

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

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STUDY QUESTION: Can additional genetic variants for circulating anti-Müllerian hormone (AMH) levels be identified through a genome-wide association study (GWAS) meta-analysis including a large sample of premenopausal women?

SUMMARY ANSWER: We identified four loci associated with AMH levels at $P < 5 \times 10^{-8}$: the previously reported *MCM8* locus and three novel signals in or near *AMH*, *TEX41* and *CDCA7*.

WHAT IS KNOWN ALREADY: AMH is expressed by antral stage ovarian follicles in women, and variation in age-specific circulating AMH levels has been associated with disease outcomes. However, the physiological mechanisms underlying these AMH-disease associations are largely unknown.

STUDY DESIGN, SIZE, DURATION: We performed a GWAS meta-analysis in which we combined summary statistics of a previous AMH GWAS with GWAS data from 3705 additional women from three different cohorts.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In total, we included data from 7049 premenopausal female participants of European ancestry. The median age of study participants ranged from 15.3 to 48 years across cohorts. Circulating AMH levels were measured in either serum or plasma samples using different ELISA assays. Study-specific analyses were adjusted for age at blood collection and population stratification, and summary statistics were meta-analysed using a standard error-weighted approach. Subsequently, we functionally annotated GWAS variants that reached genome-wide significance ($P < 5 \times 10^{-8}$). We also performed a gene-based GWAS, pathway analysis and linkage disequilibrium score regression and Mendelian randomization (MR) analyses.

MAIN RESULTS AND THE ROLE OF CHANCE: We identified four loci associated with AMH levels at $P < 5 \times 10^{-8}$: 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 single nucleotide polymorphisms for AMH levels and for age at menopause ($r_g = 0.82$, $FDR = 0.003$). Exploratory two-sample MR analyses did not support causal effects of AMH on breast cancer or polycystic ovary syndrome risk, but should be interpreted with caution as they may be underpowered and the validity of genetic instruments could not be extensively explored.

LARGE SCALE DATA: The full AMH GWAS summary statistics will be made available after publication through the GWAS catalog (<https://www.ebi.ac.uk/gwas/>).

LIMITATIONS, REASONS FOR CAUTION: Whilst this study doubled the sample size of the most recent GWAS, the statistical power is still relatively low. As a result, we may still lack power to identify more genetic variants for AMH and to determine causal effects of AMH on, for example, breast cancer. Also, follow-up studies are needed to investigate whether the signal for the *AMH* gene is caused by reduced AMH detection by certain assays instead of actual lower circulating AMH levels.

WIDER IMPLICATIONS OF THE FINDINGS: Genes mapped to the *MCM8*, *TEX41* and *CDCA7* loci are involved in the cell cycle and processes such as DNA replication and apoptosis. The mechanism underlying their associations with AMH may affect the size of the ovarian follicle pool. Altogether, our results provide more insight into the biology of AMH and, accordingly, the biological processes involved in ovarian ageing.

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Key words: anti-Müllerian hormone / AMH / genome-wide association analysis / ovarian ageing / reproductive ageing

Introduction

Anti-Müllerian hormone (AMH) is generally known for its function in sexual differentiation, during which AMH signalling is essential for the regression of internal female reproductive organs in male embryos (Jost, 1947). In women, AMH is expressed by granulosa cells of primary ovarian follicles, and AMH expression continues until the antral stage (Veenen *et al.*, 2004). AMH becomes undetectable after menopause, when the ovarian reserve is depleted, and AMH can therefore be used as a marker for reproductive ageing (Finkelstein *et al.*, 2020).

Variation in age-specific circulating AMH levels has been associated with the occurrence of several non-communicable diseases, including breast cancer (Ge *et al.*, 2018). In addition, it has been suggested that AMH may be involved in the pathogenesis of polycystic ovary syndrome (PCOS) (Homburg and Crawford, 2014). 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 aetiologies of non-communicable diseases associated with AMH levels.

Previous GWASs on circulating AMH levels included either a mixture of male and female adolescents (Perry *et al.*, 2016), a very small study population (Schuh-Huerta *et al.*, 2012a), or women of late reproductive age (Ruth *et al.*, 2019) 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) (Ruth *et al.*, 2019), which is also associated with natural age at menopause (He *et al.*, 2009; Stolk *et al.*, 2009). As the 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 20–40 years (de Kat *et al.*, 2016), the inclusion of 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 largest previous AMH GWAS meta-analysis (Ruth *et al.*, 2019) with GWAS data from 3705 additional women of early and middle reproductive age from three different cohorts.

Materials 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.* (2019) ($n = 3344$), we included data from the Doetinchem Cohort Study (Verschuren *et al.*, 2008; Picavet *et al.*, 2017) ($n = 2084$), the Study of Women's Health Across the Nation (SWAN) (Sowers *et al.*, 2000) (www.swanstudy.org) ($n = 425$) and data from adolescent daughters of the Avon Longitudinal Study of Parents and Children (ALSPAC) (www.bristol.ac.uk/alspac/) (Boyd *et al.*, 2013) ($n = 1196$). The GWAS by Ruth *et al.* included data from the Generations Study (Swerdlow *et al.*, 2011), Sister Study (Nichols *et al.*, 2015), Nurses' Health Study (Hankinson *et al.*, 1995), Nurses'

Health Study II (Eliassen *et al.*, 2006) and ALSPAC mothers (Fraser *et al.*, 2013). For the current study, we requested summary statistics excluding data from ALSPAC mothers, as we wanted to analyse data from the ALSPAC mothers separately to investigate potential bias due to first-degree relatedness. More details about participating studies and the definitions used for the assessment of menopausal status are described in the [Supplementary Materials and Methods](#) and [Supplementary Table S1](#). All studies received ethical approval from an institutional ethics committee.

AMH measurements

Included studies measured AMH in either serum or plasma using different manual AMH ELISA assays. The picoAMH assay (Ansh Labs, Webster, TX) was used to measure circulating AMH levels in the Generations Study, both Nurses' Health Studies, the Doetinchem Cohort Study and SWAN. For the Sister Study, the ultrasensitive AMH ELISA (Ansh Labs, Webster, TX, USA) was used, and for ALSPAC, AMH was measured using the Gen II AMH ELISA (Beckman Coulter UK Ltd, High Wycombe, UK). Antibodies differ between the Ansh Labs and Beckman Coulter assays (Moolhuijsen and Visser, 2020), but detailed information about these differences in antibodies is not available. The methodology for handling AMH measurements below the assay limit of detection (LOD) also differed across studies. A detailed overview of these study-specific details has been included in [Supplementary Table S1](#). 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 [Supplementary Table SII](#). Briefly, samples of the Generations Study, Sister Study and most samples of the Nurses' Health Studies, were genotyped using the OncoArray array (Ruth *et al.*, 2019). The remaining 225 samples of the Nurses' Health Studies were genotyped using Illumina HumanHap550 and HumanHap610 arrays (Ruth *et al.*, 2019). Samples of the Doetinchem Cohort Study were genotyped using the Illumina Infinium Global Screening Array-24 Kit (Illumina Inc., San Diego, CA, USA). 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 ([Supplementary Table SII](#)).

Association analyses

All studies converted AMH concentrations to pmol/l using $1 \text{ pg/ml} = 0.00714 \text{ pmol/l}$. As AMH levels are not normally distributed, AMH measurements were transformed using rank-based inverse normal transformation in all studies, as described previously (Ruth *et al.*, 2019).

In all studies, linear models were fitted, assuming additive SNP effects, adjusted for age at blood collection (years) using different software tools (Marchini *et al.*, 2007; Zhou and Stephens, 2012; Zhan *et al.*, 2016) ([Supplementary Materials and methods](#)). Analyses were

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. (2019)^a			
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		

^aIn 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, anti-Müllerian hormone; GWAS: genome-wide association analysis; SWAN, Study of Women's Health Across the Nation.

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 (Willer et al., 2010), as described elsewhere (Ruth et al., 2019). 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 (Supplementary Materials and 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; www.uni-regensburg.de/medizin/epidemiologie-praeventivmedizin/genetische-epidemiologie/software/), following a previously published protocol (Winkler et al., 2014) (Supplementary Materials and methods). No study-specific issues were identified through these QC procedures (Supplementary Figs S1, S2, S3, S4 and 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 first-degree relatedness (411 mother–daughter pairs were present in the ALSPAC data; 5.8% of the total study population). We checked this through meta-analysing only data of the two ALSPAC cohorts and both checking the corresponding QQ plot and calculating λ . Given the absence of genomic inflation ($\lambda = 1.01$, QQ plot in Supplementary Fig. S6), we included summary statistics of both ALSPAC mothers and daughters in the meta-analysis to increase statistical power.

We performed an inverse variance weighted (IVW) meta-analysis using METAL (version 2011-03-25; http://csg.sph.umich.edu/abecasis/metal/) (Willer et al., 2010). 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 whether observed effect estimates were homogeneous across studies, we additionally performed a heterogeneity analysis in METAL (Willer et al., 2010).

To identify lead and secondary SNPs within genome-wide significant associated loci, we performed an approximate conditional and joint association analysis (Yang et al., 2012). We used Genome-wide Complex Trait Analysis (GCTA) (Yang et al., 2011) (version 1.93.1f beta; https://cnsgenomics.com/software/gcta/) to run a stepwise model selection procedure to select independently associated SNPs (cojo-slc) using the summary-level data. We estimated linkage disequilibrium (LD) between SNPs using data of 4059 unrelated participants from the EPIC-NL cohort (Beulens et al., 2010) as the 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 (de Leeuw et al., 2015) implementation (v1.08) in the online Functional Mapping and Annotation of Genome-wide Association Studies (FUMA) platform (Watanabe et al., 2017) (https://fuma.ctglab.nl/) (parameter settings are listed in Supplementary Table SIII). 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 (de Leeuw et al., 2015). 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 (Watanabe *et al.*, 2017). We used the SNP2GENE process integrated into FUMA (v1.3.6a) (Watanabe *et al.*, 2017) for the characterization of genomic loci and functional gene mapping (parameter settings are listed in [Supplementary Table SIII](#)). 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 scores (Kircher *et al.*, 2014), Regulome DB scores (Boyle *et al.*, 2012) and chromatin states (Kundaje *et al.*, 2015) ([Supplementary Table SIII](#)). Positional mapping, eQTL mapping and chromatin interaction mapping were used to map SNPs to genes ([Supplementary Table SIII](#)). For chromatin states, eQTL mapping and chromatin interaction mapping, we only selected tissues and cell types that are most likely to be involved in AMH expression and signalling ([Supplementary Table SIII](#)).

Pathway analysis using DEPICT

We used the hypothesis-free pathway analysis tool DEPICT (v1; <https://github.com/perslab/depict>) (Pers *et al.*, 2015) 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 500 kb using PLINK v.1.9 as part of the DEPICT pipeline.

Linkage disequilibrium score regression

We estimated SNP heritability using the LD Hub web interface (v1.9.3; <http://ldsc.broadinstitute.org/>) (Zheng *et al.*, 2017) for LD score regression (Bulik-Sullivan *et al.*, 2015). 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 (Bulik-Sullivan *et al.*, 2015). Included phenotypes comprised reproductive traits, hormones (leptin), anthropometric traits, blood lipids, glycemic traits, metabolites, cardiometabolic traits, cancer, autoimmune diseases, bone mineral density, ageing and smoking behaviour. Of the 597 UK Biobank traits in the LD Hub 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') (R Core Team, 2017). FDR adjusted P -values < 0.05 were considered to be significant.

Mendelian randomization

In observational studies, AMH has been associated with breast cancer (Ge *et al.*, 2018), and PCOS (Laven *et al.*, 2004), amongst other diseases. As the exact function of AMH in the aetiology 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 specific instrumental variable analysis method that may provide evidence for causality of observational associations (Davey Smith and Ebrahim, 2005). Briefly, SNPs that are robustly associated with AMH can be used as instrumental variables to estimate the causal effect of AMH on other traits and/or disease outcome.

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 (Perry *et al.*, 2016), we performed MR analyses for the female-specific outcomes breast cancer and PCOS only. We performed two-sample MR analyses (Pierce and Burgess, 2013) using the R package TwoSampleMR (version 0.5.1) (Hemani *et al.*, 2018). We included identified lead SNPs as genetic instrumental variables for AMH. For the outcomes, we included summary statistics from recent large-scale GWASs for breast cancer ($n = 228\,951$; 122 977 cases) (Michailidou *et al.*, 2017) and PCOS ($n = 113\,238$; 10 074 cases) (Day *et al.*, 2018). Wald ratio estimates were calculated for individual SNPs and a random effects 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 (Burgess and Thompson, 2015). 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 (Hemani *et al.*, 2018). In addition, we tested for heterogeneity in causal effects amongst the genetic variants using Cochran's Q statistics and performed leave-one-out sensitivity analyses to assess the potential effect of outlying variants.

Post-hoc analysis: assay-specific AMH levels across rs10417628 genotype categories

A recent case report suggested that the amino acid substitution corresponding to rs10417628 (AMH locus) reduces AMH detection by the picoAMH assay from Ansh Labs without influencing AMH bioactivity (Hoyos *et al.*, 2020). 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. In addition, we requested median AMH levels across rs10417628 genotype categories from both ALSPAC mothers and daughters, because ALSPAC also used the AMH Gen II assay from Beckman Coulter.

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 II, Fig. 1 and Supplementary Figs S7 and 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 one locus on chromosome 19 (nearest gene: *AMH*) and two loci on chromosome 2 (nearest genes: *TEX4I* and *CDCA7*). The strongest signal was rs10417628 on chromosome 19, which is physically located in the *AMH* gene ($\beta = -0.34$, $SE = 0.05$, $P = 1.2 \times 10^{-11}$) (Supplementary Fig. S8A). Combined, the four lead SNPs explained 1.47% of the variance in AMH levels.

We observed no systematic deviation of associations in the ALSPAC adolescents from the associations in women of middle reproductive age (Fig. 2). Only for a locus at chromosome 5, the association in the ALSPAC adolescents was radically different than in the other cohorts. For the *MCM8* locus, estimates were weaker in both the ALSPAC adolescents and women in the Doetinchem Cohort Study compared to the remaining studies.

In the sensitivity analysis excluding ALSPAC daughters, all four loci from the main analysis remained genome-wide significant and beta estimates were very similar to those from the main analysis. However, an additional locus at chromosome 5 (rs116090962, nearest gene: *CTB99A3.1*) was identified ($\beta = 0.38$, $SE = 0.07$, $P = 6.0 \times 10^{-9}$) and *P*-values corresponding to the *MCM8*, and *CDCA7* loci were lower in this sensitivity analysis compared to the main analysis (Supplementary Table SIV).

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* (Supplementary Fig. S9).

Functional annotation using FUMA

Through the SNP2GENE process, FUMA identified 82 candidate SNPs that were in LD with the four identified lead SNPs (Supplementary Table SV). 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) (Supplementary Table SVI), of which *MCM8* and *CRLS1* were prioritized based on eQTL mapping (Fig. 3A). *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, three 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 two variants were located in intronic and intronic non-coding RNA regions. Based on the used parameter settings, FUMA mapped eight genes to the *AMH* locus (Supplementary Table SVI), of which four were highlighted by eQTL mapping (*AMH*, *C19orf35*, *SPPL2B* and *LSM7*) and one was highlighted through chromatin interactions (*ABHD17A*) (Fig. 3B).

Most of the candidate SNPs were identified for the locus on chromosome 2 near *TEX4I*. All 77 variants were located in either intronic or exonic long non-coding RNA regions. Of the 15 genes mapped to this locus (Supplementary Table SVI), no genes were prioritized based on eQTL mapping, but several genes were prioritized based on chromosome interactions, including *ZEB2-AS1* (Fig. 3C). 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.

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 and 18 loci as input for the enrichment analyses (Supplementary Table SVII). The top three prioritized gene sets were 'UR11 PPI subnetwork', 'NFYB PPI subnetwork' and 'nuclear inner membrane' (Supplementary Table SVIII). 'Induced Pluripotent Stem Cells' was identified as the highest prioritized cell type (Supplementary Table SIX). However, none of

Table II 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
<i>AMH</i>	rs10417628	19	2 251 817	T	C	0.02	7049	0.83	-0.34 (0.05)	1.2×10^{-11}	-----	0.14	0.50%
<i>TEX4I</i>	rs13009019	2	145 670 572	A	G	0.69	7049	0.95	-0.09 (0.01)	7.2×10^{-10}	-----	0.24	0.35%
<i>MCM8</i>	rs16991615	20	5 948 227	A	G	0.07	7049	0.99	0.16 (0.03)	1.2×10^{-8}	++++	0.0009	0.30%
<i>CDCA7</i>	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; AMH, anti-Müllerian hormone; SWAN, Study of Women's Health Across the Nation.

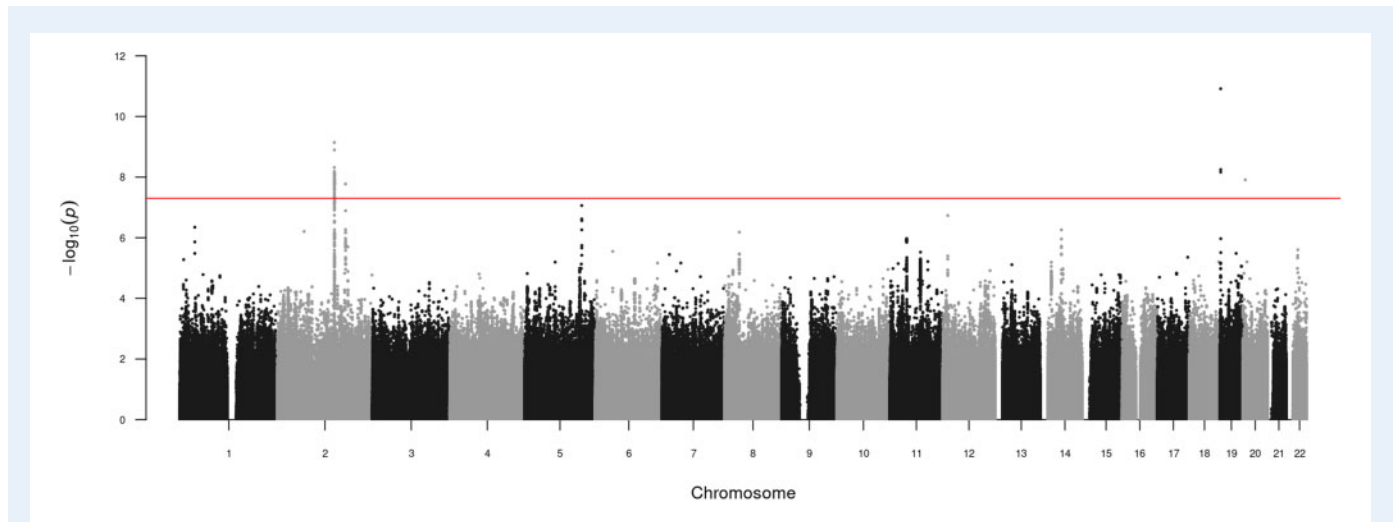


Figure 1. Manhattan plot of genome-wide association results for inverse normally transformed AMH in women. Results are association results from meta-analysis of inverse normally transformed AMH in 7049 women of European ancestry. Individual studies adjusted analyses for age at AMH measurement and population stratification (through kinship matrix or 10 principal components). AMH, anti-Müllerian hormone.

these enrichments were statistically significant (FDR values for all comparisons > 0.05).

DEPICT prioritized nine genes at $FDR < 0.05$ as most likely causal genes (Supplementary Table SX). 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 .

Linkage disequilibrium 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) (Supplementary Table SXI).

Mendelian randomization 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 III and Supplementary Fig. S10). 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 III). 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) (Supplementary Fig. S11).

For PCOS, the IVW MR estimate did not provide evidence for a causal effect either ($OR_{IVW} = 1.29$, 95% CI = 0.85–1.95; Table III and Supplementary Fig. S12), although the wide confidence interval indicated a considerable degree of uncertainty around this estimate. 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 III). 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) (Supplementary Fig. S13).

Post-hoc analysis: assay-specific AMH levels across rs10417628 genotype categories

For 1518 of the 2084 included Doetinchem Cohort Study participants, both picoAMH assay and AMH Gen II assay measurements were available. In general, median AMH levels measured using the Gen II assay were less different between women homozygous for the reference allele (C 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). Only one woman was estimated to be homozygous for the effect allele (dosage T allele = 1.9, age at measurement = 28.3 years). Whereas AMH levels for this woman were undetectable using the picoAMH assay, circulating AMH levels were detected using the less sensitive Gen II assay (318 pg/ml).

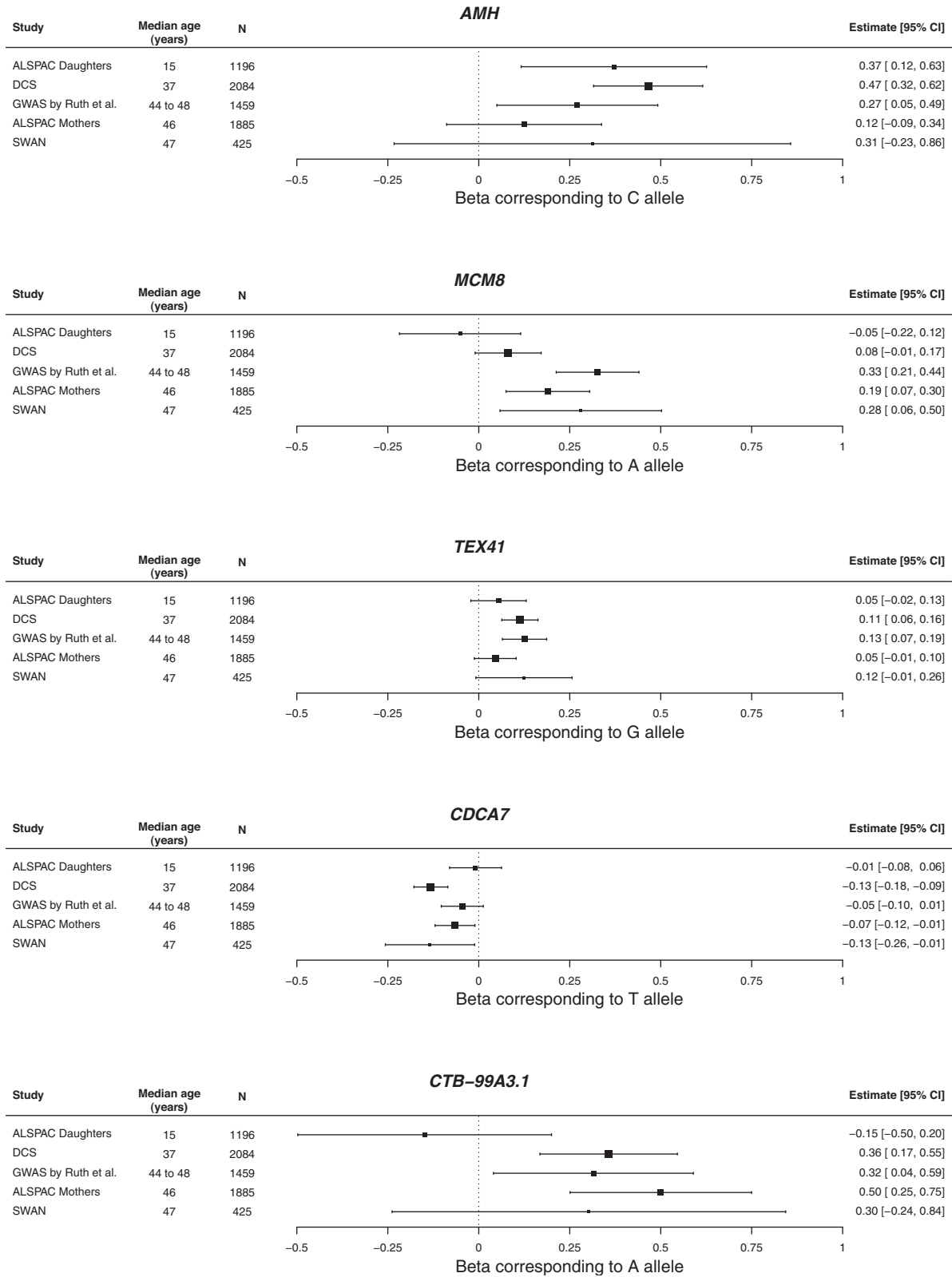


Figure 2. Effect estimates for the five SNPs that reached genome-wide significance in the main and sensitivity analyses across included studies.

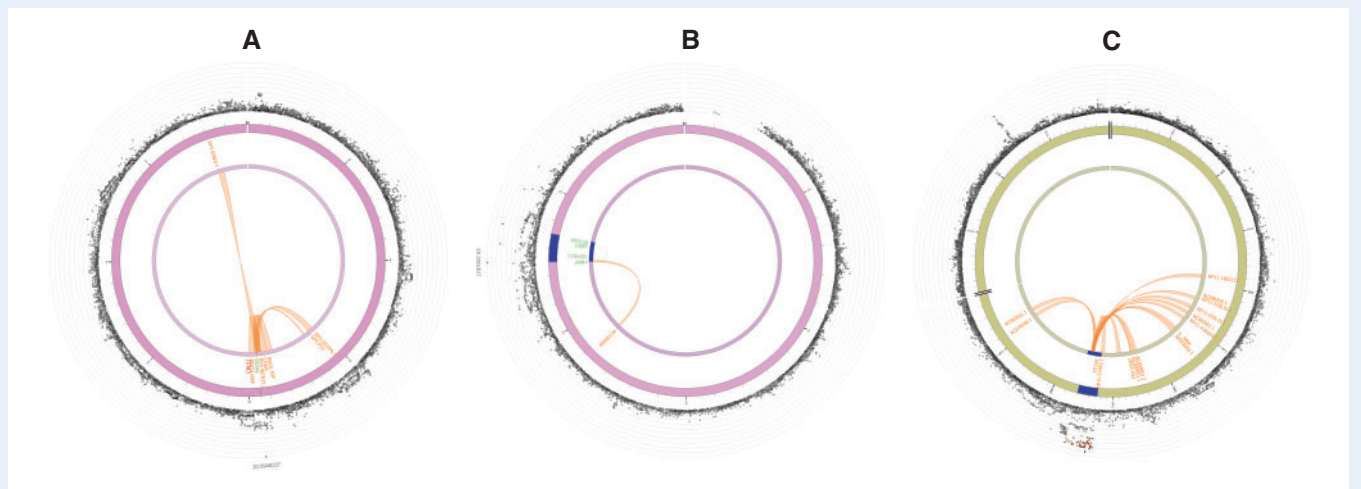


Figure 3. Circos plots for the genome-wide significant loci for inverse normally transformed AMH in women. Circos plots are presented for each of the identified loci: *MCM8* (A), *AMH* (B), *TEX41* and *CDCA7* (C). The outer layer represents the Manhattan plot. The second (including genomic positions) and third layers represent 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 (Watanabe et al., 2017). AMH, anti-Müllerian hormone; FUMA, Functional Mapping and Annotation of Genome-wide Association Studies.

Table III 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 (<i>AMH</i>)	0.99	0.87–1.12	0.85
	Wald ratio estimate for rs13009019 (<i>TEX41</i>)	0.84	0.72–0.97	0.02
	Wald ratio estimate for rs16991615 (<i>MCM8</i>)	1.60	1.37–1.87	1.79×10^{-9}
	Wald ratio estimate for rs11683493 (<i>CDCA7</i>)	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 (<i>AMH</i>)	1.27	0.64–2.56	0.49
	Wald ratio estimate for rs13009019 (<i>TEX41</i>)	1.66	0.80–3.45	0.18
	Wald ratio estimate for rs16991615 (<i>MCM8</i>)	1.75	0.83–3.69	0.14
	Wald ratio estimate for rs11683493 (<i>CDCA7</i>)	0.66	0.29–1.50	0.32

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

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

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.

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 or PCOS.

We confirmed the association between rs16991615 and circulating AMH levels, previously reported by Ruth et al. (2019). 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 homologous recombination, which is critical for DNA repair (Park et al., 2013). Previous studies have linked *MCM8* deficiency to premature ovarian failure and infertility, but also to cancer development (Michailidou et al., 2017; Griffin and Trakselis, 2019; Lutzmann et al., 2019). Associations between rs16991615 and age at menopause (Day et al., 2015) and number of ovarian follicles (Schuh-Huerta et al., 2012b) 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 has 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 ($R^2 = 0.55$) (Perry et al., 2016). 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 revealed 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 (Hoyos et al., 2020). We sought to verify this finding in a subsample of the Doetinchem Cohort Study, for which AMH was measured using both the picoAMH and the less sensitive Gen II assay from Beckman Coulter. In addition, we requested median AMH levels across genotype categories for ALSPAC, which also used this Beckman Coulter assay to measure AMH. In line with the finding of Hoyos et al., our post-hoc analyses indicated that AMH levels were undetectable using the picoAMH, but not using the Gen II assay, for the only homozygous woman in the Doetinchem Cohort Study. Moreover, median AMH levels were less different between women homozygous for the reference allele and heterozygous women in both the Doetinchem Cohort Study and the adult ALSPAC participants. In contrast, we did observe a clear difference in median AMH levels between ALSPAC adolescents homozygous for the reference allele and heterozygous adolescents. Because of this limited and inconsistent evidence, and the lack of publicly available information on the antibodies and conformational epitopes of the Gen II assay, 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 non-coding 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* (Beltran et al., 2008). *ZEB2* (also known as *SIP1*) inhibits signal transduction in TGF- β and BMP signalling through interaction with ligand-activated SMAD proteins (Postigo, 2003; Conidi et al., 2011). Among other BMP proteins, BMP4 has been reported to regulate AMH expression through activation of SMAD proteins (Estienne et al., 2015; Pierre et al., 2016). 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 hypothesis.

CDCA7 (also known as *JPO1*) is a direct target gene of the transcription factor *MYC* and is involved in apoptosis (Gill et al., 2013). 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 (Weenen et al., 2004). Ideally, future studies should explore whether the observed genetic associations may merely reflect the size of the ovarian reserve, by adjusting analyses for antral follicle count. Such analyses would also show whether we can actually use the identified variants as 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 instrumental variables for MR, SNPs have to fulfil the following three criteria (Lawlor et al., 2008): (i) SNPs have to be associated with the circulating AMH levels; (ii) SNPs cannot be associated with confounders of the studied AMH-outcome associations and (iii) 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 (Michailidou et al., 2017). Due to the limited number of identified lead SNPs, it was not possible to assess whether 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.

Initially, we decided to analyse both the ALSPAC adolescents and adult women together to increase the total number of included participants, and consequently statistical power. Previous research suggests that AMH levels in females rise during puberty, until the mid to late twenties, and after that, decreases until menopause (Kelsey et al., 2011; de Kat et al., 2016); that is AMH levels are heterogeneous across different reproductive phases. 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. However, we observed no systematic deviation of SNP-AMH associations in the adolescents from associations in the adult women. Nevertheless, our observation that the *P*-values corresponding to the *MCM8*, *CDCA7* and *CTB-99A3.1* loci were lower in the analyses excluding the ALSPAC adolescents suggests that including these samples increased heterogeneity, rather than

statistical power, even though our conclusions remained the same. A larger GWAS including older adolescents and a larger proportion of females aged 20–40, would be required to reveal potential gene–age interactions 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 the function of AMH 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 the potential overlap in participants between the current AMH GWAS and the GWAS for breast cancer (Michailidou *et al.*, 2017) (maximum $n = 1459$; 20.7% of current study) and PCOS (Day *et al.*, 2018) (maximum $n = 225$; 3.2% of current study). Overlap in participants in two-sample MR analyses may bias effect estimates and inflate Type I error rates (Burgess *et al.*, 2016).

In conclusion, we replicated the previously reported association with the *MCM8* locus and identified three 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 or PCOS, but these finding should be interpreted with caution because we could not robustly explore how valid the instruments were and because of the considerable uncertainty in effect estimates.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the NHGRI-EBI Catalog of human genome-wide association studies, at <https://www.ebi.ac.uk/gwas/>. Code underlying the analyses for this article are available via GitHub, at https://github.com/reneemgverdiesen/AMH_GWAS.

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Authors' roles

R.M.G.V., Y.T.v.d.S., C.H.v.G. and N.C.O.M. were involved in the initial study design. W.M.M.V. and F.J.M.B. (Doetinchem Cohort Study), D.A.L. (ALSPAC), A.H.E. (Nurses' Health Studies), P.K. (Nurses' Health Studies), D.P.S. (Sister Study), S.D.H. (SWAN) and A.J.S. (Generations Study) were involved in acquisition of (part of) the data. R.M.G.V., M.C.B., A.L.G.S., J.A.S. and K.S.R. carried out analyses. RMGV drafted the article. All authors (including NS, MJS and AM) gave input on the initial study design and analyses, were involved in the interpretation of the results, revising and approving the manuscript.

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Conflict of interest

F.J.M.B. has received fees and grant support from Merck Serono and Ferring BV. D.A.L. has received financial support from several national and international government and charitable funders as well as from Medtronic Ltd and Roche Diagnostics for research that is unrelated to this study. N.S. is scientific consultant for Ansh Laboratories. The other authors declare no competing interests.

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