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ORIGINAL ARTICLE ***Reproductive endocrinology***

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

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STUDY QUESTION: Is there a relationship between serum anti-Müllerian hormone (AMH) level and cardiovascular disease (CVD) risk in premenopausal women?

SUMMARY ANSWER: There are indications that premenopausal women with very low ovarian reserve may have an unfavorable CVD risk profile.

WHAT IS KNOWN ALREADY: Age at menopause is frequently linked to CVD occurrence. AMH is produced by ovarian antral follicles and provides a measure of remaining ovarian reserve Literature on whether AMH is related to CVD risk is still scarce and heterogeneous.

STUDY DESIGN, SIZE, DURATION: Cross-sectional study in 2338 women (age range of 20–57 years) from the general population, participating in the Doetinchem Cohort Study between 1993 and 1997.

PARTICIPANTS/MATERIALS, SETTING, METHODS: CVD risk was compared between 2338 premenopausal women in different AMH level-categories, with adjustment for confounders. CVD risk was assessed through levels of systolic and diastolic blood pressure, total cholesterol, high-density lipoprotein cholesterol and glucose, in addition to a summed score of CVD risk factors. Among other factors, analyses were corrected for smoking, oral contraceptive use and BMI.

MAIN RESULTS AND THE ROLE OF CHANCE: The relationship of serum AMH levels with CVD risk factor outcomes was nonlinear. Women with AMH levels <0.16 μg/l had 0.11 (95% confidence intervals (CIs) 0.01; 0.21) more metabolic risk factors compared with women with AMH levels ≥ 0.16 μg/l. There was no association of individual risk factor levels with AMH levels, besides a tendency towards lower total cholesterol levels of 0.11 mmol/l (95% CI −0.23; 0.01) in women with AMH levels <0.002 μg/l compared with women with AMH levels ≥ 0.16 μg/l. Although not statistically significant, these effect sizes were larger in women below 40 years of age.

LIMITATIONS, REASONS FOR CAUTION: Causality and temporality of the studied association cannot be addressed here. Moreover, the clinical and statistical significance of the results of this exploratory study should be interpreted with caution due to the absence of adjustment for multiple statistical testing.

WIDER IMPLICATIONS OF THE FINDINGS: This population-based study supports previous findings that premenopausal women with very low AMH levels may have an increased CVD risk. It lays the groundwork for future research to focus on this group of women. Longitudinal studies with more sensitive AMH assays may furthermore help better understand the implications of these results.

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Key words: ovarian reserve / anti-Müllerian hormone / cardiovascular disease / cardiovascular risk

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Introduction

Female reproductive lifespan is characterized by a gradual decrease of follicle quantity and quality, ultimately leading to menopause (Broekmans *et al.*, 2009). Anti-Müllerian hormone (AMH) is produced by ovarian antral follicles and its concentration in peripheral blood is a quantitative estimation of the size of the antral follicle pool, thereby providing a measure of ovarian reserve before the end of a woman's reproductive life (Hansen *et al.*, 2011). AMH has furthermore proved capable to predict individual time to menopause (Sowers *et al.*, 2008; Broer *et al.*, 2011; Tehrani *et al.*, 2013; Dolleman *et al.*, 2015).

Age at the menopause and postmenopausal states is considered to be risk factor for cardiovascular disease (CVD) occurrence, independently of chronological aging (van der Schouw *et al.*, 1996; Atsma *et al.*, 2006; Ebong *et al.*, 2014). A decrease of total cholesterol (TC) levels and relative weight were previously associated with a later age at menopause, suggesting a potential influence of CVD risk on ovarian aging (Kok *et al.*, 2006). In addition, the finding of AMH-receptor-specific mRNA in the human heart (Ricci *et al.*, 2010) suggests a direct linkage between AMH and cardiovascular physiology. To date, it is still debated whether AMH, either as a proxy variable for ovarian reserve or through direct mechanistic effects, is related to risk factors of CVD. A report in nonhuman primates (Appt *et al.*, 2012) and one study in humans (Tehrani *et al.*, 2014) provide evidence for the presence of a relationship, while others do not (Anderson *et al.*, 2013; Bleil *et al.*, 2013). The available studies used different outcomes for CVD risk, as well as varying selection criteria for their study populations, limiting their comparability. In addition, important confounders such as oral contraceptive (OC) use or smoking were dealt with differently, or not at all. In this study, we therefore aimed to provide a generalizable assessment of the association of AMH level with CVD risk, by investigating AMH levels in relation to CVD risk factors in a large population-based cohort of premenopausal women.

Materials and Methods

Study design

We performed a cross-sectional study within the second round of the Doetinchem Cohort Study (Verschuren *et al.*, 2008). The determinant of the studied association was AMH level, with CVD risk factors as the outcome.

Study population

The study population consisted of women enrolled in the Doetinchem Cohort Study. The cohort and study design were previously described in detail by Verschuren *et al.* (2008). The population-based cohort originated from an age- and gender-stratified sample of individuals from municipal registers of Doetinchem, Amsterdam and Maastricht in 1987. A random fraction ($n = 7769$) of the Doetinchem sample was subsequently invited for follow-up every 5 years, forming the Doetinchem Cohort, with the general aim of studying chronic disease risk factors (Verschuren *et al.*, 2008). At each follow-up round, lifestyle determinants, reproductive characteristics and aspects of general health were assessed through questionnaires, and biometric and laboratory measurements were performed. Written informed consent was given by all participants and ethical approval was granted by the Medical Ethics Committee of the Netherlands Organization of Applied Scientific Research.

For the current study, we included premenopausal women who participated in the second follow-up round between 1993 and 1997. Women were considered to be premenopausal if they reported having had one or more menstruations in the past year, the date of their reported last menstruation was <12 months ago, or if they were pregnant at the time of follow-up. Of the 3947 eligible women, those who were postmenopausal or reported having undergone surgery on one or both ovaries were excluded ($n = 1183$). Women who did not give informed consent for the use of their stored material for research purposes or had no available stored serum were additionally excluded ($n = 296$), as were women from whom information on reproductive status was missing ($n = 61$) or unclear (due to contradictory answers in the questionnaire) ($n = 23$). After exclusion of 46 participants from whom insufficient serum was available for AMH measurement, a population of 2338 women was eligible for inclusion. Figure 1 provides a flow chart depiction of participant selection.

AMH assessment

Nonfasting blood withdrawal occurred on a random day of the menstrual cycle, after which additional material was stored at -80°C for subsequent use. AMH was measured in stored serum samples of the participants of around two in 2011 using a Gen-II ELISA assay (Beckman-Coulter, Sinsheim, Germany) in a single laboratory (Dolleman *et al.*, 2013). The assay precision was validated with linearity-of-dilution assessment. The limit of detection was 0.08 μg/l and the limit of quantification 0.16 μg/l. The inter- and intra-assay coefficients of variation were 3.35 and 4.0%, respectively (Dolleman *et al.*, 2013). Measures with values below 0.16 μg/ml were considered to

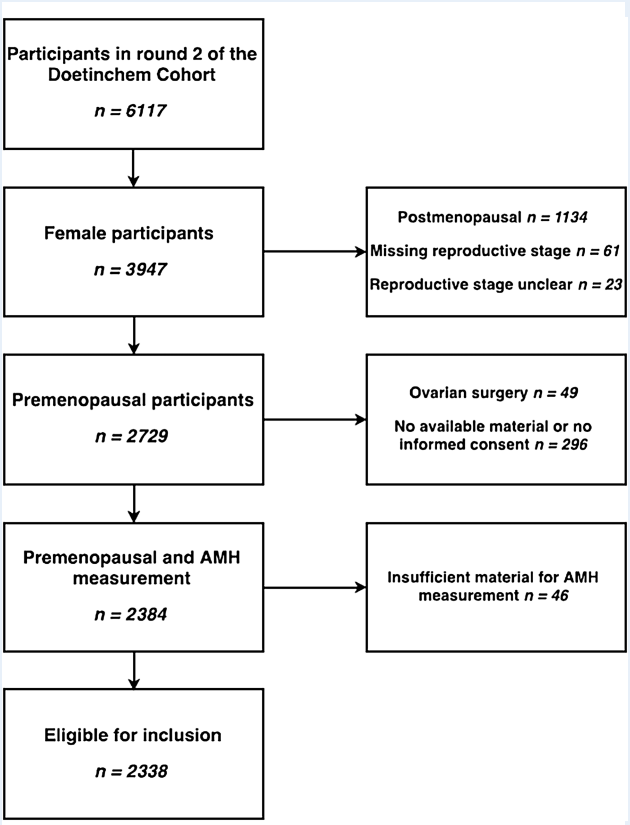

Figure 1 Flow chart of participant selection in the study of AMH and cardiovascular disease risk. AMH, anti-Müllerian hormone.

represent AMH below the limit of quantification. AMH levels were above zero and below the limit of quantification in 637 (27%) women, and in 456 (72%) of these cases the measured level was zero.

CVD risk assessment

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

SBP and DBP levels were measured twice in the supine position on the left arm, using a random zero sphygmomanometer, from which the mean value was used. Height and weight were measured by trained staff, as well as waist and hip circumferences. Directly performed laboratory measurements included nonfasting TC, HDL-c and glucose (Lipid Reference Laboratory, University Hospital Dijkzigt, Rotterdam, The Netherlands).

A summed risk factor score for CVD was estimated, henceforth referred to as 'metabolic risk score', similar to the methods described by Bleil *et al.* (2013). Risk factors were dichotomized for separate components as follows: waist circumference $\geq$80 cm (yes/no), hypertension (SBP or DBP $\geq$130 or $\geq$85 mmHg, respectively) and/or the use of antihypertensive medication (yes/no), HDL-c $<$1.1 mmol/l and/or the use of lipid-lowering drugs (yes/no), TC $>$5.6 mmol/l and/or the use of lipid-lowering drugs (yes/no) and nonfasting glucose $>$11.1 mmol/l and/or diabetes diagnosis (yes/no). The total number of risk factors present (0–5) was subsequently used as the metabolic risk score.

Assessment of potential confounders and effect modifiers

Potential confounders of the studied association were considered to be age, current OC use, current smoking status, BMI, parity, cycle regularity, socioeconomic status (SES), estrogen use at the time of follow-up and pregnancy at the time of follow-up. All factors, with the exception of BMI and SES, were associated with AMH levels in a previous cross-sectional study in this population (Dolleman *et al.*, 2013). A study from India found an association between AMH levels and SES (Surekha *et al.*, 2013), and obesity was previously associated with time to menopause (Sowers *et al.*, 2010). The factors described here were also hypothesized to be associated with CVD risk factors. The presence of polycystic ovary syndrome (PCOS) was considered to be a potential effect modifier. The likely presence of PCOS was identified by a reported irregular menstrual cycle in combination with a measured AMH level above 4.7 μg/l, based on a cut-off value proposed as a result of a meta-analysis by Iliodromiti *et al.* (2013).

Statistical analysis

In our study population, there were 2182 (93%) complete cases and the proportion of missing data of all variables did not exceed 2% per variable. Conditional multiple imputation, including determinant, outcome and confounder variables, with 10 iterations was performed in order to account for missing data and a sensitivity analysis was performed with only the complete cases.

The association of AMH with age, CVD risk factors with age and AMH with CVD risk factors was firstly visualized. The relationship of logarithmically transformed AMH with age appeared to be quadratic (Supplementary data, Fig. S1); the relationship of the CVD risk factors with age appeared to be linear or quadratic (Supplementary data, Fig. S2); and the relationship of AMH with CVD risk factors was nonlinear in most cases (Supplementary data, Fig. S3). For this reason, participants were divided into categories based on their AMH level, rather than studying AMH as a continuous parameter.

Participants were divided into the following categories based on their AMH levels as follows: AMH = 0.000 μg/l (Category 1; n = 456); AMH levels measured above zero but beneath the quantification limit of 0.16 μg/l (Category 2; n = 186); quartiles of AMH equal to or above the quantification limit (Categories 3–6; n = 424 in each quartile). The range of AMH cut-off levels was 0.161–0.643, 0.644–1.336, 1.337–2.395 and 2.398–13.67 μg/l for Categories 3–6, respectively. As the laboratory returned both AMH levels below the limit of quantification as well as values of zero, it was decided to distinguish these two groups as separate entities. However, this was done bearing in mind that the standard error of the measured AMH levels below 0.16 μg/l is larger than that of levels above this limit, rendering the former values less reliable. Supplementary data, Fig. S3 depicts the AMH category cut-off values in the plots of AMH and CVD risk factor levels.

The association of AMH level categories with CVD risk factors was studied using an analysis of variance (ANOVA) regression based on the least sums of squares, with Category 1 as the reference category. All women with AMH levels $\geq$0.16 μg/l (in Categories 3–6) were additionally pooled in a group and compared with women in Category 1. The crude models included the abovementioned AMH categories as independent dummy variables. In Model 2, age was added as a confounder. In Model 3, age^2 was also added, due to a quadratic relationship of age with some of the outcome parameters. In Model 4, current OC use (yes/no), current smoking status (smoker/nonsmoker), parity, cycle regularity (regular/nonregular), current estrogen use besides OC (yes/no), current pregnancy (yes/no), SES and BMI were added as confounders.

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

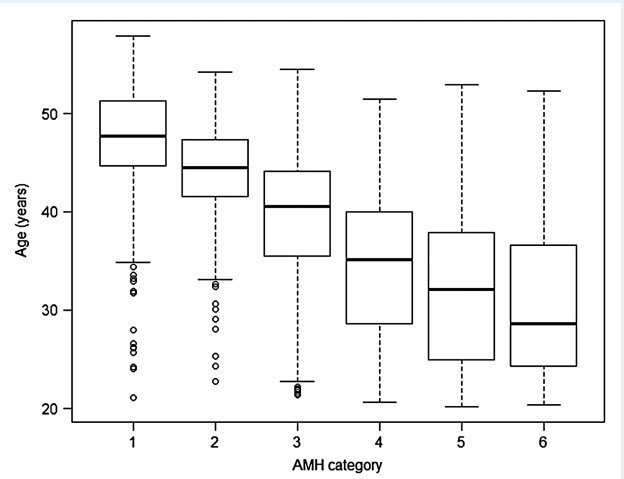

Figure 2 Boxplots of age for each AMH category. Boxes represent the median (bold horizontal line) and interquartile range (IQR) of the data. Whiskers represent the 1st quartile − 1.5 × IQR and the 3rd quartile + 1.5 × IQR. Circles represent all measured levels outside the range of the whiskers. Category 1 consists of 456 women; Category 2 of 186 women and Categories 3–6 each 424 women.

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

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

Results

Baseline characteristics

The age range of all women in the study population was 20–57 years. Women in higher AMH categories were increasingly younger compared with women in lower AMH categories, but the overall age ranges were similar in all categories (Fig. 2). BMI appeared to decrease across the six categories (Table I). The number of current and ever smokers decreased with increasing AMH quartiles, as did the number of children per participant. No clear pattern over the AMH categories was observed for SES, diabetes prevalence and cycle regularity. Women with AMH <0.16 μg/l (in Categories 1 and 2) had fewer current pregnancies and used OC less frequently than women with AMH levels ≥ 0.16 μg/l (Categories 3–6). Differences in baseline characteristics were not tested for significance.

Multivariable analyses

Tables II and III list the multivariable model summaries of the regression analyses with all outcome parameters. For all outcomes, adjustment for age and age^2 led to the largest attenuation of the differences in outcome parameters between women in the six AMH categories. Mean adjusted SBP, DBP, TC and glucose levels and the number of metabolic risk factors were nonsignificantly lower or equal in all AMH categories, compared with Category 1. On average, TC levels were 0.17 mmol/l (95% confidence intervals (CIs) −0.30; −0.04, $P = 0.01$) lower in Category 3, and 0.11 mmol/l (95% CI −0.23; 0.01, $P = 0.08$) lower in Categories 3–6 compared with Category 1. Women in Categories 3–6 had an average lower metabolic risk score of 0.11 (95% CI −0.21, −0.01; $P = 0.02$), thus 0.11 fewer metabolic risk factors, than women in

Table I Baseline and outcome characteristics for study participants by anti-Müllerian hormone (AMH) category.

	Category 1 ($n = 456$)	Category 2 ($n = 186$)	Category 3 ($n = 424$)	Category 4 ($n = 424$)	Category 5 ($n = 424$)	Category 6 ($n = 424$)
AMH range (μg/l)	0.000	0.002–0.158	0.161–0.643	0.644–1.336	1.337–2.395	2.398–13.67
Baseline parameters						
Age (years)	47.3 ± 5.5	43.8 ± 5.5	39.2 ± 7.0	34.8 ± 7.4	31.7 ± 7.5	30.2 ± 6.9
Current smoker	149 (23.7)	72 (38.7)	129 (30.4)	146 (34.4)	125 (29.5)	125 (29.4)
Ever smoker	308 (67.5)	132 (71.0)	269 (63.4)	274 (64.6)	236 (55.7)	232 (54.7)
Pack years of smoking	9.4 ± 11	8.9 ± 9.9	6.6 ± 8.4	6.7 ± 8.7	4.0 ± 6.1	3.6 ± 5.9
BMI (kg/m^2)	25.7 ± 3.8	24.7 ± 3.8	24.7 ± 4.0	24.6 ± 4.1	24.1 ± 3.7	24.0 ± 4.0
Number of children	1.9 ± 1.0	1.9 ± 1.0	1.7 ± 1.1	1.3 ± 1.2	1.1 ± 1.2	1.0 ± 1.2
SES 1	292 (64.4)	113 (60.8)	139 (56.5)	226 (53.4)	181 (43.1)	176 (51.9)
SES 2	91 (20.1)	44 (23.7)	107 (25.3)	135 (31.9)	172 (41.0)	196 (46.2)
SES 3	70 (15.4)	29 (15.6)	77 (18.2)	62 (14.7)	67 (15.9)	52 (12.2)
Current OC use	110 (24.2)	56 (30.3)	150 (35.4)	185 (43.7)	217 (51.1)	196 (46.3)
Current E_2 use[a]	17 (0.7)	2 (0.1)	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
Regular cycle	360 (80.5)	148 (81.3)	338 (80.9)	336 (81.3)	316 (75.6)	281 (67.3)
Current pregnancy	1 (0.04)	3 (0.01)	14 (3.3)	14 (3.3)	10 (2.3)	7 (1.2)
Diabetes mellitus	3 (0.7)	1 (0.5)	1 (0.2)	1 (0.2)	2 (0.5)	3 (0.7)
Outcome parameters						
SBP (mmHg)	122 [112; 134]	119 [110; 130]	115 [108; 125]	114 [107; 123]	114 [105; 121]	112 [106; 121]
DBP (mmHg)	79 [72; 85]	77 [71; 83]	75 [69; 82]	74 [68; 81]	73 [67; 79]	74 [68; 80]
TC (mmol/l)	5.45 [4.89; 6.05]	5.21 [4.65; 5.78]	4.97 [4.44; 5.60]	4.98 [4.43; 5.60]	4.79 [4.34; 5.38]	4.83 [4.31; 5.43]
HDL-c (mmol/l)	1.52 [1.29; 1.79]	1.52 [1.29; 1.78]	1.55 [0.66; 1.75]	1.45 [0.67; 1.70]	1.51 [0.81; 1.75]	1.50 [0.66; 1.78]
Glucose (mmol/l)[b]	5.2 [4.8; 5.6]	5.0 [4.6; 5.5]	4.9 [4.6; 5.4]	4.9 [4.5; 5.2]	4.8 [4.4; 5.2]	4.9 [4.4; 5.2]
Metabolic risk score	1 [0; 2]	1 [0; 1]	0 [0; 1]	0 [0; 1]	0 [0; 1]	0 [0; 1]

Values are presented as *n* (%) for categorical variables and mean ± SD or median [IQR] for continuous variables.
SES, socio-economic status; OC, oral contraceptive; E_2, estradiol; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-c, high-density lipid cholesterol; SES 1, completed primary school or lowest level of secondary education; SES 2, completed middle level of secondary education or first 3 years of highest high school education level; SES 3, completed highest form of secondary school or any university degree.
[a]Estrogen use besides OC.
[b]Nonfasting.

Table II Multivariable model estimates of mean differences in single risk factor levels for AMH categories, compared with Category 1.

		Category 1 (n = 456)	Category 2 (n = 186)	Category 3 (n = 424)	Category 4 (n = 424)	Category 5 (n = 424)	Category 6 (n = 424)	Categories 3–6 (n = 1696)
	AMH range (μg/l)	0.000	0.002–0.158	0.161–0.643	0.644–1.336	1.337–2.395	2.398–13.67	0.162–13.67
	Difference (95% CI) with reference category in Systolic blood pressure (mmHg)							
1	Crude model	Ref	**−3.8 (−6.2; −1.5)**	**−7.2 (−9.0; −5.4)**	**−8.3 (−10.1; −6.5)**	**−9.9 (−11.7; −8.1)**	**−10.8 (−12.6; −9.0)**	**−9.0 (−10.5; −7.6)**
2	+ age	Ref	**−2.7 (−5.0; −0.4)**	**−4.6 (−6.5; −2.7)**	**−4.2 (−6.3; −2.2)**	**−4.8 (−7.0; −2.7)**	**−5.2 (−7.5; −3.0)**	**−4.6 (−6.3; −2.9)**
3	+ age, age^2	Ref	−0.9 (−3.2; 1.5)	−1.8 (−3.9; 0.2)	−1.2 (−3.4; 1.0)	−2.3 (−4.5; 0.0)	**−2.8 (−5.1; −0.4)**	−1.9 (−3.7; 0.0)
4	Fully adjusted	Ref	−0.3 (−2.6; 2.0)	−1.2 (−3.1; 0.8)	−0.6 (−2.7; 1.5)	−1.1 (−3.3; 1.1)	−1.2 (−3.5; 1.1)	−1.0 (−2.8; 0.8)
	Difference (95% CI) with reference category in diastolic blood pressure (mmHg)							
1	Crude model	Ref	**−1.9 (−3.6; −0.2)**	**−3.3 (−4.6; −2.0)**	**−4.7 (−6.0; −3.4)**	**−6.1 (−7.4; −4.8)**	**−5.2 (−6.5; −3.9)**	**−4.8 (−5.9; −3.8)**
2	+ age	Ref	−1.0 (−2.7; 0.7)	−1.3 (−2.7; 0.1)	**−1.5 (−3.0; −0.0)**	**−2.2 (−3.8; −0.6)**	−0.9 (−2.5; 0.7)	**−1.4 (−2.7; −0.2)**
3	+ age, age^2	Ref	−0.8 (−2.5; 0.9)	−1.0 (−2.5; 0.5)	−1.2 (−2.8; 0.4)	**−1.9 (−3.6; −0.2)**	−0.6 (−2.3; 1.1)	−1.1 (−2.5; 0.2)
4	Fully adjusted	Ref	−0.3 (−2.0; 1.3)	−0.6 (−2.1; 0.8)	−0.9 (−2.4; 0.7)	−1.5 (−3.1; 0.2)	0.0 (−1.6; 1.7)	−0.8 (−2.1; 0.6)
	Difference (95% CI) with reference category in Total cholesterol (mmol/l)							
1	Crude model	Ref	**−0.26 (−0.41; −0.11)**	**−0.47 (−0.59; −0.35)**	**−0.44 (−0.56; −0.33)**	**−0.60 (−0.71; −0.48)**	**−0.59 (−0.70; −0.47)**	**−0.52 (−0.62; −0.43)**
2	+ age	Ref	**−0.17 (−0.32; −0.02)**	**−0.28 (−0.40; −0.16)**	**−0.15 (−0.28; −0.01)**	**−0.23 (−0.37; −0.09)**	**−0.18 (−0.33; −0.04)**	**−0.22 (−0.33; −0.11)**
3	+ age, age^2	Ref	−0.12 (−0.27; 0.03)	**−0.20 (−0.33; −0.07)**	−0.06 (−0.21; 0.08)	**−0.16 (−0.30; −0.01)**	−0.11 (−0.27; 0.04)	**−0.15 (−0.27; −0.03)**
4	Fully adjusted	Ref	−0.10 (−0.25; 0.01)	**−0.17 (−0.30; −0.04)**	−0.03 (−0.17; 0.10)	−0.10 (−0.24; 0.01)	−0.03 (−0.18; 0.13)	−0.11 (−0.23; 0.01)
	Difference (95% CI) with reference category in HDL-cholesterol (mmol/l)							
1	Crude model	Ref	−0.02 (−0.08; 0.04)	−0.01 (−0.06; 0.04)	**−0.08 (−0.13; −0.04)**	−0.03 (−0.08; 0.01)	−0.02 (−0.06; 0.03)	−0.04 (−0.08; 0.04)
2	+ age	Ref	−0.01 (−0.08; 0.05)	−0.00 (−0.05; 0.05)	**−0.07 (−0.13; −0.02)**	−0.02 (−0.08; 0.04)	0.00 (−0.06; 0.06)	−0.02 (−0.07; 0.02)
3	+ age, age^2	Ref	−0.00 (−0.06; 0.06)	0.02 (−0.04; 0.07)	−0.05 (−1.11; 0.01)	0.00 (−0.06; 0.06)	0.02 (−0.05; 0.08)	−0.00 (−0.05; 0.04)
4	Fully adjusted	Ref	−0.01 (−0.07; 0.05)	0.00 (−0.05; 0.05)	−0.05 (−0.11; 0.01)	−0.01 (−0.07; 0.05)	0.02 (−0.04; 0.01)	−0.01 (−0.06; 0.04)
	Difference (95% CI) with reference category in glucose (mmol/l)							
1	Crude model	Ref	−0.11 (−0.28; 0.06)	**−0.26 (−0.39; −0.13)**	**−0.28 (−0.41; −0.15)**	**−0.44 (−0.57; −0.31)**	**−0.41 (−0.54; −0.28)**	**−0.35 (−0.45; −0.25)**
2	+ age	Ref	−0.04 (−0.21; 0.13)	−0.10 (−0.24; 0.04)	−0.03 (−0.18; 0.11)	−0.13 (−0.29; 0.03)	−0.07 (−0.24; 0.09)	−0.08 (−0.21; 0.04)
3	+ age, age^2	Ref	−0.03 (−0.20; 0.15)	−0.08 (−0.23; 0.07)	−0.01 (−0.17; 0.15)	−0.11 (−0.28; 0.06)	−0.05 (−0.23; 0.12)	−0.07 (−0.20; 0.07)
4	Fully adjusted	Ref	−0.01 (−0.19; 0.16)	−0.07 (−0.21; 0.08)	−0.00 (−0.16; 0.16)	−0.08 (−0.25; −0.08)	−0.01 (−0.18; 0.16)	−0.05 (−0.18; 0.09)

Estimated model coefficients (95% confidence interval) indicate average difference of single risk factor levels in the respective AMH categories compared with women in Category 1. (For example: women in Category 3 had an average lower TC level of 0.17 mmol/l compared with women in Category 1 after correction for confounders.) Coefficients in bold are statistically significant ($P < 0.05$).

Model 1 was a crude model; Model 2 was adjusted for age; Model 3 was adjusted for age and age^2; Model 4 was adjusted for age, age^2, current OC use, current smoking status, parity, cycle regularity, current estrogen use besides OC, current pregnancy and BMI.

Table III Multivariable model estimates of mean differences in metabolic risk score for AMH categories, compared with Category 1.

		Category 1 (*n* = 456)	Category 2 (*n* = 186)	Category 3 (*n* = 424)	Category 4 (*n* = 424)	Category 5 (*n* = 424)	Category 6 (*n* = 424)	Categories 3–6 (*n* = 1696)
AMH range (μg/l)		0.000	0.002–0.158	0.161–0.643	0.644–1.336	1.337–2.395	2.398–13.67	0.162–13.67
Difference (95% CI) with reference category in metabolic risk score								
1	Crude model	Ref	**−0.19 (−0.33; −0.04)**	**−0.40 (−0.51; −0.29)**	**−0.37 (−0.49; −0.26)**	**−0.51 (−0.62; −0.40)**	**−0.53 (−0.64; −0.42)**	**−0.46 (−0.54; −0.37)**
2	+ age	Ref	−0.11 (−0.25; 0.03)	**−0.23 (−0.35; −0.11)**	−0.11 (−0.24; 0.02)	**−0.18 (−0.32; −0.05)**	**−0.17 (−0.31; −0.03)**	**−0.18 (−0.29; −0.08)**
3	+ age, age^2	Ref	−0.09 (−0.24; 0.05)	**−0.20 (−0.33; −0.07)**	−0.07 (−0.22; 0.06)	**−0.15 (−0.30; −0.01)**	−0.15 (−0.30; 0.00)	**−0.16 (−0.27; −0.04)**
4	Fully adjusted	Ref	−0.01 (−0.13; 0.11)	**−0.15 (−0.26; −0.04)**	−0.05 (−0.17; 0.06)	−0.09 (−0.21; 0.03)	−0.08 (−0.21; 0.04)	**−0.11 (−0.21; −0.01)**

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

Category 1. This effect size was equal when all women with AMH levels of 0.16 μg/l and higher (Categories 3–6) were compared with all women with values below this cut-off point (Categories 1 and 2), with a *P*-value of 0.01.

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

Sensitivity analyses

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

Discussion

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

Our results are in line with the findings from a study in 1015 regularly cycling Iranian women, in which changes of TC and low-density lipoprotein (LDL)-c over a follow-up time up to 12 years were more unfavorable for women in the lowest baseline age-specific AMH quartile compared with the highest quartile (Tehrani *et al.*, 2014). Additionally, two cross-sectional studies found young, regularly cycling women with lower ovarian reserve, based on cut-off points of antral follicle counts or FSH levels, to have a more unfavorable lipid status than women with normal ovarian reserve (Chu *et al.*, 2003; Verit *et al.*, 2014). The mean age in both these studies was below 40 years, likening these results to those observed in our population below 40 years of age. The unfavorable consequences of reproductive aging could thus be more evident in women in whom chronological aging has still had less of an effect on CVD risk. Alternatively, it is possible this is a group of women with a

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

	Category 1	Category 2	Category 3	Category 4	Category 5	Category 6	Category 3–6
AMH range (μg/l)	0.000	0.002–0.158	0.161–0.643	0.644–1.336	1.337–2.395	2.398–13.67	0.162–13.67
Ages 20–29 years ($n = 609$), 2% AMH levels below 0.16 μg/l (Categories 1 and 2)							
Systolic blood pressure	Ref	−6.2 (−18.4; 6.1)	−0.4 (−8.5; 7.8)	−0.8 (−8.6; 7.0)	−0.1 (−7.9; 7.6)	−2.5 (−1.0; 5.2)	−1.1 (−8.8; 6.5)
Diastolic blood pressure	Ref	−5.1 (−14.5; 4.4)	−2.9 (−9.1; 3.4)	−4.6 (−10.6; 1.4)	−5.1 (−11.0; 0.89)	−3.7 (−9.7; 2.2)	−4.3 (−10.2; 1.6)
Total cholesterol	Ref	0.06 (−0.83; 0.93)	−0.38 (−0.96; 0.21)	−0.09 (−0.65; 0.74)	−0.23 (−0.78; 0.33)	−0.15 (−0.71; 0.40)	−0.18 (−0.73; 0.37)
HDL-cholesterol	Ref	0.22 (−0.16; 0.60)	0.06 (−0.20; 0.31)	0.02 (−0.23; 0.26)	0.02 (−0.22; 0.26)	0.02 (−0.22; 0.26)	0.02 (−0.22; 0.26)
Glucose	Ref	−0.12 (−0.93; 0.69)	−0.01 (−0.55; 0.52)	−0.13 (−0.64; 0.39)	−0.19 (−0.70; 0.32)	−0.23 (−0.74; 0.28)	−0.17 (−0.68; 0.33)
Metabolic risk factors	Ref	−0.3 (−0.9; 0.3)	−0.4 (−0.8; 0.1)	−0.3 (−0.7; 0.1)	−0.3 (−0.7; 0.1)	−0.2 (−0.6; 0.2)	−0.3 (−0.7; 0.1)
Ages 30–39 years ($n = 742$), 9% AMH levels below 0.16 μg/l (Categories 1 and 2)							
Systolic blood pressure	Ref	0.6 (−5.2; 6.4)	−0.5 (−5.2; 4.1)	−0.3 (−4.8; 4.3)	−2.3 (−7.0; 2.3)	0.4 (−4.2; 5.1)	−0.7 (−5.0; 3.7)
Diastolic blood pressure	Ref	0.8 (−3.7; 5.3)	−0.3 (−3.9; 3.3)	0.8 (−2.7; 4.3)	−0.0 (−3.6; 3.6)	2.0 (−1.6; 5.6)	0.6 (−2.7; 4.0)
Total cholesterol	Ref	−0.33 (−0.76; 0.10)	−0.32 (−0.66; 0.03)	−0.25 (−0.59; 0.09)	−0.19 (−0.53; 0.16)	−0.07 (−0.42; 0.27)	−0.21 (−0.53; 0.11)
HDL-cholesterol	Ref	0.05 (−0.12; 0.23)	0.13 (−0.01; 0.27)	0.01 (−0.12; 0.15)	0.08 (−0.06; 0.22)	**0.16** (**0.02**; **0.30**)	0.09 (−0.04; 0.22)
Glucose	Ref	0.07 (−0.51; 0.64)	0.10 (−0.36; 0.57)	0.21 (−0.24; 0.67)	0.10 (−0.36; 0.56)	0.27 (−0.20; 0.74)	0.17 (−0.26; 0.60)
Metabolic risk factors	Ref	−0.1 (−0.4; 0.2)	−0.2 (−0.5; 0.1)	−0.0 (−0.3; 0.2)	−0.1 (−0.4; 0.1)	−0.2 (−0.4; 0.1)	−0.1 (−0.4; 0.1)
Ages 40–49 years ($n = 830$), 51% AMH levels below 0.16 μg/l (Categories 1 and 2)							
Systolic blood pressure	Ref	0.9 (−2.1; 3.8)	−0.7 (−3.4; 2.0)	0.9 (−2.5; 4.2)	0.5 (−3.7; 4.8)	−2.1 (−7.5; 3.4)	−0.2 (−2.7; 2.2)
Diastolic blood pressure	Ref	0.1 (−2.0; 2.2)	0.0 (−1.9; 1.9)	−0.6 (−3.0; 1.8)	−1.3 (−3.0; 1.8)	−1.9 (−5.8; 1.9)	−0.4 (−2.1; 1.3)
Total cholesterol	Ref	−0.08 (−0.26; 0.10)	−0.15 (−0.31; 0.02)	0.08 (−0.12; 0.29)	−0.22 (−0.48; 0.04)	−0.14 (−0.47; 0.19)	−0.09 (−0.24; 0.05)
HDL-cholesterol	Ref	−0.00 (−0.08; 0.07)	−0.02 (−0.09; 0.04)	−0.03 (−0.11; 0.05)	0.03 (−0.08; 0.13)	0.04 (−0.09; 0.18)	−0.01 (−0.07; 0.05)
Glucose	Ref	−0.01 (−0.20; 0.18)	−0.09 (−0.26; 0.08)	−0.05 (−0.27; 0.16)	−0.12 (−0.39; 0.15)	0.08 (−0.39; 0.15)	−0.07 (−0.22; 0.08)
Metabolic risk factors	Ref	0.1 (−0.1; 0.2)	−0.1 (−0.2; 0.0)	0.0 (−0.1; 0.2)	−0.1 (−0.3; 0.1)	0.2 (−0.5; 0.1)	−0.1 (−0.2; 0.1)
Ages 50–59 years ($n = 157$), 91% AMH levels below 0.16 μg/l (Categories 1 and 2)							
Systolic blood pressure	Ref	−9.0 (−18.8; 7.8)	−0.6 (−13.5; 12.4)	−8.6 (−29.6; 12.4)	−4.1 (−27.8; 19.7)	21.3 (−12.2; 54.9)	−0.9 (−10.8; 9.0)
Diastolic blood pressure	Ref	−3.6 (−9.9; 2.3)	−0.3 (−8.2; 7.5)	−5.8 (−18.5; 6.9)	1.3 (−13.1; 15.7)	2.0 (−18.3; 22.4)	−1.0 (−7.0; 5.0)
Total cholesterol	Ref	−0.23 (−0.74; 0.27)	0.12 (−0.55; 0.79)	−0.73 (−1.80; 0.36)	0.47 (−0.75; 1.69)	0.74 (−1.00; 2.47)	0.05 (−0.46; 0.56)
HDL-cholesterol	Ref	−0.13 (−0.36; 0.09)	−0.07 (−0.36; 0.22)	−0.46 (−0.94; 0.02)	−0.40 (−0.95; 0.14)	0.15 (−0.61; 0.92)	−0.18 (−0.41; 0.04)
Glucose	Ref	0.07 (−0.62; 0.77)	0.26 (−0.66; 1.18)	−0.00 (−1.49; 1.49)	−1.22 (−2.90; 0.47)	0.58 (−2.90; 0.47)	0.01 (−0.70; 0.71)
Metabolic risk factors	Ref	**−0.5** (**−1.0**; **−0.0**)	−0.1 (−0.8; 0.6)	−0.6 (−1.6; 0.5)	0.7 (−4.9; 1.9)	1.4 (−0.3; 3.1)	0.1 (−0.4; 0.6)

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

more extreme aging phenotype, illustrated by both quickened vascular and reproductive aging, but this currently remains conjecture.

In both an adolescent and regularly cycling adult study populations, AMH levels were not related to cardio-metabolic risk factors after correction for confounders (Anderson *et al.*, 2013; Bleil *et al.*, 2013). In the study by Bleil *et al.* (2013), BMI appeared to be an important confounder or effect mediator, whereas this effect is not supported by our results (see Supplementary data, Table SII for a comparison of multivariable regression models with and without BMI adjustment). This may be due to a more favorable BMI distribution in our population, as Bleil *et al.* (2013) reported 28.9% of their participants to have a BMI of 30 kg/m^2 or more, where in our population this was 8.9%. In a large cross-sectional population of Chinese women aged 21–64 years, AMH levels were inversely correlated with BMI and fasting glucose, but not with other CVD risk factors (Cui *et al.*, 2016). As these studies all used AMH as a continuous outcome parameter, it is possible that subtle differences with low AMH were not detected.

Recent research has suggested that endocrine changes during the menopausal transition are associated with CVD risk (El Khoudary *et al.*, 2016). Increases in LDL-c and TC were indeed previously found to be most substantial in the year surrounding the final menstrual period of 3302 participants of the SWAN study (Matthews *et al.*, 2009), which may corroborate our findings with respect to the group of women with AMH levels of zero. However, as more than 80% of the study participants were still regularly cycling, in addition to the unaltered results after exclusion of women who had their last menstrual period more than 3 months prior, it is unlikely that our results merely represent the final year preceding menopause. A higher CVD risk in premenopausal women with AMH levels of zero may thus imply that as women reach the later stages of their reproductive lifespan, CVD risk increases. *Vice versa*, a higher CVD risk could potentially influence ovarian reserve, as suggested by a study in which a 1% increase in 10-year CVD risk was associated with a 1.8-year reduction of age at menopause (Kok *et al.*, 2006). AMH could furthermore have an effect on CVD risk directly, through the regulation of vascular development for example (Dennis *et al.*, 2013). However, this cross-sectional epidemiological study does not allow to draw conclusions on whether AMH is a proxy variable for ovarian reserve or whether we observed direct mechanistic effects of AMH.

To our knowledge, we are the first to report a potential relation of undetectable AMH levels (i.e. 0.000 μg/l) with increased CVD risk. In the available studies where no relationship was found between AMH and cardio-metabolic risk factors (Anderson *et al.*, 2013; Bleil *et al.*, 2013), there were no women with AMH levels <0.16 μg/l in the study population. In the case of the study by Anderson *et al.* this is very likely due to the adolescent study population, although only the mean age was provided, rather than an age range. In the study by Bleil *et al.* (2013) women were also younger overall, with a mean age of 35 years. However, because women aged 25–45 years were included by Bleil *et al.* (2013) and AMH levels were measured with the same assay as the current study this is still surprising, as 5.2% of our participants under 40 years had undetectable AMH levels. The authors state that 27 women were excluded from their study population due to missing data on a primary variable of interest (Bleil *et al.*, 2013), which could potentially include undetectable AMH levels. Tehrani *et al.* (2014) did find an effect on CVD risk factors for women in the lowest age-corrected AMH quartile and included women with AMH levels <0.16 μg/l in their analyses, which is interesting in the light of our findings. The potential relevance of low AMH levels was previously highlighted in a population of subfertile women, in which only AMH levels up to 1 μg/l predicted live birth rates (Yarde *et al.*, 2013). The aforementioned studies, as in the current study, all used the Gen II AMH assay by Beckman Coulter. As the ability to detect very low AMH levels is increasing as more sensitive AMH assays have become available (Robertson *et al.*, 2014), it will become possible to better characterize the relationship between ovarian reserve and CVD risk in this group of women.

A limitation of our, and previous, studies is the difficulty of accurately estimating CVD risk. While we have attempted to provide a thorough representation of CVD risk here, it remains difficult to differentiate between the meaning of the various estimations. As we performed an exploratory analysis with multiple CVD risk factors, the interpretation of the statistical significance and clinical relevance of our results must be done with caution. Moreover, considering the major influence of age on both AMH levels and CVD risk, it is theoretically possible that the observed trend towards an association of AMH levels of zero with unfavorable CVD risk outcomes is still merely the consequence of chronological aging alongside the menopausal transition. By correcting for age both as a linear and quadratic term, we have circumvented this issue to the best of our ability. Another potential source of bias is the possible misclassification of premenopausal women due to the use of questionnaire information. In this case, the perceived unfavorable CVD risk in women with the lowest AMH levels could be a representation of postmenopausal status. However, as the exclusion of women with an amenorrhea with 3 months or more did not change the nature of the results, this seems less likely. Furthermore, as we expect the degree of recall bias to be comparable for all women in our study population, we do not think this greatly affected our results.

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

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

A.C.d.K.: study design, data analysis, manuscript preparation and revision. W.M.M.V.: data collection, supervision of data analysis, manuscript revision. M.J.C.E.: supervision of data analysis, revision of manuscript. Y.T.v.d.S.: conception and study design, supervision of data analysis,

revision of manuscript. F.J.M.B.: conception and study design, supervision of data analysis, revision of manuscript.

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Conflict of interest

A.C.d.K., W.M.M.V., M.J.C.E. and Y.T.v.d.S. have nothing to disclose. F.J.M.B. has received fees and grant support from Merck Serono, Gedeon Richter, Ferring BV and Roche.

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