

## Anti-Mullerian hormone and endometrial cancer: a multi-cohort study

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## **Abstract**

**Background:** The Mullerian ducts are the embryological precursors of the female reproductive tract, including the uterus; Anti-Mullerian hormone (AMH) plays a key role in the regulation of fetal sexual differentiation. AMH inhibits endometrial tumor growth in experimental models by stimulating apoptosis and cell cycle arrest. To date, there are no prospective epidemiologic data on circulating AMH and endometrial cancer risk.

**Methods:** We investigated this association among women premenopausal at blood collection in a multi-cohort study including participants from eight studies located in the United States, Europe, and China. We identified 329 endometrial cancer cases and 339 matched controls. AMH concentrations in blood were quantified using an enzyme-linked immunosorbent assay. Conditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals [CI] across tertiles and for a doubling of AMH concentrations ( $OR_{\log 2}$ ). Subgroup analyses were performed by ages at blood donation and diagnosis, oral contraceptive use, and tumor characteristics.

**Results:** AMH was not associated with the risk of endometrial cancer overall ( $OR_{\log 2}$ : 1.07 [0.99-1.17]), or any of the examined subgroups.

**Conclusions:** While experimental models implicate AMH in endometrial cancer growth inhibition, our findings do not support a role for circulating AMH in the etiology of endometrial cancer.

**Keywords:** endometrial cancer; anti-mullerian hormone; mullerian inhibiting substance; multi-cohort study

## Introduction

In early gestation, male and female embryos have both Wolffian ducts, which subsequently develop into the male genital tracts in the male fetus, and Mullerian ducts, which develop into the uterus, the fallopian tubes, and the upper vagina in female fetus (Sobel *et al*, 2004). The anti-Mullerian hormone (AMH), also known as the Mullerian inhibiting substance (MIS), is secreted by the Sertoli cells of the testes and is responsible for the regression of the Mullerian ducts during fetal life in males (MacLaughlin & Donahoe, 2010). In female fetuses, absence of AMH production during this period allows development of the female genital tracts from the Mullerian ducts.

In females, AMH is secreted by the granulosa cells of the growing ovarian follicles and serves as a sensitive marker of ovarian reserve; its concentrations are very low at birth, increase significantly at puberty, remain stable thereafter until age ~25 years, and then slowly decline to be undetectable at the onset of menopause, when the ovarian follicle pool is exhausted (Dewailly *et al*, 2014). The clinical use of AMH is well established. In females, serum AMH is utilized for monitoring patients with ovarian granulosa cell tumors, with up to 90% of cases presenting with high AMH concentrations (Geerts *et al*, 2009), and AMH concentrations can be predictive of ovarian response after in vitro fertilization treatments (Fleming *et al*, 2015). Further, AMH is under discussion to become a diagnostic criterion for patients with Polycystic Ovary Syndrome (PCOS), as AMH levels are 2-4 times higher in women with PCOS as compared to healthy women (Garg & Tal, 2016).

AMH activates down-stream pathways notable for differentiation and growth inhibition by binding to its specific type II receptor (AMHRII), and *in vitro* experimental models have shown that AMH inhibits endometrial cancer growth by apoptosis and cell cycle arrest in AMHRII-positive endometrial cancer cell lines (Kim *et al*, 2014). Renaud and colleagues provided the first evidence for a potential inhibitory effect of AMH in endometrial

cancer, showing that AMH inhibits endometrial cancer growth by apoptosis and cell cycle arrest in AMHRII-positive endometrial cancer cell lines (Renaud *et al*, 2005).

In 2012, endometrial cancer, also referred to as cancer of the corpus uteri, was diagnosed in more than 47,000 women in the U.S. (Siegel *et al*, 2012) and more than 300,000 women worldwide (Ferlay *et al*, 2014). Though this cancer is frequently diagnosed when still localized, approximately 30% of cases are diagnosed at more advanced stages (regional/distant) (Howlader *et al*, 2016) according to the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute. Established risk factors for endometrial cancer are obesity and use of exogenous estrogen after menopause; however, these factors can only explain about half of the endometrial cancer cases in Western countries (Arnold *et al*, 2015).

To date, there are no prospective epidemiologic data on the association between AMH and risk of endometrial cancer. However, given experimental data, we hypothesized that higher circulating AMH levels may confer a relative protection against the development of endometrial cancer. We investigated this hypothesis in a nested case-control study including premenopausal women from cohort studies within the Prospective Study of AMH and Gynecologic Cancer Risk.

## **Material and methods**

### *Study Population*

This nested case-control study included participants from eight prospective cohort studies located in the United States, Europe, and China. The following studies contributed to this investigation: Columbia, Missouri Serum Bank (USA) (Dorgan *et al*, 2009), the Campaign Against Cancer and Heart Disease (CLUE I/II; USA) (McSorley *et al*, 2007), the New York University Women's Health Study (NYUWHS; USA) (Clendenen *et al*, 2016), the European Prospective Investigation into Cancer and Nutrition (EPIC; Europe) (Dossus *et al*, 2010), the

Guernsey Cohort Study (UK) (Wang *et al*, 2014), the Hormones and Diet in the Etiology of Breast Cancer (ORDET; Italy) (Clendenen *et al*, 2016), the Northern Sweden Health and Disease Study (NSHDS; Sweden) (Clendenen *et al*, 2016), and the Shanghai Women's Health Study (SWHS; China) (Dorjgochoo *et al*, 2009).

In each of the cohort studies, blood samples were collected using standardized protocols. Samples were stored at  $\leq -70^{\circ}\text{C}$  in all studies except the Guernsey study; samples from this study were stored at  $-20^{\circ}\text{C}$ . Detailed information on each of the contributing cohorts is provided in **Table 1** and **Supplementary Table 1**. The study was approved by the institutional review boards of the collaborating institutions and the University of Heidelberg, Germany and the University of Maryland, Baltimore (USA).

#### *Ascertainment of cases*

Our investigation was limited to premenopausal participants, who were younger than 47 years at blood collection, as AMH concentrations decline with age and are undetectable after menopause. Cases included women diagnosed with incident, primary endometrial cancer ascertained by self-report with medical record confirmation and/or linkages to cancer registries. All cases had no history of cancer, with the possible exception of non-melanoma skin cancer, prior to the diagnosis of endometrial cancer and did not report prior hysterectomy. Tumor characteristics (i.e., histology, stage, and grade) were obtained from cancer registries, pathology reports, and medical records. We identified a total of 329 eligible endometrial cancer cases diagnosed after blood collection. Six of these cases were diagnosed with synchronous ovarian cancer (none of which were of granulosa tumors); these cases were excluded in sensitivity analyses.

#### *Control selection*

Eligible controls were premenopausal women younger than age 47 at blood collection and cancer-free (except non-melanoma skin cancer) and not reporting prior hysterectomy at the

index date of their matched case. For every cohort, except NSHDS, one control was matched to each case; NSHDS matched up to 2 controls per case. All studies matched cases and controls on age and date at blood collection; additional matching factors specific to each cohort included study center, time of day at blood collection, fasting status, and menstrual cycle phase (matching factors by study provided in **Supplementary Table 1**). A total of 339 matched controls were identified.

Participating cohorts contributed between 10 cases/10 controls (Columbia Serum Bank) to 102 cases/102 controls (CLUEI/II) (**Table 1**).

### *Case Characteristics*

Histology data were available for 309 cases (94%). The majority of cases were diagnosed with adenocarcinoma not otherwise specified (NOS; n=166, 54%), followed by endometrioid tumors (n=96, 31%) and others (n=47, 15%; e.g., serous (n=10), mucinous (n=6)). The majority of cases had data on stage (n=227; 69%) and grade (n=219; 67%) at diagnosis. Well differentiated tumors (i.e., grade 1) were classified as “low grade” (n=118; 54%), whereas moderately and poorly / undifferentiated tumors (i.e., grades 2 and 3) were classified as “high grade” (n=101; 46%). Data on histology and grade were used to classify 82% of tumors into Type I and Type II. We classified endometrioid adenocarcinoma (ICD-O-2 codes: 8380, 8381, 8382, 8383) with grades 1 and 2, adenocarcinoma NOS (8140), and adenocarcinoma with squamous differentiation (8560, 8570) as Type I (90%, n=242); endometrioid adenocarcinoma with grade 3, serous/papillary serous (8441, 8460, 8461) and mixed cell adenocarcinoma (8323) as type II tumors (Setiawan *et al*, 2013).

### *Covariate data*

Each participating cohort provided data on covariates; these data were collected at the time of blood collection and were centrally collated and harmonized. Information on demographics,

lifestyle, reproductive history, and medical history was obtained via self-report and interview (**Supplementary Table 1**).

#### *Laboratory Assays - AMH*

Blood samples from each cohort were sent to a single laboratory at the Massachusetts General Hospital (Boston, MA, USA) for AMH assays. This investigation used serum or plasma samples, depending on sample availability. AMH concentrations in paired serum and plasma samples from the same individuals are highly correlated ( $r \geq 0.98$ ) (Merhi *et al*, 2008) and we observed no difference in mean AMH concentrations among participants with serum or plasma in the two studies which provided samples in both matrices (CLUEI/II:  $p=0.84$ ; EPIC:  $p=0.88$ ). Further, matrix (serum or plasma) was the same for each case and her matched control. Specimens of individually matched case and control subjects were always included in the same laboratory batch, alongside blinded quality control samples. The technicians performing the assays were blinded to the case, control, or quality control status of the specimens. Concentrations of AMH were measured using a commercially available picoAMH enzyme-linked immunosorbent assay (ELISA; Ansh Catalog no. AL-124, Webster, TX); the assay limit of detection was 0.02 ng/mL. The overall coefficient of variation for AMH based on the study blinded pooled quality control samples was 13.9%.

#### *Laboratory Assays - Androgens and SHBG*

Androgens were measured for assessment as potential confounders. Where available, we used existing data on testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEAS), and sex hormone-binding globulin (SHBG). These data were available from previous studies in four of the participating cohorts (CLUE, EPIC, NYUWHS, and Sweden;  $n=193$ , 29%). Laboratory methods for these measurements are provided in **Supplementary Table 1**. For participants without existing data on androgens or SHBG, participating cohorts were asked to provide additional serum (or plasma) volume for these assays. Samples from 235 participants



from the Columbia, EPIC, Guernsey, NYUWHS, and ORDET cohorts were centrally assayed at the laboratory of the Division of Cancer Epidemiology at the German Cancer Research Center (DKFZ; Heidelberg, Germany). Direct radioimmunoassays (Beckman-Coulter) were used to measure testosterone, androstenedione, and DHEAS. SHBG was measured using an immunoradiometric assay (Cis-Bio). The overall coefficients of variation for samples assayed at DKFZ were <22% for all androgens and 21% for SHBG.

### *Statistical Analyses*

AMH concentrations were log<sub>2</sub>-transformed to normalize the distribution; this transformation also allows an estimation of the effect of a doubling of AMH (i.e., one-unit increase in log<sub>2</sub>-transformed AMH corresponds to a doubling). The extreme Studentized deviate (ESD) many-outlier procedure was used to identify outliers (Rosner, 1983); no outliers were identified. Tertiles of AMH were defined using the study-specific distribution in controls; p for trend was calculated using tertile medians. Given that age is a very strong determinant of AMH concentrations, cases and controls were matched on age at blood draw and in addition all models were adjusted for age. Conditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals [CI] across tertiles of AMH concentrations and for a doubling of AMH concentrations (OR<sub>log2</sub>).

To assess between-study heterogeneity we used a random effects model as proposed by DerSimonian and Laird (DerSimonian & Laird, 1986); we observed no significant between-study heterogeneity. Therefore, we present results based on the pooled participant data.

We evaluated the effect of potential confounders (i.e., age at menarche (continuous, 25% missing), body mass index (BMI; continuous; 21% missing), ever use of oral contraceptives (OC; no, yes; 23% missing), total number of pregnancies (0, 1, 2, 3, ≥4; 27% missing), smoking status (never, past, current; 4% missing)) using multiple imputation with

ten imputed datasets, adjusted OR estimates calculated in each of the multiple-imputed datasets and pooled using Rubin's rule (Raghunathan *et al*, 2010). None of the potential confounders were retained in the final models since effect estimates were not impacted by statistical adjustment (<10% change after adjustment) and statistical significance of the observed associations was not affected.

Data on circulating androgen concentrations were available for 221 cases and 207 controls (64%). Adjustments for androgens or SHBG in subjects with available data had a negligible effect on risk estimates (<10% change after adjustment) and thus these markers were not retained in the final models.

Polytomous conditional logistic regression models were used to examine heterogeneity of associations between AMH concentrations and endometrial cancer by subtype defined by tumor-related characteristics (e.g., histology, age at diagnosis). Statistical heterogeneity of associations in stratified analyses was assessed via a likelihood ratio test comparing a model allowing the association for the risk factor of interest to vary by subgroup versus one assuming the same association (Wang *et al*, 2016). We evaluated heterogeneity by oral contraceptive use and age at blood draw by including a multiplicative interaction term in the models and evaluating the Wald p value.

We conducted sensitivity analyses excluding women diagnosed  $\leq 1$  year or  $\leq 2$  years after blood donation to evaluate any effect of subclinical endometrial cancer on AMH concentrations, as well as the exclusion of women with synchronous ovarian cancer. Further sensitivity analyses excluded current OC users, as AMH levels are lower in current users compared to former or never users (Dolleman *et al*, 2013).

Given the final sample size of 329 cases and 339 controls, this study had statistical power to detect an OR of 0.61 or 1.64 with 80% power and 95% confidence when examining tertiles. This uses the observed within matched pair correlation of MIS levels of 0.16. The study was slightly better powered than the protocol specified detectable effect of 0.56 or 1.80

which had anticipated 342 matched pairs but also a larger within-matched pair correlation that would have reduced power. The study also was able to detect an  $OR_{\log 2}$  of 0.87 or 1.15 for a one-unit change in log2-transformed AMH for endometrial cancer overall based on the observed log-2 transformed AMH standard deviation of 2.26. Statistical power was more limited in small subgroups (e.g., type II disease (n=28 cases), 80% power and 95% confidence, minimum detectable  $OR_{\log 2}$  of 0.63 or 1.60).

All statistical analyses were conducted using the Statistical Analyses System (SAS) software, version 9.3 (SAS Institute, Inc., Cary, North Carolina). All statistical tests were two-sided and considered statistically significant at  $p < 0.05$ .

## Results

Median age at blood draw in the study population was 41.5 years, ranging from 38 years in Guernsey and CLUE to 44 years in SWHS (**Table 2; Supplemental Table 2**). Relative to controls, cases had a somewhat higher median BMI ( $kg/m^2$ ; cases: 24.8; controls: 23.7), a higher percentage were nulliparous (cases: 25%; controls: 20%), and a lower percentage reported ever OC use (cases: 46%; controls: 56%). The median age at diagnosis was 54 years and a median of 12 years elapsed between blood draw and diagnosis. As expected AMH was inversely correlated with age at blood collection (spearman:  $r_{cases} = -0.50$ ,  $r_{controls} = -0.43$ , both  $p < 0.01$ ). Weak correlations were observed between androgens and SHBG and AMH (Spearman:  $r_{cases} = -0.27$  (SHBG) to 0.19 (testosterone),  $r_{controls} = -0.03$  (DHEAS) to 0.15 (testosterone)).

We observed no significant association between AMH and risk of endometrial cancer overall ( $OR_{\log 2} = 1.07$  [0.99-1.17]), and results from the pooled individual-level data were similar to that from the meta-analysis ( $OR_{\log 2}$ , meta-analysis=1.05 [0.97-1.15];  $p_{het} = 0.46$ ; **Figure 1**). Similarly, we observed no association comparing extreme tertiles ( $OR_{T3vs.T1} = 1.29$  [0.82-2.03]) (**Table 3**).

Results did not significantly differ by disease subtype (e.g., by histology,  $p_{\text{het}}=0.86$ , endometrioid,  $\text{OR}_{\text{T3vs.T1}}=1.12$  [0.49-2.57],  $p_{\text{trend}}=0.46$ ; adenocarcinoma, NOS,  $\text{OR}_{\text{T3vs.T1}}=1.47$  [0.78-2.75],  $p_{\text{trend}}=0.08$ ) (**Table 3**), age at blood donation ( $p_{\text{het}}=0.13$ ) or ever OC use ( $p_{\text{het}}=0.85$ ). In analyses stratified by cancer-related characteristics, we observed no heterogeneity by age at diagnosis ( $p_{\text{het}}=0.77$ ), time between blood donation and diagnosis ( $p_{\text{het}}=0.81$ ), tumor grade ( $p_{\text{het}}=0.68$ ), stage ( $p_{\text{het}}=0.53$ ), or Type I/II classification ( $p_{\text{het}}=0.70$ ).

Results were similar when restricting analyses to women not using oral contraceptives at blood collection ( $n=291$  sets;  $\text{OR}_{\text{T3 vs. T1}}=1.19$  [0.73-1.93],  $p_{\text{trend}}=0.15$ ;  $\text{OR}_{\log 2}=1.06$  [0.98-1.16]), or to women diagnosed more than 1 year after blood draw ( $n=323$  sets) or 2 years after blood draw ( $n=315$  sets; data not shown). Exclusion of the six cases with synchronous ovarian cancer did not impact the observed effect estimates.

## Discussion

We conducted a world-wide collaborative investigation, including eight prospective cohort studies, and present the first data on pre-diagnosis AMH concentrations and subsequent risk of endometrial cancer. We observed no association between AMH concentrations and risk of endometrial cancer overall, or in analyses stratified by age at blood draw, oral contraceptive use, or cancer-related characteristics.

To date, evidence for an involvement of AMH in the development of endometrial cancer risk comes from experimental models (reviewed in (Kim *et al*, 2014)). Experimental data have shown that AMH inhibits growth of human endometrial cancer cell lines that express the AMHRII by causing cell cycle arrest in the  $G_1$  phase and inducing apoptosis. AMH regulates the proteins p107 and p130, responsible for  $G_1$ -to-S-phase transition and cell-cycle exit, respectively, as well as the transcription factor E2F1, which leads to decreased cell division (Renaud *et al*, 2005). It should be noted that the concentrations used in these experimental models were reported to be double the dose required to induce Mullerian duct

regression in culture (Renaud *et al*, 2005). Similar inhibitory effects have been observed in experimental models of endometrial stromal cells (Wang *et al*, 2009) and of endometriosis (reviewed in (Kim *et al*, 2014); (Signorile *et al*, 2014)). In terms of epidemiologic data, prior studies have noted lower AMH concentrations among women with endometriosis, although findings to date are somewhat inconsistent (reviewed in (Sanchez *et al*, 2016)), and one previous retrospective case-control study observed no difference in circulating AMH concentrations between endometrial cancer cases and controls (Dogan *et al*, 2015). In terms of another gynecologic cancer, experimental data support a role for AMH in the inhibition of epithelial ovarian cancer growth and proliferation (Chang *et al*, 2011; Chin *et al*, 1991; Donahoe *et al*, 1981; Kim *et al*, 1992; Park *et al*, 2017; Stephen *et al*, 2002), though epidemiologic data on AMH and epithelial ovarian cancer are limited (Schock *et al*, 2014). With respect to non-epithelial ovarian cancers, AMH is a marker for ovarian adult granulosa cell tumors (Farkkila *et al*, 2015; Geerts *et al*, 2009; Haltia *et al*, 2017). Epidemiologic studies to date consistently support a positive association between circulating AMH concentrations and breast cancer risk (Dorgan *et al*, 2009; Eliassen *et al*, 2016; Nichols *et al*, 2015). Our findings of a suggestive positive trend for endometrial cancer overall are in line with these observations for breast cancer.

A possible explanation for the lack of association in our study is the hypothesized AMH autocrine/paracrine system in the adult endometrium (Wang *et al*, 2009); it is plausible that circulating concentrations do not reflect AMH concentrations or activity in the endometrium, and thus do not exert an endocrine effect. This is supported by one study among 55 patients with endometriosis and 45 healthy women which observed that circulating AMH concentrations were unaffected by higher AMH mRNA and protein expression in endometriosis lesions (Carrarelli *et al*, 2014). Further support for a paracrine effect of AMH comes from the observation that AMH secreted by each testis, in genetically male embryos, induces regression of only the ipsilateral Mullerian duct (Behringer, 1995).

Strengths of our study include its prospective design, and relatively large sample size, given AMH can only be measured prior to the menopause. Individual-level data on study subjects was uniformly harmonized, standardizing covariate categorization for the statistical analysis, and all AMH assays were performed in a single laboratory using an ultrasensitive AMH assay that is valid and reproducible (Burks *et al*, 2015; Welsh *et al*, 2014). A limitation of our study is that only one AMH measurement was obtained. However, the intra-class correlation of AMH concentrations over one year is high ( $r=0.87$ ) (Dorgan *et al*, 2010), and AMH concentrations track well over time in the same woman before menopause ( $r=0.66$  for two measurements 4 years apart) (van Rooij *et al*, 2005); thus, any misclassification of AMH concentrations is likely to be small. All cases included in this study were diagnosed with incident, primary endometrial cancer and six of these cases were diagnosed with synchronous ovarian cancer; none of the cases included in this study had synchronous ovarian granulosa cell tumors, which would have caused elevated AMH concentrations. Exclusion of the six cases with synchronous ovarian cancer did not impact the observed effect estimates. Samples utilized in this study were from established biorepositories, and have been in storage for up to decades. However, no association between storage time and AMH concentrations was observed in our previous cross-sectional study (Jung *et al*, 2017). Finally, median age at diagnosis in this study was 54 years; this is younger than the median age at diagnosis in the population at large (e.g., median age at diagnosis in the United States: 62 years) (2017). It is plausible that the results observed here for endometrial cancer with younger age at diagnosis are not generalizable to women with later disease onset.

AMH has been proposed as a potential treatment for endometrial cancers (Kim *et al*, 2014). However, while experimental models demonstrate an inhibiting effect of AMH on endometrial cancer growth, our findings in premenopausal women do not support a role for circulating AMH concentrations in the etiology of endometrial cancer.

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The authors assume full responsibility for analyses and interpretation of these data.

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**Data sharing:** For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at <http://epic.iarc.fr/access/index.php>.

## Conflict of Interest

The authors declare no conflicts of interest.

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### **Titles and legends to figures**

**Figure 1.** ORs [95%-CI] for a doubling of AMH concentrations by study cohort and overall association in pooled analysis and meta-analysis: Prospective Study of AMH and Gynecologic Cancer Risk