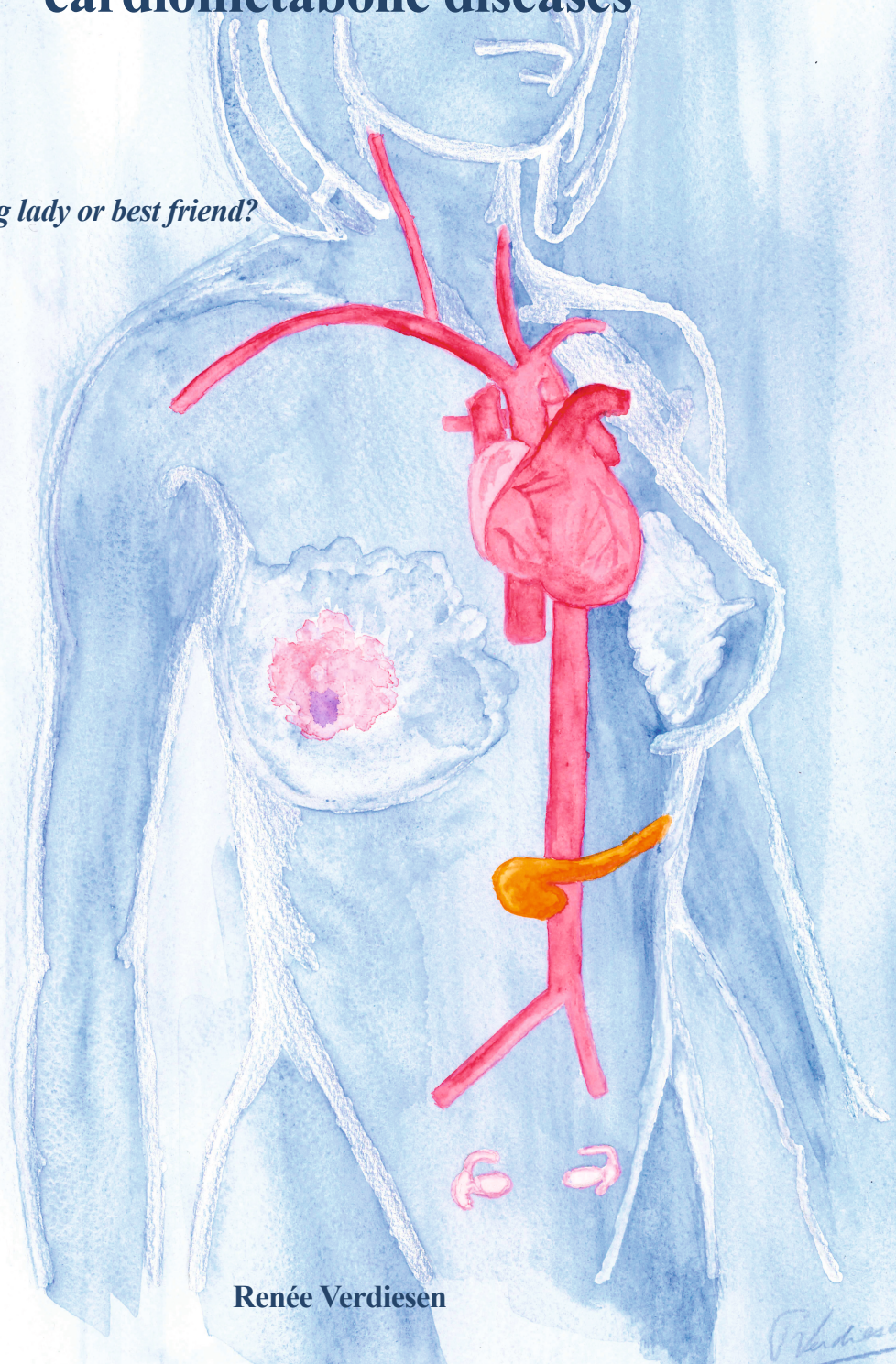
The role of anti-Müllerian hormone in the etiology of cancer and cardiometabolic diseases

Leading lady or best friend?



Renée Verdiesen

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The role of anti-Müllerian hormone in the etiology of cancer and cardiometabolic diseases; Leading lady or best friend?

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The role of anti-Müllerian hormone in the etiology of cancer and cardiometabolic diseases

Leading lady or best friend?

De rol van anti-Müller hormoon in de etiologie van kanker en cardio-metabole ziekten

Leidende dame of beste vriendin?
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
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ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

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door

Renate Maria Gerarda Verdiesen

geboren op 3 november 1990
te Oss

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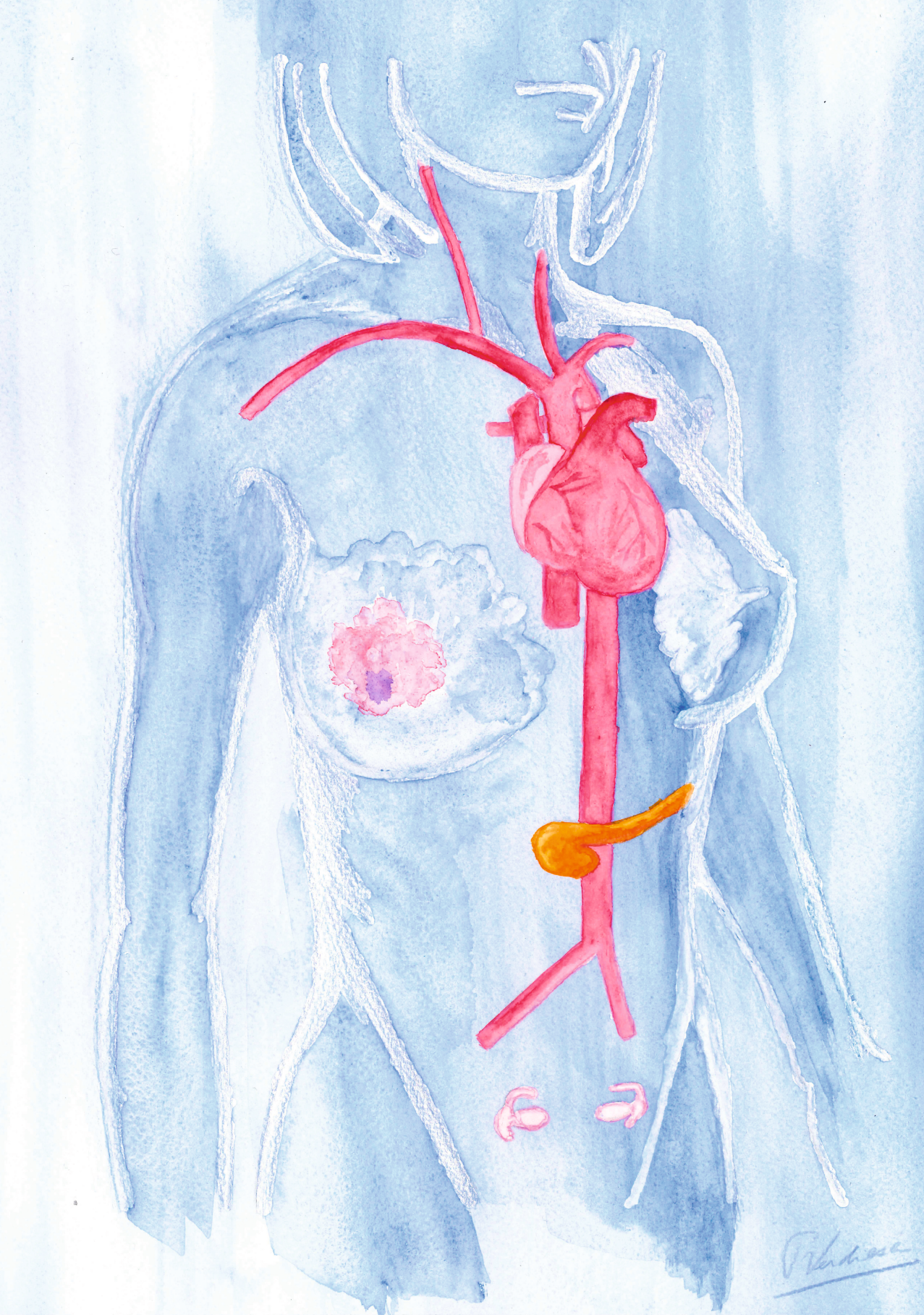

General introduction

CHAPTER 1

Advanced age is the most important risk factor for many diseases, among which most types of cancer and cardiometabolic diseases. Besides age, sex is a key determinant for many non-communicable diseases. Age-standardized incidence rates of mainly cardiovascular disease related outcomes are higher for men than for women, but only until the age of 60 years[1], which is until the menopausal transition is completed. From that moment onwards, age-standardized incidence rates of these diseases are similar for both sexes.[1] This observation has fueled research that investigated the association between female reproductive aging and risk of several non-communicable diseases.

Female reproductive aging and risk of non-communicable diseases

In epidemiological research, accelerated female reproductive aging is often quantified as an earlier age at natural menopause or as a shorter reproductive lifespan, i.e. a shorter period in which a woman would be able to conceive. Previous studies that investigated the relation between natural age at menopause and cancer suggest that accelerated female reproductive aging is associated with a decreased risk of breast[2], ovarian[3] and endometrial cancer[4]. Evidence for an association with other cancer types is less consistent.[5-8] At the same time, accelerated female reproductive aging has also been linked to an increased risk of cardiometabolic diseases, including cardiovascular disease and diabetes.[9-11]

The menopausal transition marks a period of physiological changes, which are mostly attributed to the dramatic drop in estradiol and rise in follicle-stimulation hormone levels.[12] However, the biological mechanisms that explain the association between reproductive aging and risk of non-communicable disease are not fully revealed yet. Whereas the causal role of estrogens, including estradiol, in the development of different cancers is increasingly being recogn ized[13-15], their role in the etiology of cardiometabolic diseases is less clear. Clinical trials investigating whether exogenous estrogens would prevent cardiovascular disease even suggested that supplementing these hormones may increase risk of cardiovascular disease.[16, 17] These findings contradict a protective effect of estrogens on the risk of cardiometabolic diseases. Indirectly, these findings also contest the hypothesis that accelerated reproductive aging, and thus an earlier drop in estradiol, would increase the risk of cardiometabolic diseases. Recent evidence suggests that another hormone could potentially explain the association between reproductive aging and disease risk; anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance.

Anti-Müllerian hormone as indicator of reproductive aging

Until the late 90s, AMH was primarily known for its role in sexual differentiation during embryogenesis.[18] Expression of AMH in male embryos induces regression of the Müllerian ducts, which would have developed into the fallopian tubes, uterus, cervix and upper part of the vagina in the absence of AMH.[19] Intriguingly, AMH is also produced after birth and can be measured in the circulation of both women and men.[20] In women, AMH is expressed in the antral stage ovarian follicles[21], and circulating AMH levels start to decline from age ~25 until menopause[22, 23], when the ovarian reserve is depleted. Consequently, circulating AMH levels can also be used as indicator for female reproductive aging. Accordingly, higher age-specific AMH levels correlate with a higher future age at natural menopause.[24, 25] In men, AMH is produced by Sertoli cells and also decreases with increasing age, although less prominent than in women.[26] In addition, circulating AMH levels have been linked to spermatogenesis quality, which is considered to be a marker for male fertility.[27]

In theory, AMH signaling could also take place in non-gonadal tissues, as the receptor through which AMH signals (AMH receptor type 2; AMHR2) is expressed by a wide range of tissues, including breast, prostate, liver, pancreatic, lung and arterial smooth muscle tissue.[28] Fundamental studies indeed suggest that AMH signaling can take place in cells of the ovary[29, 30], breast[31-33] and prostate[32, 34], but evidence for other tissues is currently lacking. Yet, expression of AMHR2 in tissues involved in the pathogenesis of several cancer types and cardiometabolic disease[28] suggests that AMH may be a mechanism through which reproductive aging is associated with risk of the aforementioned diseases.

Anti-Müllerian hormone as risk factor for non-communicable diseases

In both in vitro and in vivo models for cancer, administration of exogenous AMH induces apoptosis and inhibits cell growth[29-32, 35], suggesting a protective effect of AMH on tumor growth. Conversely, epidemiological studies found that higher endogenous AMH levels were associated with an increased risk of breast cancer.[36-39] These epidemiological findings are in concordance with previous research that suggested that accelerated reproductive aging, quantified as a higher age at natural menopause, is associated with a higher risk of breast cancer.[2] Epidemiological studies have also assessed whether circulating AMH levels are associated with a handful of other cancer types, but their results are less consistent.[40-43] It is therefore still unclear if, and how, circulating AMH levels are associated with risk of other cancer types, besides breast cancer.

Previous studies on ovarian and endometrial cancer mostly included women of late reproductive age in whom variation in AMH is already minimal. As a consequence, these studies were likely underpowered to detect true associations, which could explain their inconsistent findings. One strategy to provide more clarity on the role of AMH in the etiology of cancer is therefore to include a larger proportion of early reproductive women when studying risk of female-specific cancers. Besides, including a wider range of cancer diagnoses would elucidate whether AMH only has a role in the etiology of certain cancer types, or in the etiology of cancer in general. Another knowledge gap concerns the temporal association between circulating AMH levels and risk of cancer. Previous studies only included a single AMH measurement per participant, even though age-related AMH trajectories have been shown to vary between women.[23] Studies including repeated AMH measurements up to the time of cancer diagnosis could provide more insight into the association between circulating AMH levels and risk of cancer over time.

Higher age-specific AMH levels have been associated with a better cardiometabolic health[44-47], which is in concordance with the relation found in observational studies between age at natural menopause and cardiovascular disease and diabetes.[9-11] In addition, AMH levels appear to correlate with intermediate cardiovascular and metabolic outcomes, like atherosclerosis[45] and insulin resistance.[48] These findings are supported by a study in monkeys that found a correlation between higher baseline circulating AMH levels and smaller atherosclerotic plaques after ~2 years.[49] However, most previous studies have a cross-sectional study design, which makes it difficult to disentangle whether circulating AMH levels merely correlate with, or may also have a causal effect on cardiometabolic disease risk. Furthermore, research investigating these associations in men is lagging behind research in women. Finally, generalizability of research findings regarding AMH and diabetes risk to the general population remains to be investigated, since previous studies often only included participants who were already at a higher risk because of obesity or polycystic ovary syndrome.

Aims and outline of the thesis

The overall aim of this thesis was to investigate whether circulating AMH levels are (causally) associated with risk of different non-communicable diseases, including cancer, cardiovascular disease and type 2 diabetes.

The first two chapters focus on the (temporal) association between circulating AMH levels and risk of cancer. **Chapter 2** summarizes the available evidence from the literature

regarding prediagnosis and pretreatment AMH levels and risk of different types of cancer, including but not limited to breast, ovarian and prostate cancer. In **Chapter 3** we investigated the association of age-specific AMH levels with risk of cancer, and more specifically with risk of breast cancer, cancers in other AMHR2-expressing tissues and cancers in non-AMHR2-expressing tissues, using data from female participants of the Doetinchem Cohort Study. We further examined if age-related AMH trajectories were different for women who developed cancer compared to women who did not.

Chapter 4 and 5 focus on the association between circulating AMH levels and cardiometabolic disease outcomes. In **Chapter 4** we again used data from the Doetinchem Cohort Study, this time to investigate associations between age-specific AMH levels at baseline of the cohort, and age-related AMH trajectories and incident type 2 diabetes. In **Chapter 5**, we examined whether circulating AMH levels were associated with different measures of subclinical cardiovascular disease, using cross-sectional and longitudinal data of Dutch middle-aged and older men.

To gain more insight into potential biological mechanisms through which AMH could be involved in the etiology of cancer and cardiometabolic diseases we performed a genome-wide association study for circulating AMH in early and middle reproductive age women (**Chapter 6**). In **Chapter 6**, we additionally explored the causal relationship between circulating AMH levels and risk of breast cancer and polycystic ovary syndrome. In **Chapter 7**, we used summary-level data from the UK Biobank, and the Stroke Genetics Network and DIAMANTE consortia to explore the causal relationship between circulating AMH levels and risk of coronary artery disease, ischemic stroke and type 2 diabetes in women, respectively.

A brief summary of our main findings is presented in **Chapter 8**, along with a discussion of the challenges that we encountered in the interpretation of the results presented throughout this thesis and directions for future research. **Chapter 9** contains a summary of the main findings presented in this thesis.

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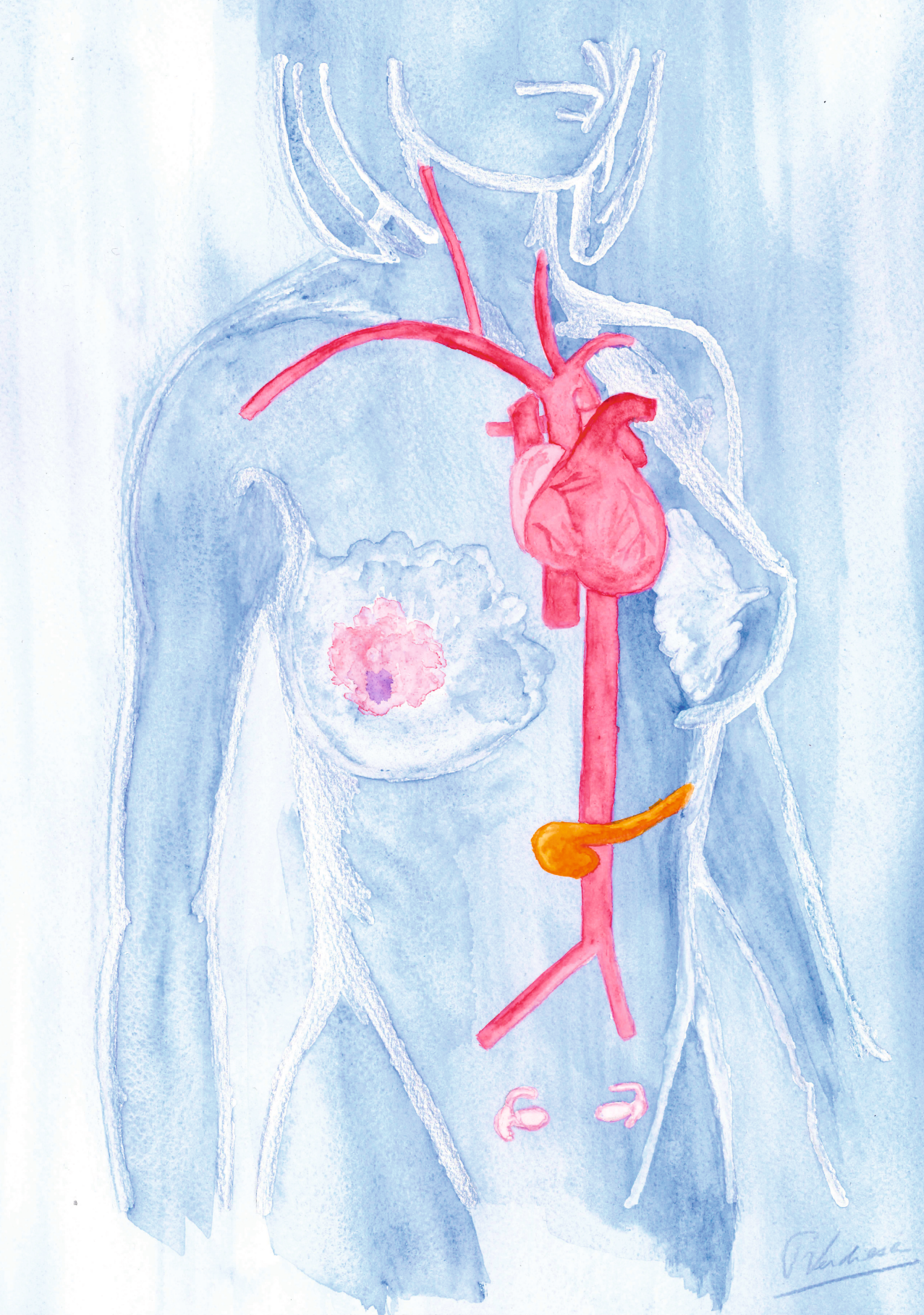

Anti-Müllerian hormone levels and risk of cancer: a systematic review

CHAPTER 2

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Maturitas, 2020; 135:53-67

Abstract

Experimental research suggests that anti-Müllerian hormone (AMH) inhibits tumor growth. Conversely, epidemiological studies suggest that higher AMH concentrations increase breast cancer risk, while associations with other cancers are inconsistent. Therefore, our aim was to provide a systematic review of current epidemiological evidence on AMH levels in relation to different cancer types. We performed a systematic search of PubMed and Embase for publications on circulating AMH in relation to cancer. Methodological quality of articles was assessed using the Study Quality Assessment Tools of the National Heart, Lung and Blood Institute. We included 12 articles on breast, ovarian and endometrial cancer, lymphomas, non-gynecological cancers, childhood cancer and prostate cancer. Five studies measured AMH prior to cancer diagnosis; the other studies measured AMH after diagnosis but prior to treatment. Higher prediagnosis AMH levels were associated with an increased risk of breast cancer. Associations with other types of cancer remained inconclusive, although analyses stratified by age hinted at an increased risk of ovarian and endometrial cancer in younger women. Pretreatment AMH levels were lower in women diagnosed with different types of cancer compared with AMH levels in healthy women. However, because we considered most of the studies that established pretreatment AMH levels to be of poor methodological quality, mainly because of inadequate correction for age at measurement and other important confounders, we refrain from definite conclusions based on these results. Future studies with young participants are needed to assess whether and how AMH affects the risk of different cancer types over time.

Introduction

Anti-Müllerian hormone (AMH) is considered to be a suitable marker for the assessment of ovarian function in women after cancer therapy, given its role in ovarian follicle development.[1] Based on experimental research, it has been suggested that AMH is a potential therapeutic agent for cancer.[2-4] In vivo and in vitro studies showed that administration of AMH induced apoptosis and inhibited tumor cell growth in models for ovarian[5], breast[6, 7] and prostate[7, 8] cancer. The involvement of AMH in processes like cell proliferation and apoptosis, raises the question whether AMH might also inhibit tumor development, especially as many different tissues are potentially responsive to AMH because of expression of the AMH type 2 receptor (AMHR2).[9]

Results from a recent individual participant data (IPD) meta-analysis of 10 studies on AMH and breast cancer suggested that women with higher plasma AMH levels are not protected, but actually have an increased risk of breast cancer.[10] This finding contests the potential protective effect of AMH in cancer biology. Studies on circulating AMH and risk of other types of cancer, including ovarian[11, 12] and prostate cancer[13], are inconsistent. We aimed to provide a systematic overview of the current epidemiological evidence on AMH levels in relation to different types of cancer.

Methods

Data sources and search strategy

We performed a systematic search of the electronic databases PubMed and Embase, last updated on April 29, 2019, for publications on circulating AMH levels in relation to cancer risk. For the identification of eligible publications MeSH and Emtree terms for "anti-Müllerian hormone" and "cancer" were used in combination with title/abstract keywords and synonyms for AMH and cancer. In addition, a combination of terms was used to restrict the search strategy to etiological research. These search strings are represented in Table 1. We additionally searched for articles that cited and were cited by included articles in Scopus and references, respectively. Authors of conference abstracts were contacted if no full-text publication was identified through this search strategy to check whether a full-text publication was available. This systematic review was conducted in adherence to the PRISMA guidelines[14] (Supplemental data).

Table 1: PubMed and Embase search strings.

PubMed search strategy	
Terms for "anti-Müllerian hormone"	(("ANTI-MULLERIAN HORMONE"[MESH]) OR ("AMH"[TIAB] OR ANTI-MULLERIAN HORMONE[TIAB] OR ANTI-MUELLERIAN HORMONE[TIAB] OR ANTIMULLERIAN HORMONE[TIAB] OR ANTIMUELLERIAN[tiab] OR (MULLERIAN INHIBITING[TIAB] OR MULLERIAN INHIBITOR[TIAB] OR MULLERIAN INHIBITORY[TIAB]) OR "ANTI-MULLERIAN FACTOR"[TIAB] OR "MULLERIAN REGRESSION FACTOR"[TIAB]))
Terms for "cancer"	(("Neoplasms"[Mesh]) OR (cancer*[tiab] OR carcinoma*[tiab] OR neoplasm*[tiab] OR tumor*[tiab] OR tumour*[tiab] OR malignan*[tiab] or sarcoma*[tiab] or lymphoma* or leukemi*[tiab] or leukaemi*[tiab] or melanoma[tiab] or oncolog*[tiab] or adenoma*[tiab]))
Terms for restriction to etiological research	((epidemiology[mesh] or "comparative study"[mesh] or comparative stud*[tiab] or risk*[Title/Abstract] OR risk[MeSH:noexp] OR cohort studies[MeSH Terms] OR group[Text Word] OR groups[Text Word] OR grouped [Text Word]) or (Validat* OR Predict*.ti. OR Rule*) OR (Predict*[tiab] AND (Outcome*[tiab] OR Risk*[tiab] OR Model*[tiab])) OR ((History[tiab] OR Variable*[tiab] OR Criteria[tiab] OR Scor*[tiab] OR Characteristic*[tiab] OR Finding*[tiab] OR Factor*[tiab]) AND (Predict*[tiab] OR Model*[tiab] OR Decision*[tiab] OR Identif*[tiab] OR Prognos*[tiab])) OR (Decision*[tiab] AND (Model*[tiab] OR Clinical*[tiab] OR logistic models[mesh])) OR (Prognostic[tiab] AND (History[tiab] OR Variable*[tiab] OR Criteria[tiab] OR Scor*[tiab] OR Characteristic*[tiab] OR Finding*[tiab] OR Factor*[tiab] OR Model*[tiab])))
Embase search strategy	
Terms for "anti-Müllerian hormone"	('Muellerian inhibiting factor'/exp) OR ('AMH':ti,ab,de OR ANTI-MULLERIAN HORMONE:ti,ab,de OR ANTI-MUELLERIAN HORMONE:ti,ab,de OR ANTIMULLERIAN HORMONE:ti,ab,de OR ANTIMUELLERIAN:ti,ab,de OR (MULLERIAN INHIBITING:ti,ab,de OR MULLERIAN INHIBITOR:ti,ab,de OR MULLERIAN INHIBITORY:ti,ab,de) OR 'ANTI-MULLERIAN FACTOR':ti,ab,de OR 'MULLERIAN REGRESSION FACTOR':ti,ab,de))
Terms for "cancer"	(('neoplasm'/exp) OR (cancer*:ti,ab,de OR carcinoma*:ti,ab,de OR neoplasm*:ti,ab,de OR tumor*:ti,ab,de OR tumour*:ti,ab,de OR malignan*:ti,ab,de or sarcoma*:ti,ab,de or lymphoma* or leukemi*:ti,ab,de or leukaemi*:ti,ab,de or melanoma:ti,ab,de or oncolog*:ti,ab,de or adenoma*:ti,ab,de))
Terms for restriction to etiological research	(('epidemiology'/exp OR 'comparative study'/exp OR comparative stud*:ti,ab,de OR risk*:ti,ab,de OR 'risk'/de OR 'cohort analysis'/exp OR group:ti,ab,de OR groups:ti,ab,de OR grouped:ti,ab,de) or (Validat* OR Predict*:ti OR Rule*) OR (Predict*:ti,ab,de AND (Outcome*:ti,ab,de OR Risk*:ti,ab,de OR Model*:ti,ab,de)) OR ((History:ti,ab,de OR Variable*:ti,ab,de OR Criteria:ti,ab,de OR Scor*:ti,ab,de OR Characteristic*:ti,ab,de OR Finding*:ti,ab,de OR Factor*:ti,ab,de) AND (Predict*:ti,ab,de OR Model*:ti,ab,de OR Decision*:ti,ab,de OR Identif*:ti,ab,de OR Prognos*:ti,ab,de)) OR (Decision*:ti,ab,de AND (Model*:ti,ab,de OR Clinical*:ti,ab,de OR 'statistical model'/exp)) OR (Prognostic:ti,ab,de AND (History:ti,ab,de OR Variable*:ti,ab,de OR Criteria:ti,ab,de OR Scor*:ti,ab,de OR Characteristic*:ti,ab,de OR Finding*:ti,ab,de OR Factor*:ti,ab,de OR Model*:ti,ab,de)))

Inclusion and exclusion criteria

For this review, observational studies that investigated pretreatment levels of circulating AMH in relation to cancer risk were considered eligible. Hence, both studies that measured AMH (years) before cancer diagnosis, and studies that measured AMH after cancer diagnosis but before initiation of cancer treatment were included. Study populations

could not comprise participants with a history of cancer, and cancer patients could not have received treatment (e.g. tumor resection, radiotherapy, chemotherapy) before blood draw for AMH measurements, as different cancer treatments have been associated with a decrease in AMH.[15] Studies in which the control group consisted of women who received IVF or were diagnosed with polycystic ovary syndrome were excluded. Studies that did not report a mean (standard deviation) or median [interquartile range] of AMH levels in cancer patients and healthy study participants and/or an odds ratio (95% CI), relative risk (95% CI) or hazard ratio (95% CI), and that did not provide data to allow calculation of an association measure, were also excluded. We additionally excluded review articles, case reports, case series, guidelines and editorials. Conference abstracts were excluded if they had not resulted in a full-text publication. Furthermore, publications in languages other than English were excluded. If the same study population was used in different publications, only the publication with the largest number of participants was included. Consequently, we included the previously mentioned IPD meta-analysis on circulating AMH in relation to breast cancer[10] which included ten nested case-control studies, among which three previously published studies[16-18] that investigated the association between prediagnosis AMH and breast cancer.

Assessment of methodological quality

The methodological quality of each included study was assessed using the Study Quality Assessment Tools developed by the National Heart, Lung and Blood Institute (NHLBI).[19] Depending on the study design, different sets of criteria were used to assess the risk of bias in each study. Granted quality rates ("good", "fair", "poor") were based upon our own judgment, and clarified for studies that we considered to be of "poor" methodological quality. As AMH levels are strongly correlated with age, and age is the most important risk factor for cancer, we considered inadequate (description of) adjustment for age as an important limitation of included studies. Accordingly, studies that did not adjust their analyses for age, lacked details on age-matching of study participants (e.g. no details on age range used for matching), or lacked appropriate statistical methods for analysis of age-matched data (e.g. conditional analyses), received a "poor quality" rating. Apart from adjustment for age, we considered adjustment for at least the following potentially confounding reproductive factors an important requirement for a "good quality" rating in studies including female participants: menopausal status at blood collection, oral contraceptive use at blood collection, hormone replacement therapy at blood collection. In addition, we considered adjustment for smoking behavior at blood collection an important criterion.

Data-extraction

The following data were extracted from each included study: (1) study characteristics (first author, year of publication, country of data collection); (2) study design and, if appropriate, median time between blood draw and cancer diagnosis; (3) description of participants (sample sizes, age range of total study population at blood draw); (4) timing of blood draw for AMH measurement (prediagnosis or pretreatment); (5) sample type and assay used for AMH measurement; (6) whether AMH was included as continuous and/or categorical variable (transformation/categories); (7) how analyses were adjusted for age at AMH measurement; (8) additional factors for which analyses were adjusted; (9) the reported measure(s) of association; (10) conclusions from stratified analyses with respect to age, time to cancer diagnosis and cancer subtypes, and (11) reported measure(s) of association for analyses stratified by age at blood collection. Results were summarized per cancer type.

Results

Search results

Figure 1 shows the flow diagram describing the study selection procedure. A total of 2093 records were identified through our systematic search of PubMed and Embase. After removal of duplicate records (n = 401), we screened titles and abstracts of 1692 records. Main reasons for exclusion at this stage were: non-relevant (e.g. studies that did not include data on AMH measurements and studies on polycystic ovary syndrome), wrong publication type (e.g. reviews and case-reports), and inclusion of unsuitable study populations (e.g. patients only and cancer survivors). Finally, we identified 49 publications that were eligible for full-text screening. Of these 49 publications we excluded 37 publications that did not meet our inclusion criteria as described in Figure 1, resulting in 12 included publications. We identified no additional publications through cross-referencing.

Description of included studies

Characteristics of the 12 included studies are presented in Table 2. Results are presented separately for the following cancer types; breast cancer (n = 4, including the IPD meta-analysis), ovarian cancer (n = 2), endometrial cancer (n = 1), Hodgkin and non-Hodgkin lymphoma (n = 2), non-gynecological cancers (n = 1), childhood cancer (n = 1), and prostate cancer (n = 1). We identified five studies that examined prediagnosis AMH levels in relation to risk of cancer, all were case-control studies nested in prospective cohort studies. We also identified seven studies that compared pretreatment AMH levels in cancer patients with AMH

levels in healthy study participants, of which six were case-control studies and one was a cross-sectional study. Studies were performed in the USA, Europe, China and India.

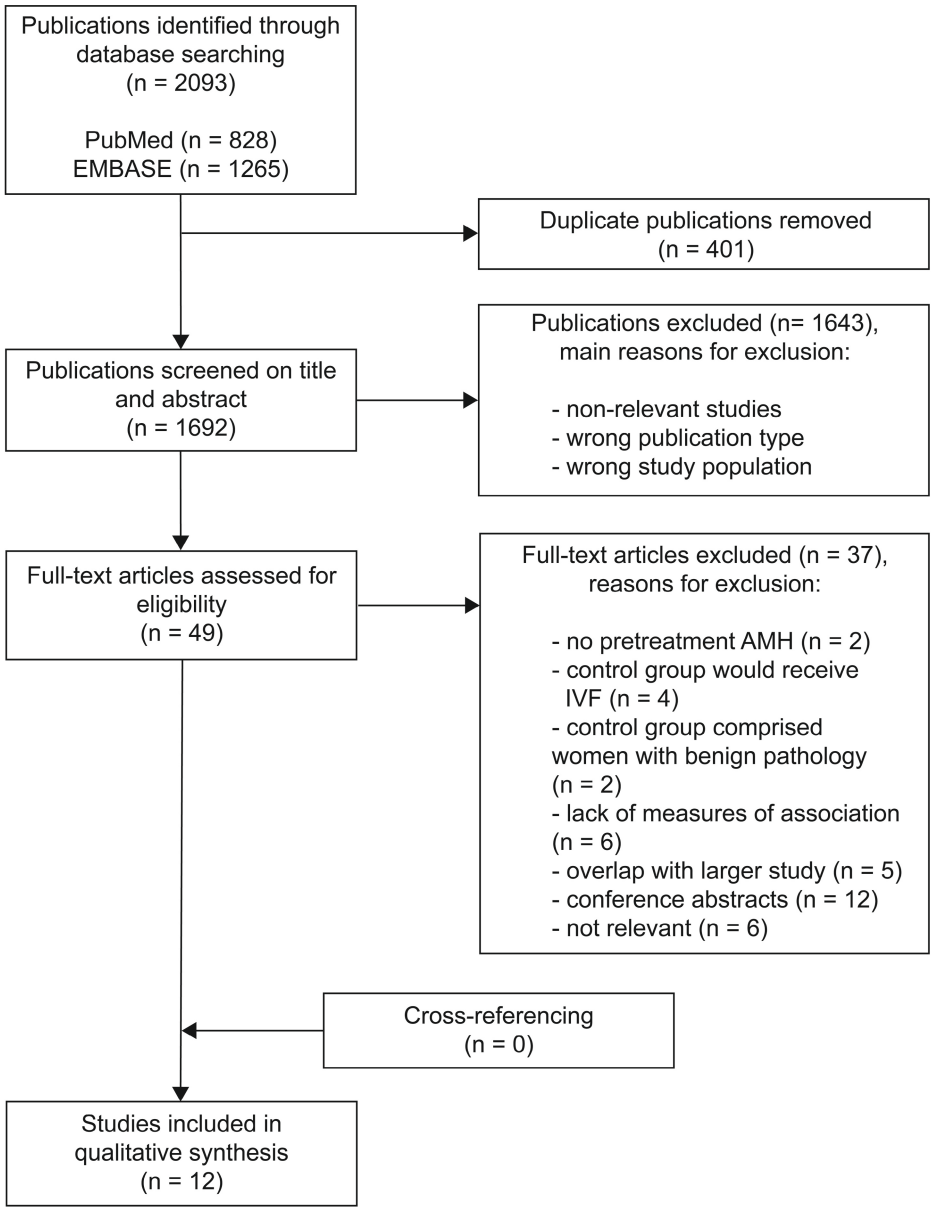

Figure 1. Flow diagram study selection.

Table 2: Study characteristics and AMH levels in relation to cancer.

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Breast cancer									
Ge et al., 2018, USA and Europe[10]	IPD meta-analysis of 10 case-control studies nested in prospective cohort studies[a] (median time to diagnosis ranged from 2.8 to 16.7 years in included studies)	5957 women, of whom 2835 diagnosed with cancer (21 - 57 years) Postmenopausal women and current or prior hormone replacement therapy users were not included	Prediagnosis	Serum or plasma, picoAMH ELISA (Ansh Labs, Webster, TX, USA) or Ultra-sensitive ELISA (Ansh Labs, Webster, TX, USA)	• Continuous (log2-transformed AMH levels) • Categorical (quartiles defined using the study-specific AMH distribution in controls, and consortium wide quartiles)	Age-matching of cases and controls (ranged from ± 6 months to ± 5 years, varied per cohort study), and appropriate analysis of age-matched data: • conditional logistic regression was performed	Race/ethnicity, education, body mass index, age at menarche parity, age at first full-term pregnancy, oral contraceptive use, partial/unilateral oophorectomy, family history of breast cancer, history of benign breast biopsy, smoking, total testosterone	OR_{Q2vsQ1} = 1.18 (1.00 - 1.39) OR_{Q3vsQ1} = 1.32 (1.10 - 1.58) OR_{Q4vsQ1} = 1.60 (1.31 - 1.94), P_{trend} < 0.0001* *Analyses not adjusted for testosterone	• Tumor characteristics: significant association for ER+ (n = 1495 cases), but not for ER- tumors (n = 380 cases); significant association for PR + (n = 1309 cases), but not for PR- tumors (n = 566 cases); other differences were not statistically significant • Age at blood collection: no significant differences ($P_{interaction}$ = 0.16) **£ 40 years:** OR_{Q4vsQ1} = 1.26 (0.93, 1.71); **41-44 years:** OR_{Q4vsQ1} = 1.22 (0.90, 1.66); **45-49 years:** OR_{Q4vsQ1} = 1.83 (1.38, 2.42); **≥50 years:** OR_{Q4vsQ1} = 1.65 (1.03, 2.65) • Age at diagnosis: no significant differences • Menopausal status at diagnosis: no significant differences

Table 2: *(continued)*

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Bala et al., 2016, India[21]	Case-control	60 women, of whom 30 diagnosed with cancer[b] (no details on age reported)	Pretreatment	Serum, Gen II AMH ELISA (Beck-man Coulter, Webster TX, USA)	• Continuous	Age-matching of cases and controls, but no appropriate analysis of age-matched data: • mean values were reported	None	Mean AMH in patients: 1.67 ng/mL ± 0.44 Mean AMH in controls: 1.90 ng/mL ± 0.37 p-value < 0.05	None
Su et al., 2013, USA[20]	Cross-sectional	207 premeno-pausal women, of whom 108 diagnosed with cancer (28 – 44 years)	Pretreatment	Serum, Gen II AMH ELISA (Beck-man Coulter, Webster TX, USA)	• Continuous (natural log-transformed)	Linear regression model was adjusted for age	Gravidity, BMI, race/ ethnicity, regularity of menstrual cycles and cigarette smoking	Predicted geometric mean AMH in patients: 0.85 ng/mL (0.66 - 1.09) Predicted geometric mean AMH in healthy women: 0.76 ng/mL (0.58 - 0.98)	• By age: significant difference ($P_{interaction}$ = 0.017) **< 37 years:** no significant association between AMH and breast cancer status (β = 0.24 (-0.24, 0.72); **≥ 37 years:** AMH levels were significantly lower in cases compared to healthy women (β = -0.85 (-1.48, -0.22)
Lutchman Singh et al., 2007, UK[22]	Case-control	27 premeno-pausal women, of whom 9 diagnosed with cancer (mean age patients 35.2 ± 1.5 years, mean age controls 34.5 ± 0.9 years)	Pretreatment	Serum, ? (Immunotech, Marseille, France)	• Continuous	Age-matching of cases and controls (no age range reported), but no appropriate analysis of age-matched data: • Unpaired t-test	None	Mean AMH in patients: 6.77 ng/mL ± 1.70 Mean AMH in controls: 7.89 ng/mL ± 1.62 p-value > 0.05	None

Table 2: *(continued)*

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Ovarian cancer									
Jung et al., 2018, USA, Europe and China[11]	Nested case-control study including participants from nine prospective cohort studies[c] (median time to diagnosis 9.2 years)	638 women, of whom 302 diagnosed with cancer (24.1 - 46.8 years) Included women were premenopausal at blood draw	Prediagnosis	Serum or plasma, picoAMH ELISA (Ansh Labs, Webster, TX, USA)	• Categorical (quartiles defined using the study-specific AMH distribution in controls, consortium wide quartiles, and quartiles of cohort-adjusted AMH concentration)	Age-matching of cases and controls (88% were matched within ± 1 years; max 2.4 years), and appropriate analysis of age-matched data: • conditional logistic regression was performed Additional adjustment for age at blood draw in regression models	Cases and controls were also matched on date of blood draw and study-specific matching factors (including menstrual cycle day, race, menopausal status and study center), analyses were additionally adjusted for age at menarche and oral contraceptive use	OR_{Q2vsQ1} = 1.03 (0.65 - 1.62) OR_{Q3vsQ1} = 1.14 (0.70 - 1.85) OR_{Q4vsQ1} = 0.99 (0.59 - 1.67), P_{trend} = 0.91	• Disease subtype: no significant differences • Age at diagnosis: no significant differences • Age at blood collection: no significant differences (Pinteraction = 0.26) < 40 years: ORQ4vsQ1 = 1.17 (0.52, 2.62); ≥ 40 years: ORQ4vsQ1 = 1.06 (0.70, 1.60) • Restriction to women diagnosed more than 1 year after blood draw: no substantial difference

Table 2: *(continued)*

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Schock et al., 2014, Finland[12]	Nested case-control study (median time to diagnosis 9.1 years)	315 women, of whom 107 diagnosed with cancer (22.4 - 43.2 years); blood samples were donated during the first trimester of pregnancy	Prediagnosis	Serum, second generation specific ELISA (Diagnostic Systems Labora-tories, Webster, USA)	• Continuous (log2-transformed AMH levels) • Categorical (tertiles defined using the frequency distribution of AMH of all controls)	Age-matching of cases and controls (± 6 months), and appropriate analysis of age-matched data: • conditional logistic regression was performed	Cases and controls were also matched on date at sample donation, parity at sample donation and parity at diagnosis, analyses were additionally adjusted for gestational age and family history of ovarian and/or breast cancer	OR_{log2} = 0.99 (0.79 - 1.24) OR_{T2vsT1} = 0.99 (0.52 - 1.87) OR_{T3vsT1} = 0.93 (0.49 - 1.77)	• By median of age at sampling (32.7 years): significant difference ($P_{homogeneity}$ = 0.002) **< 32.7 years:** OR_{log2} = 1.64 (1.06, 2.54); **≥ 32.7 years:** OR_{log2} = 0.69 (0.49, 0.96) • By median age at diagnosis (42.9 years): associations were not statistically significant for subgroups although effect estimates across subgroups appeared to be heterogeneous ($P_{homogeneity}$ = 0.02) • By median lag time to diagnosis (9.1 years): no significant differences • By tumor stage: no significant differences • Restriction to women diagnosed 2 or 5 years after blood donation: no significant differences • Restriction to cases diagnosed before age 50: no significant differences

Table 2: *(continued)*

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Endometrial cancer									
Fortner et al., 2017, USA, Europe and China[23]	Nested case-control study including partici-pants from eight prospec-tive cohort studies[d] (median time to diagnosis 12 years)	668 women, of whom 329 diagnosed with cancer (19 - 47 years) Included women were premeno-pausal at blood draw	Prediagnosis	Serum or plasma, picoAMH ELISA (Ansh Labs, Webster, TX, USA)	• Continuous (log2-transformed AMH levels) • Categorical (tertiles defined using the study-specific AMH distribution in controls)	Age-matching of cases and controls (ranged from ± 6 months to ±2 years, varied per study), and appropriate analysis of age-matched data: • conditional logistic regression was performed Additional adjustment for age at blood draw in regression models	Individual studies matched cases and controls on different factors, including: study center, time of day at blood collection, fasting status, and menstrual cycle phase, menopausal status, current use of oral contraceptive or hormone replacement therapy	OR_{log2} = 1.07 (0.99 - 1.17) OR_{T2vsT1} = 1.34 (0.89 - 2.02) OR_{T3vsT1} = 1.29 (0.82 - 2.03)	• Disease subtype: no significant differences • Age at blood donation: no significant differences ($P_{heterogeneity}$ = 0.13) **£ 40 years:** ORlog2 = 1.10 (0.94, 1.30), OR_{T3vsT1} = 1.97 (0.76, 5.12) **> 40 years:** ORlog2 = 1.07 (0.97, 1.18), OR_{T3vsT1} = 1.36 (0.79, 2.35) • Time to diagnosis: no significant differences • Cancer-related characteristics: no significant differences • Restriction to women diag-nosed more than 1 and 2 years after blood draw: no significant differences
Lymphoma (Hodgkin and non-Hodgkin)									
Lawrenz et al., 2012, Germany[24]	Case-control	No total number of participants reported[e], 38 women diagnosed with cancer (18 - 33 years)	Pretreatment	Serum, Gen II AMH ELISA (Beck-man Coulter, Webster TX, USA)	• Continuous	Age-matching of cases and controls (no age range for matching reported), and appropriate analysis of age-matched data: • Wilcoxon signed rank test	None	Mean AMH in patients: 2.06 ng/mL ± 1.52 Mean AMH in controls: 3.20 ng/mL ± 2.19 p-value < 0.05	None

Table 2: *(continued)*

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Lekovich et al., 2016, USA[25]	Case-control	429 women, of whom 64 diagnosed with cancer (17 – 40 years)	Pretreatment	Serum, Gen II AMH ELISA (Beck-man Coulter, Webster TX, USA)	• Continuous	None; age was significantly different between cases (median age: 30.5 years, IQR 28-34 years) and controls (median age: 37 years, IQR 35-39 years)	None	Mean AMH in patients: 1.08 ± 0.74 ng/mL Mean AMH in controls: 2.03 ± 1.93 ng/mL p-value < 0.001	None
Non-gynecological cancer									
Paradisi et al., 2016, Italy[26]	Case-control	234 women, of whom 191 diagnosed with cancer (12 -39 years)	Pretreatment	Serum, Gen II AMH ELISA (Beck-man Coulter, Webster TX, USA)	• Continuous	None; but age was not significantly different between cases (mean age 26.4 ± 6.9 years) and controls (mean age 28.8 ± 6.2 years)	None	Median (IQR) AMH in patients: 2.80 ng/mL (1.60 - 4.15) Median (IQR) AMH in controls: 2.80 ng/mL (1.20 - 4.90)	None

Table 2: *(continued)*

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Childhood cancer									
van Dorp et al., 2014, The Netherlands[27]	Case-control	458 girls, of whom 208 diagnosed with cancer (0 - 18 years)	Pretreatment	Serum, Gen II AMH ELISA (Beck-man Coulter, Webster TX, USA)	• Continuous (log-transformed AMH levels and standard deviation scores (SDS)) • Categorical (percentiles, based on data controls)	Age-matching of cases and controls (no age range for matching reported), calculation of SDS which is an appropriate method to adjust for age. No details on analysis of continuous age-matched data are reported	None	Difference in mean AMH SDS, cases vs controls: -0.8, p-value < 0.001 Median AMH in patients: 1.4 µg/L (0.1 - 10.2) Median AMH in controls: 3.0 µg/L (0.1 - 18.3)	• By diagnosis: no significant differences

Table 2: *(continued)*

Authors, year of publication, country	Study design (median time to diagnosis, years)	Participants (age range study population at blood draw, years)	Timing blood collection	Sample type, AMH assay	Continuous/ categorical AMH	Age-adjustment	Additional factors adjusted for	Reported measures of association (mean ± sd; median (IQR); OR (95% CI))	Stratified analyses (regarding cancer types and/ or age effects)
Prostate cancer									
Sklavos et al., 2014, USA[13]	Nested case-control study (median time to diagnosis 3 years)	1997 men, of whom 998 diagnosed with cancer (median age: 65 years)	Prediagnosis	Serum, Gen II AMH ELISA (Beckman Coulter, Webster, TX, USA)	• Continuous • Categorical (quartiles defined using the AMH distribution in controls)	Age-matching of cases and controls (matched in 5-year intervals), but no appropriate analysis of age-matched data: unconditional logistic regression Additional adjustment for age	Cases and controls were additionally matched on calendar year of enrollment and number of years of follow-up	OR_{T2vsT1} = 1.17 (0.91 - 1.50) OR_{T3vsT1} = 1.16 (0.90 - 1.49) OR_{T4vsT1} = 1.15 (0.89 - 1.48), P_{trend} = 0.13	• Disease subtype: no significant differences, but when different definitions for aggressive prostate cancer were used the second AMH quartile was associated with increased risk of aggressive prostate cancer • Restriction to men diagnosed within 2, 3, and 5 years of blood collection: no significant differences

AMH, anti-Müllerian hormone; sd, standard deviation; IQR, interquartile range; OR, odds ratio; 95% CI, 95% confidence interval; IPD, individual participant data; ER, estrogen receptor; PR, progesterone receptor; SDS, standard deviation score

[a] The ten participating cohorts were: Breakthrough Generations Study; Campaign Against Cancer and Heart Disease; Columbia, Missouri Serum Bank; Guernsey cohort; Nurses' Health Study; Nurses' Health Study II; Northern Sweden Mammography Screening Cohort; New York University Women's Health Study; Hormones and Diet in the Etiology of Breast Cancer and the Sister Study

[b] Publication did not report how many breast cancer patients were included in the pretreatment analyses; 30 patients were enrolled but divided into prechemotherapy and postchemotherapy subgroups

[c] The nine participating cohorts were: Columbia, Missouri Serum Bank; Campaign Against Cancer and Heart Disease; European Prospective Investigation into Cancer and Nutrition; Guernsey Cohort Study; New York University Women's Health Study; Nurses' Health Study and Nurses' Health Study II; Hormones and Diet in the Etiology of Breast Cancer; Northern Sweden Health and Disease Study and the Shanghai Women's Health Study

[d] The eight participating cohorts were: Columbia, Missouri Serum Bank; Campaign Against Cancer and Heart Disease; New York University Women's Health Study; European Prospective Investigation into Cancer and Nutrition; Guernsey Cohort Study; Hormones and Diet in the Etiology of Breast Cancer; Northern Sweden Health and Disease Study and the Shanghai Women's Health Study

[e] Publication did not report the number of included controls; figure 1 in publication presents 38 individual data counts for the control group, which suggests that 38 controls were included

Quality assessment

Table 3 presents the individual components on which quality assessment of the individual (nested) case-control studies was based. Overall quality rates for these studies are also presented in Table 3. Quality assessment of the included cross-sectional study is presented in Table 4. We considered four studies to be of "good", two studies to be of "fair" and six studies to be of "poor" quality. Because age-matching of cases and controls was either not conducted at all, inadequately described or not taken into account in the statistical analyses, and adjustment for other potential confounders was completely lacking, all six case-control studies that investigated pretreatment AMH levels in relation to cancer were considered to be of "poor" quality. The two studies that we considered to be of "fair" quality adjusted inadequately for important confounders. This should be taken into account when interpreting corresponding results.

Breast cancer

Of the four studies that investigated AMH levels in relation to breast cancer, one study included prediagnosis AMH levels. This large IPD meta-analysis of ten nested case-control studies calculated AMH quartiles for each participating cohort separately, as actual values are not comparable between cohorts, because of between study differences. Subsequently, they estimated breast cancer risk per cohort-specific quartile with the lowest cohort-specific quartile as the reference, and finally pooled ORs. They observed that higher AMH levels were associated with an increased risk of breast cancer (p_{trend} across AMH quartiles < 0.0001).[10] More specifically, women in the highest AMH quartile were at a 60% increased risk of breast cancer, compared to women of the same age in the lowest AMH quartile (OR_{Q4vsQ1} = 1.60, 95% CI: 1.31, 1.94). In nine of the ten included studies the direction of this association was consistent with the IPD meta-analysis result, and no significant heterogeneity between the studies was observed (I^2 = 22.7%, p-value = 0.23). In the meta-analysis, the association between AMH and breast cancer risk appeared to be strongest within women aged 45 – 49 years at blood collection (OR_{Q4vsQ1} = 1.83, 95% CI: 1.38, 2.42) compared to younger and older women (£40 years: OR_{Q4vsQ1} = 1.26, 95% CI: 0.93, 1.71; 41 – 44 years: OR_{Q4vsQ1} = 1.22, 95% CI:0.90, 1.66; ≥50 years: OR_{Q4vsQ1} = 1.65, 95% CI: 1.03, 2.65). As opposed to these findings, pretreatment AMH levels were significantly lower in breast cancer cases compared to healthy subjects in a cross-sectional study, although only in women older than 37 (threshold calculated using bootstrap procedure) at the time of blood collection (β = -0.85, 95% CI: -1.48, -0.22).[20] Two case-control studies also found lower pretreatment AMH levels in breast cancer cases compared to healthy controls.[21, 22] The cross-sectional study adjusted for age at measurement through inclusion of age in the regression models. The two case-control studies reported to have matched cases and controls on age, but only one study described the age distribution separately for cases and controls (mean age cases ± sd: 35.2 ± 1.5 years; mean age controls ± sd: 34.5 ± 0.9 years).[22] Both case-control studies lacked appropriate analyses for matched data.

Table 3: Quality assessment of (nested) case-control studies.

		Breast cancer		**Ovarian cancer**		**Endometrial cancer**	**Lymphoma**		**Non-gynecological cancer**	**Childhood cancer**	**Prostate cancer**
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
1. Was the research question or objective in this paper clearly stated and appropriate?	Yes	No*	Yes	Yes	Yes	Yes	Yes	No*	Yes	Yes	Yes
2. Was the study population clearly specified and defined?	Yes	No	No	Yes	Yes	Yes	No*	No**	Yes	No*	Yes
3. Did the authors include a sample size justification?	No	No	No*	No	No	Yes	No	No	No	No	No
4. Were controls selected or recruited from the same or similar population that gave rise to the cases (including the same timeframe)?	Yes	CD	CD	Yes	Yes	Yes	CD**	No***	Yes	Yes**	Yes

Table 3: *(continued)*

		Breast cancer		**Ovarian cancer**		**Endometrial cancer**	**Lymphoma**		**Non-gynecological cancer**	**Childhood cancer**	**Prostate cancer**
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
5. Were the definitions, inclusion and exclusion criteria, algorithms or processes used to identify or select cases and controls valid, reliable, and implemented consistently across all study participants?	Yes	CD	CD	Yes	Yes	Yes	Yes	CD	Yes	CD	Yes
6. Were the cases clearly defined and differentiated from controls?	No*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
7. If less than 100 percent of eligible cases and/or controls were selected for the study, were the cases and/or controls randomly selected from those eligible?	CD	CD	CD	CD	Yes	CD	CD	CD	CD	CD	Yes

Table 3: *(continued)*

		Breast cancer		Ovarian cancer		Endometrial cancer	Lymphoma		Non-gynecological cancer	Childhood cancer	Prostate cancer
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
8. Was there use of concurrent controls?	Yes	NR	NR	NR	NR	Yes	NR	NR	NR	No	NR
9. Were the investigators able to confirm that the exposure/ risk occurred prior to the development of the condition or event that defined a participant as a case?	Yes	No	No	Yes	Yes	Yes	No	No	No	No	Yes
10. Were the measures of exposure/risk clearly defined, valid, reliable, and implemented consistently (including the same time period) across all study participants?	No**	CD	Yes**	Yes	Yes	Yes	CD	CD	Yes	Yes	Yes

Table 3: *(continued)*

		Breast cancer		**Ovarian cancer**		**Endometrial cancer**	**Lymphoma**		**Non-gynecological cancer**	**Childhood cancer**	**Prostate cancer**
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
11. Were the assessors of exposure/ risk blinded to the case or control status of participants?	Yes	NR	NR	Yes	Yes	Yes	NR	NR	NR	NR	Yes
12. Were key potential confounding variables measured and adjusted statistically in the analyses? If matching was used, did the investigators account for matching during study analysis?	Yes	No**	No***	Yes	Yes*	Yes*	CD***	No****	CD*	CD***	No*
Overall quality rating	Good	Poor	Poor	Good	Good	Good	Poor	Poor	Poor	Poor	Fair

Table 3: *(continued)*

	Breast cancer			Ovarian cancer		Endometrial cancer	Lymphoma		Non-gynecological cancer	Childhood cancer	Prostate cancer
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
*Clarification #1	No description of identification of cases (e.g. by self-report and/or linkage to cancer and/or mortality registries).	The aim in the main text does not include the compare-son with the healthy controls, whereas the aim section in the abstract does.	Pilot study.		Inclusion of confounders in statistical models was based on their effect on effect estimates, resulting in models only adjusted for gestation-al age and family history of ovarian and/or breast cancer.	Inclusion of confounders in statistical models was based on their effect on effect estimates, resulting in models only adjusted for age at blood draw.	Number of controls is not reported.	Because there is no compari-son with men in this study. Aim in abstract and aim in main text are different.	Comparison of median AMH levels in cases compared to controls, cannot be adjusted for confounders. No matching on age was performed, although age did not significantly differ between both groups.	No time period of the inclusion of cases has been reported. In addition, it is not mentioned whether all cases were included or only a selection.	Only correction for age at randomi-zation, because univariate analyses did not identify confounding variables. Investigators performed frequency matching of cases and controls but performed uncondition-al logistic regression.

Table 3: *(continued)*

	Breast cancer		Ovarian cancer			Endometrial cancer	Lymphoma		Non-gynecological cancer	Childhood cancer	Prostate cancer
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
** Clarification #2	For one cohort a different AMH assay was used, and for two studies samples were measured at a different location. However, a calibration study showed excellent agreement between these and the other measure-ments.	Comparison of mean AMH levels in cases compared to controls, cannot be adjusted for confounders. It is not clear if matching on age was performed using a formal protocol, or whether just controls of similar age were selected. The distribu-tion of age in cases and controls is not described. No appropriate analysis of age-matched data was performed.	Time period is not reported but storage conditions of samples were the same.				Not reported where the included healthy volunteers came from, i.e. how they were recruited.	The control group is not clearly specified.		No time frame has been reported for inclusion of healthy controls either.	

Table 3: *(continued)*

		Breast cancer		Ovarian cancer		Endometrial cancer	Lymphoma		Non-gynecological cancer	Childhood cancer	Prostate cancer
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
*** Clarification #3			Comparison of mean AMH levels in cases compared to controls, cannot be adjusted for confounders. It is not clear if matching on age was performed using a formal protocol, or whether just controls of similar age were selected. Age did not significantly differ between both groups. No appropriate analysis of age-matched data was performed.				Comparison of mean AMH levels in cases compared to controls, cannot be adjusted for confounders. It is not clear if matching on age was performed using a formal protocol, or whether just controls of similar age were selected. The distribution of age in cases and controls is not described. However, appropriate analyses for age-matched data were performed.	Controls were women who underwent elective oocyte Cryopreservation, which is a selective group.		Comparison of mean AMH standard deviation scores in cases compared to controls, cannot be adjusted for confounders. It is not clear if matching on age was performed using a formal protocol, or whether just controls of similar age were selected.	

Table 3: *(continued)*

		Breast cancer		**Ovarian cancer**		**Endometrial cancer**	**Lymphoma**		**Non-gynecological cancer**	**Childhood cancer**	**Prostate cancer**
	Ge et al., 2018, USA (and Europe)[10]	Bala et al., 2016, India[21]	Lutchman Singh et al., 2007, UK[22]	Jung et al., 2018, USA (, Europe and China)[11]	Schock et al., 2014, Finland[12]	Fortner et al., 2017, USA (, Europe and China)[23]	Lawrenz et al., 2012, Germany[24]	Lekovich et al., 2016, USA[25]	Paradisi et al., 2016, Italy[26]	van Dorp et al., 2014, The Netherlands[27]	Sklavos et al., 2014, USA[13]
****Clarification #4								Investigators report that they performed logistic regression to control for age, but do not report age-adjusted odds ratios for the effect of AMH, only uncorrected mean AMH levels. Age was significantly different between cases and controls.			

CD, cannot determine; NR, not reported; N/A, not applicable

Table 4: Quality assessment of cross-sectional study.

	Breast cancer Su et al., 2013, USA[20]
1. Was the research question or objective in this paper clearly stated?	Yes
2. Was the study population clearly specified and defined?	Yes
3. Was the participation rate of eligible persons at least 50%?	CD
4. Were all the subjects selected or recruited from the same or similar populations (including the same time period)? Were inclusion and exclusion criteria for being in the study prespecified and applied uniformly to all participants?	Yes
5. Was a sample size justification, power description, or variance and effect estimates provided?	Yes
6. For the analyses in this paper, were the exposure(s) of interest measured prior to the outcome(s) being measured?	Yes
7. Was the timeframe sufficient so that one could reasonably expect to see an association between exposure and outcome if it existed?	N/A
8. For exposures that can vary in amount or level, did the study examine different levels of the exposure as related to the outcome (e.g., categories of exposure, or exposure measured as continuous variable)?	Yes
9. Were the exposure measures (independent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?	Yes
10. Was the exposure(s) assessed more than once over time?	N/A
11. Were the outcome measures (dependent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?	Yes
12. Were the outcome assessors blinded to the exposure status of participants?	NR
13. Was loss to follow-up after baseline 20% or less?	N/A
14. Were key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure(s) and outcome(s)?	No*
Overall quality rating	Fair
*Clarification #1	No adjustment for hormone replacement therapy and oral contraceptive use

CD, cannot determine; NR, not reported; N/A, not applicable

Ovarian cancer

Two independent nested-case control studies investigated the association between prediagnosis AMH levels and risk of ovarian cancer.[11, 12] In both studies, AMH levels were not associated with risk of ovarian cancer in the main analysis. However, a study which included 315 pregnant women (range age at blood draw: 22.4 -43.2 years) observed that a doubling in AMH concentration in women younger than 32.7 years (median age) at blood draw was associated with an increased risk of invasive serous ovarian cancer (OR = 1.64, 95% CI: 1.06, 2.54).[12] In contrast, in the same study a doubling in AMH concentration was associated with a decreased risk of ovarian cancer in women older than 32.7 years at blood draw (OR = 0.69, 95% CI: 0.49, 0.96). No apparent age effect was observed in the study by Jung et al. (range age at blood draw: 24.1 – 46.8), although the number of cases per AMH category for both age strata (< 40 years vs. ≥ 40 years) was limited.[11]

Endometrial cancer

One nested-case control study examined prediagnosis AMH levels in relation to risk of endometrial cancer.[23] This study included 329 endometrial cancer cases and 339 controls from eight prospective cohort studies (range age at blood draw: 19 – 47). Neither the analyses of log-transformed AMH levels, nor the analyses of AMH tertiles (OR_{T3vsT1} = 1.29, 95% CI: 0.82, 2.03) provided evidence for an association between circulating AMH levels and risk of endometrial cancer. Analyses stratified by age (£40 years vs. >40 years) did not support effect-modification by age ($p_{heterogeneity}$ = 0.13), although stronger effect estimates were observed for women up to 40 years of age compared to women older than 40 years (OR_{T3vsT1} = 1.97, 95% CI: 0.76, 5.12; and OR_{T3vsT1} = 1.36, 95% CI: 0.79, 2.35, respectively).

Lymphoma

Two case-control studies compared mean pretreatment AMH levels in lymphoma patients to mean AMH levels in healthy controls.[24, 25] In these studies, lymphoma patients included Hodgkin and non-Hodgkin lymphomas. In both studies, pretreatment AMH levels were significantly lower in lymphoma patients than in controls (2.06 ± 1.52 ng/mL versus 3.20 ± 2.19 ng/mL and 1.08 ± 0.74 ng/mL versus 2.03 ± 1.93 ng/mL, respectively). One of these studies completely lacked age-adjustment, and reported a significant difference in age between cases (median age (IQR): 30.5 years (28-34) and controls (median age (IQR): 37 years (35-39)).[25] The other study performed age-matching of cases and controls and applied appropriate statistical analyses, but lacked details on the matching procedure and age distribution of cases and controls.[24]

Non-gynecological cancer

A case-control study that included a group of non-gynecological malignancies without further specification of diagnoses, observed similar median pretreatment AMH levels in patients and healthy controls (2.80 ng/mL (1.60-4.15) and 2.80 ng/mL (1.20 – 4.90), respectively).[26] Although matching of cases and controls on age was not described, age was not significantly different between cases (mean age ± sd: 26.4 ± 6.9 years) and controls (mean age ± sd: 28.8 ± 6.2 years).

Childhood cancer

One case-control study investigated pretreatment AMH levels in relation to childhood cancer in girls.[27] Diagnoses included lymphoblastic leukemia, acute myeloid leukemia, Hodgkin and non-Hodgkin lymphoma, nephroblastoma, sarcoma and neuroblastoma. This study found significantly lower mean AMH standard deviation scores (SDS) in patients compared to controls (difference in mean AMH SDS between cases and controls: -0.8, $P < 0.001$) and additionally reported median AMH levels in cases and controls (1.4 µg/L (0.1 – 10.2) and 3.0 µg/L (0.1 – 18.3), respectively). Mean AMH SDS were comparable across different types of cancer. The use of SDS is an appropriate method to adjust for age effects in age-matched data.

Prostate cancer

Only one of the included studies focused on circulating AMH in relation to risk of cancer in men.[13] This nested case-control study investigated prediagnosis AMH levels in relation to prostate cancer in 998 cases and 999 controls. No association was observed between AMH quartiles and risk of prostate cancer ($OR_{Q4vsQ1} = 1.15$, 95% CI: 0.89, 1.48). Potential effect modification by age at blood draw was not examined in this study.

Discussion

In this systematic review we show that higher prediagnosis AMH levels are associated with an increased risk of breast cancer, whereas associations with other types of cancer are inconclusive. In contrast, pretreatment AMH levels are generally lower in women diagnosed with different types of cancer compared to AMH levels in healthy women. However, because we considered most studies including pretreatment AMH levels to be of poor methodological quality due to inadequate adjustment for age at AMH measurement and other potential confounders, we cannot draw definite conclusions based on the corresponding results.

This is the first review that presents a systematic overview of the current epidemiological evidence on the relation of AMH levels with risk of different cancer types. We performed an extensive systematic search of PubMed and Embase up to April 2019. Studies that measured AMH in either prediagnosis or pretreatment samples were included to gain more insight in differences in AMH levels between cancer cases and healthy individuals, both years before and at the moment of diagnosis. Unfortunately, given the limited number of studies for each cancer outcome, we could not perform meta-analyses. Besides, we cannot exclude the possibility of publication bias, even though we tried to limit this by contacting authors of conference abstracts for which we did not find a full-text publication.

Some limitations with regard to the studies included in this review have to be addressed as well. In both women[28, 29] and men[13, 30] AMH levels are strongly correlated with age. As a result, age is an important confounder in the association between AMH and risk of cancer, and adequate adjustment for age at blood collection is therefore very important. However, most studies that included pretreatment AMH levels did not adequately adjust their analyses for age at blood collection. Studies either did not take age at measurement in account at all, or reported to have matched their cases and controls on age but did not perform appropriate analyses for matched data. Also details on the age-matching procedures, like the age range used to match participants, were often lacking. Apart from potential confounding by age, confounding by other risk factors for cancer (e.g. smoking behavior or oral contraceptive use) cannot be excluded in most studies. Adjustment for important confounders was often lacking or selection of confounders was based on univariate analyses, even though confounder selection based on available knowledge has the preference.[31] In line with this, adjustment for circulating testosterone might be important as in women a statistically significant positive correlation between AMH and testosterone has been observed.[32, 33] Only Ge et al. retained circulating testosterone in their final analyses, but no evident confounding effect of testosterone was observed.[10]

The IPD meta-analysis on breast cancer observed that the association of AMH with breast cancer risk was strongest in middle aged women (45-49 years old), compared to younger and older women. However, the confidence intervals of the different age groups largely overlapped. This finding may be driven by the inclusion of a number of small studies that included older women and reported very large effect sizes. Ideally a one-stage analysis with inclusion of study-specific effects should be performed to adequately assess if age at measurement is indeed an effect-modifier.[34] Studies on ovarian and endometrial cancer did not observe a stronger effect of AMH in older women. On the contrary, effect estimates appeared to be stronger in younger women, although only statically significant in de study of Schock et al.[12] This could have to do with limited statistical power to detect an actual risk-increasing effect in most studies, as the proportion of women younger than 40 years was

limited. Considering its biological function, AMH levels strongly decrease from the age of 40 and become undetectable around menopause. Inclusion of mostly middle aged women could therefore explain null findings in the main analyses on prediagnosis AMH. AMH measurements at an age at which variation was already minimal, hampers the detection of a potential association. In comparison, participants in studies including pretreatment AMH measurements were in general younger than 40 years at the time of blood donation.

A limitation of studies that measured AMH at the time of cancer diagnosis is that a potential causal association between AMH and cancer cannot be proven based on their results, although in combination with the evidence from prospective studies, i.e. nested case-control studies, they can provide some insight into a potential temporal association between AMH and cancer. However, we observed no consistent relation between prediagnosis and pretreatment AMH levels and cancer. For instance, AMH levels were higher years before breast cancer diagnosis, whereas pretreatment AMH levels were lower when compared to healthy controls. Ideally, to elucidate if and how AMH is involved in tumor development, future studies should include repeated AMH measurements up to cancer diagnosis and assess how AMH trajectories differ between cancer patients and healthy individuals. In addition, studies including repeated AMH measurements are needed to complement the studies included in this review which only included single AMH measurements, as previous research has shown that AMH trajectories differ between women.[29]

Based on the wide range of tissues that express the receptor through which AMH signaling occurs (e.g. prostate and ovarian tissue), we hypothesized that if AMH would play a role in tumor development we would observe a similar effect of AMH on risk of different types of cancer. Yet we only observed an association between prediagnosis AMH and risk of breast cancer, where women with higher AMH levels were at an increased risk of breast cancer. Although the number of studies on other types of cancer is small, and they included a much smaller number of cases, it is also possible that the association between AMH and breast cancer does not involve AMHR2 signaling. Instead, circulating AMH levels might be a biomarker for ovarian reserve and, accordingly, a proxy for time to menopause. Higher AMH levels are indicative of a larger ovarian reserve, and of a later age at menopause.[35, 36] Previous studies observed that a later age at menopause is observed with an increased risk of breast cancer.[37, 38] Moreover, Mendelian randomization analyses supported that a later age at menopause has a causal effect on breast cancer risk.[39] A later age at menopause has also been associated with an increased risk of ovarian cancer[40] and endometrial cancer.[41] However, if AMH would be merely a marker for age at menopause, this would imply that higher AMH levels also would associate with an increased risk of these cancers. But the included studies did not provide evidence that supports this hypothesis.

To conclude, based on our systematic overview of the current epidemiological evidence on pretreatment AMH levels in relation to cancer we cannot conclude that AMH is actually involved in tumor development. However, only a handful of studies on prediagnosis AMH levels in relation to cancer has been published thus far, and the range of studied cancer diagnoses is limited. Moreover, most of these studies included only a small proportion of younger women. Most studies that investigated pretreatment AMH levels in relation to cancer were of poor methodological quality, therefore we refrain from drawing definite conclusions based on these results. Future research should focus on elucidating if and how AMH affects risk of different cancer types over time. This should ideally take place in large prospective studies including young participants for whom repeated AMH measurements up to the moment of cancer diagnosis are available.

Additional information

Contributors

Renée M.G. Verdiesen performed the literature search and wrote the initial draft of the manuscript.
Carla H. van Gils provided comments and critically revised the manuscript.
Yvonne T. van der Schouw provided comments and critically revised the manuscript.
N. Charlotte Onland-Moret provided comments and critically revised the manuscript.
All authors saw and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Provenance and peer review

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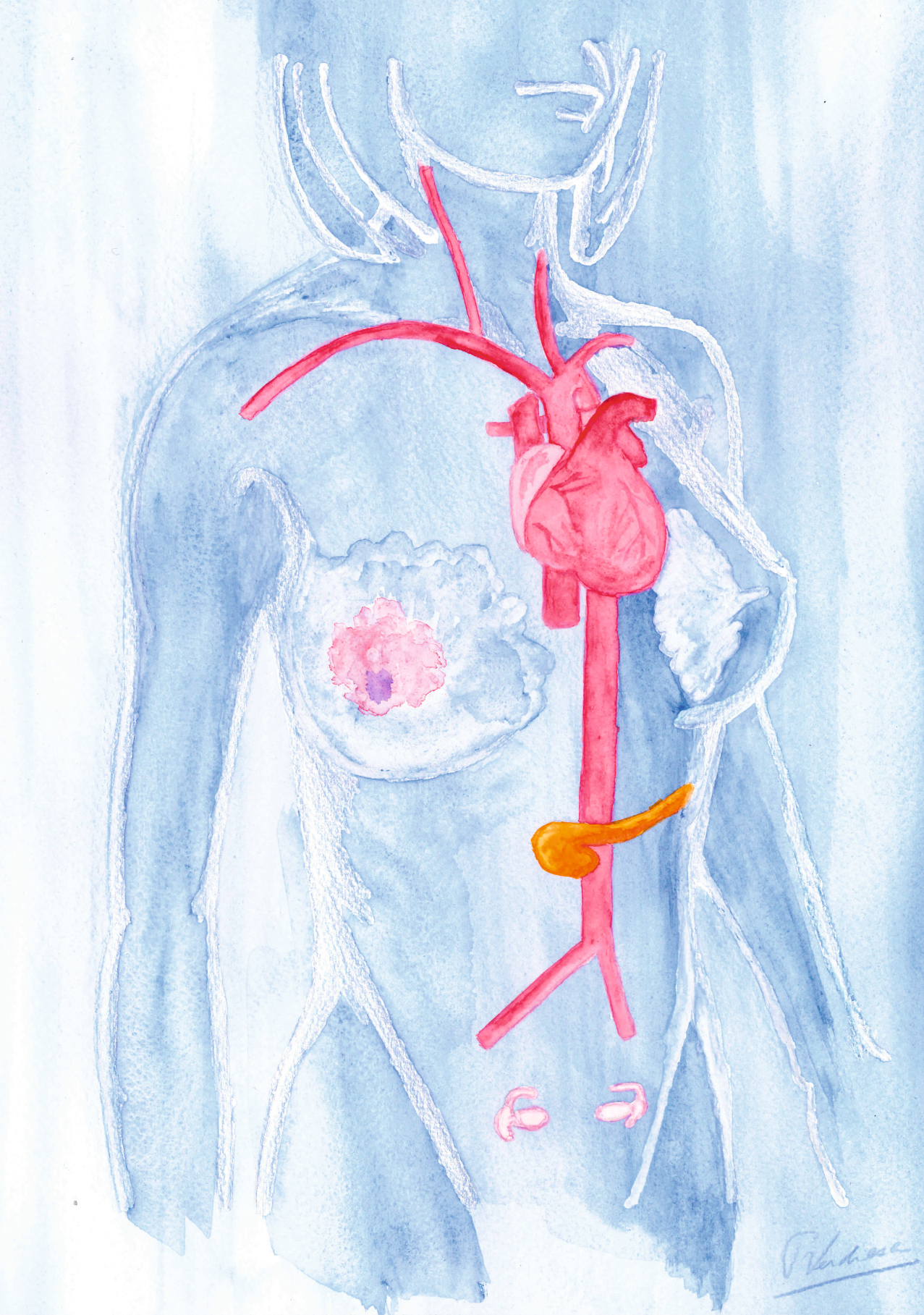
Supplemental data

Completed PRISMA 2009 checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	NA
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4-5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Table 1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4-5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5-6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	4, 6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	NA
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	NA
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	NA

Section/topic	#	Checklist item	Reported on page #
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	7 and Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 2
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Table 3 and Table 4
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Table 2
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	NA
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	NA
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12-14
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	12-15
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	16

Checklist from: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097.



Anti-Müllerian hormone levels and risk of cancer in women

CHAPTER 3

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Maturitas, 2021; 143:216-222

Abstract

Objectives: To examine if age-specific anti-Müllerian hormone (AMH) levels are associated with cancer risk; and to investigate if age-related AMH trajectories differ between women who develop cancer and women who do not. More specifically, we examined associations with breast cancer, cancers in other tissues expressing AMH receptor AMHR2, and cancers in non-AMHR2-expressing tissues.

Study design: We included longitudinal data from 3025 women in the prospective Doetinchem Cohort Study. Cox proportional hazards models were used to assess the association of baseline age-specific AMH tertiles with cancer. We applied linear mixed models to compare age-related AMH trajectories between women who were diagnosed with cancer and women who were not.

Main outcome measures: cancer (n = 385; 139 breast cancers, 112 cancers in other AMHR2-expressing tissues, 134 cancers in non-AMHR2-expressing tissues).

Results: Overall, baseline age-specific AMH levels were not associated with cancer risk, although in women £ 40 years an increased risk was suggested for breast cancer ($HR_{T2:T1}$ = 2.06, 95%CI = 0.95-4.48; $HR_{T3:T1}$ = 2.03, 95%CI = 0.91–4.50). Analysis of age-related AMH trajectories suggested that AMH levels were higher at younger ages and declined faster in women who were diagnosed with cancer compared to women who were not, but our results did not provide evidence for actual differences in trajectories.

Conclusions: Our results did not provide evidence for an association between age-specific AMH levels and age-related trajectories and risk of cancer. However, effect estimates for breast cancer were in line with risk-increasing effects found in previous studies.

Introduction

Higher circulating anti-Müllerian hormone (AMH) levels in women have been associated with increased breast cancer risk.[1] Although AMH is primarily known for its functions in sexual differentiation during embryogenesis[2] and ovarian follicle development[3], histologic evidence on the expression of AMH receptor type 2 (AMHR2) in different non-gonadal tissues[4-6] suggests responsiveness of a wide range of tissues to AMH.

This raises the question whether AMH levels are also associated with other forms of cancer, such as ovarian and lung cancer. A small number of studies examined circulating AMH levels in relation to different cancer types, but except for breast cancer results are inconsistent (see Verdiesen et al.[7] for a detailed overview). Furthermore, previous studies included a single AMH measurement per participant, although age-related AMH trajectories have been shown to vary between women.[8] Individual age-related AMH trajectories may therefore elucidate if, and how, circulating AMH levels affect cancer risk over time.

To provide more insight into the relation between circulating AMH levels and cancer risk, we examined the association of age-specific AMH levels with the risk of cancer, using data from female participants of the Doetinchem Cohort Study. We further examined if age-related AMH trajectories were different for women who developed cancer compared to women who did not. More specifically, we aimed to confirm previous findings for breast cancer and to investigate associations between circulating AMH levels and risk of cancers in other AMHR2-expressing tissues, and cancers in non-AMHR2-expressing tissues.

Methods

Study population

We used data of female participants (median age 39 years, range 20-59) from the Doetinchem Cohort Study, an ongoing prospective cohort study of 3641 men and 4128 women, who were randomly selected from the municipal register of Doetinchem, The Netherlands, between 1987 and 1991.[9, 10] Every 5 years, follow-up visits take place, during which physical examinations and questionnaires are completed. The study was approved by the Medical Ethics Committee of The Netherlands Institution of Applied Scientific Research. All participants signed informed consent prior to study inclusion.

This study included data from Round 1 (baseline; 1987-1991) to Round 5 (2008-2012). Women without any available AMH measurement (n = 802), and women whose data could

not be linked to the cancer registry (n = 224) or who were diagnosed with cancer prior to their first AMH measurement (n = 77), were excluded, leaving 3025 women with at least one available AMH measurement for analysis (Figure 1). The number of women with an AMH measurement per examination round was 2855, 2772, 2281, 2153 and 1909 for Round 1 through Round 5, respectively.

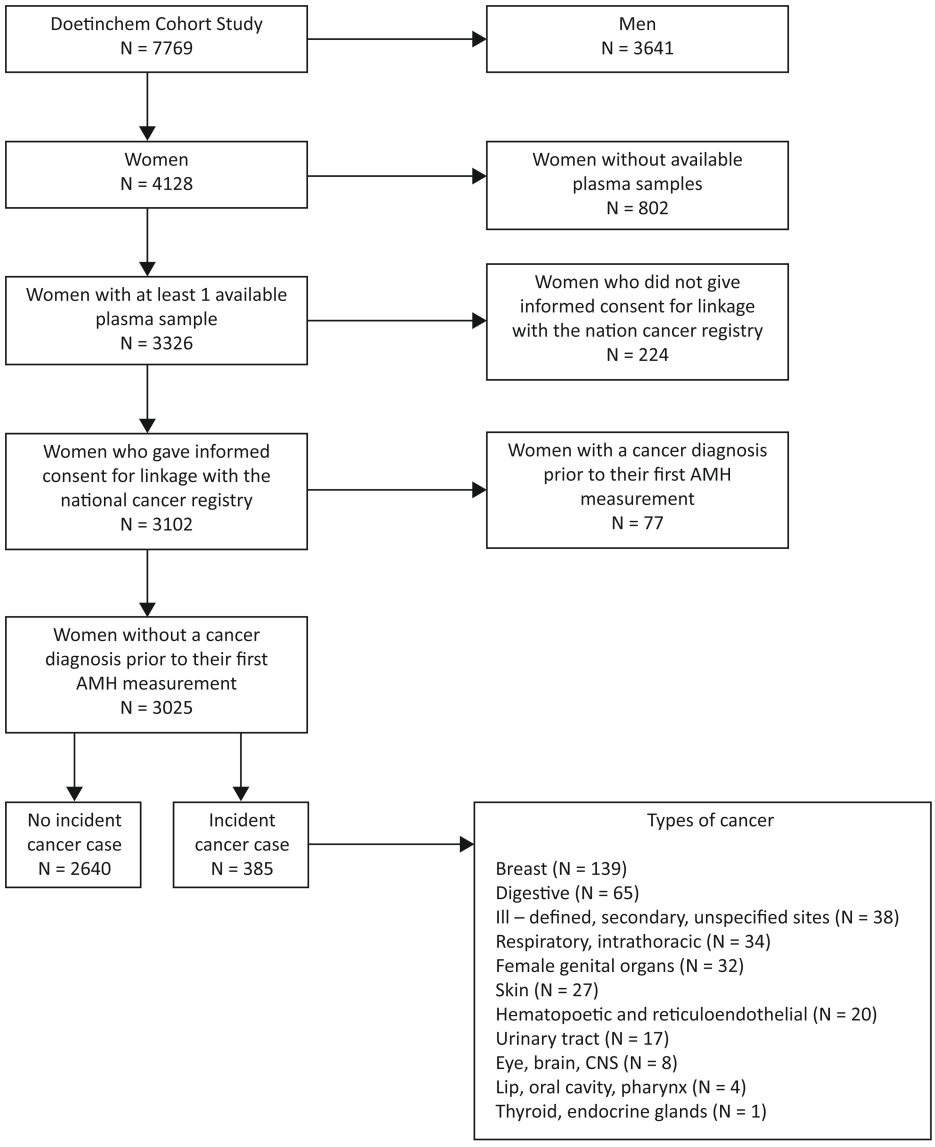

Figure 1. Flow chart study population.

AMH measurements

Details on AMH measurements and sample storage conditions have been described previously.[8, 11] Briefly, AMH was measured in all available plasma samples, collected from Round 1 to Round 5. Missing AMH measurements were the consequence of either non-attendance at certain examination rounds, no consent to blood draw at the particular examination, depletion of plasma samples because of other blood measurements, or an occasional unsuccessful AMH measurement. AMH was measured using the ultrasensitive picoAMH ELISA (Ansh Labs, Webster, Texas, USA) in the Ansh Labs laboratory. Because of its detection limit of 1.846 pg/mL (0.013 pmol/L), we were able to measure very low AMH levels in postmenopausal women in the Doetinchem Cohort Study.[8] The inter- and intra-assay coefficients of variation 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%.[11] AMH measurements below the detection limit were set to half the detection limit (0.923 pg/mL; 0.007 pmol/L).

Covariates

Information on potential confounders was collected through questionnaires and physical examinations. We included the following covariates in our analyses: age at blood collection (years), age at menarche (years), body mass index (BMI) (kg/m2), parity and age at first full-term pregnancy (AFTP) (nulliparous/ 1-2 children and AFTP <25 years/ 1-2 children and AFTP ≥25 years/ ≥3 children and AFTP <25 years/ ≥ 3 children and AFTP ≥ 25 years), current oral contraceptive (OC) use (yes/no), ever hormone replacement therapy (HRT) use (yes/no), menopausal status (premenopausal/postmenopausal), current smoking (yes/no), alcohol consumption (glasses/day), family history of breast cancer (yes/no) and educational attainment (primary education up to completing intermediate vocational education/up to higher secondary education/college degree or higher). A more detailed description of these covariates has been included in the Supplemental Methods.

Cancer outcomes

Through linkage of cohort data with the Dutch Cancer Registry, we identified 385 cases in registry data that were complete until 31 December 2014. Cancers were classified as "cancers in AMHR2-expressing tissues" based on previously published histological evidence[6] or data from the Genotype-Tissue Expression (GTEx) portal (www.gtexportal.org). As a result, the following tumors were defined as "tumors originating from AMHR2-expressing tissues": breast (n = 139), bronchus and lung (n = 32), hematopoietic and reticuloendothelial (n = 20), corpus uteri (n = 13), ovary (n = 11), kidney, except renal pelvis (n = 11), pancreas (n = 9), lymph nodes (n = 6), cervix uteri (n = 4), uterus, unspecified (n = 2), small intestine (n = 2), liver and intrahepatic bile ducts (n = 1), adrenal gland (n = 1). Breast cancer (n = 139; 127

invasive tumors and 12 with unknown behavior) and "cancers in other AMHR2-expressing tissues" (n = 112) were included as separate outcomes. We additionally included the outcome "cancers in non-AMR2-expressing tissues" (n = 134), which comprised tumors in the remaining tissues.

Statistical analyses

We calculated age-specific AMH tertiles at baseline (Round 1) using general linear modeling with CG-LMS[12] (Cole and Green, Lambda, Mu, and Sigma method; R package "gamlss"[13] version 5.1-2), as previously published.[14] Log-transformed AMH was modelled over age using splines, because of the non-linear decline of AMH with increasing age. Previous analyses showed that this model fits the AMH data in the Doetinchem Cohort Study well.[8] The CG-LMS method allows for estimation of the distribution of AMH at every age, and corresponding percentile values (for 33.3% and 66.7%) were used to create age-specific tertiles. Accordingly, women could be classified as having either low (1st age-specific tertile), normal (2nd age-specific tertile), or high (3rd age-specific tertile) AMH levels given their age.

Characteristics for women with an available AMH measurement at baseline (n = 2855) were described using mean (standard deviation), median [interquartile range (IQR)], or frequency (%). We summarized these baseline characteristics by age-specific AMH tertiles.

Missing information on most baseline and time-varying covariates was below 2%. Data on menopausal status was missing for up to 24.9% in Round 3, due to the relatively high proportion of OC users. Missing values for baseline age-specific AMH tertiles and baseline and time-varying covariates were imputed with multiple imputation (50 iterations, 10 imputed datasets) using the R package "mice" (version 3.3.0)[15] (Supplemental Methods). Subsequent regression analyses were performed in each imputed dataset; regression coefficients and standard errors of the mean were pooled according to Rubin's Rule of combination[16] using the pool function in "mice".

Baseline age-specific AMH tertiles and cancer risk

We investigated associations between baseline age-specific AMH tertiles and incident cancer, by estimating hazard ratios (HRs) and 95% confidence intervals (95% CIs) from Cox proportional hazards models. We used follow-up time in years as underlying time scale (t_0 = baseline examination, t_{max} = linkage of data with cancer registry; 31 December 2014), and adjusted models for known risk factors for cancer: age at baseline, age at menarche, current OC use, parity and AFTP, menopausal status, BMI, educational attainment, current smoking, alcohol consumption and family history of breast cancer.

Mean AMH trajectories in women who developed cancer compared to women who did not

To assess whether age-related AMH trajectories differed between women who were diagnosed with cancer and women who were not diagnosed with cancer during follow-up, we used linear mixed models (R package "nlme"[17]; version 3.1-139). Measurements from examination Rounds 1-5 were used to construct AMH trajectories. In total, we analyzed 11,655 AMH measurements performed in the period from baseline until cancer diagnosis, censoring, or end of follow-up, whichever came first. Of these measurements, 4223 (36.2%) were below the limit of detection (< 1.846 pg/mL). Missing AMH measurements were not imputed as this is not needed for linear mixed model analyses.[18] Imputed values were included for the covariates described below.

Linear mixed models included repeated log transformed AMH measurements as dependent variable and age in years, modelled with natural splines (2 knots: 36 and 45 years, upper boundary: 65 years), as the underlying timescale. To assess whether models including incident cancer status (yes/no) and interaction terms of this case variable and the spline terms were a better fit to the data compared with models without these variables, a global likelihood ratio test was applied[19] using the testModels function (method "D3") implemented in R package "mitml"[20] (version 0.3-7). All models additionally included the following fixed effects: age at blood collection (time-dependent), current OC use (time-dependent), current smoking (time-dependent), BMI (time-dependent), menopausal status (time-dependent), alcohol consumption (time-dependent), age at menarche, parity and AFTP, educational level and family history of breast cancer. We also included random intercepts and random slopes for each woman. We used the estimated fixed effects from the fitted models to calculate predicted geometric mean AMH trajectories over age, which were adjusted for the described potential confounders. Predicted AMH trajectories and standard errors of the mean were also pooled using Rubin's Rule. All analyses were performed in R (version 3.4.3).[21]

Sensitivity analyses

Because AMH is known to strongly decrease from age 40 and because less variation is found in AMH levels after this age[8], we performed sensitivity analyses restricted to women younger than 40 years at baseline (n = 1543). We additionally performed sensitivity analyses in which we excluded (1) AMH measurements within two years prior to diagnosis, (2) women who were current OC users at baseline of the cohort (n = 766, on average across 10 imputation sets), and (3) women who had ever reported hormone replacement therapy (HRT) use (n = 923, on average across 10 imputation datasets). Sensitivity analyses excluding current OC users at baseline were only performed for Cox proportional hazards models, since current OC use was included as time-varying covariate in the linear mixed models.

Results

Baseline characteristics of women with an available AMH measurement at Round 1 are presented by age-specific AMH tertiles in Table 1. Women in the lowest age-specific AMH tertile were older than women in the middle and highest age-specific AMH tertiles. Women in the highest age-specific AMH tertile were more likely to be premenopausal, and less likely to be current OC user, ever HRT user or current smoker compared to women in the lowest age-specific AMH tertile. In addition, women in the highest age-specific AMH tertile were more likely to have attained a higher educational level and to have a positive family history of breast cancer. Alcohol consumption was also higher among women in the highest age-specific AMH tertile.

Baseline age-specific AMH tertiles and cancer risk

We observed no increased risk of cancer in women with higher age-specific AMH levels ($HR_{T2:T1}$ = 1.00, 95% CI = 0.77 – 1.28 and $HR_{T3:T1}$ = 1.12, 95% CI = 0.86 – 1.46; Table2). Restricting our analyses to breast cancer resulted in somewhat stronger risk-increasing effect estimates, but confidence intervals were wide and included the null (Table 2). Associations between age-specific AMH levels and risk of cancers in other AMHR2-expressing tissues were similar to those for risk of total cancer, whereas a risk-decreasing effect of higher AMH levels was suggested for cancers in non-AMHR2-expressing tissues ($HR_{T2:T1}$ = 0.74, 95% CI = 0.49 – 1.14 and $HR_{T3:T1}$ = 0.96, 95% CI = 0.62 – 1.49; Table2). Restricting analyses to women £ 40 years at baseline (n = 1543) resulted in stronger effect estimates for breast cancer, although corresponding confidence intervals still indicated considerable uncertainty: $HR_{T2:T1}$ = 2.06, 95% CI = 0.95 – 4.48 and $HR_{T3:T1}$ = 2.03, 95% CI = 0.91 – 4.50 (Table 2). Effect estimates for cancers in non-AMHR2-expressing tissues were also more extreme in women ≤ 40 age at baseline due to increased uncertainty (Table 2). Exclusion of AMH measurements within two years prior to diagnosis, exclusion of current OC users at baseline, and exclusion of women that ever-used HRT did not change our conclusions (Supplemental Table 1).

Table 1. Baseline characteristics of women with an available AMH measurement at Round 1 of the Doetinchem Cohort Study (n = 2855) presented by age-specific AMH tertiles.

		Tertiles of age-specific AMH levels		
		1st age-specific AMH tertile (n = 859)	2nd age-specific AMH tertile (n = 1048)	3rd age-specific AMH tertile (n = 948)
AMH (pg/mL)[a]		29.7 [0.9, 747.2]	1313.4 [154.9, 2734.6]	3796.3 [1036.8, 6405.0]
Age (years)[a]		42.1 [32.7, 51.5]	38.1 [31.6, 45.7]	39.0 [32.2, 46.0]
BMI (kg/m²)[a]		23.7 [21.8, 26.3]	23.7 [21.6, 26.1]	23.3 [21.5, 25.8]
Educational attainment[c,e]				
	primary education up to completing intermediate vocational education	71.5 (612)	68.3 (714)	63.8 (604)
	up to higher secondary education	16.9 (145)	18.9 (198)	20.9 (198)
	college degree or higher	11.6 (99)	12.7 (133)	15.2 (144)
Reproductive factors				
Age at menarche (years)[b,e]		13.4 (1.5)	13.4 (1.5)	13.4 (1.4)
Parity and age at first full-term pregnancy[c,e]				
	Nulliparous	21.3 (183)	24.5 (256)	21.4 (203)
	1-2 children and <25 years	20.1 (173)	23.5 (246)	22.6 (214)
	1-2 children and ≥25 years	33.5 (288)	30.8 (322)	33.7 (319)
	≥3 children and <25 years	12.5 (107)	10.9 (114)	12.6 (119)
	≥3 children and ≥25 years	12.6 (108)	10.3 (108)	9.7 (92)
Premenopausal[c,e]		72.9 (555)	84.0 (816)	95.6 (856)
Current OC use[c,e]		28.5 (243)	27.5 (288)	19.1 (181)
Ever HRT use[c,d,e]		35.3 (196)	25.5 (167)	27.4 (176)

Table 1. *(continued)*

		Tertiles of age-specific AMH levels		
		1st age-specific AMH tertile (n = 859)	2nd age-specific AMH tertile (n = 1048)	3rd age-specific AMH tertile (n = 948)
Lifestyle factors				
Current smoker[c,e]		35.0 (301)	35.1 (368)	29.5 (279)
Current alcohol consumption[c,e]				
	No	20.8 (179)	19.8 (208)	17.3 (164)
	<1 glass/week	31.8 (273)	32.0 (335)	29.6 (280)
	≥1 glass/week	47.3 (406)	48.1 (504)	53.1 (503)
Family history of disease				
Ever reported family history of breast cancer[c,d]		12.2 (105)	14.8 (155)	17.0 (161)

AMH, anti-Müllerian hormone; OC, oral contraceptive, HRT; hormone replacement therapy

[a] Median [interquartile range]

[b] Mean (standard deviation)

[c] Percentage (n)

[d] Ever variables are presented because of absent data on HRT use and family history of breast cancer in Round 1.

[e] Missing values (n): educational attainment (8); age at menarche (10); parity and age at first full-term pregnancy (3), menopausal status (228), current OC use (7), ever HRT use (1001), current smoking (1), current alcohol consumption (3)

Table 2. Associations between age-specific AMH tertiles and total cancer, breast cancer, cancers in other AMHR2-expressing tissues and cancers in non-AMHR2-expressing tissues in women of the Doetinchem Cohort Study (upper panel; n = 3025) and in women ≤40 years at baseline (lower panel; n = 1543).

	Total cancer[a,b]		Breast cancer[a,b]		Cancers in other AMHR2-expressing tissues[a,b]		Cancers in non-AMHR2-expressing tissues[a,b]	
Age-specific AMH tertiles	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)
Total study population (n = 3025)	**385 cases**		**139 cases**		**112 cases**		**134 cases**	
1st age-specific tertile	1	-	1	-	1	-	1	-
2nd age-specific tertile	1.00	(0.77, 1.28)	1.27	(0.83, 1.95)	1.05	(0.65, 1.70)	0.74	(0.49, 1.14)
3rd age-specific tertile	1.12	(0.86, 1.46)	1.27	(0.80, 2.01)	1.18	(0.71, 1.95)	0.96	(0.62, 1.49)
Women ≤40 years at baseline (n = 1543)	**131 cases**		**59 cases**		**34 cases**		**38 cases**	
1st age-specific tertile	1	-	1	-	1	-	1	-
2nd age-specific tertile	1.02	(0.65, 1.59)	2.06	(0.95, 4.48)	1.10	(0.46, 2.66)	0.42	(0.19, 0.94)
3rd age-specific tertile	1.13	(0.71, 1.79)	2.03	(0.91, 4.50)	1.09	(0.43, 2.78)	0.66	(0.31, 1.42)

AMH, anti-Müllerian hormone; AMHR2, anti-Müllerian hormone receptor type 2; HR, hazard ratio; CI, confidence interval

[a] Cox proportional hazards models adjusted for age at baseline (years), age at menarche (years), parity and age at first birth (nulliparous/ 1-2 children and AFTP <25 years/ 1-2 children and AFTP ≥25 years/ ≥3 children and AFTP <25 years/ ≥ 3 children and AFTP ≥ 25 years), menopausal status (premenopausal/postmenopausal), current OC use (yes/no), ever reported family history of breast cancer (yes/no), BMI (kg/m²), educational attainment (primary education up to completing intermediate vocational education/up to higher secondary education/college degree or higher), current smoking (yes/no), alcohol consumption (g/day).

[b] Cox proportional hazards models were not adjusted for menopausal status as only 1 woman of the 1543 women ≤40 years at baseline was classified as postmenopausal.

Mean AMH trajectories in women who developed cancer compared to women who did not

On average, 3.9 AMH measurements were available per woman (see Supplemental Table 2 for details on repeated AMH measurements). Figure 2 presents predicted geometric mean AMH trajectories in women who were diagnosed with cancer during follow-up and women who were not, averaged across the ten imputed datasets. These plots suggested that AMH levels were higher around age 30 and subsequently declined faster in women who were later diagnosed with cancer compared to women who were not, but our results did not provide evidence for an actual difference in trajectories (p-value global likelihood ratio tests > 0.05 for each outcome; Supplemental Table 3). Sensitivity analyses restricted to women younger than 40 years at baseline, exclusion of AMH measurements within two years prior to cancer diagnosis, and exclusion of women who reported ever having used HRT also did not provide evidence for differences in trajectories (p-value for each global likelihood ratio test > 0.05).

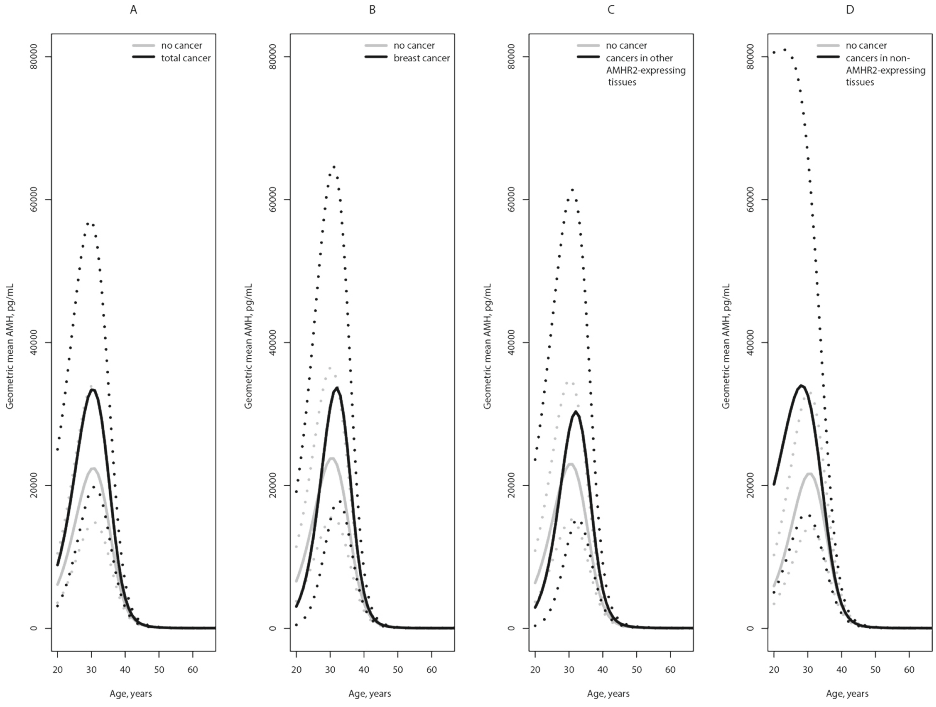

Figure 2. Predicted geometric mean AMH trajectories (solid lines) and 95% confidence intervals (dashed lines) over age in women who developed (A) total cancer, (B) breast cancer, (C) cancers in other AMHR2-expressing tissues, and (D) cancers in non-AMHR2-expressing tissues compared to women who did not develop cancer during follow-up. Plots show average predicted AMH trajectories across 10 imputed datasets. Trajectories are adjusted for the time-varying covariates current oral

Figure 2. *(continued)*

contraceptive use, current smoking, body mass index, menopausal status, alcohol consumption; and the time-invariant covariates age at menarche, parity and age at first full-term pregnancy, educational level and family history of breast cancer.

Discussion

This study found no evidence for associations between baseline age-specific AMH and cancer risk, although the risk-increasing effect estimates for breast cancer were in line with previously published findings. Examination of AMH trajectories indicated that AMH levels around age 30 may be higher, and may decline faster, in women who are diagnosed with cancer compared to women who are not. However, our results did not provide strong evidence for an actual difference in age-related AMH trajectories.

The main strength of this study is that we were the first to investigate the association between age-related AMH trajectories and risk of cancer, whereas previous studies included only one AMH measurement for each participant. Also, this is the first study to investigate the effect of AMH on the risk of total cancer, and on cancer types subdivided based on expression of AMHR2. Additional strengths of this study are its large study population, with a median follow-up period of 25 years, and time-varying information on risk factors for cancer. Nevertheless, the current analyses are mostly exploratory in nature because of the limited number of cancer cases, and the limited number of measurements at younger ages. As a result, we cannot rule out that age-related AMH trajectories do differ between women who later develop cancer and women who do not. Moreover, the heterogeneous nature across and within cancer types most likely also limited statistical power to detect associations.

Following our objective to investigate whether AMH trajectories differed for women who were or were not diagnosed with cancer during follow-up, we used linear mixed models in which AMH was included as dependent variable. An evident disadvantage of this approach is that time until cancer diagnosis is not taken into account. Although various methods that can model repeated measurements and time to event data are available (e.g. Cox proportional hazards models including a time-varying exposure or joint models), these approaches test whether AMH levels at, or near, the moment of diagnosis are associated with risk of cancer, whereas we were specifically interested in the complete AMH trajectory over time up to the moment at which women were diagnosed with cancer.

Even though not statistically significant, our finding for breast cancer is in line with a previous individual participant data meta-analysis, reporting that women in the highest

AMH quartile were at a 60% increased risk of breast cancer compared to women in the lowest AMH quartile.[1] Interestingly, in this meta-analysis the relation of baseline circulating AMH levels with breast cancer was strongest in women aged 45-49 years at blood draw, whereas in our longitudinal analyses AMH levels were not different between future breast cancer cases and healthy women in that age range. A possible explanation for this difference may be the fact that the study by Ge et al. included a number of small studies in older women, which reported very large effect sizes. In contrast to previous studies on AMH and female specific cancers, we could not assess potential confounding of our results by estradiol and/or testosterone levels, as these were not measured in our study population. However, as correction for endogenous estradiol and/or testosterone did not influence results of previous studies[1, 22-24], we do not expect a large confouding effect of these hormones in our study.

Our results provide no answer to the question whether AMH is merely a proxy for time until menopause, or whether AMH has a direct effect on tissues that express its receptor, AMHR2. Performing a formal mediation analysis for age at menopause was not feasible in the current study population, due to the limited number of cases that underwent the menopausal transition (for breast cancer only 72 cases; i.e. 52%). We hypothesized that if AMH regulates cell growth in AMHR2-expressing tissues, we would observe a stronger effect of high AMH levels on risk of cancers in AMHR2-expressing tissues than for total cancer, and absence of an association with cancers in non-AMHR2-expressing tissues. However, apart from supporting the association between high AMH and an increased risk of breast cancer, our results do not support an association with cancers in other AMHR2-expressing tissues. Due to the low number of cases in this latter group, we could not examine the association between AMH and individual cancer types, such as ovarian and endometrial cancer. Similarly, we were not able to investigate associations with different breast cancer subtypes.

In conclusion, plasma AMH levels were not associated with risk of cancer, although our findings are in agreement with previous evidence suggesting that higher circulating AMH levels are associated with an increased risk of breast cancer. Our longitudinal analyses suggested that AMH levels may be higher around age 30 and may decline faster in women who later develop cancer, but our results did not provide clear evidence for an actual difference in trajectories. Prospective studies with repeated AMH measurements in a larger population of young women are required to establish if, and at which age, AMH could be considered a risk factor for cancer, and specifically for breast cancer.

Additional information

Contributors

Renée M.G. Verdiesen contributed to data analysis, data interpretation, preparation of first draft of the manuscript and critical revision of the manuscript. Carla H. van Gils contributed to data interpretation and critical revision of the manuscript. Rebecca K. Stellato contributed to data analysis, data interpretation and critical revision of the manuscript. W.M. Monique Verschuren contributed to data collection and critical revision of the manuscript. Frank J.M. Broekmans contributed to data collection and critical revision of the manuscript. Annelien C. de Kat contributed to critical revision of the manuscript. Yvonne T. van der Schouw contributed to data collection, data interpretation and critical revision of the manuscript. N. Charlotte Onland-Moret contributed to data interpretation and critical revision of the manuscript.

Conflict of interest

FJMB has received fees and grant support from Merck Serono, Gedeon Richter, Ferring BV, and Roche. The other authors declare that they have no conflict of interest.

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Ansh Labs performed the AMH measurements free of charge. Ansh Labs was not involved in the data analysis, interpretation, or reporting, nor was it financially involved in any aspect of the study.

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Ethical approval

The Doetinchem Cohort Study was conducted according to the principles of the World Medical Association Declaration of Helsinki and its amendments since 1964, and in accordance with the Medical Research Involving Human Subject Act (WMO). The Doetinchem Cohort Study received ethical approval from the Medical Ethics Committee

of The Netherlands Institution of Applied Scientific Research and all study participants gave written informed consent prior to study inclusion.

Research data (data sharing and collaboration)

There are no linked research datasets for this paper. The full dataset and statistical code are available on request, in liaison with the National Institute of Public Health and the Environment.

Provenance and peer review

This article was not commissioned. Peer review was directed by Martina Dören independently of Yvonne T. van der Schouw, an author and Maturitas editor, who was blinded to the process.

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Supplemental data

Supplemental methods

Details on the assessment of covariates and the multiple imputation procedure are provided below.

Covariates

BMI was calculated using weight and height measurements obtained during physical examinations. Current oral contraceptive use was assessed through the categorical questions: "Have you ever used oral contraceptives?" (Yes, currently/Yes, ever/No, never) in Round 1, and "Are you currently using oral contraceptives?" (Yes/ No) in Rounds 2-5.

Ever and current use of HRT was not asked in examination Round 1. Therefore, we used data from Round 2-5 to classify women as ever or never HRT users. Women were classified as ever HRT users when they had answered the question "Have you ever used estrogens or female hormones because of menopausal complaints?" at least once with "Yes" in Round 2-5. If women answered this question each round with either "No" or "Not applicable" they were classified as never HRT users, in all other situations this variable was set to missing. We performed a sensitivity analysis excluding women who were classified as "ever had HRT".

Menopausal status was assessed using data on cycle regularity, date of last menstrual period and reproductive surgery, as previously described.[1] Women who had amenorrhea for at least 12 consecutive months were considered postmenopausal, as defined by the World Health Organization.[2] Women who underwent a bilateral oophorectomy were considered postmenopausal from the moment they had surgery. Menopausal status was set to missing for women who had a hysterectomy without bilateral oophorectomy, and imputed subsequently as described in the statistical analyses section. In addition, menopausal status was set to missing and subsequently imputed in current OC users because Dutch guidelines recommend the use of OCs in perimenopausal women with vasomotor complaints. Also, Dutch women use OCs up to the age of 52 when OCs are the preferred method of birth control.

Current smoking was assessed in each examination round using the question "Are you currently smoking cigarettes?" to which women could reply (1) "Yes, I smoke on average 1 or more cigarettes a month"; (2) Yes, but I smoke less than 1 cigarette a month"; (3) No, I smoked cigarettes in the past, but quit"; and (4) "No, I never smoked". For the current study, women were classified as current smokers if they smoked on average ≥ 1 cigarette

per month. Alcohol consumption was calculated in women who reported to consume on average more than 1 glass of alcohol per week.

Family history of breast cancer was based on data obtained over the complete follow-up period of the Doetinchem Cohort Study; women were classified as having a family history of breast cancer if they reported that their mother and/or sister(s) were diagnosed with breast cancer before or during follow-up.

Educational attainment was classified as previously reported[3] (low: primary education up to completing intermediate vocational education; middle: up to higher secondary education; high: college degree or higher).

Multiple imputation

Imputation models were dependent on the type of variable: predictive mean matching, logistic regression, multinomial logit, and ordered logit models were used for continuous, binary, nominal and ordinal categorical variables, respectively. Predictor variables were selected based on their presence in subsequent analyses, their mutual correlations and their correlation with the imputed variables. For imputation of repeated variables we used variables for the previous and following examination rounds as predictors.

References supplemental methods

1. de Kat AC, van der Schouw YT, Eijkemans MJ, Herber-Gast GC, Visser JA, Verschuren WM, et al. Back to the basics of ovarian aging: a population-based study on longitudinal anti-Mullerian hormone decline. BMC Med. 2016;14(1):151.

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Supplemental Table 1. Results sensitivity analyses for associations between age-specific AMH tertiles and total cancer, breast cancer, cancers in other AMHR2-expressing tissues and cancers in non-AMHR2-expressing tissues.

	Total cancer[a]		Breast cancer[a]		Cancers in other AMHR2-expressing tissues[a]		Cancers in non-AMHR2-expressing tissues[a]	
Age-specific AMH tertiles	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)
Excluding AMH measurements performed in plasma samples collected within two years prior to cancer diagnosis (n = 3013)								
	373 cases		**134 cases**		**110 cases**		**129 cases**	
1st age-specific AMH tertile	1	-	1	-	1	-	1	-
2nd age-specific AMH tertile	1.05	(0.81, 1.36)	1.38	(0.89, 2.15)	1.08	(0.67, 1.76)	0.78	(0.51, 1.20)
3rd age-specific AMH tertile	1.15	(0.88, 1.52)	1.38	(0.86, 2.22)	1.19	(0.71, 1.98)	0.96	(0.61, 1.51)
Excluding women who were current OC users at baseline of the cohort (n = 2259)[b]								
	315 cases[b]		**110 cases[b]**		**97 cases[b]**		**108 cases[b]**	
1st age-specific AMH tertile	1	-	1	-	1	-	1	-
2nd age-specific AMH tertile	0.93	(0.70, 1.23)	1.09	(0.68, 1.75)	0.88	(0.52, 1.49)	0.81	(0.50, 1.31)
3rd age-specific AMH tertile	1.03	(0.77, 1.38)	0.96	(0.57, 1.62)	1.06	(0.62, 1.83)	1.07	(0.65, 1.76)
Excluding women who were hormone replacement therapy users (n = 2102)[c]								
	218 cases		**95 cases**		**47 cases**		**77 cases**	
1st age-specific AMH tertile	1	-	1	-	1	-	1	-
2nd age-specific AMH tertile	1.15	(0.80, 1.65)	1.43	(0.80, 2.55)	1.76	(0.76, 4.07)	0.71	(0.39, 1.32)
3rd age-specific AMH tertile	1.21	(0.79, 1.84)	1.40	(0.72, 2.72)	1.65	(0.64, 4.29)	0.90	(0.47, 1.74)

AMH, anti-Müllerian hormone; AMHR2, anti-Müllerian hormone receptor type 2; HR, hazard ratio; CI, confidence interval

[a] Cox Proportional hazards models adjusted for age at baseline (years), age at menarche (years), parity and age at first birth (nulliparous/ 1-2 children and AFTP <25 years/ 1-2 children and AFTP ≥25 years/ ≥3 children and AFTP <25 years/ ≥ 3 children and AFTP ≥ 25 years), menopausal status (premenopausal/postmenopausal), current OC use (yes/no), ever reported family history of breast cancer (yes/no), BMI (kg/m²), educational attainment (primary education up to completing intermediate vocational education/up to higher secondary education/ college degree or higher), current smoking (yes/no), alcohol consumption (g/day)

[b] Numbers differ between imputation sets, as the variable for current OC use was imputed; presented numbers are average sample sizes and average numbers of cases

[c] Numbers differ between imputation sets, as the variable for ever HRT use was imputed; presented numbers are average sample sizes and average numbers of cases

Supplemental Table 2. AMH measurement related characteristics stratified by menopausal status for Round 1 to Round 5 of the Doetinchem Cohort Study.[a]

	Premenopausal women	Postmenopausal women
Available AMH measurement at Round 1[b]	2355 (82.5)	500 (17.5)
Age (years)[c,d]	37.0 [31.1 - 42.7]	55.5 [51.9 - 57.3]
AMH (pg/mL)[c]	1625 [441 – 3686]	0.92 [0.92 - 0.92]
Measurements above the LOD (%)	95.6	12.8
Available AMH measurement at Round 2[b]	1958 (70.6)	814 (29.4)
Age (years)[c,d]	41.8 [35.7 - 46.9]	57.8 [54.2 - 62.2]
AMH (pg/mL)[c]	798 [125 – 2 229]	0.92 [0.92 - 0.92]
Measurements above the LOD (%)	91.1	10.1
Available AMH measurement at Round 3[b]	1339 (58.7)	942 (41.3)
Age (years)[c,d]	44.0 [39.4 - 48.5]	59.7 [54.6 - 64.3]
AMH (pg/mL)[c]	350 [25.4 – 1 397]	0.92 [0.92 - 0.92]
Measurements above the LOD (%)	85.6	6.4
Available AMH measurement at Round 4[b]	750 (34.8)	1403 (65.2)
Age (years)[c,d]	45.2 [41.8 - 48.7]	59.1 [54.4 - 66.4]
AMH (pg/mL)[c]	267 [33.1 - 924]	0.92 [0.92 - 1.85]
Measurements above the LOD (%)	87.2	10.3
Available AMH measurement at Round 5[b]	408 (21.4)	1501 (78.6)
Age (years)[c,d]	47.6 [43.9 - 50.3]	62.0 [56.9 - 68.2]
AMH (pg/mL)[c]	130 [9.9 - 532]	0.92 [0.92 - 0.92]
Measurements above the LOD (%)	81.7	8.1

[a] Numbers differed between imputation sets, as menopausal status was imputed; presented values are averages

[b] Number (%)

[c] Median [IQR]

[d] Median age does not increase with 5 years over rounds because women shift from premenopausal to postmenopausal status over examination rounds. Also, women could skip examination rounds resulting in different groups of women in different examination rounds

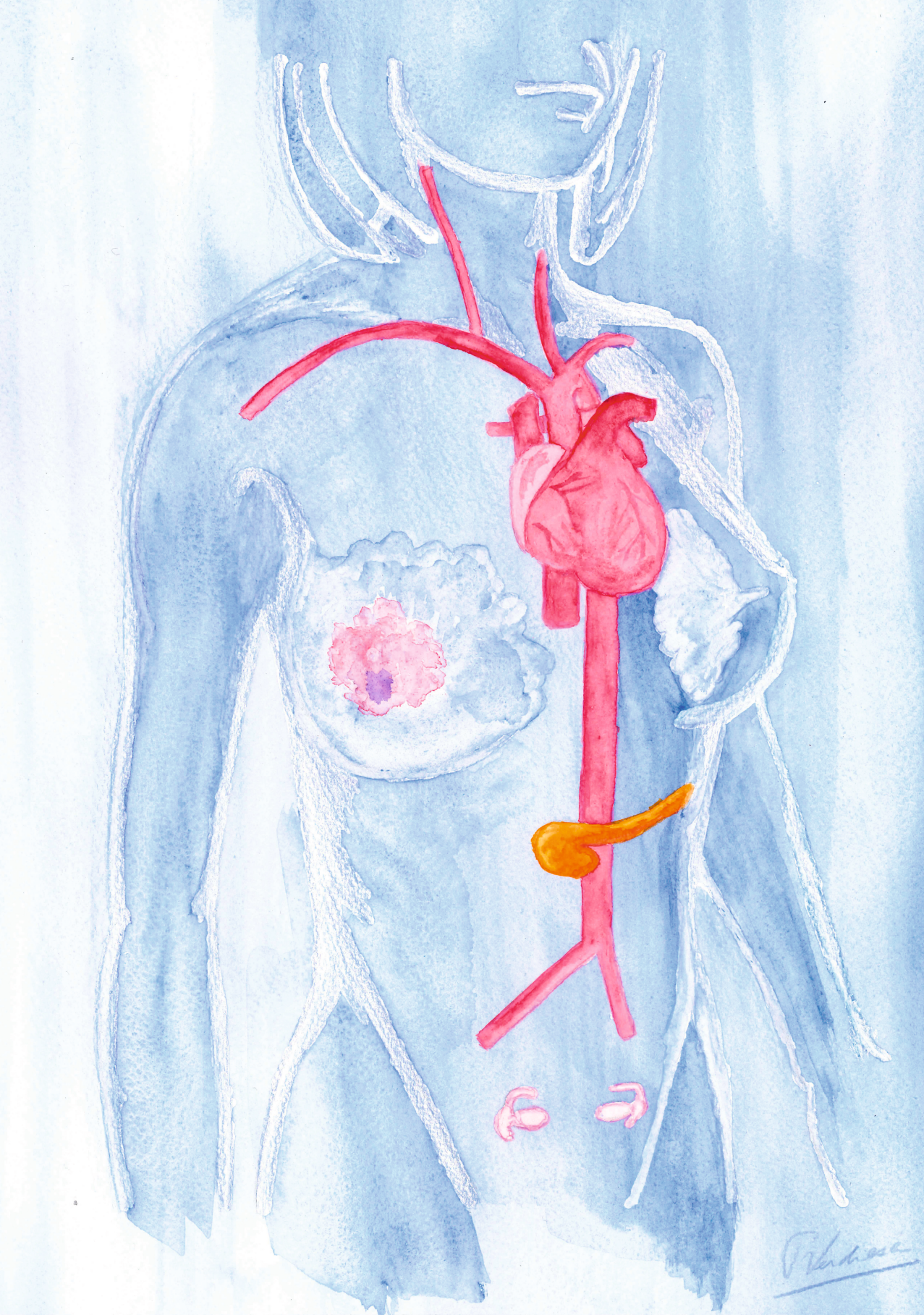
Supplemental Table 3. Fixed effects of the Linear Mixed Models with change in log transformed anti-Müllerian hormone (AMH) levels as outcome in women of the Doetinchem Cohort Study.

		Total cancer[a]		Breast cancer[a]		Cancers in other AMHR2-expressing tissues[a]		Cancers in non-AMHR2-expressing tissues[a]	
		Estimate	(95% CI)	Estimate	(95% CI)	Estimate	(95% CI)	Estimate	(95% CI)
Total study population[b]		**385 cases**		**139 cases**		**112 cases**		**134 cases**	
Fixed intercept		9.85	(9.21, 10.50)	9.88	(9.22, 10.55)	9.82	(9.16, 10.48)	9.70	(9.03, 10.36)
Cancer vs. no cancer		0.37	(-0.58, 1.32)	-0.75	(-2.54, 1.04)	-0.77	(-2.81, 1.28)	1.21	(-0.08, 2.50)
Age									
	1st spline	-5.63	(-5.89, -5.38)	-5.64	(-5.90, -5.38)	-5.64	(-5.91, -5.38)	-5.63	(-5.89, -5.37)
	2nd spline	-2.78	(-3.31, -2.24)	-2.80	(-3.34, -2.26)	-2.80	(-3.34, -2.27)	-2.77	(-3.31,-2.24)
	3rd spline	-6.37	(-6.58, -6.16)	-6.38	(-6.59, -6.16)	-6.38	(-6.60, -6.16)	-6.37	(-6.58, -6.16)
1st spline age x cancer		-0.40	(-1.01, 0.22)	-0.10	(-1.21, 1.00)	0.46	(-0.81, 1.74)	-0.70	(-1.63, 0.23)
2nd spline age x cancer		-0.31	(-2.18, 1.57)	1.98	(-1.56, 5.52)	2.15	(-1.93, 6.22)	-2.23	(-4.74, 0.29)
3rd spline age x cancer		-0.44	(-0.92, 0.04)	-0.19	(-1.04, 0.67)	0.05	(-0.93, 1.02)	-0.59	(-1.32, 0.14)
P-value global likelihood ratio test		0.19		0.09		0.16		0.49	

AMHR2, anti-Müllerian hormone receptor type 2; CI, confidence interval

[a] All models included additionally the following fixed effects: age at blood collection (time-dependent), current OC use (time-dependent), current smoking (time-dependent), BMI (time-dependent), menopausal status (time-dependent), alcohol consumption (time-dependent), age at menarche, parity and AFTP, educational level and family history of breast cancer

[b] Sample size analyses total cancer, N= 3024; breast cancer, N = 2778; cancers in other AMHR2-expressing tissues, N = 2751; cancers in non-AMHR2-expressing tissues, N = 2773



Anti-Müllerian hormone levels and risk of type 2 diabetes in women

CHAPTER 4

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Abstract

Aims/hypothesis: Given its role in ovarian follicle development, circulating anti-Müllerian hormone (AMH) is considered to be a marker of reproductive ageing. Although accelerated reproductive ageing has been associated with a higher risk of type 2 diabetes, research on the relationship between AMH and type 2 diabetes risk is scarce. Therefore, we aimed to investigate whether age-specific AMH levels and age-related AMH trajectories are associated with type 2 diabetes risk in women.

Methods: We measured AMH in repeated plasma samples from 3293 female participants (12,460 samples in total), aged 20–59 years at recruitment, from the Doetinchem Cohort Study, a longitudinal study with follow-up visits every 5 years. We calculated age-specific AMH tertiles at baseline to account for the strong AMH–age correlation. Cox proportional hazards models adjusted for confounders were used to assess the association between baseline age-specific AMH tertiles and incident type 2 diabetes. We applied linear mixed models to compare age-related AMH trajectories for women who developed type 2 diabetes with trajectories for women who did not develop diabetes.

Results: During a median follow-up of 20 years, 163 women developed type 2 diabetes. Lower baseline age-specific AMH levels were associated with a higher type 2 diabetes risk (HR_{T2vsT3} 1.24 [95% CI 0.81, 1.92]; HR_{T1vsT3} 1.62 [95% CI 1.06, 2.48]; $p_{trend} = 0.02$). These findings seem to be supported by predicted AMH trajectories, which suggested that plasma AMH levels were lower at younger ages in women who developed type 2 diabetes compared with women who did not. The trajectories also suggested that AMH levels declined at a slower rate in women who developed type 2 diabetes, although differences in trajectories were not statistically significant.

Conclusions/interpretation: We observed that lower age-specific AMH levels were associated with a higher risk of type 2 diabetes in women. Longitudinal analyses did not show clear evidence of differing AMH trajectories between women who developed type 2 diabetes compared with women who did not, possibly because these analyses were underpowered. Further research is needed to investigate whether AMH is part of the biological mechanism explaining the association between reproductive ageing and type 2 diabetes.

Introduction

Female reproductive ageing has been associated with risk of chronic diseases, including type 2 diabetes, in later life [1]. Women with an earlier menopause have been found to be at a higher risk of postmenopausal type 2 diabetes [2]. This association appears to be independent from the effect of BMI [3, 4]. Yet, the biological mechanisms underlying the association between reproductive ageing and type 2 diabetes remain to be established. A potential causal candidate explaining this association is anti-Müllerian hormone (AMH), a gonadal hormone expressed by early-stage ovarian follicles in premenopausal women [5]. From birth onwards, the ovarian follicle pool decreases until menopause [6]. Accordingly, circulating AMH levels decline with age until they become undetectable after menopause. AMH can therefore be used as marker for reproductive ageing in women [7, 8].

To date, the relationship between circulating AMH and type 2 diabetes has been examined in one small study in pregnant women [9]. Several studies investigated AMH in relation to conditions, such as insulin resistance, that predispose to type 2 diabetes but their results are inconsistent [10-14]. Furthermore, most of these studies had a cross-sectional design and/or included only women with polycystic ovary syndrome (PCOS). As a result, reverse causation could not be excluded in previous studies and generalisability of their results to healthy women is limited.

Therefore, the aim of the current study was to investigate the association between AMH and type 2 diabetes using data from women in the population-based Doetinchem Cohort Study. Specifically, we investigated associations between age-specific AMH levels at baseline of the cohort and age-related AMH trajectories and incident type 2 diabetes.

Methods

Study population

The Doetinchem Cohort Study is an ongoing prospective cohort study, which has been described in more detail previously [15, 16]. Briefly, the Doetinchem Cohort Study included 3641 men and 4128 women, aged 20–59 years at recruitment, who were randomly selected from the municipal register of Doetinchem, the Netherlands, between 1987 and 1991. Every 5 years, study participants are invited for a follow-up visit during which physical examinations are conducted, extensive questionnaires are completed and blood samples are collected. Invitations for the follow-up visits are sent irrespective of attendance at previous follow-up rounds. The Doetinchem Cohort Study received approval from the Medical Ethics

Committee of the Netherlands Institution of Applied Scientific Research and all study participants signed an informed consent prior to study inclusion. For the current study we only used data from female participants (median age at recruitment 39 years, range 20–59 years) from examination Round 1 (baseline 1987–1991) to examination Round 5 (2008–2012).

Exclusion criteria

For 3326 of the 4128 female participants in the Doetinchem Cohort Study, at least one AMH measurement was available for any of the five included examination rounds. For this study we excluded women who were diagnosed with diabetes prior to their first available AMH measurement (*n*=33) (Fig. 1). We included data for the remaining 3293 women in subsequent analyses. The number of women with an AMH measurement per examination round was 3104, 2888, 2488, 2305 and 2038 for Rounds 1, 2, 3, 4 and 5, respectively.

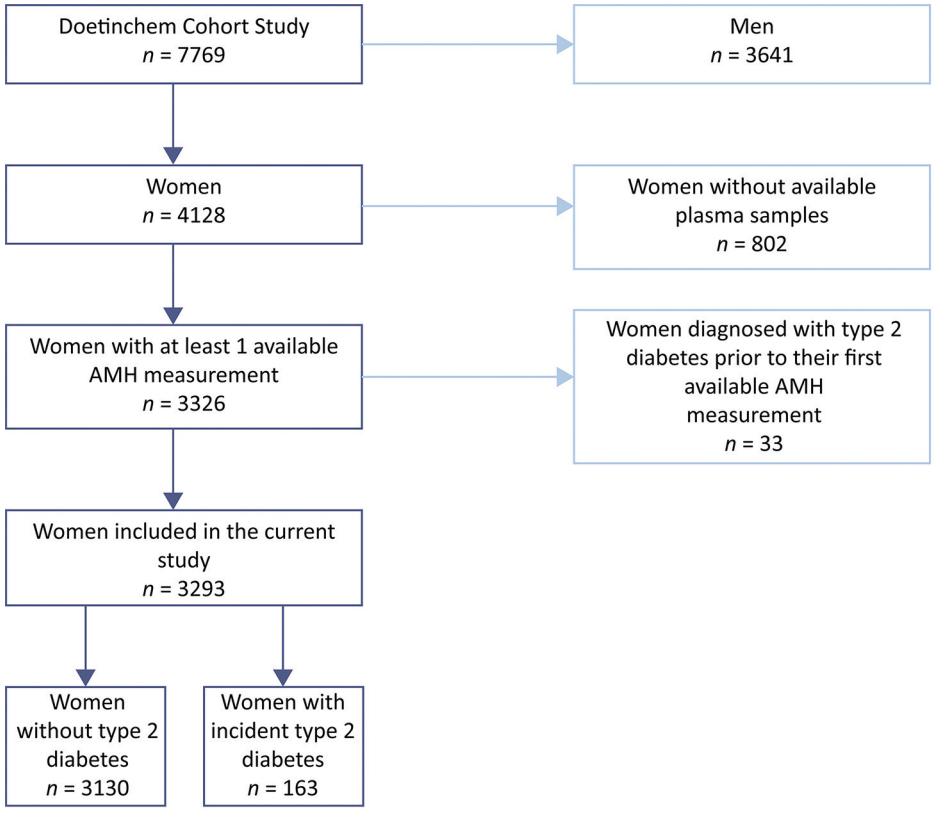

Figure 1. Flow chart for study population.

AMH measurements

Approval for AMH measurements was given by the Ethical Committee for Biobank Studies of the University Medical Center Utrecht. Details of these measurements and sample storage conditions have been described previously[17, 18]. In short, AMH was measured in all available plasma samples, collected from baseline to examination Round 5, from each female study participant. Missing AMH measurements were the consequence of either non-attendance at certain follow-up visits, no consent to blood draw at the particular examination, depletion of plasma samples because of other blood measurements, or an occasional unsuccessful AMH measurement. AMH was measured using the picoAMH ELISA (Ansh Labs, Webster, TX, USA) in the Ansh Labs laboratory. This AMH assay has a lower detection limit of 0.013 pmol/l. AMH measurements below the limit of detection were set to half this value (0.007 pmol/l).

Covariates

Data on age at blood collection (years), educational attainment (low, middle, high), current smoking (yes, no), alcohol consumption (glasses/day), physical activity (inactive, active), parity (nulliparous, parous), current oral contraceptive use (yes, no), ever hormone replacement therapy (HRT) use (yes, no) and menopausal status (premenopausal, postmenopausal) were collected through questionnaires. Time-varying data was available for age at blood collection, BMI, current smoking, alcohol consumption, physical activity, hypertension, total cholesterol, current oral contraceptive use and menopausal status.

Educational attainment was classified using the following categories: primary education up to completing intermediate vocational education (low); up to higher secondary education (middle); and higher vocational education and university (high) [19]. Women were classified as current smokers if they reported smoking on average ≥1 cigarette per month. Total alcohol consumption (glasses/day) was calculated in women who reported consuming on average more than one glass of alcohol per week. Physical activity was assessed using the validated Cambridge Physical Activity Index [20]. Because data on physical activity at baseline was completely missing, we assumed that physical activity at baseline was equal to data from Round 2. Questions on current and ever HRT use were only included in questionnaires from Rounds 2–5. Consequently, women were classified as ever HRT users when they reported HRT use on at least one of these questionnaires. Women who reported no HRT use on any of the questionnaires were classified as never HRT users. Menopausal status was assessed as previously described [17]; women who had amenorrhea for at least 12 consecutive months were considered postmenopausal. Women who underwent a bilateral oophorectomy were considered postmenopausal from the moment they had surgery. Menopausal status was set to ‘missing’ for women who had a hysterectomy without bilateral oophorectomy and for

current oral contraceptive users, and imputed subsequently as described in the statistical analyses section. We imputed menopausal status in current oral contraceptive users because Dutch guidelines state that oral contraceptive use is preferable in perimenopausal women with vasomotor complaints. In addition, women with birth control wishes use oral contraceptives as the preferred method up to the age of 52.

BMI (kg/m^2) was calculated using standardised weight and height measurements obtained during physical examinations. Hypertension (yes, no) was classified according to the guidelines of the WHO (systolic BP ≥140 mmHg and/or diastolic BP ≥90 mmHg) and/or use of BP-lowering medication. Total cholesterol (mmol/l) was measured in non-fasting EDTA–plasma until 1998 and in serum from 1998 onwards, using standardised enzymatic methods [16].

Ascertainment of type 2 diabetes

Women reporting that they had been diagnosed with diabetes for the first time at Rounds 2–5 were classified as incident type 2 diabetes cases. In addition, non-fasting glucose measurements were available in Rounds 2–5, and women with at least one glucose measurement ≥11.1 mmol/l were also classified as incident cases. Previous research has shown that 86% of the self-reported diabetes cases in the Doetinchem Cohort Study could be confirmed by general practitioner or pharmacy registries [21]. In total, we identified 163 incident type 2 diabetes cases over a median follow-up period of 20 years. For women who reported their age at diabetes diagnosis, we set their diagnosis date to the first day of January of the corresponding year. For the remaining women, we set their diagnosis date to the first day of January of the year in which the examination during which they first reported to have been diagnosed with diabetes or at which their glucose was ≥11.1 mmol/l took place.

Statistical analyses

We calculated age-specific baseline AMH tertiles using general linear modelling with the Cole and Green, Lambda, Mu and Sigma (CG–LMS) method [22] (R package 'gamlss', version 5.1-2 [23]), as previously described [24]. $_{\log}$AMH at examination Round 1 was modelled over age using splines because of the non-linear decline in AMH with increasing age. Previous analyses showed that this model fits the AMH data in the Doetinchem Cohort Study well [17]. The CG–LMS method allows for estimation of the distribution of AMH at every age, and corresponding percentile values (for 33.3% and 66.7%) were used to create age-specific tertiles. Accordingly, women could be classified as having either low (first age-specific tertile), normal (second age-specific tertile) or high (third age-specific tertile) AMH levels given their age.

Characteristics for women with an available AMH measurement at baseline (*n*=3104) were described using medians (IQR) or percentages (*n*). We summarised these baseline characteristics by age-specific AMH tertiles. In addition, we compared baseline characteristics and the proportion of incident diabetes cases between women with and without an AMH measurement at each round, to assess whether missing AMH measurements were potentially associated with these characteristics.

Missing values for baseline age-specific AMH tertiles and baseline and time-varying covariates were imputed with multiple imputation (100 iterations, ten imputed datasets) using the R package 'mice' (version 3.3.0) [25] (ESM Methods). We based the number of imputed datasets on the average proportion of missing values on variables included in the association analyses (8.0%), as recommended previously [26]. Subsequent regression analyses were performed in each imputed dataset; regression coefficients and 95% CIs were pooled according to Rubin's Rule of combination [27] using the pool function in 'mice'.

Baseline age-specific AMH tertiles and type 2 diabetes risk

We assessed associations between baseline age-specific AMH tertiles and incident type 2 diabetes by estimating HRs and 95% CIs from Cox proportional hazards models. We used follow-up time in years as underlying time scale (t_0 represented baseline examination; t_{max} represented either date on which participant last attended an examination or date at diabetes diagnosis), and adjusted models for known risk factors for type 2 diabetes and reproductive factors. Fully adjusted models included the following baseline variables: age; BMI; educational attainment; current smoking; alcohol consumption; physical activity; hypertension; total cholesterol; current oral contraceptive use; parity; and menopausal status. We visually checked the proportional hazards assumption using scaled Schoenfeld residuals and statistically tested it using the cox.zph function in R (R package 'survival', version 2.44-1.1 [28]), which consistently indicated that the proportional hazards assumption was not violated.

Mean AMH trajectories in women who develop type 2 diabetes compared with women who do not

To assess whether age-related AMH trajectories differed between women with and without incident type 2 diabetes, we used linear mixed models (R package 'nlme', version 3.1-139 [29]). AMH trajectories were constructed using available measurements from examination Rounds 1–5. We included non-imputed AMH values in the linear mixed model analyses, as these analyses provide unbiased estimates when outcomes are missing at random [30]. Imputed values were included for the covariates described below. We excluded AMH measurements after diabetes diagnosis. In women with incident type 2 diabetes, the earliest age at which

AMH was measured was 21.4 years. Accordingly, we excluded 79 AMH measurements that were available at earlier ages for women without diabetes, as differences in AMH trajectories between both groups cannot be assessed at ages for which no measurements were available in one of the groups. Two women without diabetes were completely excluded from these analyses due to these excluded measurements. In addition, one woman with incident type 2 diabetes was excluded from our longitudinal analyses because no AMH measurements were available before her diagnosis. As a result, we included data from 3290 women, among which there were 162 incident cases of type 2 diabetes, in our longitudinal analyses. In total, we included 12,460 AMH measurements performed in the period from baseline until diabetes diagnosis or last-attended examination round. Of these measurements, 4587 (36.8%) were below the limit of detection (<0.013 pmol/l).

Models included repeated $_{\log}$AMH levels as dependent variable and age in years, modelled with natural splines (2 knots, 36 and 45 years; upper boundary, 65 years), as the underlying timescale. To assess whether models including incident type 2 diabetes status (yes, no) and interaction terms of this case variable and the spline terms were a better fit to the data compared with models without these variables, a global likelihood ratio test was applied [31] using the testModels function (method 'D3') implemented in R package 'mitml' (version 0.3-7 [32]). Linear mixed models additionally included the following fixed effects: age at blood collection; BMI; educational attainment; current smoking; alcohol consumption; physical activity; hypertension; total cholesterol; current oral contraceptive use; parity; and menopausal status. Except for educational attainment and parity, all included covariates were time-varying. We also included random intercepts and random slopes for age for each woman. We used the estimated fixed effects from these models to calculate predicted geometric mean AMH trajectories adjusted for the described potential confounders over age per imputation set. Predicted AMH trajectories and corresponding 95% CIs were pooled using Rubin's Rule. All analyses were performed in R, version 3.6.0 [33].

Sensitivity analyses

To rule out a potential effect of undiagnosed type 2 diabetes on AMH measurements included in our analyses, we repeated our main analyses after excluding AMH measurements in samples collected within 2 years prior to diabetes diagnosis (n=8). We also explored how imputation of baseline age-specific AMH tertiles influenced our survival analyses through excluding women with missing AMH data at baseline (n = 189). Current HRT use has been shown to affect both AMH levels and risk of diabetes. As current HRT use was not assessed at Round 1, we could not model this variable as a time-varying covariate. Instead, we assessed a potential effect of HRT use on our main results by performing analyses excluding women who reported any use of HRT at Rounds 2–5 (n = 1490 on average over

ten imputed datasets). In addition, we performed sensitivity analyses in which we excluded women who never reported having had regular menstrual cycles during follow-up (n = 268), as this could be an indication that these women had PCOS. Although an irregular menstrual cycle in itself is not sufficient to diagnose PCOS, no other data was available that allowed us to assess whether women potentially had PCOS.

Results

Characteristics of the women with an available AMH measurement at baseline are presented by age-specific tertile in Table 1. Women in the middle and highest tertiles were younger, more often premenopausal, less likely to ever have used HRT, and more physically active than women in the lowest age-specific AMH tertile. In addition, women in the highest age-specific tertile were more likely to be highly educated and consume more alcohol but were less likely to be a current oral contraceptive user, current smoker or to be hypertensive compared with women in the middle and lowest age-specific AMH tertiles. Baseline characteristics and the proportion of incident diabetes cases were mostly comparable between women with and women without a missing AMH measurement, for each of the five examination rounds (ESM Table 1).

Baseline age-specific tertiles and risk of type 2 diabetes

We observed that women with lower age-specific AMH levels had a higher risk of type 2 diabetes (HR_{T2vsT3} 1.24 [95% CI 0.81, 1.92]; HR_{T1vsT3} 1.62 [95% CI 1.06, 2.48]; p_{trend} across tertiles = 0.02) (Table 2). Sensitivity analyses excluding AMH measurements performed in plasma samples collected within 2 years prior to diabetes diagnosis and analyses excluding women with missing AMH data at baseline did not change these results (Table 2). Exclusion of women with potential PCOS did not considerably change effect estimates either, although associations were no longer statistically significant (Table 2). Exclusion of women who ever had HRT resulted in wider CIs and decreased effect estimates for both the first and second age-specific AMH tertile.

Table 1. Characteristics of women with an available AMH measurement at baseline of the Doetinchem Cohort Study (*n* = 3104) presented by age-specific AMH tertiles.

Characteristic	Lowest age-specific AMH tertile (*n* = 907)	Middle age-specific AMH tertile (*n* = 1184)	Highest age-specific AMH tertile (*n* = 1013)
AMH, pmol/l	0.21 (0.01–5.38)	8.90 (0.85–19.29)	26.85 (7.07–45.31)
Age, years	42.0 (32.6–51.4)	38.5 (31.6–46.2)	39.1 (32.1–45.8)
BMI, kg/m²	23.7 (21.7–26.3)	23.7 (21.7–26.2)	23.3 (21.4–25.7)
Educational attainment[a]			
Low	70.9 (641)	69.3 (818)	64.0 (646)
Middle	16.7 (151)	19.1 (226)	21.0 (212)
High	12.4 (112)	11.6 (137)	15.0 (152)
Reproductive factors			
Parous, yes	77.1 (699)	75.1 (889)	78.6 (796)
Premenopausal[a]	73.9 (588)	83.0 (906)	96.2 (917)
Current OC use[a]	29.8 (269)	27.6 (327)	18.8 (190)
Ever HRT use[a,b]	36.5 (206)	27.2 (190)	27.8 (184)
Lifestyle factors			
Current smoker, yes[a]	35.2 (319)	35.4 (419)	30.3 (307)
Current alcohol consumption[a]			
No	20.7 (188)	19.8 (235)	17.6 (178)
<1 glass/week	31.7 (287)	31.4 (372)	29.8 (302)
≥1 glass/week	47.6 (431)	48.7 (576)	52.6 (532)
Physical activity[a,c]			
Inactive	31.5 (224)	26.2 (242)	27.0 (225)
Active	68.5 (486)	73.8 (682)	73.0 (608)
Total cholesterol, mmol/l[a]	5.4 (4.7–6.2)	5.3 (4.6–6.0)	5.2 (4.6–5.8)
Hypertension, yes	15.4 (140)	14.4 (171)	10.8 (109)

AMH, anti-Müllerian hormone; OC, oral contraceptive; HRT, hormone replacement therapy

Data are presented as median (IQR) or percentage (*n*)

[a] Missing values (*n*): educational attainment (9); menopausal status (263), current oral contraceptive use (7), ever HRT use (1179), current smoking (1), current alcohol consumption (3), physical activity (637), total cholesterol (1)

[b] Ever variable presented because of absent data on HRT use at baseline

[c] Physical activity at examination Round 2 due to absent data on physical activity at baseline

Table 2. HRs (95% CIs) for the association between baseline age-specific AMH tertiles and risk of type 2 diabetes in women of the Doetinchem Cohort Study.

Population	Lowest age-specific AMH tertile	Middle age-specific AMH tertile	Highest age-specific AMH tertile (reference)	*p* value for trend
Total study population (*n* = 3293, 163 cases)	1.62 (1.06, 2.48)*	1.24 (0.81, 1.92)	1.00	0.02
Exclusion of AMH measurements within 2 years prior to type 2 diabetes diagnosis (*n* = 3285, 155 cases)	1.55 (1.00, 2.40)*	1.19 (0.76, 1.85)	1.00	
Exclusion of women with a missing AMH measurement at baseline (*n*= 3104, 148 cases)	1.62 (1.04, 2.52)*	1.29 (0.83, 2.00)	1.00	
Exclusion of women who ever used HRT (*n*= 1803, 95 cases)[a]	1.26 (0.72, 2.20)	0.74 (0.41, 1.32)	1.00	
Exclusion of women who potentially had PCOS (*n* = 3025, 138 cases)	1.57 (0.97, 2.54)	1.13 (0.71, 1.83)	1.00	

Cox proportional hazards models were adjusted for the following baseline variables: age (years), parity (nulliparous, parous), current oral contraceptive use (yes, no), menopausal status (premenopausal, postmenopausal), BMI (kg/m²), educational attainment (low, middle, high), current smoking (yes, no), alcohol consumption (glasses/day), physical activity (inactive, active), hypertension (yes, no), total cholesterol (mmol/l)

[a] Numbers differed between imputation sets, as the variable ever HRT use itself was imputed; presented numbers are average sample sizes and average numbers of cases

*p <0.05

Mean AMH trajectories in women who are diagnosed with type 2 diabetes compared with women who are not

On average, 3.8 AMH measurements were available per woman. Figure 2 presents predicted geometric mean AMH trajectories in incident type 2 diabetes cases and women without type 2 diabetes averaged across the ten imputation sets. This plot suggests that AMH levels were lower until approximately 37 years of age and that from the age of 30 years onwards AMH levels declined more slowly in women who developed type 2 diabetes compared with women who did not develop type 2 diabetes. However, neither the type 2 diabetes case variable nor interaction terms of this case variable with splines for age were statistically significant (ESM Table 2). Comparing models including these diabetes variables with models that did not include them did not indicate that age-related AMH trajectories differed between women with and without type 2 diabetes either (p value global likelihood ratio test = 0.58). Exclusion of AMH measurements within 2 years prior to diagnosis, exclusion of women who reported ever having used HRT and exclusion of women who potentially had PCOS did not change these results (ESM Table 2).

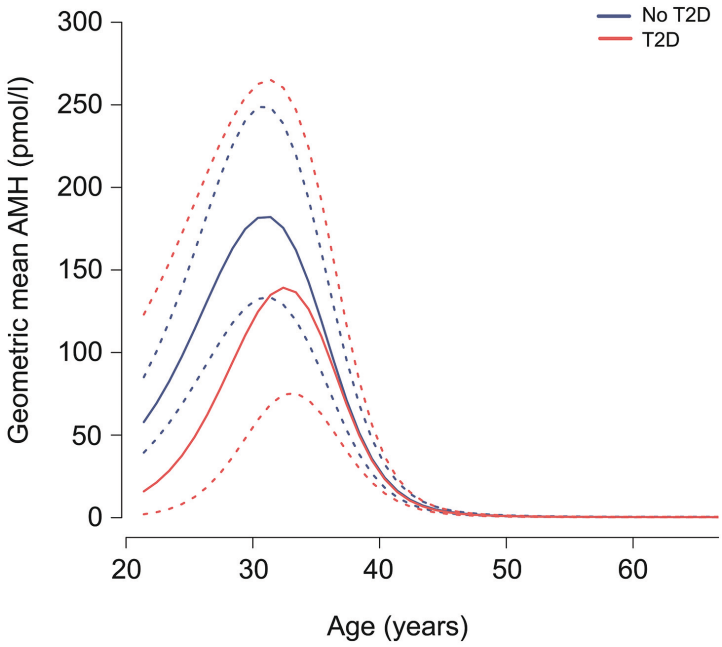

Figure 2. Predicted geometric mean AMH trajectories (pmol/l) (solid lines) and 95% CIs (dashed lines) over age in women who were diagnosed with type 2 diabetes compared with women who were not diagnosed with type 2 diabetes during follow-up. Plots show average predicted AMH trajectories across ten imputed datasets. Trajectories are adjusted for current oral contraceptive use, current smoking, BMI, menopausal status, alcohol consumption, physical activity, hypertension, total cholesterol, parity and educational level. T2D, type 2 diabetes

Discussion

In this prospective cohort study we observed that lower age-specific AMH levels were associated with a higher risk of type 2 diabetes in women. Longitudinal analyses that included multiple AMH measurements per woman did not show clear evidence of a difference in age-related trajectories between women with and without incident diabetes, possibly because of the limited number of AMH measurements at younger ages, particularly in women diagnosed with type 2 diabetes.

The main strength of this study is that we investigated the association between age-specific AMH and age-related trajectories and risk of type 2 diabetes in women in a large longitudinal population-based cohort study with a median follow-up of 20 years. To date, just one small study (n=69) examined AMH in relation to type 2 diabetes in women, and

only included pregnant women [9]. Additional strengths of the current study are its time-varying information on AMH as well as a wide array of potential confounders, including BMI. Nevertheless, residual confounding cannot be ruled out completely.

A potential limitation of this study is that type 2 diabetes case ascertainment was based on self-report and non-fasting glucose measurements. Accordingly, we made an assumption about the date of type 2 diabetes diagnosis, which we set to the first day of January of the year in which a woman first reported that she had been diagnosed with diabetes and/or in which her glucose was ≥11.1 mmol/l. This approach may have resulted in some misclassification, although diagnosis dates obtained from hospital discharge or general practitioner registries are not precise either, because diabetes develops over several years. However, sensitivity analyses in which we excluded AMH measurements performed in plasma samples collected within 2 years prior to the assumed type 2 diabetes diagnosis date did not change our findings, suggesting that our assumption did not induce reverse causation bias. Furthermore, previous research has shown that most of the self-reported diabetes cases in the Doetinchem Cohort Study (86%) could be verified with data from general practitioner or pharmacy registries [21].

The only previous study examining AMH in relation to type 2 diabetes in women [9] observed no difference in AMH levels between women with type 2 diabetes, women with gestational diabetes and a healthy control group of women during the second and third trimester of pregnancy. The generalisability of this finding may be limited, as it has been suggested that circulating AMH levels temporarily drop during late-stage pregnancy [34]. In line with our results, a previous study in men observed a lower risk of type 2 diabetes in overweight individuals with higher AMH levels [35]. In men, AMH is produced by Sertoli cells and also decreases with increasing age [36], although to a lesser degree than in women. Lower AMH levels have also been observed in men with the metabolic syndrome [37] and in obese boys with insulin resistance [38], conditions that are both associated with an increased risk of type 2 diabetes. In women, lower AMH levels have also been reported to correlate with higher HOMA-IR [11] and higher fasting insulin [39], although other studies could not replicate this [10, 13, 40] (see de Kat et al [41] for a more detailed discussion). Similarly, results of studies on the relationship between AMH and conditions that predispose to type 2 diabetes in women diagnosed with PCOS are inconsistent [12, 14, 41].

Because AMH levels are higher in women with PCOS [42], and these women are at an increased risk of type 2 diabetes [43], we performed a sensitivity analysis in which we excluded women who potentially had PCOS. Based on the positive associations between AMH and PCOS and between PCOS and type 2 diabetes, we hypothesised that if PCOS was a confounder in our analyses, we would observe an even lower risk of type 2 diabetes

in women with higher AMH levels after exclusion of those with PCOS. However, our effect estimates did not change. A likely explanation for this is that we classified women as potentially having PCOS when they reported never having had regular menstrual cycles, whereas in practice PCOS is diagnosed based on a set of criteria that additionally include clinical and/or biochemical hyperandrogenism and/or polycystic ovaries [44]. Future studies including data on actual PCOS diagnosis should indicate whether PCOS acts as confounder in the observed association between AMH and type 2 diabetes.

Given its role in ovarian follicle development and its expression in these follicles [5], AMH is considered to be a proxy for ovarian ageing and, accordingly, lower AMH levels have been associated with an earlier age at menopause [45]. Previous studies observed that an earlier age at menopause was associated with a higher risk of type 2 diabetes [2-4], which is in accordance with our results. However, the question remains as to whether ovarian ageing is indeed causally associated with risk of diabetes or whether residual confounding by biological ageing influenced our and previous findings. Future studies including data on both proxies for ovarian (e.g. AMH and/or age at menopause) and biological ageing (e.g. epigenetic clock) may provide more insight into this matter. In addition, functional studies may investigate if AMH signalling actually takes place in the pancreas and how this might be related to the pathophysiology of type 2 diabetes, since the receptor through which AMH signals (AMHR2) is expressed in pancreatic tissue [46].

In conclusion, we observed that women with lower age-specific AMH levels were at a higher risk of type 2 diabetes. Longitudinal analyses also indicated that AMH levels may be lower in women who develop type 2 diabetes compared with women who do not, although our results did not provide clear evidence for an actual difference in age-related AMH trajectories. Future studies that investigate the association between age-specific AMH (trajectories) and type 2 diabetes should ideally include a larger proportion of younger women and, if possible, include proxies for biological ageing.

Additional information

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Data availability

The full dataset and statistical codes are available on request, in liaison with the National Institute of Public Health and the Environment.

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Authors' relationships and activities

FJMB has received fees and grant support from Merck Serono, Gedeon Richter, Ferring BV and Roche. All other authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement

HSJP and WMMV were involved in data collection for the Doetinchem Cohort Study. RMGV, NCOM, CHvG and YTvdS conceptualised and designed the current study. RMGV conducted the statistical analyses and prepared the first draft of the manuscript. RMGV, NCOM, CHvG, RKS, AMWS, FJMB, WMMV and YTvdS interpreted the data. All authors critically revised the manuscript for important intellectual content and approved the published version of the manuscript. YTvdS is responsible for the integrity of the work as a whole.

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Supplemental data

Electronic supplementary material (ESM) methods

Missing values in baseline age-specific AMH tertiles and baseline and time-varying covariates and were imputed with multiple imputation (100 iterations, 10 imputed datasets) using the R package "mice". Imputation models were dependent on the type of variable: predictive mean matching, logistic regression, and ordered logit models were used for continuous (BMI, alcohol consumption and total cholesterol), binary (current smoking, physical activity, hypertension, current oral contraceptive use, menopausal status, ever hormone replacement therapy use), and ordinal categorical (age-specific AMH tertiles, educational attainment) variables, respectively. Predictor variables were selected based on their presence in subsequent analyses, their mutual correlations and their correlation with the imputed variables. For imputation of repeated variables we used variables for the previous and following examination rounds as predictors.

ESM Table 1. Baseline characteristics presented by availability of AMH measurements per round.

	Round 1		Round 2		Round 3		Round 4		Round 5	
Baseline characteristics										
	Women with AMH measurement at Round 1 (*n* = 3104)	Women without AMH measurement at Round 1 (*n* = 189)	Women with AMH measurement at Round 2 (*n* = 2888)	Women without AMH measurement at Round 2 (*n* = 405)	Women with AMH measurement at Round 3 (*n* = 2488)	Women without AMH measurement at Round 3 (*n* = 805)	Women with AMH measurement at Round 4 (*n* = 2305)	Women without AMH measurement at Round 4 (*n* = 988)	Women with AMH measurement at Round 5 (*n* = 2038)	Women without AMH measurement at Round 5 (*n* = 1255)
Age, years[a]	39.5 (32.0, 47.9)	37.9 (31.3, 44.7)	39.5 (32.2, 47.4)	38.2 (30.2, 48.5)	39.0 (31.8, 46.8)	41.0 (32.9, 51.0)	38.5 (31.6, 46.3)	41.5 (33.1, 51.3)	37.7 (31.3, 45.6)	41.8 (33.8, 51.4)
BMI, kg/m^2[a]	23.6 (21.6, 26.1)	23.8 (21.6, 26.5)	23.6 (21.6, 26.1)	23.7 (21.6, 26.1)	23.5 (21.6, 25.9)	24.1 (21.8, 26.9)	23.3 (21.4, 25.7)	24.3 (21.9, 27.4)	23.2 (21.4, 25.5)	24.3 (22.0, 27.0)
Educational attainment, %										
Low	68.0	71.8	68.3	67.9	65.5	76.8	63.9	78.3	61.5	79.2
Middle	19.0	19.1	18.9	20.0	20.1	15.6	21.1	14.3	22.7	13.1
High	13.0	9.0	12.8	12.1	14.4	7.6	15.0	7.4	15.8	7.7
Reproductivefactors										
Parous, %	76.8	68.8	76.7	73.6	76.4	76.1	75.7	77.9	75.6	77.6
Postmeno-pausal,%	15.1	15.0	15.0	16.0	13.8	19.4	12.6	21.2	10.8	22.5
Current OC use, %	25.4	30.2	25.0	30.3	26.3	23.5	26.6	23.5	27.6	22.5
Lifestyle factors										
Current smoker, %	33.7	37.6	34.0	32.9	31.8	40.5	31.3	40.0	29.9	40.3
Current alcohol consumption, %										
No	19.4	19.1	19.3	20.3	17.4	25.3	17.3	24.2	16.8	23.5

ESM Table 1. *(continued)*

	Round 1		Round 2		Round 3		Round 4		Round 5	
Baseline characteristics										
	Women with AMH measurement at Round 1 (n = 3104)	Women without AMH measurement at Round 1 (n = 189)	Women with AMH measurement at Round 2 (n = 2888)	Women without AMH measurement at Round 2 (n = 405)	Women with AMH measurement at Round 3 (n = 2488)	Women without AMH measurement at Round 3 (n = 805)	Women with AMH measurement at Round 4 (n = 2305)	Women without AMH measurement at Round 4 (n = 988)	Women with AMH measurement at Round 5 (n = 2038)	Women without AMH measurement at Round 5 (n = 1255)
<1 glass/week	31.0	33.5	30.9	32.7	31.5	30.0	31.8	29.6	32.0	29.8
≥1 glass/week	49.6	47.3	49.8	47	51.1	44.6	50.9	46.1	51.3	46.6
Physically active, %	72.0	65.3	71.9	61.3	73.6	66.1	73.6	67.2	74.3	67.4
Total cholesterol, mmol/l[a]	5.3 (4.6, 6.0)	5.3 (4.6, 6.1)	5.3 (4.6, 6.0)	5.2 (4.6, 6.0)	5.2 (4.6, 6.0)	5.3 (4.7, 6.1)	5.2 (4.6, 5.9)	5.5 (4.8, 6.2)	5.2 (4.5, 5.8)	5.5 (4.8, 6.3)
Hypertension, %	13.5	15.9	13.6	13.8	12.4	17.6	11.0	19.8	9.6	20.2
Outcome										
Incident diabetes, %	4.8	7.9	4.8	5.9	5.5	3.4	5.5	3.6	5.3	4.4

AMH, anti-Müllerian hormone; BMI, body mass index; OC, oral contraceptive

[a] Median (IQR)

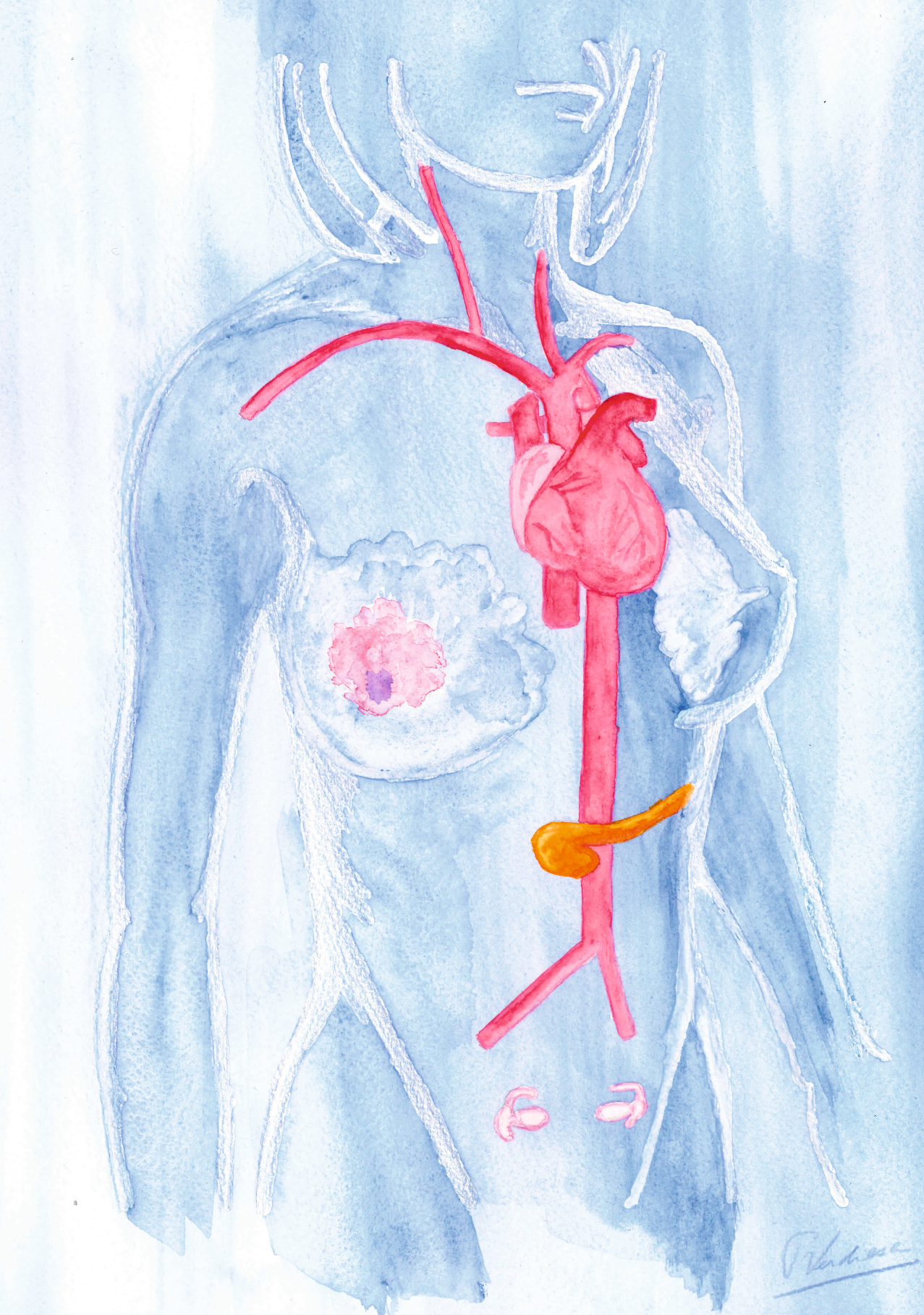
ESM Table 2. Fixed effects of linear mixed models with change in $_{log}$AMH (pmol/l) over age as outcome.

		Total study population (*n* = 3290, 162 cases)	**Exclusion of AMH measurements within 2 years prior to type 2 diabetes diagnosis** (*n* = 3279, 151 cases)	**Exclusion of women who reported ever to have used HRT[a]** (*n* = 1801, 95 cases)	**Exclusion of women who potentially had PCOS** (*n* = 3023, 138 cases)
		Estimate (95% CI)	**Estimate (95% CI)**	**Estimate (95% CI)**	**Estimate (95% CI)**
Fixed intercept		5.64 (5.16, 6.12)	5.66 (5.17, 6.15)	6.05 (5.43, 6.66)	5.74 (5.24, 6.24)
T2D vs. no T2D		-1.29 (-3.32, 0.74)	-1.09 (-3.14, 0.96)	-0.71 (-2.73, 1.30)	-1.55 (-3.63, 0.53)
Age					
	1st spline	-5.74 (-5.91, -5.56)	-5.73 (-5.91, -5.56)	-5.53 (-5.72, -5.34)	-5.71 (-5.89, -5.54)
	2nd spline	-3.05 (-3.46, -2.65)	-3.06 (-3.46, -2.65)	-3.25 (-3.72, -2.79)	-3.17 (-3.58, -2.76)
	3rd spline	-6.31 (-6.48, -6.14)	-6.31 (-6.47, -6.14)	-6.09 (-6.29, -5.90)	-6.32 (-6.49, -6.15)
1st spline * T2D		0.55 (-0.69, 1.80)	0.53 (-0.75, 1.80)	0.38 (-0.86, 1.62)	0.83 (-0.44, 2.10)
2nd spline * T2D		2.42 (-1.65, 6.48)	2.01 (-2.11, 6.12)	1.14 (-2.99, 5.27)	2.97 (-1.21, 7.15)
3rd spline * T2D		0.51 (-0.45, 1.47)	0.33 (-0.66, 1.33)	0.31 (-0.64, 1.26)	0.60 (-0.39, 1.58)
P-value likelihood ratio tests		0.58	0.67	0.84	0.65

AMH, anti-Müllerian hormone; HRT, hormone replacement therapy; PCOS, polycystic ovary syndrome

Models additionally included the following fixed effects: current oral contraceptive use, current smoking, body mass index, menopausal status, alcohol consumption, physical activity, hypertension, total cholesterol, parity and educational level

[a] Numbers differed between imputation sets, as the variable ever HRT use itself was imputed; presented numbers are averages



CHAPTER 5

Circulating anti-Müllerian hormone levels and markers of subclinical cardiovascular disease in middle-aged and older men

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Submitted

Abstract

Context: Recent research suggests that higher circulating anti-Müllerian hormone (AMH) levels are associated with lower occurrence of (subclinical) cardiovascular disease (CVD) in women, but evidence in men is limited.

Objective: We aimed to investigate whether circulating AMH levels are associated with measures of subclinical CVD in middle-aged and older men.

Design: Prospective cohort study with a median follow-up time of 8.7 years. Serum AMH was measured at baseline. We assessed both cross-sectional and longitudinal associations using linear regression models adjusted for confounders.

Setting: Dutch middle-aged and older men from the community.

Participants: 394 men (aged 40-80 years) with an available AMH measurement at baseline.

Main Outcome Measures: At baseline (2001-2002): carotid intima-media thickness (CIMT), pulse wave velocity (PWV), abdominal aortic diameter, and Framingham risk score (FRS) predictions. At follow-up (2010-2011): CIMT, mean carotid aortic plaque score, PWV, and FRS predictions. All outcomes were transformed using rank-based inverse normal transformation to meet the normality assumption.

Results: Higher AMH levels were associated with lower CIMT at baseline (β = -0.04; 95%CI = 0.07, -0.01), but not with the other baseline subclinical CVD measures. Longitudinal analyses suggested that higher baseline AMH levels were associated with lower mean plaque scores at follow-up (β = -0.03, 95%CI = -0.07, 0.00), but not with the other follow-up outcomes.

Conclusions: Our results suggested that AMH is associated with current CIMT and future carotid aortic plaque burden in men, implying that circulating AMH is potentially associated with structural rather than with functional changes of the arterial wall.

Introduction

Anti-Müllerian hormone (AMH) is a gonadal hormone that is primarily known for its crucial role in sexual differentiation during embryogenesis.[1] In adult men and women, AMH is produced by immature Sertoli cells and antral stage ovarian follicles[2], respectively. Based on few fundamental studies[3, 4], it has been proposed that AMH may also have a role in the pathogenesis of cardiovascular diseases (CVD).

Recent epidemiological research indeed suggests that higher circulating AMH levels may be associated with a lower risk of both subclinical CVD[5, 6] and clinical CVD[7, 8], but studies in men are scarce[9, 10]. In addition, most previous studies had a cross-sectional design and did not adjust for potential confounding by circulating sex hormones, which hampers establishing whether AMH could play a causal role in CVD pathology in both sexes.

To gain more insight into the mechanisms through which AMH levels may affect CVD risk, we aimed to investigate whether circulating AMH levels are associated with different subclinical CVD measures in middle-aged and older men. More specifically, we examined associations of AMH with markers of atherosclerosis, arterial stiffness, abdominal aortic dilation, and 10-year risk of coronary heart disease (CHD), using cross-sectional and longitudinal data.

Materials and Methods

Study population

We used data from a Dutch population-based cohort study, which included 400 middle-aged and older men aged 40 to 80 years. Details about the scope of this cohort and about recruitment of study participants have been described into detail previously.[11] Briefly, participants were recruited through either convenience sampling or a random selection of the municipal register in 2001 – 2002. After a median follow-up period of 8.7 years, all study participants who were still alive and not living abroad (n = 346) were invited for a follow-up visit in 2010-2011. Of these 346 study participants, 270 were re-examined at follow-up (participation rate 68%). Reasons for non-attendance at follow-up examinations were being physically or mentally unable to visit the study center (n = 40), not interested (n = 22), and non-response (n = 14). During baseline and follow-up visits, questionnaires were completed, fasting blood samples were collected, and physical examinations were conducted. This study received approval from the Institutional Review Board of the University Medical Center Utrecht, and all study participants signed an informed consent prior to study inclusion. For

the current study we excluded study participants without an available serum sample (n = 6). As a result 394 men were included in subsequent analyses.

AMH measurements

Serum samples collected at baseline were stored at -80°C. In September 2019, AMH was measured in serum samples using the cobas e 411 analyzer (Roche Diagnostics) by the Clinical Chemistry Laboratory at the University Medical Center Utrecht. The cobas e 411 is a fully automated analyzer that uses a ElectroChemiLuminescence technology for immunoassay analysis. This method has a variation coefficient of £ 6%, and for AMH its lower limit of quantification is 0.03 µg/L. Of the 394 samples, one measurement exceeded the upper limit of quantification (23 µg/L). For this sample AMH was remeasured in a diluted aliquot. None of the AMH measurements were below the lower limit of quantification.

Subclinical cardiovascular disease measures

Atherosclerosis

We included carotid intima-media thickness (CIMT) and carotid artery plaque burden as markers of atherosclerosis. CIMT (mm) was measured at baseline and follow-up through ultrasonography of both the left and right carotid arteries using a 7.5-MHz linear array transducer (at baseline: Acuson Aspen, Siemens; at follow-up: Acuson Sequoia, Siemens). For each study participant, the average CIMT of eight predefined angles (90°, 120°, 180°, and 150° for the right carotid artery and 180°, 210°, 240°, and 270° for the left carotid artery) was included as measure of CIMT. Carotid artery plaque burden was only assessed at follow-up, using ultrasound images of 12 arterial sites (near and far walls of right and left common carotid artery, the bifurcation and internal carotid artery). A 4-level rating scale was used to quantify plaque burden at each of these sites; 0 = no plaque, 1= minimal plaque, 2 = moderate plaque and 3 = severe plaque. We used these 12 scores to calculate mean plaque scores for each participant, which we included as outcome in subsequent analyses.

Arterial stiffness

We included pulse wave velocity (PWV) as marker of arterial stiffness. PWV (m/s) was measured at baseline and follow-up using using a SphygmoCor device (PWV Medical, Sydney, Australia), as described previously.[12] To cover a complete respiratory cycle, an average of ten successive waveforms was measured. The whole procedure was repeated three times per participant and average PWV values were included as measure of PWV. We set two biologically implausible baseline PWV measurements (2.75 and 30.51 m/s) to missing and subsequently imputed.

Abdominal aortic dilation

We included abdominal aortic diameter as indicator of abdominal aortic dilation, which can be used as indicator for abdominal aortic aneurysms. Abdominal aortic diameter (cm) was measured at both baseline and follow-up visits, but due to the high proportion of missing aortic diameter data at follow-up (91%) we only included aortic diameter at baseline in our analyses. Aortic diameter was measured through ultrasonography (Acuson Aspen, Siemens).

10-year risk of CHD

For each participant, 10-year risk of CHD was calculated using the Framingham risk score formula published by Wilson et al. (1998).[13] Framingham risk score probabilities were calculated using both baseline data and follow-up data.

Covariates

Information on age (years), smoking status (current/former/never) and educational attainment (low/middle/high/university) were collected through questionnaires. Body mass index (BMI) (kg/m²) was calculated using height and weight measurements obtained during physical examination. Systolic blood pressure (mm Hg) was measured in supine position using a semi-automated oscillometric method. We included the average of the two systolic blood pressure measurements in subsequent analyses.

Total cholesterol was measured in serum samples using an automatic enzymatic procedure (Synchron LX Systems, Beckman Coulter, Mydrecht, The Netherlands). Details on serum sex hormone measurements have been described previously.[11] Briefly, total testosterone was measured using an in-house competitive radioimmunoassay employing a polyclonal anti-testosterone (Dr JH Pratt, Indianapolis, IN, USA). The lower limit of detection of this assay was 0.24 nmol/L. Sex hormone binding globuline (SHBG) was measured using an immunometric technique on an Immulite Analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). The lower limit of detection of this method was 5 nmol/L. Dihydroepiandrosterone sulphate (DHEAS) was measured on an Advantage Chemiluminescence System (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), which has a lower limit of detection of 0.1 mmol/L. Total estradiol was measured using an in-house competitive radioimmunoassay employing a polyclonal anti-E2 antibody (Dr F de Jong, Erasmus MC, Rotterdam, The Netherlands). The lower limit of detection was 20 pmol/L. Free testosterone, and free estradiol were calculated using algorithms previously described by Vermeulen et al.[14] and Södergard et al.[15], respectively.

Statistical analyses

Baseline characteristics of study participants were described using median [interquartile range (IQR)], or percentage (n). We summarized baseline characteristics by AMH tertiles. In addition, we calculated Spearman's rank correlation coefficients to assess correlations between age, and AMH, free testosterone, free estradiol and DHEAS. We also calculated correlations between AMH levels and levels of the three sex hormones.

Imputation procedure

Missing information on baseline variables was below 6%, but missing follow-up data ranged from 34% for CIMT to 52% for mean aortic plaque scores. Previous research showed that multiple imputation of missing follow-up data yields unbiased effect estimates if the follow-up data is missing at random.[16] To assess if our missing data met this assumption, we compared baseline characteristics of men without missing values on relevant variables (n = 164) to characteristics of men with missing values on at least one of these variables (n = 230) prior to imputation. We imputed missing values on both baseline and follow-up variables using multiple imputation (100 iterations, 50 imputed datasets), for which we used the R-package "mice" (version 3.6.0).[17] Subsequent regression analyses were performed in each imputed dataset; regression coefficients and standard errors were pooled according to Rubin's Rule of combination[18], using the pool function implemented in "mice".

Cross-sectional analyses

Because of their non-normal distributions, we transformed CIMT, PWV, aorta diameter and Framingham risk score predictions using rank-based inverse normal transformation (INT) implemented in R-package "RNOmni" (version 0.7.1).[19] This approach first transforms observations onto the probability scale using the empirical cumulative distribution function. As a second step, the observations are transformed into Z-scores using the probit function.[19]

We investigated associations of continuous AMH and AMH tertiles with baseline INT CIMT, INT PWV, INT aorta diameter and INT Framingham risk score predictions using linear regression models. First, we performed unadjusted analyses (Model 1). Second, we adjusted regression models for age (Model 2) and, third, for other known CVD risk factors (Model 3); BMI, smoking status, educational attainment, systolic blood pressure and total cholesterol. Finally, we conducted analyses additionally adjusted for levels of free testosterone, free estradiol and DHEAS (Model 4). Analyses including INT Framingham risk score predictions as outcome were not adjusted for age and Model 3 only included BMI and educational level, as age, smoking status, systolic blood pressure and total cholesterol, were already used to calculate Framingham risk score predictions.

Longitudinal analyses

Follow-up CIMT, mean plaque burden scores, PWV and Framingham risk score predictions were also transformed using rank-based INT. We assessed the association between AMH and each INT outcome using linear regression models. Models were adjusted for baseline measurements of the corresponding outcome and for the same baseline covariates as described for the cross-sectional analyses. Because we did not have baseline measurements for carotid artery plaque burden, we fitted an additional model for mean plaque scores, in which we additionally included CIMT at baseline (Model 5). All analyses were performed using R (version 3.5.1).[20]

Sensitivity analyses

Since higher circulating AMH levels have been associated with a lower risk of diabetes and CVD, we performed sensitivity analyses excluding men with prevalent diabetes or CVD, to assess how this affected our results. Sensitivity analyses excluding men with prevalent diabetes were not performed for analyses including Framingham risk score predictions as outcome, as diabetes is included as predictor in the Framingham risk score itself. In addition, we performed sensitivity analyses excluding two outlying AMH measurements (> 15 μg/L) to assess their effect on our results. We also performed a sensitivity analysis in which we truncated follow-up outcome measures prior to imputation for participants who died during follow-up (n = 51). Excluding these participants completely from the longitudinal analyses potentially could have introduced selection bias. If imputations in participants who died were done on the basis of the distribution of CIMT, PWV, mean plaque score and FRS predictions in participants who survived, this could also have biased subsequent regression analyses. Besides, such an approach would create an immortal study population, which is not realistic. Therefore, we performed worst-case scenario analyses for which we assigned arbitrary values representing death due to CVD to each outcome at follow-up to assess how this influenced our results. We truncated CIMT, mean plaque score, PWV and FRS proportions at follow-up to 2.0 mm, 3 (representing severe plaque at each measured site), 25.0 m/s and 1.0, respectively. Subsequently, we repeated multiple imputation and association analyses using the same approach as described for the main analysis.

Results

Baseline characteristics of the study population are presented by AMH tertiles in Table 1. Men with AMH levels in the middle and highest tertiles were younger and less likely to have prevalent CVD than men with AMH levels in the lowest tertile. In addition, men in

the highest AMH tertile were less likely to be current smokers or prevalent diabetes cases compared to men in the middle and lowest AMH tertiles.

Table 1. Baseline characteristics of the study population (n = 394) presented by AMH tertiles.

		AMH tertiles		
		1st AMH tertile (n = 129)	2nd AMH tertile (n = 134)	3rd AMH tertile (n = 131)
AMH (µg/L)[a]		1.9 [1.4, 2.2]	3.2 [2.8, 3.6]	5.9 [4.8, 7.2]
Age (years)[a]		65.0 [56.0, 72.0]	59.0 [48.0, 70.0]	59.0 [48.0, 66.5]
BMI (kg/m²)[a]		26.2 [24.0, 28.2]	26.5 [24.2, 28.6]	25.5 [23.8, 27.3]
Educational attainment[b]				
	Low	17.1 (22)	16.4 (22)	16.8 (22)
	Middle	26.4 (34)	27.6 (37)	32.8 (43)
	High	35.7 (46)	40.3 (54)	27.5 (36)
	University	20.9 (27)	15.7 (21)	22.9 (30)
Smoking status[b]				
	Current	26.4 (34)	26.9 (36)	19.1 (25)
	Former	59.7 (77)	49.3 (66)	55.0 (72)
	Never	14.0 (18)	23.9 (32)	26.0 (34)
Systolic blood pressure (mm Hg)[a,c]		142.0 [124.8, 157.0]	139.0 [127.0, 156.0]	140.0 [129.0, 157.0]
Total cholesterol (mmol/L)[a,c]		5.6 [4.9, 6.2]	6.0 [5.3, 6.7]	5.8 [5.2, 6.4]
Prevalent diabetes[b]		7.0 (9)	7.5 (10)	5.3 (7)
Prevalent CVD[b]		25.6 (33)	13.4 (18)	13.0 (17)
Free testosterone (pmol/L)[a]		316.5 [266.7, 374.6]	343.1 [293.6, 407.3]	360.2 [309.3, 443.8]
Free estradiol (pmol/L)[a]		1.5 [1.3, 1.8]	1.5 [1.3, 1.8]	1.5 [1.3, 1.7]
DHEAS (pmol/L)[a]		5.5 [3.6, 8.2]	6.4 [4.5, 9.1]	6.9 [4.3, 9.1]

AMH, anti-Müllerian hormone; BMI, body mass index; CVD, cardiovascular disease; DHEAS, dihydroepiandrosterone sulphate

[a] Median [IQR]

[b] Percentage (n)

[c] Missing values (n): systolic blood pressure (21), total cholesterol (1)

We observed a weak negative correlation between age and AMH levels (Spearman's rho = -0.21). Higher age was also correlated with lower free testosterone (Spearman's rho = -0.50), free estradiol (Spearman's rho = -0.18) and DHEAS levels (Spearman's rho = 0.54) (Figure 1). We observed weak positive correlations between AMH and free testosterone (Spearman's rho = 0.23) and DHEAS levels (Spearman's rho = 0.13). AMH levels were not correlated with levels of free estradiol.

Comparison of baseline characteristics among study participants with (n = 230) and without complete data (n = 164) indicated that men with one or more missing values on variables included in association analyses were older, less likely to be never smokers, had a higher systolic blood pressure, and were more likely to have prevalent diabetes or CVD

(Supplemental Table 1). In general, participants with missing data were less healthy than participants without missing data. We observed a similar pattern of differences in baseline characteristics between men who attended and who did not attend the follow-up visit.

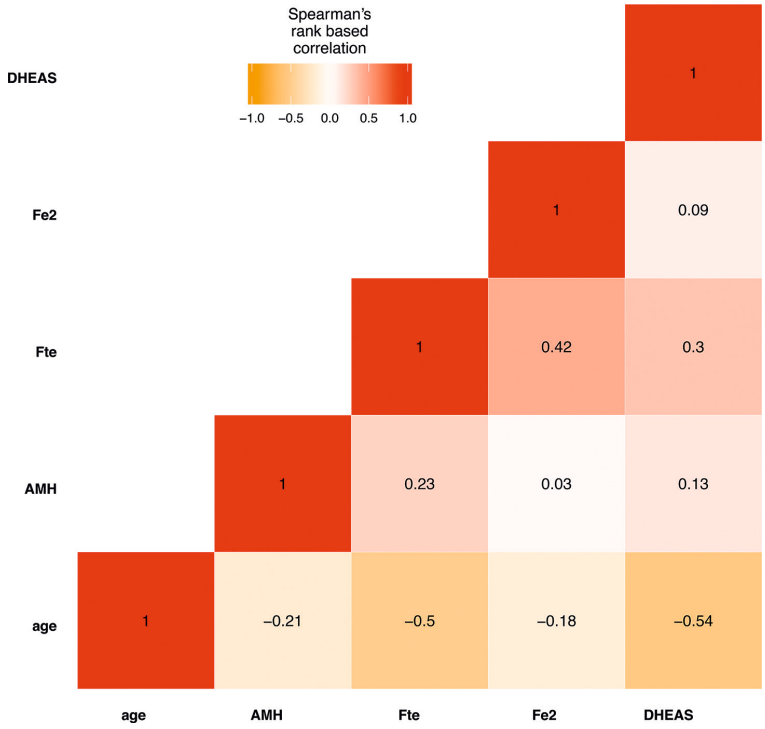

Figure 1. Spearman's rank-based correlation coefficients for age, AMH and sex hormones.

AMH, anti-Müllerian hormone; Fte, free testosterone; Fe2, free estradiol; DHEAS, dihydroepiandrosterone sulphate

Cross-sectional analyses

After adjustment for age and other risk factors for CVD, higher AMH levels were associated with lower baseline INT CIMT ($\beta_{\text{continuous AMH}}$ = -0.04; 95% CI = -0.07, -0.01), but not with INT PWV, or INT aorta diameter measured at baseline (Table 2). Analyses including AMH tertiles supported a linear association between circulating AMH levels and INT CIMT (p_{trend} = 0.01) Adjustment for free testosterone, free estradiol and DHEAS levels did not change these results. In addition, we observed that higher AMH levels were associated with lower INT Framingham risk score predictions ($\beta_{\text{continuousAMH}}$ = -0.05; 95% CI = -0.09, -0.02; Table 2), but effect estimates attenuated after adjustment for free testosterone, free estradiol and DHEAS ($\beta_{\text{continuousAMH}}$ = -0.03; 95% CI = -0.06, 0.00; Table 2). Stepwise adjustment for these sex hormones showed that free testosterone affected effect estimates most.

Table 2. Cross-sectional associations between circulating AMH levels and rank-based inverse normal transformed measures for subclinical CVD measured at baseline.

	Continuous AMH (µg/L)	**AMH tertiles**			
		T1 (< 2.5 µg/L)	**T2 (2.5– 4.0 µg/L)**	**T3 (> 4.0 µg/L)**	
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	p-value for trend
Subclinical CVD measures at baseline					
CIMT					
Model 1: unadjusted	**-0.07 (-0.10, -0.03)**	ref	**-0.41 (-0.65, -0.17)**	**-0.49 (-0.72, -0.25)**	
Model 2: adjusted for age	**-0.04 (-0.07, -0.01)**	ref	-0.17 (-0.35, 0.02)	**-0.20 (-0.38, -0.01)**	
Model 3: model 2 + adjustment for CVD risk factors[a]	**-0.04 (-0.07, -0.01)**	ref	**-0.21 (-0.39, -0.03)**	**-0.24 (-0.43, -0.06)**	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	**-0.04 (-0.07, -0.01)**	ref	**-0.21 (-0.39, -0.03)**	**-0.24 (-0.43, -0.06)**	**0.01**
PWV					
Model 1: unadjusted	-0.03 (-0.07, 0.00)	ref	**-0.31 (-0.55, -0.07)**	**-0.29 (-0.53, -0.04)**	
Model 2: adjusted for age	0.00 (-0.03, 0.02)	ref	-0.06 (-0.25, 0.14)	0.01 (-0.18, 0.20)	
Model 3: model 2 + adjustment for CVD risk factors[a]	-0.01 (-0.04, 0.01)	ref	-0.12 (-0.28, 0.05)	-0.09 (-0.26, 0.08)	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	-0.01 (-0.03, 0.01)	ref	-0.11 (-0.27, 0.06)	-0.08 (-0.25, 0.09)	0.34
Abdominal aortic diameter					
Model 1: unadjusted	-0.02 (-0.06, 0.01)	ref	-0.15 (-0.39, 0.09)	-0.16 (-0.39, 0.08)	
Model 2: adjusted for age	-0.01 (-0.04, 0.03)	ref	-0.05 (-0.28, 0.19)	-0.03 (-0.27, 0.20)	
Model 3: model 2 + adjustment for CVD risk factors[a]	0.00 (-0.04, 0.03)	ref	-0.04 (-0.27, 0.19)	0.02 (-0.22, 0.26)	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	0.00 (-0.04, 0.03)	ref	-0.03 (-0.26, 0.20)	0.03 (-0.21, 0.27)	0.78

Table 2. *(continued)*

	Continuous AMH (µg/L)	AMH tertiles			
		T1 (< 2.5 µg/L)	T2 (2.5– 4.0 µg/L)	T3 (> 4.0 µg/L)	
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	p-value for trend
Framingham risk score predictions					
Model 1: unadjusted	**-0.05 (-0.09, -0.02)**	ref	-0.20 (-0.44, 0.04)	**-0.40 (-0.64, -0.16)**	
Model 2: adjusted for age	N/A	N/A	N/A	N/A	
Model 3: model 2 + adjustment for CVD risk factors[b]	**-0.05 (-0.09, -0.02)**	ref	-0.21 (-0.44, 0.02)	**-0.39 (-0.63, -0.16)**	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	-0.03 (-0.06, 0.00)	ref	-0.05 (-0.26, 0.16)	-0.20 (-0.42, 0.02)	0.07

AMH, anti-Müllerian hormone; CVD, cardiovascular disease; CIMT, carotid intima-media thickness; PWV, pulse wave velocity; DHEAS, dihydroepiandrosterone sulphate
Figures in bold indicate that corresponding p-values were < 0.05

[a] Model adjusted for: age (years), body mass index (kg/m²), educational attainment (low/middle/high/university), smoking status (current/former/never), systolic blood pressure (mm Hg), and total cholesterol (mmol/L)

[b] Model adjusted for: body mass index and educational attainment

Sensitivity analyses excluding men with prevalent diabetes attenuated the association between the middle AMH tertile and baseline INT CIMT, but did not affect other estimates (Supplemental Table 2). Exclusion of men with prevalent CVD and exclusion of outlying AMH measurements did not change results from fully adjusted models either.

Longitudinal analyses

Over a median follow-up period of 8.7 years, CIMT, PWV and FRS predictions increased compared to baseline (Table 3). We observed no associations between AMH and INT CIMT at follow-up (Table 4). However, our results suggested that higher continuous AMH levels were associated with lower INT mean plaque scores, independent of CIMT at baseline ($\beta_{continuousAMH}$ = -0.03, 95% CI = -0.07, 0.00). Effect estimates for the AMH tertiles were in accordance with this, but associations were no longer statistically significant after adjustment for baseline CIMT. AMH levels were not associated with INT PWV or INT FRS at follow-up (Table 4). Sensitivity analyses excluding prevalent diabetes, CVD and the two outlying AMH measurements did not change our conclusions regarding CIMT, PWV and FRS predictions, but the association between AMH and mean plaque score was no longer statistically significant. Analyses including truncated values for participants that died during follow-up did also not change any of our conclusions (Supplemental Table 3).

Table 3. Median [IQR] measures for subclinical CVD measured at baseline and follow-up presented by AMH tertiles.

	AMH tertiles		
	T1 (< 2.5 µg/L)	**T2 (2.5– 4.0 µg/L)**	**T3 (> 4.0 µg/L)**
Subclinical CVD measures[a]			
Atherosclerosis			
CIMT at baseline (mm)	0.85 [0.76, 0.97]	0.78 [0.71, 0.91]	0.76 [0.67, 0.92]
CIMT at follow-up (mm)	0.94 [0.85, 1.09]	0.90 [0.80, 1.01]	0.88 [0.77, 1.01]
Average plaque score at follow-up	0.58 [0.33, 0.92]	0.33 [0.17, 0.75]	0.42 [0.08, 0.67]
Arterial stiffness			
PWV at baseline (m/s)	9.47 [8.17, 10.93]	8.71 [7.31, 10.25]	8.77 [7.40, 10.45]
PWV at follow-up (m/s)	9.80 [8.25, 11.90]	9.25 [7.85, 10.80]	9.10 [7.55, 11.15]
Abdominal aortic diameter at baseline (cm)	1.90 [1.70, 2.00]	1.80 [1.60, 2.00]	1.80 [1.60, 2.00]
10-year risk of coronary heart disease			
Framingham risk score prediction at baseline (proportion)	0.18 [0.13, 0.27]	0.15 [0.10, 0.25]	0.14 [0.08, 0.22]
Framingham risk score prediction at follow-up (proportion)	0.22 [0.13, 0.41]	0.18 [0.12, 0.31]	0.17 [0.10, 0.37]

IQR, interquartile range; CVD, cardiovascular disease; AMH, anti-Müllerian hormone; CIMT carotid intima-media thickness; PWV, pulse wave velocity

[a] Presented median values [IQR] are averages across 50 multiply imputed datasets

Table 4. Longitudinal associations between circulating AMH levels and rank-based inverse normal transformed CIMT and PWV.

	Continuous AMH (µg/L)	**AMH tertiles**			
		T1 (< 2.5 µg/L)	**T2 (2.5 – 4.0 µg/L)**	**T3 (> 4.0 µg/L)**	
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	p-value for trend
Subclinical CVD measures at follow-up					
CIMT					
Model 1: adjusted for baseline CIMT[a]	-0.01 (-0.03, 0.02)	ref	0.07 (-0.08, 0.22)	0.01 (-0.15, 0.18)	
Model 2: model 1 + adjustment for age	-0.01 (-0.03, 0.02)	ref	0.08 (-0.08, 0.23)	0.02 (-0.15, 0.18)	
Model 3: model 2 + adjustment for CVD risk factors[b]	-0.01 (-0.03, 0.02)	ref	0.05 (-0.10, 0.20)	0.01 (-0.15, 0.17)	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	-0.01 (-0.03, 0.02)	ref	0.04 (-0.11, 0.20)	0.00 (-0.17, 0.16)	0.96
Mean plaque score					
Model 1: unadjusted	**-0.06 (-0.10, -0.03)**	ref	**-0.32 (-0.58, -0.06)**	**-0.44 (-0.69, -0.18)**	
Model 2: adjusted for age	**-0.04 (-0.08, -0.01)**	ref	-0.13 (-0.37, 0.11)	-0.22 (-0.45, 0.02)	
Model 3: model 2 + adjustment for CVD risk factors[b]	**-0.04 (-0.08, -0.01)**	ref	-0.17 (-0.4, 0.06)	**-0.26 (-0.49, -0.03)**	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	**-0.04 (-0.08, -0.01)**	ref	-0.17 (-0.4, 0.07)	**-0.25 (-0.48, -0.02)**	
Model 5: model 4 + adjustment for CIMT at baseline[a]	**-0.03 (-0.07, 0.00)**	ref	-0.11 (-0.33, 0.12)	-0.19 (-0.41, 0.04)	0.10

Table 4. *(continued)*

	Continuous AMH (µg/L)	AMH tertiles			
		T1 (< 2.5 µg/L)	T2 (2.5 – 4.0 µg/L)	T3 (> 4.0 µg/L)	
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	p-value for trend
PWV					
Model 1: adjusted for baseline PWV[a]	-0.02 (-0.05, 0.01)	ref	-0.05 (-0.26, 0.16)	-0.12 (-0.32, 0.09)	
Model 2: model 1 + adjustment for age	-0.01 (-0.04, 0.01)	ref	-0.01 (-0.22, 0.20)	-0.05 (-0.26, 0.15)	
Model 3: model 2 + adjustment for CVD risk factors[b]	-0.01 (-0.04, 0.02)	ref	0.00 (-0.21, 0.21)	-0.02 (-0.23, 0.18)	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	-0.01 (-0.04, 0.02)	ref	0.00 (-0.21, 0.22)	-0.01 (-0.22, 0.20)	0.93
Framingham risk score predictions					
Model 1: adjusted for baseline Framingham risk score predictions[a]	0.00 (-0.04, 0.03)	ref	-0.13 (-0.34, 0.09)	-0.04 (-0.26, 0.18)	
Model 2: model 1 + adjustment for age	N/A	ref	N/A	N/A	
Model 3: model 2 + adjustment for CVD risk factors[b]	0.00 (-0.03, 0.03)	ref	-0.14 (-0.36, 0.07)	-0.03 (-0.24, 0.19)	
Model 4: model 3 + adjustment for free testosterone, free estradiol and DHEAS	0.00 (-0.03, 0.03)	ref	-0.12 (-0.33, 0.10)	-0.01 (-0.22, 0.21)	0.97

AMH, anti-Müllerian hormone; CIMT, carotid intima-media thickness; PWV, pulse wave velocity; CVD, cardiovascular disease; DHEAS, dihydroepiandrosterone sulphate

[a] Rank-based INT baseline measurements were included

[b] CVD risk factors included were: body mass index (kg/m²), educational attainment (low/middle/high/university), smoking status (current/former/never), systolic blood pressure (mm Hg), and total cholesterol (mmol/L)

Discussion

In the current study, cross-sectional analyses suggested that higher AMH levels are associated with lower CIMT, independent of risk factors for CVD and circulating sex hormones. Higher AMH levels were also associated with lower Framingham risk score predictions, but adjustment for free testosterone, DHEAS and free estradiol attenuated this association. Our results did not provide evidence for associations between AMH and aorta diameter or PWV. Longitudinal analyses did not provide evidence for associations between circulating AMH levels and CIMT at follow-up, but our results indicated that higher AMH is potentially associated with a lower plaque score after a median follow-up period of 8.7 years. Circulating AMH levels were not associated with PWV and FRS predictions at follow-up.

The main strength of this study is its prospective design, which enabled us to investigate both cross-sectional and longitudinal associations between AMH and different measures of subclinical CVD. Most previous studies only studied these associations cross-sectionally, and mostly in women.[5-10] Furthermore, previous research on the relation of AMH with CVD related outcomes did not examine potential confounding by testosterone, estradiol and DHEAS. A third strength is the relatively large sample size of this study compared to previous studies that investigated the association between circulating AMH and subclinical CVD.

A limitation of the current study is its selective loss to follow-up. Due to the age distribution of the study population at baseline (25% >70 years), a considerable proportion of the participants was not able to attend the follow-up examination after ~9 years. Extra effort was taken to maximize the participation rate at follow-up, amongst others through house calls, although follow-up measurements for CIMT, PWV and plaque scores could not be performed outside of the outpatients' clinic. To limit bias due to this selective loss to follow-up, we imputed missing data using a multiple imputation approach, which is preferable over not addressing missing follow-up data at all.[16] In addition, we performed a worst-case scenario sensitivity analysis in which we assigned participants who died during follow-up values reflecting death due to CVD. Results from this analysis did not indicate that the results of our main analyses were biased.

Our findings regarding AMH and CIMT at baseline and mean carotid artery plaque score at follow-up are in concordance with a previous cross-sectional study that reported that higher circulating AMH levels were associated with lower CIMT in 70 healthy women.[5] In addition, higher baseline circulating AMH levels correlated with smaller atherosclerotic plaques after ~2 years in female monkeys.[4] Our findings add to these findings that AMH

is also associated with CIMT in men, and that this association appears to be independent of circulating sex hormones. Other previous studies examining the relation between AMH and CIMT included a small number of women with type 1 diabetes[6, 21], which limits direct comparison to findings in healthy individuals.

Similar to two previous studies[5, 21], our results do not support that AMH is associated with PWV. In other words, our results suggest that circulating AMH is associated with structural but not with functional changes of the arterial wall. We did also not observe an association between AMH and abdominal aortic diameter, whereas Dennis et al. reported that lower AMH levels were correlated with a larger infrarenal aortic diameter.[10] An explanation for this discrepancy may be the difference in locations at which the aortic diameter was measured, since the study by Dennis et al. did not find a difference in AMH levels between men with an abdominal aortic aneurysm and healthy men.[10] Another explanation may be that the number of participants with an aortic diameter larger than 3.0 cm, which is indicative of a dilated abdominal aorta, in the current study was too small to detect an association (n = 12).

Finally, our results did not provide evidence for an association of circulating AMH levels with 10-year risk predictions for CHD after adjustment for circulating sex hormones. To date, no other studies have directly investigated this association, but our finding is in agreement with a previous longitudinal study that did not find an association between AMH and silent CHD[8], which was quantified as possible or probable CHD on an electrocardiogram. On the other hand, higher AMH levels have been found to be associated with lower incidence of CHD and total CVD in women.[7] Research investigating the association between circulating AMH levels and clinical cardiovascular disease in men is currently lacking.

Future research is required to confirm whether the observed associations between AMH and cardiovascular health reflect a direct effect of AMH or merely correlation. Since higher circulating AMH levels have also been independently associated with lower ~10-year all-cause mortality in men (HR = 0.94; 95 %CI = 0.90−0.98)[22], future studies should be conducted to disentangle whether AMH may be a proxy for biological aging instead of a cardiovascular hormone. Ideally, such studies would examine if the relation between circulating AMH levels and cardiovascular outcomes is confounded by biological aging through inclusion of, for example, epigenetic data.

In conclusion, our results indicate that AMH is associated with current CIMT and potentially with future mean carotid aortic plaque score. However, future studies are required to confirm if our findings are clinically relevant and if AMH is causally associated with, or merely a biomarker for, atherosclerosis.

Disclosure statement

The authors have nothing to disclose.

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Data Availability

Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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Supplemental data

Supplemental Table 1. Baseline characteristics of study participants with and without missings on variables relevant for association analyses.

		Participants with at least one missing (n = 230)	Participants without missings (n = 164)
AMH (µg/L)[a]		3.2 [2.1, 4.7]	3.3 [2.4, 4.9]
Age (years)[a]		65.0 [55.0, 72.0]	56.5 [48.0, 65.0]
BMI (kg/m^2)[a]		26.3 [24.2, 28.6]	25.6 [23.2, 27.6]
Educational attainment[b]			
	Low	17.8 (41)	15.2 (25)
	Middle	30.9 (71)	26.2 (43)
	High	34.8 (80)	34.1 (56)
	University	16.5 (38)	24.4 (40)
Smoking status[b]			
	Current	20.9 (48)	28.7 (47)
	Former	59.6 (137)	47.6 (78)
	Never	19.6 (45)	23.8 (39)
Systolic blood pressure (mm Hg)[a]		144.0 [131.0, 159.0]	136.5 [123.8, 148.3]
Total cholesterol (mmol/L)[a]		5.8 [5.0, 6.4]	5.7 [5.2, 6.3]
Prevalent diabetes[b]		7.8 (18)	4.9 (8)
Prevalent CVD[b]		23.0 (53)	9.1 (15)
Free testosterone (pmol/L)[a]		332.3 [279.0, 389.4]	352.8 [305.8, 431.1]
Free estradiol (pmol/L)[a]		1.5 [1.3, 1.8]	1.5 [1.3, 1.8]
DHEAS (pmol/L)[a]		5.5 [3.8, 8.1]	7.0 [4.9, 9.6]
CIMT at baseline (mm)[a]		0.84 [0.74, 0.96]	0.76 [0.69, 0.86]
PWV at baseline (m/s)[a]		9.52 [8.24, 11.54]	8.32 [7.31, 9.50]
Abdominal aortic diameter (cm)[a]		1.90 [1.70, 2.10]	1.80 [1.60, 2.00]
Framingham risk score predictions (proportions)[a]		0.19 [0.12, 0.27]	0.13 [0.08, 0.19]

AMH, anti-Müllerian hormone; BMI, body mass index; CVD, cardiovascular disease; DHEAS, dihydroepiandrosterone sulphate; CIMT, carotid intima-media thickness; PWV, pulse wave velocity

[a] Median [IQR]

[b] Percentage (n)

Supplemental Table 2. Results of cross-sectional sensitivity analyses; associations between circulating AMH levels and rank-based inverse normal transformed measures for subclinical CVD measured at baseline after (1) exclusion of prevalent diabetes cases, (2) exclusion of prevalent CVD cases, and (3) exclusion of two outlying AMH measurements.

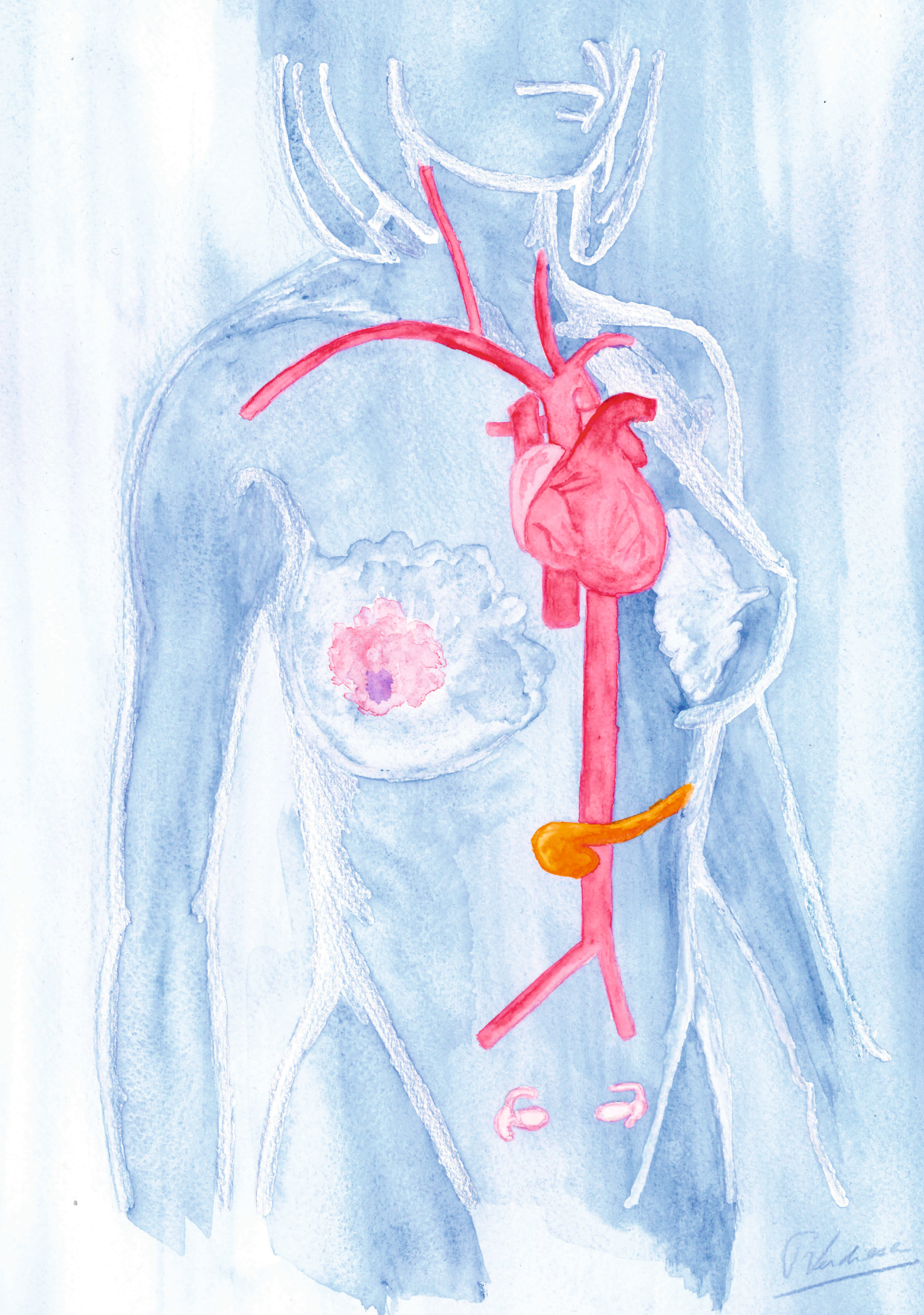
	Continuous AMH (µg/L)	**AMH tertiles**		
		T1	**T2**	**T3**
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Subclinical CVD measures				
CIMT				
Main analysis	**-0.04 (-0.07, -0.01)**	ref	**-0.21 (-0.39, -0.03)**	**-0.24 (-0.43, -0.06)**
Excluding prevalent diabetes	**-0.04 (-0.07, -0.01)**	ref	-0.18 (-0.37, 0.01)	**-0.23 (-0.42, -0.04)**
Excluding prevalent CVD	**-0.04 (-0.07, -0.01)**	ref	**-0.22 (-0.42, -0.03)**	**-0.24 (-0.45, -0.04)**
Excluding outlying AMH measurements	**-0.03 (-0.07, 0.00)**	ref	**-0.21 (-0.39, -0.03)**	**-0.23 (-0.42, -0.04)**
PWV				
Main analysis	-0.01 (-0.03, 0.01)	ref	-0.11 (-0.27, 0.06)	-0.08 (-0.25, 0.09)
Excluding prevalent diabetes	-0.01 (-0.04, 0.01)	ref	-0.15 (-0.32, 0.02)	-0.11 (-0.28, 0.07)
Excluding prevalent CVD	-0.01 (-0.04, 0.02)	ref	-0.11 (-0.29, 0.08)	-0.07 (-0.26, 0.11)
Excluding outlying AMH measurements	0.00 (-0.03, 0.02)	ref	-0.11 (-0.27, 0.06)	-0.08 (-0.25, 0.09)
Aorta diameter				
Main analysis	0.00 (-0.04, 0.03)	ref	-0.03 (-0.26, 0.20)	0.03 (-0.21, 0.27)
Excluding prevalent diabetes	0.00 (-0.04, 0.03)	ref	-0.04 (-0.28, 0.20)	0.03 (-0.22, 0.27)
Excluding prevalent CVD	0.00 (-0.04, 0.03)	ref	-0.02 (-0.27, 0.23)	0.02 (-0.23, 0.28)
Excluding outlying AMH measurements	0.00 (-0.04, 0.04)	ref	-0.03 (-0.26, 0.20)	0.03 (-0.21, 0.28)
Framingham risk score predictions				
Main analysis	-0.03 (-0.06, 0.00)	ref	-0.05 (-0.26, 0.16)	-0.20 (-0.42, 0.02)
Excluding prevalent diabetes	N/A	ref	N/A	N/A
Excluding prevalent CVD	-0.03 (-0.06, 0.01)	ref	0.03 (-0.21, 0.28)	-0.18 (-0.42, 0.07)
Excluding outlying AMH measurements	-0.04 (-0.07, 0.00)	ref	-0.05 (-0.27, 0.16)	-0.20 (-0.41, 0.02)

AMH, anti-Müllerian hormone; CVD, cardiovascular disease; CIMT, carotid intima-media thickness; PWV, pulse wave velocity
Presented estimates correspond to fully adjusted models (Model 4), and thus included the following potential confounders: age, body mass index, educational attainment, smoking status, systolic blood pressure, and total cholesterol, free testosterone, free estradiol and dihydroepiandrosterone sulphate (DHEAS)

Supplemental Table 3. Results of sensitivity analyses including truncated values for participants who died during follow-up.

	Continuous AMH (µg/L)	**AMH tertiles**		
		T1	**T2**	**T3**
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Subclinical CVD measures at follow-up[a]				
CIMT	-0.01 (-0.04, 0.02)	ref	0.14 (-0.03, 0.31)	0.07 (-0.10, 0.25)
Mean plaque score	**-0.03 (-0.06, 0.00)**	ref	-0.05 (-0.25, 0.14)	-0.11 (-0.32, 0.09)
PWV	-0.02 (-0.05, 0.01)	ref	0.08 (-0.11, 0.28)	0.03 (-0.18, 0.23)
Framingham risk score predictions	0.00 (-0.03, 0.03)	ref	0.03 (-0.17, 0.22)	0.04 (-0.15, 0.24)

AMH, anti-Müllerian hormone; CVD, cardiovascular disease, CIMT, carotid intima-media thickness; PWV, pulse wave velocity
[a] Presented estimates correspond to fully adjusted models for each outcome (Model 4 for CIMT, PWV and Framingham risk score predictions and model 5 for mean plaque score)



CHAPTER 6

Genome-wide association study meta-analysis identifies three novel loci for circulating anti-Müllerian hormone levels in women

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Abstract

Anti-Müllerian hormone (AMH) is expressed by antral stage ovarian follicles in women. Consequently, circulating AMH levels are detectable until menopause. Variation in age-specific AMH levels has been associated with breast cancer and polycystic ovary syndrome (PCOS), amongst other diseases. Identification of genetic variants underlying variation in AMH levels could provide clues about the physiological mechanisms that explain these AMH-disease associations. To date, only one variant in *MCM8* has been identified to be associated with circulating AMH levels in women. We aimed to identify additional variants for AMH through a GWAS meta-analysis including data from 7049 premenopausal women of European ancestry, which more than doubles the sample size of the largest previous GWAS. We identified four loci associated with AMH levels at $p < 5x10^{-8}$: the previously reported *MCM8* locus and three novel signals in or near *AMH, TEX41,* and *CDCA7.* The strongest signal was a missense variant in the *AMH* gene (rs10417628). Most prioritized genes at the other three identified loci were involved in cell cycle regulation. Genetic correlation analyses indicated a strong positive correlation among SNPs for AMH levels and for age at menopause ($r_g = 0.82$, FDR=0.003). Exploratory Mendelian randomization analyses did not support a causal effect of AMH on breast cancer or PCOS risk, but should be interpreted with caution as they may be underpowered and the validity of genetic instruments could not be extensively explored. In conclusion, we identified a variant in the *AMH* gene and three other loci that may affect circulating AMH levels in women.

Introduction

Anti-Müllerian hormone (AMH) is generally known for its function in sexual differentiation, during which AMH signaling is essential for the regression of internal female reproductive organs in male embryos.[1] In women, AMH is expressed by granulosa cells of primary ovarian follicles, and AMH expression continues until the antral stage.[2] AMH becomes undetectable after menopause, when the ovarian reserve is depleted, and AMH can therefore be used as a marker for reproductive aging.[3]

Variation in age-specific circulating AMH levels has been associated with the occurrence of several non-communicable diseases, including breast cancer.[4] In addition, it has been suggested that AMH may be involved in the pathogenesis of polycystic ovary syndrome (PCOS).[5] Gaining more insight into genetic variation and biological mechanisms underlying inter-individual variation in AMH expression through genome-wide association studies (GWASs) could provide new clues regarding postnatal functions of AMH, and possibly, the etiologies of non-communicable diseases associated with AMH levels.

Previous GWASs on circulating AMH levels included either a mixture of male and female adolescents[6], a very small study population[7], or women of late reproductive age[8] in whom AMH levels are generally very low. Of the previous GWASs, only the largest (n = 3344) identified a single genetic variant for AMH levels in premenopausal women, at chromosome 20 (rs16991615)[8], which is also associated with natural age at menopause.[9; 10] As sample sizes of previous GWASs were relatively small, a larger GWAS meta-analysis might lead to detection of more AMH variation loci. Moreover, as most variation in AMH levels in women is observed at ages 20 to 40 years[11], including younger women will increase power to identify additional loci. Therefore, we aimed to identify additional genetic variants for AMH through a GWAS meta-analysis including 7049 premenopausal female participants. For that, we combined summary statistics from the AMH GWAS meta-analysis by Ruth et al.[8] with GWAS data from 3705 additional women of early and middle reproductive age from 3 different cohorts.

Subjects and Methods

Study population

We included data from 7049 premenopausal female participants (median age ranged from 15.3 to 48 years across cohorts; Table 1) of European ancestry. In addition to the data from the AMH GWAS meta-analysis by Ruth et al.[8] (n = 3344), we included data from

the Doetinchem Cohort Study[12; 13] (n = 2084), the Study of Women's Health Across the Nation (SWAN)[14] (n = 425), and data from adolescent daughters of the Avon Longitudinal Study of Parents and Children (ALSPAC)[15] (n = 1196). The GWAS by Ruth et al. included data from the Generations Study[16], Sister Study[17], Nurses' Health Study[18], Nurses' Health Study II[19], and ALSPAC mothers[20]. For the current study, we requested summary statistics excluding data from ALSPAC mothers, as we wanted to analyze data from the ALSPAC mothers separately to investigate potential bias due to cryptic relatedness. More details about participating studies and the definitions used for the assessment of menopausal status are described in the Supplemental Methods and Supplemental Table 1. All studies received ethical approval from an institutional ethics committee.

Table 1. Distributions of AMH and age per participating study.

Study	N	AMH, pmol/L (median (IQR))	Age at blood collection, years (median (IQR))
Studies contributing to summary statistics GWAS Ruth et al.[8]*			
Generations Study	379	3.9 (0.8, 11.7)	44 (40, 48)
Sister Study	438	1.2 (0.1, 6.0)	48 (45, 51)
Nurses' Health Studies	642	6.1 (2.0, 13.9)	44 (41, 47)
Additional studies			
Doetinchem Cohort Study	2084	10.9 (2.9, 25.6)	37.2 (31.2, 42.9)
ALSPAC mothers	1885	2.0 (0.4, 5.2)	46 (44, 49)
ALSPAC daughters	1196	26.1 (18.2, 39.8)	15.3 (15.3, 15.5)
SWAN	425	1.1 (0.2, 3.3)	47.3 (45.3, 49.3)
Total	7049		

* In the original study ALSPAC mothers were included as well, but in the current analyses summary statistics from ALSPAC were included separately to assess potential genomic inflation due to inclusion of both ALSPAC mothers and daughters. Therefore, we treated the ALSPAC mothers as individual study.

AMH measurements

Included studies measured AMH in either serum or plasma using different AMH ELISA assays. Also, the methodology for handling AMH measurements below the assay limit of detection (LOD) differed across studies. A detailed overview of these study-specific details has been included in Supplemental Table 1. Across studies, the percentage of measurements under the assay-specific LODs ranged from 0% to 24.2%.

Genotyping and imputation

Extensive details on genotyping and imputation procedures for each participating study are presented in Supplemental Table 2. Briefly, samples of the Generations Study, Sister Study, and most samples of the Nurses' Health Studies, were genotyped using the OncoArray

array.[8] The remaining 225 samples of the Nurses' Health Studies were genotyped using Illumina HumanHap550 and HumanHap610 arrays.[8] Samples of the Doetinchem Cohort Study were genotyped using the Illumina Infinium Global Screening Array-24 Kit (Illumina Inc., San Diego, California, United States of America). For genotyping of samples from ALSPAC mothers and daughters, the Illumina Human660W-Quad array and Illumina HumanHap550 quad genome-wide SNP genotyping platform were used, respectively. SWAN participants were genotyped using the Illumina Multi-Ethnic Global Array (MEGA A1). All participating studies performed sample and SNP QC prior to imputation, which was done using the Haplotype Reference Consortium (HRC) panel version r1.1 2016 (Supplemental Table 2).

Association analyses

All studies converted AMH concentrations to pmol/L using 1 pg/mL = 0.00714 pmol/L. As AMH levels are not normally distributed, AMH measurements were transformed using rank-based inverse normal transformation in all studies, as previously described.[8]

In all studies linear models were fitted, assuming additive SNP effects, adjusted for age at blood collection (years) (Supplemental Methods). Analyses were further adjusted for population stratification by inclusion of either 10 principal components (ALSPAC, SWAN) or a kinship matrix (Generations Study, Sister Study, Nurses' Health Studies, Doetinchem Cohort Study). In addition, we included summary statistics of the meta-analysis of the Generations Study, Sister Study, and Nurses' Health Studies, which was performed using METAL, as described elsewhere.[8] Separate association analyses were conducted for the ALSPAC mothers and daughters, because of the large differences in both age and AMH distributions between these groups (Supplemental Methods).

Prior to meta-analysis, we performed file-level and meta-level QC on all summary statistics files to clean and check the data, and to identify potential study-specific problems. File-level and meta-level QC were conducted using the R package EasyQC (v9.2), following a previously published protocol[21] (Supplemental Methods). No study-specific issues were identified through these QC procedures (Figure S1-S5). In addition, we sought to confirm that inclusion of ALSPAC mothers and daughters as separate cohorts would not result in inflation of effect estimates due to cryptic relatedness (411 mother-daughter pairs were present in the ALSPAC data). We checked this through meta-analyzing only data of the two ALSPAC cohorts and checking both the corresponding QQ plot and calculating λ. Given the absence of genomic inflation ($\lambda = 1.01$, QQplot in Figure S6) we included summary statistics of both ALSPAC mothers and daughters in the meta-analysis.

We performed an inverse variance weighted meta-analysis using METAL (version 2011-03-25). Genomic control was applied for all included studies. SNPs with a minor allele frequency (MAF) < 1% and/or poor imputation quality (info score < 0.4 or r^2 < 0.3, depending on which metric was provided) were excluded. As a result, 8,298,138 autosomal SNPs were included in this AMH GWAS meta-analysis. To assess if observed effect estimates were homogeneous across studies, we additionally performed a heterogeneity analysis in METAL.

To identify lead and secondary SNPs within genome-wide significant associated loci, we performed an approximate conditional and joint association analysis.[22] We used Genome-wide Complex Trait Analysis (GCTA)[23] (version 1.93.1f beta) to run a stepwise model selection procedure to select independently associated SNPs (cojo-slct) using the summary-level data. We estimated linkage disequilibrium (LD) between SNPs using data of 4059 unrelated participants from the EPIC-NL cohort[24] as LD reference panel.

Because of the strong correlation between AMH and age, and the difference in both the AMH and age distributions in the ALSPAC daughters compared to the other included cohorts, we performed a sensitivity analysis in which we excluded the ALSPAC daughters. Furthermore, this sensitivity analysis served as an additional check that inclusion of both ALSPAC mothers and daughters did not cause identification of false-positive hits.

Gene-based genome-wide association analysis

We performed a gene-based genome-wide association analysis using the MAGMA[25] implementation (v1.08) in the online Functional Mapping and Annotation of Genome-wide Association Studies (FUMA) platform (FUMA)[26] (parameter settings are listed in Supplemental Table 3). For this analysis, SNPs located in gene bodies were aggregated to 18,896 protein coding genes (Ensembl build 92). MAGMA tests the joint association of all SNPs in each gene with inverse normal transformed AMH levels using a multiple linear regression approach, which takes LD between SNPs into account.[25] FUMA considered genes to be significantly associated with circulating AMH levels if $p < 2.65 \times 10^{-6}$ (Bonferroni corrected p-value; 0.05/ 18,896).

Functional annotation using FUMA

FUMA is an integrative web-based platform that uses 18 biological resources and can be used to functionally annotate lead variants from GWAS, and to prioritize the most likely causal SNPs and genes[26]. We used the SNP2GENE process integrated into FUMA (v1.3.6a)[26] for the characterization of genomic loci and functional gene mapping (parameter settings are listed in Supplemental Table 3). We included SNPs identified in our approximate conditional and joint analysis as predefined lead SNPs for the characterization of genomic risk loci.

SNPs that were in LD with these lead SNPs ($r^2 > 0.6$) within a 500kb window based on the 1000G Phase 3 European reference panel population in FUMA, and a GWAS meta-analysis p-value < 0.05 were selected as candidate SNPs. Non-GWAS-tagged SNPs from the 1000G Phase 3 European reference that met these LD and distance criteria were also selected as candidate SNPs. Candidate SNPs were annotated based on Combined Annotation Dependent Depletion (CADD) scores[27], Regulome DB scores[28], and chromatin states[29] (Supplemental Table 3). Positional mapping, eQTL mapping and chromatin interaction mapping were used to map SNPs to genes (Supplemental Table 3). For chromatin states, eQTL mapping and chromatin interaction mapping we only selected for tissues and cell types that are most likely to be involved in AMH expression and signaling (Supplemental Table 3).

Pathway analysis using DEPICT

We used the hypothesis-free pathway analysis tool DEPICT (v1)[30] to prioritize the most likely causal genes at associated loci, to highlight gene sets enriched in genes within associated loci, and to identify tissues/cell types that are implicated by the associated loci. For these analyses, we included all suggestive significant SNPs ($p < 5 \times 10^{-6}$), which were clumped at LD $r^2 < 0.1$ and a physical distance of 500kb using PLINK v.1.9 as part of the DEPICT pipeline.

LD Score Regression

We estimated SNP heritability using the LD Hub web interface (v1.9.3)[31] for LD score regression[32]. In addition, we used LD Hub to estimate SNP-based genetic correlations between AMH and phenotypes that have been associated with AMH in observational studies. These genetic correlation analyses make use of GWAS summary statistics for all SNPs to estimate genetic covariance among SNPs for two traits.[33] Included phenotypes comprised reproductive traits, hormones (leptin), anthropometric traits, blood lipids, glycemic traits, metabolites, cardiometabolic traits, cancer, autoimmune diseases, bone mineral density, aging and smoking behavior. Of the 597 UK Biobank traits in the LDHub database, we only included traits that corresponded to these phenotype categories, resulting in 345 comparisons. To correct for multiple testing, we calculated false discovery rates (FDR), using the p.adjust function in R (R package "stats").[34] FDR adjusted p-values < 0.05 were considered to be significant.

Mendelian randomization

In observational studies, AMH has been associated with breast cancer[4], and PCOS[35], amongst other diseases. As the exact function of AMH in the etiology of these diseases is unclear, and actual AMH levels are associated with predicted future age at menopause and current menopausal status, causality of these AMH-disease associations remains to be determined.

Mendelian randomization (MR) is a method that may provide evidence for causality of observational associations.[36] Because our AMH GWAS meta-analysis only included women, and previous research suggests that genetic variants for inter-individual differences in AMH levels differ between males and females[6], we performed MR analyses for the female-specific outcomes breast cancer and PCOS only. We performed two-sample MR analyses[37] using the R package "TwoSampleMR" (version 0.5.1)[38]. We included identified lead SNPs as genetic instruments for AMH. For the outcomes, we included summary statistics from the most recent largest GWASs for breast cancer (n = 228,951; 122,977 cases)[39] and PCOS (n = 113,238; 10,074 cases)[40]. Wald ratio estimates were calculated for individual SNPs and a random effects inverse variance weighted (IVW) meta-analysis approach was used to combine these estimates. To assess the strength of included genetic variants for AMH we calculated F-statistics corresponding to the IVW analyses, using the proportion of variance in AMH explained by the genetic variants, the sample size of the outcome GWASs, and the number of variants included.[41] We compared the overall MR estimate (i.e. IVW) to SNP-specific MR estimates (i.e. Wald ratio) since inconsistent estimates are indicative of horizontal pleiotropy, which is a violation of the MR assumptions.[38] In addition, we tested for heterogeneity in causal effects amongst the genetic instruments using Cochrane's Q statistics and performed leave-one-out sensitivity analyses to assess the potential effect of outlying variants.

Results

Descriptive statistics on age and AMH levels of the study participants included in this GWAS meta-analysis are presented per study in Table 1. Median AMH ranged from 1.1 pmol/L in SWAN to 26.1 pmol/L in ALSPAC daughters. Median age ranged from 15.3 years in ALSPAC daughters to 48 years in the Sister Study.

Genome-wide association analysis

We identified four genome-wide significant lead SNPs ($p < 5 \times 10^{-8}$) for inverse normally transformed AMH, in four loci (Table 2, Figure 1, Figure S7-S8). Approximate conditional and joint analysis did not reveal secondary signals. In addition to the previously reported locus on chromosome 20 (rs16991615, nearest gene: *MCM8*), we identified 1 locus on chromosome 19 (nearest gene: *AMH*) and 2 loci on chromosome 2 (nearest genes: *TEX41* and *CDCA7*). The strongest signal was rs10417628 on chromosome 19, which is physically located in the *AMH* gene ($\beta = -0.34$, se = 0.05, $p = 1.2 \times 10^{-11}$) (Figure S8A). Combined the four lead SNPs explained 1.47% of the variance in AMH levels.

Table 2. Loci significantly associated ($p < 5 \times 10^{-8}$) with inverse normally transformed AMH in women.

Nearest gene	SNP	Chr	Pos	EA	OA	EAF	N	Imputation quality	Effect (SE)	P	Direction	P_{het}	Percentage of variance in AMH explained
AMH	rs10417628	19	2,251,817	T	C	0.02	7049	0.83	-0.34 (0.05)	1.2×10^{-11}	-----	0.14	0.50 %
TEX41	rs13009019	2	145,670,572	A	G	0.69	7049	0.95	-0.09 (0.01)	7.2×10^{-10}	-----	0.24	0.35%
MCM8	rs16991615	20	5,948,227	A	G	0.07	7049	0.99	0.16 (0.03)	1.2×10^{-8}	++-++	0.0009	0.30%
CDCA7	rs11683493	2	174,259,325	T	C	0.57	7049	0.97	-0.08 (0.01)	1.7×10^{-8}	-----	0.03	0.32%

Definition of columns: nearest gene, nearest gene identified using DEPICT tool (Subjects and Methods); SNP, genetic variant identified as lead SNP; Chr, chromosome; Pos, base pair position genomic build GRCh37; EA, effect allele; OA, other allele; EAF, effect allele frequency; N, number of samples contributing to estimate; Imputation quality, mean imputation quality over the included studies; Effect (SE); effect size and corresponding standard error; P, p-value; Direction, direction of effect for previous GWAS, ALSPAC mothers, ALSPAC daughters, Doetinchem Cohort Study and SWAN, respectively; P_{het}, p-value for heterogeneity of effect across studies.

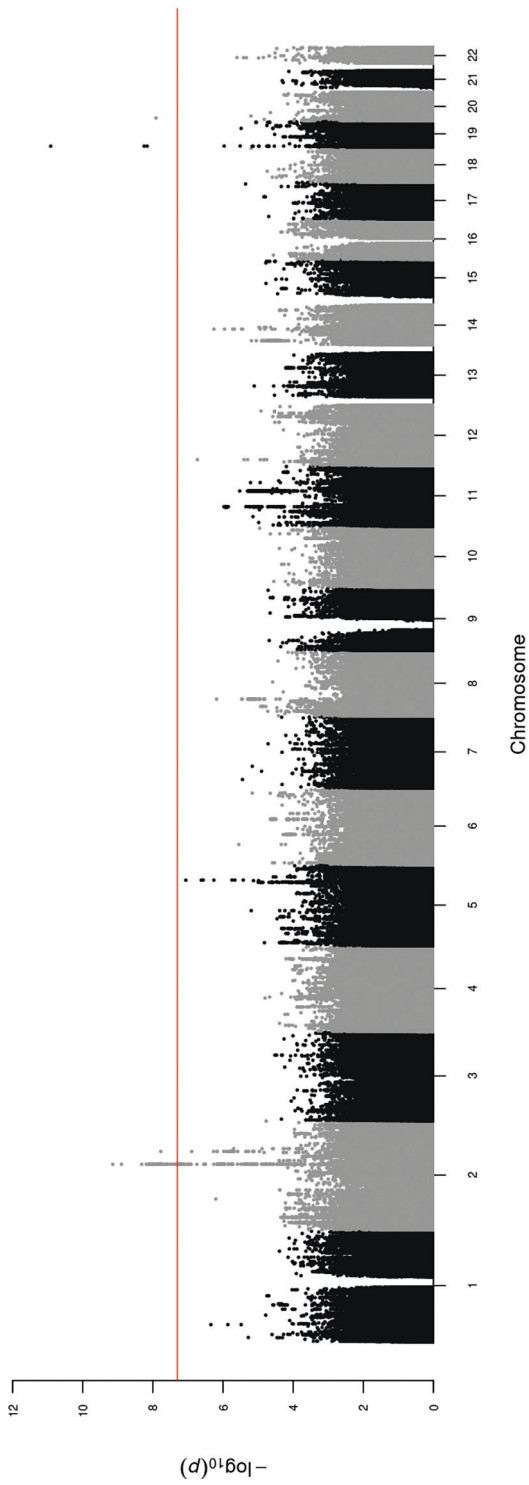

Figure 1. Manhattan plot of genome-wide association results for inverse normally transformed AMH in women.

Results are association results from meta-analysis of inverse normally transformed AMH in 7049 women of European ancestry. Individual studies adjusted analyses for age at AMH measurement and population stratification (through kinship matrix or 10 principal components).

In the sensitivity analysis excluding ALSPAC daughters, all four loci from the main analysis remained genome-wide significant, and an additional locus at chromosome 5 (rs116090962, nearest gene: *CTB-99A3.1*) was identified ($\beta = 0.38$, se = 0.07, $p = 6.0 \times 10^{-9}$) (Supplemental Table 4).

Gene-based genome-wide association analysis

Gene-based genome-wide association analysis, which tested associations between 18,896 protein coding genes and inverse normal transformed AMH, highlighted the following two significant genes: *AMH* and *BMP4* (Figure S9).

Functional Annotation using FUMA

Through the SNP2GENE process, FUMA identified 82 candidate SNPs that were in LD with the four identified lead SNPs (Supplemental Table 5). These candidate SNPs were used for the prioritization of genes.

In total, 12 genes were mapped to the locus of the previously identified SNP on chromosome 20 (rs16991615) (Supplemental Table 6), of which *MCM8* and *CRLS1* were prioritized based on eQTL mapping (Figure 2A). *CRLS1* was the only gene prioritized based on both eQTL mapping and chromatin interactions. However, as rs16991615 is a missense variant located in exon 9 of the *MCM8* gene, and this was the only SNP identified for this locus, *MCM8* is the most likely gene causing this signal.

For the locus on chromosome 19, 3 candidate SNPs (rs10417628, rs12462821, rs7247495) were identified. The lead SNP in this locus (rs10417628) is also a missense variant, located in exon 5 of the *AMH* gene, making this the most likely causal gene at this locus. The other 2 variants were located in intronic and intronic non-coding RNA regions. Based on the used parameter settings, FUMA mapped 8 genes to the *AMH* locus (Supplemental Table 6), of which 4 were highlighted by eQTL mapping (*AMH, C19orf35, SPPL2B and LSM7*) and 1 through chromatin interactions (*ABHD17A*) (Figure 2B).

Most of the candidate SNPs were identified for the locus on chromosome 2 near *TEX41*. All 77 variants were located in either intronic or exonic long noncoding RNA regions. Of the 15 genes mapped to this locus (Supplemental Table 6), no genes were prioritized based on eQTL mapping, but several genes were prioritized based on chromosome interactions, including *ZEB2-AS1* (Figure 2C). In the other locus on chromosome 2, for which *CDCA7* is the nearest gene, no additional candidate SNPs were identified and no genes were mapped to this locus.

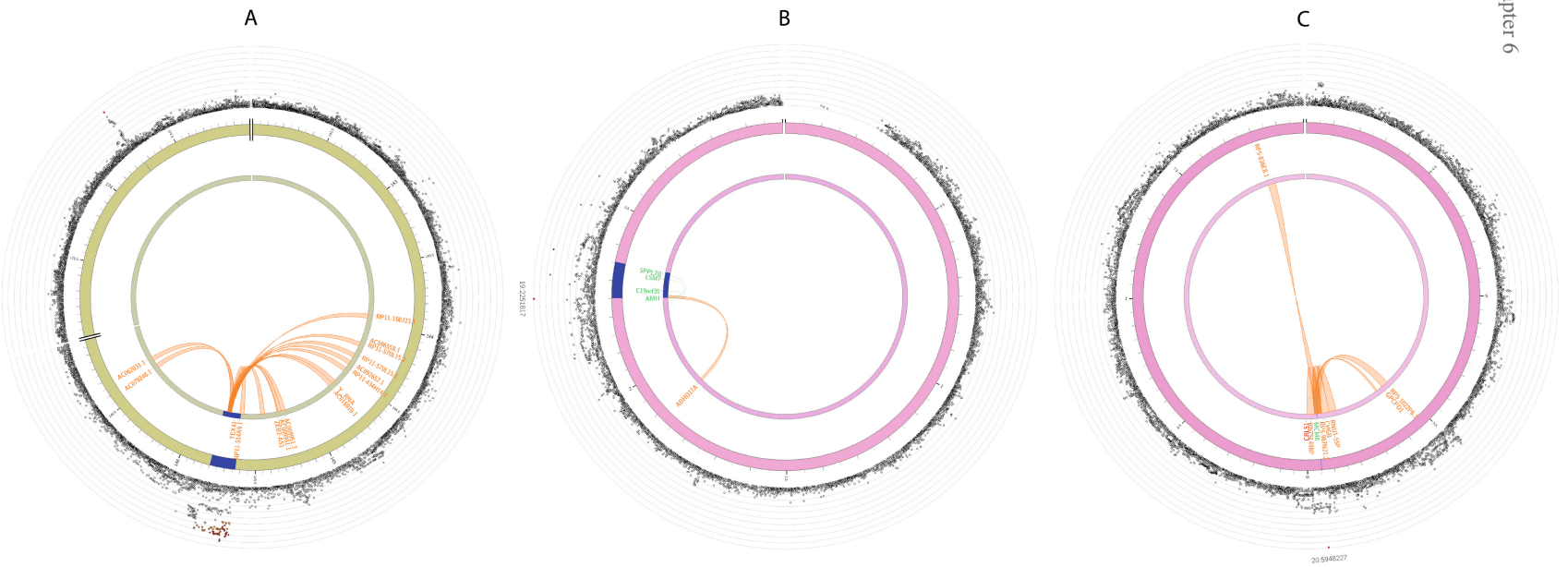

Figure 2. Circos plots for the genome-wide significant loci for inverse normally transformed AMH in women.

Circos plots are presented for each of the identified loci: *MCM8* (panel A), *AMH* (panel B), *TEX41* and *CDCA7* (panel C). The outer layer represents the Manhattan plot. The second (including genomic positions) and third layers represents the chromosome ring, genomic risk loci are depicted in blue. Only genes mapped by either eQTLs or chromatin mapping are plotted. Genes only mapped by eQTLs are green, genes only mapped by chromatin interactions are orange, and genes mapped by both have a red colour. Orange coloured lines represent chromatin interactions, green coloured lines are eQTL links. Plots were created using the FUMA platform.[26]

Pathway analysis using DEPICT

Using the DEPICT tool, 188 suggestive associated SNPs ($p < 5 \times 10^{-6}$) were clumped at LD $r^2 < 0.1$ and a physical distance of 500 kb, resulting in 24 clumps as input for the enrichment analyses (Supplemental Table 7). The top three prioritized gene sets were "URI1 PPI subnetwork", "NFYB PPI subnetwork" and "nuclear inner membrane" (Supplemental Table 8). "Induced Pluripotent Stem Cells" was identified as the highest prioritized cell type (Supplemental Table 9). However, none of these enrichments were statistically significant (FDR > 0.05).

DEPICT prioritized nine genes at FDR < 0.05 as most likely causal genes (Supplemental Table 10). Of the genome-wide associated loci, only *MCM8* (rs16991615), and *CDCA7* (rs11683493) were prioritized at this FDR threshold. *AMH* and *BMP4* were also prioritized by DEPICT, but FDR values were > 0.20.

LD Score Regression

We used LD score regression implemented in LD Hub to calculate SNP heritability for AMH based on the meta-analysis summary statistics. Total SNP heritability (h_g^2) on the observed scale was estimated to be 15% (se = 7%). We additionally performed genetic correlations analyses between AMH and 345 traits on LD Hub. After correction for multiple testing, AMH was only significantly correlated with age at menopause ($r_g = 0.82$, se = 0.19, FDR = 0.003) (Supplemental Table 11).

MR analyses

IVW MR estimates did not indicate a causal effect of circulating AMH on breast cancer risk ($OR_{IVW} = 1.00$, 95%CI: 0.74 – 1.36; Table 3). Results from the single SNP analysis including the variant in the *AMH* locus also did not support a causal association with breast cancer ($OR_{IVW} = 0.99$, 95%CI = 0.87 – 1.12), whereas analyses for the remaining variants suggested a risk decreasing effect of the SNPs in the *TEX41* and *CDCA7* loci and a risk increasing effect of the variant in the *MCM8* locus (Table 3). In agreement with these findings, a formal heterogeneity test for the IVW estimate indicated heterogeneity in causal effects amongst the four genetic variants (2.13×10^{-11}), although the interpretation of this heterogeneity p-value is limited due to the small number of included SNPs. Leave-one-out sensitivity analyses supported the outlying effect of rs16991615 (*MCM8* locus) (Figure S10).

Table 3. Mendelian randomization estimates for causal effects of circulating AMH on breast cancer and PCOS risk.

Outcome	Method	Odds Ratio	95% CI	p
Breast Cancer	IVW	1.00	0.74 - 1.36	0.98
	Wald ratio estimate for rs10417628 (*AMH*)	0.99	0.87 - 1.12	0.85
	Wald ratio estimate for rs13009019 (*TEX41*)	0.84	0.72 - 0.97	0.02
	Wald ratio estimate for rs16991615 (*MCM8*)	1.60	1.37 - 1.87	1.79×10^{-9}
	Wald ratio estimate for rs11683493 (*CDCA7*)	0.76	0.65 - 0.89	9.41×10^{-4}
PCOS	IVW	1.29	0.85 - 1.95	0.23
	Wald ratio estimate for rs10417628 (*AMH*)	1.27	0.64 - 2.56	0.49
	Wald ratio estimate for rs13009019 (*TEX41*)	1.66	0.80 - 3.45	0.18
	Wald ratio estimate for rs16991615 (*MCM8*)	1.75	0.83 - 3.69	0.14
	Wald ratio estimate for rs11683493 (*CDCA7*)	0.66	0.29 - 1.50	0.32

AMH, anti-Müllerian hormone; PCOS, polycystic ovary syndrome; IVW, inverse variance weighted; MR, Mendelian randomization

Odds ratio and 95%CI are per 1 unit increase in inverse normally transformed AMH

For PCOS, the IVW MR estimate suggested that higher genetically predicted AMH levels are potentially associated with an increased risk of PCOS, but confidence intervals were wide and included the null (OR_{IVW} = 1.29, 95%CI = 0.85 – 1.95; Table 3). Single SNP analyses resulted in a similar effect estimate for the variant in the *AMH* locus (OR_{IVW} = 1.27, 95%CI = 0.64 – 2.56), risk increasing effects for the SNPs in the *TEX41* and *MCM8* loci, and a risk decreasing effect of the variant in the *CDCA7* locus (Table 3). The heterogeneity test did not suggest heterogeneous effects of the individual SNPs (p = 0.30), most likely because of the high uncertainty in individual SNP estimates, but again interpretation of this p-value is limited with only four SNPs. Leave-one-out sensitivity analyses indicated rs11683493 (*CDCA7* locus) affected the IVW estimate most, and that exclusion of this variant resulted in a positive association (OR_{IVW} = 1.53, 95%CI = 1.01 – 2.33) (Figure S11).

Discussion

We identified four loci for circulating AMH levels in women of European ancestry. In addition to confirming a previously reported signal in the *MCM8* locus, we discovered three new signals in and near the *AMH*, *TEX41* and *CDCA7* genes. In total, 35 genes were prioritized for these loci based on physical position, eQTL mapping and chromatin interactions, but pathway analyses did not reveal enrichments of gene-sets, tissues or cell types for genes annotated to suggestive associated SNPs. Genetic correlation analyses supported a shared genetic architecture between AMH levels and age at menopause. Exploratory MR analyses did not provide strong evidence of a causal effect of circulating AMH on breast cancer and PCOS.

We confirmed the association between rs16991615 and circulating AMH levels, previously reported by Ruth et al.[8]. This SNP is a missense variant located in exon 9 of the *MCM8* gene, rendering *MCM8* the most likely causal gene at this locus. In humans, *MCM8* plays a role in in homologous recombination, which is critical for DNA repair.[42] Previous studies have linked *MCM8* deficiency to premature ovarian failure and infertility, but also to cancer development.[39; 43; 44] Associations between rs16991615 and age at menopause[45] and number of ovarian follicles[46] have also been reported, which suggests that this locus is associated with circulating AMH levels because of its influence on the number of antral follicles.

Our GWAS study is the first AMH GWAS that identified a missense SNP (rs10417628) in the *AMH* gene in women. A previous AMH GWAS including adolescents from ALSPAC identified three SNPs in the *AMH* gene that were only significantly associated with AMH levels in male adolescents, and of which one (rs2385821) was in moderate LD with our lead SNP rs10417628 ($R^2 = 0.55$).[6] However, approximate conditional and joint analyses suggested that these variants represent the same signal at the *AMH* locus. Although identification of a genetic variant in the gene encoding for AMH itself suggests that we reveal an actual signal for circulating AMH concentrations, a recent case report suggests that the amino acid substitution corresponding to rs10417628 reduces AMH detection by the picoAMH assay from Ansh Labs without influencing AMH bioactivity.[47] We sought to verify this finding in a subsample of the Doetinchem Cohort Study, for which AMH was measured using both the picoAMH assay and the less sensitive Gen II assay from Beckman Coulter. For the only woman who was estimated to be homozygous for the T allele (dosage T allele = 1.9, age at measurement = 28.3 years), AMH levels were indeed undetectable using the picoAMH assay, whereas circulating AMH levels were detected using the Gen II assay (318 pg/mL). In addition, median AMH levels measured using the Gen II assay were less different between women homozygous for the reference allele and heterozygous women (median AMH $levels_{homozygousrefallele}$ = 953.0 pg/mL, IQR: 428.0 - 1999.0; median AMH $levels_{heterozygous}$ = 848.0 pg/mL, IQR: 509.0 - 1310.0), compared to AMH levels measured using the picoAMH assay (median AMH $levels_{homozygousrefallele}$ = 1485.9 pg/mL, IQR: 704.4 - 3150.0; median AMH $levels_{heterozygous}$ = 811.0 pg/mL, IQR: 462.3 - 1480.9). For ALSPAC, which also used the Gen II assay to measure AMH, the distribution of AMH levels was similar across adult women homozygous for the reference allele and heterozygous women as well. However, in the ALSPAC daughters median AMH levels were clearly higher in adolescents homozygous for the reference allele compared to heterozygous adolescents. Among the ALSPAC participants, only one adolescent was homozygous for the T allele, but her AMH levels could not be shared due to disclosure risk. Because of the lack of publicly available information on the antibodies and conformational epitopes of the Gen II assay, and the limited and inconsistent evidence in the current study, we do not want to draw any definite conclusions about this yet.

For the associated loci on chromosome 2 it is more challenging to assign possible causal genes, as *TEX41* is a long noncoding RNA and the SNP in the *CDCA7* locus was located in an intergenic region. Gene mapping based on chromatin interactions with *TEX41* highlighted several genes, including the long non-coding RNA *ZEB2-AS1* (ZEB2 antisense RNA 1). *ZEB2-AS1* up-regulates expression of the protein ZEB2.[48] ZEB2 (also known as SIP1) inhibits signal transduction in TGF-β and BMP signaling through interaction with ligand-activated SMAD proteins.[49; 50] Among other BMP proteins, BMP4 has been reported to regulate AMH expression through activation of SMAD proteins.[51; 52] Based on identification of *BMP4* in our gene-based association analysis and its prioritization by DEPICT, we hypothesize that BMP4 induced AMH expression may be regulated by ZEB2 interaction. However, fundamental laboratory research is needed to prove this.

CDCA7 (also known as *JPO1*) is a direct target gene of the transcription factor *MYC* and is involved in apoptosis.[53] Functional annotation did not map any genes to this locus and thus the mechanism through which this locus affects circulating AMH levels remains to be elucidated. Based on the involvement of *CDCA7* in apoptosis, it may be possible that this gene affects the number of antral follicles, which are the main producers of AMH in women.[2] Ideally, future studies should explore whether the observed genetic associations may merely reflect the size of the ovarian reserve, through adjusting analyses for antral follicle count. Such analyses would also show if we can actually use the identified variants are instruments for circulating AMH levels itself or for the quantity of antral follicles in MR analyses.

We did not find support for a causal effect of circulating AMH levels on breast cancer and PCOS risk in our exploratory MR analyses. To be valid genetic instruments for MR, SNPs have to fulfil the following three criteria[54]: (1) SNPs have to be associated with circulating AMH levels; (2) SNPs cannot be associated with confounders of the studied AMH-outcome associations, and (3) SNPs cannot influence the outcomes through mechanisms that do not involve circulating AMH levels. Because rs10417628 in the *AMH* gene potentially reflects AMH detection instead of AMH expression, analyses including this variant should be interpreted with caution. However, leave-one-out analyses excluding this variant did not affect IVW MR estimates. Based on the function of genes mapped to the loci on chromosomes 20 and 2, it is likely that these variants affect breast cancer and PCOS risk through mechanisms independent of AMH (e.g. DNA replication and apoptosis), in particular the *MCM8* locus, which has also been identified in breast cancer GWAS.[39] Due to the limited number of identified lead SNPs it was not possible to assess if our results were indeed biased by horizontal pleiotropy. Furthermore, weak instrument bias may still have biased MR results towards the null, since the F statistics may be overestimated in this GWAS (853.9 for breast cancer, and 422.4 for PCOS). Consequently, we should be cautious about excluding a causal effect of AMH on the studied outcomes.

Previous research suggests that AMH levels in females rise during puberty, until the mid to late twenties, and after that decrease until menopause.[11; 55] Based on these observations and the differences in both age and AMH distributions between the ALSPAC adolescents and other participants, we performed a sensitivity analysis excluding the adolescents from ALSPAC. This analysis revealed an additional locus on chromosome 5 (rs116090962, nearest gene: *CTB-99A3.1*), although we could not find clues for its association with circulating AMH levels in adult women only, nor with AMH levels in general. Study-specific betas revealed an opposite effect for the *MCM8* locus and a minimal effect for the *CDCA7* locus in adolescents compared to effects in adult women. A larger GWAS including older adolescents and a larger proportion of females aged 20 to 40, would be required to reveal potential gene-age interactions that explain variation in AMH expression.

The main strengths of the current GWAS meta-analysis are its size, which is twice the size of the previous GWAS meta-analysis, and its larger proportion of women of early-reproductive age. Given AMH's function in ovarian follicle development, circulating levels and variation in AMH levels decrease with age. As a result, statistical power to identify genetic variants for circulating AMH increases if younger women are included. Still, our sample size remains relatively small for a GWAS, and future larger studies may lead to the detection of additional variants for circulating AMH levels. This is supported by our chip heritability estimate of 15% (se = 7%), which indicates that there are likely more SNPs that contribute to variability in AMH levels. Identification of additional genetic variants will also facilitate increased power to identify pathways and tissues enriched for genes involved in AMH expression. A second limitation of this study is potential overlap in participants between the current AMH GWAS and the GWAS for breast cancer[39] (maximum n = 1459; 20.7% of current study) and PCOS[40] (maximum n = 225; 3.2% of current study). Overlap in participants in two-sample MR analyses may bias effect estimates and inflate Type 1 error rates.[56]

In conclusion, we replicated the previously reported association with the *MCM8* locus and identified 3 novel loci for circulating AMH levels in women, including the *AMH* locus. The strongest signal in this locus possibly affects AMH detection by specific assays rather than AMH bioactivity, but further research is required to confirm this hypothesis. Genes mapped to the *MCM8*, *TEX41* and *CDCA7* loci are involved in the cell cycle and processes like DNA replication and apoptosis. The mechanism underlying their associations with AMH may affect the size of the ovarian follicle pool. MR analyses did not support a causal effect of AMH on breast cancer and PCOS, but these finding should be interpreted with caution because we could not robustly explore how valid the instruments were and weak instrument bias may have biased estimates towards the null.

Supplemental data

Document S1: Supplemental Methods.
Document S2: Figures S1 – S13.
Document S3: Supplemental Tables 1 – 11.

Declarations of interest

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Web resources

ALSPAC: http://www.bristol.ac.uk/alspac/
SWAN: https://www.swanstudy.org/
EASYQC: https://www.uni-regensburg.de/medizin/epidemiologie-praeventivmedizin/genetische-epidemiologie/software/
METAL: http://csg.sph.umich.edu/abecasis/metal/
GCTA: https://cnsgenomics.com/software/gcta/
FUMA: https://fuma.ctglab.nl/
DEPICT: https://github.com/perslab/depict
LD Hub: http://ldsc.broadinstitute.org/

Data and code availability

Code generated for this study is available at Github [https://github.com/reneemgverdiesen/AMH_GWAS]. The full AMH GWAS summary statistics will made available through the GWAS catalog (https://www.ebi.ac.uk/gwas/)

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Supplemental methods

Study population

Studies included in the previous GWAS study: Generations Study, Sister Study, Nurses' Health Study and Nurses' Health Study II

We included AMH GWAS summary statistics from the most recent previous AMH GWAS analysis.[1] We requested summary statistics excluding data from ALSPAC, resulting in meta-analysis summary statistics for the Generations Study (n = 379, median age: 44 years, IQR: 40, 48), Sister Study (n = 438, median age: 48 years, IQR: 45, 51), Nurses' Health Studies (n = 642, median age: 44, IQR: 41, 47). These population-based cohort studies have been described in more detail previously.[2-5] Details on the premenopausal women included in the previous AMH GWAS by Ruth et al. are presented in Supplemental Table 1 and are described into more detail elsewhere.[1; 6]

Doetinchem Cohort Study

The Doetinchem Cohort Study is an ongoing prospective cohort study that included 3641 men and 4128 women, aged 20-59 years at recruitment, who were randomly selected from the municipal register of Doetinchem, The Netherlands, between 1987 and 1991. Every five years, study participants are invited for a follow-up visit, during which physical examinations and extensive questionnaires are completed, and blood samples are collected. The Doetinchem Cohort Study received ethical approval from the Medical Ethics Committee of The Netherlands Institution of Applied Scientific Research and all study participants signed an informed consent prior to study inclusion. For more details see previous reports.[7; 8] Details on the included participants are presented in Supplemental Table 1.

Avon Longitudinal Study of Parents and Children

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a longitudinal birth cohort, which has been described in detail elsewhere.[9; 10] In short, 14,541 women who were expected to give birth between 1st April 1991 and 31st December 1992 from the South West of England were enrolled in ALSPAC between 1990 and 1992.[10] Initially, 14,676 fetuses were included in ALSPAC. When the oldest children were approximately 7 years old, an attempt was made to bolster the initial sample with eligible cases who had failed to join the study originally. The total sample size for analyses using any data collected after the age of seven is therefore 15,454 pregnancies, resulting in 15,589 foetuses. Of these 14,901 were alive at 1 year of age.

Ethical approval for the study was obtained from the Avon Longitudinal Study of Parents and Children Ethics and Law Committee and the Local Research Ethics Committees. Written informed consent was obtained from all adult participants in the study. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004). Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool and reference the following webpage: <http://www.bristol.ac.uk/alspac/researchers/our-data/>. Details on the included participants are presented in Supplemental Table 1.

Study of Women's Health Across the Nation

The **S**tudy of **W**omen's Health **A**cross the **N**ation (SWAN) is a multi-site, multiracial/ethnic longitudinal study of women's health designed to describe the biological, behavioral, and psychosocial characteristics that occur during midlife and the menopausal transition. Briefly, the SWAN cohort was enrolled in 1996-97 and consists of 3302 community-based women from seven sites with data from five race/ethnic groups: Black (n=935), Chinese (n=250), Hispanic (n=286), Japanese (n=281), and White (n=1550). To be eligible for enrollment women had to be aged 42 to 52 years old, have an intact uterus and at least one ovary, have had a menstrual period in the previous three months, and not be taking hormones. Subsequently, 1757 participants consented to provide genetic materials. Immortalized cell lines were developed successfully for 1588, with 1536 processed into distributable diluted, extracted DNA and 1464 successfully genotyped. A total of 738 were of European ancestry, 425 of whom had AMH measures and contributed to this analysis. The study protocol was approved by the Institutional Review Boards at each study site. All participants provided written, informed consent at each visit. Details of SWAN are described elsewhere.[11] Details on the included participants are presented in Supplemental Table 1.

Study-specific association analyses

The Doetinchem Cohort Study, ALSPAC, and SWAN performed association analyses based on a standardized analysis plan, which was distributed in advance. The analyses described in this analyses plan were in agreement with the analyses conducted for the previous AMH GWAS study.[1] All studies assumed an additive model and adjusted analyses for age at blood collection (years) and population stratification, either by including principal components (ALSPAC, SWAN) or a kinship matrix (Doetinchem Cohort Study, Generations Study, Sister Study, Nurses' Health Studies).

Doetinchem Cohort Study

We used rvtests[12] (version 20170210) to perform association analyses in the Doetinchem Cohort Study. Linear mixed model analyses were adjusted for age at blood collection and

a kinship matrix was included to adjusted for cryptic relatedness. This kinship matrix was calculated using the vcf2kinship script provided by rvtests.

Avon Longitudinal Study of Parents and Children

Because of the large differences in both age and AMH distribution between the ALSPAC mothers and daughters, we considered it inappropriate to analyze them together using a linear mixed model method to correct for relatedness. Consequently, separate association analyses were conducted for the ALPSAC mothers and daughters. For both groups, linear regression analyses were performed in SNPTEST v2.5[13], and models were adjusted for age at blood collection and 10 principal components.

Study of Women's Health Across the Nation

Rvtests (version 20190205) was used to perform association testing in SWAN. Linear regression analyses were adjusted for age at blood draw and 10 principal components.

Generations Study, Sister Study, Nurses' Health Study and Nurses' Health Study II

For the Generations Study, Sister Study, and Nurses' Health Studies, linear mixed model association analyses were performed using GEMMA 0.94.1[14], which calculates a kinship matrix. Analyses were adjusted for age at blood draw. In the current meta-analysis we included summary statistics of the meta-analysis of these studies, which was performed using METAL[15], as described elsewhere.[1]

File-level and meta-level QC prior to meta-analysis

Prior to meta-analysis, we performed file-level QC on all summary statistics files to clean and check the data, as described elsewhere.[16] File-level QC consisted of (1) removal of rows with missing data on alleles, p-values, betas, standard errors or allele frequency; (2) removal of rows with unrealistic values (e.g. p-values < 0 or > 1); (3) exclusion of monomorphic SNPs; (4) harmonizing alleles; and (5) removal of duplicated SNPs. Subsequently, we performed meta-level QC to identify potential study-specific problems following a previously published protocol.[16] Meta-level QC comprised creation of five plots: (1) SE-N plot, which reveals potential issues with trait transformation; (2) P-Z scatter plot, which reveals potential issues with betas, standard errors and p-values; (3) Allele frequency plot, to check for issues with allele frequencies or strand; (4) QQ plots, to assess genomic inflation, and (5) λ_{GC} plot, also to assess genomic inflation. Both file-level and meta-level QC were performed using the R package EasyQC (v9.2).[16] No study-specific issues were identified through these QC procedures (Figure S1-S5).

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Supplemental figures

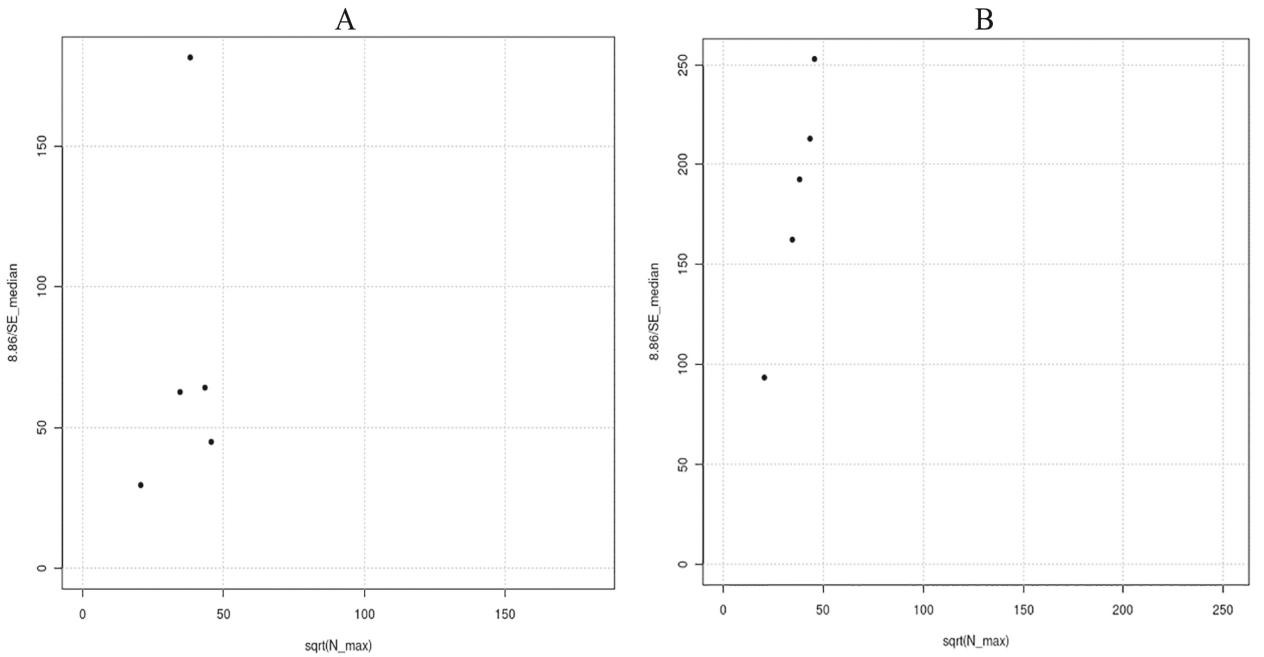


Figure S1. SE-N plot to detect issues with trait transformations.

The individual points represent, for each included study, the inverse of the median standard error of the beta estimates across all SNPs against the square root of the sample size. If there are no issues with trait transformations the data points are more or less on a straight line. (A) Before filtering on minor allele frequency; the data point for the GWAS by Ruth et al. appears an outlier compared to the data points of the other studies. (B) After filtering on minor allele frequency previous (MAF > 1%); data points are more or less on a straight line. Plots were created using the EasyQC R-package.[1]

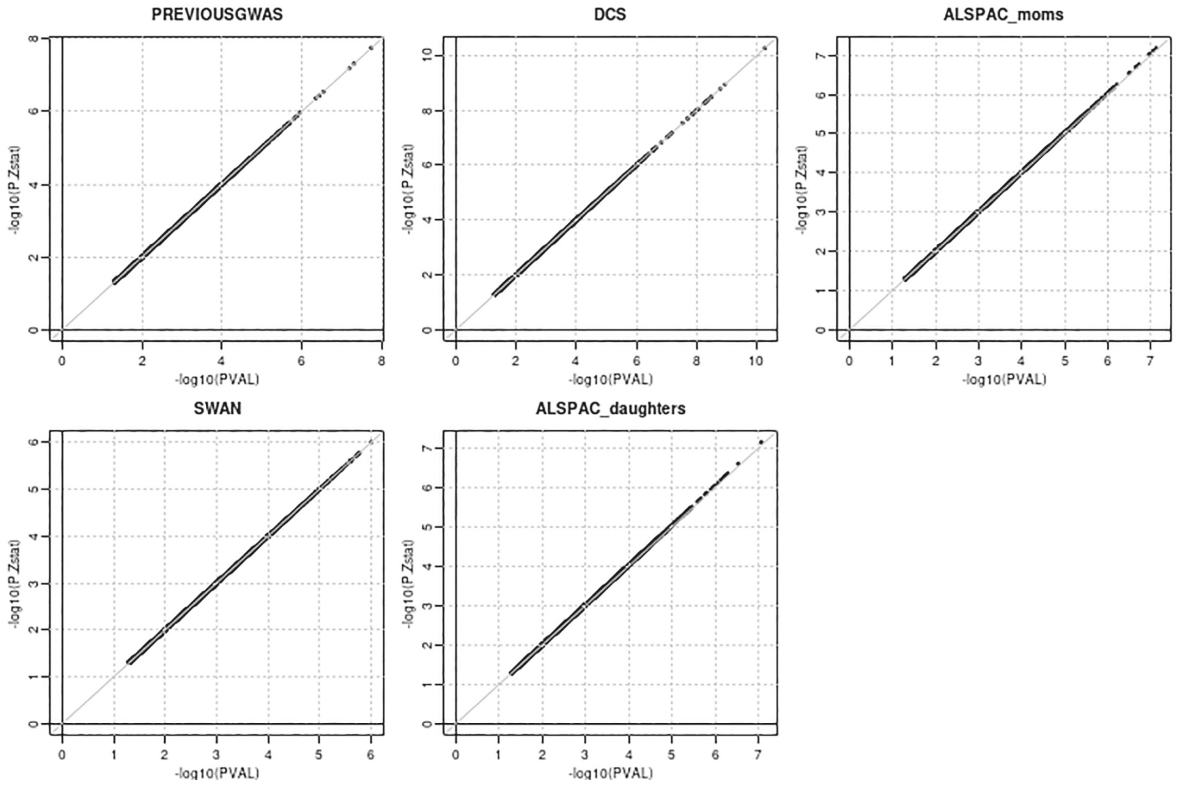


Figure S2. P-Z plots to detect analytical issues with beta, standard errors and P-values.

For each participating study, observed p-values are compared with P-values calculated from the Z-statistics based on the observed beta-estimates and standard errors. A straight line indicates that observed and calculated p-values are in agreement, and thus no analytical issues with betas standard errors and p-values are present for the included studies. Plots were created using the EasyQC R-package.[1]

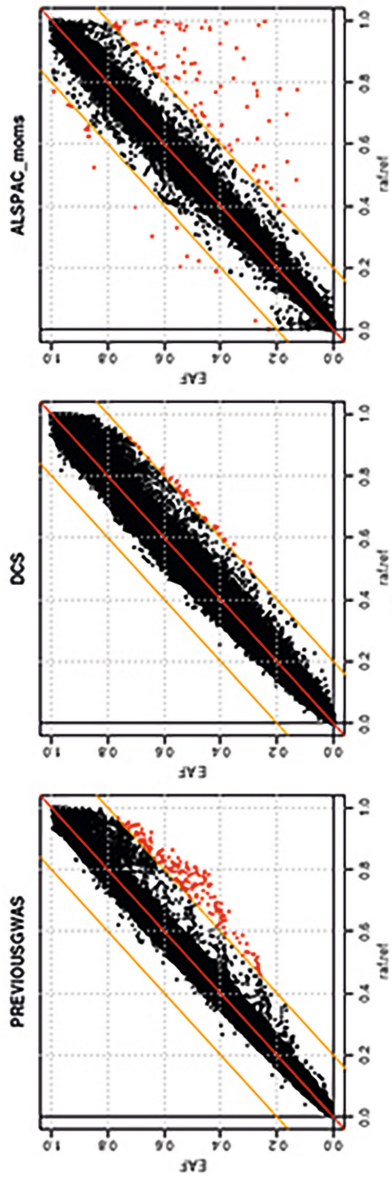


Figure S3. Allele frequency plots to check for issues with allele frequency.

For each included study, effect allele frequencies (EAF) are plotted against the HRC reference panel. For the GWAS by Ruth et al., the Doetinchem Cohort Study, and ALSPAC data are very consistent with the reference panel. Although the plot for SWAN indicates various differences with the HRC panel, none of the previously described specific patterns for systematic deviations are observed. Most likely the SWAN population have a somewhat different ancestry than the reference. Plots were created using the EasyQC R-package.[1]

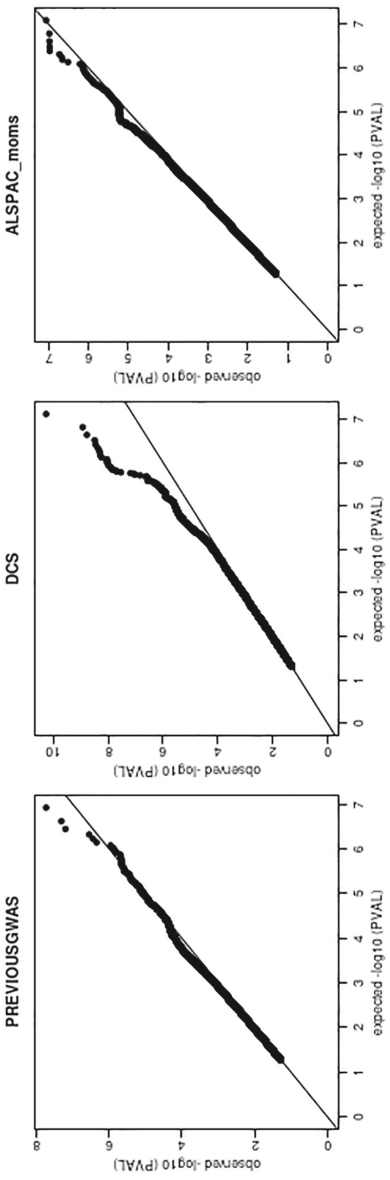


Figure S4. QQ plots per included study before removal of SNPs with MAF < 1% and poor imputation quality.

For each study, observed –log10 p-values for each SNP are plotted against expected –log10 p-values from a theoretical χ^2 distribution. Plots were created using the EasyQC R-package.[1]

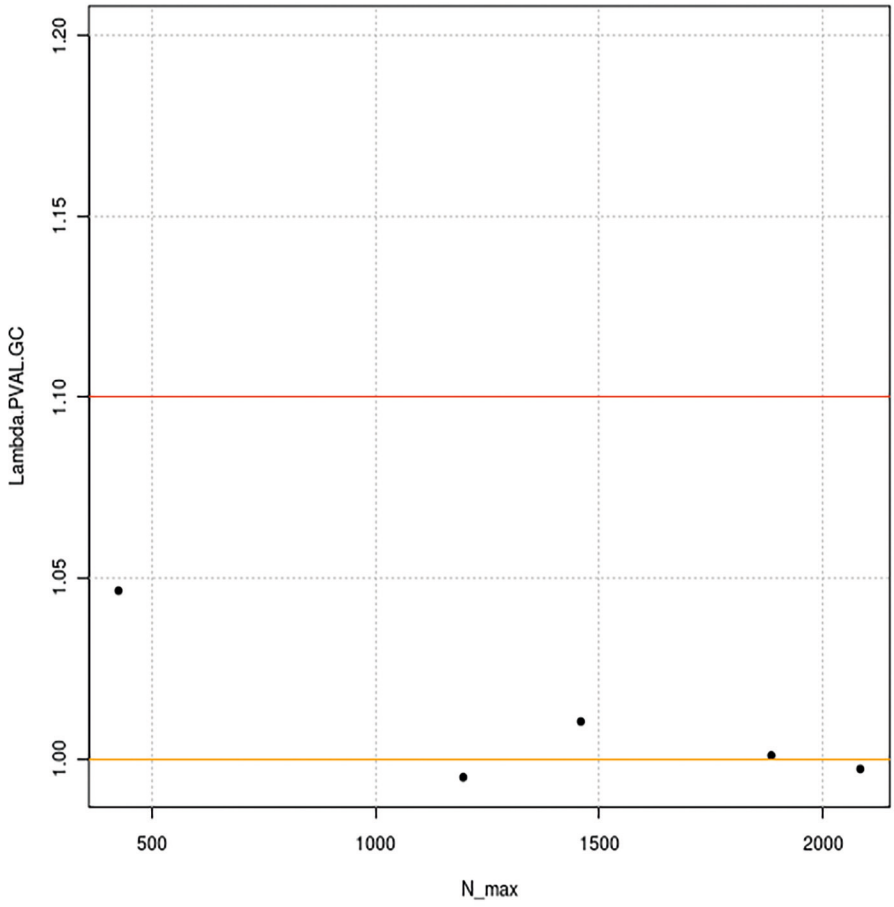


Figure S5. Lambda-N plot to reveal issues with population stratification.

For each study, the lambda for genomic control is plotted against the maximum sample size. The orange line indicates the optimal lambda; $l_{GC} = 1.0$. The red line indicates the threshold for values that indicate problems with population stratification; $l_{GC} = 1.1$. This plot suggests that none of the included studies has population stratification issues. Plot was created using the EasyQC R-package.[1]

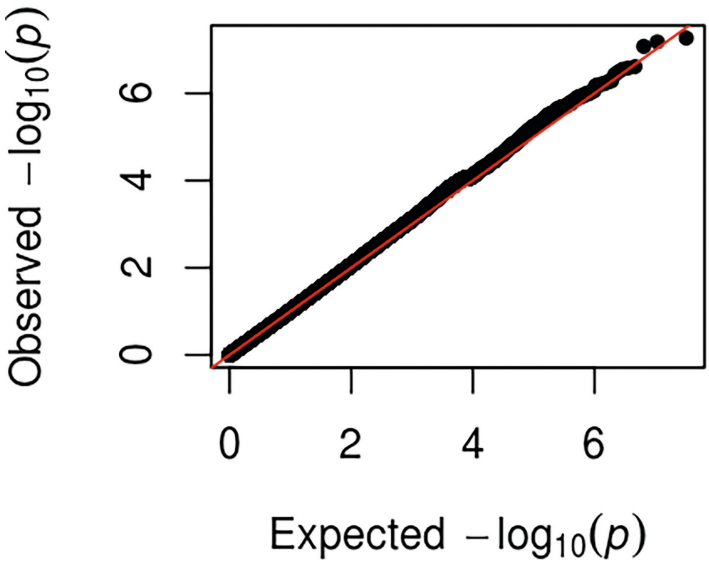


Figure S6. QQ plot of meta-analysis p-values of ALSPAC mothers and daughters only.

Observed –log10 p-values from the meta-analysis including only ALSPAC mothers and daughters are plotted against expected –log10 p-values from a theoretical χ^2 distribution. Corresponding lambda is 1.009.

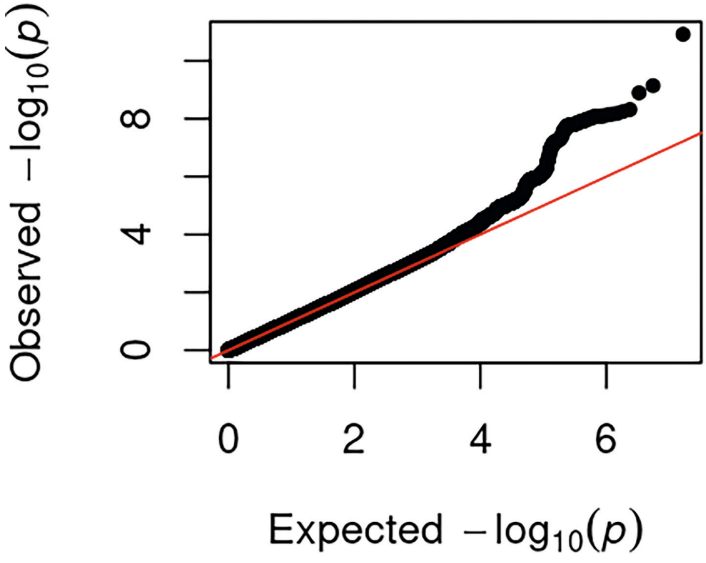
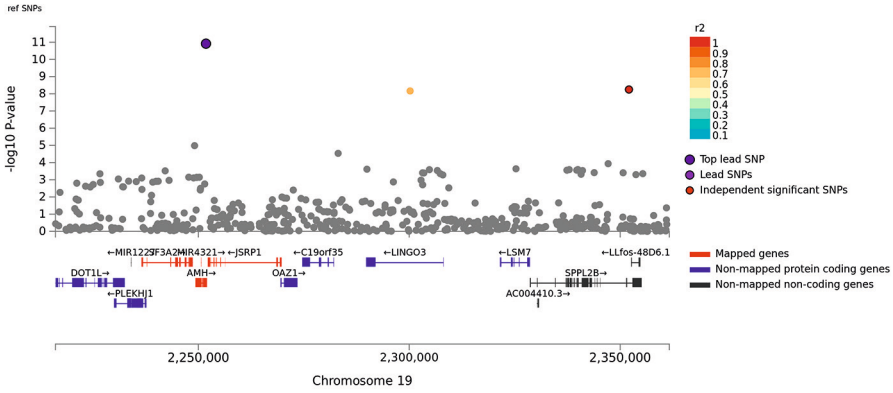


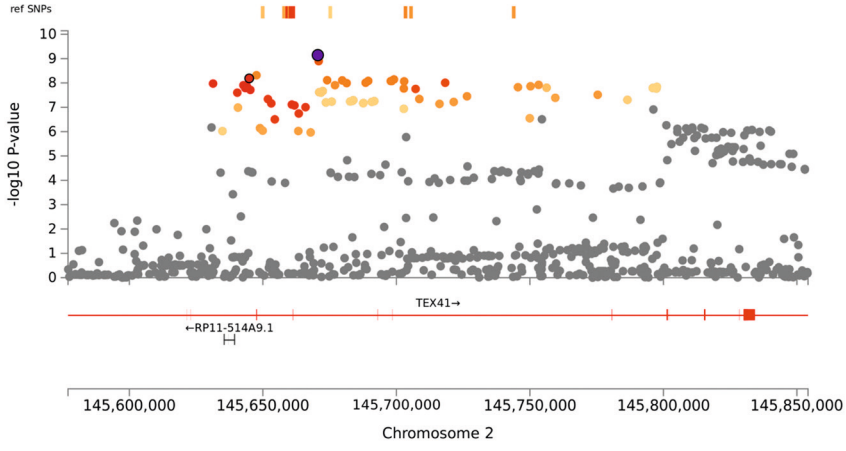
Figure S7. QQ plot of meta-analysis p-values for inverse normally transformed AMH in women.

Observed –log10 p-values for each of the 8,298,138 SNPs included in the meta-analysis are plotted against expected –log10 p-values from a theoretical χ^2 distribution. Corresponding lambda is 1.006.

A


ref SNPs
-log10 P-value
r2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Top lead SNP
Lead SNPs
Independent significant SNPs
←MIR1227
SF3A2
MIR4321→
←JSRP1
←C19orf35
←LINGO3
←LSM7
←LLfos-48D6.1
DOT1L→
AMH→
OAZ1→
SPPL2B→
←PLEKHJ1
AC004410.3→
Mapped genes
Non-mapped protein coding genes
Non-mapped non-coding genes
2,250,000
2,300,000
2,350,000
Chromosome 19

B


ref SNPs
-log10 P-value
TEX41→
←RP11-514A9.1
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145,700,000
145,750,000
145,800,000
145,850,000
Chromosome 2

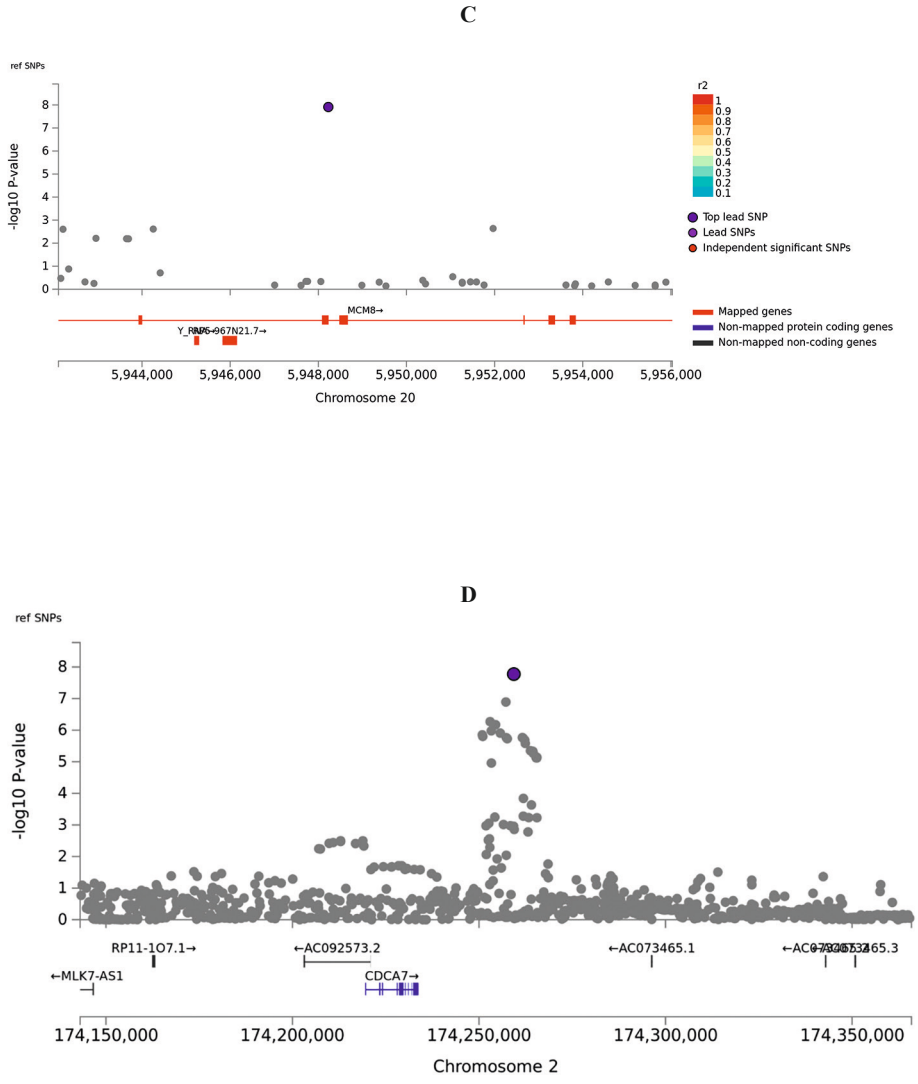

Figure S8. Regional association plots for genome-wide significant loci for inverse normally transformed AMH in women.

Regional plots for the *AMH* locus (panel A), *TEX41* locus (panel B), *MCM8* locus (panel C), and *CDCA7* locus (panel D) show SNPs plotted by their position and –log10 P-value for association with inverse normally transformed AMH. Nearby genes are depicted below each plot. Plots were created using FUMA.[2]

Figure S9. Manhattan plot of gene-based genome-wide association results for inverse normally transformed AMH in women.

Plot was created using FUMA.[2]

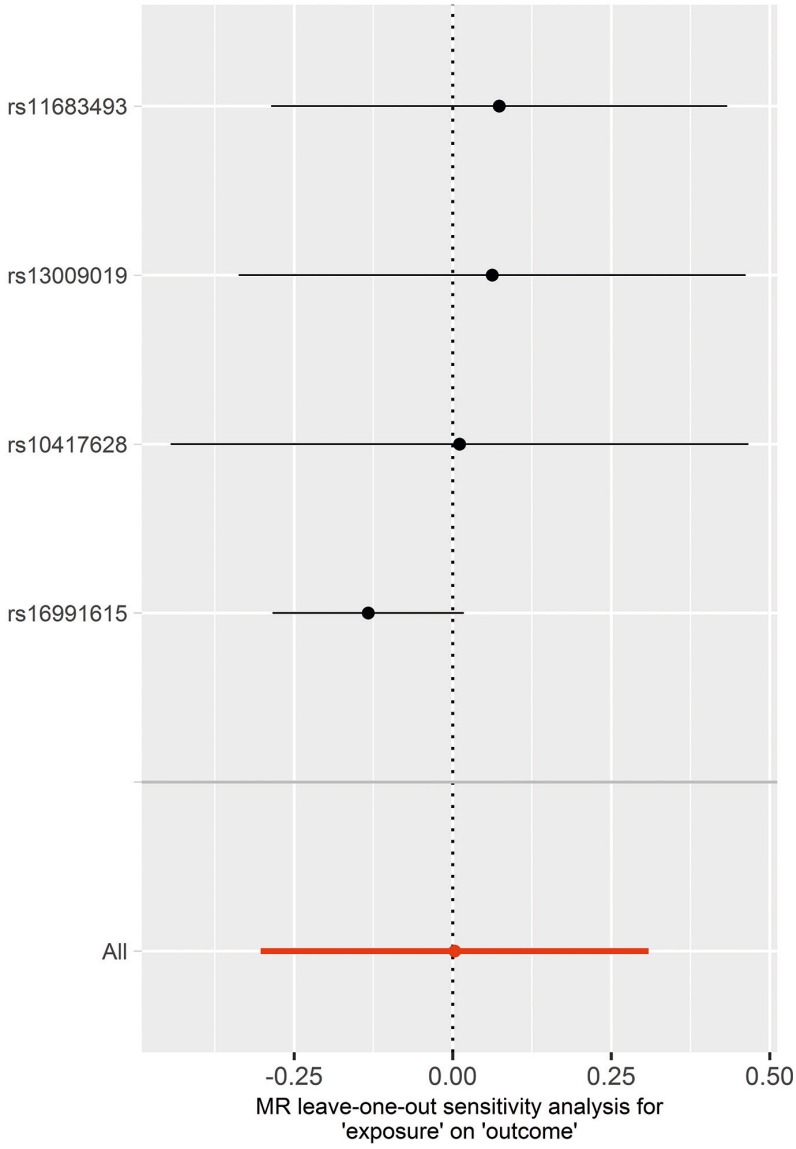

Figure S10. Estimates leave-one-out analyses for the association between circulating AMH and risk of breast cancer.

Plot was created using the TwoSampleMR R-package.[3]

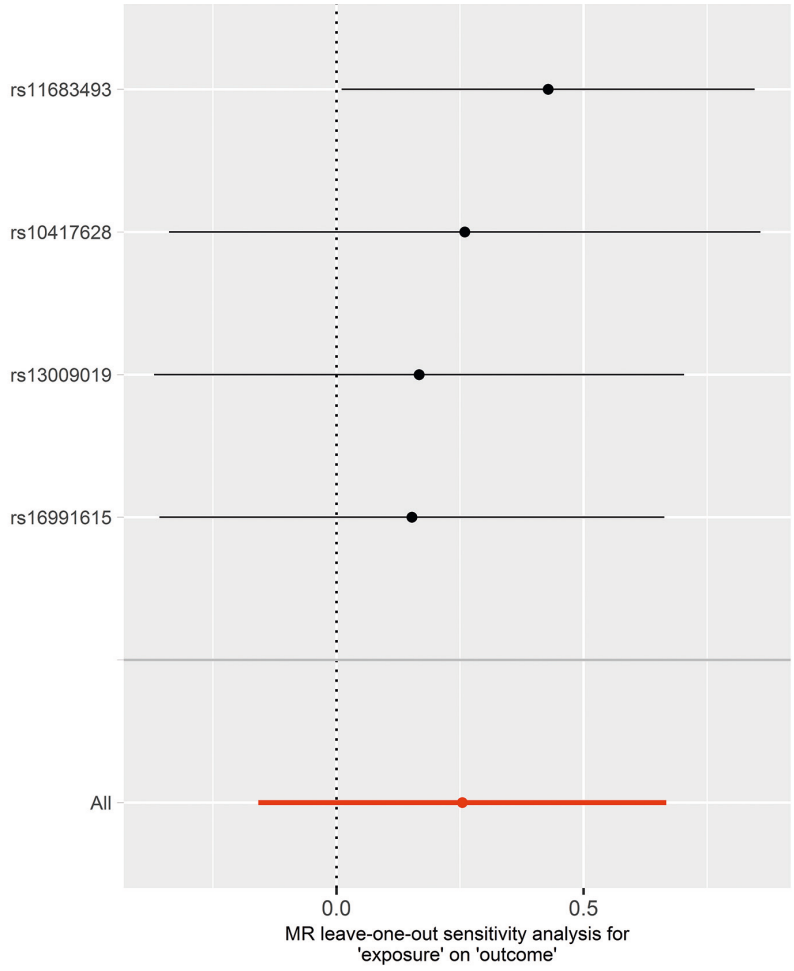

Figure S11. Estimates leave-one-out analyses for the association between circulating AMH and risk of PCOS.

Plot was created using the TwoSampleMR R-package.[3]

References supplemental figures

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Supplemental tables

Supplemental Table 1. Description of included participants and AMH measurements in participating studies.

Study populations	Studies included in AMH GWAS by Ruth et al.			Doetinchem Cohort Study	ALSPAC mothers	ALSPAC daughters	SWAN
	Generations Study	Sister Study	Nurses' Health Studies				
N	379	438	642	2084	1885	1196	425
Age, years (median (IQR))	44 (40, 48)	48 (45, 51)	44 (41, 47)	37.2 (31.2, 42.9)	46 (44, 49)	15.3 (15.3, 15.5)	47.3 (45.3, 49.3)
Definition premenopausal status	Reporting to be not postmenopausal at blood donation. Women with unknown menopausal status were categorized as premenopausal based on age < 50	Reporting one or more menstrual cycles in the prior 12-month period on the enrollment questionnaire. Women whose only reason for not experiencing menses was hysterectomy (without bilateral oophorectomy) were categorized as premenopausal based on age ≤55	Women who were still having menstrual periods, or had at least one ovary remaining and were younger than 46 (for smokers) or 48 (for non-smokers)	Women who did not have amenorrhea for at least 12 consecutive months, nor cessation of menses due to if they reported to had an bilateral ovariectomy	Being in the reproductive stage according to the STRAW criteria	Not applicable	Being in the reproductive stage according to the STRAW criteria
AMH measurements							
Type of sample	Serum	Serum	Serum	Plasma	Fasting serum	Fasting serum	Fasting serum
AMH assay	picoAMH ELISA (Ansh Labs, Webster, TX)	ultrasensitive ELISA (Ansh Labs, Webster, TX)	picoAMH ELISA (Ansh Labs, Webster, TX)	picoAMH ELISA (Ansh Labs, Webster, TX)	AMH Gen II ELISA assay (Beckman Coulter UK Ltd, High Wycombe, UK)	AMH Gen II ELISA assay (Beckman Coulter UK Ltd, High Wycombe, UK)	picoAMH ELISA (Ansh Labs, Webster, TX)

Supplemental Table 1. *(continued)*

Study populations	Studies included in AMH GWAS by Ruth et al.			Doetinchem Cohort Study	ALSPAC mothers	ALSPAC daughters	SWAN
	Generations Study	Sister Study	Nurses' Health Studies				
Limit of detection (LOD) of assay	0.01642 pmol/L	0.5 pmol/L	2.038 pg/mL	1.85 pg/mL	0.01 ng/mL	0.01 ng/mL	3.00 pg/mL in batch 1; 1.85 pg/mL in batch 2
Handling of measurements under LOD	Set to half the LOD (= 0.00821 pmol/L)	Remeasuring with picoAMH ELISA (Ansh Labs, Webster, TX), missing values were set to 0.0015 ng/mL	Excluded from analyses (n = 24)	Set to half the LOD (= 0.923 pg/mL)	Set to LOD	Set to LOD	batch 1 set to 1/2 LOD, batch 2 set by SWAN to 1.45 pg/mL
Percentage of measurements under LOD	3.7%	24.2%	0%	4.1%	10.9%	0%	8.84%

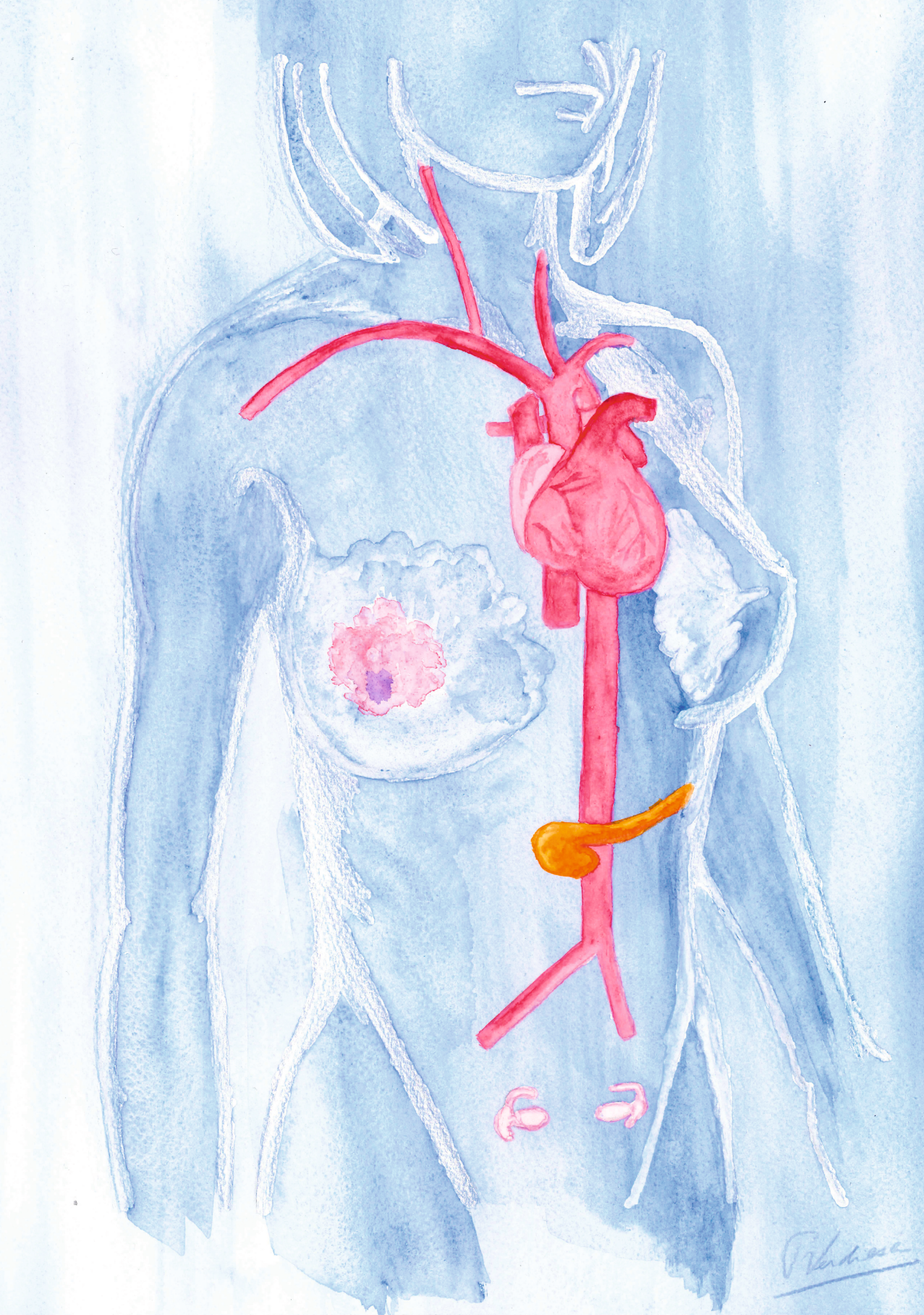
Supplemental Table 2. Description of genotyping and imputation procedures in participating studies.

	Studies included in AMH GWAS by Ruth et al.			Doetinchem Cohort Study	ALSPAC mothers	ALSPAC daughters	SWAN
	Generations Study	Sister Study	Nurses' Health Studies				
Genotyping							
Sample for DNA extraction	Blood	Blood	Blood	Blood	Blood	Blood	Epstein-Barr Virus Transformed Beta Lymphocytes
Genotyping array	OncoArray	OncoArray	OncoArray (n = 417) and Illumina HumanHap550 and HumanHap610 arrays (n = 225)	Illumina Infinium Global Screening Array-24 Kit (Illumina Inc., San Diego, California, United States of America)	the Illumina Human660W-Quad array at Centre National de Génotypage	Illumina HumanHap550 quad genome-wide SNP genotyping platform by the Wellcome Trust Sanger Institute (Cambridge, UK) and the Laboratory Corporation of America (Burlington, North Carolina, US)	Illumina Multi-Ethnic Global Array (MEGA A1)
QC procedure							
Used software (version)	PLINK v1.9	PLINK v1.9	PLINK v1.9	GenABEL (1.8-0)	PLINK v1.07	PLINK v1.07	GWASTools 3.6, GENESIS 3.6, SNPRelate 3.6
Sample QC thresholds for exclusions							
Sex mismatch	Yes	Yes	Yes	Yes	Not applicable	Yes	Yes
Call rate	< 95%	< 95%	< 95%	< 95%	<95%	< 97%	< 95%

Supplemental Table 2. *(continued)*

	Studies included in AMH GWAS by Ruth et al.			Doetinchem Cohort Study	ALSPAC mothers	ALSPAC daughters	SWAN
	Generations Study	Sister Study	Nurses' Health Studies				
Outlying heterozygosity	Minimal or excessive heterozygosity	Minimal or excessive heterozygosity	Minimal or excessive heterozygosity	FDR < 1%	indeterminate X chromosome heterozygosity or extreme autosomal heterozygosity	Minimal or excessive heterozygosity	Identified using VerifyBamID software
Duplicated or related individuals	3rd degree relatives or closer	3rd degree relatives or closer	3rd degree relatives or closer	IBD > 0.98	IBD > 0.125	IBD > 0.1	Kinship coefficient > 1/32
Outlying ancestry	Principal component analysis using FlashPCA software	Principal component analysis using FlashPCA software	Principal component analysis using FlashPCA software	Visual inspection of principal components	Multidimensional scaling analysis and comparison with Hapmap populations	Multidimensional scaling analysis and comparison with Hapmap II (release 22) populations	Visual inspection of principal components
SNP QC thresholds for exclusions							
Minor allele frequency	< 1%	< 1%	< 1%	<0.01%	< 0.01%	< 0.01%	< 0.01%
Call rate	< 95%	< 95%	< 95%	< 98%	< 95%	< 95%	< 98%
Hardy-Weinberg equilibrium	P < 1 × 10−6	P < 1 × 10−6	P < 1 × 10−6	P < 1 × 10−6	P < 1 × 10−7	p < 5 x 10-7	p < 1 x 10-4
Imputation details							
Imputation software (version)	Michigan Imputation Server (Minimac3)	Michigan Imputation Server (Minimac3)	Michigan Imputation Server (Minimac3)	Michigan Imputation Server (Minimac 3)	Michigan Imputation Server (Minimac 3)	Michigan Imputation Server (Minimac 3)	Michigan Imputation Server (Minimac 3)
Imputation panel (version)	HRC v1.1	HRC v1.1	HRC v1.1	HRC v1.1	HRC v1.1	HRC v1.1	HRC v1.1
Genomic build	CRCh37/hg19	CRCh37/hg19	CRCh37/hg19	CRCh37/hg19	CRCh37/hg19	CRCh37/hg19	CRCh37/hg19

Supplemental Tables 3 to 11 have not been included in this thesis because of their size, but can be found online at https://doi.org/10.1101/2020.10.29.20221390



CHAPTER 7

Anti-Müllerian hormone and cardiometabolic disease in women: a two-sample Mendelian randomization study

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Abstract

Context: Higher age-specific circulating AMH levels have been linked to a lower risk of cardiometabolic outcomes. However, whether AMH has a casual role in the etiology of these diseases is currently unknown.

Objective: To explore if circulating AMH levels have a causal effect on risk of coronary artery disease (CAD), ischemic stroke and type 2 diabetes (T2D) in women.

Design: a two-sample Mendelian randomization (MR) approach.

Methods: We used four single nucleotide polymorphisms (SNPs) from the most recent AMH GWAS meta-analysis as instrumental variables. Summary-level data for CAD, ischemic stroke and T2D were extracted from the UK Biobank, the Stroke Genetics Network, and DIAMANTE consortia, respectively. To assess potential pleiotropy we tested if the four AMH SNPs, either individually or combined as weighted genetic risk score, were associated with a range of traits in the UK Biobank.

Results: MR estimates did not support a causal effect of circulating AMH levels on CAD (OR_{IVW} = 1.13, 95%CI: 0.95 – 1.35), ischemic stroke (OR_{IVW} = 1.11, 95%CI: 0.83 – 1.49), and T2D (OR_{IVW} = 0.98, 95%CI = 0.87 - 1.10). After adjustment for multiple testing, we observed associations between genetically predicted AMH and age at menopause and age at menarche in the UK Biobank, but not with intermediate traits on the causal pathway between AMH and cardiometabolic health, such as atherosclerosis or glucose levels.

Conclusions: This study does not provide evidence for a causal effect of circulating AMH levels on CAD, ischemic stroke and T2D in women, although weak instrument bias cannot be excluded.

Introduction

In women, anti-Müllerian hormone (AMH) is expressed by early antral stage ovarian follicles.[1] AMH declines with age, and becomes undetectable after menopause, when the ovarian reserve is depleted, and AMH can therefore be used as a marker for reproductive aging.[2] Accelerated female reproductive aging, often quantified as an earlier age at menopause, has been linked to a higher risk of cardiometabolic diseases[3-5], but the causal mechanisms underlying these associations remain to be established. Based on recent observational studies that provided evidence for an association between higher circulating AMH levels and lower risk of cardiovascular disease[6] and diabetes[7] in women, it has been postulated that AMH may have a causal role in the etiology of these diseases. However, a potential causal effect of AMH on risk of cardiometabolic disease is difficult to establish in observational studies.

Mendelian randomization (MR) analysis uses genetic variants as instrumental variables for the risk factor of interest to estimate causal effects on outcomes that are not influenced by confounding, and are not altered by disease occurrence (reverse causation).[8] In two-sample MR, summary-level data from independent genome-wide association studies (GWAS) for the exposure and outcome(s) are used instead of individual data from one study population, with generally a larger number of participants, increasing statistical power to detect a causal association.[9] For AMH, we have recently identified four genetic variants in ~7000 premenopausal women.[10]

Using these genome-wide significant genetic variants for AMH levels, we aimed to explore if circulating AMH levels could have a causal effect on risk of cardiometabolic disease in women, using a two-sample MR approach and summary-level data of large GWAS for coronary artery disease (CAD), ischemic stroke and type 2 diabetes (T2D).

Materials and Methods

Instrumental variable selection

Recently, we have identified four single nucleotide polymorphisms (SNPs) in an AMH GWAS meta-analysis that included data of 7049 European ancestry premenopausal women.[10] One of the variants is a missense variant located in the *AMH* gene (rs10417628). However, for this SNP the possibility that it is associated with AMH levels through impaired detection by specific AMH assays instead of reduced AMH bioactivity cannot be excluded.[10, 11] Therefore, and because inclusion of multiple genetic instruments increases statistical power to detect

a causal association[12], we included all four SNPs associated with circulating AMH levels in premenopausal women at genome-wide significance ($p < 5 \times 10^{-8}$). Combined, these four SNPs explained 1.47% of the variance in circulating AMH levels (i.e. $R^2 = 0.0147$). In the GWAS, AMH levels (pmol/L) were transformed using rank-based inverse normal transformation. As a result, presented odds ratios (ORs) for outcomes correspond to one unit increase in inverse normally transformed circulating AMH levels. AMH GWAS analyses were adjusted for population stratification (either by inclusion of the first 10 principal components or a genetic relationship matrix) and age at AMH measurement.

Outcome data sources

We included summary-level data for genetic associations of the four AMH variants with CAD, ischemic stroke and T2D in women of European descent from the UK Biobank[13], and the Stroke Genetics Network (SiGN)[13] and DIAMANTE[14] consortia, respectively.

The UK Biobank is a large, population-based cohort study established to study the interrelationships between environment, lifestyle, and genes. The UK Biobank (www.ukbiobank.ac.uk) recruited over 500,000 men and women between 2006 and 2010[13], aged between 37 and 73 years at baseline. The UK Biobank was approved by the North West Multi-Centre Research Ethics Committee, and all participants provided written informed consent to participate in the UK Biobank study. Prevalence of CAD was determined using self-reported data as per prior analysis.[15] Additionally, we used the Hospital Episode Statistics "Spell and Episode" category with hospital in-patient stay diagnoses. CAD was defined using the International classification of disease (ICD) version 9 codes 410, 412 and 414, ICD version 10 codes I21-I25, Z951 and Z955, and the Office of Population Censuses and Surveys Classification of Interventions and Procedures, version 4 (OPCS-4) codes K40-K46, K49, K50 and K75. Controls were excluded if their father, mother or sibling was reported to suffer from any heart disease in order to reduce biological misclassification. CAD GWAS analyses were performed using linear mixed models implemented in BOLT-LMM software[16] (v2.3.1), and adjusted for age at inclusion, genotyping array (UK Biobank Axiom or UK BiLEVE Axiom), and the first 30 principal components provided by the UK Biobank. BOLT-LMM effect estimates and standard errors were transformed to log odds ratios and corresponding standard errors as previously described.[17]

The SiGN consortium is a previously compiled dataset consisting of 14,549 ischemic stroke cases of several cohorts and publicly available controls.[18] The SiGN study population has been described previously, together with details on genetic quality control and genotype imputation methodology.[19] Different procedures were used to establish ischemic stroke diagnosis, which have been described into detail elsewhere.[19] Female sex was defined as the presence of XX chromosomes. GWAS analyses for ischemic stroke were performed

using BOLT-LMM[16] (v2.3.1), and adjusted for population stratification, by inclusion of a genetic relation matrix, and age. BOLT-LMM estimates for ischemic stroke were also transformed to log odds ratios and corresponding standard errors using a previously published approximation.[17]

The DIAMANTE consortium included 74,124 T2D cases and 824,006 controls from 32 GWASs and has been described into detail elsewhere.[14] Studies included in DIAMANTE based T2D diagnosis on different criteria, including but not limited to, fasting glucose and HbA1c levels, hospital discharge diagnosis, use of diabetes medication, and self-report. For the current study, we requested results from sex-specific GWAS analyses, which were adjusted for population stratification and study-specific covariates.[14]

There was no overlap in participants between the UK Biobank and the AMH GWAS. However, there may be some overlap in participants between SiGN and DIAMANTE and the AMH GWAS, since all three studies included participants from the Nurses' Health Study (maximum overlap n = 642). An additional 127 participants of EPIC-Interact[20] may overlap between the AMH GWAS and DIAMANTE (total maximum overlap n = 769).

Statistical analysis

We calculated MR estimates for the individual SNPs in relation to each disease outcome using the Wald ratio method. Individual Wald ratio estimates were meta-analyzed using a random-effects inverse-variance weighted (IVW) method. To assess the strength of included genetic variants for AMH we calculated F-statistics corresponding to the IVW analyses, using the proportion of variance in AMH explained by the genetic variants, the sample size of the outcome GWASs, and the number of variants included.[21] We compared overall MR estimates (i.e. IVW) to SNP-specific MR estimates (i.e. Wald ratio) since inconsistent estimates are indicative of horizontal pleiotropy. In addition, we tested for heterogeneity in causal effects amongst the individual SNPs using Cochrane's Q statistics and performed leave-one-out sensitivity analyses to assess the influence of outlying variants. For stroke, we examined whether causal associations were affected by exclusion of early onset cases (age < 50 years at diagnosis), because early onset stroke is suggested to have a different etiology than stroke at older ages.[22] All MR analyses were performed using the "TwoSampleMR" package (version 0.4.25)[23] in R (version 3.5.1).[24]

To assess potential pleiotropy (i.e. whether genetic variants are associated with multiple traits) we tested if the four AMH SNPs, either individually or combined as a genetic risk score, were associated with a range of traits in the UK Biobank. For this analysis, we selected 44 traits that were either likely to be confounders or that could affect cardiometabolic health through pathways not involving AMH (i.e. horizontal pleiotropy;

e.g. active smoking and body mass index), and traits that could be mediators on the causal pathway between AMH and cardiometabolic disease (i.e. vertical pleiotropy; e.g. markers for subclinical atherosclerosis and glycemic traits). An overview of the 44 investigated traits has been included in Supplemental Table 1. Depending on the type of trait linear or logistic regression models were fitted. We created a heatmap of z-scores aligned with higher genetically predicted AMH levels to visually represent potential pleiotropy. To correct for multiple testing, we considered false discovery rate (FDR) values < 0.05 to be statistically significant.

Results

Descriptive data outcome data sources

The included number of cases and controls for each outcome are presented in Table 1.

Table 1. Number of cases and controls for each outcome.

Outcome	Study	Number of cases	Number of controls
Coronary artery disease	UK Biobank	11,802	137,950
Ischemic stroke	SiGN	4678	12,863
• Age at onset ≥ 50 years		4247	12,863
Type 2 diabetes	DIAMANTE	30,053	434,336

SiGN, Stroke Genetics Network

CAD

We did not find evidence for a causal association between circulating AMH levels and CAD risk (OR_{IVW} = 1.13, 95%CI: 0.95 – 1.35) (Table 2). Results from single SNP analyses for the variants in the *AMH*, *CDCA7* and *MCM8* loci also did not support a causal association with CAD (Table 2), but we observed a risk increasing effect of the SNP in the *TEX41* locus (OR = 1.43, 95%CI: 1.07 - 1.91). The heterogeneity test for the IVW estimate did not indicate heterogeneous effects of the individual SNPs (Cochran's Q = 4.42, p = 0.22). Leave-one-out sensitivity analyses showed that exclusion of the SNP in the *CDCA7* locus resulted in a significant association between genetically predicted circulating AMH levels and CAD risk, although the IVW effect estimate did not change considerably (OR_{IVW} = 1.19, 95%CI: 1.00, 1.42; Supplemental Figure 1).

Table 2. Mendelian randomization estimates for causal effects of circulating AMH levels on coronary artery disease, ischemic stroke and type 2 diabetes in women.

Outcome	Method	F-statistic	Odds Ratio	95% CI	p-value
Coronary artery disease	IVW	558.5	1.13	0.95 – 1.35	0.18
	Wald ratio estimate for rs10417628 (*AMH*)		1.06	0.82 – 1.37	0.65
	Wald ratio estimate for rs13009019 (*TEX41*)		1.43	1.07 – 1.91	0.02
	Wald ratio estimate for rs16991615 (*MCM8*)		1.15	0.85 -1.57	0.37
	Wald ratio estimate for rs11683493 (*CDCA7*)		0.92	0.67 – 1.26	0.60
Ischemic stroke	IVW	65.4	1.11	(0.83 - 1.49)	0.48
	Wald ratio estimate for rs10417628 (*AMH*)		1.31	(0.78 - 2.20)	0.30
	Wald ratio estimate for rs13009019 (*TEX41*)		0.97	(0.55 - 1.70)	0.90
	Wald ratio estimate for rs16991615 (*MCM8*)		0.85	(0.46 - 1.59)	0.62
	Wald ratio estimate for rs11683493 (*CDCA7*)		1.35	(0.71 - 2.56)	0.35
Type 2 diabetes	IVW	1732.1	0.98	(0.87 - 1.10)	0.74
	Wald ratio estimate for rs10417628 (*AMH*)		1.01	(0.83 - 1.23)	0.93
	Wald ratio estimate for rs13009019 (*TEX41*)		0.91	(0.72 - 1.15)	0.43
	Wald ratio estimate for rs16991615 (*MCM8*)		0.99	(0.77 - 1.26)	0.93
	Wald ratio estimate for rs11683493 (*CDCA7*)		1.01	(0.79 - 1.30)	0.93

AMH, anti-Müllerian hormone; IVW, inverse variance weighted
Odds ratio and 95%CI are per 1 unit increase in inverse normally transformed AMH

Ischemic stroke

The IVW estimate did not provide clear evidence for a causal association between higher genetically predicted AMH levels and risk of ischemic stroke (OR_{IVW} = 1.11, 95%CI = 0.83 - 1.49). Wald ratio estimates for the individual genetic variants did also not support a causal association with ischemic stroke (Table 2). Causal effects across the four genetic variants were not heterogeneous (Cochran's Q = 1.69, p = 0.64). Leave-one-out analyses suggested that IVW results would not change after exclusion of any of the SNPs (Supplemental Figure 1).

Exclusion of women younger than 50 years of age at stroke diagnosis attenuated IVW estimates (OR_{IVW} = 0.95, 95%CI: 0.70 - 1.27) and effect estimates for the SNPs in the *AMH*, *CDCA7* and *TEX41* loci (Supplemental Table 2). The effect estimate for the *MCM8* locus changed to a risk increasing effect on ischemic stroke in women aged older than 50 at diagnosis, but its confidence interval was very wide and still included the null (OR = 1.14, 95%CI = 0.60 - 2.17).

T2D

IVW MR estimates did not support an association between genetically predicted AMH and T2D (OR_{IVW} = 0.98, 95%CI = 0.87 - 1.10). Results from the single SNP analyses also did not indicate causal associations with T2D risk (Table 2). The heterogeneity test statistic did not suggest heterogeneous effects amongst the four SNPs (Cochran's Q = 0.54, p = 0.91), and leave-one-out analyses indicated that none of the SNPs had outlying effects (Supplemental Figure 1).

Associations between genetic instruments for AMH and possible pleiotropic traits

Associations between the individual AMH SNPs and the weighted genetic risk score including all four variants are presented in Figure 1. After correction for multiple testing, we observed a positive significant association between the SNP in the *MCM8* locus (rs16991615) and age at menopause and age at menarche. The weighted genetic risk score was only associated with age at menopause. We did not find associations with intermediate traits on the causal pathway between AMH and cardiometabolic health, such as subclinical atherosclerosis or HbA1c and glucose levels.

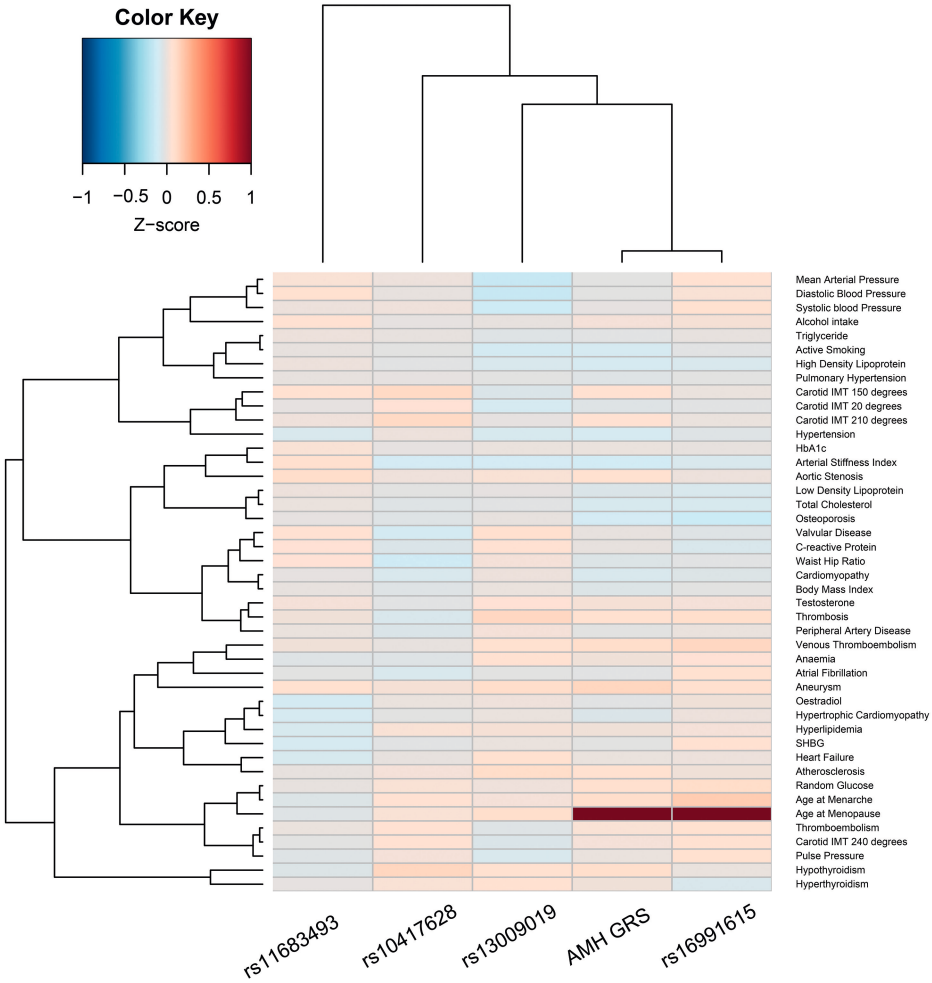

Figure 1. Heatmap of associations between the individual genetic variants for AMH and the weighted genetic risk score (AMH GRS) and 44 traits of the UK Biobank.

The heatmap presents z-scores for 44 UK Biobank traits that correspond to higher genetically predicted AMH levels. Only associations between rs16991615 (*MCM8* locus) and age at menopause and age at menarche, and the association between the AMH GRS and age at menopause were statistically significant at false discovery rate < 0.05. Abbreviations: IMT, intima-media thickness; SHBG, sex hormone binding globulin.

Discussion

Our MR analyses did not provide evidence for causal effects of circulating AMH levels on the risk of CAD, ischemic stroke and T2D in women. However, due to the limited number of genetic instruments, these findings should be interpreted with caution.

Genetic instruments used for MR analyses have to meet the following assumptions to yield valid MR estimates: (1) genetic variants have to be strongly associated with the exposure; (2) genetic variants cannot be associated with confounders of the studied associations; and (3) genetic variants cannot affect the studied outcomes through mechanisms that do not involve the exposure.[25] To meet the first criterion we only included SNPs associated with circulating AMH levels at genome-wide significance as genetic instruments. We also quantified the strength of the combination of these four SNPs through calculation of F-statistics for each outcome (558.5 for CAD, 65.4 for ischemic stroke, and 1732.1 for T2D). Although a F-statistic higher than 10 is considered to indicate a strong genetic instrument, the estimated F statistics may be overestimated due to the use of the R^2 from the discovery AMH GWAS. It is therefore still possible that weak instrument bias may have biased our MR estimates towards the null and reduced statistical power to detect a causal effect.[26] Due to the limited number of genetic variants we were not able to assess violation of the second and the third MR assumption using methods such as MR-Egger and MR-PRESSO.

We did assess potential pleiotropy of the genetic instruments for AMH with 44 traits in the UK Biobank. These analyses did not provide evidence for associations of the genetic variants, either individually or combined into a genetic risk score, with intermediate traits on the causal pathway between AMH and cardiometabolic health, such as subclinical atherosclerosis or HbA1c and glucose levels. We also did not observe associations between genetically predicted AMH and potential confounders like body mass index and active smoking. Heterogeneity tests and leave-one-out analyses did not support bias due to horizontal pleiotropy, although their results should also be interpreted with caution due to the limited number of SNPs. Our results suggested that higher genetically predicted AMH levels are associated with age and menarche and age at menopause. Indeed, previous GWAS identified rs16991615 at the *MCM8* locus as genetic variant for age at menopause.[27, 28] Whether these associations reflect horizontal or vertical pleiotropy remains difficult to disentangle since AMH, age at menarche and age at menopause are all linked to the functional ovarian reserve.[27, 29, 30]

Potential overlap in study participants between the exposure and outcome GWAS from which summary-level data were used, could bias MR estimates towards the observational association.[31] For both SiGN and DIAMANTE, numbers of overlapping participants were small compared to the total numbers in the study (642 and 769, respectively). We assessed the magnitude of potential bias due to sample overlap in the current study using a web application developed by Burgess et al. (https://sb452.shinyapps.io/overlap), and observed that, if anything, this bias would have been minimal for both ischemic stroke and T2D. Moreover, MR estimates for each outcome indicated null effects, whereas previous observational studies showed that higher AMH levels were associated with a lower risk of

cardiometabolic disease.[6, 7] Therefore, the effect of this type of bias on the MR estimates seems negligible.

We are aware of one previous MR study on AMH, looking at the association with ischemic heart disease in men and women[32], using genetic variants that were significant in male adolescents only.[33] In contrast with our results, this MR provided some evidence for an association of higher genetically predicted AMH levels with a lower risk of ischemic heart disease in women and men combined, yet the validity of this finding is questionable since the used genetic instruments violated the first MR assumption of being strongly related to AMH levels in females. In addition, no details about possible heterogeneous effects across the individual SNPs were described.

Our findings are not in agreement with observational studies that found that women with higher age-specific AMH levels had a lower risk of these cardiometabolic diseases[6, 7]. On the other hand, previous MR studies investigating the causal effect of age at menopause, another indicator for reproductive aging, on CAD also did not find evidence for a causal association.[34, 35] To date, no MR studies investigated whether age at menopause may be causally associated with stroke or diabetes.

An explanation for the discrepancy between the observational and MR findings for the relation between AMH, but also other indicators of reproductive aging, and cardiometabolic disease may be residual confounding by (biological) aging. Given its role in ovarian follicle development and the expression of AMH in these follicles, lower AMH levels are strongly correlated with higher age in women. Also, decelerated reproductive aging, corresponding to higher age-specific AMH levels, has been linked to longevity.[36, 37] Future studies in which both circulating AMH levels and markers for biological aging (e.g. DNA methylation) are available could explore this hypothesis. Another explanation for the discrepancy with observational findings may be that signaling factors that are either upstream or downstream of AMH in the same pathway, instead of AMH itself, are causally associated with risk of cardiovascular disease. Among the suggested upstream regulators of AMH is BMP4[38], and reported downstream targets of AMH include NF-κB[39-41], which have both been linked to cardiovascular disease.[42, 43] One approach to disentangle these relationships would be to perform a mediation MR analysis including separate genetic instruments for AMH, BMP4 and NF-kB.[44]

In conclusion, our results do not support a causal effect of circulating AMH levels on CAD, ischemic stroke and T2D in women. These results should be interpreted carefully, since bias towards the null due to weak instrument bias in our analyses cannot be excluded.

Additional information

Disclosures statement

As of January 2020, AM is an employee of Genentech, and a holder of Roche stock. The other authors have no competing interests to report.

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Supplemental Data

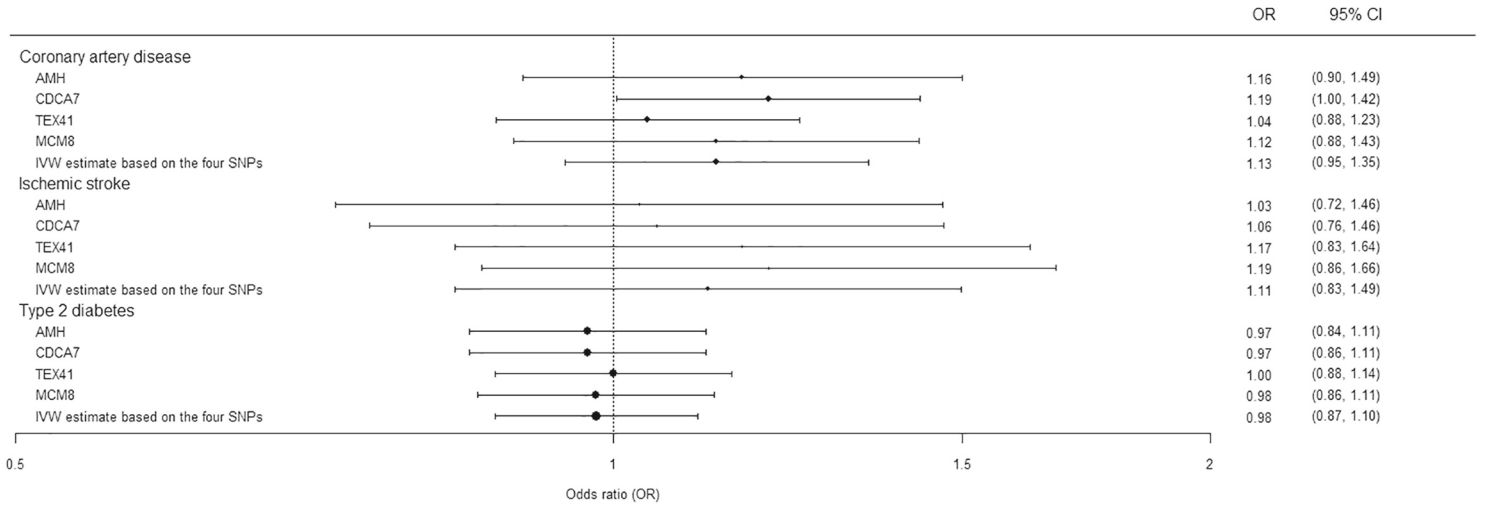
Supplemental Table 1: Overview of the 44 UK Biobank traits tested for an association with the individual genetic variants for AMH and the weighted genetic risk score.

UK Biobank trait
Pulmonary hypertension
Cardiomyopathy
Valvular disease (incl. endocarditis)
Aortic stenosis
Peripheral artery disease in extremities (incl. aneurysms)
Atrial fibrillation/Atrial flutter
Heart failure
Aneurysm (any location)
Hypertrophic cardiomyopathy
Thromboembolism
Thrombosis
Venous thromboembolism
Osteoporosis (incl medication)
Anaemia
Hypertension (incl. touchscreen medication)
Atherosclerosis
Hyperlipidemia (incl. medication)
Active smoker
Hyperthyroidism
Hypothyroidism
Diastolic blood pressure, mean across manual & automatic
Systolic blood pressure, mean across manual & automatic
Pulse pressure, mean across manual & automatic
Mean arterial pressure, mean across manual & automatic
Arterial Stiffness
Carotid intima-media thickness, mean at 120 degrees
Carotid intima-media thickness, mean at 150 degrees
Carotid intima-media thickness, mean at 210 degrees
Carotid intima-media thickness, mean at 240 degrees
C-reactive protein (high-sensitivity) in mg/L
Body mass index (kg/m2)
Waist hip ratio
Low density lipoprotein in mmol/L
High density lipoprotein in mmol/L
Total cholesterol in mmol/L
Triglyceride in mmol/L
HbA1c mmol/L
Glucose in mmol/L
Log2 Interpolated Alcohol in UK Units (8 mg or 10 mL Alc) per week
Age at Menarche
Age at Menopause
Oestradiol in pmol/L
Sex hormome binding globulin in nmol/L
Testosterone in nmol/L

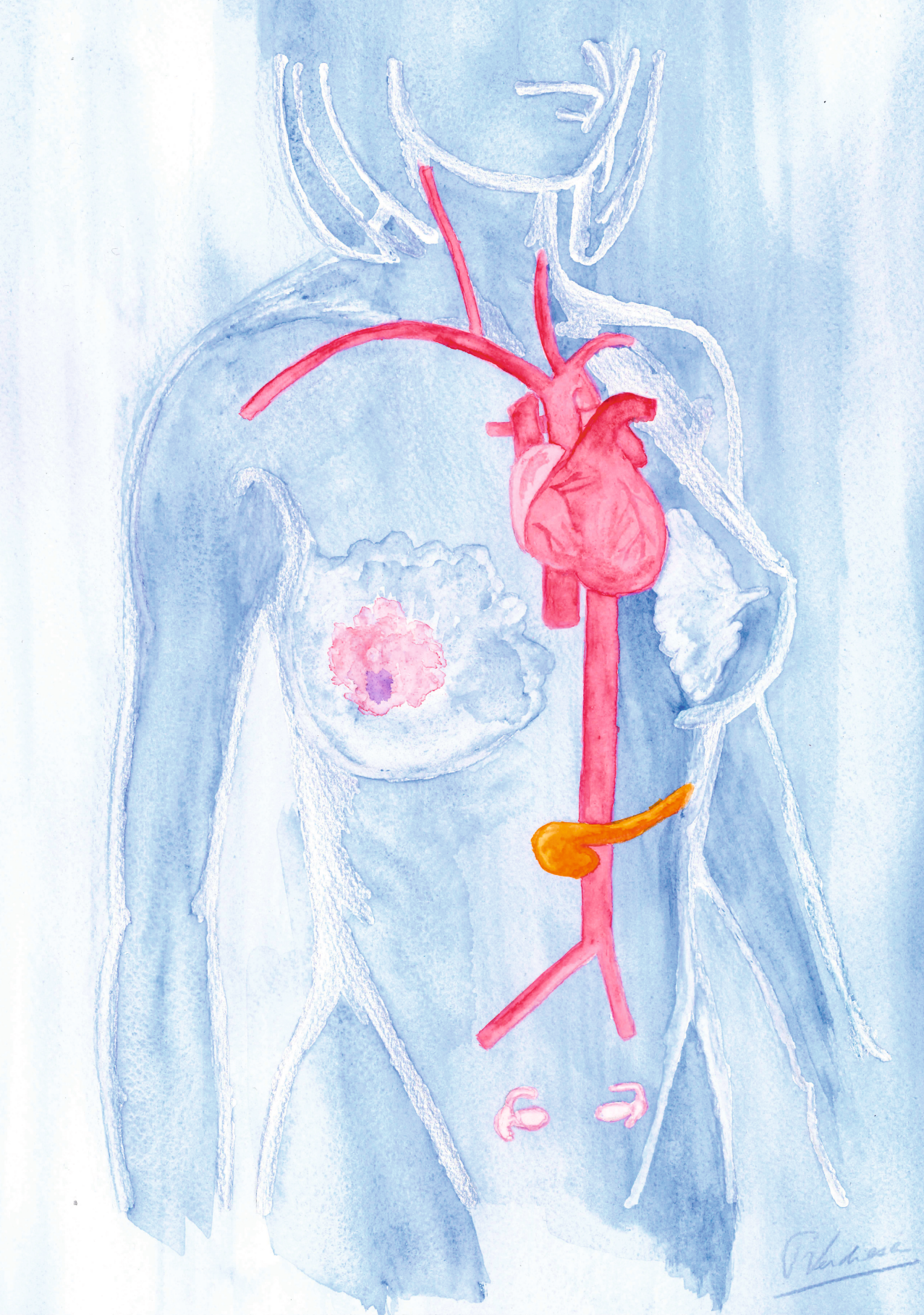
Supplemental Table 2. Mendelian Randomization estimates for causal effects of circulating AMH levels on ischemic stroke in women ≥ 50 years at diagnosis.

Outcome	Method	Odds Ratio	95% CI	p
Ischemic stroke in age onset ≥ 50 years	IVW	0.95	0.70 - 1.27	0.72
	Wald ratio estimate for rs10417628 (*AMH*)	0.87	0.52 - 1.46	0.60
	Wald ratio estimate for rs13009019 (*TEX41*)	0.86	0.48 - 1.54	0.61
	Wald ratio estimate for rs16991615 (*MCM8*)	1.14	0.60 - 2.17	0.70
	Wald ratio estimate for rs11683493 (*CDCA7*)	1.01	0.53 - 1.96	0.97

AMH, anti-Müllerian hormone; IVW, inverse variance weighted
Odds ratio and 95%CI are per 1 unit increase in inverse normally transformed AMH



Supplemental Figure 1: Estimates of leave-one-out analyses for the association between circulating AMH and coronary artery disease, ischemic stroke and type 2 diabetes in women.



General discussion

CHAPTER 8

In this thesis we used diverse methodological approaches to investigate whether circulating anti-Müllerian hormone (AMH) levels are causally associated with risk of cancer and cardiometabolic diseases. Whereas evidence from previous studies indicates a relation between higher AMH levels and risk of breast cancer (**Chapter 2**), we did not find clear evidence for a causal effect of AMH on (breast) cancer risk (**Chapter 3 and 6**). Results from **Chapter 4 and 5** indicated that lower AMH levels are associated with a higher risk of type 2 diabetes in women and potentially with a higher degree of subclinical atherosclerosis in men, respectively. On the other hand, Mendelian randomization (MR) analyses did not support a causal effect of AMH on risk of several cardiometabolic diseases (**Chapter 7**), potentially due to weak genetic instruments for AMH, which may have biased our results towards the null. Accordingly, our results do not provide conclusive evidence in favor of, nor against, a causal relation between endogenous AMH levels and risk of cancer and cardiometabolic diseases. Challenges commonly faced in etiological epidemiology, such as residual confounding and reverse causation, are a main thread running through the previous thesis chapters. In this chapter, these challenges are discussed in the light of the research presented in this thesis. We also discuss directions for future research on the role of AMH in the etiology of cancer and cardiometabolic diseases.

Challenges in etiological epidemiology

Etiological epidemiology aims to identify risk factors that are *causally* associated with the outcome of interest. Although the randomized controlled trial is the paradigm for such research, observational studies are often used instead because of ethical and practical reasons. For the studies presented in this thesis, for example, randomized controlled trials are not feasible since endogenous AMH levels cannot be allocated. Inferring causal associations from observational data faces challenges that are not encountered in randomized controlled trials, such as (residual) confounding. Additionally, it remains difficult in observational studies to establish whether exposures have an actual causal role in the etiology of the outcome of interest.

Triangulation

To overcome these challenges in etiological epidemiology and strengthen causal inferences from observational data, Lawlor and colleagues recently advocated the need for a "triangulation framework in etiological epidemiology".[1] The term "triangulation" originates from navigation and cartography in which it refers to determining a difficult to measure location through the use of the angles of at least two known locations.[2] In the context of

etiological epidemiology, the following definition has been proposed: "The practice of strengthening causal inferences by integrating results from several different approaches, where each approach has different (and assumed to be largely unrelated) key sources of potential bias".[1]

If we look at the work in this thesis, where we use different methodologies to address the same causal research questions, it perfectly fits in this triangulation framework. Cross-sectional analyses such as described in **Chapter 5**, may be prone to reverse causation, which means that the outcome affects the exposure instead of the other way around. Longitudinal analyses (**Chapter 3, 4 and 5**) allow the possibility to investigate and limit the risk of bias by reverse causation. MR analyses (**Chapter 6 and 7**) can also be used for this purpose, since the genetic exposure is present since conception and thus will be present before the onset of disease in adulthood. Moreover, MR analyses can be used to limit risk of bias by (residual) confounding, which is a common source of bias in observational studies (**Chapter 3, 4, and 5**). As long as the genetic instrumental variables meet the three MR assumptions*, results are unlikely to be biased due to confounding. On the other hand, MR estimates are prone to weak instrument bias (i.e. bias because of differences in confounders between exposure subgroups if the genetic instrumental variables explain little variation in the exposure[3]) and bias caused by population stratification (i.e. differences in the frequency of genetic variants across different ethnic (sub)populations).[4]

Although we used these diverse methodological approaches to assess the causal relation between AMH and cancer, and AMH and cardiometabolic diseases, a bottleneck in some of the studies was limited statistical power to detect true (causal) associations. For example for type 2 diabetes, results from our longitudinal analyses in **Chapter 4** indicated that lower AMH levels are associated with a higher risk of type 2 diabetes in women, but our MR analyses in **Chapter 7** did not support a causal effect of AMH on the risk of type 2 diabetes. Combined these results do not provide strong evidence for a causal relation. However, our MR analyses were possibly biased toward the null due to the small proportion of variation in circulating AMH levels explained by the four genetic variants (1.47%). The lack of triangulation of our results could therefore be the consequence of reduced statistical power, rather than the true absence of a causal effect of AMH.

Accordingly, it is not possible to establish if AMH has a causal role in the etiology of cancer and cardiometabolic diseases based on the findings presented in this thesis. In addition to the reduced statistical power in **Chapter 3, 6 and 7**, the relation between AMH and female

* MR assumptions: (1) genetic variants have to be strongly associated with the exposure; (2) genetic variants cannot be associated with confounders of the studied associations; and (3) genetic variants cannot affect the studied outcomes through mechanisms that do not involve the exposure.

reproductive aging complicates making causal inferences regarding the role of AMH. The link between reproductive aging and biological aging adds an additional layer of complexity to the interpretation of our results.

Disentangling effects of AMH and reproductive aging

Given the biological link between circulating AMH levels and ovarian aging, it is difficult to establish whether AMH itself has a causal role in the pathophysiology of non-communicable diseases or if AMH is merely a marker of female reproductive aging. Moreover, AMH may also be a marker of male reproductive aging. Circulating AMH levels have been linked to spermatogenesis quality[5], which is considered to be a marker for male fertility. Here we describe three possible scenarios through which AMH could affect disease risk, using the association between AMH and type 2 diabetes in women (**Chapter 3**) as example (Figure 1).

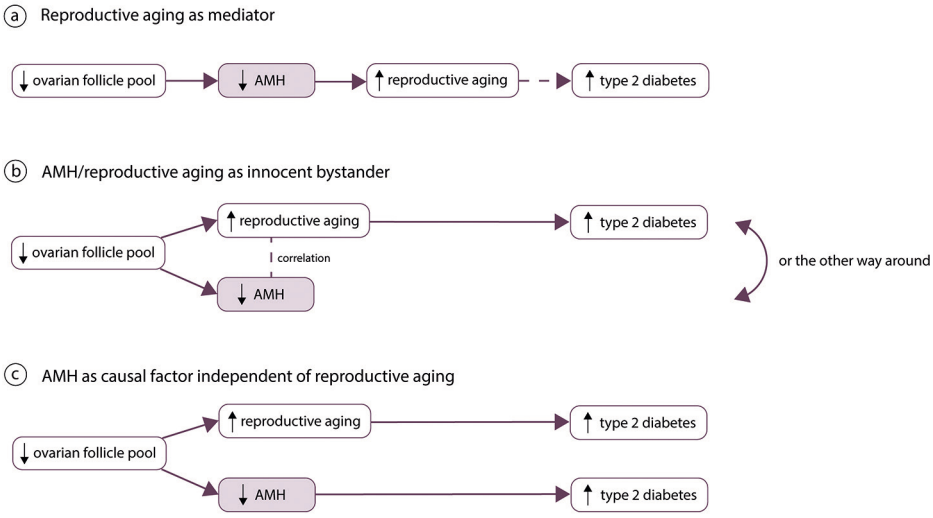


Figure 1. Schematic overview of three possible scenarios through which AMH could be associated with risk of type 2 diabetes in women.

Female reproductive aging is driven by depletion of the pool of antral follicles, which are the main producers of AMH in women.[6] Hence, decreasing numbers of antral follicles result in decreased AMH production, and therefore lower levels of circulating AMH. AMH acts as inhibitor of primordial follicle recruitment, and it has been suggested that this reduction in AMH production results in more activated primordial follicles. This would provoke a more rapid depletion of the ovarian follicle pool, and accordingly an earlier age

at menopause.[7] Women with an earlier menopause have been found to be at a higher risk of postmenopausal type 2 diabetes.[8] Following this lead, the first possible scenario is that AMH indirectly affects risk of type 2 diabetes through its role in female reproductive aging, i.e. that reproductive aging is a mediator (Figure 1a). A second possibility is that circulating AMH levels are merely a marker of the reproductive aging process, but that AMH signaling is not involved in the pathophysiology of type 2 diabetes, i.e. that AMH is a so-called "innocent bystander", or the other way around (Figure 1b). On the other hand, the receptor through which AMH signaling takes place (AMHR2) is expressed in several non-gonadal tissues, among which pancreatic tissue[9]. The third possible scenario is therefore that AMH can have a causal role in the pathophysiology of diabetes, potentially *independent* of reproductive aging (Figure 1c). Finally, it might very well be possible that actual biology involves a more complex combination of these simplified scenarios and that additional processes are involved, like biological aging, as we discuss later in this chapter.

One of the main difficulties in disentangling whether AMH itself has a causal role in the etiology of breast cancer, type 2 diabetes and cardiovascular disease lies in the quantification of the reproductive aging process. Reproductive aging is a complex exposure that represents a wide range of physiological processes, including, but not limited to, various hormonal changes.[10] Ideally, repeated data on all relevant components that reproductive aging encompasses should be used to determine in which reproductive phase women are. However, the most recent Stages of Reproductive Aging Workshop (STRAW) criteria only include specific menstrual criteria for some, and not even for all, of the defined reproductive stages.[11] Although the STRAW criteria clearly recognize the importance of the inclusion of quantitative criteria for endocrinological parameters, including AMH and estradiol, the lack of standardized assays forms a serious obstacle to actual inclusion of information about hormonal levels into the current criteria.[11] As a result, it is hardly possible to establish which of the scenarios presented in Figure 1 best represent(s) biology based on epidemiological research only.

Functional studies could provide more insight into this subject; for instance through the use of experimental models in which *AMHR2*, the gene encoding the receptor that is required for AMH signaling, is knocked-out in target tissues only. For type 2 diabetes, for example, the absence of AMH signaling in pancreatic tissue may reveal if AMH is involved in pathophysiology. More robust epidemiological evidence on the causal role of AMH could potentially be obtained through MR analyses that include more genetic instrumental variables for AMH (i.e. a stronger genetic instrument). However, in order to identify more genetic variants for AMH, genome-wide association studies (GWAS) including a larger number of participants should be conducted first. Most large studies with available AMH and genotyping data to date have been included in our meta-analysis in **Chapter 6**. Hence,

conducting a much larger GWAS for AMH requires that AMH levels are measured in additional large cohort studies that include sufficient women of early reproductive age, and preferably also adult men, which requires both financial and time investments. Moreover, the aforementioned considerations raise the question whether we should study AMH as independent risk factor at all, or rather as part of a more complex exposure. We elaborate on this latter issue in the paragraph "Directions for future research".

Excluding potential residual confounding by biological aging

Because of the relation between AMH and reproductive aging, and the link between reproductive aging and biological aging, the results of **Chapter 4** (type 2 diabetes) **and Chapter 5** (markers of subclinical cardiovascular disease) are potentially biased due to residual confounding by biological aging. The link between reproductive and biological aging has been acknowledged for decades. Because of this link, it has been postulated that the observational relation between accelerated reproductive aging and higher risk of cardiovascular diseases may be confounded by accelerated biological aging. This hypothesis is indirectly supported by recent MR analyses that dispute a causal association between age at menopause, and (risk factors for) cardiovascular disease.[12, 13]

Similar to reproductive aging, biological aging is a complex exposure that encompasses chronological age but also functional aspects, such as cognitive functioning and physical fitness.[14] As a consequence, adjusting association analyses for chronological age alone will only partly remove a potential confounding effect by biological aging. Currently, several biomarkers are available to quantify human biological aging on the molecular level, of which the epigenetic/DNA methylation clock and leukocyte telomere length are most frequently used.

There is some evidence that DNA methylation and leukocyte telomere length are correlated with circulating AMH levels in men[15] and in women[16], although the direction of this correlation is inconsistent across studies. Higher AMH levels in men were associated with a higher odds of having a short leukocyte telomere length, which was defined as a telomere length shorter than the 75th percentile in the cohort.[15] This finding suggests an association between higher AMH levels and accelerated biological aging. In contrast, accelerated biological aging based on DNA methylation has been linked to lower circulating AMH levels in women undergoing ovarian stimulation, but these analyses were not adjusted for other factors.[16] In other words, clear evidence for an association between circulating AMH levels and molecular markers of biological aging has yet to emerge.

We did not have data available to investigate if the associations studied in this thesis were potentially confounded by biological aging. However, our GWAS meta-analysis (**Chapter**

6) provided some circumstantial evidence for a shared genetic architecture between circulating AMH levels and DNA methylation, since *CDCA7* has also been identified as a DNA methylation regulating gene.[17] Besides, genes mapped to the *CDCA7, MCM8* and *TEX41* loci are involved in the cell cycle and processes like DNA replication and apoptosis, which can be linked to the "genomic instability" and "cellular senescence" hallmarks of aging.[18] Consequently, residual confounding by biological aging should be excluded in future studies before claims about causal relations between AMH and risk of cancer, and AMH and risk of cardiometabolic diseases, can be made.

Directions for future research

In the previous paragraphs, we formulated suggestions for future research to disentangle effects of AMH and reproductive aging on disease risk, and highlighted the need for additional research to establish whether biological aging may (partly) explain the observational relation between AMH and disease risk. Additionally, we formulated three topics that should be considered during the conceptual design of future studies on the role of AMH in the etiology of cancer and cardiometabolic diseases.

Modelling AMH as part of a hormone profile rather than as simple exposure

Investigating individual hormones in relation to disease outcomes in epidemiological research is a tremendous oversimplification of biology. As the Britannica encyclopedia states, a hormone is an "organic substance secreted by plants and animals that functions in the regulation of physiological activities and in maintaining homeostasis".[19] To maintain homeostasis, hormones interact with and regulate one another in a time- and context-dependent manner. Also for AMH, interactions with other reproductive hormones have been reported in a time- and context-dependent manner.[20-22] Accordingly, investigating AMH as part of a hormone profile in relation to disease risk, instead of as a single risk factor, would very likely better represent actual biology.

Modelling AMH as part of a complex hormone exposure corresponds to the concept of the exposome, which refers to the total exposure to internal (e.g. endogenous hormones) and external factors (e.g. lifestyle factors) over the life-time.[23] The internal exposome can be quantified using so-called -omics data, which refers to the complete set of certain molecules; e.g. genomics refers to all genes in the genome and their interactions.[24] For the analysis of (multi)-omics data, data-reduction methods (e.g. principal component analysis) and clustering methods (e.g. hierarchical cluster analysis and k-means cluster analysis), either on their own or combined, are used to model mixtures of exposures rather

than single exposures.[25] Although the use of clustering techniques to construct hormone profiles is not novel[26, 27], previous studies that investigated hormone profiles as complex exposure in relation to risk of cancer and cardiometabolic disease are scarce[28, 29] and did not include AMH data. An explanation for this may be that for most study populations only a subset of the relevant reproductive hormones is measured due to relatively high costs of multiple hormone measurements. Interestingly, high-throughput -omics technologies allow for the measurement of a wide range of molecules in a single biological sample. Due to the reducing costs of these technologies, collecting complete hormone data will become increasingly feasible for large study populations in the near future. Ideally, repeated hormone measurements would be performed for each study participant to investigate hormone profiles based on age-related hormone trajectories in relation to disease risk.

Complementary research could involve studies that include participants in whom hormone profiles are known to be different, like women with polycystic ovary syndrome and patients with disorders of sex development (e.g. persistent Müllerian duct syndrome and Klinefelter syndrome). Such studies would mimic natural experiments, in which combinations of hormones naturally differ between patient groups and healthy participants, and could be investigated in relation to the risk of different diseases.

Investigating time-specific associations between AMH and disease risk

In addition to considering to model AMH as complex exposure, it would also be worth to consider investigating age-specific effects of AMH on disease risk. It is perfectly possible that AMH only affects disease risk during a critical period, like AMH's time-specific role during embryogenesis.[30] Another possibility is that the magnitude of an effect of AMH differs over the life course. For breast cancer, a previous study indeed suggested that the association between AMH and breast cancer differs by age at AMH measurement.[31] However, it is not clear to what extent this finding was driven by a number of small studies including mostly older women, which reported very large effect sizes.

Even though we aimed to assess the temporal association between AMH and risk of cancer, and AMH and risk of type 2 diabetes, our trajectory analyses suffered from the limited number of AMH measurements at younger ages. AMH has been measured in cohorts including children and adolescents (e.g. ALSPAC)[32, 33], but mostly in female study populations with a median age of 40 years or higher.[31, 34-39] Studies with a substantial proportion of study participants aged 20 to 35 years are currently lacking (Figure 2). In male study populations, the median age at which AMH was measured was even higher compared to female study populations.[15, 40]

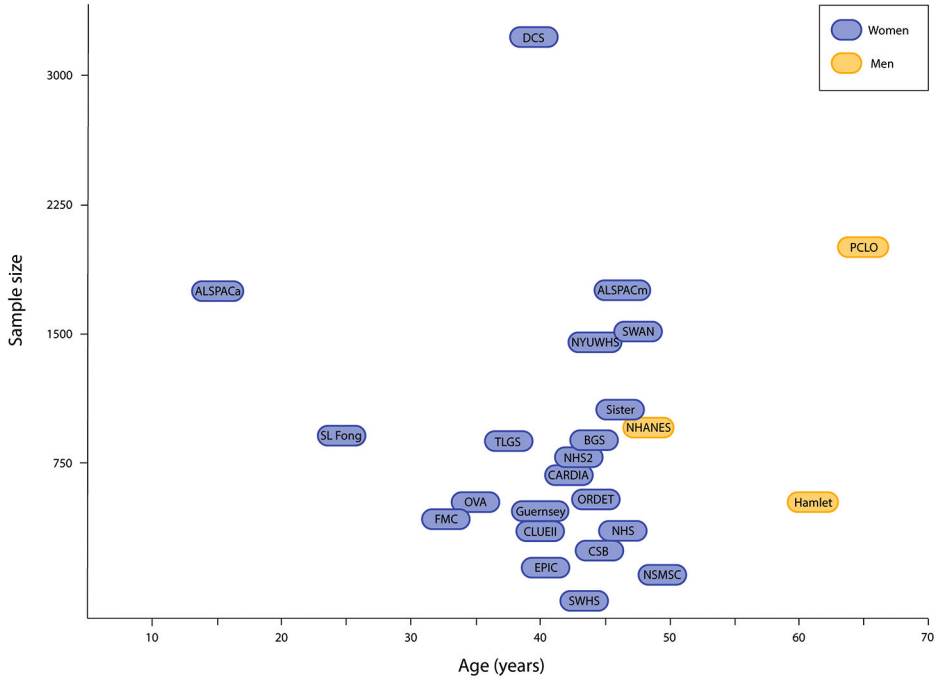


Figure 2. Schematic overview of studies that measured AMH in women (purple) or men (yellow) presented by the median/mean age at AMH measurement.**

As a result, large-scale analyses investigating if AMH, either as single exposure or as component of a hormone profile, has a time-specific effect on disease risk are currently only feasible in middle-aged to older study participants. Additional AMH data-collection in younger study populations is thus needed to accurately estimate temporal associations between AMH and non-communicable diseases. The main challenge that cohort studies will face is that long periods of follow-up are needed until the number of incident disease cases is large enough to ensure that analyses have sufficient statistical power to detect true associations. Therefore, collecting AMH data for case-control sets nested in existing cohorts with sufficient follow-up, as has been done

** ALSPACa, Avon Longitudinal Study of Parents and Children – adolescent participants; SL Fong, study population publication SL Fong et al. (2012); FMC, Finnish Maternity Cohort; OVA, Ovarian Aging Study; TLGS, Tehran Lipid and Glucose Study; DCS, Doetinchem Cohort Study; Guernsey, Guernsey Cohort; CLUEII, Campaign Against Cancer and Heart Disease; EPIC, European Prospective Investigation into Cancer and Nutrition; SWHS, Shanghai Women's Health Study; CSB, Columbia, Missouri Serum Bank; NHS, Nurses' Health Study; ORDET, Hormones and Diet in the Etiology of Breast Cancer; CARDIA, Coronary Artery Risk Development in Young Adults; NHS2, Nurses' Health Study II; BGS, Breakthrough Generations Study; Sister, Sister Study; NYUWHS, New York University Women's Health Study; SWAN, Study of Women's Health Across the Nation; ALSPACm, Avon Longitudinal Study of Parents and Children – adult participants; NHANES, National Health and Nutrition Examination Survey; PCLO, Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial.

previously for several cancer types[31, 35, 36, 40-43], will be the most efficient approach. Combining all relevant studies into living individual participant data meta-analyses[44], i.e. continuously updated analyses, would increase efficiency to investigate age-specific effects of AMH even further.

Integrating epidemiological research with fundamental research to answer the same research question

Most previous studies that investigated circulating AMH levels in relation to disease risk, including ours, based their hypothesis on circumstantial evidence from a handful of fundamental studies. For example, our hypothesis that endogenous AMH may be involved in tumorigenesis was based on a limited number of fundamental studies in which the effect of recombinant AMH on (breast) cancer cells was tested, using in vitro as well as in vivo model systems.[45-48] However, these experiments do not directly translate to the function of endogenous AMH in humans. Besides, such experiments do not address the question whether and how AMH signaling plays a role in the *development* of cancer. We should therefore be very careful not to extrapolate certain findings from fundamental studies (e.g. inhibiting tumor cell growth) to a different setting (e.g. tumor development), which we subsequently only further investigate in epidemiological research. Instead, it would be more powerful if epidemiologists and wet-lab biologists would collaborate more intensively. Such collaborations can tackle the same research question from different perspectives and therefore generate even more valuable insights, as has been proven by similar initiatives in genetic epidemiology in translating GWAS findings to function. Moreover, integration of fundamental findings with the research presented in this thesis is essential to elucidate if circulating AMH has a causal role in the etiology of cancer and cardiometabolic diseases.

Concluding remarks

In this thesis, we used a triangulation framework by applying diverse methodological approaches to investigate the (causal) associations between circulating AMH levels and cancer, (subclinical) cardiovascular disease and type 2 diabetes. Nevertheless, given the biological link between AMH and reproductive aging in women, and reproductive function in men, it is practically impossible to disentangle whether AMH has a causal role in the etiology of these diseases based on epidemiological findings alone. The link between reproductive aging and biological aging adds an additional layer of complexity in establishing the causal relation between AMH and non-communicable disease risk. We therefore advocate collaborative initiatives between epidemiologists and wet-lab biologists to reveal whether AMH is a *leading lady or best friend* in the etiology of cancer and cardiometabolic diseases.

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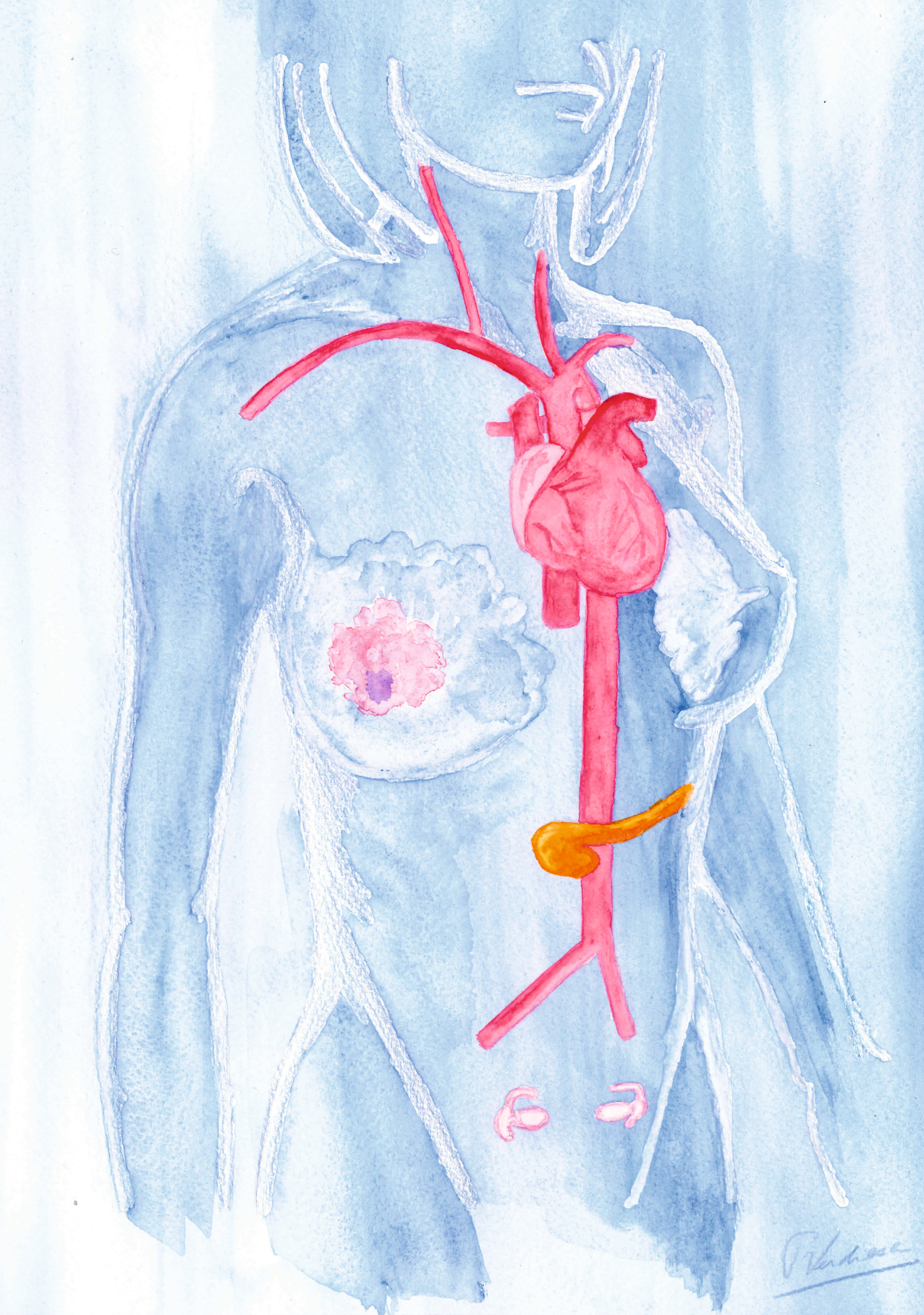


Summary

CHAPTER 9

Reproductive aging has been linked to the risk of several non-communicable diseases, including breast cancer, cardiovascular disease and type 2 diabetes. However, the causality of these associations and the underlying biological mechanisms are not fully revealed yet. A potential causal candidate explaining the association between reproductive aging and risk of the aforementioned diseases is anti-Müllerian hormone (AMH). The aim of this thesis was to investigate whether circulating AMH levels are (causally) associated with the risk of cancer, type 2 diabetes, and cardiovascular disease.

The first two chapters focus on the (temporal) association between circulating AMH levels and risk of cancer. In **Chapter 2** we provide a systematic overview of the current epidemiological evidence on endogenous AMH levels in relation to risk of different cancer types. We included 12 studies on breast, ovarian and endometrial cancer, lymphomas, non-gynecological cancers, childhood cancer and prostate cancer. Of these, five studies measured AMH prior to cancer diagnosis; the others measured AMH after diagnosis but prior to cancer treatment. Altogether, we found that higher AMH levels were associated with an increased risk of breast cancer, whereas there was little evidence for associations with other cancer types. Analyses stratified by age at AMH measurement hinted at an increased risk of ovarian and endometrial cancer in younger women with higher AMH levels, but these three studies included too few women to provide a definite answer. Postdiagnosis-pretreatment AMH levels were lower in women diagnosed with different types of cancer compared with AMH levels in healthy women. However, we considered most of the studies that included postdiagnosis-pretreatment AMH levels to be of poor methodological quality, because of inadequate correction for age and other important confounders. We therefore refrained from drawing definite conclusions regarding the relation between postdiagnosis-pretreatment AMH levels and cancer.

To gain more insight into the relation between circulating AMH levels and cancer, we subsequently examined the association of age-specific AMH levels with the risk of cancer in **Chapter 3**. Previous studies included a single AMH measurement per participant, although age-related AMH trajectories have been shown to vary between women. We therefore explored the temporal association between AMH and cancer through investigating if age-related AMH trajectories were different for women who developed cancer compared to women who did not. For this purpose, we used data of 3025 female participants of the Doetinchem Cohort Study. AMH was repeatedly measured in blood samples collected at 5-year intervals over a period of 20 years, resulting in 11,655 measurements available for analyses. We calculated age-specific AMH tertiles at baseline to account for the strong AMH-age correlation. In addition to overall cancer, we separately investigated associations between circulating AMH levels and breast cancer, cancers in other AMHR2-expressing tissues, and cancers in non-AMHR2-expressing tissues. Age-specific AMH levels measured

at baseline of the study were not associated with any of these cancer outcomes. Because AMH is known to strongly decrease from age 40, and because less variation is found in AMH levels after this age, we performed additional analyses restricted to women younger than 40 years at baseline of the cohort. Analyses among these 1543 younger women supported previous studies that found higher AMH levels to be associated with a higher risk of breast cancer (HR_{T2vsT1} = 2.06, [95% CI 0.95, 4.48]; HR_{T3vsT1} = 2.03, [95% CI 0.91, 4.50]). Examination of AMH trajectories indicated that AMH levels around age 30 may be higher, and may decline faster, in women who are diagnosed with cancer compared to women who are not, but our results did not provide strong evidence for an actual difference in age-related AMH trajectories.

Few studies have investigated endogenous AMH in relation to cardiometabolic diseases. Their results are inconsistent and most of these studies had a cross-sectional design. We therefore used data from the Doetinchem Cohort Study and a similar approach as in **Chapter 3**, to investigate associations between age-specific AMH levels at baseline of the cohort, and age-related AMH trajectories, and the risk of incident type 2 diabetes. The results of this analysis are reported in **Chapter 4**. During a median follow-up of 20 years, 163 women developed type 2 diabetes. Lower baseline age-specific AMH levels were associated with a higher risk of type 2 diabetes (HR_{T2vsT3} = 1.24, [95% CI 0.81, 1.92]; HR_{T1vsT3}= 1.62, [95% CI 1.06, 2.48]). Trajectory analyses, including 12,460 AMH measurements, did not show clear evidence of different AMH trajectories in women who developed type 2 diabetes compared with women who did not. Yet, it remains to be elucidated whether AMH is indeed causally associated with risk of diabetes, or whether residual confounding influenced our findings. Further research is therefore needed to investigate whether AMH is part of the biological mechanism explaining the association between reproductive aging and type 2 diabetes.

The association between circulating AMH levels and risk of cardiometabolic diseases has been studied most frequently in women. However, as AMH is also measurable in men, AMH could potentially play a role in cardiovascular disease pathology in both sexes. Therefore, we investigated whether circulating AMH levels were associated with measures of subclinical cardiovascular disease in middle-aged and older participants of a Dutch population-based cohort study in **Chapter 5**. Among 394 men (aged 40-80 years) we examined cross-sectional associations between AMH levels and carotid intima-media thickness (CIMT), pulse wave velocity (PWV), abdominal aortic diameter and Framingham risk score (FRS) predictions (i.e. predicted 10-year risk of coronary heart disease) at baseline. We additionally assessed longitudinal associations with CIMT, carotid aortic plaque score, PWV and FRS predictions. Our results indicated that higher AMH levels were associated with a lower CIMT at baseline (estimates for inverse-normally transformed CIMT; β = -0.04, [95% CI 0.07, -0.01]). In addition, our results suggested that higher AMH levels are potentially

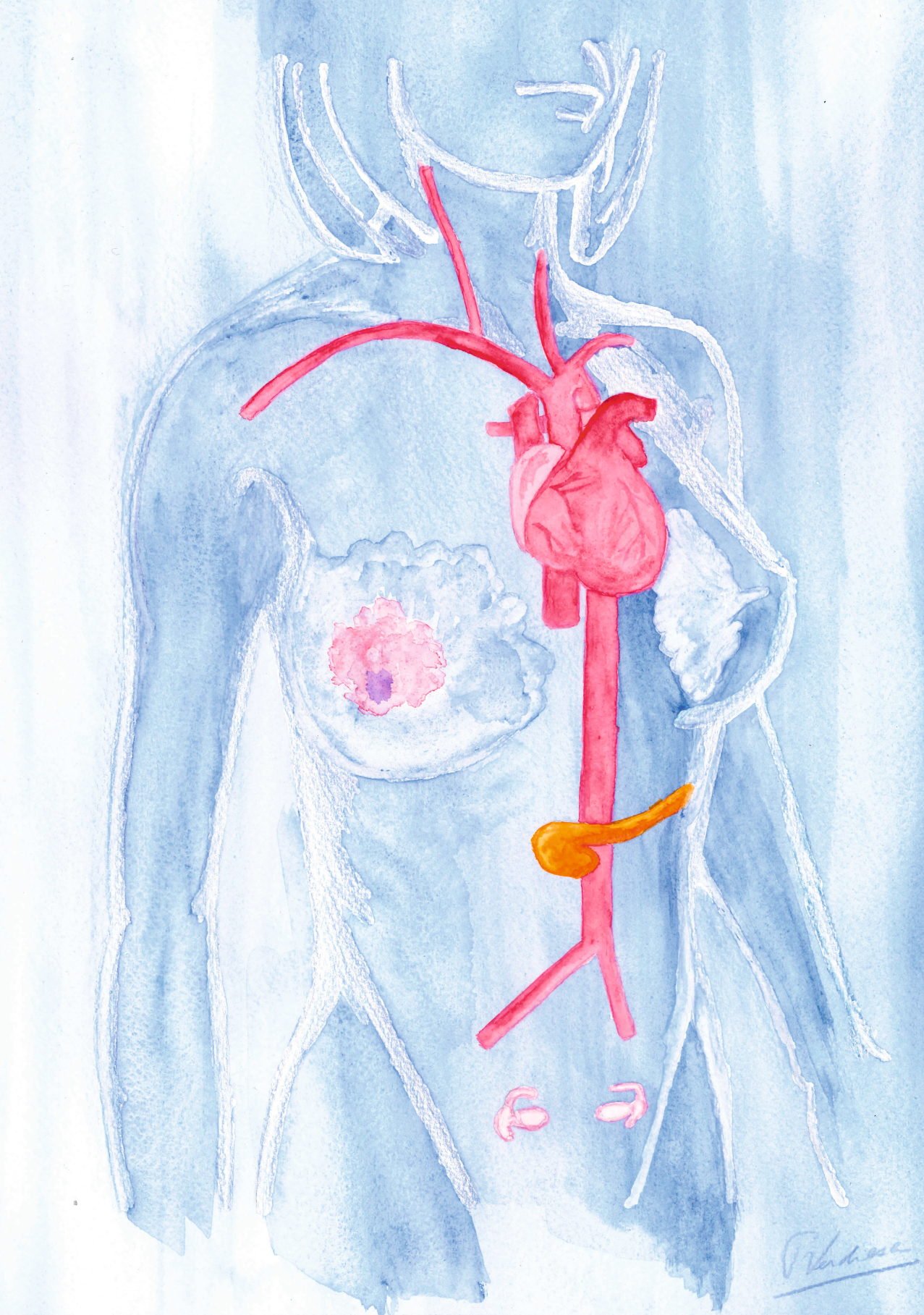
associated with a lower mean carotid aortic plaque score after a median follow-up time of 8.7 years, independent of CIMT at baseline (estimates for inverse-normally transformed mean plaque score; β = -0.03, [95%CI = -0.07, 0.00]). Although higher AMH levels were associated with lower baseline FRS predictions, this effect attenuated after adjustment for circulating sex hormone levels. Circulating AMH levels were not associated with aorta diameter and PWV at baseline, and also not with PWV and FRS predictions at follow-up. In conclusion, AMH may be associated with structural (i.e. atherosclerosis) but not with functional changes (i.e. arterial stiffness) of the arterial wall.

A question that remained after completion of the aforementioned studies, was whether the role of AMH in the etiology of the investigated diseases is actually causal. Gaining more knowledge about the genetic variation and biological mechanisms underlying inter-individual variation in circulating AMH levels could provide new clues about the functions of AMH. As a result, more insight into the mechanisms through which AMH is involved in the etiology of cancer, cardiovascular disease and type 2 diabetes can be gained. Moreover, genetic variants for circulating AMH levels enable the assessment of potential causal effects of AMH on disease outcomes using a Mendelian randomization (MR) approach. To identify such genetic variants, we performed a genome-wide association study (GWAS) meta-analysis for AMH in **Chapter 6**. We used data from seven cohorts, and in total from 7049 premenopausal women of European ancestry, which more than doubled the sample size of the largest previous AMH GWAS. We identified four genetic loci associated with AMH levels at the genome-wide significance level: the previously reported *MCM8* locus and three novel signals in or near *AMH*, *TEX41*, and *CDCA7*. The strongest signal was a missense variant in the *AMH* gene (rs10417628). Most prioritized genes at the other identified loci were involved in processes related to cell cycle regulation, such as apoptosis. Genetic correlation analyses indicated a strong positive correlation among SNPs for AMH levels and SNPs for age at menopause (r_g = 0.82, FDR = 0.003). Exploratory MR analyses did not support a causal effect of AMH on breast cancer or polycystic ovary syndrome risk, but should be interpreted with caution as they may be underpowered and validity of genetic instruments could not be extensively explored. In conclusion, we identified one variant in the *AMH* gene and three other loci that are associated with inter-individual variation in circulating AMH levels in women.

In **Chapter 7** we used these four AMH SNPs to explore whether the relation between circulating AMH levels and cardiometabolic disease risk could be causal, using a MR approach. We included outcome data for coronary artery disease, ischemic stroke and type 2 diabetes of female participants of the UK Biobank, the Stroke Genetics Network, and DIAMANTE consortia, respectively. Our results did not support a causal effect of circulating AMH levels on coronary artery disease (OR_{IVW} = 1.13, [95% CI: 0.95 – 1.35]),

ischemic stroke (OR_{IVW} = 1.11, [95% CI: 0.83 – 1.49]), or type 2 diabetes (OR_{IVW} = 0.98, [95% CI = 0.87 - 1.10]) in women. After adjustment for multiple testing, we observed that higher genetically predicted AMH levels were associated with a later age at menopause and a later age at menarche in the UK Biobank, but not with intermediate traits on the causal pathway between AMH and cardiometabolic disease, such as subclinical atherosclerosis or HbA1c and glucose levels. These results do not provide evidence for a causal effect of circulating AMH levels on coronary artery disease, ischemic stroke and type 2 diabetes in women, although we cannot exclude the possibility of weak instrument bias that may have biased our results towards the null.

In **Chapter 8** we discuss the main challenges that we encountered during the interpretation of the results from the preceding chapters. We also provide recommendations for future research on the role of AMH in the etiology of cancer, and cardiometabolic diseases. In our view, future epidemiological research should go hand in hand with fundamental research to reveal whether AMH actually plays a role in the pathophysiology of these diseases. The findings presented in this thesis lay a valuable scientific foundation for such research, but in itself do not provide definitive evidence in favor, nor against, a causal role of endogenous AMH in the etiology of different cancer types and cardiometabolic diseases.



Nederlandse samenvatting

Dankwoord

About the author

APPENDICES

Nederlandse samenvatting

De term 'reproductieve veroudering' verwijst naar het afnemen van zowel het aantal eicellen als de kwaliteit daarvan tot het moment dat vrouwen in de overgang komen. Reproductieve veroudering is in verband gebracht met het risico op verschillende ziekten, waaronder borstkanker, hart- en vaatziekten en diabetes type 2. Of reproductieve veroudering daadwerkelijk een oorzakelijke rol speelt in het ontstaan van deze ziekten, en welke biologische processen hieraan ten grondslag liggen, is nog niet helemaal duidelijk. Anti-Müller hormoon (AMH) is een hormoon dat bij vrouwen geproduceerd wordt door de eiblaasjes in de eierstokken. AMH speelt tijdens de zwangerschap een belangrijke rol bij de geslachtsontwikkeling van het embryo. In eerste instantie werd gedacht dat AMH geen functie meer had na dit proces. Echter, receptoren waaraan AMH kan binden blijken aanwezig te zijn in verschillende organen. Het is daarom mogelijk dat AMH de relatie tussen reproductieve veroudering en het risico op bovenstaande ziekten verklaart. In dit proefschrift is onderzocht of AMH-concentraties gemeten in het bloed een (oorzakelijk) verband hebben met het risico op kanker, diabetes type 2 en hart- en vaatziekten.

De eerste twee hoofdstukken in dit proefschrift gaan over het verband tussen AMH-concentraties in het bloed en het krijgen van kanker. In **hoofdstuk 2** wordt een systematisch overzicht gegeven van de huidige epidemiologische bevindingen met betrekking tot de relatie tussen AMH-concentraties en het risico op verschillende soorten kanker. In dit overzicht hebben we 12 eerder gepubliceerde studies opgenomen die één van de volgende kankersoorten onderzochten: borst-, eierstok- en baarmoederkanker, lymfomen, niet-gynaecologische tumoren, kanker bij kinderen en prostaatkanker. Vijf van deze studies maten AMH-concentraties jaren voordat de diagnose kanker gesteld werd en de andere zeven studies maten AMH-concentraties na de diagnose, maar voor het begin van de behandeling. Op basis van deze twaalf studies concludeerden we dat er bewijs is voor een relatie tussen hogere AMH-concentraties en het risico op borstkanker, en dat er momenteel weinig bewijs is voor een relatie met andere kankersoorten. Analyses binnen verschillende leeftijdsgroepen suggereerden dat hogere AMH-concentraties in jongere vrouwen mogelijk ook geassocieerd zijn met een hoger risico op eierstok- en baarmoederkanker, hoewel op basis van het kleine aantal vrouwen in deze analyses geen definitieve conclusies getrokken kunnen worden. AMH-concentraties gemeten tussen diagnose en behandeling waren lager in vrouwen die gediagnosticeerd waren met kanker vergeleken met gezonde vrouwen. Echter, omdat de meeste studies die AMH-concentraties maten in de periode tussen diagnose en behandeling een slechte studie-opzet hadden, konden we op basis van deze zeven studies niet tot harde conclusies komen over de relatie tussen kanker en AMH-concentraties gemeten ten tijde van de diagnose.

Omdat er dus nog veel onduidelijkheid is over de relatie tussen AMH en kanker, hebben wij dit verder onderzocht in **hoofdstuk 3.** Eerst onderzochten we de relatie tussen leeftijdsspecifieke AMH-concentraties en het risico op kanker. Daarnaast gebruikten we meerdere AMH metingen per studiedeelnemer, om per persoon te schatten hoe AMH afneemt tijdens het ouder worden. Hoewel is aangetoond dat deze leeftijd-gerelateerde AMH-trajecten verschillen tussen vrouwen, namen voorgaande studies slechts één AMH meting per studiedeelnemer mee. Vanwege deze individuele verschillen in AMH-trajecten, hebben we in **hoofdstuk 3** onderzocht of leeftijd-gerelateerde AMH-trajecten verschilden tussen vrouwen die kanker ontwikkelden en vrouwen die geen kanker ontwikkelden. Voor dit onderzoek gebruikten we gegevens van 3.025 vrouwelijke deelnemers uit de Doetinchem Cohort Studie. AMH-concentraties werden iedere vijf jaar gemeten in het bloed gedurende een periode van 20 jaar, wat resulteerde in een totaal van 11.655 metingen die geanalyseerd konden worden. We onderzochten het verband tussen AMH-concentraties en het ontstaan van alle vormen van kanker samen, maar ook apart voor borstkanker, tumoren in andere weefsels waarin de AMH-receptor AMHR2 aanwezig is, en tumoren in weefsels waarin AMHR2 *niet* aanwezig is. Leeftijdsspecifieke AMH-concentraties gemeten in bloed afgenomen aan het begin van de studie toonden geen verband met deze vier uitkomsten. Omdat AMH-concentraties sterk beginnen te dalen vanaf 40 jaar, en omdat er minder variatie zit in AMH-concentraties vanaf deze leeftijd, onderzochten we de relatie tussen AMH en het ontstaan van kanker ook in de subgroep vrouwen die 40 jaar of jonger was aan het begin van de studie. Analyses binnen deze groep van 1.543 jongere vrouwen bevestigden resultaten van voorgaande studies die aantoonden dat hogere AMH-concentraties geassocieerd waren met een hoger risico op borstkanker. Analyses waarin we leeftijd-gerelateerde AMH-trajecten vergeleken tussen vrouwen die kanker kregen en vrouwen die geen kanker kregen, suggereerden dat AMH-concentraties rond de leeftijd van 30 jaar mogelijk hoger zijn, en daarna mogelijk sneller dalen in vrouwen die gediagnosticeerd worden met kanker. Echter, een daadwerkelijk verschil in leeftijd-gerelateerde AMH-trajecten tussen deze twee groepen kunnen we op basis van onze resultaten niet bewijzen.

Slechts een beperkt aantal studies hebben de relatie tussen AMH-concentraties in het bloed en het risico op hart- en vaatziekten en diabetes onderzocht, en produceerden tegenstrijdige resultaten. Daarnaast maten voorgaande studies AMH-concentraties en deze ziekte-uitkomsten op hetzelfde moment, waardoor het onduidelijk blijft of AMH daadwerkelijk een rol kan spelen in het *ontstaan* van hart- en vaatziekten en diabetes. Daarom onderzochten we de verbanden tussen leeftijdsspecifieke AMH-concentraties aan het begin van de studie, en leeftijd-gerelateerde AMH-trajecten, en het risico op diabetes type 2 met behulp van gegevens van de Doetinchem Cohort Studie. We gebruikten hiervoor dezelfde aanpak als beschreven voor hoofdstuk 3. De resultaten van deze analyses zijn beschreven in **hoofdstuk 4**. In de

totale groep van 3.293 vrouwen ontwikkelden er 163 vrouwen diabetes type 2, gedurende een periode van 20 jaar. Vrouwen met lagere leeftijdsspecifieke AMH-concentraties aan het begin van de studie hadden een hoger risico op het krijgen van diabetes type 2. Er was geen duidelijk verschil in leeftijd-gerelateerde AMH-trajecten te zien tussen vrouwen die diabetes type 2 ontwikkelden en vrouwen die geen diabetes ontwikkelden. Hoewel onze resultaten suggereerden dat er een relatie tussen AMH-concentraties en diabetes type 2 is, moet toekomstig onderzoek uitwijzen of AMH daadwerkelijk een oorzakelijke rol speelt in het ontstaan van diabetes.

Het verband tussen AMH-concentraties en het risico op hart- en vaatziekten en diabetes is met name in vrouwen onderzocht. Echter, omdat AMH ook meetbaar is bij mannen, is het mogelijk dat AMH een rol zou kunnen spelen in het ontstaan van hart- en vaatziekten bij zowel mannen als vrouwen. Om deze reden hebben we in **hoofdstuk 5** onderzocht of er een verband is tussen AMH-concentraties in het bloed en een beginnend stadium van hart- en vaatziekten in Nederlandse middelbare en oudere mannen. We gebruikten gegevens van 394 studiedeelnemers om de relaties tussen AMH en slagadervernauwing, vaatstijfheid, diameter van de buikslagader en het 10-jaars risico op coronaire hartziekten, alle gemeten aan het begin van de studie, te onderzoeken. Daarnaast onderzochten we de relatie tussen AMH-concentraties gemeten in bloed afgenomen aan het begin van de studie en slagadervernauwing, slagaderverkalking, vaatstijfheid en 10-jaars risico op coronaire hartziekte gemeten na ruim 8,5 jaar. Onze resultaten suggereerden dat hogere AMH-concentraties geassocieerd waren met een mindere mate van slagadervernauwing aan het begin van de studie. Verder waren hogere AMH-concentraties geassocieerd met minder slagaderverkalking na ruim 8,5 jaar, onafhankelijk van de mate van slagadervernauwing aan het begin van de studie. Hoewel hogere AMH-concentraties ook verband hielden met een lager 10-jaars risico op coronaire hartziekte, verdween dit verband na correctie voor geslachtshormonen. AMH-concentraties waren niet geassocieerd met diameter van de buikslagader en vaatstijfheid gemeten aan het begin van de studie, en ook niet met vaatstijfheid en 10-jaars risico op coronaire hartziekte na ruim 8,5 jaar. Kortom, er is mogelijk wel een verband tussen AMH-concentraties en veranderingen in de structuur van de vaatwand, maar onze resultaten duiden niet op een verband met veranderingen in de functie van de vaatwand.

Een vraag die onbeantwoord bleef na afronding van bovenstaande studies was, of AMH daadwerkelijk een oorzakelijke rol speelt bij het ontstaan van de onderzochte ziekte-uitkomsten. Het vergaren van meer kennis over de genetische variatie en biologische mechanismen die ten grondslag liggen aan de variatie in AMH-concentraties tussen personen, kan meer inzicht opleveren in de mogelijke processen waardoor AMH betrokken is bij het ontstaan van kanker, hart- en vaatziekten en diabetes type 2. Bovendien kunnen genetische varianten die geassocieerd zijn met AMH-concentraties gebruikt worden om effecten van AMH op ziekte-

uitkomsten te besturen met behulp van Mendeliaanse randomisatie analyses. Om dergelijke genetische varianten voor AMH-concentraties te vinden, hebben we in **hoofdstuk 6** een genoomwijde associatie studie (GWAS) uitgevoerd. Voor deze studie gebruikten we gegevens van zeven studies, en in totaal van 7.049 premenopauzale vrouwen van Europese afkomst; meer dan twee keer zoveel als in de vorige AMH GWAS. We vonden vier posities op het DNA die samenhingen met AMH-concentraties: het reeds bekende signaal in het *MCM8* gen, en drie nieuwe signalen in of in buurt van de *AMH, TEX41* en *CDCA7* genen. Het sterkste signaal was een genetische variant in het *AMH* gen (rs10417628). Van de genen die mogelijk de andere drie signalen veroorzaakten, waren de meeste betrokken in celcyclus processen, zoals celdood. Verder vonden we een sterke positieve correlatie tussen genetische varianten voor AMH-concentraties en genetische varianten voor menopauzeleeftijd. Verkennende Mendeliaanse randomisatie analyses duidden niet op een oorzakelijk effect van AMH op het risico op borstkanker of polycysteus ovarium syndroom. Echter, deze resultaten moet voorzichtig geïnterpreteerd worden vanwege de mogelijk beperkte statistische power om een echt effect aan te kunnen tonen, en vanwege de beperkte mogelijkheid om validiteit van de analyses te garanderen. Kortom, we hebben een genetische variant in het *AMH* gen en drie andere genetische varianten voor AMH-concentraties in vrouwen ontdekt.

In **hoofdstuk 7** hebben we de vier genetische varianten uit hoofdstuk 6 gebruikt om, met behulp van Mendeliaanse randomisatie analyses, te verkennen of AMH-concentraties mogelijk oorzakelijk geassocieerd zijn met hart- en vaatziekten en diabetes type 2. Voor deze analyses hebben we gegevens van de UK Biobank, the Stroke Genetics Network en DIAMANTE studies gebruikt om het effect van AMH op coronaire hartziekten, ischemische beroerte en diabetes type 2, respectievelijk, te onderzoeken. We vonden geen bewijs voor een oorzakelijk effect van AMH-concentraties op deze drie ziekte-uitkomsten bij vrouwen. Onze resultaten op basis van gegevens van de UK Biobank suggereerden wel dat genetische varianten voor hogere AMH-concentraties geassocieerd waren met een hogere menopauzeleeftijd, en met een latere leeftijd waarop de eerste menstruatie plaatsvond, maar niet met factoren die het risico op hart- en vaatziekten en diabetes beïnvloeden, zoals slagadervernauwing, HbA1c en glucose.

In **hoofdstuk 8** bespreken we de voornaamste uitdagingen met betrekking tot de interpretatie van de resultaten beschreven in hoofdstuk 2 tot en met 7. Daarnaast doen we aanbevelingen voor toekomstig onderzoek naar de rol van AMH bij het ontstaan van kanker en hart- en vaatziekten. Wat ons betreft, moet toekomstig epidemiologisch onderzoek hand in hand gaan met fundamenteel onderzoek om te achterhalen of AMH inderdaad een effect heeft op het ontstaan van deze ziekten. De bevindingen beschreven in dit proefschrift leggen een waardevolle wetenschappelijke fundering voor dergelijk onderzoek, maar vormen opzichzelfstaand geen doorslaggevend bewijs voor, óf tegen, een oorzakelijk verband tussen AMH en het ontstaan van verschillende soorten kanker en hart- en vaatziekten en diabetes.

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Curriculum vitae

Renée M.G. Verdiesen was born November 3rd, 1990, in Oss, the Netherlands. She graduated in 2009 from the Ubbo Emmius in Stadskanaal, after which she started studying Art History at the University of Groningen. After two years, Renée decided to switch to the Bachelor's programme Health and Life sciences at the Vrije Universiteit Amsterdam. In 2014, she started her Master in Epidemiology at Utrecht University, during which she specialized in Clinical Epidemiology. For her research project, Renée studied the causal relation between metabolic syndrome and mammographic density using a Mendelian randomization approach, under supervision of dr. Carla van Gils and dr. Charlotte Onland-Moret, at the Julius Center for Primary Care and Health Sciences, University Medical Center Utrecht. As part of the Honours Track of the Master Epidemiology, she wrote the PhD proposal entitled 'The role of anti-Müllerian hormone (AMH) in the development of cancer, cardiovascular disease and type 2 diabetes', which was awarded funding through the NWO graduate programme. After completing her Master in Epidemiology in 2016, she started with this PhD project under supervision of prof. dr. ir. Yvonne van der Schouw, prof. dr. Carla van Gils, and dr. Charlotte Onland-Moret, which resulted in the research papers that comprise this thesis. In June 2020, Renée joined the group of prof. dr. ir. Marjanka Schmidt at the Netherlands Cancer Institute (NKI), Amsterdam, to focus on the genetic epidemiology of breast cancer (subtypes).

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