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ASSISTED REPRODUCTION TECHNOLOGIES

Follicular fluid anti-Müllerian hormone (AMH) concentrations and outcomes of in vitro fertilization cycles with fresh embryo transfer among women at a fertility center

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Abstract

Purpose To enhance the understanding of the clinical significance of anti-Müllerian hormone (AMH) in follicular fluid, we aimed to determine the variability of AMH concentrations in follicular fluid within and across IVF cycles and whether high follicular fluid AMH concentrations are associated with improved clinical IVF outcomes.

Methods This was a retrospective cohort study of companion follicular fluid and serum samples from 162 women enrolled in the Environment and Reproductive Health (EARTH) Study between 2010 and 2016. AMH concentrations were quantified using a sandwich enzyme-linked immunosorbent assay. Spearman correlation and intra-class correlation (ICC) were calculated to assess variability of follicular fluid AMH, and generalized linear mixed models were used to evaluate the associations of FF AMH with IVF outcomes.

Results The median (interquartile range, IQR) age of the 162 women was 34.0 years (32.0, 37.0). Follicular fluid AMH concentrations were highly correlated between follicles within each IVF cycle (Spearman $r = 0.78$ to 0.86) and across cycles for each woman (ICC 0.87 (95% CI 0.81 to 0.92)). Compared with women in the highest tertile of FF AMH (mean AMH = 2.3 ng/ml), women in the lowest tertile (mean AMH = 0.2 ng/ml) had lower serum AMH (T1 = 0.1 ng/ml vs. T3 = 0.6 ng/ml, $p < 0.0001$). In adjusted models, higher tertiles of follicular fluid AMH concentrations were associated with lower mean endometrial thickness and higher probability of clinical pregnancy.

Conclusions Follicular fluid AMH concentrations show little variability between pre-ovulatory follicles, and higher pre-ovulatory follicular fluid AMH may predict a higher probability of clinical pregnancy.

Keywords Follicular fluid · Müllerian inhibiting substance/anti-Müllerian hormone · IVF/ICSI outcome · Pregnancy

Introduction

Serum anti-Müllerian hormone (AMH), or MIS (Müllerian inhibiting substance), has emerged as an important, currently indispensable, indicator of ovarian reserve in reproductive-age women. AMH is produced by ovarian granulosa cells, primarily in pre-antral and small antral follicles less than 8 mm in diameter, and thus, serum concentration appears to reflect the numbers of these growing follicles [1–3]. Serum levels are particularly helpful in predicting a woman's response to ovarian stimulation during IVF [4, 5]. In fertile women, follicular AMH production declines precipitously with a follicular diameter over 10 mm, and AMH concentration in pre-ovulatory follicles is low. The decrease in AMH as the cycle progresses releases the follicles from its inhibitory effect on folliculogenesis and premature maturation [1, 6, 7].

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Our understanding of the regulation of AMH in pre-ovulatory follicular fluid (FF), however, is limited. Schenk et al. attempted to address AMH variation between follicles by studying AMH concentrations in all individual follicles over 10 mm retrieved from 20 women [8]. In this small cohort of patients undergoing IVF, the mean level of FF AMH from all follicles correlated with serum AMH, as did individual FF AMH levels from the first five dominant follicles retrieved. Although a large range of follicle sizes were assessed, the authors concluded that FF AMH levels from individual follicles reflect serum AMH. Whether this low inter-follicle variability is present in a larger cohort of patients and whether FF AMH varies over time across IVF cycles in the same woman has not yet been explored.

Efforts to elucidate the clinical significance of FF AMH have been similarly limited by small study populations. FF AMH levels appear to be elevated in women with polycystic ovarian syndrome (PCOS) due to both a larger number of small antral follicles and evidence of elevated intrafollicular AMH production for a given follicle diameter [9–11]. Women with anovulation have also been shown to have higher pooled FF AMH on average than those with other infertility diagnoses [12]. However, literature regarding the correlation between FF AMH and oocyte yield, embryo quality, and pregnancy outcomes is sparse and conflicted. Mashiach et al. reported a non-significant association between FF AMH levels from one dominant follicle and embryo quality in 11 women with PCOS but found that FF AMH did not predict mature oocyte yield or fertilization rate in those women [13]. In contrast, a study by Kim et al. reported that FF AMH levels in a dominant pre-ovulatory follicle in normally ovulating women are positively associated with improved embryo quality and low FF AMH may be associated with a lower fertilization rate, suggesting that FF AMH may be reflective of oocyte quality [14]. Finally, Fanchin et al. found that higher FF AMH from a single dominant follicle, but not serum AMH, was associated with improved implantation rate and clinical pregnancy rate [15].

Given the mixed data produced by these studies, we aimed to investigate both the correlation of FF AMH levels between pre-ovulatory follicles within and across IVF cycles and the associations of FF AMH with reproductive characteristics and clinical IVF outcomes among women treated at an academic fertility center.

Materials and methods

Study design

Participants included in this analysis were women seeking fertility care at the Massachusetts General Hospital (MGH) Fertility Center who enrolled in the Environment and Reproductive Health (EARTH) Study, a prospective cohort study established in 2004 that aimed to investigate environmental exposures in relation to reproductive health [16]. Women between the ages of 18 and 45 years old were eligible to enroll. Our center additionally has a maximum body mass index (BMI) threshold of 40 kg/m^2 to undergo treatment. The EARTH Study is approved by the institutional review boards at MGH (Partners IRB #1999P008167), Harvard T.H. Chan School of Public Health, and the Centers for Disease Control and Prevention (CDC) [16]. Participants signed an informed consent prior to study enrollment.

FF was analyzed from 162 women (EARTH study participants between 2010 and 2016) contributing 217 IVF cycles, who also had a serum sample available. Both FF and serum AMH concentrations were measured by the same laboratory. A total of 304 women who underwent 433 fresh IVF cycles between 2010 and 2016 were excluded since they did not have at least two FF samples and serum available. These excluded women had similar demographic and reproductive characteristics compared with women included in this analysis (Table 1).

Ovarian stimulation protocols

All study participants underwent a standard infertility work-up as previously described [16, 17]. Infertility diagnosis was assigned according to previously described definitions of the Society for Assisted Reproductive Technology (SART) [18]. SART diagnoses include (1) male factor infertility which included poor semen quantity/quality; (2) female factor infertility which included endometriosis, diminished ovarian reserve, tubal or ovulatory disorders, or other causes; and (3) unexplained infertility (idiopathic).

Patients then underwent controlled ovarian hyperstimulation by luteal-phase gonadotropin-releasing hormone (GnRH) agonist, GnRH antagonist downregulation, or GnRH agonist flare protocol, with follicular synchronization and pituitary downregulation as clinically indicated and previously described [17, 19–21]. During treatment with recombinant gonadotropins (follitropin beta, Follistim, Merck, Kenilworth, NJ, or follitropin alpha, Gonal-F, EMD-Serono; and menotropins, Menopur or Repronex, Ferring Pharmaceuticals, Parsippany, NJ), patients were serially monitored with transvaginal ultrasound and serum estradiol (E_2) to assess follicular measurements and endometrial thickness. Once at least three follicles reached 16 mm or more in diameter and the E_2 level was > 600 pg/mL, intramuscular human chorionic gonadotropin (hCG) (10,000 IU, Novarel, Ferring Pharmaceuticals or 10,000 IU, Pregnyl, Merck) was administered to induce final oocyte maturation. The peak serum E_2 concentration was defined as the highest level of E_2 preceding the oocyte retrieval and obtained on the day of hCG administration. The patients underwent a transvaginal ultrasound-

Table 1 Demographic and reproductive characteristics of women with and without FF AMH concentrations between 2010 and 2016 in the EARTH Study

	162 women included (217 IVF cycles)	304 women excluded (433 IVF cycles)
Age, years[a]	34.0 (32.0, 37.0)	35.0 (32.0, 38.0)
Race, *N* (%)		
White	135 (83)	250 (83)
Black	5 (3)	11 (4)
Asian	16 (10)	32 (10)
Other	6 (4)	12 (4)
Body mass index[a], kg/m^2	22.7 (21.0, 25.8)	23.2 (21.3, 26.3)
Ever smoked, *N* (%)	38 (24)	78 (26)
Education, *N* (%)		
High school/some college	12 (7)	22 (7)
College graduate	48 (30)	86 (28)
Graduate degree	102 (63)	196 (64)
Infertility diagnosis, *n* (%)		
Male factor	77 (35)	122 (28)
Female factor	55 (26)	138 (32)
Diminished ovarian reserve	11 (5)	38 (9)
Endometriosis	7 (3)	28 (6)
Ovulatory disorders	20 (9)	37 (9)
Tubal	15 (7)	28 (6)
Uterine	2 (1)	7 (2)
Idiopathic	85 (39)	173 (40)
Treatment protocol, *n* (%)		
Antagonist	34 (16)	67 (16)
Flare	33 (15)	78 (18)
Luteal-phase agonist	150 (69)	288 (67)
Peak E_2, pmol/L[a]	2026 (1544, 2597)	1940 (1512, 2697)
ICSI cycles, *n* (%)	121 (56)	220 (53)
Day of embryo transfer[a]	3 (3, 5)	3 (3, 5)
Number of embryos transferred[a]	2 (1, 2)	2 (1, 2)
Serum AMH concentrations, ng/ml[a]	0.4 (0, 0.7)	–
Day 3 FSH levels, IU/L[a]	6.6 (5.9, 8.0)	6.9 (5.9, 8.1)
Implantation, *n* (%)	127 (58)	243 (56)
Clinical pregnancy, *n* (%)	112 (52)	211 (49)
Live birth, *n* (%)	91 (42)	196 (39)

[a] Median (IQR)

guided oocyte retrieval 35–37 h later [19]. Intramuscular progesterone, 50 mg per day, was started on the day after oocyte retrieval and continued until 10 weeks of gestation if the patient conceived.

Serum sample collection

Serum follicle stimulating hormone (FSH) was measured in a blood sample collected on the third day of the menstrual cycle using an automated electrochemiluminescence immunoassay at the MGH Core Laboratory as previously described (LOD = 0.1 U/L) [22]. Peak E_2 levels were also measured at the MGH Core Laboratory as previously described [22]. Blood samples collected during the monitoring (follicular) phase of the IVF cycle were used for AMH measurement using the methods described below.

Follicular fluid collection

FF was collected from the first three ($N = 116$ women, 72%) or two ($N = 46$ women, 28%) dominant (> 16 mm) follicles punctured during each patient's egg retrieval to minimize

contamination with blood during the later stages of the procedure. The fluid from each follicle was independently aspirated into a tube with 1 ml sterile culture media so that FF volume could be accurately calculated. After identification and transfer of the oocytes by the embryologist, the FF from each follicle was transferred to a 15-ml collection tube and the collection time and sample volumes logged. The FF was then centrifuged at 3000 rpm for 10 min, and the supernatant was stored in aliquots at − 80C, taking care not to pool follicular fluid from different follicles.

Detection of serum and follicular fluid AMH using sandwich enzyme-linked immunosorbent assay

AMH concentration was quantified in serum and FF of the study patients using a sandwich enzyme-linked immunosorbent assay (ELISA) with mouse monoclonal anti-human recombinant antibody (6E11), which binds to the AMH homodimer, as the primary antibody, and rabbit polyclonal anti-AMH antibody (MGH6), as the secondary antibody [23, 24]. The standard curve was created using a recombinant AMH protein "LRMIS" (created from AMH/MIS cDNA with human albumin leader sequence (L) and modified cleavage site (R)) in blocking buffer (1% BSA/PBS and Tween 20 (PBST)), which has been recommended as an international standard by the WHO [25, 26]. This ELISA produces absolute AMH concentrations that correlate closely with the Gen II Elisa commercial assay used at our center during the study time period (Spearman $r^2 = 0.81$, evaluated for a subset of 22 women with serum AMH levels previously performed for their fertility work-up). Intra-assay variability was 12.0% and inter-assay variability was 15.5% with a limit of detection of 0.034–0.068 ng/ml. Values below this level represent extrapolation from the standard curve. Results were corrected for sample culture media volume.

Clinical outcomes

Embryologists determined the total and mature oocyte yield per cycle. Oocytes underwent either conventional IVF or intracytoplasmic sperm injection (ICSI) as clinically indicated. The fertilization rate was determined 17–20 h after insemination as the number of oocytes with two pronuclei divided by the number of MII oocytes inseminated. We classified embryo quality based on morphology and number of blastomeres, ranging from 1 (best) to 5 (worst) on days 2 and 3. High-quality embryos were defined as having 4 cells on day 2, 8 cells on day 3, and a morphologic quality score of 1 or 2 on days 2 and 3 [27, 28]. An overall score of 1 or 2 was considered high quality; 3 was considered intermediate-quality and 4 or 5 indicated poor-quality embryos.

Embryo transfer was performed on day 2, 3, or 5 based on number of embryos and their quality according to clinic protocol. Implantation was defined as a serum β-hCG level > 6 mIU/ml, typically measured 17 days (range 15–20 days) after oocyte retrieval. An elevation in β-hCG with the confirmation of an intrauterine pregnancy on an ultrasound at 6 weeks was considered a clinical pregnancy. A live birth was defined as the birth of a neonate on or after 24 weeks of gestation. Clinical information was abstracted from the patient's electronic medical record by research staff.

Statistical analyses

Demographic and reproductive characteristics of the study participants were presented using median ± interquartile ranges (IQRs) or counts (%). Mean intra-cycle FF AMH concentrations were calculated as the sum of FF AMH concentrations in the two or three selected follicles, divided per cycle by the number of follicles. Spearman correlations were used to assess the correlation of AMH concentrations between FF and serum as well as between the follicles evaluated within each IVF cycle. The intra-class correlation coefficient (ICC) from a multivariable mixed model was calculated to assess variability of mean intra-cycle FF AMH concentrations across cycles within women. Mean intra-cycle FF AMH concentrations were divided into tertiles (T1–T3) to minimize the effect of outliers. Associations between demographics and reproductive characteristics across tertiles of mean FF AMH concentrations per cycle were evaluated using Kruskal–Wallis tests for continuous variables and chi-squared tests for categorical variables (or Fisher's exact test where appropriate). We used generalized linear mixed models to evaluate the associations of tertiles of FF AMH concentrations with IVF outcomes, with a random intercept to account for within-women correlation in outcomes over multiple IVF cycles, and adjustment for potential confounders. A binomial distribution and logit link function were specified for outcomes of implantation, clinical pregnancy, and live birth.

Tests for linear trends across tertiles of FF AMH concentrations were conducted using ordinal level indicator variables for each tertile and the first tertile was considered the reference group. To allow for better interpretation of the results, population marginal means [29] are presented adjusting for all the covariates in the model (at the mean level for continuous variables and weighted according to their relative frequencies for categorical variables). Confounding was assessed using prior knowledge on biological relevance and descriptive statistics from our study population. The variables considered as potential confounders included factors previously related to female reproductive endpoints [30, 31], and factors associated with FF AMH concentrations and reproductive outcomes in this study. Final models were adjusted for age, race, body mass index (BMI), and stimulation protocol type. Statistical analyses were conducted with SAS (version

9.4; SAS Institute Inc., Cary, NC, USA). Statistical tests were two-tailed and all p values < 0.05 were regarded as statistically significant.

Results

Study population

Participants' baseline demographic and clinical characteristics are shown in Table 1. The median (interquartile range, IQR) age and body mass index (BMI) of the women were 34.0 years (32.0, 37.0) and 22.7 kg/m^2 (21.0, 25.8), respectively. The majority of patients were white (83%), with a college or graduate degree (93%). The most common infertility diagnoses were idiopathic (39%) and male factor (35%). Most patients underwent a luteal-phase GnRH agonist stimulation protocol (69%), and over half underwent ICSI (56%). Day 3 and day 5 embryo transfers occurred in 47% and 42% of cycles, respectively. The median number of embryos transferred was 2 on day 3.

The median and mean FF AMH concentration from 217 IVF cycles were 0.55 and 1.20 ng/ml, respectively (range = 0 to 24.0 ng/ml, Supplemental Table 1). Of the 162 women, 47 underwent 2 IVF cycles during the study period, and 7 underwent 3 cycles. Cycles ranged from 2 to 13 months apart. Follicular fluid AMH concentrations were highly correlated between follicles within each IVF cycle (range of Spearman $r = 0.78$ to 0.86), indicating low variability within each IVF cycle (Table 2). Moreover, the ICC indicated low within-woman variability in mean intra-cycle FF AMH levels across IVF cycles (ICC 0.87 (95% CI 0.81 to 0.92); Table 2).

Demographic and clinical predictors of follicular fluid AMH concentration

The tertiles of mean IVF cycle FF AMH levels are shown in Table 3. Compared with women in T3 of FF AMH concentrations (high follicular fluid AMH, mean = 2.3 ng/ml), women in T1 (low follicular fluid AMH, mean = 0.2 ng/ml) were older (median age in T1 = 36.0 years vs. T3 = 33.5, $p = 0.04$) and heavier (median BMI in T1 = 24.5 kg/m^2 vs. T3 = 22.4 kg/m^2, $p = 0.04$). There were no differences in smoking history or education level across tertiles of FF AMH concentrations.

Women in the highest tertile of FF AMH (T3) were more likely to have undergone a luteal-phase GnRH agonist protocol compared with women in T1 (T3 = 79% vs. T1 = 54%, $p = 0.01$; Table 3). Consistent with this finding, women in T3 also had significantly higher serum AMH concentrations (median in T3 = 0.6 ng/ml vs. T1 = 0.1 ng/ml; $p < 0.0001$; Table 3) and lower day 3 follicular stimulating hormone (FSH) levels (median T3 = 6.4 IU/L vs. T1 = 7.0 IU/L, $p = 0.03$). Peak E_2 levels and intracytoplasmic sperm injection (ICSI) usage were not significantly associated with pre-ovulatory FF AMH concentrations. Although most diagnoses were similar across tertiles of FF AMH concentrations, women in T3 were more often diagnosed with ovulatory disorders compared with women in T1 (T3 = 17% vs. T1 = 4%, $p = 0.30$), an expected finding given the known association of PCOS with higher FF AMH. However, this did not reach statistical significance due to low power (14 cycles had a PCOS diagnosis). Overall, serum and pre-ovulatory mean FF AMH concentrations were moderately correlated in our population of women undergoing IVF (Spearman $r = 0.48$; Table 2).

Table 2 Comparison of mean values between AMH concentrations (ng/ml) in follicular fluid and serum among 162 women contributing 217 IVF cycles in the EARTH Study

AMH concentrations in follicle 1 (F1) (mean, 95% CI)	1.13 (0, 2.15)
AMH concentrations in follicle 2 (F2) (mean, 95% CI)	1.27 (0, 4.40)
AMH concentrations in follicle 3 (F3) (mean, 95% CI)	1.19 (0, 5.18)
Spearman correlations between AMH concentrations in different follicles	F1 vs F2 = 0.86 ($p < 0.0001$)
	F1 vs F3 = 0.78 ($p < 0.0001$)
	F2 vs F3 = 0.85 ($p < 0.0001$)
Mean of follicular fluid AMH concentrations per cycle (mean, 95% CI)	1.20 (0, 4.35)
ICC of mean follicular fluid AMH concentrations per cycle (mean, 95% CI)	0.87 (0.81, 0.92)
Serum AMH concentrations (mean, 95% CI)	0.55 (0, 1.73)
Spearman correlation between mean FF and serum AMH concentrations (r, p value)	0.48 ($p < 0.0001$)

Table 3 Demographic and reproductive characteristics (median (IQR) or *N* (%)) by tertiles of mean follicular fluid AMH concentrations

	Tertiles of total follicular fluid AMH concentrations			
	T1	T2	T3	*p* value[a]
FF AMH concentrations, ng/ml	0.2 (0.1, 0.2)	0.6 (0.5, 0.9)	2.3 (1.5, 3.7)	0.0001
Demographics				
Age, years	36.0 (33.0, 39.0)	33.0 (31.0, 37.0)	33.5 (32.0, 37.0)	0.04
Race, *N* (%)				0.03
White	46 (80)	54 (91)	35 (76)	
Black	2 (4)	3 (5)	0 (0)	
Asian	7 (12)	1 (2)	8 (17)	
Other	2 (4)	1 (2)	3 (7)	
Body mass index, kg/m^2	24.5 (21.4, 27.5)	22.6 (20.6, 24.5)	22.4 (20.5, 25.2)	0.04
Ever smoked, *N* (%)	14 (25)	15 (25)	9 (20)	0.75
Education, *N* (%)				0.12
High school/some college	3 (5)	3 (5)	6 (13)	
College graduate	12 (21)	23 (39)	13 (28)	
Graduate degree	42 (74)	33 (56)	27 (59)	
Cycle-reproductive characteristics				
Infertility diagnosis, *n* (%)				0.30
Male factor	26 (36)	26 (36)	25 (35)	
Female factor	16 (22)	18 (26)	19 (27)	
Diminished ovarian reserve	5 (7)	2 (3)	2 (3)	
Endometriosis	3 (4)	2 (3)	2 (3)	
Ovulatory disorders	3 (4)	5 (7)	12 (17)	
Tubal	5 (7)	7 (10)	3 (4)	
Uterine	0 (0)	2 (3)	0 (0)	
Idiopathic	30 (42)	27 (38)	28 (38)	
Treatment protocol, *n* (%)				0.01
Antagonist	15 (21)	11 (15)	8 (11)	
Flare	18 (25)	8 (11)	7 (10)	
Luteal-phase agonist	39 (54)	54 (74)	57 (79)	
Peak E_2, pmol/L	1982 (1484, 2612)	1979 (1591, 2643)	2110 (1557, 2443)	0.81
ICSI cycles, *n* (%)	42 (59)	34 (47)	45 (63)	0.12
Serum AMH concentrations, ng/ml	0.1 (0, 0.3)	0.4 (0.1, 0.8)	0.6 (0.3, 1.2)	0.0001
Day 3 FSH levels, IU/L	7.0 (6.3, 8.6)	6.5 (5.7, 8.0)	6.4 (5.6, 7.5)	0.03

Follicular fluid AMH and clinical IVF outcomes

Total oocyte yield (T1 = 11.4 (95% CI 10.2, 12.9); T2 = 11.7 (95% CI 10.5, 13.1); T3 = 12.0 (95% CI 10.7, 13.5), *p* trend = 0.51), mature oocyte yield (T1 = 9.4 (95% CI 8.4, 10.6); T2 = 9.6 (95% CI 8.6, 10.8); T3 = 9.9 (95% CI 8.9, 11.2), *p* trend = 0.49), fertilization rate (T1 = 0.73 (95% CI 0.68, 0.78); T2 = 0.71 (95% CI 0.66, 0.76); T3 = 0.71 (95% CI 0.65, 0.76), *p* trend = 0.51), high-quality blastocyst yield (T1 = 0.45 (95% CI 0.32, 0.59); T2 = 0.36 (95% CI 0.24, 0.50); T3 = 0.32 (95% CI 0.21, 0.46), *p* trend = 0.20), and probability of implantation (T1 = 0.55 (95% CI 0.43, 0.67); T2 = 0.57 (95% CI 0.45, 0.68); T3 = 0.64 (95% CI 0.52, 0.74), *p* trend = 0.29) did not differ significantly by FF AMH concentration in the adjusted models (Table 4). However, higher FF AMH concentrations were associated with lower mean endometrial thickness (T1 = 10.9 mm (95% CI 10.2, 11.5); T2 = 10.2 mm (95% CI 9.6, 10.8); T3 = 10.0 mm (95% CI, 9.3, 10.6), *p* trend = 0.03) and higher probability of clinical pregnancy (T1 = 0.41 (95% CI 0.30, 0.53); T2 = 0.53 (95% CI 0.41, 0.64); T3 = 0.60 (95% CI 0.48, 0.70), *p* trend = 0.03) in adjusted models. These findings remained when models were additionally adjusted for PCOS diagnosis and when FF AMH was treated as a continuous variable (data not shown). Although non-significant, higher probability of live birth across tertiles of FF AMH concentrations was observed in the crude models (T1 = 0.29

Table 4 Crude and adjusted early developmental and pregnancy outcomes (adjusted mean, 95% CI) by tertiles of serum FF AMH concentrations among 161 women undergoing 217 IVF cycles in the EARTH Study

	Total oocyte yield (*n*)	MII oocyte yield (*n*)	Endometrial wall thickness (mm)	Fertilization (rate)	High-quality blastocyst yield (proportion)	Implantation (probability)	Clinical pregnancy (probability)	Live birth (probability)
Crude								
T1	11.9 (10.4, 13.5)	9.3 (8.3, 10.5)	10.9 (10.3, 11.5)	0.72 (0.67, 0.77)	0.46 (0.33, 0. 59)	0.53 (0.42, 0.64)	0.40 (0.30, 0.51)	0.29 (0.20, 0.41)
T2	12.1 (10.6, 13.7)	9.7 (8.7, 10.9)	10.2 (9.7, 10.8)	0.72 (0.67, 0.77)	0.36 (0.25, 0.50)	0.59 (0.47, 0.69)	0.54 (0.43, 0.65)	0.52 (0.40, 0.63
T3	13.2 (11.8, 14.9)	10.1 (9.00, 11.3)	9.96 (9.4, 10.6)	0.71 (0.65, 0.75)	0.32 (0.02, 0.45)	0.64 (0.52, 0.74)	0.59 (0.48, 0.70)	0.44 (0.33, 0.56)
p t-r-0.07	end	0.38	0.34	0.02	0.65	0.13	0.19	0.02
Adjusted								
T1	11.4 (10.2, 12.9)	9.4 (8.4, 10.6)	10.9 (10.2, 11.5)	0.73 (0.68, 0.78)	0.45 (0.32, 0.59)	0.55 (0.43, 0.67)	0.41 (0.30, 0.53)	0.31 (0.21, 0.43)
T2	11.7 (10.5, 13.1)	9.6 (8.6, 10.8)	10.2 (9.6, 10.8)	0.71 (0.66, 0.76)	0.36 (0.24, 0.50)	0.57 (0.45, 0.68)	0.53 (0.41, 0.64)	0.49 (0.37, 0.61)
T3	12.0 (10.7, 13.5)	9.9 (8.9, 11.2)	10.0 (9.3, 10.6)	0.71 (0.65, 0.76)	0.32 (0.21, 0.46)	0.64 (0.52, 0.74)	0.60 (0.48, 0.70)	0.43 (0.32, 0.55)
p t-r-0.19	end	0.51	0.49	0.03	0.51	0.20	0.29	0.03

Models were adjusted for maternal age, body mass index, race, and protocol type

(95% CI 0.20, 0.41); T2 = 0.52 (95% CI 0.40, 0.63); T3 = 0.44 (95% CI 0.33, 0.56), *p* trend = 0.07); these differences were attenuated in adjusted models (T1 = 0.31 (95% CI 0.21, 0.43); T2 = 0.49 (95% CI 0.37, 0.61); T3 = 0.43 (95% CI 0.32, 0.55), *p* trend = 0.19).

Discussion

We investigated variability of FF anti-Müllerian hormone (AMH) concentrations within and across IVF cycles, and its association with clinical IVF outcomes among 162 women contributing 217 IVF cycles and attending a fertility center. We observed low within-woman variability in pre-ovulatory follicular fluid AMH within and across IVF cycles and higher probabilities of clinical pregnancy with higher pre-ovulatory FF AMH concentrations. While AMH has long been known to be present within ovarian FF, its function in pre-ovulatory follicles, its correlation with serum AMH, and its association with pregnancy outcomes are not yet fully understood. Thus, our findings in a large cohort of women add novel information regarding the predictive value of FF AMH and how the follicular environment may be altered in women with infertility.

Our results showing low variability of FF AMH concentration between pre-ovulatory follicles within a cycle are consistent with those of Schenk et al. (2017) [8], though we demonstrate this finding in a much larger and demographically diverse cohort of women who were pursuing IVF for a range of diagnoses. FF AMH concentration was not only consistent across IVF cycles for a given women but also appeared to be associated with the woman's age and serum AMH, and inversely associated with day 3 FSH. Additionally, women with a higher BMI tended to have lower FF AMH, suggesting that body habitus, regardless of concomitant ovulatory dysfunction, may impact the intrafollicular hormonal milieu. There was no significant association between FF AMH and infertility diagnoses, including PCOS. Furthermore, adjusting for this diagnosis did not alter our clinical findings. The lack of association between higher FF AMH and PCOS diagnosis is likely a consequence of inadequate power given the consistency of prior literature on this subject.

FF AMH was not associated with total or mature oocyte yield, which was consistent with another previous report [13]. An association between FF AMH and total oocyte yield could be masked by protocol adjustments by clinicians to avoid hyperstimulation in patients with a high response to gonadotropins, although we attempted to control for stimulation protocol given the evidence that exposure to GnRH agonists can alter serum and FF AMH [32, 33]. Although some studies report a potential association between pre-ovulatory FF

AMH and embryo quality, fertilization rate, and implantation rate [13–15], we did not observe an association with these outcomes.

On the other hand, higher FF AMH concentration was associated with a higher probability of clinical pregnancy in our study, after controlling for age, race, BMI, and stimulation protocol. In contrast, serum AMH has been repeatedly correlated with ovarian response to stimulation in IVF cycles and resulting oocyte yield in many studies [4, 5, 34–36], but has been shown to be an unreliable predictor of clinical pregnancy and live birth [37–39]. Mean FF AMH reflected other markers of ovarian reserve for the women in our cohort, including a moderate correlation with serum AMH, but the finding that FF AMH is associated with probability of clinical pregnancy suggests that it may play a distinct role locally in oocyte development and quality. The moderate, rather than high, correlation between FF and serum AMH may be further explained by AMH production from all follicles contributing to circulating AMH levels and the measurement of serum AMH during a variable time point in the follicular phase of the IVF cycle.

We also found an association between higher FF AMH concentration and lower endometrial thickness at the time of trigger. An association between AMH production and endometrial lining thickness should be explored further, as no studies have examined this to our knowledge. However, several studies suggest that AMH may inhibit endometrial development and that high endogenous AMH could affect uterine function during pregnancy in women with PCOS [40–42]. In the present cohort, this finding likely has little clinical significance in each cycle because the reduction in thickness was less than 1 mm and on average patients had excellent lining development.

A main strength of this study is the evaluation of FF AMH in a large cohort of women treated at a single institution with a consistent method for follicular fluid collection per EARTH Study protocols. There were at least two follicles per patient and 55 patients had follicles from multiple IVF cycles, allowing us to assess both within cycle and across cycle variability in pre-ovulatory FF AMH. The same assay for both serum and follicular fluid detection of AMH was used to reduce potential inter-assay variability, which can greatly alter results [26]. The AMH values reported here are best interpreted as relative values between study tertiles rather than absolute values, as they differ from those obtained from commercial assays. While prior studies focused disproportionately on women with PCOS, our cohort included all infertility diagnoses. Importantly, the AMH concentrations in pre-ovulatory follicles established across this population of women undergoing IVF allowed us to explore how follicular fluid AMH correlates with IVF outcomes, including clinical pregnancy and live birth outcomes, improving upon prior conflicting small studies.

A limitation of our analysis is the lack of follicle diameter measurements, though we assumed that precise volumes can serve as a proxy for follicle size. We were also not able to track IVF outcomes for the individual oocytes retrieved from the dominant follicles. Stimulation and transfer protocols have changed over time, and so data from our earliest study patients may not be generalizable to the present population. Due to limited specimen volumes, fluid from each follicle was assessed in duplicate. The in-house ELISA used to measure AMH concentrations was highly sensitive, but all assays involve inherent variability. Sample contamination with blood from the puncture procedure cannot be ruled out, and importantly is a limitation common to all studies analyzing components of FF obtained during oocyte retrieval. We took maternal age, race, BMI, and stimulation protocol in account as likely confounding variables, but there may be other patient or cycle characteristics affecting FF AMH levels or IVF outcomes that we were not able to control. Because patients were referred by their primary fertility physician to participate in the EARTH Study and had to agree to a time-intensive study, selection bias may affect our results. While it may not be possible to generalize our findings to women in the general population, subfertile couples are an important public health subpopulation given the decreasing birth rates in the US general population [43] and growing number of babies born using medically assisted reproduction in the USA, estimated to be > 250,000 births per year and over 1 million over the next 10 years [44–46].

This study assessed within and across cycle variability in pre-ovulatory FF AMH concentrations, as well as the association of FF AMH with IVF outcomes, in the largest cohort of women undergoing IVF yet studied. We conclude that, since variability in pre-ovulatory FF AMH concentrations is low within and across IVF cycles, a dominant follicle's AMH concentration may reflect the overall FF AMH concentration of the pre-ovulatory follicular cohort. Furthermore, serum AMH correlates moderately with FF AMH, prompting a need for additional investigation of the local role of AMH in pre-ovulatory follicles and how the transport of AMH from follicles into the circulation is regulated. Finally, higher FF AMH appears to be associated with a significantly higher probability of clinical pregnancy, an observation that should be explored further in both fertile and subfertile women.

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interpretation, and revision of the manuscript. All authors approved of the manuscript in its final form.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This study was approved by the applicable institutional review boards and was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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