POSTER A-074

Novel Anti-Müllerian Hormone ELISAs: Help Diagnose Polycystic Ovary Syndrome*.

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ABSTRACT

Objective: The aim of this study was to measure circulating anti-Müllerian hormone (AMH) levels in women with polycystic ovary syndrome (PCOS) and normal controls using well-characterized AMH ELISAs.

Relevance: There is a growing interest in the role and measurement of AMH in PCOS. It is a syndrome characterized by hyperandrogenism, ovulatory dysfunction and polycystic ovary morphology. Since the assessment of ovarian morphology requires ultrasonography, there has been considerable interest in identifying biochemical proxies for PCOS-associated changes in folliculogenesis. AMH may be such a proxy. Within the ovary, highest levels of AMH are expressed by the granulosa cells of small antral follicles <4 mm in diameter. A high circulating AMH concentration identifies women with an unusually high number of small antral follicles. Classically, these will be women with polycystic ovaries. Accordingly, it may be possible to use AMH as a diagnostic tool to differentiate PCOS from its age-matched healthy subjects. Current commercial AMH immunoassays are designed to measure pro-mature AMH complex and may not detect the cleaved pro-region fragment if present in circulation. The development of new immunological methods to measure AMH isoforms in circulation is needed to better understand the role of AMH in PCOS.

Methodology: Two independent ELISA methods (24/32 & 10/24) based on antibody pairing against linear epitopes in the mid-region capture and mature-region detection, and pro-region capture and mid-region detection have been developed to measure circulating levels of AMH. Serum from 368 PCOS and 192 aged matched control subjects were studied and the diagnostic accuracy was calculated dividing the sum of true positives and true negatives by the total number of subjects.

Results: The limit of detection of 24/32 and 10/24 ELISAs were 1.0 and 0.5 pg/mL, respectively. Total imprecision measured on two controls (70.4 pg/mL, 221.4 pg/mL) using 24/32 and 10/24 AMH ELISAs over 22 runs were 6.6%, 6.8% and 4.1%, 5.0%, respectively. Linearity of dilution plot (multiple dilutions of 5 samples) resulted in a slope of 1.0 and a p value of <0.0001 in both ELISAs. The median AMH levels for the 24/32 ELISA and 10/24 ELISAs showed significant difference between the control and the PCOS subjects (10.14 vs 2.71 ng/ml, and 6.05 vs 1.78 ng/mL, respectively). ROC analysis for each ELISA was used to establish the cut-offs for diagnosing PCOS subjects (characterized by NIH criteria). The sensitivity, specificity and diagnostic accuracy of 0.84, 0.83, 83.6 at a cut-off of 5.0 ng/mL and 0.85, 0.83, 84.3 at a cut-off of 3.0 ng/mL were observed for 24/32 and 10/24 ELISA, respectively. Higher prevalence of PCOS was observed in sisters of PCOS subjects (43 out of 113 subjects) using the ELISAs.

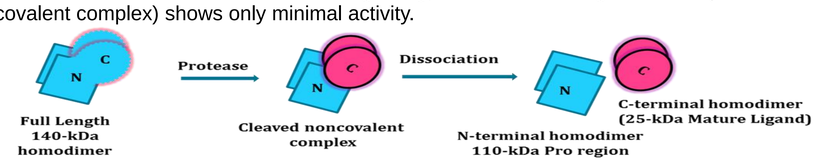
Conclusions: Highly sensitive, specific and precise AMH ELISAs have been developed to measure circulating forms of AMH in PCOS subjects. AMH levels in PCOS subjects were highly elevated and were significantly different than the control group. The diagnostic accuracy of 85% was obtained by the novel ELISAs where subjects were characterized by NIH criteria.

WHY IS AMH TEST NEEDED?

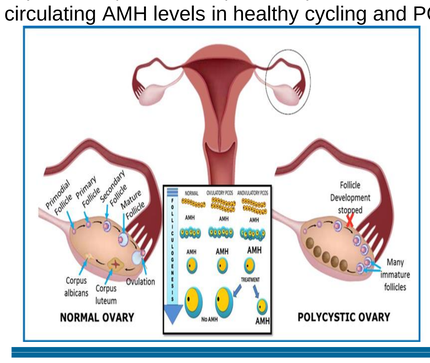
- There is no single diagnostic criterion which is sufficient for diagnosis, as by definition it must combine ovarian dysfunction, including polycystic morphology with other features, clinical or biochemical, of hyperandrogenism.
- Polycystic morphology determined by ultrasound is not reliable in the diagnosis of polycystic ovaries in adolescent and young women. Up to 70% of young women may have polycystic ovaries on ultrasound. In young women, menstrual cycles may take up to 2 years to regulate after menarche.
- Hirsutism is difficult to assess as most women treat this, so it is not obvious on examination. Moreover, the measurements of free testosterone by immunological methods are not very accurate and precise as use of hormonal contraceptive affects the free testosterone concentration in circulation.
- AMH in circulation strongly correlates to the Antral Follicle Count (AFC) and may replace the invasive and expensive vaginal ultrasound measurements.
- The test may also help accurately assess females' ovarian reserve (egg counts, reproductive years and time to menopause) and prevent ovarian hyper stimulation during in-vitro fertilization procedure (high occurrence in PCOS).

HYPOTHESIS

The associated pro-mature AMH (cleaved noncovalent complex) has shown to bind to AMHRII and stimulates intracellular signaling, whereas full-length AMH (uncleaved covalent complex) shows only minimal activity.



If isomeric forms of AMH exist in circulation, then they can be accurately measured by the ELISA method (epitope mapped, region specific antibody pairs) and chemical separation (dissociation) techniques. The method upon validation can quantitate the circulating AMH levels in healthy cycling and PCOS subjects.



PCOS subjects have defect in folliculogenesis (follicles are mostly small and immature). **Since AMH is produced by small growing follicles, the PCOS subjects should have higher levels of circulating AMH than the control subjects (age-matched normal subjects).**

If AMH measurement between the two groups shows statistically significant differentiation, then the method can help diagnose PCOS.

METHOD

Epitope Mapping: 12 antibodies raised in AMH knockout mice were screened on 80 overlapping biotinylated AMH Peptides (1-560aa, 12 amino acid long with an overlap of 4 aa and additional 4aa spacer consisting of SGSG. The antibodies were selected based on distinct linear epitope.

Antibody clone / Isotype	Affinity (Region)
AMH Capture (24), IgG2b	+++ (Midpro)
AMH Detection (32), IgG1	+++ (Mature)
AMH Detection (37), IgG2b	+++ (Midpro)
AMH Detection (10), IgG2b	+++ (Pro)
AMH Detection (33), IgG2b	+++ (Mature)

ASSAY DEVELOPMENT: Four independent ELISA methods based on different antibody pairs were developed to measure circulating levels of AMH isoforms in 20µL of the sample under dissociation (chemically treated) and non dissociation (non-treated) buffer conditions..

NT=Non-Treated T=Chemically Treated	24/32 NT	24/32 T	24/37 10/24 NT	24/37 T	32/33 NT	32/33 T
Pro+Mature (Associated)	✓	×	✓	✓	✓	✓
Pro-Mature (Covalent)	✓	✓	✓	✓	✓	✓
Pro Fragment	×	×	✓	✓	×	×
Mature Fragment	×	×	×	×	✓	✓

Study-1: Proof of concept using various ELISAs on recombinant AMH preparations. Multiple pediatric boys <1 year of age and five post menopausal female serum samples were used to verify the accuracy of AMH measurement.

ND=Non Detectable	% Detection						
NT=Non-Treated T=Treated	24/32 NT	24/32 T	10/24 NT	24/37 NT	24/37 T	32/33 NT	32/33 T
Pro + Mature (Associated)	100	ND	100	100	36	100	55.3
Pro-Mature (Covalent)	100	69	100	< 1	ND	<3	<1
Pro-Fragment	ND	ND	100	100	39	6	28
Mature-Fragment	< 1	ND	ND	ND	ND	100	255

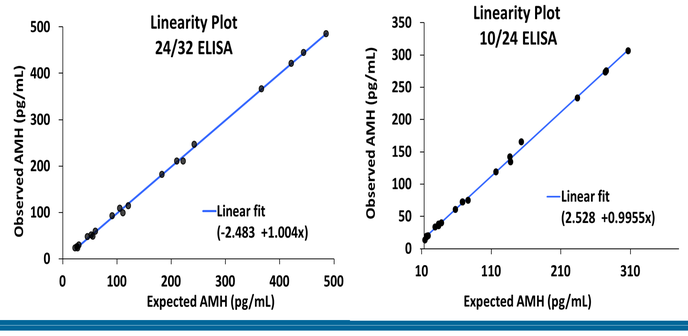
- 24/32 ELISA method under non-treated condition detected full length AMH (140kD, associated and covalent) and under treated condition detected only full length pro-mature covalent complex.
  - 24/32 T ELISA AMH = 0.69 (Covalent Pro-Mature AMH)
  - 24/32 NT ELISA AMH = Total Full length (uncleaved + cleaved associated Pro-Mature AMH forms)
  - Pro+Mature Associated AMH (In Circulation) = 24/32 NT AMH results - (1.45 X 24/32 T AMH results).
- 41 boys (<1 year) when tested under non-treated and treated 24/32 ELISA resulted in median AMH of 92 ng/mL and 8.6 ng/mL, respectively. The results indicated that the cleaved Pro+Mature Associated is the major circulating (91%) AMH isoform.
- 24/37 detects the pro-region fragment and 32/33 detects the mature region fragment in addition to the full length associated AMH. Both the assays did not detect the pro-mature covalent AMH and require further analysis.

ANALYTICAL CHARACTERISTICS

Analytical Sensitivity	24/32 ELISA	10/24 ELISA
	0.982 pg/mL	0.52 pg/mL

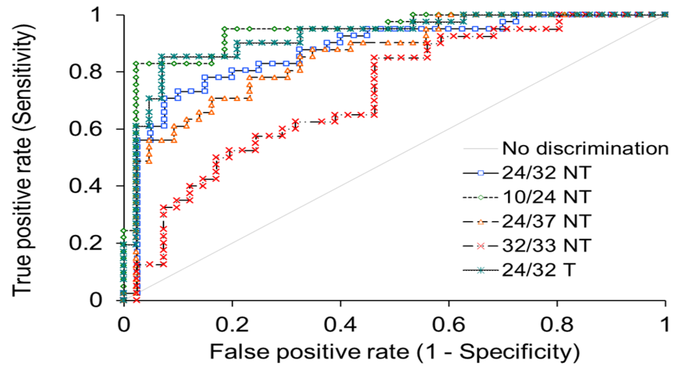
Imprecision		24/32 ELISA		10/24 ELISA	
	Conc. (pg/mL)	*SD*	*% CV*	*SD*	*% CV*
Control-1	70.4	4.53	6.4	2.9	4.1
Control-2	221.4	15.1	6.8	11.1	5.0

LINEARITY OF DILUTION: Multiple dilutions of the five serum samples containing various AMH levels were assayed and the observed concentration was plotted against the expected concentration.



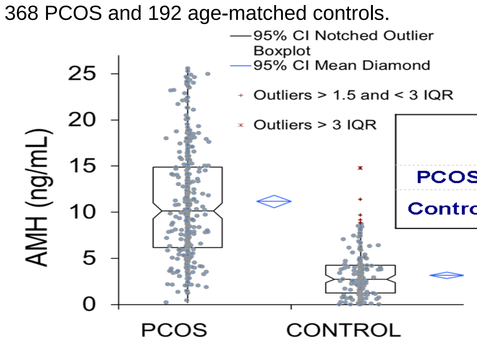
RESULTS

Study-2: Comparative ROC analysis (accuracy) for 43 PCOS and 41 controls was studied in different ELISA conditions.



Test	AUC
24/32 NT	0.88
10/24 NT	0.94
24/37 NT	0.85
32/33 NT	0.72
24/32 T	0.92

Study-3: Diagnostic accuracy of the 24/32 NT AMH test methods was determined for 368 PCOS and 192 age-matched controls.



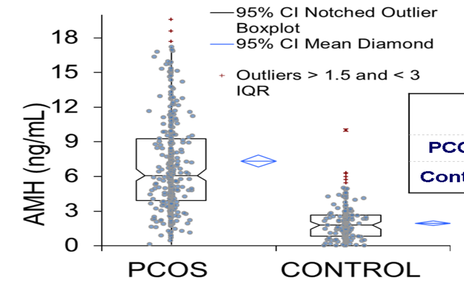
	n	Mean (ng/mL)	Median (ng/mL)
PCOS	368	11.162	10.142
Control	192	3.153	2.711

Diagnostic Accuracy	83.6%

	Subject Category		
24/32 AMH ELISA	PCOS	CONTROL	Total
Positive test ≥ 5	308	32	340
Negative test < 5	60	160	220
Total	368	192	560

Sensitivity TP proportion	0.837
Specificity TN proportion	0.833

Study-4: Diagnostic accuracy of the 10/24 NT AMH test methods was determined for 368 PCOS and 192 age-matched controls.



	n	Mean (ng/mL)	Median (ng/mL)
PCOS	368	7.331	6.057
Control	192	1.957	1.782

Diagnostic Accuracy	84.3%

	Subject Category		
10/24 AMH ELISA	PCOS	CONTROL	Total
Positive test ≥ 3	312	32	344
Negative test < 3	56	160	216
Total	368	192	560

Sensitivity TP proportion	0.848
Specificity TN proportion	0.833

Study-5: Percent prevalence of PCOS in sisters of PCOS were studied using optimized AMH ELISAs on 109 subjects.

Prevalence of PCOS subjects (Total = 20)		Prevalence of irregular menses (normal androgens) subjects (Total = 8)	
24/32 assay <5.0 ng/mL	35%	24/32 assay <5.0 ng/mL	50%
24/32 assay ≥5.0 ng/mL	65%	24/32 assay ≥5.0 ng/mL	50%
10/24 assay <3.0 ng/mL	30%	10/24 assay <3.0 ng/mL	38%
10/24 assay ≥3.0 ng/mL	70%	10/24 assay ≥3.0 ng/mL	62%
Prevalence of Hyperandrogenemic (with regular menses) subjects (Total = 21)		Prevalence of unaffected status (normal menses and androgens) subjects (Total = 61)	
24/32 assay <5.0 ng/mL	57%	24/32 assay <5.0 ng/mL	75%
24/32 assay ≥5.0 ng/mL	43%	24/32 assay ≥5.0 ng/mL	25%
10/24 assay <3.0 ng/mL	67%	10/24 assay <3.0 ng/mL	80%
10/24 assay ≥3.0 ng/mL	33%	10/24 assay ≥3.0 ng/mL	20%

CONCLUSIONS

1. AMH in circulation is processed and the cleaved AMH non-covalent complex is the major isoform. Uncleaved AMH complex was found to be less than 10% in circulation.
2. AMH measured by these ELISAs showed that the adolescence and adult PCOS subjects have >2 folds increased AMH levels than the controls. The results showed excellent diagnostic accuracy (83.6 and 84.3%, respectively) as a single marker.
3. The sisters of PCOS subjects are at a higher risk (>3 folds) of having PCOS and early diagnosis will help manage the symptoms.
4. AMH measurements should be included in the Rotterdam or NIH PCOS
5. AMH measurements serve as an non-invasive and objective biochemical marker of ovarian volume and should be considered essential for determination of PCOS in adolescents.