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Anti-Müllerian hormone measurement for the diagnosis of polycystic ovary syndrome

Running title: AMH in PCOS

¹Thozhukat Sathyapalan*, ¹Ahmed Al-Qaissi, ²Eric S. Kilpatrick, MD, ³Soha R. Dargham, ⁴Stephen L. Atkin,

¹ Department of Diabetes and Endocrinology, University of Hull, Hull, UK

² Division of Pathology, Sidra Research Centre, Doha, Qatar

³ Infectious Disease Epidemiology Group, Weill Cornell Medicine, Doha, Qatar

⁴Research Faculty, Weill Cornell Medicine, Doha, Qatar. Email: sla2002@qatar-med.cornell.edu

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Summary

Objective: Anti-Müllerian hormone (AMH) is derived from the small antral follicles and an elevated level has been suggested to add value to the Rotterdam criteria for the diagnosis of PCOS in cases of diagnostic uncertainty. Therefore, the role of AMH in the classical phenotype of PCOS was defined within a Caucasian population.

Design: This was a cross sectional study

Patients: 65 women without PCOS and 110 women with PCOS fulfilling all 3 diagnostic Rotterdam criteria.

Measurements: The main outcomes were the utility of serum AMH for the diagnosis of PCOS and its relationship to the metabolic parameters.

Results. AMH was increased in PCOS compared to controls ($p < 0.001$) Areas under the receiver operator curve showed AMH to be predictive of PCOS (0.76) using a cut off AMH of 46pmol/l, derived from the 95th percentile of the controls gave a 41% sensitivity and 86% specificity; an AMH cut off of 35pmol/l gave a 55% sensitivity and 79% specificity. Age and BMI adjusted multiple logistic regression showed that AMH was more predictive of PCOS independently of either serum testosterone (T) (OR = 4.04; 95% CI 1.42-11.11; $p = 0.007$) or free androgen index (FAI) (OR = 3.90; 95% CI 1.40-10.83; $p = 0.009$).

Conclusion. Whilst an elevated AMH has poor sensitivity, it is four fold more likely to be associated with a diagnosis of PCOS, and supplementary to biochemical parameters will make a positive diagnosis of PCOS in 22% of patients when neither serum testosterone or FAI are elevated.

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders and affects 6-20 % of reproductive-aged women^{1,2}. Currently PCOS is diagnosed using the Rotterdam, or Androgen Excess Society or NIH criteria, but it still remains a diagnosis to be made only after the exclusion of other conditions^{3,4}. A diagnostic test that would positively identify a patient with PCOS and add value to the current diagnostic criteria would be of great benefit, and anti-Müllerian hormone (AMH) measurement has been suggested to add value to the Rotterdam criteria. AMH is produced in the granulosa cells by the pre-antral and small antral follicles whose mechanism appears to be to inhibit the action of FSH on aromatase so assisting with the development of a single follicle for ovulation⁵. AMH is reported to be elevated in PCOS due to the increased small antral follicle count, where that increase in AMH may be a combination of the increased number of small antral follicles and the increased secretion of AMH per follicle⁶. It has been suggested that AMH levels are a good marker for the size of the antral follicle pool. Ultrasound is used to measure the antral pool size but concerns over operator dependency are its main limitation and therefore a single serum measurement of AMH would be attractive⁷. Several studies have been done on the utility of AMH in the diagnosis of PCOS^{8,9} with differing diagnostic thresholds being proposed, though a meta-analysis suggested a cut off of 35pmol/l with a sensitivity of 79.4% and a specificity of 82.8%⁸: however, it was noted that this may not be applicable to all PCOS subgroups and that the populations tended to be from fertility clinics rather than a general population and differing assays for AMH undertaken⁸. A raised AMH is particularly associated with PCOS patients with all three diagnostic criteria¹⁰; therefore the aim of this study was to look specifically at the utility of AMH for the diagnosis of PCOS within a well-defined cohort of PCOS patients from the general population that fulfilled all 3 of the Rotterdam criteria to determine if it could be complementary to accepted androgen markers.

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Materials and methods

This was a cross sectional study involving 105 well characterised women with PCOS and 65 women without PCOS who presented sequentially to the Department of Endocrinology and were recruited to the local PCOS biobank (ISRCTN70196169). All patients gave written informed consent. This study was approved by the Newcastle & North Tyneside Ethics committee. The diagnosis of PCOS was based on all three diagnostic criteria of the Rotterdam consensus, namely clinical and/or biochemical evidence of hyperandrogenemia (Ferriman-Gallwey score >8; free androgen index >4 respectively), oligomenorrhea or amenorrhea and polycystic ovaries on transvaginal ultrasound¹¹, described as the type A phenotype in which AMH levels are highest. Study participants had no concurrent illness, were not on any medication for the preceding 9 months except study medications and were not planning to conceive. Non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease and androgen-secreting tumours were excluded by appropriate tests. All of the control women had regular periods, no clinical or biochemical hyperandrogenemia, no significant background medical history and none of them were on any medications including oral contraceptive pills or over the counter medications. All women underwent a 75g oral glucose tolerance test to exclude impaired glucose tolerance and type 2 diabetes. All women with PCOS and control women were Caucasian. Height, weight and waist circumference and body mass index (BMI) were performed according to WHO guidelines¹².

Collection and analysis of blood samples

Blood samples were collected immediately (within 5 min) and was stored frozen at -80°C pending analysis. Serum T was measured by LC/MS/MS on an Acquity UPLC system

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coupled to a Quattro Premier XE mass spectrometer (Waters, Manchester, UK). Sex hormone binding globulin (SHBG) was measured by an immunometric assay with fluorescence detection on the DPC Immulite 2000 analyzer using the manufacturer's recommended protocol. The free androgen index (FAI) was calculated as the total testosterone x 100/SHBG. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK). The analytical sensitivity of the insulin assay was 2µU/ml, the coefficient of variation was 6%, and there was no stated cross-reactivity with proinsulin. Plasma glucose was measured using a Synchron LX 20 analyzer (Beckman-Coulter), using the manufacturer's recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 5.3 mmol/liter during the study period. The insulin resistance was calculated using the HOMA method [$HOMA-IR = (\text{insulin} \times \text{glucose}) / 22.5$]. Anti-Müllerian hormone was measured using a Beckman Coulter Access automated immunoassay. Between run precision was <3% across the range measured.

Statistical analysis

Data trends were visually evaluated for AMH and each androgen and non-parametric tests were applied on data that violated the assumptions of normality when tested using Kolmogorov-Smirnov Test. Accordingly, comparative analysis evaluating androgen levels between PCOS cases and controls were performed using the non-parametric Mann-Whitney tests. Pearson's correlations were also estimated to assess any linear relationship between AMH and the different parameters. Finally multivariable logistic regressions adjusting for age and BMI, serum testosterone or FAI were conducted to assess the effects of AMH in the diagnoses PCOS, firstly with a AMH categorical value of greater than 46 (based on the 95th percentile sensitivity of the ROC) and secondly based on AMH with a categorical value of greater than 35 according to the literature⁸. Significance was defined at $\alpha=0.05$. All analyses

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were done using IBM-SPSS version 24.0. All values are given as (mean \pm SD) unless specified.

Results

The baseline demographics of patients are given in Table 1. Of the 170 subjects with a serum AMH, 4 of 65 controls and 3 of 105 PCOS subjects did not have a FAI level available. PCOS women were significantly younger (27.7 ± 6.3 years) than the controls (30.2 ± 6.30 years).

Their body mass index (BMI), waist and hip circumferences were also significantly higher ($p < 0.0001$) than the controls (Table 1). Patients with PCOS showed greater insulin, HOMA-IR, 2 hour glucose post oral glucose tolerance test (OGTT) values ($p < 0.001$) (Table 1).

Serum T (1.5 ± 0.9 vs. 1.0 ± 0.5 nmol/L) and FAI (6.3 ± 5.7 vs. 2.5 ± 1.6) were significantly elevated in PCOS compared to control ($p < 0.001$).

All 105 PCOS women were categorized into a single phenotype according to the combination of all 3 major Rotterdam's Consensus Criteria.

The diagnostic upper threshold for AMH was calculated using the 95th percentile of the control for this population and was 46pmol/l (Figure 1). Taking the categorical cut off of AMH greater than 46pmol/l, AMH was positive in 8 of 61 (13%) controls of whom 1 had both a raised FAI and serum T and 7 had neither. Of the 42/102 PCOS patients positive for an AMH with cut off of greater than 46pmol/l, 16/42 (38%) were positive for clinical hyperandrogenism (neither serum T nor FAI elevated) and 26/42 (62%) were positive if serum T and or FAI were elevated. Conversely, of the 60/102 with an AMH less than 46pmol/l, 29/60 (48%) were positive for clinical hyperandrogenism (neither serum T nor FAI elevated) and 31/60 (52%) were positive if serum T and or FAI were elevated.

Therefore, for those with neither a raised T nor FAI, AMH picked up an additional 16 of 102 (16%) PCOS subjects. This gave a 41% sensitivity and 86% specificity for AMH at 46pmol/l.

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It is suggested from the literature that an AMH greater than 35pmol/l is suggestive of a diagnosis of PCOS⁸. Therefore, taking the categorical cut off of AMH greater than 35pmol/l, AMH was positive in 13 of 61 (21%) controls of whom 1 had both a raised FAI and serum T and 12 had neither. Of the 57/102 PCOS patients positive for an AMH with cut off of greater than 35pmol/l, 23/57 (40%) were positive for clinical hyperandrogenism (neither serum T nor FAI elevated) and 34/57 (60%) were positive if serum T and or FAI were elevated. Conversely, of the 45/102 with an AMH less than 35pmol/l, 22/45 (49%) were positive for clinical hyperandrogenism (neither serum T nor FAI elevated) and 23/45 (51%) were positive if serum T and or FAI were elevated. Therefore, for those with neither a raised T nor FAI, AMH picked up an additional 23 of 102 (22%) PCOS subjects. This gave a 55% sensitivity and 79% specificity for AMH at 35pmol/l.

The PCOS groups with biochemical hyperandrogenemia showed a more metabolic profile with increased BMI, insulin and HOMA values ($p < 0.01$) compared to those with clinical hyperandrogenism, but AMH levels did not differ between the 2 groups (Table 3). When the metabolic parameters were compared between those patients with an AMH of 35 or 46pmol/l with those less than 35pmol/l there were no differences in any of the metabolic parameters (Supplemental Table 1). AMH as a quantitative measure, did not correlate to insulin, HOMA-IR or CRP in either the control or PCOS subjects, but it did correlate to serum T and FAI in controls ($r = 0.3$, $p < 0.05$; $r = 0.35$, $P < 0.01$, respectively), but only to serum testosterone ($r = 0.2$, $p < 0.05$), and not FAI in the PCOS subjects.

As individual tests, the age and BMI adjusted multiple logistic regression showed that for AMH at thresholds greater than either 35 or 46pmol/L, added significantly to both serum T and FAI in the prediction of PCOS, and when serum T and FAI were both included in the

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model that AMH remained predictive of PCOS with an OR 3.8 and 4.5 ($p < 0.01$) for a AMH cut off of 46 and 35pmol/l, respectively of (Table 2).

Discussion

It has been suggested that AMH may be a useful initial diagnostic test for PCOS with a cut off value of around 35pmol/l^{8,13}. In accord with others, we found a raised AMH was strongly associated with a diagnosis of PCOS with a 4 fold odds ratio. Thus, in an analogous way to making a diagnosis of diabetes – where a diagnosis can be made using either a raised fasting plasma glucose, a raised 2 hour glucose value or a raised HbA1c¹⁴ – then a raised serum testosterone, or a raised FAI or a raised AMH will identify an additional 22% more PCOS patients positively with a AMH threshold of 35 (16% with an AMH cut off of 46pmol/l). Therefore, there is additional discriminatory benefit for making the diagnosis of PCOS by measuring AMH in addition to the traditional androgen markers. The sensitivity of AMH for a diagnosis of PCOS was low with either cut off (46pmol/l; 41% and 35pmol/l; 55%) and lower than that reported of 79%, though with a similar specificity and likely reflected the general population of study rather than from fertility clinics⁸

The number of patients with clinical hyperandrogenism who had a raised AMH but had no biochemical evidence of hyperandrogenaemia (as evidenced by normal serum T and FAI) was 16/42 (38%) v 23/57 (40%) using 46pmol/L and 35pmol/L thresholds respectively. If one then applies the AMH levels at either cut off to that of the NIH or Androgen excess society guidelines for the diagnosis of PCOS^{4,15} AMH measurement would have added additional value in making a positive diagnosis of PCOS. Those PCOS patients with biochemical hyperandrogenemia had a more metabolic profile with a higher BMI than those with clinical hyperandrogenism, but AMH levels were no different between the 2 groups

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showing that AMH was independent of the metabolic parameters. This was confirmed when no difference between PCOS groups were seen for those metabolic factors when those with elevated AMH (greater than 35 and 46pmol/l) were compared to those with levels below 35pmol/l.

Positive aspects of this study included the significant number of subjects in the study of a homogeneous Caucasian population, the type A phenotype for PCOS all with the same 3 diagnostic criteria fulfilled where AMH noted to be the highest in this population^{10, 16}. All patients had polycystic ovaries on ultrasound and therefore it might have expected all of the AMH values to be elevated. Why this was not the case is likely due to the observation that AMH values have been shown to be lower in anovulation and many of these patients may have been anovulatory, though this was not determined in this study. AMH levels have been correlated with serum testosterone, but not to BMI or to insulin resistance i.e. AMH is likely to be a marker of the consequence of PCOS rather than the instigator¹⁷. Limitations of this study were in part the converse of the strengths. This was a narrow group of individuals all with the same 3 diagnostic criteria fulfilled for the diagnosis of PCOS, and other PCOS phenotypes not included. In addition, this was a narrow ethnic group and therefore more detailed studies including the other PCOS phenotypes with other ethnicities are required. In conclusion, whilst an elevated AMH has poor sensitivity, it is four fold more likely to be associated with a diagnosis of PCOS which, in this study, results in its measurement identifying an additional 16% or 22% of patients (using AMH cut offs of 35 or 46pmol/l, respectively) as having PCOS when routine measurement of androgen concentrations are not elevated. This means there may be merit in considering AMH as a marker which complements androgen measurement in the biochemical assessment of PCOS.

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Author's roles

T. Sathyapalan was involved in protocol development, patient recruitment, data analysis and manuscript drafting

A. Al-Qaissi was involved in patient recruitment, data analysis and manuscript drafting

ES Kilpatrick was involved in protocol development, data analysis and manuscript drafting

SR Dargham was the statistician responsible for the overall data analysis and manuscript drafting

SL Atkin was involved in protocol development, data analysis and manuscript drafting

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Legend to Table 1.

Demographics of the 170 subjects involved in the study, 65 women without PCOS and 105 women with PCOS. The diagnosis of PCOS was based on all three diagnostic criteria of the Rotterdam consensus, namely clinical and biochemical evidence of hyperandrogenemia (Ferriman-Gallwey score >8; free androgen index >4 respectively), oligomenorrhea or amenorrhea and polycystic ovaries on transvaginal ultrasound. These women therefore represented the phenotype with the greatest metabolic features.

Legend to Table 2

Logistic regression analysis for the diagnosis of PCOS for either serum testosterone or Free Androgen Index (FAI).

A, anti-Müllerian hormone as categorical data with a value of greater than 46pmol/L (95th percentile on ROC).

B, anti-Müllerian hormone as categorical data with a value of greater than 35pmol/L according to the literature⁸. There was a four fold prediction of PCOS by AMH and both cut off values appeared equally predictive.

Legend to Table 3. The PCOS groups with biochemical hyperandrogenemia showed a more metabolic profile with increased BMI, insulin and HOMA-IR values compared to those PCOS women with clinical hyperandrogenism, but AMH levels did not differ between the 2 groups. (BMI=body mass index; FAI= free androgen index; T = serum testosterone; HOMA-IR =Homeostasis model of assessment – insulin resistance).

Legend to supplemental Table 1. A. Comparison of the metabolic parameters between those PCOS women with a AMH greater than 46pmol/l compared to those with a AMH less than

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35pmol/l. B, A. Comparison of the metabolic parameters between those PCOS women with a AMH greater than 35pmol/l compared to those with a AMH less than 35pmol/l. In either case it can be seen that there is no difference between the groups showing that AMH is independent of the metabolic parameters.

Legend to Figure 1

Receiver operator curves for anti-Müllerian hormone showing that AMH is predictive of the diagnosis of PCOS with an area under the curve of $0.76 \pm 0.04SE$.