The original Beckman Coulter Generation II assay significantly underestimates AMH levels compared with the revised protocol

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Abstract

Purpose Anti-Müllerian hormone (AMH) is used as a marker for ovarian reserve. Since 2011, the standard test for AMH has been the Beckman Coulter Generation (Gen) II assay. However, in July 2013, the protocol was revised due to falsely low readings. The aim of this study was to compare AMH levels measured with the original and revised Gen II assay and to establish a fertile female reference range for the revised protocol.

Methods Serum AMH levels were measured for 492 natural conception first trimester pregnant women using the original and revised Gen II assay.

Results The original protocol significantly underestimated AMH levels compared with the revised protocol ($p < 0.001$), the median being 8.4 and 14.2 pmol/L, respectively. In all samples with detectable AMH levels, the revised protocol yielded a higher concentration compared with the original protocol, the magnitude shift ranging from 3.4 to 283.3 % (median 68.0 %). AMH levels measured with the revised protocol were collated to generate an age-specific reference range, with median levels peaking at 27 years then declining with advancing age. The median AMH concentration for ages 20–24 was 17.3 pmol/L, ages 25–29 was 20.5 pmol/L, ages

30–34 was 17.8 pmol/L, ages 35–39 was 10.8 pmol/L, and ages 40–44 was 6.1 pmol/L.

Conclusions Our study demonstrated that the original Gen II assay significantly underestimated AMH levels, suggesting caution is required when interpreting literature and testing results achieved with this assay. We also established the revised Gen II assay reference range for AMH in women with unassisted proven fertility.

Keywords Anti-Müllerian hormone · Generation II assay · Ovarian reserve · Reference range

Introduction

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein and a member of the transforming growth factor beta family [1]. It has a critical role in male fetal sex differentiation, with its production by immature Sertoli cells inducing the regression of the Müllerian ducts [2]. In females, AMH is produced in the ovary by the granulosa cells of pre-antral and small antral follicles [3] where its main role appears to be inhibition of the early stages of follicular development and recruitment by follicle stimulating hormone (FSH) [4, 5]. There is also evidence to suggest that AMH plays a role as a follicular “gatekeeper” by decreasing estrogen production in small antral follicles prior to selection [5].

AMH, released from the granulosa cells of antral follicles, can be measured in serum and has been shown to correlate with the number of pre-antral and antral follicles in the ovaries [6]. AMH levels also correlate strongly with female age [7] and do not vary significantly in the first trimester of pregnancy [8–10]. Measurement of serum AMH is used for a number of clinical applications, the most common being to predict the ovarian response to stimulation in infertility treatment

Capsule The original Gen II assay significantly underestimates AMH levels suggesting caution is required when interpreting literature and testing results achieved with this assay.

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programs [11]. Additionally, AMH assessment may be useful to aid diagnosis of ovarian disorders including polycystic ovarian syndrome (PCOS) and premature ovarian failure, to evaluate ovarian damage from medical interventions such as cancer therapies [5] and has also been suggested as a predictor of reproductive lifespan [12, 13].

A number of assays and protocols have been used over the years to measure serum AMH levels, complicating the interpretation of AMH results and literature. The first-generation commercial AMH enzyme-linked immunoassays (ELISAs) were the Diagnostic Systems Lab (DSL) assay and Immunotech (IOT) assay (also called the Immunotech Beckman Coulter (IBC) assay) and used different primary antibodies and standards. These two assays were superseded in 2011 by the Beckman Coulter AMH Generation (Gen) II ELISA, an amalgamation using the DSL antibody and the IOT standard calibrations [14]. However, there have been a number of protocol issues with this new assay resulting in inaccurate readings and leading to urgent protocol revisions [15, 16]. This has caused considerable confusion among testing laboratories and clinicians, being unsure how to interpret past and present AMH results, and has thrown into question the body of AMH literature published with the unrevised Gen II assay [17]. Furthermore, there is a need for new AMH reference ranges for the normal fertile female population so that results achieved with the revised Gen II protocol can be appropriately interpreted.

This study investigated the effect of the July 2013 Gen II assay protocol revision [16] on AMH results and established the age-specific fertile female AMH reference range for this assay.

Materials and methods

Study design

This study measured AMH levels using the original and revised Gen II assay for 492 consenting women aged between 20 and 44 years attending the Genea (previously Sydney IVF) biochemistry laboratory for first trimester pregnancy screening between February 2011 and January 2012. Participants were eligible if they had a natural conception without the use of ovarian stimulation and were between 9 and 13 weeks of pregnancy (median 12 weeks, IQR 11–12 weeks). Serum samples were collected, frozen within 2 h, and stored at -80°C until assayed. This study was approved by Genea's ethics committee, an independent institutional human research ethics committee registered with Australia's National Health and Medical Research Council.

AMH ELISA

AMH levels were measured on serum samples using the Beckman Coulter AMH Gen II ELISA both prior to (original protocol) and after (revised protocol) the July 2013 Field Safety Notice [16] according to the manufacturer's instructions. The difference between the two protocols relates to serum sample dilution; the original protocol used undiluted (neat) samples [15], whereas the revised protocol used 1/6 diluted serum samples [16]. Both protocols were performed in Genea's commercial biochemistry laboratory on identical samples (same aliquot), with measurement with the revised protocol occurring approximately one to one and a half years after that of the original protocol using samples stored at -80°C . All assays were measured using the ELx800™ Absorbance Microplate Reader and evaluated using Gen5 Data Analysis Software (BioTek). The minimal detectable AMH concentration with the AMH Gen II assay is 0.9 pmol/L, as advised by the manufacturer, and samples with undetectable levels were recorded as 0.9 pmol/L. Note that 1 ng/ml AMH is equivalent to 7.14 pmol/L.

Statistical analysis

The distribution of continuous variables was explored using the one sample Kolmogorov-Smirnov test. A Wilcoxon signed-rank test was conducted to explore the difference in AMH values using the original and revised Gen II protocol, and non-linear regression models were used to explore the fit

Fig. 1 Serum AMH concentration determined using the original and revised Gen II assay. Four hundred ninety-two patient samples are represented by circles. The line shows the quadratic relationship ($R^2=0.92$). Note that there were 14 samples with undetectable levels (designated 0.9 pmol/L) using both the original and revised Gen II assay

Table 1 Age-specific AMH reference intervals for the revised Gen II assay

Age (years)	n	Percentile						
		5	10	25	50	75	90	95
20–24	63	7.1	8.8	12.0	17.3	26.2	47.8	56.9
25–29	65	7.5	9.4	13.5	20.5	27.8	50.8	60.5
30–34	169	4.4	6.0	10.0	17.8	29.8	39.5	46.8
35–39	109	1.2	2.7	6.0	10.8	18.4	31.4	36.9
40–44	86	0.9	0.9	2.8	6.1	9.9	15.3	21.1

AMH values are in picomole per liter

between the two. The relationship between AMH and age was assessed using the Spearman's rho correlation (r_s). Curvilinear regression analyses were conducted to determine non-linear models of best fit for the relationships between AMH values using the original and revised Gen II protocol, and between AMH and age, with explained variance (R^2) reported. All analyses were conducted using SPSS v21. The significance level was set at $\alpha=0.05$. All graphs were plotted using SigmaPlot 11.0.

Results

AMH concentration using the original and revised Gen II assay

This study measured serum AMH concentrations using the original and revised Gen II assay for 492 first trimester pregnant women who had conceived naturally (Supplementary Table 1). There was a significant positive correlation between AMH levels measured using the original and the revised

protocol ($r_s=0.97$, $p<0.001$, $n=492$). The median AMH concentration for the original protocol was 8.4 pmol/L and for the revised protocol 14.2 pmol/L. Of the 492 samples, 34 had undetectable (≤ 0.9 pmol/L) AMH levels using the original protocol, and of these, 20 (58.8 %) had detectable levels using the revised protocol (ranging from 1.2 to 4.5 pmol/L, median 1.95 pmol/L). In all 458 samples with detectable AMH levels using both protocols, the revised protocol yielded a higher AMH concentration compared with the original protocol (Wilcoxon signed-rank test $z=-18.54$, $p<0.001$), with the median magnitude shift being 68.0 % (min 3.4 %, max 283.3 %). Curvilinear regression of all samples indicated that AMH concentrations (pmol/L) measured using the original protocol were best converted to the revised protocol using a quadratic equation ($F(2,489)=2,731.50$, $p<0.001$, $R^2=0.92$, Fig. 1): estimated revised protocol AMH value = $0.929 + 1.658(\text{original value}) - 0.006(\text{original value})^2$.

Age-specific AMH reference range for the revised Gen II assay

We next established the age-specific AMH reference range for our fertile female population measured using the revised Gen II protocol. As expected, there was a correlation between AMH levels and age ($r_s=-0.46$, $p<0.001$, $n=492$). The relationship was curvilinear ($F(1,490)=128.771$, $p<0.001$; $R^2=0.21$ for compound, growth, exponential and logistic models), with median levels peaking at age 27 (23.0 pmol/L) and then subsequently decreasing with advancing age (Supplementary Table 2 and Fig. 2). All of the 14 participants with undetectable AMH levels were greater than 35 years of age, and nine (64.3 %) of these were greater than 40 years of age. The participants were grouped by age intervals of 5 years from 20 to 44 years and AMH concentration reference intervals determined (Table 1). The median

Fig. 2 Age-specific reference range using the revised Gen II assay. Regression of percentiles for ages 20 to 44 years (492 patient samples; Supplementary Table 2)

Table 2 Published age-specific AMH reference ranges based on a healthy female cohort

Publication	Participants	Assay	Age range	Peak median AMH	Example median AMH for age
Current study	492 first trimester pregnant females (attending screening)	Gen II (revised)	20–44 years	27 years=23 pmol/L	20–24 years=17.3 pmol/L 25–29 years=20.5 pmol/L 30–34 years=17.8 pmol/L 35–39 years=10.8 pmol/L 40–44 years=6.1 pmol/L
Han et al. [23]	179 first trimester pregnant females (attending screening)	Gen II (revised)	20–45 years	Not reported	20–25 years=22.2 pmol/L 26–30 years=19.6 pmol/L 31–35 years=17.0 pmol/L 36–40 years=11.1 pmol/L 41–45 years=8.1 pmol/L
Kelsey et al. [26]	3,260 healthy premenopausal females (data from 20 published studies)	IBC and DSL	Birth–50 years	24.5 years=not stated	25 years=mean ~36 pmol/L ^b 30 years=mean ~26 pmol/L ^b 35 years=mean ~20 pmol/L ^b 40 years=mean ~14 pmol/L ^b 45 years=mean ~5 pmol/L ^b
La Marca et al. [24]	277 healthy females (51 % parous)	IBC	18–50 years	not reported	≤30 years=29.3 pmol/L 31–35 years=24.7 pmol/L 36–40 years=21.4 pmol/L 41–45 years=9.9 pmol/L
Masse et al. [21]	340 first trimester pregnant females (attending abortion service)	Gen II (original)	15–50 years	29 years=not stated	20–2 years=21.3 pmol/L 25–29 years=24.5 pmol/L 30–34 years=10.7 pmol/L 35–39 years=8.4 pmol/L
Shebl et al. [25]	1,105 females attending infertility clinic on the basis of male factor infertility	IBC ^a	17–60 years	28 years=30.3 pmol/L	≤40 years=5.8 pmol/L 25 years=27.9 pmol/L 30 years=25.1 pmol/L 35 years=22.1 pmol/L 40 years=11.6 pmol/L
Tehrani et al. [22]	1,015 healthy parous females	Gen II (original ^a)	18–50 years	18 years=39.3 pmol/L	25 years=23.8 pmol/L 30 years=15.0 pmol/L 35 years=8.7 pmol/L 40 years=4.6 pmol/L 45 years=2.3 pmol/L

^a Presumed^b Mean IBC values estimated from a graph

AMH concentration using the revised protocol for ages 20 to 24 years was 17.3 pmol/L, ages 25 to 29 years was 20.5 pmol/L, ages 30 to 34 years was 17.8 pmol/L, ages 35 to 39 years was 10.8 pmol/L, and ages 40 to 44 years was 6.1 pmol/L.

Discussion

Since 2011, the standard clinical test used to measure AMH has been the Gen II assay [14]. However, in July 2013, the protocol was revised due to concerns around interference from complement, potentially resulting in falsely low readings [16]. Our study comparing AMH results measured with the original and revised assay for 492 pregnant first trimester samples has validated those concerns, with all samples reading higher using the revised protocol. Here, the median magnitude shift was 68.0 % (range 3.4 to 283.3 %), although if interference from complement is to blame, this shift may not be as large for non-pregnant samples as complement levels are higher during pregnancy [18]. Our study is supported by a recent publication by Craciunas et al. [19], who compared 156 samples from women undergoing subfertility investigation and found that samples with the revised protocol read on average 51.4 % higher than the original protocol. In contrast, Welsh et al. [20] found insubstantial differences for 37 non-pregnant serum samples measured with the original and revised Gen II protocol.

Our finding that the original Gen II protocol underestimated AMH levels questions the reliability of literature that draw conclusions based on this assay. The degree to which AMH levels measured using the original Gen II protocol were affected by complement ultimately depends on the amount of complement in the sample and the way in which samples were handled prior to processing, with fresh or quickly frozen samples (in our case) likely being the most affected. Given these variables, we would not recommend simply converting AMH measurements reported in the literature using the original protocol to that of the revised protocol. It should also be noted that additional confusion is likely to arise from which protocol version a laboratory has used given that the manufacturer has not clearly labelled the protocol change, either by updating the kit name or protocol version. Consequently, Craciunas et al. [19] have suggested distinguishing protocol versions by naming the revised Gen II protocol as Gen II_m (for Gen II modified).

This study also established an AMH reference range for the revised Gen II assay using naturally fertile women (Fig. 2). While there are studies reporting AMH age-specific reference ranges for fertile [21–23] or presumably fertile populations [24–26], all but one used first-generation AMH assays or the Gen II assay with the original protocol (Table 2). That study, by Han et al. [23], reported very similar age-grouped median AMH values using the revised Gen II assay, despite having a much smaller reference population and slightly different age

groupings. As AMH levels are stable from pre-pregnancy through to the first trimester of pregnancy [8–10], our AMH reference range is applicable to the non-pregnant fertile population. Furthermore, it is also relevant to samples processed automatically with the Access2 Immunoassay System (Beckman Coulter), as we have found a strong correlation with manually processed samples (152 samples; $R^2=0.99$; unpublished data). This reference range may not be applicable to other AMH assay such as the new automated Elecsys AMH (Roche) and the AMH ELISA (Ansh Labs).

The AMH reference range reported here is of clinical value for women planning and attempting conception and for the diagnosis and management of infertility issues. For women whose AMH levels are above the 90th centile, we would recommend PCOS should be considered and confirmed using standard diagnostic criteria. This is supported by a meta-analysis examining the diagnosis value of AMH for PCOS using first-generation AMH assays and a single threshold [27]. Whether using an age-specific AMH threshold for PCOS is more appropriate remains to be determined. Those women whose AMH levels fall below the 10th centile for their age group should be counseled for diminished ovarian reserve which may indicate a reduced reproductive lifespan [12, 28, 29], although it should be made clear that there is no substantial evidence to suggest reduced fecundability during their fertile life. Additionally, fertility specialists will be able to use the AMH reference range in conjunction with antral follicle count, ovarian volume, age, and weight to determine the dose of FSH to prescribe for assisted conception cycles.

In summary, we show that the Gen II protocol revision of July 2013 had a significant impact on AMH measurements, suggesting caution is required when interpreting literature and testing results achieved with the original Gen II protocol. Additionally, this study established the reference range for the revised AMH Gen II assay using women with proven unassisted fertility.

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Compliance with ethical standards This study was approved by Genea's ethics committee. Informed consent was obtained from all individuals included in the study.

Conflict of interest The authors declare that they have no conflicts of interests.

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