highest percentage (64%) in the surgical menopause group. In the premenopausal group, 203 (0.5%) women said to have ever used HT, but reported a currently regular menstrual cycle. In the naturally postmenopausal group, median age [interquartile range, IQR] at menopause was 51 [46-53] years.

For all CVD risk factors studied, the association between age and risk factor level was significantly non-linear (p-value for non-linearity <0.001 in all cases), so all models included restricted cubic splines for age. In addition, for all CVD risk factors besides SBP and glucose there was a significant interaction between age and menopausal status (p-values for the interaction term ranged between <0.001 and 0.01), indicating that the magnitude of the differences in these risk factor levels between menopausal status groups varied with age. The models including cubic splines and the interaction term had a better fit than the models without, assessed by comparison of the Akaike's Information Criterion (AIC). All model residuals were furthermore normally distributed. Since a single regression coefficient cannot be estimated due to the splines and interactions, the fully adjusted mean levels of with 95% confidence interval (CI) bands of all CVD risk factors are displayed for each menopausal status group with age in Figure 1 (A-H).

Table 1. *Number of study participants in each menopausal status group per annual age stratum*

Age stratum	Pre-menopausal	Peri-menopausal	Naturally post-menopausal	Surgically post-menopausal	Total
18	726	32	1	0	759
19	561	19	2	0	582
20	552	35	3	0	590
21	638	29	8	1	676
22	655	50	4	0	709
23	670	50	7	0	727
24	704	51	14	0	769
25	813	79	10	0	902
26	1138	119	26	1	1284
27	1064	120	25	0	1209
28	982	101	19	0	1102
29	971	119	19	1	1110
30	950	107	27	0	1084
31	995	116	28	2	1141
32	1028	119	42	3	1192
33	1070	110	32	1	1213
34	1118	99	39	2	1258
35	1151	113	54	1	1319
36	1235	138	70	5	1448
37	1369	134	83	8	1594
38	1464	145	63	6	1678
39	1606	153	108	15	1882
40	1731	187	95	7	2020
41	1796	245	119	11	2171
42	1860	248	121	16	2245
43	1783	352	135	22	2292
44	1781	380	137	19	2317
45	1740	490	157	28	2415
46	1577	535	208	40	2360
47	1489	659	290	39	2477
48	1337	743	392	43	2515

Table 1. *Continued*

Age stratum	Pre-menopausal	Peri-menopausal	Naturally post menopausal	Surgically post-menopausal	Total
49	1148	788	508	61	2505
50	898	795	703	51	2447
51	467	565	691	37	1760
52	134	233	464	18	849
53	69	169	556	22	816
54	57	123	653	32	865
55	29	62	802	24	917
56	14	32	886	23	955
57	4	16	870	28	918
58	1	6	902	38	947
59	3	1	876	47	927
60	1	1	900	29	931
61	0	0	883	30	913
62	0	1	817	52	870
63	0	0	821	37	858
64	0	0	790	48	808
65	0	0	84	56	140
Total	39397	8669	14514	904	63466

Between ages 29-52 mean SBP levels adjusted for hormonal contraception use, smoking and BMI were significantly lower in the naturally postmenopausal group compared to the three other menopausal status groups, as there was no overlap of CIs (Figure 1A). Compared to the premenopausal group, fully adjusted SBP levels were between 2.6-4.0 mm Hg lower in the naturally postmenopausal group. Similar results were found with the regression analyses within each age stratum (Supplementary Table 1 displays the regression coefficients with 95% CI for the linear regression analyses in each age stratum for SBP). With regard to chronological aging, compared to age 45, adjusted SBP levels at age 50 were between 3.0 to 3.8 mm Hg higher on average (Table 3). No distinct pattern of differences between menopausal stages within the age bands was observed for DBP (Figure 1B, Supplementary Table 2). Adjusted DBP levels in all menopausal status groups were between 0.9-1.6 mm Hg higher at age 50 compared to age 45 (Table 3).

Fully adjusted mean TC and LDL-c levels were 0.1 mmol/L higher in the peri-

menopausal group compared to the premenopausal group, and 0.2-0.4 mmol/L higher in the naturally postmenopausal group compared to the premenopausal group across the range of 45-55 years, which reached statistical significance (Figure 1C). Between 37-49 years, adjusted TC levels were between 0.2-0.4 mmol/L higher in the surgically postmenopausal group compared to women in the premenopausal group, and significantly higher than all three other groups (Figure 1C). Between 46-55 years, adjusted LDL-c levels in the peri- and naturally postmenopausal groups were 0.1 and 0.3 mmol/L, respectively. Surgically postmenopausal women had significantly higher adjusted LDL-c levels than all other women between the ages 38-49. Linear regression analyses within the age strata echoed these results (Supplementary Tables 3 and 4). With respect to chronological aging, the average adjusted difference in TC and LDL-c levels between 45-50 years ranged between 0.2-0.5 and 0.2-0.4 mmol/L, respectively (Table 3).

No clear differences were observed in mean adjusted HDL-c or glucose levels between the menopausal status groups at all ages (Figure 1E and F, Supplementary Tables 5 and 6). Compared to women aged 45 years, mean adjusted HDL-c and glucose levels were 0.0-0.1 mmol/L higher at age 50, dependent on menopausal status group (Table 3). Fully adjusted mean TG levels were up to 12% higher in surgically postmenopausal women compared to premenopausal women between the ages 42 and 53. Between the ages 32 and 52, BMI levels were up to 3.2 kg/m^2 higher in surgically postmenopausal compared to premenopausal women. In these age ranges, TG and BMI levels were significantly higher in surgically postmenopausal women compared to women in all other menopausal status groups (Figure 1G and H). In contrast, compared to premenopausal women, TG levels were 5-22% lower in postmenopausal women between the ages 30-48. Similar results were found in the linear regression analyses in each age stratum, although the differences with the surgically postmenopausal group were not significant, possibly due to lack of power (Supplementary Tables 5 and 6). At age 50, TG levels were 0.1 mmol/L higher in all menopausal status groups compared to age 45 (Table 3). Adjusted BMI levels were either the same or between 0.1-0.4 kg/m^2 lower at age 50 compared to age 45, depending on the menopausal status group (Table 3).

Sensitivity analyses

The sensitivity analyses are summarized for each outcome in Supplementary Figures 1-8. First, inclusion of the 5,293 women with an age-based reproductive status did not alter the results. Second, the exclusion of women who used cholesterol- or blood pressure-lowering medication (n=1,880 and n=4,705, respectively) also did not alter the results, although the confidence interval of the surgical menopause group became wider. Third, excluding 1,260 women in the perimenopausal group with an irregular cycle since

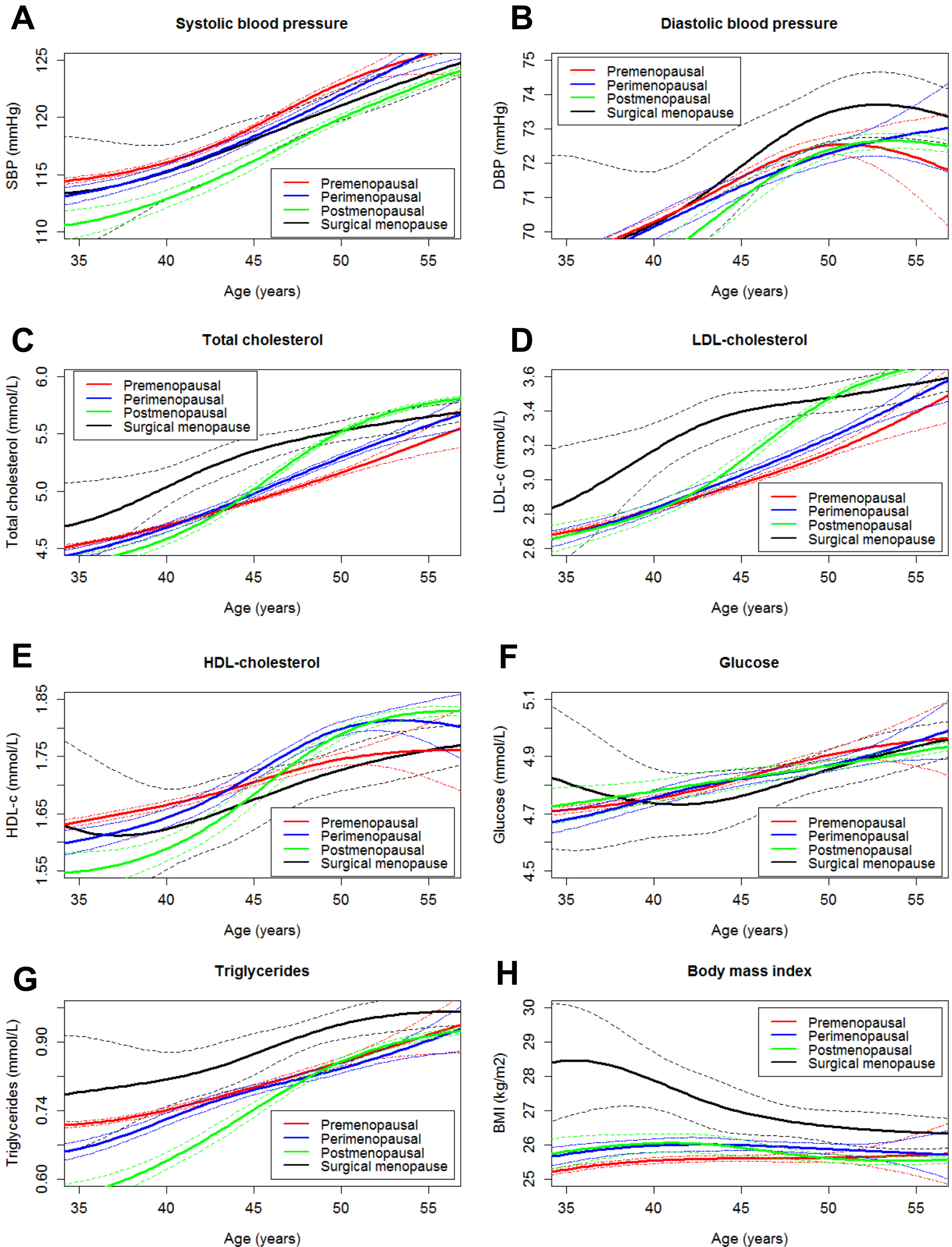

Figure 1. *Associations of adjusted cardiovascular risk factors with age per menopausal status group. Cardiovascular risk factor levels were adjusted for age, oral contraceptive use, smoking status and body mass index. The premenopausal status group comprised a total of 39,379 women, the perimenopausal group 8,669 women; the naturally postmenopausal group 14,514 women; and the surgically postmenopausal group 904 women.*

Table 2. *Characteristics per menopausal status group*

	Pre-menopausal *n = 39,397*	Peri-menopausal *n = 8,669*	Naturally post-menopausal *n = 14,514*	Surgically post-menopausal *n = 904*
	Baseline			
Age (years)	36.9 ± 8.1	45.0 ± 8.1	55.3 ± 7.4	52.7 ± 8.1
Age range (years)	18-60	18-62	18-65	21-65
Current hormonal contraception use	18,526 (47.6)	1,938 (22.7)	1,787 (12.6)	825 (2.6)
Current smoker	8,125 (21.0)	1,969 (22.9)	2,751 (19.1)	165 (18.4)
Antihypertensive medication	1,559 (4.0)	608 (7.0)	2,356 (20.3)	182 (20.3)
Lipid-lowering medication	388 (1.0)	178 (2.1)	1,222 (8.4)	92 (10.2)
Ever HT use	203 (0.5)*	275 (3.2)	1315 (9.1)	253 (28.4)
	Outcome			
BMI (kg/m²)	25.2 ± 4.6	26.0 ± 4.9	26.2 ± 4.5	27.3 ± 5.0
SBP (mm Hg)	119 ± 13	121 ± 14	125 ± 16	126 ± 16
DBP (mm Hg)	71 ± 9	72 ± 9	73 ± 9	72 ± 9
TC (mmol/L)	4.7 ± 0.8	5.0 ± 0.9	5.6 ± 1.0	5.5 ± 1.0
LDL-c (mmol/L)	2.9 ± 0.8	3.1 ± 0.8	3.6 ± 0.9	3.5 ± 0.9
HDL-c (mmol/L)	1.6 ± 0.4	1.6 ± 0.4	1.7 ± 0.4	1.6 ± 0.4
TG (mmol/L)	1.0 ± 0.5	1.0 ± 0.6	1.1 ± 0.6	1.2 ± 0.7
Glucose (mmol/L)	4.8 ± 0.6	4.9 ± 0.7	5.0 ± 0.8	5.1 ± 1.0

Values given in mean ± SD or n (%). HT hormone replacement therapy; BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; TC total cholesterol; LDL-c low-density lipoprotein cholesterol; HDL-c high-density lipoprotein cholesterol; TG triglycerides

**All reported a currently regular menstrual cycle*

several years additionally did not alter the nature of the results for the perimenopausal group. Fourth, exclusion of women using hormonal contraception (n=23,076) and HT (n=2,056) caused an expected widening of the confidence intervals due to the reduced power. This did not affect the overall results, with the exception of a more marked difference in TC, LDL-c and TG levels between young pre- and postmenopausal women (Supplementary Figures 3, 4 and 6).

Table 3. *Average absolute differences in adjusted risk factors between women aged 45 and aged 50 years.*

	Pre-menopausal	Peri-menopausal	Naturally post-menopausal	Surgically post-menopausal
Difference in adjusted risk factor levels (95% CI) between women aged 45 and 50 years				
SBP (mm Hg)	3.8 (3.6 to 3.9)	3.6 (3.6 to 3.7)	3.7 (3.4 to 4.1)	3.0 (2.5 to 3.5)
DBP (mm Hg)	0.9 (0.8 to 1.0)	1.0 (1.0 to 1.0)	1.4 (1.2 to 1.6)	1.6 (1.2 to 1.9)
TC (mmol/L)	0.2 (0.2 to 0.3)	0.3 (0.3 to 0.3)	0.5 (0.5 to 0.5)	0.2 (0.1 to 0.2)
LDL-c (mmol/L)	0.2 (0.2 to 0.2)	0.2 (0.2 to 0.2)	0.4 (0.3 to 0.4)	0.1 (0.0 to 0.1)
HDL-c (mmol/L)	0.0 (0.0 to 0.0)	0.1 (0.1 to 0.1)	0.1 (0.1 to 0.1)	0.1 (0.0 to 0.1)
Glucose (mmol/L)	0.1 (0.1 to 0.1)	0.0 (0.0 to 0.0)	0.0 (0.0 to 0.1)	0.1 (0.1 to 0.1)
TG (mmol/L)	0.1 (0.1 to 0.1)	0.1 (0.1 to 0.1)	0.1 (0.1 to 0.1)	0.1 (0.1 to 0.1)
BMI (kg/m²)	0.0 (-0.0 to 0.1)	-0.1 (-0.1 to -0.1)	-0.3 (-0.4 to -0.2)	-0.4 (-0.6 to -0.2)

Discussion

This study presents a unique view of reproductive aging, independently of biological aging. We observed an association of CVD risk factors with menopausal status within several clusters of annual age strata, indicating that this relationship cannot be explained by the effects of chronological aging alone. The magnitude of differences in CVD risk factors between menopausal status groups did vary with age, highlighting the added role of chronological aging. Based on these results, it seems likely that both chronological aging and menopausal status contribute to the CVD risk profile of aging women.

Naturally postmenopausal women had lower adjusted SBP levels across a large age range than pre-, peri or surgically postmenopausal women. Prior reports found a later reproductive stage to be associated with increased blood pressure (50, 58, 183), while others reported a lack of any association after adjustment for age (44,

45, 173, 184, 185). A longitudinal study in 193 women was the first to detect a decreased SBP level in post- compared to premenopausal women (186), hypothesizing that a diminishing ovarian reserve exhibits a protective effect on increasing SBP levels. By design we cannot confirm this hypothesis, but our results do contest previous reports of an adverse blood pressure milieu in a peri- and postmenopausal state (50, 58, 183).

Where lipid levels are concerned, previous findings are less ambiguous and correspond well to our results. LDL-c and TC levels are widely thought to be influenced by the menopausal transition (45) or associated with menopausal status (43, 44, 47, 48, 187-190). In fact, the approximate difference in LDL-c levels of 11 mg/dL (0.28 mmol/L) observed by Matthews *et al.* (45) between the year preceding and following the final menstrual period fits well within the range of our observations. The decrease of estradiol throughout the menopausal transition may not play a role in this regard, as TC and LDL-c levels did not correlate with total or free estradiol in 99 postmenopausal women (191). On the other hand, post-menopausal hormone therapy was associated with a better lipid profile compared to placebo in a meta-analysis of 28 trials (192). Another explanation is the reduced activity of LDL-c receptors or lipoprotein lipase in a postmenopausal state (193, 194).

In our population, differences in LDL-c and TC levels between menopausal status groups only became evident after the age of 45, after which LDL-c and TC levels more sharply increased in the peri- and postmenopausal groups. While a rapid increase in lipid levels was previously linked to the menopausal transition (45, 47), our results do suggest that chronological aging is equally involved. Indeed, the adjusted difference in TC and LDL-c values in the interval of 45-50 years was equal to the maximum observed differences between the menopausal status groups. It may be possible that with increasing age, the availability of compensatory mechanisms to neutralize hyperlipidemia diminishes.

Surgically postmenopausal women, having undergone a bilateral oophorectomy, had consistently higher BMI and TG levels than the remaining women in the same age stratum, the latter even after adjusting for BMI. Others observed similar results (173, 195-199), with the odds of becoming obese specifically increasing after bilateral oophorectomy (198). Interestingly, the adjusted BMI of pre-, peri- and naturally postmenopausal women hardly differed throughout the study population, which is in line with previous findings (195), but at odds with the observation that the menopausal transition influences fat distribution (175, 189).

For the past two decades, the relationship of menopause with CVD risk factors has been studied extensively through a myriad of ways. As most previous research was based on smaller study populations, often with significantly differing ages between pre- and postmenopausal groups, we hope to provide a substantial contribution to this age-old question with our study. Its strengths are the use of a large study population,

with the ability to compare menopausal status groups and CVD risk factors within yearly age strata, over a wide age range. The protocolled assessment of study parameters and relative lack of missing information limit the chance of bias. Unfortunately, this was not quite the case for the classification of menopausal status. It is likely that some postmenopausal women using hormonal contraception or HT were classified as premenopausal due to the report of a regular cycle, and that some premenopausal women with an irregular cycle were wrongly classified as peri- or postmenopausal (200).

The exclusion of women using exogenous hormones did not have an obvious impact on the overall results, with the exception that the lipid profile of young postmenopausal women appeared notably more unfavorable than the other groups in this analysis. It is possible that this difference is due to the putative benefits of hormone supplementation in young women in particular (53), or incorrect classification of premenopausal women using hormonal contraception as postmenopausal. In order to be considered postmenopausal, women had to report in the questionnaire that they had entered menopause in addition to reporting an amenorrhea of at least a year, which makes large-scale misclassification in this category less likely. Moreover, the finding of very young women with non-iatrogenic menopause corresponds to our observations in clinical practice and other Dutch cohort studies and could therefore well be a realistic representation. Lastly, due to the small number of women with surgical menopause, there is insufficient power to separately compare this group in all yearly strata. However, as this group of women represents a different clinical entity than natural menopause, we chose to maintain this classification.

As this was a cross-sectional study, our observations are limited to associations without drawing conclusions on causality. A previous study was able to longitudinally follow CVD risk factors (45), providing important information on the changes surrounding the menopausal transition. It is by definition impossible to distinguish these changes from general aging throughout the menopausal transition in the same participant, however, which is why our current study provides an important contribution from a different perspective. Although we are able to confirm previous reports of differences in lipid parameters based on menopausal status, the clinical implications of the observed differences may be limited. A reduction of LDL-c of 1.0 mmol/L was associated with a 22% decreased rate of major vascular events in an extensive meta-analysis of individual patient data (201), but this is difference is 2.5 to 10 times larger than menopause-related differences in this study or the study by Matthews et al. (45), and in fact more approximate to the differences found with 20 years of chronological age. It may be that the increased risk of cardiovascular events observed in post-menopausal women, the causality of which is a matter of debate in itself (171, 172, 202), is mediated through other pathways, such as oxidative stress and inflammation (203). A previ-

ous proposal of lipid screening of women entering the menopausal transition (45) may therefore not prove beneficial. That being said, vigilance of changing lipid parameters in high-risk women as they pass both biological and reproductive aging thresholds may be worthwhile.

Conclusion

In conclusion, we observed independent associations of both age and menopausal status with selected CVD risk factors, mainly at the level of lipid metabolism, in a large population-based cohort. The clinical ramifications of a more unfavorable CVD risk factor profile with the transition to menopause may be limited, however.

Supplementary Table 1. *Differences (95% CI) in adjusted systolic blood pressure levels (mm Hg) per age stratum, in reference to premenopausal participants*

Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally postmenopausal	Surgically post-menopausal
≤34	0.0 (Ref)	-1.5 (-2.0 to -0.9)	-3.6 (-4.7 to -2.4)	-0.16 (-6.4 to 5.9)
35	0.0 (Ref)	-1.5 (-3.5 to 0.5)	-3.0 (-5.9 to -0.1)	8.5 (-12.0 to 28.9)
36	0.0 (Ref)	-2.3 (-4.2 to -0.5)	-2.7 (-5.3 to -0.1)	2.3 (-6.8 to 11.5)
37	0.0 (Ref)	-2.3 (-4.2 to -0.4)	-4.5 (-7.0 to -2.1)	2.3 (-5.1 to 10.0)
38	0.0 (Ref)	0.6 (-1.3 to 2.6)	-5.0 (-7.9 to -2.0)	-2.2 (-11.4 to 7.0)
39	0.0 (Ref)	-0.4 (-2.4 to 1.5)	-2.5 (-4.9 to -0.1)	-3.6 (-9.8 to 2.6)
40	0.0 (Ref)	-0.7 (-2.5 to 1.1)	-2.6 (-5.2 to -0.1)	-6.2 (-15.2 to 2.7)
41	0.0 (Ref)	-1.1 (-2.7 to 0.5)	-4.6 (-6.9 to -2.3)	-5.1 (-12.2 to 2.0)
42	0.0 (Ref)	-1.2 (-2.8 to 0.5)	-2.7 (-5.1 to 0.4)	0.5 (-5.7 to 6.7)
43	0.0 (Ref)	-0.1 (-1.6 to 1.3)	-3.8 (-6.1 to -1.6)	1.7 (-3.7 to 7.0)
44	0.0 (Ref)	-1.0 (-2.5 to 0.5)	-3.1 (-5.5 to -0.8)	1.2 (-4.9 to 7.2)
45	0.0 (Ref)	0.1 (-1.3 to 1.5)	-3.0 (-5.3 to -0.7)	-1.7 (-6.9 to 3.5)
46	0.0 (Ref)	-1.0 (-2.4 to 0.3)	-3.7 (-5.7 to -1.7)	-3.2 (-7.5 to 1.1)
47	0.0 (Ref)	-1.3 (-2.6 to -0.1)	-2.7 (-4.4 to -0.9)	-0.0 (-4.4 to 4.4)
48	0.0 (Ref)	-2.2 (-3.6 to -0.9)	-2.5 (-4.1 to -0.9)	-0.9 (-5.2 to 3.5)
49	0.0 (Ref)	-1.0 (-2.4 to 0.3)	-4.2 (-5.8 to -2.7)	-3.6 (-7.4 to 0.1)
50	0.0 (Ref)	0.2 (-1.2 to 1.7)	-1.6 (-3.1 to -0.1)	-1.6 (-5.8 to 2.5)
51	0.0 (Ref)	-0.5 (-2.4 to 1.4)	-2.6 (-4.5 to -0.8)	-1.0 (-6.1 to 4.1)
52	0.0 (Ref)	0.6 (-2.7 to 3.9)	-2.2 (-5.2 to 0.8)	2.6 (-4.9 to 10.2)
53	0.0 (Ref)	-1.8 (-6.1 to 2.6)	-4.5 (-8.5 to -0.4)	-7.1 (-14.3 to 0.1)
54	0.0 (Ref)	1.3 (-3.5 to 6.2)	-1.7 (-6.0 to 2.7)	1.7 (-4.8 to 8.1)
55	0.0 (Ref)	-4.2 (-11.2 to 2.9)	-6.3 (-12.4 to -0.2)	-6.1 (-14.8 to 2.5)
≥56	0.0 (Ref)	-1.5 (-9.7 to 6.8)	-1.9 (-9.1 to 5.2)	-0.5 (-7.8 to 6.8)

Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Orange cells indicate p-value <0.0001, green cells indicate p-value <0.001, blue cells indicate p-value <0.05.

Supplementary Table 2. *Differences (95% CI) in adjusted diastolic blood pressure levels (mm Hg) per age stratum, in reference to premenopausal participants*

Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally postmenopausal	Surgically post-menopausal
≤34	0.0 (Ref)	-0.4 (-0.8 to -0.0)	-1.2 (-2.0 to -0.3)	2.1 (-2.1 to 6.3)
35	0.0 (Ref)	-0.8 (-2.3 to 0.6)	-1.4 (-3.5 to 0.7)	2.9 (-11.7 to 17.6)
36	0.0 (Ref)	-0.0 (-1.4 to 1.3)	-1.0 (-2.9 to -0.8)	0.2 (-6.4 to 6.8)
37	0.0 (Ref)	-0.9 (-2.3 to 0.5)	-2.3 (-4.1 to -0.5)	0.2 (-5.1 to 5.6)
38	0.0 (Ref)	0.9 (-0.4 to 2.4)	-2.5 (-4.6 to -0.3)	0.2 (-6.3 to 6.8)
39	0.0 (Ref)	0.7 (-0.6 to 2.1)	-1.5 (-3.1 to 0.2)	-2.0 (-6.3 to 2.3)
40	0.0 (Ref)	-0.5 (-1.7 to 0.7)	-1.5 (-3.2 to 0.2)	-3.1 (-9.1 to 2.8)
41	0.0 (Ref)	0.1 (-1.0 to 1.3)	-2.4 (-4.1 to -0.8)	-4.0 (-9.0 to 0.5)
42	0.0 (Ref)	-0.8 (-1.9 to 0.3)	-0.9 (-2.5 to 0.7)	3.6 (-0.6 to 7.7)
43	0.0 (Ref)	0.0 (-1.0 to 1.0)	-0.4 (-1.9 to 1.1)	3.0 (-0.6 to 6.6)
44	0.0 (Ref)	-0.4 (-1.3 to 1.6)	-1.2 (-2.8 to 0.3)	2.9 (-1.0 to 6.8)
45	0.0 (Ref)	0.2 (-0.7 to 1.1)	-0.7 (-2.2 to 0.7)	-0.3 (-3.6 to 3.0)
46	0.0 (Ref)	-0.7 (-1.6 to 0.1)	-0.5 (-1.8 to 0.7)	-1.5 (-4.2 to 1.3)
47	0.0 (Ref)	-0.8 (-1.6 to 0.0)	-0.7 (-1.8 to 1.4)	0.6 (-2.2 to 3.5)
48	0.0 (Ref)	-0.4 (-1.2 to 0.5)	0.4 (-0.7 to 1.4)	-0.6 (-3.3 to 2.2)
49	0.0 (Ref)	-0.8 (-1.6 to 0.1)	-0.9 (-1.9 to 0.0)	0.3 (-2.1 to 2.7)
50	0.0 (Ref)	0.6 (-0.3 to 1.5)	0.6 (-0.4 to 1.5)	0.3 (-2.3 to 2.9)
51	0.0 (Ref)	-0.8 (-1.9 to 0.4)	0.0 (-1.1 to 1.2)	2.0 (-1.0 to 5.1)
52	0.0 (Ref)	2.2 (0.2 to 4.2)	1.5 (-0.3 to 3.3)	3.2 (-1.3 to 7.7)
53	0.0 (Ref)	-0.3 (-3.0 to 2.4)	-0.5 (-3.0 to 1.9)	-1.5 (-5.6 to 2.9)
54	0.0 (Ref)	1.4 (-1.6 to 4.4)	0.5 (-2.2 to 3.2)	3.0 (-1.0 to 7.0)
55	0.0 (Ref)	-4.2 (-8.4 to 0.1)	-3.9 (-7.5 to -0.2)	-3.9 (-9.0 to 1.3)
≥56	0.0 (Ref)	-0.3 (-4.8 to 4.2)	-0.2 (-4.2 to 3.7)	0.6 (-3.4 to 4.6)

Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Green cells indicate p-value <0.001.

Supplementary Table 3. *Differences (95% CI) in adjusted total cholesterol levels (mmol/L) per age stratum, in reference to premenopausal participants*

Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally postmenopausal	Surgically post-menopausal
≤34	0.0 (Ref)	-0.1 (-0.1 to -0.1)	-0.2 (-0.3 to -0.1)	0.1 (-0.4 to 0.7)
35	0.0 (Ref)	-0.3 (-0.4 to -0.1)	-0.2 (-0.4 to 0.0)	-0.0 (-1.5 to 1.5)
36	0.0 (Ref)	-0.1 (-0.2 to 0.1)	-0.2 (-0.4 to 0.0)	0.6 (-0.1 to 1.2)
37	0.0 (Ref)	0.0 (-0.1 to 0.2)	-0.3 (-0.5 to -0.1)	0.5 (-0.1 to 1.2)
38	0.0 (Ref)	-0.1 (-0.2 to 0.1)	-0.3 (-0.5 to 0.1)	-0.2 (-0.8 to 0.5)
39	0.0 (Ref)	0.0 (-0.1 to 0.2)	-0.2 (-0.3 to -0.0)	0.3 (-0.3 to 0.8)
40	0.0 (Ref)	0.0 (-0.1 to 0.2)	-0.1 (-0.3 to 0.1)	-0.2 (-0.8 to 0.4)
41	0.0 (Ref)	0.0 (-0.1 to 0.1)	-0.0 (-0.2 to 0.1)	0.5 (0.0 to 1.0)
42	0.0 (Ref)	-0.1 (-0.2 to 0.0)	-0.1 (-0.2 to 0.1)	0.7 (0.3 to 1.1)
43	0.0 (Ref)	0.0 (-0.1 to 0.1)	0.1 (-0.1 to 0.2)	0.5 (0.1 to 0.8)
44	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.0 (-0.1 to 0.2)	0.5 (0.1 to 0.8)
45	0.0 (Ref)	0.1 (0.0 to 0.2)	0.1 (-0.0 to 0.3)	0.6 (0.3 to 1.0)
46	0.0 (Ref)	0.1 (0.0 to 0.2)	0.1 (-0.0 to 0.2)	0.1 (-0.2 to 0.3)
47	0.0 (Ref)	0.0 (-0.0 to 0.1)	0.3 (0.2 to 0.4)	0.6 (0.3 to 0.9)
48	0.0 (Ref)	0.1 (0.1 to 0.2)	0.3 (0.2 to 0.4)	0.1 (-0.1 to 0.4)
49	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.3 (0.2 to 0.4)	0.3 (0.0 to 0.5)
50	0.0 (Ref)	0.1 (-0.0 to 0.2)	0.3 (0.2 to 0.4)	0.3 (0.1 to 0.6)
51	0.0 (Ref)	0.1 (-0.1 to 0.2)	0.3 (0.2 to 0.4)	0.1 (-0.3 to 0.4)
52	0.0 (Ref)	0.2 (0.0 to 0.4)	0.4 (0.2 to 0.6)	0.2 (-0.2 to 0.7)
53	0.0 (Ref)	0.2 (-0.1 to 0.5)	0.4 (0.1 to 0.6)	0.5 (0.0 to 0.9)
54	0.0 (Ref)	0.0 (-0.3 to 0.3)	0.2 (-0.1 to 0.5)	0.3 (-0.1 to 0.8)
55	0.0 (Ref)	-0.2 (-0.6 to 0.2)	0.0 (-0.4 to 0.4)	-0.0 (-0.6 to 0.5)
≥56	0.0 (Ref)	0.2 (-0.3 to 0.7)	0.5 (0.1 to 1.0)	0.5 (0.1 to 0.9)

Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Orange cells indicate p-value <0.0001, green cells indicate p-value <0.001, blue cells indicate p-value <0.05.

Supplementary Table 4. *Differences (95% CI) in adjusted LDL-cholesterol levels (mmol/L) per age stratum, in reference to premenopausal participants*

Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally postmenopausal	Surgically post-menopausal
≤34	0.0 (Ref)	-0.1 (-0.1 to -0.0)	-0.1 (-0.2 to -0.0)	0.2 (-0.3 to 0.7)
35	0.0 (Ref)	-0.2 (-0.3 to -0.1)	-0.1 (-0.3 to 0.1)	-0.1 (-1.5 to 1.3)
36	0.0 (Ref)	-0.0 (-0.2 to 0.1)	-0.0 (-0.2 to 0.1)	0.4 (-0.2 to 1.0)
37	0.0 (Ref)	0.1 (-0.1 to 0.2)	-0.1 (-0.3 to 0.0)	0.4 (-0.2 to 1.0)
38	0.0 (Ref)	-0.0 (-0.2 to 0.1)	-0.1 (-0.3 to 0.1)	-0.1 (-0.7 to 0.5)
39	0.0 (Ref)	0.0 (-0.1 to 0.2)	-0.0 (-0.2 to 0.1)	0.3 (-0.2 to 0.8)
40	0.0 (Ref)	0.0 (-0.1 to 0.1)	-0.1 (-0.2 to 0.1)	-0.3 (-0.9 to 0.3)
41	0.0 (Ref)	0.1 (-0.0 to 0.2)	0.1 (-0.0 to 0.3)	0.6 (0.1 to 1.0)
42	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.0 (-0.1 to 0.2)	0.5 (0.1 to 0.9)
43	0.0 (Ref)	0.0 (-0.1 to 0.1)	0.1 (-0.0 to 0.3)	0.5 (0.2 to 0.8)
44	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.0 (-0.1 to 0.2)	0.4 (0.1 to 0.8)
45	0.0 (Ref)	0.1 (0.0 to 0.2)	0.2 (0.0 to 0.3)	0.5 (0.3 to 0.8)
46	0.0 (Ref)	0.1 (0.0 to 0.2)	0.1 (0.0 to 0.2)	0.1 (-0.1 to 0.4)
47	0.0 (Ref)	-0.0 (-0.1 to 0.7)	0.2 (0.1 to 0.4)	0.6 (0.3 to 0.9)
48	0.0 (Ref)	0.1 (0.0 to 0.2)	0.2 (0.1 to 0.3)	0.1 (-0.2 to 0.3)
49	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.3 (0.2 to 0.3)	0.2 (0.0 to 0.5)
50	0.0 (Ref)	0.1 (-0.0 to 0.1)	0.3 (0.2 to 0.4)	0.3 (0.1 to 0.6)
51	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.3 (0.1 to 0.4)	0.2 (-0.1 to 0.5)
52	0.0 (Ref)	0.2 (0.0 to 0.4)	0.3 (0.1 to 0.5)	0.1 (-0.3 to 0.6)
53	0.0 (Ref)	0.2 (-0.0 to 0.5)	0.4 (0.1 to 0.6)	0.5 (0.1 to 0.9)
54	0.0 (Ref)	-0.0 (-0.3 to 0.3)	0.1 (-0.2 to 0.4)	0.3 (-0.2 to 0.7)
55	0.0 (Ref)	-0.1 (-0.6 to 0.3)	0.0 (-0.3 to 0.4)	-0.0 (-0.5 to 0.5)
≥56	0.0 (Ref)	0.2 (-0.3 to 0.7)	0.5 (0.1 to 0.9)	0.5 (0.0 to 0.9)

Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Orange cells indicate p-value <0.0001, green cells indicate p-value <0.001, blue cells indicate p-value <0.05.

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Supplementary Table 5. *Differences (95% CI) in adjusted HDL-cholesterol levels (mmol/L) per age stratum, in reference to premenopausal participants*

Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally postmenopausal	Surgically post-menopausal
≤34	0.0 (Ref)	-0.0 (-0.0 to -0.0)	-0.0 (-0.1 to 0.0)	-0.0 (-0.2 to 0.2)
35	0.0 (Ref)	-0.1 (-0.1 to 0.0)	-0.1 (-0.2 to 0.0)	-0.0 (-0.7 to 0.6)
36	0.0 (Ref)	0.0 (-0.1 to 0.1)	-0.1 (-0.2 to 0.0)	0.1 (-0.2 to 0.4)
37	0.0 (Ref)	-0.0 (-0.1 to 0.0)	-0.1 (-0.2 to -0.0)	0.1 (-0.2 to 0.3)
38	0.0 (Ref)	0.0 (-0.1 to 0.1)	-0.1 (-0.2 to -0.1)	-0.1 (-0.4 to 0.2)
39	0.0 (Ref)	-0.0 (-0.1 to 0.1)	-0.1 (-0.2 to -0.1)	0.0 (-0.2 to 0.2)
40	0.0 (Ref)	0.0 (-0.0 to 0.1)	-0.0 (-0.1 to 0.0)	-0.1 (-0.4 to 0.1)
41	0.0 (Ref)	-0.0 (-0.1 to 0.0)	-0.1 (-0.2 to -0.0)	-0.1 (-0.3 to 0.1)
42	0.0 (Ref)	-0.0 (-0.1 to 0.0)	-0.1 (-0.1 to 0.0)	0.2 (0.0 to 0.3)
43	0.0 (Ref)	-0.0 (-0.1 to 0.0)	-0.0 (-0.1 to 0.0)	-0.1 (-0.2 to 0.1)
44	0.0 (Ref)	-0.0 (-0.0 to 0.0)	0.0 (-0.1 to 0.1)	-0.0 (-0.2 to 0.1)
45	0.0 (Ref)	0.0 (-0.0 to 0.1)	-0.0 (-0.1 to 0.0)	0.0 (-0.1 to 0.2)
46	0.0 (Ref)	0.0 (-0.0 to 0.0)	0.0 (-0.0 to 0.1)	-0.1 (-0.2 to 0.0)
47	0.0 (Ref)	0.0 (0.0 to 0.1)	0.0 (-0.0 to 0.1)	-0.0 (-0.2 to 0.1)
48	0.0 (Ref)	0.1 (0.0 to 0.1)	0.0 (-0.0 to 0.1)	-0.0 (-0.1 to 0.1)
49	0.0 (Ref)	0.0 (-0.0 to 0.1)	0.0 (-0.0 to 0.1)	-0.1 (-0.2 to 0.0)
50	0.0 (Ref)	0.0 (0.0 to 0.1)	0.0 (-0.0 to 0.1)	-0.1 (-0.2 to 0.0)
51	0.0 (Ref)	0.1 (0.0 to 0.1)	0.0 (-0.0 to 0.1)	-0.1 (-0.2 to 0.0)
52	0.0 (Ref)	0.0 (-0.1 to 0.1)	0.0 (-0.0 to 0.1)	-0.0 (-0.2 to 0.2)
53	0.0 (Ref)	0.0 (-0.1 to 0.1)	0.1 (-0.1 to 0.2)	0.1 (-0.1 to 0.2)
54	0.0 (Ref)	0.1 (-0.1 to 0.2)	0.1 (-0.0 to 0.2)	0.1 (-0.1 to 0.3)
55	0.0 (Ref)	-0.1 (-0.2 to 0.1)	0.0 (-0.1 to 0.2)	-0.0 (-0.3 to 0.2)
≥56	0.0 (Ref)	0.1 (-0.1 to 0.3)	0.1 (-0.1 to 0.3)	0.1 (-0.1 to 0.3)

Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Green cells indicate p-value <0.001, blue cells indicate p-value <0.05.

Supplementary Table 6. *Differences (95% CI) in adjusted glucose levels (mmol/L) per age stratum, in reference to premenopausal participants*

Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally post-menopausal	Surgically post-menopausal
≤34	0.0 (Ref)	-0.0 (-0.0 to 0.0)	0.1 (-0.0 to 0.1)	0.6 (0.3 to 0.9)
35	0.0 (Ref)	0.1 (-0.0 to 0.2)	0.0 (-0.2 to 0.2)	-0.4 (-1.6 to 0.8)
36	0.0 (Ref)	-0.1 (-0.2 to 0.1)	0.0 (-0.2 to 0.2)	-0.0 (-0.7 to 0.6)
37	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.0 (-0.1 to 0.1)	-0.2 (-0.5 to 0.1)
38	0.0 (Ref)	-0.0 (-0.1 to 0.0)	-0.0 (-0.2 to 0.1)	0.1 (-0.3 to 0.5)
39	0.0 (Ref)	-0.0 (-0.1 to 0.1)	0.0 (-0.1 to 0.2)	0.0 (-0.3 to 0.3)
40	0.0 (Ref)	0.0 (-0.1 to 0.1)	-0.0 (-0.2 to 0.1)	-0.3 (-0.8 to 0.2)
41	0.0 (Ref)	-0.0 (-0.1 to 0.1)	-0.0 (-0.1 to 0.1)	-0.0 (-0.5 to 0.4)
42	0.0 (Ref)	-0.0 (-0.1 to 0.0)	0.1 (-0.0 to 0.2)	-0.2 (-0.4 to 0.1)
43	0.0 (Ref)	0.1 (-0.0 to 0.1)	0.1 (-0.0 to 0.2)	-0.1 (-0.3 to 0.2)
44	0.0 (Ref)	0.0 (-0.1 to 0.1)	0.1 (-0.0 to 0.2)	-0.1 (-0.5 to 0.2)
45	0.0 (Ref)	-0.1 (-0.1 to 0.0)	0.0 (-0.1 to 0.1)	-0.1 (-0.4 to 0.2)
46	0.0 (Ref)	-0.0 (-0.1 to 0.0)	0.0 (-0.0 to 0.1)	-0.1 (-0.3 to 0.1)
47	0.0 (Ref)	0.0 (-0.0 to 0.1)	-0.1 (-0.2 to 0.0)	0.3 (0.1 to 0.5)
48	0.0 (Ref)	-0.0 (-0.1 to 0.0)	-0.1 (-0.1 to -0.0)	-0.1 (-0.2 to 0.1)
49	0.0 (Ref)	-0.0 (-0.1 to 0.1)	-0.0 (-0.1 to 0.1)	-0.1 (-0.3 to 0.0)
50	0.0 (Ref)	-0.0 (-0.1 to 0.0)	-0.0 (-0.1 to 0.0)	-0.1 (-0.3 to 0.1)
51	0.0 (Ref)	-0.1 (-0.2 to 0.0)	-0.1 (-0.1 to 0.0)	0.1 (-0.1 to 0.4)
52	0.0 (Ref)	-0.0 (-0.1 to 0.1)	-0.0 (-0.2 to 0.1)	-0.0 (-0.3 to 0.3)
53	0.0 (Ref)	-0.1 (-0.3 to 0.1)	0.0 (-0.2 to 0.2)	-0.1 (-0.5 to 0.2)
54	0.0 (Ref)	0.0 (-0.2 to 0.2)	-0.0 (-0.2 to 0.2)	-0.1 (-0.4 to 0.1)
55	0.0 (Ref)	0.1 (-0.3 to 0.4)	-0.1 (-0.3 to 0.3)	-0.1 (-0.5 to 0.3)
≥56	0.0 (Ref)	0.1 (-0.3 to 0.6)	0.1 (-0.3 to 0.4)	0.1 (-0.3 to 0.5)

Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Green cells indicate p-value <0.001, blue cells indicate p-value <0.05.

Supplementary Table 7. *Proportional differences (95% CI) in adjusted log triglyceride levels (mmol/L) per age stratum, in reference to premenopausal participants*

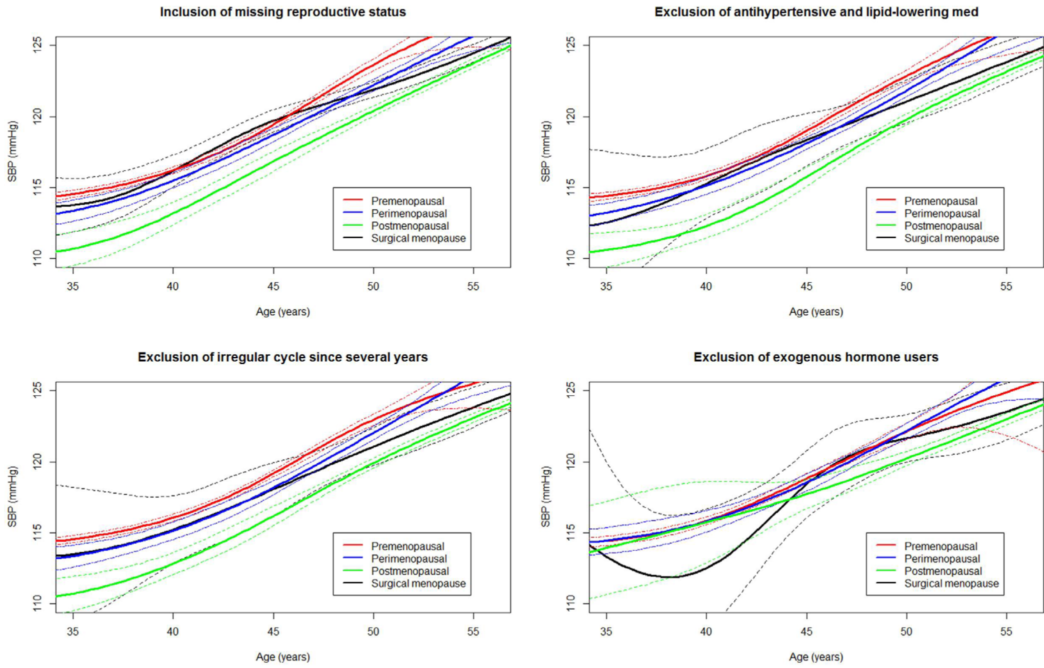
Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally post-menopausal	Surgically post-menopausal
≤34	1.0 (Ref)	0.9 (0.9 to 0.9)	0.8 (0.7 to 0.8)	1.0 (0.8 to 1.3)
35	1.0 (Ref)	0.9 (0.8 to 1.0)	0.8 (0.7 to 0.9)	1.5 (0.7 to 3.4)
36	1.0 (Ref)	0.9 (0.9 to 1.0)	0.8 (0.8 to 0.9)	1.0 (0.7 to 1.4)
37	1.0 (Ref)	1.0 (0.9 to 1.1)	0.8 (0.7 to 0.8)	1.4 (1.0 to 1.9)
38	1.0 (Ref)	0.9 (0.9 to 1.0)	0.7 (0.7 to 0.8)	0.9 (0.7 to 1.3)
39	1.0 (Ref)	1.0 (0.9 to 1.0)	0.9 (0.8 to 0.9)	1.0 (0.8 to 1.3)
40	1.0 (Ref)	1.0 (0.9 to 1.0)	0.8 (0.7 to 0.9)	1.3 (0.9 to 1.7)
41	1.0 (Ref)	1.0 (0.9 to 1.0)	0.9 (0.8 to 1.0)	1.1 (0.9 to 1.4)
42	1.0 (Ref)	1.0 (0.9 to 1.0)	0.9 (0.8 to 1.0)	1.1 (0.9 to 1.4)
43	1.0 (Ref)	1.0 (0.9 to 1.0)	0.9 (0.8 to 1.0)	1.1 (0.9 to 1.3)
44	1.0 (Ref)	1.0 (0.9 to 1.0)	0.9 (0.8 to 1.0)	1.1 (0.9 to 1.3)
45	1.0 (Ref)	1.0 (0.9 to 1.0)	0.9 (0.9 to 1.0)	1.1 (0.9 to 1.3)
46	1.0 (Ref)	1.0 (0.9 to 1.0)	0.9 (0.9 to 1.0)	1.1 (0.9 to 1.2)
47	1.0 (Ref)	1.0 (1.0 to 1.0)	1.0 (0.9 to 1.0)	1.1 (0.9 to 1.2)
48	1.0 (Ref)	1.0 (0.9 to 1.0)	1.0 (1.0 to 1.0)	1.1 (0.9 to 1.2)
49	1.0 (Ref)	0.9 (0.9 to 1.0)	1.0 (0.9 to 1.0)	1.1 (1.0 to 1.2)
50	1.0 (Ref)	1.0 (0.9 to 1.0)	1.0 (1.0 to 1.1)	1.1 (1.0 to 1.3)
51	1.0 (Ref)	0.9 (0.9 to 1.0)	1.0 (0.9 to 1.0)	1.1 (0.9 to 1.2)
52	1.0 (Ref)	1.1 (1.0 to 1.2)	1.0 (0.9 to 1.1)	1.2 (1.0 to 1.5)
53	1.0 (Ref)	0.8 (0.7 to 1.0)	0.9 (0.8 to 1.0)	0.9 (0.7 to 1.1)
54	1.0 (Ref)	1.0 (0.8 to 1.1)	1.0 (0.8 to 1.1)	1.0 (0.8 to 1.2)
55	1.0 (Ref)	1.0 (0.8 to 0.2)	0.9 (0.8 to 1.1)	1.0 (0.8 to 1.3)
≥56	1.0 (Ref)	0.98 (0.6 to 1.0)	0.8 (0.7 to 1.0)	0.8 (0.7 to 1.0)

Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Orange cells indicate p-value <0.0001, green cells indicate p-value <0.001, blue cells indicate p-value <0.05.

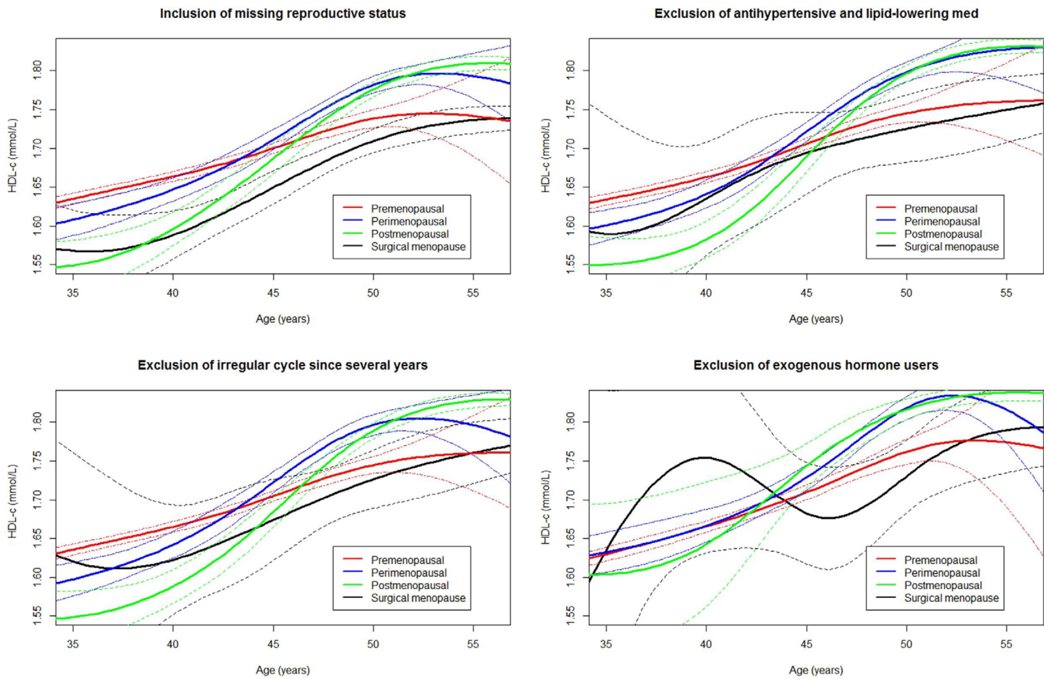
Supplementary Table 8. *Proportional differences (95% CI) in adjusted BMI levels (kg/m²) per age stratum, in reference to premenopausal participants*

Age (years)	Pre-menopausal (reference)	Perimenopausal	Naturally postmenopausal	Surgically post-menopausal
≤34	0.0 (Ref)	0.5 (0.3 to 0.8)	1.1 (0.6 to 1.6)	2.9 (0.3 to 5.5)
35	0.0 (Ref)	-0.1 (-1.0 to 0.8)	0.5 (-0.8 to 1.8)	1.9 (-7.1 to 10.9)
36	0.0 (Ref)	0.8 (-0.0 to 1.6)	0.3 (-0.8 to 1.5)	1.1 (-3.0 to 5.2)
37	0.0 (Ref)	0.4 (-0.4 to 1.3)	0.9 (-0.2 to 2.0)	3.7 (0.4 to 7.0)
38	0.0 (Ref)	0.2 (-0.6 to 1.0)	1.2 (-0.1 to 2.4)	0.7 (-3.2 to 4.5)
39	0.0 (Ref)	0.6 (-0.2 to 1.4)	-0.2 (-1.2 to 0.7)	3.7 (1.3 to 6.2)
40	0.0 (Ref)	0.5 (-0.2 to 1.2)	0.7 (-0.3 to 1.7)	7.8 (4.3 to 11.3)
41	0.0 (Ref)	0.4 (-0.2 to 1.1)	0.2 (-0.7 to 1.2)	0.4 (-2.4 to 3.2)
42	0.0 (Ref)	0.6 (0.0 to 1.3)	-0.1 (-1.0 to 0.7)	0.7 (-1.6 to 2.9)
43	0.0 (Ref)	0.5 (-0.0 to 1.0)	0.2 (-0.6 to 1.1)	1.2 (-0.8 to 3.2)
44	0.0 (Ref)	0.2 (-0.3 to 0.7)	0.4 (-0.4 to 1.2)	2.4 (0.3 to 4.5)
45	0.0 (Ref)	0.0 (-0.4 to 0.5)	0.5 (-0.3 to 1.3)	2.1 (0.3 to 0.9)
46	0.0 (Ref)	0.9 (0.4 to 1.3)	0.7 (0.1 to 1.4)	0.8 (-0.6 to 2.2)
47	0.0 (Ref)	0.5 (0.0 to 0.9)	0.2 (-0.4 to 0.8)	1.2 (-0.3 to 2.6)
48	0.0 (Ref)	0.0 (-0.4 to 0.4)	0.1 (-0.5 to 0.6)	1.2 (-0.2 to 2.6)
49	0.0 (Ref)	0.6 (0.1 to 1.0)	0.2 (-0.3 to 0.7)	1.0 (-0.1 to 2.2)
50	0.0 (Ref)	0.4 (-0.1 to 0.8)	0.0 (-0.4 to 0.5)	1.0 (-0.2 to 2.2)
51	0.0 (Ref)	0.1 (-0.5 to 0.6)	-0.0 (-0.6 to 0.5)	0.2 (-1.3 to 1.7)
52	0.0 (Ref)	0.7 (-0.3 to 1.6)	0.3 (-0.6 to 1.2)	2.2 (0.1 to 4.4)
53	0.0 (Ref)	0.3 (-1.0 to 1.6)	-0.1 (-1.3 to 1.1)	-0.1 (-2.2 to 2.0)
54	0.0 (Ref)	-0.2 (-1.6 to 1.2)	-0.2 (-1.5 to 1.1)	0.4 (-1.5 to 2.3)
55	0.0 (Ref)	0.6 (-1.4 to 2.5)	0.3 (-1.3 to 2.0)	1.1 (-1.3 to 3.4)
≥56	0.0 (Ref)	-1.4 (-3.6 to 0.7)	-1.2 (-3.1 to 0.6)	-0.5 (-2.4 to 1.5)

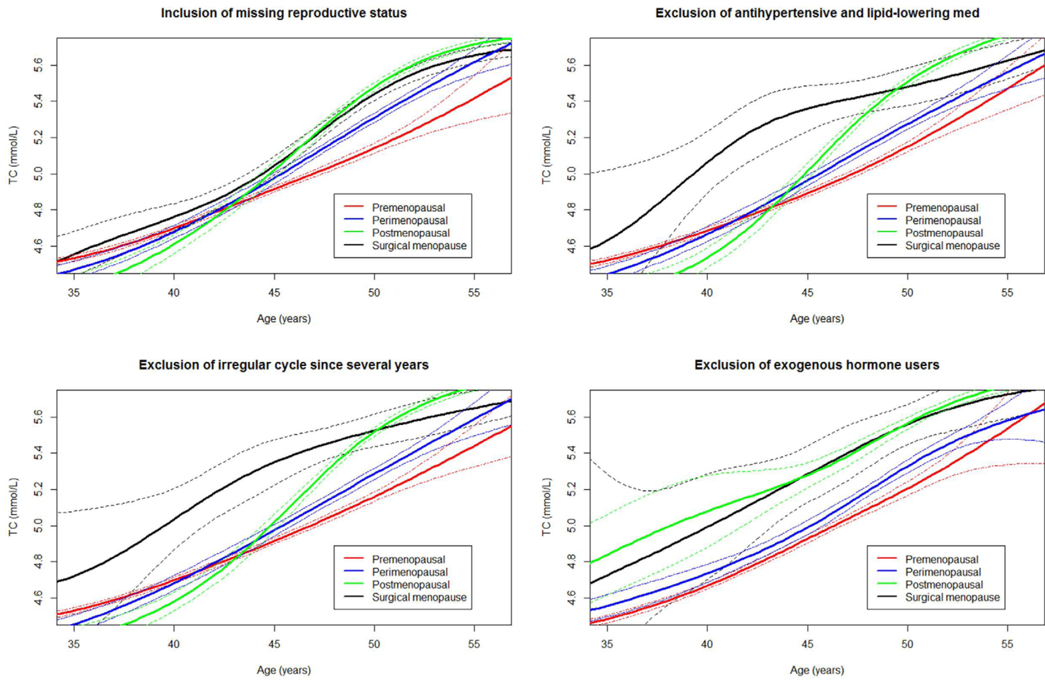
Values reflect coefficients of linear regression analyses in each age stratum, adjusted for OC use, smoking status and BMI. Premenopausal women are the reference category. Orange cells indicate p-value <0.0001, green cells indicate p-value <0.001, blue cells indicate p-value <0.05.



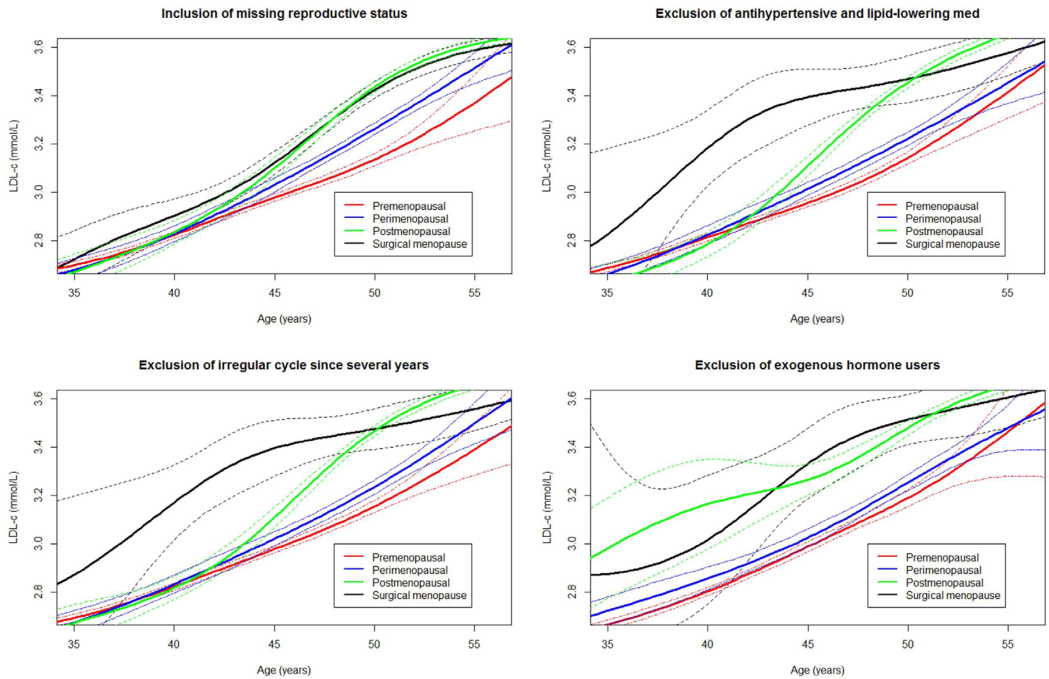
Supplementary Figure 1. *Associations of adjusted systolic blood pressure levels with age per menopausal status group with sensitivity analyses.*



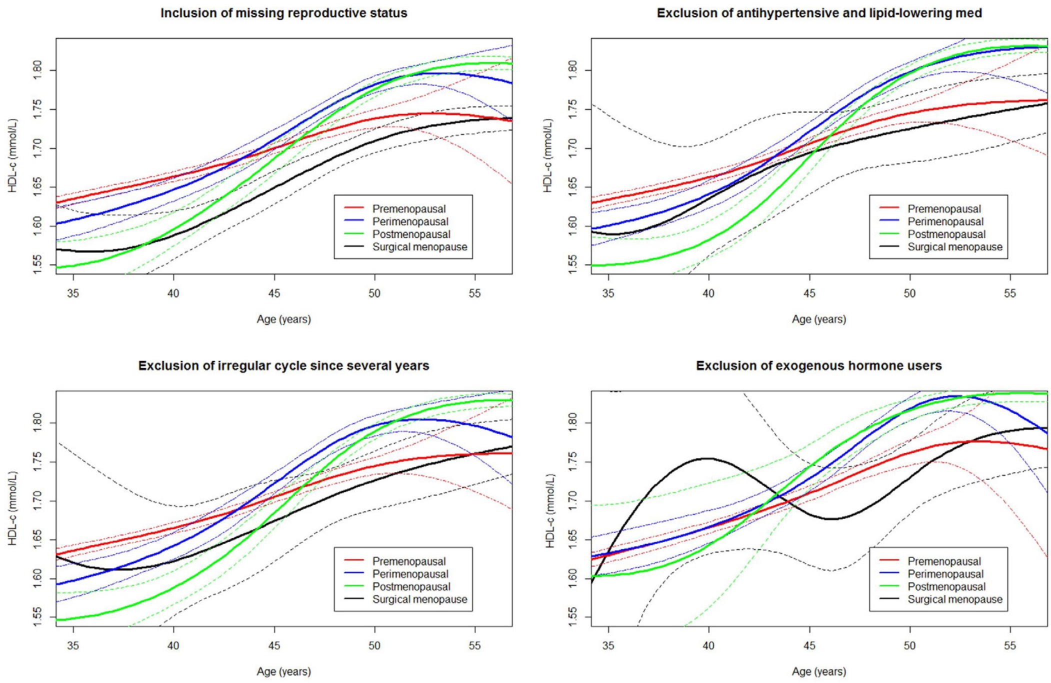
Supplementary Figure 2. *Associations of adjusted diastolic blood pressure levels with age per menopausal status group with sensitivity analyses.*



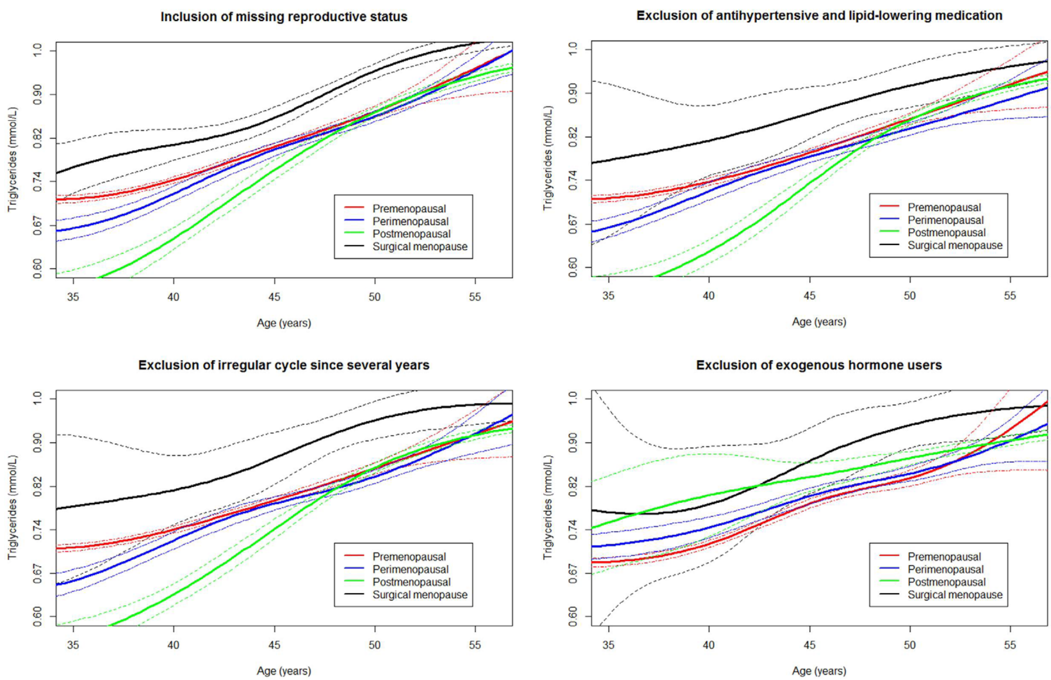
Supplementary Figure 3. *Associations of adjusted total cholesterol levels with age per menopausal status group with sensitivity analyses.*



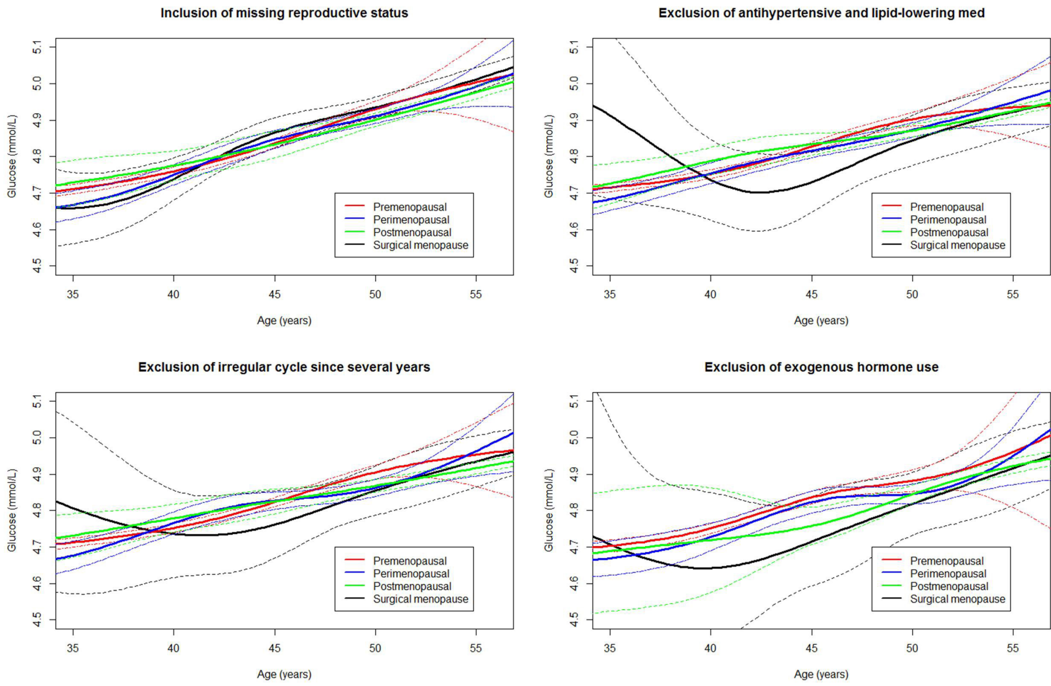
Supplementary Figure 4. *Associations of adjusted LDL-cholesterol levels with age per menopausal status group with sensitivity analyses.*



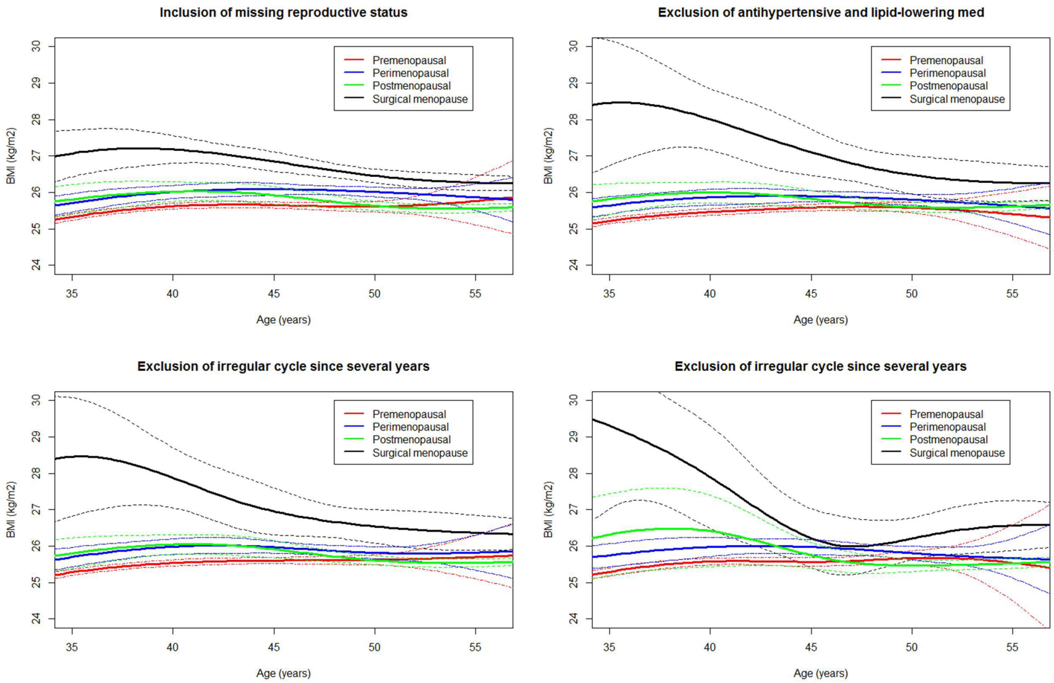
Supplementary Figure 5. *Associations of adjusted HDL-cholesterol levels with age per menopausal status group with sensitivity analyses.*



Supplementary Figure 6. *Associations of adjusted triglyceride levels with age per menopausal status group with sensitivity analyses.*



Supplementary Figure 7. *Associations of adjusted glucose levels with age per menopausal status group with sensitivity analyses.*



Supplementary Figure 8. *Associations of adjusted body mass index levels with age per menopausal status group with sensitivity analyses.*

6

Chapter 7

Chapter 7

Anti-Müllerian hormone in relation to cardio-metabolic health: a narrative review

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Abstract

The final hallmark of diminishing ovarian reserve is menopause, a state known to be inextricably linked to the deterioration of female cardiovascular health. The menopausal transition is associated with an increased risk of future cardiovascular morbidity and mortality, irrespective of chronological age. The aim of this narrative review is to identify studies investigating the association between Anti-Müllerian Hormone (AMH), a marker of ovarian reserve status, and factors of cardio-metabolic risk. Both for regularly cycling women and women with polycystic ovary syndrome (PCOS), current reports are conflicting and heterogeneous, with some indicating presence and others absence of a correlation between AMH and cardio-metabolic risk factors. The occurrence of hypertensive complications in pregnancy, known to increase the risk of later cardiovascular sequelae, is associated with reduced AMH levels in various study populations. Further research remains a prerequisite in order to further elucidate a possible common mechanism for ovarian and cardiovascular decline. More knowledge of the temporal or causal association between ovarian and cardiovascular decline may enable timely identification of women with increased risk of cardiovascular disease or early onset ovarian aging. Following this, AMH may in the future play a role beyond the scope of female reproduction.

Introduction

Menopause is the final hallmark of diminishing ovarian reserve. It has long been known that the process of ovarian aging and the deterioration of female cardiovascular health are inextricably linked. The menopausal transition has been shown to be associated with cardio-metabolic risk factors, such as a more unfavorable lipid status or glycemic control, irrespective of age (44). Results from the SWAN study support this notion, by demonstrating that the deterioration of lipid status is associated with the perimenopausal transition, rather than chronological aging (45). Arterial atherosclerosis furthermore increases with the onset of menopause, again independent of age (204-206). Large prospective studies have demonstrated a link between age at menopause and risk of morbidity and mortality due to cardiovascular events (41, 207-209), which was later confirmed in a meta-analysis (171). In addition, early menopause and a shorter reproductive lifespan are associated with an increased post-menopausal incidence of type 2 diabetes (210). These findings are indicative of a process influencing cardio-metabolic risk apart from chronological age, possibly resulting from a changing endocrine environment as a result of decreased ovarian function.

Quantification of the remainder of the ovarian reserve before the onset of menopause has received ample attention during recent years. Developments within this field primarily evolved from their use for assisted reproduction techniques (106). As a result, Anti-Müllerian Hormone (AMH) has recently emerged as the most promising single marker of ovarian reserve quantification. A recent review by Broer et al. aptly summarized current implications and limitations of (clinical) AMH use and analysis, describing AMH to be the best currently available determinant of ovarian reserve (22). AMH is produced by antral follicles in the ovaries (21, 211, 212), and has been shown to give a reliable estimation of the remaining size of the primordial follicle pool (ovarian reserve) (25), and of age at menopause (18, 30). Applications of AMH as a biomarker include the prediction of ovarian response in assisted reproduction (22, 213) and use as a potential diagnostic test for the distinction of polycystic ovary syndrome (PCOS), replacing the ultrasound based criterion of Polycystic Ovarian Morphology, discussed in a recent meta-analysis (214). AMH can additionally play an important role in (the diagnosis of) ovarian insufficiency conditions, for example due to cancer treatment (22).

More knowledge on the exact nature of the relationship between ovarian and cardiovascular decline could enable clinicians to gain more insight into the consequences of early onset ovarian aging or cardiovascular disease. Thus, AMH might be able to play a role in the early identification of women with a higher risk for earlier and possibly more severe cardiovascular disease. Vice versa, early manifestations of cardiovascular disease could help identify women with limited ovarian reserve, and lead to the advice

of not unnecessarily prolonging their age of childbearing. In addition, it would provide an opportunity to further explore the pathophysiology of accelerated ovarian decline and its role in increased risk of cardiovascular disease. In this review, we discuss currently available literature in which the relationship of ovarian reserve with cardiovascular risk was addressed, by studying AMH in relation to factors of the metabolic syndrome or hypertensive disorders of pregnancy.

Relationship of AMH with metabolic parameters

The metabolic syndrome is a constellation of multiple cardiovascular risk factors, associated with increased arterial atherosclerosis and subsequent cardiovascular morbidity and mortality (215-218). These risk factors include dyslipidemia, elevated blood pressure, elevated plasma glucose (and/or decreased insulin sensitivity), a prothrombotic and/or proinflammatory state and increased waist circumference (218-220). In recent years, several studies have sought to study the relationship of ovarian reserve with the full metabolic syndrome (with the presence of 3 out of 5 items) or single metabolic risk factors. The results of these studies are summarized in Table 1. In a cross-sectional study of 951 healthy, regularly-cycling women, Bleil et al. (64) found a low AMH tertile to be significantly associated with lower low-density lipoprotein cholesterol (LDL-c), higher blood glucose level, higher waist circumference and hypertension. However, after adding body mass index (BMI) to the regression analyses, all associations became non-significant (64), identifying BMI as an important confounding factor. A recent longitudinal study with premenopausal participants reported women in the lowest baseline age-specific AMH quartile to exhibit a worsening lipid profile over a mean follow-up time of 12 years (i.e. increased TC and LDL-c levels, although interestingly also higher HDL-c levels) compared to women in the highest AMH quartile (61). However, there was no correction for confounders besides age and BMI, such as smoking status (61). In cross-sectional research conducted in a large cohort of adolescent females, AMH quintile was not associated with lipid and blood glucose levels and insulin resistance, nor with C-reactive protein (CRP) levels (63). This was supported by another study among adolescents (221), including subjects with a diagnosis of PCOS (5.3%). In this study, AMH was not related to any metabolic parameters (221). When researching the degree of insulin resistance or level of fasting insulin alone in relation to AMH, a significant correlation was found by two studies (222, 223), whereas another study was not able to confirm this correlation (224).

In summary, results regarding the relationship between AMH and factors for cardio-metabolic risk in healthy adults and adolescents are inconclusive, but overall do not strongly support the existence of such a relationship.

Relationship of AMH with metabolic parameters in women with PCOS

Further attention has been given to the association between AMH and metabolic indices in the context of the polycystic ovary syndrome (PCOS). PCOS is characterized by oligomenorrhea or amenorrhea, hyperandrogenism and/or polycystic ovaries in women with WHO class 2 anovulation (225). Women with PCOS, especially those with hyperandrogenism and/or obesity (226), more frequently have metabolic disturbances, making them more prone to developing the metabolic syndrome and type 2 diabetes (225). In these women, AMH has been described to be elevated (214), and therefore the relationship between AMH and cardio-metabolic risk factors may also differ compared to regularly cycling women.

For an overview of included studies, see Table 2. In an adult population with prior exclusion of women with decreased ovarian reserve (based on FSH and estrogen levels), AMH was significantly positively correlated to levels of cholesterol and insulin resistance, both in PCOS and control groups (227). In a different, large group of PCOS and non-PCOS patients undergoing IVF treatment, AMH was not correlated to insulin resistance or cholesterol and triglyceride levels (228).

In addition to the degree of insulin resistance being studied as a composite of the metabolic syndrome, several studies have assessed whether there is a direct relationship between AMH as a marker of ovarian reserve and indices of insulin resistance in patients with PCOS. Again, conflicting results were found: in some cases a significant relationship between AMH and insulin resistance was observed (227, 229, 230), whereas in others there was no direct correlation (231-234). An association or lack thereof appeared to be independent of the presence of PCOS when comparing to controls in some studies (224, 228, 231), suggesting that the relationship between ovarian reserve and insulin resistance may only be partially influenced by the metabolic and endocrine disorders encountered in PCOS patients. On the other hand, some of the included case-control studies (227, 230, 232, 233) pooled the results of all study subjects for the correlation of AMH with metabolic indices, which may have diluted any observed effect inherently related to the pathophysiology of PCOS. In all mentioned studies, AMH was shown to be elevated in women with PCOS compared to healthy controls (221, 222, 227, 228), in correspondence with other literature (214).

In conclusion, the comparison of AMH levels to parameters of cardio-metabolic risk becomes more complicated in the case of polycystic ovary syndrome, due to the more frequent occurrence of metabolic disturbances in this group of women, as well as increased AMH levels compared to regularly cycling women. Both a positive and

***Table 1.** Overview of included studies regarding the relationship between AMH and cardio-metabolic risk*

	Study design	Main results	Results indicative of relationship AMH and CV risk or metabolic disturbance?
AMH and cardio-metabolic risk – adult women			
Bleil et al., 2013 (64)	Cross-sectional Regularly cycling women (n=951)	Low AMH tertile associated with ↓HDL-c, ↑HOMA-IR, ↑BP, ↓TG, but not significant after correction for BMI	No
Tehrani et al., 2014 (61)	Cohort Regularly cycling women (n=755) 9-year follow-up	Lowest age-specific AMH quartile: ↑TC, ↑LDL-c, ↑HDL-c	Yes
Park et al., 2010 (222)	Cross-sectional Regularly cycling women (n=120)	Negative correlation between AMH and HOMA-IR	Yes
Van Dorp et al., 2013 (223)	Cohort Survivors of childhood cancer (n=191)	AMH inversely associated with BMI and high fasting insulin	Yes
Nardo et al., 2009 (224)	Cohort Women referred for IVF; with male factor, tubal factor or unexplained infertility (n=183)	AMH positively associated with HOMA-IR and insulin level, but not significant after correction for age	No
AMH and cardio-metabolic risk – adolescents			
Pinola et al., 2014 (221)	Cross-sectional Adolescents from birth cohort age 16 (n=400) (Follow-up at age 26)	No correlation between AMH levels and WHR, fasting plasma glucose, serum insulin, hsCRP, TC, HDL-c, LDL-c or TG	No
Anderson et al., 2013 (63)	Cross-sectional Adolescents from birth cohort (n=1308)	No correlation between AMH levels and insulin level, fasting plasma glucose, HDL-c, LDL-c, TG, or CRP	No

Table 2. *Overview of included studies regarding the relationship between AMH and cardio-metabolic risk in women with PCOS*

	Study design	Main results	Results indicative of relationship AMH and CV risk or metabolic disturbance?
AMH and cardio-metabolic risk – adults and adolescents with PCOS			
Skalba et al., 2011 (227)	Case-control Women with PCOS (n=87) and regularly cycling controls (n=50)	Significant inverse correlation of AMH with LDL-c, serum glucose, serum insulin and positive correlation with HDL-c for all study subjects	Yes
Cui et al., 2014 (228)	Case-control Women with PCOS (n=304) and infertile controls (n=1896)	AMH levels not related to HOMA-IR, TC, TG, LDL-c or HDL-c levels in both groups	No
Cengiz et al., 2014 (234)	Case-control Adolescents with PCOS (n=58) and adolescent controls (n=28)	AMH levels not correlated to insulin level or HOMA-IR, positive correlation with 2-h glucose after OGTT	No
Chen et al., 2008 (229)	Cohort Women with PCOS (n=99)	Negative association of AMH with BMI and HOMA-IR	Yes
Nardo et al., 2009 (224)	Cohort Women referred for IVF; PCOS (n=232)	AMH positively associated with HOMA-IR and insulin level, but not significant after correction for age	No
La Marca et al., 2004 (230)	Case-control Women with PCOS (n=14) and controls (n=15)	Positive correlation between HOMA-IR and AMH in all study subjects	Yes
Begawy et al., 2010 (231)	Case-control Women with PCOS (n=35) and controls (n=35)	No correlation between AMH level and insulin resistance in all study subjects	No
Caglar et al., 2013 (232)	Case-control Women with PCOS (n=34) and controls (n=21)	No correlation between AMH level and HOMA-IR in all study subjects	No
Tian et al., 2014 (233)	Case-control Women with PCOS (n=160) and controls (n=40)	No correlation between AMH and HOMA-IR	No

Table 3. *Overview of studies regarding the relationship between AMH and hypertensive disorders of pregnancy*

	Study design	**Main results**	**Results indicative of relationship AMH and CV risk or metabolic disturbance?**
	AMH and hypertensive pregnancy complications		
Yarde et al., 2014 (240)	Retrospective cohort Women with PE history (n=336) and normotensive controls (n=329)	AMH significantly lower in patients with history or PE	Yes
Birdir et al., 2014 (241)	Case-control Women with PE (n=50) and controls (n=100)	Mean AMH at 11-13 weeks gestation significantly higher in patients with PE, no difference in median levels	No
Shand et al., 2014 (242)	Cohort Pregnant women with known outcome >20 weeks gestation (n=331)	Low AMH (<p10) in first trimester associated with higher risk of hypertensive pregnancy complications	Yes
Tokmak et al., 2014 (243)	Case-control Women with PE (n=45) and controls (n=42)	AMH level lower in pregnant women with PE compared to controls	Yes

negative correlation between AMH and parameters of cardio-metabolic risk were shown among women with PCOS, impeding an overall interpretation. In addition, the design and sample size of the discussed studies varied considerably. Similar to the outcome of the studies presented in the previous section, results regarding the relationship of AMH and cardio-metabolic risk when including women with PCOS are inconsistent. Hence, it is not possible to draw any firm conclusions on the basis of these results.

AMH and hypertensive complications of pregnancy

Pregnancy poses a major challenge to the maternal vasculature, which can in some cases lead to pregnancy-induced hypertension or preeclampsia. A history of hypertensive pregnancy complications leads to a higher risk of later cardiovascular sequelae, such as hypertension, ischemic heart disease, and deaths due to cardiovascular causes

(235-239). Recently, the occurrence of hypertensive pregnancy complications has been studied in relation to AMH. For an overview of the concerning studies, see Table 3. Two retrospective cohort studies found median AMH levels measured on average 10 years after pregnancy or from 1st trimester of pregnancy serum samples, respectively to be significantly lower or similar in women with a preeclamptic or hypertensive pregnancy in comparison to women with an uncomplicated pregnancy (240, 241). In another retrospective cohort study, AMH measured in the first trimester of pregnancy in the lowest 10th percentile was significantly associated with the occurrence of subsequent gestational hypertension or preeclampsia (242). Lastly, when measuring AMH at the time of diagnosis of preeclampsia in a prospective study, AMH level was significantly lower in patients with preeclampsia compared to normotensive pregnant controls (243).

The relationship between AMH and the occurrence of preeclampsia or pregnancy-induced hypertension thus far mostly appears to be inverse, both at the time of diagnosis as well as years later. It is therefore possible to conclude that decreased ovarian reserve may increase the chance of gestational hypertensive complications and thus cardiovascular complications at a later stage, or vice versa.

Discussion

Despite inconsistent results presented in this review, the majority of the studies comparing components of the metabolic syndrome to ovarian reserve in healthy adults found a mostly inverse association between these factors to be present. This suggests that the process of ovarian aging and cardiovascular decline may be part of a simultaneous process occurring before the onset of the perimenopausal transition. In line with these findings, Verit et al. demonstrated that diminished ovarian reserve (determined by antral follicle count (AFC) and estrogen level) was associated with increased LDL-c level, triglyceride, and CRP levels, in addition to decreased insulin sensitivity and decreased HDL-c levels (62). This statement is further supported by the finding of lower AFC in women with type 2 diabetes compared to healthy controls over several age groups in a cross-sectional study (244). Ovarian volume measured by ultrasound was similarly reduced in women with metabolic syndrome compared to healthy controls, although similar FSH levels between the groups raise doubt on the implications for true differences in ovarian reserve status (245). Finally, in cynomolgus monkeys, baseline AMH was inversely associated with subsequent atherosclerosis development, providing longitudinal data in support of a relationship between ovarian and cardiovascular deterioration (246).

Due to the heterogeneous nature of the study designs, study populations and outcomes, it is not possible to draw a singular conclusion from the results described here. Moreover, because most studies are of a cross-sec-

tional nature, it is not possible to assess any kind of causality. In the studies among adolescents no association was found, possibly resulting from a mostly unaffected cardiovascular profile in all adolescents, including those with PCOS.

Interestingly, in settings comprised of groups of women with and without PCOS, the occurrence of PCOS did not appear to influence the relationship between AMH and indices of the metabolic syndrome or insulin resistance alone. The outcomes of the studies comparing the degree of insulin resistance as a single parameter to AMH were greatly divided. This might indicate the lack of any relationship between ovarian reserve and insulin resistance directly, or that other factors, besides BMI, are associated with both AMH level and insulin sensitivity. As described by Daan et al., the extent of hyperandrogenism appears to be of influence on cardio-metabolic risk in patients with PCOS (226). It can thus be hypothesized that this holds true for women without PCOS as well, suggesting that other hormone pathways (such as the adrenal axis) may play an additional role.

In the comparison between AMH and hypertensive disorders of pregnancy, the results seem somewhat more straightforward. Reduced ovarian reserve is shown to be paired with a greater incidence of hypertension or preeclampsia in pregnancy, and thus with a greater risk of subsequent cardiovascular disease.

In the study by Bleil et al. (64), BMI was shown to play an important role in attenuating the perceived association between cardio-metabolic risk factors and AMH levels. BMI is by its nature closely linked to parameters of the metabolic syndrome. In addition, AMH is described to be significantly decreased in obese premenopausal women compared to women of normal weight (247, 248), an effect that was confirmed in a group of women with PCOS(249). Other studies were not able to find a correlation between BMI and AMH levels, however (250-252). Indeed, the studies presented in this review both demonstrated a relationship between AMH and BMI, as well as the absence of one. The mechanism through which obesity may influence AMH levels has been suggested to take place on the level of the ovary directly. This is illustrated by an increase of apoptosis and altered gene expression in the mouse ovary after being fed a high-fat diet or exposure to increased lipids in utero (253, 254). It is thus plausible that BMI acts as a confounder in the relationship between cardiovascular risk and ovarian aging. Importantly, in all discussed studies the BMI of the participants was taken into account, and was rightly corrected for in the analyses.

A challenge of clinical usage of AMH lies, among other factors, in the assay technique, with several distinct available assay methods and the lack of an international guideline for AMH measurement (22). Despite recent developments, direct comparison of results from various assays and clinical interpretation of AMH still await improvement.

There are different theories that could explain a relationship between ovarian reserve and cardiovascular risk. One such theory is a changing endocrine environment

as a result of ovarian reserve decline and subsequent loss of cyclic ovarian function, exerting its effects on the vasculature and metabolic parameters, thus increasing the risk of (post-menopausal) cardiovascular disease. A different hypothesis ensued from a study by Kok et al., with a group of 695 women enrolled in the Framingham Heart Study (59). Premenopausal serum cholesterol levels were associated with age at menopause, suggesting that cardiovascular risk may dictate ovarian aging rather than the reverse (59). A possible pathophysiological basis for this process could be impaired vascularization of the ovaries, causing end-organ hypoxia, and leading to accelerated ovarian decline. Chemotherapy has been shown to significantly reduce ovarian blood flow with simultaneously diminishing AMH levels (255), supporting this idea. Another possible explanation for the simultaneous decline of ovarian and cardiovascular function could be increased somatic aging in general. In support of this notion, granulosa cells of women with diminished ovarian reserve (defined by elevated FSH levels) were shown to have shorter telomeres and reduced telomerase activity compared to women with normal ovarian reserve (256). In addition, gene loci associated with age at menopause are related to DNA repair, an important contributor to somatic aging (257, 258).

As stated earlier, an important limitation of the majority of the discussed studies is their cross-sectional design. Furthermore, the amount of available literature on this subject is scarce. In order to truly understand the relationship between ovarian reserve and cardiovascular risk, it is necessary to perform additional research. Preferably, an animal model of vascular and ovarian impairment should be used in order to establish the potential existence of such a relationship and its nature. This should then be confirmed by prospective studies in humans with long-term follow-up. Following this, more may be said on its possible temporal and causal nature.

In conclusion, there are subtle indications that the process of ovarian aging and cardiovascular decline may share a common mechanism, occurring before the onset of menopause. Further research remains a prerequisite in order to further elucidate this possible connection. In a clinical setting, more extensive knowledge regarding the pathophysiology of accelerated ovarian aging could potentially help prevent complications from cardiovascular disease, while vascular dysfunction markers may indicate accelerated ovarian decline in an earlier stage. It can then well be that in the future, biomarkers of ovarian reserve such as AMH will play a role beyond the scope of female reproduction.

7

Chapter 08

Chapter 8

The association of low ovarian reserve with cardiovascular disease risk: a cross-sectional population-based study

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Abstract

Study question Is there a relationship between AMH level and cardiovascular disease (CVD) risk in premenopausal women?

Summary answer There are indications that premenopausal women with very low ovarian reserve may have a more unfavorable CVD risk profile.

What is known already Age at menopause is frequently linked to cardiovascular disease (CVD) occurrence. Anti-Müllerian hormone (AMH) is produced by ovarian antral follicles and provides a measure of ovarian reserve before the end of the reproductive lifespan. Literature on whether AMH is related to CVD risk is still scarce and heterogeneous.

Study design, size, duration Cross-sectional study in the general population.

Participants/materials, setting, methods Cardiovascular disease risk was compared between 2,338 premenopausal women in different AMH level-categories, with adjustment for confounders. CVD risk was assessed through levels of single risk factors and a summed score of CVD risk factors.

Main results and the role of chance The relationship of AMH levels with CVD risk factor outcomes was non-linear. Women with AMH levels below 0.16 µg/L had 0.11 (95% CI 0.01;0.21) more metabolic risk factors compared to women with AMH levels ≥0.16 µg/L. There was no association of individual risk factor levels with AMH levels, besides a tendency towards lower total cholesterol levels of 0.11 mmol/L (95% CI -0.23;0.01) in women with AMH levels <0.002 µg/L compared to women with AMH levels ≥0.16 µg/L. Although non-significant, these effect sizes were larger in women below 40 years of age.

Limitations, reasons for caution Causality and temporality of the studied association cannot be addressed here. Moreover, the significance of the results of this exploratory study should be interpreted with caution due to the use of multiple statistical analyses.

Wider implications of the findings This population-based study supports previous findings that young premenopausal women in a late stage of their reproductive lifespan may have an increased CVD risk. It lays the groundwork for future research to focus on this group of women. Longitudinal studies with more sensitive AMH assays may furthermore help better understand the implications of these results.

Introduction

The female reproductive lifespan is characterized by a gradual decrease of follicle quantity and quality, ultimately leading to menopause (1). Anti-Müllerian hormone (AMH) is produced by ovarian antral follicles and its concentration in peripheral blood is a quantitative estimation of the size of the antral follicle pool, thereby providing a measure of ovarian reserve before the end of the reproductive lifespan (26). AMH has furthermore proven capable to predict individual time to menopause (18, 29, 33, 38).

Age at menopause and the postmenopausal state are considered to be risk factors for cardiovascular disease (CVD) occurrence, independently of chronological aging (41, 171, 209). A decrease of total cholesterol (TC) levels and relative weight were previously associated with a later age at menopause, suggesting a potential influence of CVD risk on ovarian aging (59). In addition, the finding of AMH-receptor-specific mRNA in the human heart (65) suggests a direct linkage between AMH and cardiovascular physiology. To date, it is still debated whether AMH, either as a proxy variable for ovarian reserve or through direct mechanistic effects, is related to risk factors of CVD. A report in non-human primates (60) and one study in humans (61) provide evidence for the presence of a relationship, while others do not (63, 64). The available studies used different outcomes for CVD risk, as well as varying selection criteria for their study populations, limiting their comparability. In addition, important confounders such as oral contraceptive (OC) use or smoking were dealt with differently, or not at all. With this study, we therefore aimed to provide a generalizable assessment of the association of AMH level with CVD risk, by investigating AMH levels in relation to CVD risk factors in a large population-based cohort of premenopausal women.

Materials and Methods

Study design

We performed a cross-sectional study within the second round of the Doetinchem Cohort Study (132). The determinant of the studied association was AMH level, with CVD risk factors as the outcome.

Study population

The study population consisted of women enrolled in the Doetinchem Cohort Study. The cohort and study design were previously described in detail by Verschuren et al. (132). The population-based cohort originated from an age- and gender stratified sample of individuals from municipal registers of Doetinchem, Amsterdam and Maastricht in 1987. A random fraction (n=7,769) of the Doetinchem sample was subsequently invited for fol-

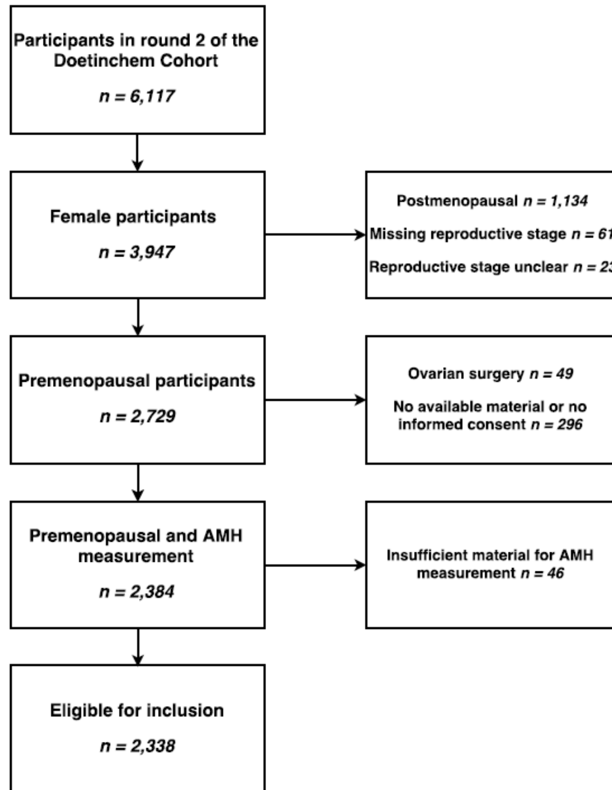


Figure 1. *Flow chart of participant selection*

low-up every 5 years, forming the Doetinchem Cohort, with the general aim of studying chronic disease risk factors (132). At each follow-up round, lifestyle determinants, reproductive characteristics and aspects of general health were assessed through questionnaires and biometric and laboratory measurements were performed. Written informed consent was given by all participants and ethical approval was granted by the Medical Ethics Committee of the Netherlands Organization of Applied Scientific Research.

For the current study, we included premenopausal women who participated in the second follow-up round between 1993-1997. Women were considered to be premenopausal if they reported having had 1 or more menstruations in the past year, the date of their reported last menstruation was less than 12 months ago, or if they were pregnant at the time of follow-up. Of the 3,947 eligible women, those who were postmenopausal or reported having undergone surgery of one or more ovaries were excluded (n=1,183). Women who did not give informed consent for the use of their stored material for research purposes or had no available stored serum were additionally excluded (n=296), as were women from whom information on the reproductive status was missing (n=61) or unclear (due to contradictory answers in the questionnaire)

(n=23). After exclusion of 46 participants from whom insufficient serum was available for AMH measurement, a population of 2,338 women was eligible for inclusion. Figure 1 provides a flow chart depiction of participant selection.

AMH assessment

Non-fasting blood withdrawal occurred on a random day of the menstrual cycle, after which additional material was stored for subsequent use at -80°C. AMH was measured in stored serum samples of the participants of round 2 in 2011 using a Gen-II ELISA assay (Beckman-Coulter, Sinsheim, Germany) in a single laboratory (133). The assay precision was validated with linearity-of-dilution assessment. The limit of detection was 0.08 µg/L and the limit of quantification 0.16 µg/L. The inter-assay and intra-assay coefficients of variation were 3.35% and 4.0%, respectively (133). Measures with values below 0.16 µg/mL were considered to represent AMH below the limit of quantification. AMH levels were below the limit of quantification in 637 (27%) women, and in 456 (72%) of these cases the measured level was zero.

Cardiovascular disease risk assessment

Participants' CVD risk was assessed with two approaches: 1) single risk factors: systolic blood pressure (SBP), diastolic blood pressure (DBP), TC, high-density lipoprotein cholesterol (HDL-c), and glucose levels; and 2) a summed score of adverse CVD risk factors.

SBP and DBP levels were measured twice in supine position on the left arm, using a random zero sphygmomanometer, from which the mean value was used. Height and weight was measured by trained staff, as well as waist and hip circumference. Laboratory measurements included non-fasting TC, HDL-c and glucose.

A summed risk factor score for CVD was estimated, henceforth referred to as "metabolic risk score", similar to the methods described by Bleil et al. (64). Risk factors were dichotomized for separate components as follows: waist circumference ≥80 cm (yes/no), hypertension (systolic or diastolic blood pressure ≥130 or ≥85 mm Hg) and/or use of antihypertensive medication (yes/no), HDL-c <1.1 mmol/L and/or use of lipid-lowering drugs (yes/no), TC >5.6 mmol/L and/or use of lipid-lowering drugs (yes/no), and non-fasting glucose >11.1 mmol/L and/or diabetes diagnosis (yes/no). The total number of present risk factors (0-5) was subsequently used as the metabolic risk score.

Assessment of potential confounders and effect modifiers

Potential confounders of the studied association were considered to be age, current OC use, current smoking status, body mass index (BMI), parity, cycle regularity, socio-economic status (SES), estrogen use at the time of follow-up and pregnancy at the time of

follow-up. All factors, with the exception of BMI and SES, were associated with AMH levels in a previous cross-sectional study in this population (133). A study from India found an association between AMH levels and SES (259), and obesity was previously associated with time to menopause (37). The factors described here were also hypothesized to be associated with CVD risk factors. The presence of polycystic ovary syndrome (PCOS) was considered to be a potential effect modifier. The likely presence of PCOS was identified by a reported irregular menstrual cycle in combination with a measured AMH level above 4.7 µg/L, based on a cut-off value proposed as a result of a meta-analysis by Iliodromiti *et al* (214).

Statistical analysis

In our study population, there were 2,182 (93%) complete cases and the proportion of missing data of all variables did not exceed 2% per variable. Conditional multiple imputation, including determinant, outcome and confounder variables, with 10 iterations was performed in order to account for missing data and a sensitivity analysis was performed with only the complete cases.

The association of AMH with age, CVD risk factors with age, and AMH with CVD risk factors was firstly visualized. The relationship of logarithmically transformed AMH with age appeared to be quadratic (Supplementary Figure 1); the relationship of the CVD risk factors with age appeared to be linear or quadratic (Supplementary Figure 2); and the relationship of AMH with CVD risk factors was non-linear in most cases (Supplementary Figure 3). For this reason, participants were divided into categories based on their AMH level, rather than studying AMH as a continuous parameter.

Participants were divided into the following categories based on their AMH levels as follows: AMH=0.000 µg/L (category 1; n=456); AMH levels measured above zero but beneath the quantification limit of 0.16 µg/L (category 2; n=186); quartiles of AMH equal to or above the quantification limit (category 3 to 6; n=424 in each quartile). AMH cut-off levels ranged from 0.161-0.643 µg/L, 0.644-1.336 µg/L, 1.337-2.395 µg/L and 2.398-13.67 µg/L for category 3-6, respectively. As the laboratory returned both AMH levels below the limit of quantification as well as values of zero, it was decided to distinguish these two groups as separate entities. However, this was done bearing in mind that the standard error of the measured AMH levels below 0.16 µg/L is larger than that of levels above this limit, rendering the former values less reliable. Supplementary Figure 3 depicts the AMH category cut-off values in the plots of AMH and CVD risk factor levels.

The association of AMH level categories with CVD risk factors was studied using an analysis of variance (ANOVA) regression based on the least sums of squares, with category 1 as the reference category. All women with AMH levels ≥0.16 µg/L (in

categories 3-6) were additionally pooled in a group and compared to women in category 1. The crude models included the abovementioned AMH categories as independent dummy variables. In model 2, age was added as a confounder. In model 3, age^2 was also added, due to a quadratic relationship of age with some of the outcome parameters. In model 4, current OC use (yes/no), current smoking status (smoker/non-smoker), parity, cycle regularity (regular/non-regular), current estrogen use besides OC (yes/no), current pregnancy (yes/no), SES and BMI were added as confounders.

For the single risk factors and metabolic risk score, regression model residuals were normally distributed. Homoscedasticity was assessed by plotting the model residuals against the fitted values. Multicollinearity was assessed with the use of variance inflation factors. In all models, the variance inflation factors of all variables were close to 1, with the exception of the AMH category variable, which approximated 2 in the presence of age. The Spearman rank correlation coefficient of the AMH categories with age was -0.67. As we aimed to assess the association of AMH with CVD risk factors independently of age, we included both variables in the analyses.

The regression analyses were performed for the group as a whole, as well as in separate age groups. A sensitivity analysis was performed by excluding potential women with PCOS from the analyses. The analyses were furthermore repeated with the exclusion of women with an amenorrhea of more than 3 months, in order to account for potential peri- or post-menopausal study participants.

All analyses were performed with SPSS for Windows Version 21 (SPSS INC., Chicago, IL) and R version 3.0.3. (http://www.r-project.org), with an α of 0.05.

Results

Baseline characteristics

The age range of all women in the study population was 20-57 years. Women in higher AMH categories were increasingly younger compared to women in lower AMH categories, but the overall age ranges were similar in all categories (Figure 2). BMI appeared to decrease across the six categories (Table 1). The number of current and ever smokers decreased with increasing AMH quartiles, as did the number of children per participant. No clear pattern over the AMH categories was observed for SES, diabetes prevalence and cycle regularity. Women with AMH <0.16 µg/L (in categories 1 and 2) had fewer current pregnancies and used OC less frequently than women with AMH levels ≥0.16 µg/L (categories 3-6).

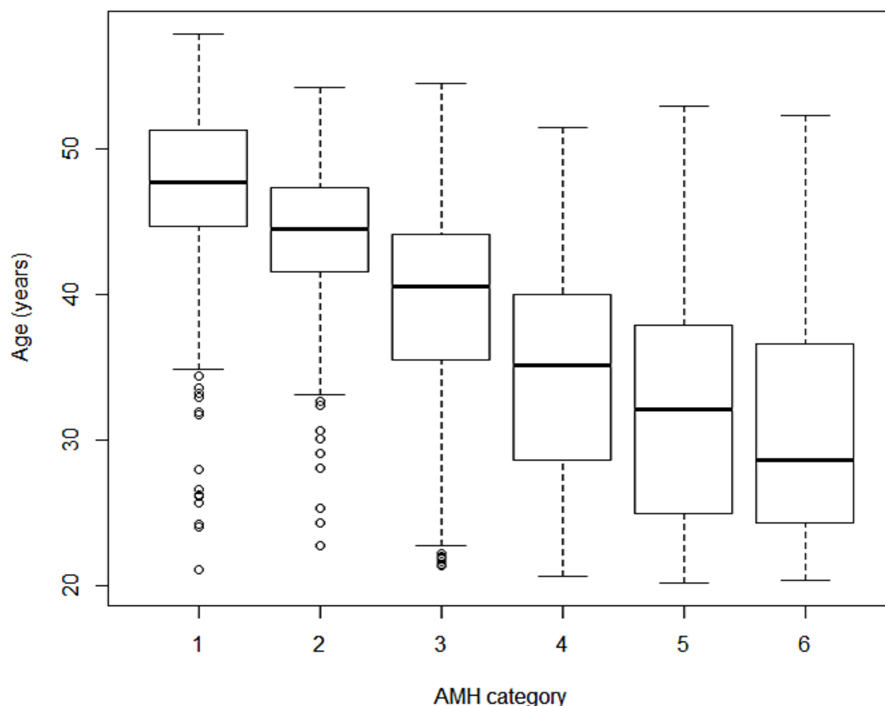


Figure 2. *Boxplots of age for each AMH category*

Multivariable analyses

In Tables 2 and 3 the multivariable model summaries of the regression analyses with all outcome parameters have been listed. For all outcomes, adjustment for age and age^2 led to the largest attenuation of the differences in outcome parameters between women in the six AMH categories. Mean adjusted SBP, DBP, TC and glucose levels and number of metabolic risk factors were mostly non-significantly lower in all AMH categories, compared to category 1. On average, TC levels were 0.17 mmol/L (95% CI -0.30;-0.04, p=0.01) lower in category 3, and 0.11 mmol/L (95% CI -0.23;0.01, p=0.08) lower in categories 3-6 compared to category 1. Women in categories 3-6 had an average lower metabolic risk score of 0.11 (95% CI -0.21;-0.01, p=0.02), thus 0.11 fewer metabolic risk factors, than women in category 1. This effect size was equal when all women with AMH levels of 0.16 μg/L and higher (categories 3-6) were compared to all women with values below this cut-off point (categories 1 and 2), with a p-value of 0.01.

When the multivariable regression analyses were repeated with stratification

Table 1. *Baseline and outcome characteristics for study participants per AMH category*

	Category 1 *n=456*	Category 2 *n=186*	Category 3 *n=424*	Category 4 *n=424*	Category 5 *n=424*	Category 6 *n=424*
AMH range (µg/L)	0.000	0.002-0.158	0.161-0.643	0.644-1.336	1.337-2.395	2.398-13.67
Baseline parameters						
Age (years)	47.3 ± 5.5	43.8 ± 5.5	39.2 ± 7.0	34.8 ± 7.4	31.7 ± 7.5	30.2 ± 6.9
Current smoker	149 (23.7)	72 (38.7)	129 (30.4)	146 (34.4)	125 (29.5)	125 (29.4)
Ever smoker	308 (67.5)	132 (71.0)	269 (63.4)	274 (64.6)	236 (55.7)	232 (54.7)
Pack years of smoking	9.4 ± 11	8.9 ± 9.9	6.6 ± 8.4	6.7 ± 8.7	4.0 ± 6.1	3.6 ± 5.9
BMI (kg/m²)	25.7 ± 3.8	24.7 ± 3.8	24.7 ± 4.0	24.6 ± 4.1	24.1 ± 3.7	24.0 ± 4.0
Number of children	1.9 ± 1.0	1.9 ± 1.0	1.7 ± 1.1	1.3 ± 1.2	1.1 ± 1.2	1.0 ± 1.2
SES 1†	292 (64.4)	113 (60.8)	139 (56.5)	226 (53.4)	181 (43.1)	176 (51.9)
SES 2†	91 (20.1)	44 (23.7)	107 (25.3)	135 (31.9)	172 (41.0)	196 (46.2)
SES 3†	70 (15.4)	29 (15.6)	77 (18.2)	62 (14.7)	67 (15.9)	52 (12.2)
Current OC use	110 (24.2)	56 (30.3)	150 (35.4)	185 (43.7)	217 (51.1)	196 (46.3)
Current E2 use‡	17 (0.7)	2 (0.1)	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
Regular cycle	360 (80.5)	148 (81.3)	338 (80.9)	336 (81.3)	316 (75.6)	281 (67.3)
Current pregnancy	1 (0.04)	3 (0.01)	14 (3.3)	14 (3.3)	10 (2.3)	7 (1.2)
Diabetes mellitus	3 (0.7)	1 (0.5)	1 (0.2)	1 (0.2)	2 (0.5)	3 (0.7)
Outcome parameters						
SBP (mm Hg)	122 [112;134]	119 [110;130]	115 [108;125]	114 [107;123]	114 [105;121]	112 [106;121]
DBP (mm Hg)	79 [72;85]	77 [71;83]	75 [69;82]	74 [68;81]	73 [67;79]	74 [68;80]
TC (mmol/L)	5.45 [4.89;6.05]	5.21 [4.65;5.78]	4.97 [4.44;5.60]	4.98 [4.43;5.60]	4.79 [4.34;5.38]	4.83 [4.31;5.43]
HDL-c (mmol/L)	1.52 [1.29;1.79]	1.52 [1.29;1.78]	1.55 [0.66;1.75]	1.45 [0.67;1.70]	1.51 [0.81;1.75]	1.50 [0.66;1.78]
Glucose (mmol/L)*	5.2 [4.8;5.6]	5.0 [4.6;5.5]	4.9 [4.6;5.4]	4.9 [4.5;5.2]	4.8 [4.4;5.2]	4.9 [4.4;5.2]
Metabolic risk factors	1 [0;2]	1 [0;1]	0 [0;1]	0 [0;1]	0 [0;1]	0 [0;1]

*Values are presented as n (%) for categorical variables and mean ± SD or median [IQR] for continuous variables. BMI = body mass index; SES = socio-economic status; OC = oral contraceptive; E2 = estrogen; SBP = systolic blood pressure; DBP = diastolic blood pressure; TC = total cholesterol; HDL-c = high-density lipid cholesterol. †SES 1 = Completed primary school or lowest level of secondary education; SES 2 = Completed middle level of secondary education or first three years of highest high school education level; SES 3 = Completed highest form of secondary school or any university degree; ‡Estrogen use besides OC; * Non-fasting*

Table 2. *Multivariable Model estimates of mean differences in Single risk factor levels for AMH categories, compared to Category 1*

	Cat. 1 *n=456*	**Cat. 2** *n=186*	**Cat. 3** *n=424*	**Cat. 4** *n=424*	**Cat. 5** *n=424*	**Cat. 6** *n=424*	**Cat. 3-6** *n=1,696*
AMH range (μg/L)	0.000	0.002-0.158	0.161-0.643	0.644-1.336	1.337-2.395	2.398-13.67	0.162-13.67
Difference (95% CI) with reference category in Systolic blood pressure (mm Hg)							
1. Crude model	Ref	**-3.8 (-6.2;-1.5)**	**-7.2 (-9.0;-5.4)**	**-8.3 (-10.1;-6.5)**	**-9.9 (-11.7;-8.1)**	**-10.8 (-12.6;-9.0)**	**-9.0 (-10.5;-7.6)**
2. + age	Ref	**-2.7 (-5.0;-0.4)**	**-4.6 (-6.5;-2.7)**	**-4.2 (-6.3;-2.2)**	**-4.8 (-7.0;-2.7)**	**-5.2 (-7.5;-3.0)**	**-4.6 (-6.3;-2.9)**
3. + age, age²	Ref	-0.9 (-3.2;1.5)	-1.8 (-3.9;0.2)	-1.2 (-3.4;1.0)	-2.3 (-4.5;0.0)	**-2.8 (-5.1;-0.4)**	-1.9 (-3.7;0.0)
4. Fully adjusted	Ref	-0.3 (-2.6;2.0)	-1.2 (-3.1;0.8)	-0.6 (-2.7;1.5)	-1.1 (-3.3;1.1)	-1.2 (-3.5;1.1)	-1.0 (-2.8;0.8)
Difference (95% CI) with reference category in Diastolic blood pressure (mm Hg)							
1. Crude model	Ref	**-1.9 (-3.6;-0.2)**	**-3.3 (-4.6;-2.0)**	**-4.7 (-6.0;-3.4)**	**-6.1 (-7.4;-4.8)**	**-5.2 (-6.5;-3.9)**	**-4.8 (-5.9;-3.8)**
2. + age	Ref	-1.0 (-2.7;0.7)	-1.3 (-2.7;0.1)	**-1.5 (-3.0;-0.0)**	**-2.2 (-3.8;-0.6)**	-0.9 (-2.5;0.7)	**-1.4 (-2.7;-0.2)**
3. + age, age²	Ref	-0.8 (-2.5;0.9)	-1.0 (-2.5;0.5)	-1.2 (-2.8;0.4)	**-1.9 (-3.6; -0.2)**	-0.6 (-2.3;1.1)	-1.1 (-2.5;0.2)
4. Fully adjusted	Ref	-0.3 (-2.0;1.3)	-0.6 (-2.1;0.8)	-0.9 (-2.4;0.7)	-1.5 (-3.1;0.2)	0.0 (-1.6;1.7)	-0.8 (-2.1;0.6)
Difference (95% CI) with reference category in Total cholesterol (mmol/L)							
1. Crude model	Ref	**-0.26 (-0.41; -0.11)**	**-0.47 (-0.59; -0.35)**	**-0.44 (-0.56; -0.33)**	**-0.60 (-0.71; -0.48)**	**-0.59 (-0.70; -0.47)**	**-0.52 (-0.62; -0.43)**
2. + age	Ref	**-0.17 (-0.32;- 0.02)**	**-0.28 (-0.40; -0.16)**	**-0.15 (-0.28; -0.01)**	**-0.23 (-0.37; -0.09)**	**-0.18 (-0.33; -0.04)**	**-0.22 (-0.33; -0.11)**
3. + age, age²	Ref	-0.12 (-0.27;0.03)	**-0.20 (-0.33; -0.07)**	-0.06 (-0.21;0.08)	**-0.16 (-0.30; -0.01)**	-0.11 (-0.27;0.04)	**-0.15 (-0.27; -0.03)**
4. Fully adjusted	Ref	-0.10 (-0.25;0.01)	**-0.17 (-0.30; -0.04)**	-0.03 (-0.17;0.10)	-0.10 (-0.24;0.01)	-0.03 (-0.18;0.13)	-0.11 (-0.23;0.01)

Table 2. *Continued*

	Cat. 1 *n=456*	Cat. 2 *n=186*	Cat. 3 *n=424*	Cat. 4 *n=424*	Cat. 5 *n=424*	Cat. 6 *n=424*	Cat. 3-6 *n=1,696*
AMH range (μg/L)	0.000	0.002-0.158	0.161-0.643	0.644-1.336	1.337-2.395	2.398-13.67	0.162-13.67
Difference (95% CI) with reference category in HDL-cholesterol (mmol/L)							
1. Crude model	Ref	-0.02 (-0.08;0.04)	-0.01 (-0.06;0.04)	**-0.08 (-0.13; -0.04)**	-0.03 (-0.08;0.01)	-0.02 (-0.06;0.03)	-0.04 (-0.08;0.04)
2. + age	Ref	-0.01 (-0.08;0.05)	-0.00 (-0.05;0.05)	**-0.07 (-0.13; -0.02)**	-0.02 (-0.08;0.04)	0.00 (-0.06; 0.06)	-0.02 (-0.07;0.02)
3. + age, age²	Ref	-0.00 (-0.06; 0.06)	0.02 (-0.04;0.07)	-0.05 (-1.11;0.01)	0.00(- 0.06;0.06)	0.02 (-0.05;0.08)	-0.00 (-0.05;0.04)
4. Fully adjusted	Ref	-0.01 (-0.07;0.05)	0.00 (-0.05;0.05)	-0.05 (-0.11;0.01)	-0.01 (-0.07;0.05)	0.02 (-0.04;0.01)	-0.01 (-0.06;0.04)
Difference (95% CI) with reference category in Glucose (mmol/L)							
1. Crude model	Ref	-0.11 (-0.28;0.06)	**-0.26 (-0.39; -0.13)**	**-0.28 (-0.41; -0.15)**	**-0.44 (-0.57; -0.31)**	**-0.41 (-0.54; -0.28)**	**-0.35 (-0.45; -0.25)**
2. + age	Ref	-0.04 (-0.21;0.13)	-0.10 (-0.24;0.04)	-0.03 (-0.18;0.11)	-0.13 (-0.29;0.03)	-0.07 (-0.24;0.09)	-0.08 (-0.21;0.04)
3. + age, age²	Ref	-0.03 (-0.20;0.15)	-0.08 (-0.23;0.07)	-0.01 (-0.17;0.15)	-0.11 (-0.28;0.06)	-0.05 (-0.23;0.12)	-0.07 (-0.20;0.07)
4. Fully adjusted	Ref	-0.01 (-0.19;0.16)	-0.07 (-0.21;0.08)	-0.00 (-0.16;0.16)	-0.08 (-0.25; -0.08)	-0.01 (-0.18;0.16)	-0.05 (-0.18;0.09)

Estimated model coefficients (95% CI) indicate average difference of single risk factor levels in the respective AMH categories compared to women in category 1. (For example: women in category 3 had an average lower TC level of 0.17 mmol/L compared to women in category 1 after correction for confounders). Coefficients in bold are statistically significant ($p<0.05$). Model 1 was a crude model; Model 2 was adjusted for age; Model 3 was adjusted for age and age²; Model 4 was adjusted for age, age², current OC use, current smoking status, parity, cycle regularity, current estrogen use besides OC, current pregnancy and BMI.

Table 3. *Multivariable Model estimates of mean differences in Metabolic risk score for AMH categories, compared to Category 1*

	Cat. 1 *n=456*	**Cat. 2** *n=186*	**Cat. 3** *n=424*	**Cat. 4** *n=424*	**Cat. 5** *n=424*	**Cat. 6** *n=424*	**Cat. 3-6** *n=1,696*
AMH range (µg/L)	0.000	0.002-0.158	0.161-0.643	0.644-1.336	1.337-2.395	2.398-13.67	0.162-13.67
Difference (95% CI) with reference category in Metabolic risk score							
1. Crude model	Ref	**-0.19 (-0.33; -0.04)**	**-0.40 (-0.51; -0.29)**	**-0.37 (-0.49; -0.26)**	**-0.51 (-0.62; -0.40)**	**-0.53 (-0.64; -0.42)**	**-0.46 (-0.54; -0.37)**
2. + age	Ref	-0.11 (-0.25;0.03)	**-0.23 (-0.35; -0.11)**	-0.11 (-0.24;0.02)	**-0.18 (-0.32; -0.05)**	**-0.17 (-0.31; -0.03)**	**-0.18 (-0.29; -0.08)**
3. + age, age²	Ref	-0.09 (-0.24;0.05)	**-0.20 (-0.33; -0.07)**	-0.07 (-0.22;0.06)	**-0.15 (-0.30; -0.01)**	-0.15 (-0.30;0.00)	**-0.16 (-0.27; -0.04)**
4. Fully adjusted	Ref	-0.01 (-0.13;0.11)	**-0.15 (-0.26; -0.04)**	-0.05 (-0.17;0.06)	-0.09 (-0.21;0.03)	-0.08 (-0.21;0.04)	**-0.11 (-0.21; -0.01)**

Estimated model coefficients (95% CI) indicate average difference in metabolic risk score in the respective AMH categories compared to women in category 1. (For example: women in category 3 had an average 0.15 lower metabolic risk score than women in category 1 after correction for confounders). Coefficients in bold are statistically significant ($p<0.05$). Model 1 was a crude model; Model 2 was adjusted for age; Model 3 was adjusted for age and age^2; Model 4 was adjusted for age, age^2, current OC use, current smoking status, parity, cycle regularity, current estrogen use besides OC, current pregnancy and BMI.

Table 4. *Multivariable model estimates of mean differences of all outcome parameters for AMH categories, stratified by age decades.*

	Cat. 1	Cat. 2	Cat. 3	Cat. 4	Cat. 5	Cat. 6	Cat. 3-6
AMH range (µg/L)	0.000	0.002-0.158	0.161-0.643	0.644-1.336	1.337-2.395	2.398-13.67	0.162-13.67
Ages 20-29 (n=609), 2% AMH levels below 0.16 µg/L (category 1 & 2)							
Systolic blood pressure	Ref	-6.2 (-18.4;6.1)	-0.4 (-8.5;7.8)	-0.8 (-8.6;7.0)	-0.1 (-7.9;7.6)	-2.5 (-1.0;5.2)	-1.1 (-8.8;6.5)
Diastolic blood pressure	Ref	-5.1 (-14.5;4.4)	-2.9 (-9.1;3.4)	-4.6 (-10.6;1.4)	-5.1(-11.0;0.89)	-3.7 (-9.7;2.2)	-4.3 (-10.2;1.6)
Total cho-lesterol	Ref	0.06 (-0.83; 0.93)	-0.38 (-0.96; 0.21)	-0.09 (-0.65; 0.74)	-0.23 (-0.78; 0.33)	-0.15 (-0.71; 0.40)	-0.18 (-0.73; 0.37)
HDL-cho-lesterol	Ref	0.22 (-0.16; 0.60)	0.06 (-0.20; 0.31)	0.02 (-0.23; 0.26)	0.02 (-0.22; 0.26)	0.02 (-0.22; 0.26)	0.02 (-0.22; 0.26)
Glucose	Ref	-0.12 (-0.93; 0.69)	-0.01 (-0.55; 0.52)	-0.13 (-0.64; 0.39)	-0.19 (-0.70; 0.32)	-0.23 (-0.74; 0.28)	-0.17 (-0.68; 0.33)
Metabolic risk factors	Ref	-0.3 (-0.9;0.3)	-0.4 (-0.8; 0.1)	-0.3 (-0.7;0.1)	-0.3 (-0.7;0.1)	-0.2 (-0.6;0.2)	-0.3 (-0.7;0.1)
Ages 30-39 (n=742), 9% AMH levels below 0.16 µg/L (category 1 & 2)							
Systolic blood pressure	Ref	0.6 (-5.2; 6.4)	-0.5 (-5.2;4.1)	-0.3 (-4.8;4.3)	-2.3 (-7.0;2.3)	0.4 (-4.2;5.1)	-0.7 (-5.0;3.7)
Diastolic blood pressure	Ref	0.8 (-3.7;5.3)	-0.3 (-3.9;3.3)	0.8 (-2.7;4.3)	-0.0 (-3.6;3.6)	2.0 (-1.6;5.6)	0.6 (-2.7;4.0)
Total cho-lesterol	Ref	-0.33 (-0.76; 0.10)	-0.32 (-0.66; 0.03)	-0.25 (-0.59; 0.09)	-0.19 (-0.53; 0.16)	-0.07 (-0.42; 0.27)	-0.21 (-0.53; 0.11)
HDL-cho-lesterol	Ref	0.05 (-0.12; 0.23)	0.13 (-0.01; 0.27)	0.01 (-0.12; 0.15)	0.08 (-0.06; 0.22)	**0.16 (0.02; 0.30)**	0.09 (-0.04; 0.22)
Glucose	Ref	0.07(-0.51;0.64)	0.10(-0.36;0.57)	0.21(-0.24;0.67)	0.10(-0.36;0.56)	0.27(-0.20;0.74)	0.17(-0.26;0.60)
Metabolic risk factors	Ref	-0.1 (-0.4;0.2)	-0.2 (-0.5;0.1)	-0.0 (-0.3;0.2)	-0.1 (-0.4;0.1)	-0.2 (-0.4;0.1)	-0.1 (-0.4;0.1)

Table 4. *Continued*

	Cat. 1	Cat. 2	Cat. 3	Cat. 4	Cat. 5	Cat. 6	Cat. 3-6
AMH range (μg/L)	0.000	0.002-0.158	0.161-0.643	0.644-1.336	1.337-2.395	2.398-13.67	0.162-13.67
Ages 40-49 (n=830), 51% AMH levels below 0.16 μg/L (category 1 & 2)							
Systolic blood pressure	Ref	0.9 (-2.1;3.8)	-0.7 (-3.4;2.0)	0.9 (-2.5;4.2)	0.5 (-3.7;4.8)	-2.1 (-7.5;3.4)	-0.2 (-2.7;2.2)
Diastolic blood pressure	Ref	0.1 (-2.0;2.2)	0.0 (-1.9;1.9)	-0.6 (-3.0;1.8)	-1.3 (-3.0;1.8)	-1.9 (-5.8;1.9)	-0.4 (-2.1;1.3)
Total cholesterol	Ref	-0.08(-0.26;0.10)	-0.15(-0.31;0.02)	0.08(-0.12;0.29)	-0.22(-0.48;0.04)	-0.14(-0.47;0.19)	-0.09(-0.24;0.05)
HDL-cholesterol	Ref	-0.00(-0.08;0.07)	-0.02(-0.09;0.04)	-0.03(-0.11;0.05)	0.03(-0.08;0.13)	0.04(-0.09;0.18)	-0.01(-0.07;0.05)
Glucose	Ref	-0.01(-0.20;0.18)	-0.09(-0.26;0.08)	-0.05(-0.27;0.16)	-0.12(-0.39;0.15)	0.08(-0.39;0.15)	-0.07(-0.22;0.08)
Metabolic risk factors	Ref	0.1 (-0.1;0.2)	-0.1 (-0.2;0.0)	0.0 (-0.1;0.2)	-0.1 (-0.3;0.1)	0.2 (-0.5;0.1)	-0.1 (-0.2;0.1)
Ages 50-59 (n=157), 91% AMH levels below 0.16 μg/L (category 1 & 2)							
Systolic blood pressure	Ref	-9.0 (-18.8;7.8)	-0.6(-13.5;12.4)	-8.6(-29.6;12.4)	-4.1(-27.8;19.7)	21.3(-12.2;54.9)	-0.9 (-10.8;9.0)
Diastolic blood pressure	Ref	-3.6 (-9.9;2.3)	-0.3 (-8.2;7.5)	-5.8 (-18.5;6.9)	1.3 (-13.1;15.7)	2.0(-18.3;22.4)	-1.0 (-7.0;5.0)
Total cholesterol	Ref	-0.23(-0.74;0.27)	0.12(-0.55;0.79)	-0.73(-1.80;0.36)	0.47(-0.75;1.69)	0.74(-1.00;2.47)	0.05(-0.46;0.56)
HDL-cholesterol	Ref	-0.13(-0.36;0.09)	-0.07(-0.36;0.22)	-0.46(-0.94;0.02)	-0.40(-0.95;0.14)	0.15(-0.61;0.92)	-0.18(-0.41;0.04)
Glucose	Ref	0.07(-0.62;0.77)	0.26(-0.66;1.18)	-0.00(-1.49;1.49)	-1.22(-2.90;0.47)	0.58(-2.90;0.47)	0.01(-0.70;0.71)
Metabolic risk factors	Ref	**-0.5 (-1.0;-0.0)**	-0.1 (-0.8;0.6)	-0.6 (-1.6;0.5)	0.7 (-4.9;1.9)	1.4 (-0.3;3.1)	0.1 (-0.4;0.6)

Estimated model coefficients (95% CI) indicate average difference of risk factor levels in the respective AMH categories compared to women in category 1. (For example: in women aged between 20-29 years, those in category 3 had an average (non-significant) lower TC level of 0.38 mmol/L compared to women in category 1 after correction for confounders). Coefficients in bold are statistically significant ($p<0.05$). Model 1 was a crude model; Model 2 was adjusted for age; Model 3 was adjusted for age and age^2; Model 4 was adjusted for age, age^2, current OC use, current smoking status, parity, cycle regularity, current estrogen use besides OC, current pregnancy and BMI.

in 10-year age groups (Table 4), the mean differences in risk factor levels between AMH categories were largely non-significant. The differences in mean number of metabolic risk factors appeared increasingly larger in women between 20-29, 30-39 and 40-49 years old, although none of these differences reached statistical significance, probably due to the lower power. In all women under 40 years old, women in category 3 had a lower TC level of 0.35 mmol/L (95% CI -0.64;-0.06) and 0.24 (95% CI -0.46;-0.02) fewer metabolic risk factors than women in category 1, which are larger differences than in the group as a whole. Of the 70 women under 40 years old with AMH levels below 0.16 µg/L, 51% was a current OC user. In all women aged 40 and higher, no differences were found for any of the outcome parameters between any of the AMH categories.

Sensitivity analyses

There were 46 (2%) participants with potential PCOS. Excluding these women from the analyses did not change the values or significance level of any model coefficients. After excluding 112 (5%) women with an amenorrhea of 3 or more months from the analyses, some model coefficients were somewhat attenuated, but there was no difference of effect direction or significance level (see supplementary Table 1 for the adjusted model summaries of this sensitivity analysis).

Discussion

In this cross-sectional study, women with AMH levels ≥0.16 µg/L had fewer metabolic risk factors than women with AMH levels of zero. No associations were found between single CVD risk factor levels and AMH levels, although a tendency was seen towards more unfavorable TC levels in women with AMH levels of zero. The observed effect was not linear, implying that higher premenopausal AMH levels werenot associated with a more favorable cardiovascular risk profile. Altogether, these results suggest that premenopausal women with very low ovarian reserve may have a more unfavorable CVD risk profile, compared to women with AMH levels above the quantification limit with the same age and reproductive profile.

Our results are in line with the findings from a study in 1,015 regularly cycling Iranian women, in which changes of TC and LDL-c over a follow-up time up to 12 years were more unfavorable for women in the lowest baseline age-specific AMH quartile compared to the highest quartile (61). Additionally, two cross-sectional studies found young, regularly cycling women with lower ovarian reserve, based on cut-off points of antral follicle counts or follicle-stimulating hormone levels, to have a more unfavorable lipid status than women with normal ovarian reserve (62, 260). The mean age in both these studies was below 40 years, likening these results to those observed in our population below 40

years of age. The unfavorable consequences of reproductive aging could thus be more evident in women in whom age-related changes are still less prominent. Alternatively, it is possible this is a group of women with a more extreme aging phenotype, illustrated by both quickened vascular and reproductive aging, but this currently remains conjecture.

In both an adolescent and regularly cycling adult study population, AMH levels were not related to cardio-metabolic risk factors after correction for confounders (63, 64). In the study by Bleil et al. (64), BMI appeared to be an important confounder or effect mediator, whereas this effect is not supported by our results (see Supplementary Table II for a comparison of multivariable regression models with and without BMI adjustment). This may be due to a more favorable BMI distribution in our population, as Bleil *et al* reported 28.9% of their participants to have a BMI of 30 kg/m^2 or more (64), where in our population this was 8.9%. In a large cross-sectional population of Chinese women aged between 21-64 years, AMH levels were inversely correlated with BMI and fasting glucose, but not with other CVD risk factors (146). As these studies all used AMH as a continuous outcome parameter, it is possible that subtle differences with low AMH were not detected.

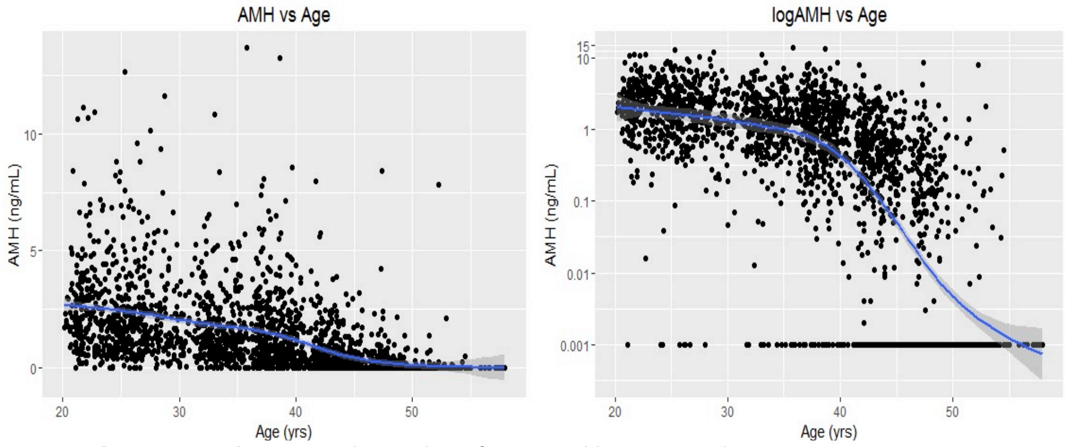
Recent research has suggested that endocrine changes during the menopausal transition are associated with CVD risk (49). Increases in LDL-c and TC were indeed previously found to be most substantial in the year surrounding the final menstrual period of 3,302 participants of the SWAN study (45), which may corroborate our findings with respect to the group of women with AMH levels of zero. However, as more than 80% of the study participants was still regularly cycling, in addition to the unaltered results after exclusion of women who had their last menstrual period more than 3 months prior, it is unlikely that our results merely represent the final year preceding menopause. A higher CVD risk in premenopausal women with AMH levels of zero may thus imply that as women reach the later stages of their reproductive lifespan, CVD risk increases. Vice versa, a higher CVD risk could potentially influence ovarian reserve, as suggested by a study in which a 1% increase in 10-year CVD risk was associated with a 1.8-year reduction of age at menopause (59). AMH could furthermore have an effect on CVD risk directly, through the regulation of vascular development for example (261). However, this cross-sectional epidemiological study does not allow to draw conclusions on whether AMH is a proxy variable for ovarian reserve or whether we observed direct mechanistic effects of AMH.

To our knowledge, we are the first to report a potential relation of undetectable AMH levels (i.e. 0.000 µg/L) with increased CVD risk. In the available studies where no relationship was found between AMH and cardio-metabolic risk factors (63, 64), there were no women with AMH levels <0.16 µg/L in the study population. In the case of the study by Anderson *et al*. this is very likely due to the adolescent study population, although only the mean age was provided, rather than additionally including an age range. In the study by Bleil *et al.* women were also younger overall, with a mean

age of 35 years. However, because women aged between 25-45 years were included and AMH levels were measured with the same assay this is still surprising, as 5.2% of our participants under 40 had undetectable AMH levels. The authors state that 27 women were excluded from their study population due to missing data on a primary variable of interest (64), which could potentially include undetectable AMH levels. Tehrani et al. (61) did find an effect on CVD risk factors for women in the lowest age-corrected AMH quartile and included women with AMH levels <0.16 μg/L in their analyses, which is interesting in the light of our findings. The potential relevance of low AMH levels was previously highlighted in a population of subfertile women, in which only AMH levels up to 1 μg/L predicted live birth rates (262). As the ability to detect very low AMH levels is increasing with more available sensitive AMH assays (122), it will become possible to better characterize the relationship between ovarian reserve and CVD risk in this group of women.

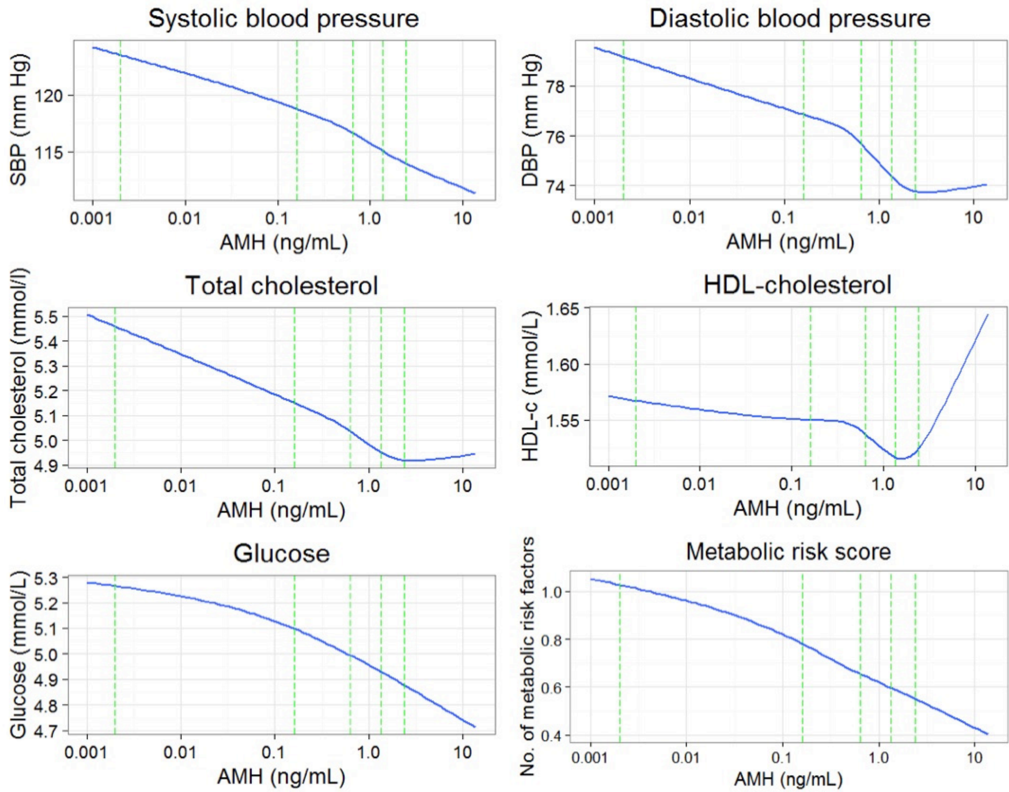
A limitation of our, and previous, studies is the difficulty of accurately estimating CVD risk. While we have attempted to provide a thorough representation of CVD risk here, it remains difficult to differentiate between the meaning of the various estimations. As we performed an exploratory analysis with multiple CVD risk factors, the interpretation of the significance and clinical relevance of our results must be done with caution. Moreover, considering the vast influence of age on both AMH levels and CVD risk, it is theoretically possible that the observed trend towards an association of AMH levels of zero with unfavorable CVD risk outcomes is still merely the consequence of chronological aging alongside the menopausal transition. By correcting for age both as a linear and quadratic term, we have circumvented this issue to the best of our ability. Another potential source of bias is the possible misclassification of premenopausal women due to the use of questionnaire information. In this case, the perceived unfavorable CVD risk in women with the lowest AMH levels could be a representation of post-menopausal status. However, as the exclusion of women with an amenorrhea with three months or more did not change the nature of the results, this seems less likely. As we furthermore expect the degree of recall bias to be comparable for all women in our study population, we do not think this greatly affected our results.

In summary, this is the largest population-based study to investigate the relationship between AMH and CVD risk in premenopausal women. Our results underline previous reports that premenopausal ovarian reserve may be inversely related to CVD risk, but suggest that this effect may primarily be present in women with AMH levels of zero. Future research with further focus on this group of women can help assess the significance and clinical relevance of the results presented here. Longitudinal research and use of more sensitive AMH-assays may then be the next step to fully understanding their implications.



Supplementary Figure 1. *Relationship of AMH and logAMH with age*

Supplementary Figure 2. *Relationship of cardiovascular risk outcomes with age*



Supplementary Figure 3. *Relationship between CVD risk factors and AMH. Dashed green lines represent boundaries of AMH categories.*

Supplementary Table 1. *Fully adjusted Model estimates of differences in all outcomes for AMH categories compared to Category 1, with the exclusion of women with an amenorrhea of ≥3 months*

	Cat. 1 *n=385*	Cat. 2 *n=178*	Cat. 3 *n=406*	Cat. 4 *n=402*	Cat. 5 *n=414*	Cat. 6 *n=411*	Cat. 3-6 *n=1633*
Difference (95% CI) with reference category							
SBP (mm Hg)	Ref	-0.4 (-2.7;2.0)	-1.3 (-3.3;0.8)	-0.7 (-2.9;1.5)	-1.4 (-3.7;0.8)	-1.6 (-4.0;0.7)	-1.2 (-3.0;0.7)
DBP (mm Hg)	Ref	-0.5 (-2.2;1.2)	-0.5 (-1.9;1.0)	-0.7 (-2.3;0.9)	-1.4 (-2.3;0.9)	0.1 (-1.6;0.8)	-0.6 (-2.0;0.7)
TC (mmol/L)	Ref	-0.10 (-0.26;0.05)	**-0.14 (-0.27;-0.00)**	0.02 (-0.13;0.16)	-0.08 (-0.22;0.08)	-0.00 (-0.16;0.16)	-0.07 (-0.20;0.05)
HDL-c (mmol/L)	Ref	-0.01 (-0.07;0.06)	0.02 (-0.04;0.07)	-0.03 (-0.09;0.02)	0.00 (-0.06;0.07)	0.03 (-0.03;0.09)	0.00 (-0.05;0.05)
Glucose (mmol/L)	Ref	-0.01 (-0.19;0.16)	-0.06 (-0.21;0.09)	0.01 (-0.15;0.17)	-0.08 (-0.25;0.09)	-0.02 (-0.19;0.16)	-0.04 (-0.18;0.10)
Metabolic risk factors	Ref	-0.02 (-0.14;0.11)	**-0.15 (-0.26;-0.04)**	-0.06 (-0.17;0.06)	-0.11 (-0.23;0.02)	-0.08 (-0.21;0.04)	**-0.11 (-0.21;-0.01)**

Estimated model coefficients indicate average difference in outcome parameters in the respective groups compared to women in AMH Category 1. Models were corrected for age, age2, current OC use, current smoking status, parity, cycle regularity, current estrogen use besides OC, current pregnancy and BMI. Coefficients in bold are statistically significant ($p<0.05$). SBP = systolic blood pressure; DBP = diastolic blood pressure; TC = total cholesterol; HDL-c = high-density lipid cholesterol.

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Supplementary Table 2. *Comparison of multivariable Model coefficients with and without adjustment for BMI*

	Cat. 1 *n=456*	Cat. 2 *n=186*	Cat. 3 *n=424*	Cat. 4 *n=424*	Cat. 5 *n=424*	Cat. 6 *n=424*	Cat.3-6 *n=424*
Difference (95% CI) with reference category in Systolic blood pressure (mm Hg)							
1. Without BMI†	Ref	-0.7 (-3.1;1.6)	-1.4 (-3.4;0.6)	-0.7 (-2.9;1.5)	-1.4 (-3.7;0.8)	-1.6 (-4.0;0.8)	-1.3 (-3.1;0.6)
2. Fully adjusted†	Ref	-0.3 (-2.6;2.0)	-1.2 (-3.1;0.8)	-0.6 (-2.7;1.5)	-1.1 (-3.3;1.1)	-1.2 (-3.5;1.1)	-1.0 (-2.8;0.8)
Difference (95% CI) with reference category in Diastolic blood pressure (mm Hg)							
1. Without BMI†	Ref	-0.7 (-2.4;1.0)	-0.8 (-2.3;0.6)	-1.0 (-2.6;0.6)	**-1.7 (-3.4;-0.0)**	-0.2 (-2.0;1.5)	-1.0 (-2.3;0.4)
2. Fully adjusted†	Ref	-0.3 (-2.0;1.3)	-0.6 (-2.1;0.8)	-0.9 (-2.4;0.7)	-1.5 (-3.1;0.2)	0.0 (-1.6;1.7)	-0.8 (-2.1;0.6)
Difference (95% CI) with reference category in Total cholesterol (mmol/L)							
1. Without BMI†	Ref	-0.12 (-0.27;0.03)	**-0.18 (-0.31;-0.05)**	-0.03 (-0.17;0.11)	-0.10 (-0.25;0.04)	-0.03 (-0.19;0.12)	-0.12 (-0.24;0.00)
2. Fully adjusted†	Ref	-0.10 (-0.25;0.01)	**-0.17 (-0.30;-0.04)**	-0.03 (-0.17;0.10)	-0.10 (-0.24;0.01)	-0.03 (-0.18;0.13)	-0.11 (-0.23;0.01)
Difference (95% CI) with reference category in HDL-cholesterol (mmol/L)							
1. Without BMI†	Ref	-0.00 (-0.06;0.06)	0.01 (-0.04;0.07)	-0.05 (-0.10;0.01)	0.00 (-0.06;0.06)	0.03 (-0.04;0.09)	-0.00 (-0.05;0.05)
2. Fully adjusted†	Ref	-0.01 (-0.07;-0.05)	0.00 (-0.05;0.05)	-0.05 (-0.11;0.01)	-0.01 (-0.07;0.05)	0.02 (-0.04;0.01)	-0.01 (-0.06;0.04)
Difference (95% CI) with reference category in Glucose (mmol/L)							
1. Without BMI†	Ref	-0.03 (-0.21;0.14)	-0.08 (-0.23;0.07)	-0.01 (-0.17;0.15)	-0.10 (-0.27;0.07)	-0.03 (-0.20;0.15)	-0.06 (-0.20;0.08)
2. Fully adjusted†	Ref	-0.01 (-0.19;0.16)	-0.07 (-0.21;0.08)	-0.00 (-0.16;0.16)	-0.08 (-0.25;-0.08)	-0.01 (-0.18;0.16)	-0.05 (-0.18;0.09)
Difference (95% CI) with reference category in Metabolic risk factors							
1. Without BMI†	Ref	-0.09 (-0.24;0.05)	**-0.20 (-0.33;-0.07)**	-0.07 (-0.21;0.06)	-0.14 (-0.28;0.01)	-0.13 (-0.27;0.02)	**-0.15 (-0.27;-0.03)**
2. Fully adjusted†	Ref	-0.01 (-0.13;0.11)	**-0.15 (-0.26;-0.04)**	-0.05 (-0.17;0.06)	-0.09 (-0.21;0.03)	-0.08 (-0.21;0.04)	**-0.11 (-0.21;-0.01)**

Estimated model coefficients (95% CI) indicate average difference of outcome parameters in the respective AMH categories compared to women in category 1. Coefficients in bold are statistically significant (p<0.05).
†Model 1 was adjusted for age, age2,current OC use, current smoking status, parity, cycle regularity, current estrogen use besides OC and current pregnancy. Model 2 was additionally adjusted for BMI.

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Chapter 9

Chapter 9

Anti-Müllerian hormone trajectories are associated with cardiovascular disease in women: results from the Doetinchem Cohort Study

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Abstract

Background Earlier age at menopause is widely considered to be associated with an increased risk of cardiovascular disease. However, the underlying mechanisms of this relationship remain undetermined. There are indications that anti-Müllerian hormone (AMH), an ovarian reserve marker, plays a physiological role outside of the reproductive system. We therefore investigated whether longitudinal AMH decline trajectories are associated with an increased risk of cardiovascular disease (CVD) occurrence.

Methods This study included 3,108 female participants, aged between 20-60 years at baseline, of the population-based Doetinchem Cohort. Participants completed at least 1 of 5 consecutive quinquennial visits between 1987 and 2010, resulting in a total follow-up time of 20 years. AMH was measured in 8,507 stored plasma samples. Information on total CVD, stroke and coronary heart disease was obtained through hospital discharge registry linkage. The association of AMH trajectories with CVD was quantified with joint modeling, with adjustment for age, smoking, oral contraceptive use, body mass index, menopausal status, postmenopausal hormone therapy use, diastolic blood pressure, total cholesterol, high-density lipoprotein cholesterol and glucose levels.

Results By the end of follow-up, 8.2% of the women had suffered from CVD, 4.9% had suffered from CHD, and 2.6% had experienced a stroke. After adjustment, each ng/mL lower $_{log}$AMH *level* was associated with a 21% higher risk of CVD (HR 1.21 [95% CI 1.07 – 1.36]) and a 26% higher risk of CHD (hazard ratio (HR) 1.25 [95% CI 1.08 – 1.46]) . Each additional ng/mL/year *decrease* of $_{log}$AMH was associated with a significantly higher risk of CVD (HR 1.46 [95% CI 1.14 – 1.87]) and CHD (HR 1.56 [95% CI 1.15-2.12]). There was no association of AMH with stroke.

Conclusions These results indicate that AMH trajectories in women are independently associated with CVD risk. We therefore postulate that the decline of circulating AMH levels may be part of the pathophysiology of the increased cardiovascular risk of earlier menopause. The confirmation of this association and elucidation of its underlying mechanisms are needed to place these results in a clinical perspective.

Introduction

Age at menopause is widely considered to be associated with the risk of cardiovascular disease (CVD). Women with an earlier menopause have an increased risk of CVD compared to women with a later age at menopause, as summarized in a recent meta-analysis (42), and each postponed year of menopause decreases the risk of CVD mortality by 2% (41). Proposed mechanisms of this association include the sudden decrease of estrogen exposure with putative effects on vasculature and cardiac function (263, 264). Although this theory is often accepted as fact, the available evidence regarding the role of estrogens remains contradictory (52, 265, 266). It is therefore plausible that there are still other, unknown, factors contributing to the increased CVD risk associated with menopause.

The onset of menopause is dictated by the depletion of the ovarian oocyte pool, also known as ovarian reserve (1). The decline of ovarian reserve can be measured with proxy markers of ovarian reserve, such as anti-Müllerian hormone (AMH), which is produced by ovarian follicles in an early stage of their cyclic development (106). While AMH is mainly thought to play a local role in ovarian follicle recruitment and development (153, 154), it is also available in the peripheral circulation. The identification of the AMH receptor in tissues such as the neuronal system and lungs (267, 268) suggest a function of circulating AMH outside of the reproductive system. In particular, recent experimental and observational studies have identified AMH as a potential influencing factor of cardiovascular function or risk factors of CVD (65, 137, 240, 261, 269). To date, there are no studies that have investigated a direct link of AMH to clinical CVD occurrence. In this study, we therefore aimed to determine whether there is an independent relationship between longitudinal trajectories of AMH and clinically manifest CVD in women from the general population.

Methods

Study population

Data from female participants of the Doetinchem Cohort Study were included in the current analysis. Details of the Doetinchem Cohort Study have been described in detail elsewhere (132). In brief, the Doetinchem Cohort Study is an ongoing prospective cohort study in the Netherlands for which a sex- and age-stratified sample of participants (3,641 men and 4,128 women) was randomly recruited from the general population. The study commenced with the baseline visit (round 1) in 1987, after which participants were invited back for a follow-up visit every 5 years. At the time of the current study, data up until round 5 were available, resulting in a maximum follow-up time of 20 years. At each visit, participants answered an extensive questionnaire, anthropometric mea-

surements were taken and venous blood withdrawal occurred. Non-fasting blood was drawn, from which plasma and other fractions were stored for future use. All participants gave written informed consent and ethical approval was granted by the Medical Ethics Committee of the Netherlands Institution of Applied Scientific Research. Anti-Müllerian hormone levels were measured in all available stored plasma samples (details described below). Ethical approval for the AMH measurements was granted by the Ethical Committee for Biobank Studies of the University Medical Center Utrecht.

For the present study, all 4,128 participating women of the Doetinchem Cohort Study were eligible. We excluded women without available plasma samples (n=802). We additionally excluded women with no AMH measurement *before* the occurrence of a CVD outcome or censoring (n=211 for stroke, n=214 for CHD and n=218 for total CVD), leaving 3,115 women for analysis of stroke, 3,112 women for analysis of CHD and 3,108 women for analysis of total CVD.

Anti-Müllerian hormone measurement

Anti-Müllerian hormone levels were measured with in all available stored plasma samples with the picoAMH assay (AnshLAbs, Webster, Texas, USA), after approval from the Ethical Committee for Biobank Studies of the University Medical Center Utrecht. The details of these measurements were previously described by de Kat et al. (160). The plasma samples from round 1 were stored at –30 °C and the samples from rounds 2-5 were stored at –80 °C. Prior to the current study, the samples were thawed once for additional measurements and immediately refrozen. In March 2015, all the available samples of each participant were retrieved from storage and were shipped on dry ice to AnshLabs (Webster, Texas, USA), where they were temporarily stored at –20 °C until the analyses were performed. The inter- and intra-assay coefficient of variation were 4.4 and 3.9%, respectively. There was no indication of plate drift, as all CVs within plate columns and rows were below 5%. For the purpose of log-transformation, levels of AMH below the limit of detection of 1.8 pg/mL were censored at this level to avoid the use of null-values. The analytical range of the picoAMH assay is 3-11,000 pg/mL, with a clinically reportable range up to 280 ng/mL. The total number of available AMH measurements before the occurrence of total CVD or censoring was 8,507, with 2,431 (28.9%) samples below the limit of detection. At baseline, women were divided into four categories based on their age-standardized AMH level. This was done by use of the CG-LMS method, previously described by Dólleman and de Kat et al. (133, 160). A model was made of the nonlinear distribution of AMH with standardization for age, using smoothing splines for AMH and taking into account skewness, the median and coefficient of variation. This fitted model was then applied to predict in which quartile

the AMH level of a woman belonged, given her age. The women who were predicted to have an AMH level in the lower 25% of their age were grouped into the lowest age-specific AMH category, and so on. As a result of the differing baseline ages, the number of women in each age-specific AMH category was not equal.

Cardiovascular disease outcomes

Cause of death was ascertained through linkage with Statistics Netherlands, and morbidity data were collected through the Dutch Hospital Discharge Registry (270). In the current study, CVD endpoints included nonfatal and fatal occurrences of cerebrovascular events (stroke), coronary heart disease (CHD) and a combination of all manifestations of CVD (total CVD). Stroke comprised both hemorrhagic and ischemic stroke and was defined with the 9th (up to 1996) and 10th (from 1996 onwards) edition of the International Classification of Diseases (ICD-9 and ICD-10, respectively) as follows: 430-438 (ICD-9) and I60-I67; I69; G45 (ICD-10). Coronary heart disease was defined with ICD-9 codes 410-414; 427.5; 798.1; 798.2; 798.9 and ICD-10 codes I20-I25; I46; R96. Total CVD was defined with ICD-9 codes 410-414; 427.5; 428; 415.1; 443.9; 430-438; 440-442; 444; 798.1; 798.2; 798.9 and ICD-10 codes I20-I26; I46; R96; G45; I60-I67; I69; I70-I74; I50. Follow-up was complete until January 1, 2011.

Covariates

Other information that was taken into account in the current study for each follow-up round was menopausal status, smoking, oral contraceptive (OC) use, postmenopausal hormone therapy (HT) use, body mass index (BMI), systolic and diastolic blood pressure (SBP and DBP), total cholesterol (TC) levels, high-density lipoprotein cholesterol (HDL-c) and glucose levels. Menopausal status was assessed through information on current cycle regularity, date of the last menstrual period and reproductive surgery (for a more detailed description see de Kat et al. (160)). Information of current smoking status at each follow-up round was derived from the questionnaires, where a participant was regarded as a current smoker if they reported to smoke at least one cigarette per month. Current OC and HT users were identified by the question 'Are you currently using .. [OC or estrogens for climacterial symptoms]'? Body mass index was assessed using the standardized measurement of height and weight by trained staff. Systolic and diastolic blood pressure was measured twice in a seated position with a random zero sphygmomanometer (Hawksley and Sons, Lancing, UK) by trained staff. Non-fasting TC and HDL-c levels were measured in EDTA plasma (until 1998) or serum (from 1998 onwards) at the Lipid Reference Laboratory, using standardized enzymatic methods(270).

Missing data

At each round, there was a small proportion of missing information in the study population. For OC use, smoking, BMI, SBP, DBP, TC and HDL-c this was <0.1%. The proportion of missing information regarding HT use and nonfasting glucose was <5% per round (with the exception of round 1, where it was not included in the questionnaire or laboratory analyses). Missing cycle status information averaged 17% over all rounds, due to the inclusion of women with a hysterectomy and missing information of the last menstrual period in rounds 2 and 3. Missing data were imputed through multiple imputation using 10 iterations and predictive mean matching. Multiple imputation was performed in SPSS Statistics (IBM), version 21.

Joint modeling

First, we characterized decline trajectories of $_{log}$AMH with the use of a linear mixed model analysis, taking into account varying AMH levels and decline rates between individuals by adding a random intercept and slope, respectively for each individual (details described extensively by de Kat et al.(160)). In this way, a separate effect for an individual AMH level and slope at time point could be modeled. The -$_{log}$AMH was set as the outcome, in order to be able to give an interpretation of declining, rather than increasing, AMH levels. The time axis of the linear mixed effects models was set as the follow-up time in years (t=0 at the baseline visit), and baseline age was added as a covariate in the model. The follow-up time of a participant either ended at the time of the event or diagnosis of CVD, CHD or stroke, or at the time of leaving the study (including censoring at the last follow-up visit). Natural splines were added to the time variable in order to take into account the individual non-linear decline of AMH (160). Models were subsequently adjusted for the following time-varying covariates: current OC use, current smoking, menopausal status (pre- or postmenopausal based on cycle status), BMI, DBP, TC, HDL-c, glucose, HT use, and use of blood pressure- or lipid-lowering medication. Diastolic blood pressure was chosen to represent blood pressure, as it was normally distributed where SBP was slightly right-skewed, even after logarithmic transformation. Models were not adjusted for both SBP and DBP due to the strong, linear relationship (R = 0.74) between the two variables in each participant. HDL-cholesterol was log-transformed in order to achieve a normal distribution. Linear mixed model analyses were performed in R (http://www.R-project.org), using the 'lme4' library.

In order to relate the longitudinal decline trajectories of AMH to the CVD outcomes, the linear mixed effects models were combined with a Cox proportional hazards model with a Weibull baseline hazard distribution (library 'survival', R), using a

joint model analysis (library 'JM', R) (271). The outcomes of the Cox proportional hazards models were total CVD, CHD and stroke with the corresponding follow-up times as a time variable. In accordance with the methods used for the linear mixed models, the Cox proportional hazards model was adjusted for baseline age with the inclusion of natural splines. The final joint models included either time-varying AMH levels, a time-varying slope (i.e. speed of AMH decline), or both time-varying AMH levels and a time-varying slope as potential predictors for the hazard of CVD.

Sensitivity analysis

In order to exclude a potential effect of polycystic ovary syndrome (PCOS) or surgical menopause, the analyses of total CVD were separately repeated with exclusion of women who never had a regular menstrual cycle (n=647) and exclusion of women with a bilateral oophorectomy (n=50).

Results

Population characteristics

On average, included participants completed 3.9 visits (including the baseline visit) with available AMH measurements. The baseline characteristics of participants per age-standardized baseline AMH category are listed in Table 1. Hormone replacement therapy use was not assessed in the baseline questionnaire, but declined from 4% in round 2 to 1.5% in round 5. In rounds 2-5, mean glucose levels were 5 ± 1 mmol/L. The population characteristics stratified by round are presented in Supplementary Table 1.

Outcome characteristics

By the end of follow-up, 255 (8.2%) women had total CVD, 152 (4.9%) women had CHD and 81 (2.6%) women had experienced a stroke. The incidence of total CVD was compared between age-standardized AMH categories, as shown in Table 2. The number of women with CVD decreased with the decline of age-standardized AMH.

AMH trajectories in relation to total cardiovascular disease

The results of the joint models in regard to total CVD are presented in Table 3. After adjustment for all covariates, each unit (ng/mL) lower $_{log}$AMH *level* at any time point during the trajectory was associated with a 21% higher risk of total CVD during follow-up (HR [95% CI] 1.21 [1.07-1.36]. Each additional unit (ng/mL/year) *decrease* in $_{log}$AMH, i.e. a steeper negative slope, was associated with a 46% higher risk of total

CVD. When models were accounted for both time-varying AMH levels and time-varying slopes, both HRs [95% CI] were attenuated to 1.12 [0.98-1.28] and 1.42 [1.09-1.86], respectively. The fully adjusted relationship between AMH levels and the relative hazard of CVD was non-linear and is provided in Figure 1.

Table 1. *Population characteristics at round 1† compared between women in different baseline age-specific AMH categories.*

	Lowest age-specific AMH (1st category) *n=1,050*	**Second-lowest age-specific AMH (2nd category)** *n=593*	**Second-highest age-specific AMH (3rd category)** *n=297*	**Highest age-specific AMH (4th category)** *n=1,000*	**p for trend**
AMH (ng/mL)	0.02 [0.00-0.34]	0.96 [0.02-2.12]	2.37 [0.63-3.63]	3.62 [1.84-6.06]	<0.01
Age (years)	47.9 ± 8.1	40.8 ± 9.5	36.0 ± 7.8	33.1 ± 6.7	0.02
OC use (% (n))	12 (130)	23 (136)	31 (92)	36 (364)	0.43
Current smoker (% (n))	29 (308)	34 (199)	39 (117)	36 (358)	0.20
BMI (kg/m²)	25.6 ± 3.9	24.9 ± 3.9	23.8 ± 3.6	23.6 ± 3.4	0.03
Premenopausal (% (n))	62 (558)	85 (450)	99 (283)	100 (969)	0.07
Systolic blood pressure (mm Hg)	122 ± 16	117 ± 14	115 ± 13	113 ± 12	0.03
Diastolic blood pressure (mm Hg)	77 ± 11	76 ± 10	74 ± 10	74 ± 9	0.05
Total cholesterol (mmol/L)	5.7 ± 1.1	5.4 ± 1.0	5.1 ± 0.9	5.1 ± 0.9	0.06
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	0.74

†Total number of women with an available AMH measurement in round 1: n= 2,940; Values presented in mean ± SD, median [IQR] or % (n). AMH = anti-Müllerian hormone; OC = oral contraceptive; BMI = body mass index

Table 2. *Incidence of nonfatal and fatal CVD per age-standardized AMH category.*

	Lowest age-specific AMH (1st category) *n=1,050*	**Second-lowest age-specific AMH (2nd category)** *n=593*	**Second-highest age-specific AMH (3rd category)** *n=297*	**Highest age-specific AMH (4th category)** *n=1,000*	**p for trend**
Total CVD *(total n=255)*	13.3 (140)	7.9 (47)	5.1 (15)	4.1 (41)	0.05
Coronary heart disease *(total n=152)*	8.1 (85)	5.1 (30)	3.4 (10)	2.3 (23)	0.02
Stroke *(total n= 81)*	4.3 (45)	2.7 (16)	1.0 (3)	1.1 (11)	0.06

Values presented in % (n). CVD=cardiovascular disease; AMH = anti-Müllerian hormone

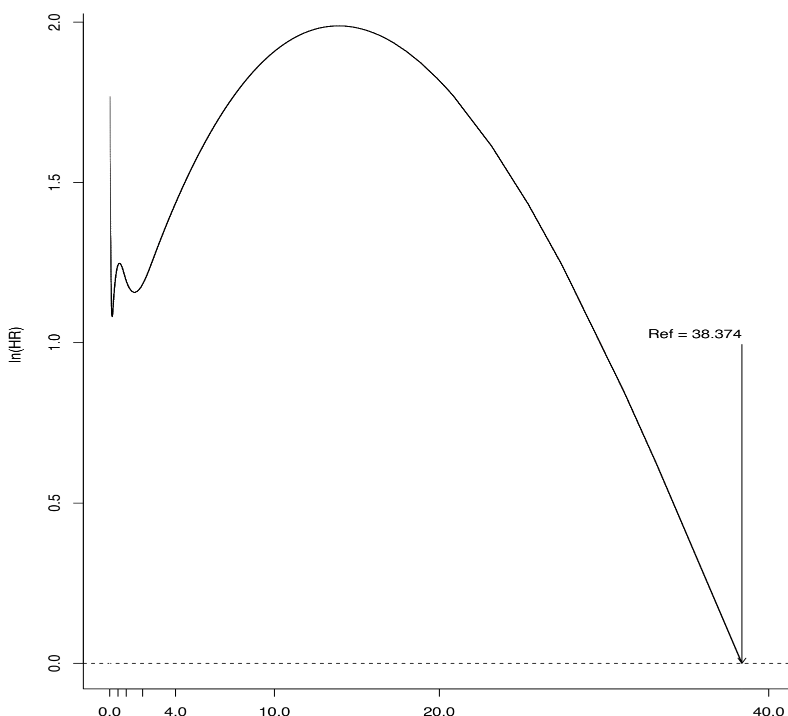


Figure 1. *Relative hazard of cardiovascular disease by AMH levels after full adjustment. The relationship between adjusted AMH and hazard of cardiovascular disease is non-linear. An adjusted AMH level of 38.4 ng/mL is associated with the lowest risk of cardiovascular disease and is therefore the reference value.*

Table 3. *Hazard ratios for the association of fully adjusted AMH levels and AMH decline trajectories with the risk of total CVD, CHD and stroke.*

	HR (95% CI) ↓AMH level	**p-value**	**HR (95% CI) AMH decline rate**	**p-value**
	Total cardiovascular disease			
Only time-varying AMH level	1.21 (1.07 to 1.37)	<0.01		
Only time-varying decline rate			1.46 (1.14 to 1.87)	<0.01
Time-varying AMH level and decline rate	1.12 (0.98 to 1.28)	0.09	1.42 (1.09 to 1.86)	0.01
	Coronary heart disease			
Only time-varying AMH level	1.26 (1.08 to 1.46)	<0.01		
Only time-varying decline rate			1.56 (1.15 to 2.12)	<0.01
Time-varying AMH level and decline rate	1.16 (0.98 to 1.37)	0.09	1.49 (1.07 to 2.07)	0.02
	Stroke			
Only time-varying AMH level	1.03 (0.82 to 1.30)	0.78		
Only time-varying decline rate			1.17 (0.94 to 1.45)	0.16
Time-varying AMH level and decline rate	1.00 (0.78 to 1.25)	0.93	1.14 (0.94 to 1.37)	0.17

Models are adjusted for age, OC use, smoking, BMI, menopausal status, TC, DBP, $_{log}$HDL-c, HT, glucose, lipid-lowering medication, and blood pressure-lowering medication. OC = oral contraceptive; BMI = body mass index; TC = total cholesterol; DBP = diastolic blood pressure; HDL-c = high-density lipoprotein cholesterol; HT = hormone replacement therapy

Table 4. *Association of fully adjusted AMH decline trajectories with the risk of total CVD after exclusion of women with an irregular menstrual cycle or surgical menopause.*

	HR (95% CI) ↓AMH level	p-value	HR (95% CI) AMH decline rate	p-value
After exclusion of irregular menstrual cycle (n=2,461)				
Only time-varying AMH level	1.17 (1.02 to 1.34)	0.03		
Only time-varying decline rate			1.39 (1.04 to 1.86)	0.03
Time-varying AMH level and decline rate	1.09 (0.93 to 1.27)	0.28	1.43 (1.04 to 1.95)	0.03
After exclusion of surgical menopause (n=3,054)				
Only time-varying AMH level	1.21 (1.07 to 1.36)	<0.01		
Only time-varying decline rate			1.55 (1.19 to 2.03)	<0.01
Time-varying AMH level and decline rate	1.22 (0.98 to 1.28)	0.09	1.48 (1.11 to 1.97)	0.01

Models are adjusted for age, OC, smoking, BMI, menopausal status, TC, DBP, $_{log}$HDL-c, HT, glucose, lipid-lowering medication, and blood pressure-lowering medication. OC = oral contraceptive; BMI = body mass index; TC = total cholesterol; DBP = diastolic blood pressure; HDL-c = high-density lipoprotein cholesterol; HT = hormone replacement therapy

AMH trajectories in relation to coronary heart disease and stroke

The results of the joint models with regard to CHD and stroke are presented in Table 3 . After adjustment for all covariates, each ng/mL lower $_{log}$AMH *level* at any time point during the trajectory was associated with a 26% higher risk of CHD during follow-up (hazard ratio (HR) [95% CI] 1.25 [1.08-1.46]). Each additional ng/mL/year *decrease* in $_{log}$AMH, i.e. a steeper negative slope, was associated with a 56% higher risk of CHD (HR [95% CI 1.55 [1.14-2.10]) by the end of follow-up. When models were accounted for both time-varying AMH levels and time-varying slopes, both HRs [95% CI] were attenuated to 1.16 [0.98-1.37] and 1.49 [1.07-2.07], respectively. Both AMH levels and decline rates were not associated with the occurrence of nonfatal or fatal stroke in this study. When the models were run with both the inclusion of both time-varying AMH levels *and* time-varying slopes, the HRs [95% CI] of $_{log}$AMH levels and slopes were 1.00 [0.78-1.25] and 1.14 [0.94-1.37], respectively.

Sensitivity analysis

Of the 2,461 women with ever a regular cycle, there were 182 (7.4%) women who developed any manifestation of CVD by the end of follow-up. The results of the joint model analyses in this group of women are presented in Table 4. After full adjustment, each ng/mL lower *level* of $_{log}$AMH was associated with a 17% higher risk of total CVD (HR [95% CI] 1.17 [1.02-1.35]). Each additional ng/mL/year *decline* was associated with a 38% increased risk of total CVD (HR [95% CI] 1.38 [1.03-1.85]). Of the 3,054 women without a bilateral oophorectomy, there were 246 (8.1%) women who developed any manifestation of CVD. The coefficient estimates of AMH levels and rate of decline approximated those in the whole study group (Table4).

Discussion

In the present study, we observed an association of AMH levels and the rate of AMH decline with the incidence of CHD and total CVD in women, independently of menopausal status and metabolic risk factors. There was no clear evidence for an association of AMH level with stroke, although there was an inverse relationship between the speed of AMH decline and the risk of stroke. Both a lower AMH level and swifter rate of decline were individually associated with an increased hazard of CHD and total CVD, but the corresponding effect estimates were attenuated when models were mutually adjusted for time-varying levels and slopes.

Before interpreting our findings, strengths and limitations of the present study should be addressed. Strengths of the present study include the availability of AMH data of up to 5 study visits over a 20-year period, enabling the modeling of longitudinal AMH trajectories. Furthermore, with the use of national morbidity and mortality registries we were able to associate AMH to clinically relevant outcomes, rather than just risk factors. The population-based design and long follow-up time are uniquely suited to study this association. Moreover, the adjustment for relevant, time-varying, confounders strengthens the observation of an independent relationship. The fact that this relationship was upheld after the exclusion of women with an irregular cycle or bilateral oophorectomy, affirms that the results presented here are likely generalizable to the general female population.

An observational design is suitable to detect associations, but does not allow for conclusions to be drawn on causality. It also does not rule out residual confounding. Due to the low incidence of stroke, our study had limited power of 35% to detect an association with AMH, also impeding the distinction between ischemic and hemorrhagic stroke. We have no data on the presence of PCOS in our cohort and therefore chose to repeat our analyses only in women with ever a regular cycle. It is therefore possible that we misclassified non-PCOS women as PCOS and vice versa. As we excluded almost 20% of the

women in the study population with an irregular cycle, more than the expected prevalence of PCOS (272), and adjusted for cardiometabolic risk factors, it is likely that we captured an association between AMH and CVD independently from PCOS. Moreover, women with missing AMH measurements smoked more often and had a higher BMI at baseline. It is therefore possible that our results are based on a relatively healthier selection of the general population, but as there were no differences in incidence of CVD this is not likely to influence the association between AMH and CVD. It would have furthermore been interesting to take into account other hormones such as estrogen in addition to cycle status, but as these measurements were not performed in the Doetinchem Cohort Study, this was not possible for the current study. The lack of an association between endogenous sex steroid concentrations and cardiovascular disease in prospective studies suggests that this would not influence our results (273). Lastly, the joint models assume that study censoring is independent of the random effects. Although study participation in each round remained >75%, the reasons for loss to follow-up were not fully known and could therefore not be taken into account in the analyses, which could have caused a slight overestimation of our results.

The results of the present study are in accordance with prior observations of a relationship between AMH and subclinical CVD. In a longitudinal study, female premenopausal *cynomolgus* macaques with the lowest AMH levels at baseline had the largest atherosclerotic plaques after two years of follow-up (60). A cross-sectional evaluation of both healthy and HIV-infected women concurred that premenopausal women with undetectable AMH levels had larger atherosclerotic plaques than premenopausal women with detectable AMH levels (274). While these observations could potentially be explained by a relationship of either ovarian reserve status or AMH levels with lipid measures (61, 62, 137, 227), the results of the current study add to the evidence suggesting that the association of AMH with CVD occurrence is independent from cholesterol levels (60, 274). Interestingly, a previous study did not find any association between age-specific AMH levels and silent coronary artery disease (CAD), based on a composite factor of various electrocardiography-based ischemic changes, in a 10-year follow-up period (275). The high reported rate of silent CAD in this population (14%, compared to 4.8% (276) and 3.6/10000 person-years (277) in a Dutch population) suggests that the used classification of silent CAD may not be entirely able to distinguish the women with clinically relevant coronary-based ischemia from this population (275). A recent study reported an association between lower AMH levels and higher all-cause mortality in population-based sample of 989 men (278), suggesting that this effect may not be limited to women alone, although the study in question was underpowered to assess a relationship with CVD-related mortality.

As there are no previous studies that have studied the association of AMH tra-

jectories with CVD risk or outcomes, it is challenging to interpret the meaning of the rate of AMH decline. We previously found the level of AMH to be associated with the rate of AMH decline (160), which explains the attenuation of the coefficients when mutually adjusted. It is likely that processes that cause a swifter decrease of AMH, such as accelerated general aging (258, 279), also influence CVD risk. On the other hand, it can be speculated that the faster decline of AMH could also be involved in the pathophysiology of CVD. This is potentially represented by the increased CVD risk of women with surgical menopause compared to women with natural menopause (280). In our study the small number of women with surgical menopause precluded separate analysis of this group.

It is relevant to consider variation in AMH levels in order to place AMH decline into perspective. To date, there is a relative lack of studies that have studied longitudinal decline and short-term variation of AMH. Two recent reports found AMH levels to remain stable throughout the menstrual cycle (166, 167), while others reported absolute differences up to 0.5 ng/mL (168) and 0.8 ng/mL (281). The average yearly decline of AMH, also measured with an AnshLabs assay, was estimated to be 6-8% (168). Given the fact that in the current study AMH measurements were spaced 5 years apart, the absolute AMH decline would likely be greater than the observed short-term variation. Another potential source of AMH variation arises from the storage of samples. Two-week sample storage of serum aliquots at -20°C and -80°C was previously associated with a difference in detected AMH levels in the magnitude of 0.1 ng/mL (129). A prior study did not find any effects of freeze-thaw cycles on AMH measurements, albeit with a different assay (282). Although there are no long-term data on the stability of specimen storage, it is likely that there is some variation due to storage effects in our study. However, as samples were treated the same way within each round, we found no significant variations in between-round age groups and a correlation >90% with prior measurements in round 2 (data not shown), it is unlikely that this variation greatly affects our results of relative AMH change. Lastly, the recent emergence of high-sensitive assays, such as the picoAMH assay used in this study, has enabled measurement of AMH in women in and after the menopausal transition (122, 160). However, the relevance of AMH detection and variation in very low ranges still remains a subject of speculation and requires further research.

To date, there are some indications that AMH may be directly involved in cardiovascular physiology. The AMH molecule is part of the same family as bone morphogenetic proteins (BMP), which are thought to play an important role in vascular homeostasis (269, 283). Moreover, cardiac tissue from neonates with hypoplastic left heart syndrome exhibited fewer AMH receptors than controls, suggesting a role for AMH in (early) myocardial development (65). Women with hypertensive pregnancy disorders, which are thought to originate from impaired vascularization and cause

increased cardiovascular risk later in life (284), were previously shown to have lower AMH levels than controls (240, 242, 243). Taking these results together, we speculate that AMH may exert an influence on cardiovascular tissue function throughout reproductive life, with decreasing AMH levels leaving the vasculature more prone to injury and atherosclerosis. This notion requires further investigation in experimental and epidemiological studies, before being considered as a likely hypothesis.

There has been much debate as to what governs the association between menopause and increased CVD risk. It was long believed that the reduction of estrogen as a result of the ovarian reserve depletion was responsible for the rise in CVD. However, this theory may be "stronger than the evidence" (52), as there is no conclusive proof that estrogen therapy can eliminate the increased CVD risk of menopause, despite an improvement in lipid paramers (52, 265). Another hypothesis is the 'aging soma' theory. Processes that influence general aging, such as impaired DNA repair (257, 258), may lead to both an earlier menopause and aging of the cardiovascular system (285). The reverse may also be true. Transplanting ovaries of young mice into older mice significantly increased their lifespan, suggesting that ovarian function dictates general aging (286). Within this context, it is possible that lower AMH levels and a swifter rate of AMH decline are illustrative of a deterioration of ovarian function, with potential effects on cardiovascular aging. Whether this could be mediated through AMH requires further research. We are the first to report on the association between AMH and CVD and must therefore wait for others to confirm our results.

In conclusion, this is the first study to identify an independent relationship of AMH levels and rate of AMH decline with CVD in women. The potential role of AMH in cardiovascular health may provide a missing link to the CVD risk associated with menopause, for which an unequivocal explanation is still lacking. We eagerly await future studies to confirm and elaborate on our results for the transition to clinical practice.

Supplementary Table 1. *Population characteristics per follow-up round*

	Round 1	*Round 2*	*Round 3*	*Round 4*	*Round 5*
	N=2,940	*N=2,848*	*N=2,330*	*N=2,195*	*N=1,944*
AMH (ng/mL)	0.99 [0.04-3.07]	0.18 [0.00-1.47]	0.00 [0.00-0.52]	0.00 [0.00-0.05]	0.00 [0.00-0.00]
Age (years)	40.2 ± 10.1	46.1 ± 10.0	50.7 ± 9.9	55.2 ± 9.8	59.6 ± 9.6
OC use (% (n))	25 (722)	20 (580)	16 (380)	9 (205)	6 (112)
Current smoker (% (n))	33 (982)	31 (875)	26 (601)	22 (479)	18 (339)
BMI (kg/m^2)	24.6 ± 3.8	25.6 ± 4.2	26.2 ± 4.3	26.6 ± 4.6	27.0 ± 4.8
Premenopausal (% (n))	84 (2,260)	70 (1,472)	59 (1,046)	32 (532)	18 (278)
Systolic blood pressure (mm Hg)	117 ± 15	122 ± 17	126 ± 18	128 ± 19	129 ± 18
Diastolic blood pressure (mm Hg)	75 ± 10	78 ± 11	80 ± 11	80 ± 10	79 ± 10
Total cholesterol (mmol/L)	5.4 ± 1.0	5.5 ± 1.0	5.7 ± 1.1	5.7 ± 1.0	5.7 ± 1.1
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.5 ± 0.4	1.5 ± 0.4	1.6 ± 0.4	1.6 ± 0.4

Values presented in mean ± SD, median [IQR] or % (n). AMH = anti-Müllerian hormone; OC = oral contraceptive; BMI = body mass index; HDL=high-density lipoprotein

Chapter 10

Chapter 10

General Discussion

As women age, they experience continuous changes in their reproductive physiology. After reaching a point of optimal fertility in her early 20s, a woman's reproductive capabilities gradually wane until she is no longer able to conceive a natural pregnancy in hear early 40s. With the following onset of menopause women are thought to become more at risk for chronic diseases, among which cardiovascular disease (CVD). This thesis aimed to provide more insight into the assessment and cardiovascular consequences of reproductive health changes of the aging woman. The following questions and sub-questions were therefore addressed:

1. Can knowledge of individual AMH decline improve the prediction of time to menopause?
   a. Is the measurement of AMH comparable between different assays?
   b. Do individual AMH trajectories differ from one another?
2. Is there a relationship between ovarian and cardiovascular aging?
   a. Through which mechanisms is this relationship mediated?

Part I: Quantification of ovarian reserve decline

AMH measurement

In **Chapter 3**, the comparability of the AMH measurements performed in this thesis was assessed by comparing the measurements of two different AMH assays used in this thesis; the Gen II (Beckman Coulter) and picoAMH (AnshLabs) assays. The observed correlation coefficient between both methods of 0.92 was similar to previous comparisons (125, 129), and reassuring for the use of both methods in this thesis. However, the systematic (69%) higher picoAMH assay levels indicated that the absolute levels of both assays cannot be compared to one another. Reasons for this difference can be hypothesized to originate from factors such as binding affinity of the antibodies or differences in incubation time, for example. Furthermore, the Gen II assay measurements were performed at a time when the new pre-dilution Gen II protocol had not yet been implemented (134). The levels measured by Gen II could therefore have been underestimated due to complement interference with the samples (282). The proportional difference between the Gen II and picoAMH measurements has furthermore varied per research site (125, 129). This suggests that in addition to the lack of comparability of absolute AMH levels between various assays, the inter-laboratory variability may also be considerable. This has important consequences for the interpretation of AMH levels both for research and clinical purposes. For research of AMH decline, for example, a percentage rather than

absolute unit change should be reported for the purpose of comparison. Furthermore, the translation to clinical practice from AMH research can be hindered. For example, if levels of AMH may replace ultrasound findings for the diagnosis of polycystic ovary syndrome (PCOS) in the near future, with a current lack of a gold standard of AMH measurement, each assay type and laboratory site should still be validated individually.

While the measurements of the Gen II and picoAMH assay were comparable to one another, the picoAMH assay was able to detect AMH concentrations in 78% of the undetectable samples of the Gen II assay. Because such high-sensitive assays have only recently been introduced, little is still known about the added value to clinical practice of detecting AMH levels in a very low range. Moreover, the picoAMH was able to detect AMH levels in a proportion of postmenopausal women (**Chapter 3**), which is a novel finding. It can be hypothesized that postmenopausal women with detectable AMH levels have some, or more, remaining ovarian function in contrast to women with undetectable levels with high-sensitive assays. The estimated remaining follicle number at menopause was previously estimated to be approximately 1000 (2), indicating that there could still be hormone-producing follicles after this time. The next step could thus be to investigate whether women who have detectable AMH measurements after menopause differ from women with undetectable levels, with regard to levels of sex steroids, climacteric complaints, osteoporosis and cardiovascular health, for example. Such investigations may furthermore aid to refine the definition of menopause, which is now retrospectively diagnosed after an arbitrary period of 12 months cessation of menses. Perhaps, at least for research purposes, it would in the future be more appropriate to classify women based on the presence of ovarian function, with proxy tools such as AMH.

The comparability of the measurements of the Gen II and picoAMH assays is thus reassuring for the interpretation of AMH levels in this thesis. However, it deserves some thought to consider what is actually being measured. In the granulosa cells, AMH is first present as a precursor called proAMH. It is thought that cleavage of most of the available proAMH to the biologically active $AMH_{N,C}$ occurs before both forms enter the peripheral circulation (287). The capture antibodies of the Gen II and picoAMH ELISAs are directed at a linear epitope on the mature C-ligand of the AMH molecule (123) (Figure 1), which is present on both proAMH and $AMH_{N,C}$ forms. The advantage of this binding site is the minimized risk of detecting proteolytic components of the AMH molecule, which may arise during long-term storage (119). It is therefore unlikely that an 'untrue' fraction of AMH was measured in this research, either by the Gen II or picoAMH assay. As a result, though, a combination of proAMH and $AMH_{N,C}$ is detected with the Gen II and picoAMH assays (24). Interestingly, two recent reports found a higher fraction of biologically $AMH_{N,C}$ in the follicular fluid of 13 PCOS patients compared to 9 regularly cycling controls (24 vs 8%) (288), and a higher

fraction in adult women compared to girls (289). Both studies found at least twofold higher degrees of cleaved AMH in men compared to women (288, 289). The degree of receptor binding of the follicular fluid $AMH_{N,C}$ was furthermore higher than that of $AMH_{N,C}$ in serum (288). This indicates that the ratio of $AMH_{N,C}$ to proAMH could have clinical implications and raises several questions. For example, if women with PCOS have more biologically active AMH, could follicular receptor desensitization be a cause of their above average follicular recruitment? Do other tissues have binding sites for $AMH_{N,C}$? Is the cleavage of proAMH to $AMH_{N,C}$ hormone- or age-dependent? Do women with high fractions of uncleaved AMH in follicular fluid also have higher fractions in their peripheral circulation? These questions may be answered by the continued development of proAMH- or $AMH_{N,C}$-specific AMH assays (290) and comparison of the proAMH/ $AMH_{N,C}$ ratio in subgroups of women based on menstrual cycle stage, age and fertility status. For the current use of the Gen II and picoAMH assays in this thesis it may be assumed that both detect a similar proportional fraction of proAMH to $AMH_{N,C}$.

In summary, the reliability of the Gen II and picoAMH assays in this thesis is underscored by the high correlation coefficient of both assays, although a comparison of absolute AMH levels is not possible. Increasing knowledge of the circulating AMH molecule suggests that the concentration of circulating AMH measured by either the Gen II or picoAMH assay may not be directly indicative of its biological function.

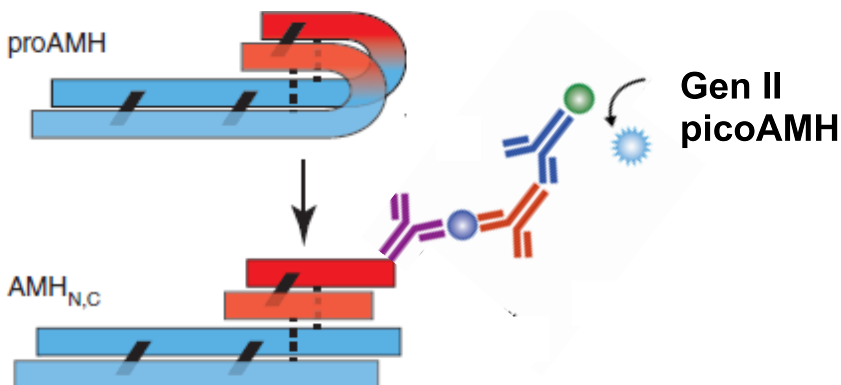


Figure 1. *Schematic representation of AMH measurement with the Gen II and picoAMH ELISAs. Capture and detection antibodies of the Gen II and picoAMH assays are directed at the mature C-terminal region of the molecule, which is present on both circulating proAMH and AMHN,C molecules. Modified from McLennan et al (269).*

Measurement of AMH in relation to ovarian reserve decline

In order to study the effect of multiple AMH measurements on the prediction of age at menopause, it was first necessary to evaluate how AMH levels decline in individual women. It was previously debated whether age-specific AMH levels declined in parallel fashion (18, 143, 144), or converged with age (141, 142, 145-147, 162). We observed that women with an initial high AMH level, and slow speed of decline at an early age, had a more rapid speed of AMH decline at a later age than women with initially lower AMH levels (**Chapter 4**), resulting in converging AMH trajectories at later ages.

It is important to question whether the observed individual AMH decline trajectories are indeed reflective of the decline of the primordial follicle pool, or true ovarian reserve. AMH levels were previously found to be correlated to the size of the primordial follicle pool (26). By measuring the number of non-growing follicles (NGFs) in ovaries obtained through elective surgery, organ donation or autopsy in 122 women between the ages 26-52 years, it was estimated that the decline rate of the primordial follicle pool continuously accelerates with increasing age (291). Although biologically plausible, this is at odds with our finding of a sigmoidal pattern of AMH decline in **Chapter 4**, where AMH levels overall remained relatively stable or declined slowly until a rapid decrease at approximately 35-40 years. An explanation for this difference may therefore lie in follicular recruitment, as small developing follicles are the source of AMH production (21). The rate of NGF recruitment is thought to increase until a mean age of 14 years in women with an average age at menopause (292) (Figure 2). Primary follicles are furthermore thought to achieve later stages of maturation until a peak at age 25 (293). Although these findings are purely based on mathematical models of cross-sectional studies, it may be that up to a certain age, AMH levels can indeed be expected to increase or decline more slowly than the decline of follicle numbers. This is underscored by cross-sectional population studies that observed increasing AMH levels until after puberty (142, 162). Until the number of primordial follicles can be assessed without the use of invasive techniques, it is unfortunately not possible to confirm this theory with a prospective evaluation of NGF counts and AMH levels in humans.

Thus, AMH may not be reflective of true ovarian reserve throughout the entire reproductive lifespan, with variations in this correlation especially to be expected at younger ages. This does not yet explain why women have varying decline rates of AMH with age, and whether this can be an indication of a swifter decline of true ovarian reserve. The explanation for this disagreement may partly be found in the results of a study in which both AMH levels and NGF numbers were measured in female mice (25). In these animals, AMH levels strongly correlated with NGF numbers, in line with previous findings in humans (26). As expected based on the findings above, there was an ini-

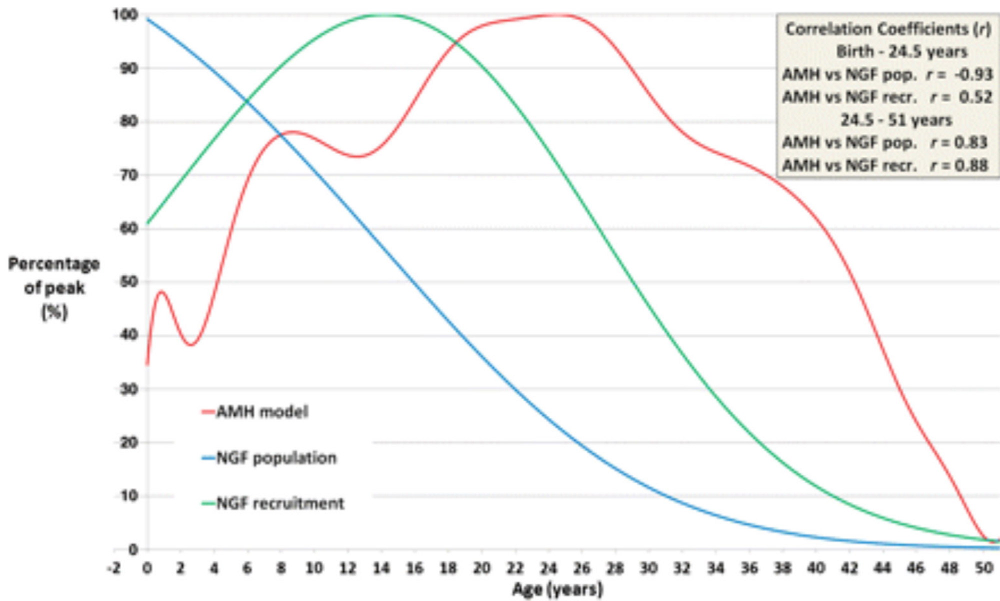


Figure 2. *Schematic representation of NGF pool decline with age (blue line), NGF recruitment with age (green line) and changes of AMH with age (red line). From (295).*

tial lag in AMH decline in young (4-month old) mice, where NGF numbers were already decreasing. The authors ascribed this contradictory result to a compensatory function in mice, which allowed a continuous number of follicles to be recruited for development in fertile mice despite a diminishing primordial follicle pool. If this same mechanism exists in humans, this is an interesting notion, as those with a lesser endowment of primordial follicles may be able to recruit proportionally more follicles in order to extend the duration of their fertile lifespan. As discussed, theoretical models of follicle recruitment suggest that women with an earlier age at menopause reach their peak of NGF recruitment earlier (293). It can therefore be theorized that women with an early age at menopause have initially slower decline rates of AMH or a sharper increase, with a subsequent swifter decline. Perhaps it is this mechanism that partially explains the shorter duration of 4 years between the last pregnancy and final amenorrhea in a population of women with menopause before 40 years (294), compared to the estimated 10-year interval for the general population (12). The latter finding could also be ascribed to a better oocyte quality in these relatively younger women, however.

Individual AMH decline in age at menopause prediction

The differences in individual decline trajectories of AMH suggested that the decline rate of AMH could potentially be of influence for the characterization of individual ovarian reserve decline, and thereby prediction of age at menopause. Addition of the AMH de-

cline rate between the ages 20-25 years led to a slightly better prediction of age at menopause, whereas the decline rate between 25-30 years did not lead to an improvement. The decline rate of AMH was not able to improve the prediction of early menopause.

In **Chapter 4**, rather than declining in parallel, individual trajectories of AMH decline with age converged towards one another with increasing age. Combined with the expected finding that overall, lower AMH levels were associated with an earlier age at menopause, a paradigm shift can be proposed from the prevailing theory of parallel AMH decline in relation to age and age at menopause (Figure 3) to converging AMH decline trajectories (Figure 4).

At any given time before the occurrence of menopause, there was ~30% variability between the AMH levels of individuals, which appeared greatest at a longer time before of menopause, and decreased as time progressed. In other words, the decline rate of AMH in an early stage of reproductive aging could be expected to refine the estimation of remaining time to menopause in addition to AMH level at that time. A prior study in 50 women with a relatively high mean age of 42 years did not find an association between the decline rate of AMH and menopause when taking into account AMH level (29), which supports this notion. This could perhaps explain why the prediction models for early menopause had an overall better predictive capacity than the models for all ages at menopause, and AMH measurements before age 40 were better predictors than at a later age (**Chapter 5**). Another explanation may lie in the non-proportionality of the hazard of AMH for menopause with age (39, 159): as women approach their 50s, their risk of menopause becomes intrinsically higher, thereby leaving less variability in age at menopause to be explained by predictors such as AMH.

At age 20, a *higher* AMH level was associated with a *higher* risk of early menopause, and the highest AMH levels at age 20 were associated with a larger short-term *increase* in AMH levels. It could therefore be possible that in some women, an increased number of follicles is continually recruited, ultimately resulting in a swifter depletion of the ovarian reserve pool and earlier age at menopause. AMH is thought to play an inhibiting role in follicular recruitment and development (153-155). Moreover, interactions of polymorphisms in the genes encoding AMH and the AMH receptor were previously linked to age at menopause (296). It can thus be speculated that impaired feedback mechanisms or insensitivity to the inhibitory role of AMH could contribute to early menopause, of which the causes still remain largely unexplained (297). Altogether, these results indicate that AMH levels and decline rates are more variable at an earlier stage of the reproductive lifespan, which is in line with the thought that AMH may not be representative of true ovarian reserve in young women (Figure 4). This emphasizes that AMH as a tool for menopause prediction is still impaired by unknown mechanisms causing its variability and variability in age at menopause. These

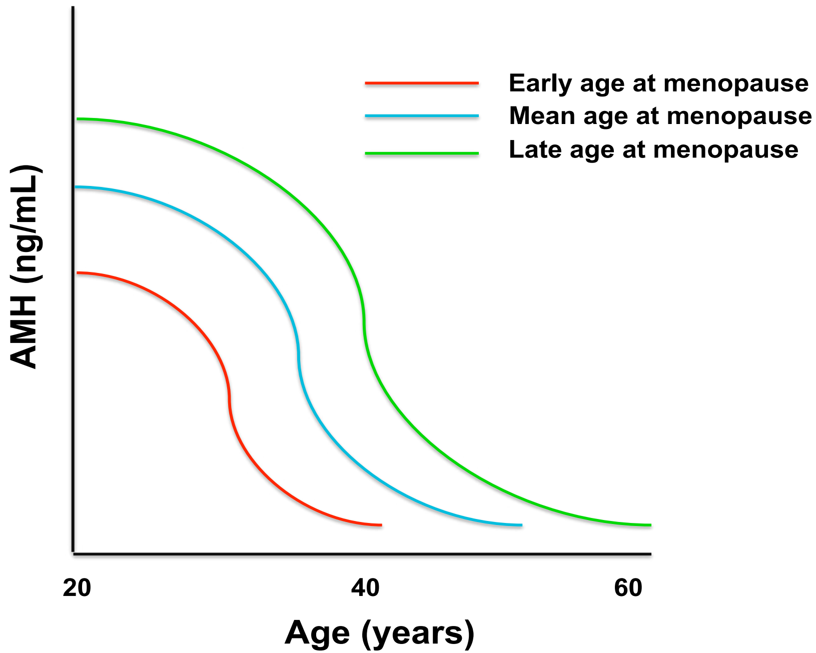


Figure 3. *Schematic representation of the original theorized relationship between AMH levels with age in women with early, mean and late ages at menopause.*

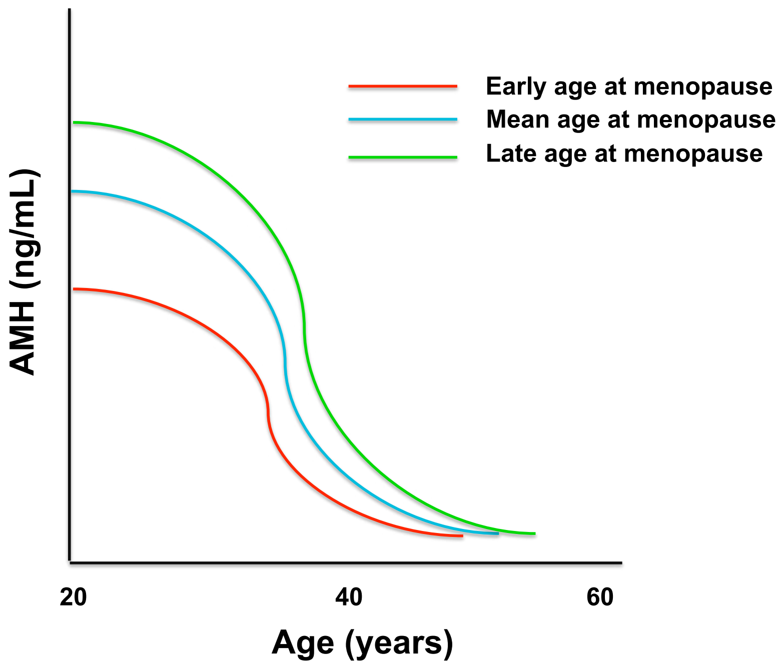


Figure 4. *Schematic representation of the proposed putative relationship between AMH levels with age in women with early, mean and late ages at menopause with converging trajectories.*

mechanisms require additional research before they can be placed in clinical context.

The findings in the studies presented in this thesis are based on the use of adjusted AMH levels with 5-year age intervals. It would be interesting to examine the value of AMH decline between measurements of a shorter time interval of 1-2 years for example, and compare the effect of 3 versus 2 measurements. However, the results indicate that the predictive capacity of AMH for age at menopause increase with AMH measurements at a later age. As it is most relevant for women to have an indication of the remaining duration of their reproductive lifespan before their 30s, it is unlikely that the addition of multiple AMH measurements with a shorter time interval will have a very large effect on the prediction of age at menopause for women below the age of 30.

In summary, the use of multiple AMH measurements was at best only able to slightly improve age at menopause prediction, and did not improve the accurate discrimination of women with an early age at menopause. For a woman aged between 20-30 years in clinical practice it is therefore unlikely that she can receive an accurate estimate of her risk of becoming postmenopausal at an early age. Furthermore, AMH was previously found not to be correlated to fecundity (109, 112), indicating that it may perform even more poorly as a predictor of future reproduction.

Part II: The relationship between ovarian and cardiovascular aging

The relationship of AMH and menopause with cardiovascular risk factors

Reports of the relationship between menopause and cardiovascular disease (CVD) date back to the 1950s (298). Since then, associations of (age at) menopause with varying forms of cardiovascular disease have consistently been described both in animal and human studies. The underlying mechanisms of this relationship have remained largely unidentified, however. It was, and often remains, a common notion that this association stems from the cardiovascular protective effect of estrogen, which diminishes with the advent of the menopausal transition, making the vasculature more prone to injury and plaques. While this may in part be true, estrogen supplementation to date does not conclusively lower CVD risk, despite an improvement in lipid parameters (52, 265, 266, 299). Circulating endogenous levels of estrogen were furthermore repeatedly not linked to CVD occurrence (273). These results suggest that at the least, other processes are also involved.

If menopause is a causative factor of CVD, this could potentially be mediated through a deterioration of risk factors of CVD such as cholesterol and blood pressure. However, as menopause is a process that occurs over time, and can only be established at least 12 months after the last menstrual cycle, it is challenging to differentiate the effects of menopause from chronological aging (**Chapter 6**). From **Chapter 6**, it became clear that both chronological age and menopausal status were independently as-

sociated with more harmful lipid levels particularly after the age of 45, indicating that the increased CVD risk of menopause could potentially be mediated through these traditional risk factors. The association of very low AMH levels with an unfavorable CVD risk factor profile in **Chapter 8** suggests that this could indeed be the case, with a common pathophysiology of reproductive and cardiovascular aging potentially occurring before the onset of menopause. Other studies corroborate these findings. Lipid and blood pressure changes over 12 years were most unfavorable for women in the lowest age-specific AMH quartile (61), and lipid parameters appeared to increase at an accelerated pace in the years surrounding the final menstrual period (45). AMH levels were furthermore related to atherosclerotic plaque size at a later time point in a primate study (60). Other studies made a case for an inverse relationship, where an atherogenic status could influence ovarian reserve (59, 300). The observational design of the studies in **Chapter 6** and **8** prevents a confirmation of the direction of this relationship. Either way, when considering the observed differences in lipid levels of 0.1-0.4 mmol/L in our studies, with a 1.0 mmol/L low-density-lipoprotein (LDL-c) cholesterol reduction previously associated with a 22% lower risk of cardiovascular events (201), it is questionable whether lipid levels alone can explain the increased CVD risk associated with menopause. To sum up, these results indicate that there is a relationship between ovarian aging and cardiovascular risk, which could be mediated through an adverse lipid profile and atherosclerosis.

The relationship of AMH with cardiovascular disease

When considering AMH in the light of reproductive aging, it is largely viewed as a proxy variable of ovarian reserve. The AMH molecule shares common binding sites with bone morphogenetic proteins (BMPs), which, like AMH, are part of the TGFβ superfamily. BMPs are thought to have a broad effect on embryonic vascular development and adult cardiovascular homeostasis (283). It could therefore be speculated that AMH may play a direct physiological role in cardiovascular homeostasis. The results from **Chapter 9** indeed suggest that AMH could have an effect on cardiovascular health, independently of traditional cardiovascular risk factors. That this may not be limited to women is illustrated by a study that found AMH levels to be associated with a higher risk of all-cause mortality in men (278), further strengthening this hypothesis. Theoretically, AMH could thus prove to play a role both in- and outside of the female reproductive tract, and provide the missing link of the association with CVD.

Mechanisms for the relationship between ovarian and cardiovascular aging

The results presented in this thesis provide evidence for the relationship between ovarian and cardiovascular aging. Taking this research into account, several causative mechanisms of this relationship can be conceived and are summarized in Figure 5. First, as already discussed, the hormone alterations accompanying menopause could make the vasculature prone to atherosclerosis and thrombosis, although evidence on this topic is contradictory (265). Second, an adverse cardiovascular milieu could potentially influence the rate of ovarian aging, for example through diminished ovarian blood flow due to plaques in the ovarian artery. Third, the decline of AMH with reproductive aging could potentially have an effect on cardiovascular function directly. Fourth, any association between ovarian and cardiovascular health could be the result of an overall accelerated aging process, also known as the 'aging soma theory' (285). This is supported by the association of genes encoding for DNA repair and immune function with menopause (257). These pathways are thought to play a role in cardiovascular aging (301). The results described in this thesis confirm the plausibility of either one or, more likely, a combination of these four theories.

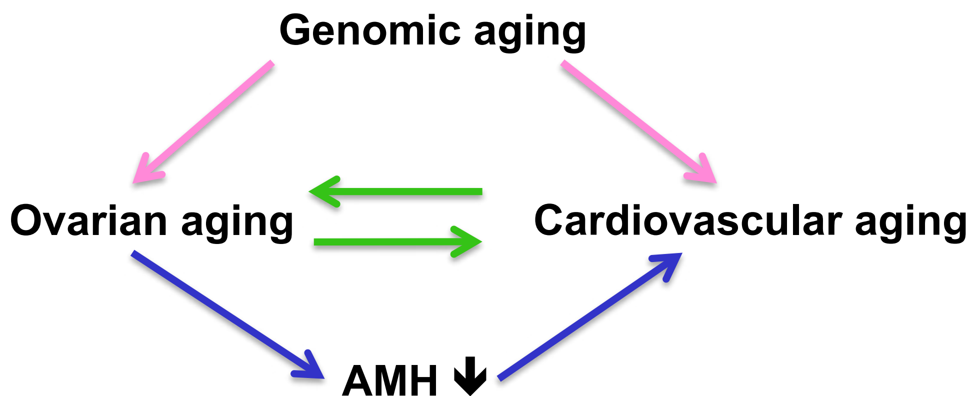


Figure 5. *Overview of potential causal mechanisms of the association between ovarian and cardiovascular aging. Green arrows: the decline of ovarian reserve with accompanying hormonal changes may signify the disappearance of a cardioprotective factor; or, cardiovascular impairment may induce accelerated decrease of ovarian function. Pink arrows: accelerated genomic aging may be a causative factor of simultaneous ovarian and cardiovascular aging. Blue arrows: decreased circulating AMH concentrations accompanying the decline of ovarian reserve may exert a direct influence on cardiovascular tissues.*

Implications for clinical practice and avenues for future research

Quantification of ovarian reserve decline

After the discovery of AMH as a reliable ovarian reserve marker, it was perceived to be the potential holy grail of age at menopause prediction. In the research presented in this thesis, a modest improvement of prediction models of age at menopause with the addition of AMH decline rate was observed at best. Moreover, the chance to correctly predict which of two women would become postmenopausal before age 45, based on a measurement at age 25 or 30, was 73%, with an a priori chance of 4%. The results described in this thesis do not suggest that it would be useful to use AMH levels and decline rates as a screening test for early menopause in young women.

There are some possibilities for clinical implementation of these results. The observation that AMH levels of women with an early menopause can be higher at an early age, warrant a more cautious interpretation of seemingly reassuring AMH levels. Moreover, the almost 50% likelihood of early menopause with an AMH level and decline rate in the bottom 5%, indicates that if individuals are identified with such extreme levels, they may be viewed to be at high risk. However, this is likely to be a very rare situation, as illustrated by the 2.2% of the women identified with this combination in our study population. A potential approach to study AMH trajectories in women with a high risk of early menopause could be to follow up young women whose mothers had an early age at menopause. It would be interesting to evaluate whether AMH levels and/or decline rates of AMH at a young age more often fall into this extreme category and whether they are indicative of the risk of early menopause in this population. By nature such a design would require an abundance of time and resources, however.

A potential strategy to refine the prediction models for age at menopause could be to further investigate the causes of AMH variability within and between women. As discussed above, the age of menopause may not coincide with the same follicular depletion for all women. Perhaps an assay-specific null-value should be used in prediction studies, rather than self-reported age at menopause. Our results should furthermore be repeated in other cohorts to assess their external validity. Future research efforts should further focus on the time interval between the cessation of natural sterility and menopause, in order to assess whether prediction of early menopause is truly effective for advice regarding the postponement of reproduction. This is a challenging objective due to the current widespread use of contraceptives. One alternative would be to prospectively assess whether levels and rate of AMH decline are associated with time to pregnancy.

Ultimately, women want advice on how to best achieve a viable pregnancy in their situation and desired time frame. In this context, oocyte quality is equally as

important as oocyte quantity. Little is known about the determinants of oocyte quality, and future research could thus focus on whether markers of general aging, such as epigenetic changes or telomere length, can give an indication of oocyte quality. Furthermore, it is possible that age at menopause prediction may simply prove not to be the most effective strategy to guide women in family planning. The focus may need to shift to educating women on reproductive potential with age in general and factors influencing ovarian reserve, such as smoking, and fertility, such as body weight. Finally, efforts should focus on making it easier for women to start a family whilst successfully starting or maintaining a career. Herein lies an important role for policy makers and society as a whole.

The relationship between ovarian and cardiovascular aging

The results pertaining to the relationship of AMH and cardiovascular disease risk provide an important contribution to the confirmation of the relationship between ovarian and cardiovascular aging. However, the direct clinical implications of this relationship will remain unclear until its causative mechanisms are further uncovered. Although the Doetinchem Cohort Study and other such population studies provide a wealth of information, an important downside is the lack of inference potential of causality. The difficulty of extracting the effects of ovarian aging from cardiovascular aging furthermore lies in the inherent long-term development of both; neither menopause nor a myocardial infarction occur overnight. Taking our results into account, there are several feasible avenues that could be explored to overcome these limitations.

With regard to the theory of ovarian reserve decline induced by cardiovascular disease, the effect of progressive ovarian artery blockage in an animal model could be studied, to mimic the potential effect of ovarian atherosclerosis. It would furthermore be interesting to prospectively evaluate the progression of AMH levels in women with extreme phenotypes of cardiovascular risk; for example women from families with monozygotous hypercholesterolemia, women with a history of hypertensive pregnancy disorders (242, 262), or women with type 1 diabetes (302-304); using the partners of siblings as controls, for example.

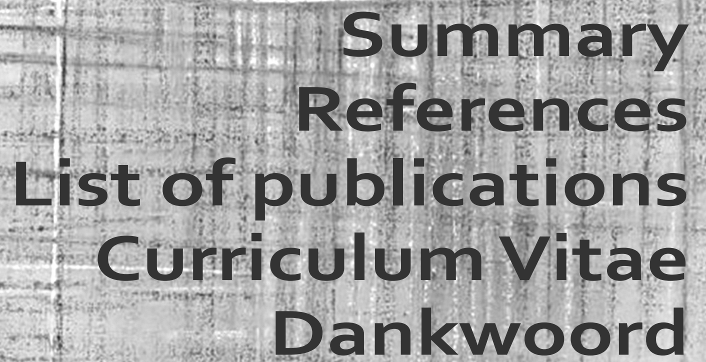
The association of AMH levels with cardiovascular events should be investigated by others, to strengthen our results. The causal effect of AMH polymorphisms, evaluated by genome-wide association studies (GWAS), on cardiovascular health could then potentially be studied in large cohorts with the use of mendelian randomization. Furthermore, in vitro research can focus on identifying target sites for AMH and expression patterns of AMH receptors on other human cardiovascular tissue. To date, one study found AMH type 2 receptors on cardiac tissue of human fetuses with hypoplastic heart

syndrome (65). Eventually, if a causal role of AMH is indeed identified, it would be interesting to study the effects of recombinant AMH on parameters of cardiovascular health, such as vessel relaxation. AMH-knockout mice models may prove useful in this respect.

Lastly, the studies in this thesis were not able to address to which extent accelerated general and cardiovascular aging are associated with ovarian aging. One strategy could be to investigate the link between age at menopause and longevity, for instance by studying the timing of menopause in women reaching exceptionally high ages, as a theoretical model for slow genomic aging. Another strategy could be to study DNA methylation patterns, also known as an 'epigenetic clock' as an indicator of biological aging (305), in women with low and high ovarian reserve with the same chronological age band.

Conclusion

In summary, this thesis provided evidence for the presence of individualized AMH decline. Knowledge of individual AMH decline may slightly improve the prediction of age at menopause, although the results do not warrant the use of AMH levels and decline rates as a screening tool for early menopause. Ovarian reserve decline was furthermore found to be associated with risk factors and clinical manifestations of cardiovascular disease. The results of this thesis indicate that female reproductive and cardiovascular health, whether identified or assessed at an early or later age, deserve an integrated approach.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Summary

Nederlandse Samenvatting

Summary

This thesis was driven by two main research objectives. First, it aimed to investigate whether AMH levels of individual women decline differently from one another, and whether this knowledge can help improve age at menopause prediction. Second, the relationship between ovarian and cardiovascular aging was assessed.

Part I: Quantification of ovarian reserve decline

Female reproductive aging is a function of the decline of both oocyte quantity and quality, which has consequences for the menstrual cycle and fertility as women age. Anti-Müllerian hormone (AMH) is correlated with the size of the remaining primordial follicle pool and is used as a proxy marker of remaining quantitative ovarian reserve. Prior to this thesis, long-term AMH decline trajectories of individuals were a subject of speculation. It was furthermore unknown whether knowledge of short-term changes of current quantitative reserve could improve the prediction of age at menopause.

In **Chapter 3**, we first focused on the comparison of two different AMH assays used in this thesis; the Gen II (Beckman Coulter) and picoAMH (AnshLabs) assays. Measurements by both assays in a sample of 1,985 premenopausal women were highly correlated, with a correlation coefficient of 0.92. The picoAMH assay was furthermore able to detect 78% of the samples that were undetectable with the Gen II assay and reported 69% higher values compared to the Gen II assay. These results confirmed the reliability of the use of both assays for the measurement of peripherally circulating AMH. However, the systematic difference indicated that absolute AMH levels of both assays could not directly be compared to one another.

Longitudinal individual AMH decline trajectories were quantified in **Chapter 4**. AMH decline rates were sigmoid-shaped and different between women, and were furthermore dependent on a woman's age and AMH level. The intraclass correlation coefficient (ICC) of 0.87 indicated that there was little intra-individual variation of AMH levels across a trajectory with age, meaning that in general, AMH levels remained consistently low or high until the trajectories converged around the age of 50 years. The potential relevance of AMH decline rate in addition to AMH levels was further explored by relating individual AMH decline trajectories to age at menopause in **Chapter 5**. For a 25-year-old woman, knowledge of her prior 5-year AMH decline rate would result in her having a 5% higher chance of an accurate overall age at menopause prediction, whereas this would not affect the predictive accuracy (73%) of her risk of becoming postmenopausal before the age of 45 years. Counter-intuitively, higher AMH levels at 20 years were associated with an earlier age at menopause. In the early adult stages of the reproductive lifespan, AMH levels may therefore not directly reflect the true ovari-

an reserve decline. The accelerated decline of AMH may furthermore represent the loss of compensatory follicle recruitment mechanisms in the ovary. Altogether, these results indicate that screening of AMH levels and AMH decline rates in young women will not likely help identify the 4% who are at risk for becoming postmenopausal by the age of 45 years.

Part II: The relationship between ovarian and cardiovascular aging

Menopause has often been related to an increased risk of cardiovascular disease (CVD), but the mechanisms through which this occurs remain largely unknown. In **Chapter 6**, an independent association of both chronological and reproductive aging with alterations in cardiovascular risk factors was observed. Specifically, after the age of 45, peri- and postmenopausal women had more unfavorable lipid levels compared to premenopausal women in their same age stratum. These results are in accordance with others and indicate that the higher risk of CVD with the onset of menopause could potentially be mediated through the deterioration of lipid levels and increased risk of atherosclerosis. However, the modest magnitude of up to 0.4 mmol/l higher cholesterol suggests additional involvement of other mechanisms.

In **Chapter 8**, premenopausal women with AMH levels of zero had more risk factors for CVD compared to women with AMH levels above 0.16 ng/mL (measured by the Gen II assay). These results suggest that the transition to menopause and the postmenopausal state are associated with a deterioration of cardiovascular risk factors, independently from chronological aging. Repeating the analysis only in women with a regular cycle and last menstruation in the prior 3 months did not alter the results. This suggests that it is possible that this association occurs before the decline of ovarian reserve becomes clinically apparent from changes in cycle regularity. This would mean that ovarian aging influences cardiovascular aging or vice versa, before the onset of menopause.

The AMH molecule is a member of the Transforming Growth Factor-beta family, and AMH activates a BMP-like signaling pathway through its specific type II receptor (AMHR2). Activation of the TGF-β and BMP pathways has been reported to be an early event in the cascade preceding an atherosclerotic lesion. AMH could thus be viewed both as an indicator of ovarian aging, as well as a biomarker hypothetically involved in atherosclerotic disease. Each lower ng/mL $_{log}$AMH was associated with a 21% increase of total clinical CVD and 26% increase of coronary heart disease occurrence in **Chapter 9**. Each additional ng/mL/year decline of $_{log}$AMH was furthermore associated with a 46% increase of total CVD and 56% increase of coronary heart disease. These associations were independent from menopausal status and traditional risk factors of CVD such as lipid- and blood pressure levels, suggesting that AMH could indeed influence cardiovascular phys-

iology through mechanisms other than an increase in circulating lipids. Future experimental and clinical research is necessary to confirm and further elucidate this hypothesis.

In conclusion, AMH decline trajectories differ between women and may slightly improve the prediction of age at menopause. Strategies to enable women to achieve their wish of starting a family should shift towards a focus on the assessment of oocyte quality, as well as educating women on the importance of timely reproduction and factors influencing this potential. Ovarian aging is furthermore related to cardiovascular aging, for which there are several putative causative mechanisms. For the aging woman, reproductive health may thus be viewed in the context of cardiovascular health, and vice versa.

Nederlandse samenvatting

Twee hoofddoelstellingen vormden de aanleiding voor dit proefschrift. Ten eerste had het tot doel te onderzoeken of het concentratieverval van anti-Müller hormoon (AMH) verschilt tussen individuele vrouwen en of deze kennis kan bijdragen aan een verbeterde voorspelling van menopauzeleeftijd. Ten tweede werd de relatie tussen ovariële en cardiovasculaire veroudering onderzocht.

Deel I: Het kwantificeren van de afname van ovariële reserve

Vrouwelijke reproductieve veroudering is het resultaat van de kwantitatieve en kwalitatieve afname van de voorraad eicellen waarmee de vrouw wordt geboren. Dit heeft consequenties voor de menstruatiecyclus en vruchtbaarheid van de ouder wordende vrouw. Concentraties van circulerend AMH hangen samen met de hoeveelheid primordiale follikels in de ovaria en worden gebruikt als proxy-maat voor kwantitatieve ovariële reserve. Voorafgaand aan het onderzoek van dit proefschrift was het onbekend hoe AMH niveaus over een langere termijn afnemen in de individuele vrouw. Voorts was het onbekend of kennis over de individuele snelheid van AMH afname op een korte termijn zou kunnen leiden tot een verbetering van de voorspelling van menopauzeleeftijd.

In **Hoofdstuk 3** richtten wij ons eerst op de vergelijking tussen twee AMH analysemethoden die werden gebruikt in dit proefschrift; de Gen II (Beckman Coulter) en picoAMH (AnshLabs) *assays*. Metingen van beide *assays* waren sterk aan elkaar gecorreleerd, met een correlatiecoëfficiënt van 0.92. De picoAMH *assay* was in staat AMH niveaus te detecteren in 78% van monsters die onder de detectiegrens van de Gen II *assay* vielen en observeerde gemiddeld 69% hogere AMH-waarden dan de Gen II *assay*. Deze resultaten bevestigden de betrouwbaarheid van het gebruik van beide analysemethoden voor het meten van circulerend AMH. Het relatieve verschil in geobserveerde waarden gaf aan dat de absolute AMH-waarden van beide *assays* niet direct met elkaar vergeleken kunnen worden.

Longitudinale trajecten van AMH afname werden gekwantificeerd in **Hoofdstuk 4**. De individuele afnametrajecten van AMH hadden een sigmoïdale vorm en de afnamesnelheid van AMH verschilde per individu, afhankelijk van leeftijd en hoogte van het AMH niveau. Er was weinig intra-individuele variatie van AMH waarden over een afnametraject met leeftijd, aangegeven door de *intraclass correlation coefficient* (ICC) van 0.87. Dit wil zeggen dat de AMH-waarden consistent laag of hoog bleven met de leeftijd, tot het moment dat de trajecten samenvielen rond de leeftijd van 50 jaar. De potentiële relevantie van de afnamesnelheid van AMH naast een AMH-waarde voor het voorspellen van de menopauzeleeftijd werd onderzocht in **Hoofdstuk 5**, waar individuele afnametrajecten in verband werden gebracht met menopauzeleeftijd. Voor een 25-jarige vrouw leidde

kennis van de snelheid van haar AMH verval over de laatste 5 jaar tot een 5% toename naar 69% kans op het correct voorspellen van haar menopauzeleeftijd. De kans op het correct voorspellen van een vroege overgang (≤45 jaar) van 73% werd niet beïnvloed door de toevoeging van snelheid van AMH afname tussen 20-25 jaar. Het was een contra-intuïtieve bevinding dat een hogere AMH-waarde op 20-jarige leeftijd was geassocieerd met een *vroegere* menopauzeleeftijd. In de vroeg volwassen jaren van de vruchtbare levensloop zijn AMH niveaus derhalve mogelijk geen goede reflectie van het aantal primordiale follikels in de eierstokken. Mogelijk is een versneld verval van AMH ook een indicatie van de afgenomen mogelijkheid om een constant aantal follikels te rekruteren voor ontwikkeling in de ovaria. Over het geheel genomen geven deze resultaten aan dat het screenen van AMH niveaus en –afnamesnelheden niet een belangrijke bijdrage kunnen leveren om de 4% vrouwen met een overgang voor hun 45ste op jonge leeftijd te identificeren.

Deel II: De relatie tussen ovariële en cardiovasculaire veroudering

De menopauze is al vaak in verband gebracht met een verhoogd risico op hart- en vaatziekten (HVZ), maar de achterliggende mechanismen zijn nog grotendeels onverklaard. Een onafhankelijk verband van chronologische en reproductieve veroudering met veranderingen van risicofactoren van HVZ werd waargenomen in **Hoofdstuk 6**. In het bijzonder hadden peri- en postmenopauzale vrouwen een meer ongunstig lipidenprofiel na hun 45ste jaar, vergeleken met premenopauzale vrouwen van dezelfde leeftijd. Deze resultaten bevestigen eerder onderzoek en suggereren dat het hogere risico op HVZ in de menopauze mogelijk wordt veroorzaakt door een verslechtering van cholesterolwaarden, leidend tot een hoger risico op atherosclerose. Het bescheiden geobserveerde verschil in cholesterolwaarden tot maximaal 0.4 mmol/L suggereert echter dat er ook andere mechanismen betrokken zijn bij dit verband.

In **Hoofdstuk 8** hadden premenopauzale vrouwen met AMH-waarden van 0.00 ng/mL een groter aantal risicofactoren voor HVZ dan vrouwen met AMH-waarden boven 0.16 ng/mL (gemeten met de Gen II *assay*). Deze resultaten suggereren dat de transitie naar de menopauze al gepaard kan gaan met een verslechtering van risicofactoren van HVZ, onafhankelijk van chronologische leeftijd. Herhaling van de resultaten met alleen vrouwen met een regulaire cyclus en laatste menstruatie in de afgelopen 3 maanden leidde niet tot een verschil in de resultaten. Dit zou kunnen betekenen dat het verband tussen ovariële en cardiovasculaire veroudering al optreedt voordat de afname van ovariële reserve klinisch merkbaar is middels een toegenomen irregulariteit van de cyclus. Mogelijk wordt cardiovasculaire veroudering dus al beïnvloed door ovariële veroudering, of andersom, voorafgaand aan het optreden van de menopauze.

AMH is onderdeel van de *Transforming Growth Factor-beta* (TGF-β) familie. Door binding aan de AMH-specifieke type II AMH receptor (AMHR2) wordt een signaalroute in gang gezet die verwant is aan de signalering door *Bone Morphogenetic Proteins* (BMP's). Activering van de TGF-β en BMP signaalroutes is eerder in verband gebracht met de cascade leidend tot atherosclerotische plaques. Zo zou AMH dus kunnen worden gezien zowel als een biomarker die mogelijk betrokken is bij de pathofysiologie van atherosclerose, als een indicator van ovariële veroudering. In **Hoofdstuk 9** was iedere lagere ng/mL $_{log}$AMH waarde geassocieerd met een 21% hoger risico op totale HVZ en een 26% hoger risico klinische uitingen van coronaire hartziekten. Iedere ng/mL/jaar snellere afname van $_{log}$AMH was voorts geassocieerd met een 46% hoger risico op totale HVZ en een 56% hoger risico op coronaire hartziekten. Deze associaties waren onafhankelijk van menopauzale status, alsmede van klassieke risicofactoren van HVZ zoals cholesterol en bloeddruk. Dit suggereert dat AMH mogelijk betrokken zou kunnen zijn bij de pathofysiologie van HVZ via een andere wijze dan een verslechterd lipidenprofiel. Verder experimenteel en klinisch onderzoek is noodzakelijk om deze hypothese te bevestigen en verder te verhelderen.

Concluderend verschillen AMH afnametrajecten per individu en kan kennis van een individueel afnametraject mogelijk leiden tot een subtiele verbetering van de voorspelling van menopauzeleeftijd. Strategieën om vrouwen te helpen hun zwangerschapswens te voltooien zouden ook gericht moeten worden op het meten van eicelkwaliteit, alsmede informatievoorziening over het belang van tijdige voortplanting en factoren die de vruchtbaarheid kunnen beïnvloeden. Dit proefschrift toont aan dat er een verband bestaat tussen ovariële en cardiovasculaire veroudering, waar meerdere vermeende mechanismen aan ten grondslag kunnen liggen. Voor de ouder wordende vrouw kan reproductieve veroudering derhalve worden bestudeerd in de context van cardiovasculaire veroudering en andersom.

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Annelien de Kat was born in Berkeley, California on December 21st in 1987. Thereafter, she lived in Bennekom and Washington D.C. with her parents and younger siblings, Julian and Helena. After graduating high school in 2006, she studied Pharmacy at the University of Groningen, from which she obtained a BSc degree. Having then decided to pursue a career in medicine, she moved to Utrecht in 2009 to start with the Selective Utrecht Medical Master (SUMMA) program. Obstetrics and Gynecology quickly caught her interest, leading to multiple chosen internships in the field, among which a research project under supervision of prof. dr. Frank Broekmans and dr. Felicia Yarde. After completing her medical degree in 2013 she worked as a clinical resident (not in training) in Obstetrics and Gynecology in the St. Antonius Ziekenhuis under supervision of dr. J.H. Schagen van Leeuwen. In 2014, she started her PhD research in Reproductive Medicine and Epidemiology under supervision of prof. dr. Frank Broekmans and prof. dr. ir. Yvonne van der Schouw at the UMC Utrecht. During this time she completed an Epidemiology Postgraduate degree at Utrecht University. To continue her research, she received a KNAW Ter Meulen Grant to start a postdoctoral research project in Obstetrics at the University of Oxford in January 2017. She will then return to the Netherlands to commence with her specialization in Obstetrics and Gynaecology in July 2017. She is currently living with Jelke Fros in Oxford.

It is done, ik hoop dat u net zo van de voorgaande hoofdstukken heeft genoten als ik!

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