

Full length article

Evaluation of the Elecsys[®] anti-Müllerian hormone assay for the prediction of hyper-response to controlled ovarian stimulation with a gonadotrophin-releasing hormone antagonist protocol



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ABSTRACT

Objective: This non-interventional study aimed to validate a pre-specified anti-Müllerian hormone (AMH) cut-off of 15 pmol/L (2.10 ng/mL) for the prediction of hyper-response to controlled ovarian stimulation (COS) using the fully automated Elecsys[®] AMH immunoassay.

Study design: One hundred and forty-nine women aged <44 years with regular menstrual cycles underwent COS with 150 IU/day follicle-stimulating hormone in a gonadotrophin-releasing hormone (GnRH) antagonist protocol. Response to COS (poor vs normal vs hyper-response) was defined by number of oocytes retrieved and occurrence of ovarian hyper-stimulation syndrome (OHSS).

Results: Significant differences were seen between response classes for the number of follicles prior to follicle puncture ($p < 0.001$), the number of retrieved oocytes ($p < 0.001$) and the occurrence of OHSS ($p < 0.001$), which were all highest in hyper-responders. The area under the receiver operating characteristic curve for AMH to predict hyper-response was 82.1% (95% confidence interval [CI]: 72.5–91.7). When applying the AMH cut-off of 15.0 pmol/L, a sensitivity of 81.3% (95%CI: 54.4–96.0) to predict hyper-response and a specificity of 64.7% (95%CI: 55.9–72.8) to identify poor/normal responders was reached.

Conclusion: The Elecsys[®] AMH assay can reliably predict hyper-response to COS in women undergoing a GnRH antagonist treatment protocol.

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Introduction

Controlled ovarian stimulation (COS) with exogenous gonadotropin, an essential step of in-vitro fertilization (IVF) and intra-cytoplasmic sperm injection protocols, is used to achieve a reasonable number of mature oocytes for IVF/intra-cytoplasmic sperm injection [1]. During COS, spontaneous ovulation is suppressed using a gonadotrophin-releasing hormone (GnRH) agonist (agonist protocol) or a GnRH antagonist (antagonist protocol) [2,3]. The magnitude of ovarian response is influenced by the type of downregulation protocol [2,3].

There is individual variability in response to COS, including poor response with inherent lower prognosis for live birth or hyper-response with a potentially serious adverse event (ovarian hyper-stimulation syndrome [OHSS]). There is also an unmet medical need to improve the markers of response, such as antral follicle count (AFC) and anti-Müllerian hormone (AMH) [4], which are currently the most reliable biomarkers for prediction of response to COS. Studies can then be conducted to see whether adjusting the follicle-stimulating hormone (FSH) dose, based on the chosen marker(s) of ovarian reserve, leads to better outcomes [5,6]. Poor response is defined as less than four oocytes retrieved or cancellation of stimulation cycle due to insufficient number of follicles [7,8], and hyper-response is defined as more than 15 oocytes retrieved or cancellation of stimulation cycle due to too many follicles [7]. These definitions are consistent with those used by Hamdine et al. [9], which were adapted from the commonly used definitions for both high and low response with GnRH

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agonists (as ovarian response definitions for GnRH antagonists are lacking).

AMH is a dimeric glycoprotein from the transforming growth factor β family produced by ovarian granulosa cells of pre-antral and small antral follicles [10,11]. Release of AMH from ovarian granulosa cells results in measurable serum levels, which are proportional to the number of developing antral follicles in the ovaries [12]. AMH is rapidly becoming the preferred biomarker of ovarian response to COS [12,13]; levels are predictive of the number of retrieved oocytes and poor vs excessive response in patients who received a GnRH antagonist protocol [9,14–16].

AMH cut-offs can be selected to achieve high sensitivity for predicting hyper-response, which is important to reliably detect patients who are at risk of developing OHSS. Two previous studies, one using an agonist protocol and the other an antagonist protocol, have proposed a 15 pmol/L AMH cut-off using the AMH Gen 2 ELISA (Beckman-Coulter) and DSL AMH ELISA assays [16,17]. Systematic variation has been observed between the two most commonly used AMH assays, the AMH Gen 2 ELISA and Elecsys[®] AMH assay (Roche Diagnostics), so cut-offs derived on one assay are not directly transferable [18,19].

We selected a subgroup of the OPTIMIST study population who had received a standard fixed FSH dose of 150 IU/day and an antagonist protocol [7] for inclusion in a non-interventional study designed to investigate the performance of the Elecsys[®] AMH assay for the prediction of response to COS. We aimed to validate an AMH cut-off of 15.0 pmol/L (2.10 ng/mL) for the prediction of hyper-response during GnRH antagonist protocol cycles, specifically using the Elecsys[®] AMH assay. We focused on the prediction of hyper-response as FSH dose adjustments may produce most benefit for patient safety by reducing the risk of OHSS.

Materials and methods

Study design

This was a non-interventional study designed to investigate the performance of the Elecsys[®] AMH assay for the prediction of response to COS. Serum samples and data from patients treated with antagonist cycles were obtained retrospectively from the control arm of the OPTIMIST study [7] (registered at <http://www.trialregister.nl>; trial number NTR2657). The study was conducted

in accordance with Good Clinical Practice and the principles of the Declaration of Helsinki. All study participants provided written, informed consent for both the OPTIMIST study and the present study. The OPTIMIST study protocol has been approved by the Institutional Review Board of the University Medical Center Utrecht (MEC 10-273) and by the board of directors of all participating centers.

Study participants

The study population comprised subfertile women aged <44 years who received the standard stimulation dose of 150 IU/day FSH, had blood samples available and were treated with a GnRH antagonist in the control arm of the OPTIMIST study [7]. Additional inclusion criteria were: a regular menstrual cycle (average 25–35 days), no major uterine or ovarian abnormalities detected by transvaginal ultrasound and no previous IVF cycles. Patients with polycystic ovary syndrome, endocrine or metabolic abnormalities, medical contraindication for pregnancy or IVF treatment or undergoing oocyte donation were excluded. Response to COS was not evaluated as an inclusion criterion in the present study.

Blood samples were collected at cycle day 1–3 either during the menstrual cycle within which stimulation treatment was received, or during the previous cycle. Blood samples were excluded from the analysis if they were taken after the start of FSH administration, taken from patients who underwent FSH dose adjustment or taken from cycles following the first stimulation cycle.

Study endpoints

The primary endpoint was response to COS. Responses were defined as: poor, <4 oocytes retrieved or cancellation of stimulation cycle (<2 growing follicles >12 mm, or <3 follicles of ≥ 17 mm) [7,8]; normal, 4–15 oocytes retrieved; hyper-response, >15 oocytes retrieved or cancellation of the stimulation cycle (>20 growing follicles >12 mm and estradiol levels >11 700 pmol/L, or >30 growing follicles >12 mm) [7].

Secondary endpoints included: number of oocytes retrieved; number of follicles between 12 mm and 16 mm on last ultrasound before follicle puncture; number of follicles >16 mm on last ultrasound before follicle puncture; and occurrence of OHSS after COS.

Table 1
Demographic characteristics of patients receiving a GnRH antagonist.

	All patients (N = 149)		Poor response (n = 45)		Normal response (n = 88)		Hyper-response (n = 16)		p value ^a
	SD		SD		SD		SD		
Mean age, years	33.9	4.5	36.4	3.7	33.0	4.5	32.3	4.0	<0.001
Mean BMI, kg/m ²	24.0	4.0	25.0	3.9	23.9	4.2	22.1	2.7	<0.05
Mean AFC, n	15.2	7.9	10.8	7.8	15.6	6.3	25.6	6.5	<0.001
Mean AMH, pmol/L	14.4	9.9	7.7	6.6	16	8.3	25	12.7	<0.001
	n	%	n	%	n	%	n	%	
Race									0.08
White	99	66.4	35	77.8	55	62.5	9	56.3	
Asian	3	2.0	2	4.4	1	1.1	0		
Black or African American	2	1.3	1	2.2	0		1	6.3	
Other	16	10.7	2	4.4	13	14.8	1	6.2	
Missing	29	19.5	5	11.1	19	21.6	5	31.2	
Smoking status									0.13
Yes	24	16.1	11	24.4	12	13.6	1	6.2	
No	123	82.6	33	73.3	75	85.2	15	93.8	
Unknown	2	1.3	1	2.2	1	1.1	0		

AFC = antral follicle count; AMH = anti-Müllerian hormone; ANOVA = analysis of variance; BMI = body mass index; GnRH = gonadotrophin-releasing hormone; SD = standard deviation.

^a p values were calculated by one-way ANOVA F-test for continuous variables (age and AFC), or by Kruskal-Wallis rank sum test for BMI, or by χ^2 -test for smoking status, or by Fisher's exact test for race. AMH mean values were reported as original data. F-test was performed on the square-root transformed AMH values to fit the assumptions of the F-test. Raw p values were reported without adjustment for multiple testing.

Table 2

Association of response class with outcomes and AMH classification.

	Poor response (n = 45)		Normal response (n = 88)		Hyper-response (n = 16)		p value ^a
	n	SD	n	SD	n	SD	
Duration of stimulation, mean number of days	8.9	2.0	8.7	1.5	8.9	1.1	0.65
Follicles on last ultrasound before follicle puncture							
>12 mm	3.0	1.6	7.5	3.4	13.9	4.7	<0.001
12–16 mm	1.2	1.3	4.4	3.0	10.3	4.4	<0.001
>16 mm	1.8	1.2	3.1	1.4	3.6	1.2	<0.001
Retrieved oocytes	2.1	0.9	8.1	2.9	21.3	4.7	<0.001
	n	%	n	%	n	%	
Cancelled cycles							
Due to poor response	17	37.8	0		0		
Due to hyper-response	0		0		0		
Occurrence of OHSS							
Yes	0		0		5	31.3 ^b	<0.001
AMH classification							
≤15 pmol/L	40	88.9	46	52.3	3	18.8	
>15 pmol/L	5	11.1	42	47.7	13	81.2	

ANOVA = analysis of variance; OHSS = ovarian hyper-stimulation syndrome; SD = standard deviation.

^a p values were calculated by one-way ANOVA F-test for continuous variables, or by Fisher's exact test for the occurrence of OHSS. Number of retrieved oocytes was log-transformed to fit the assumption of F-test. Raw p values were reported without adjustment for multiple testing. ^b Of the patients who developed OHSS, two mild and three moderate cases were observed.

OHSS included mild, moderate and severe OHSS as defined in the OPTIMIST trial protocol and by Nederlandse Vereniging voor Obstetrie & Gynaecologie Guidelines (mild OHSS: abdominal bloating, mild abdominal pain, ovarian size usually <8 cm; moderate OHSS: moderate abdominal pain, nausea with/without vomiting, ultrasound evidence of ascites, ovarian size usually 8–12 cm; severe OHSS: clinical ascites [occasionally hydrothorax], oliguria, hematocrit >45%, hypoproteinemia, ovarian size usually >12 cm) [20].

An archived serum aliquot of ≥0.6 mL of a blood sample taken for the OPTIMIST study, along with a subset of data, was transferred from the University Medical Center Utrecht to Roche Diagnostics for this study. Serum samples were shipped on dry ice and stored at –20 °C until analyzed. Investigators and laboratory staff at the measuring site were blinded to the identities, demographics and clinical data of participants associated with these samples.

Sample measurement and statistical analysis

AMH measurements were carried out using the Elecsys[®] AMH assay on a cobas e 601 analyzer (single determination as per intended use of Elecsys[®] AMH assay) at one central measuring site (Universitair Ziekenhuis Brussel, Belgium). Based on measurement of control samples, the coefficient of variation for intermediate precision was <3.0%. Results from AMH measurement were captured by WinCAEv. Clinical data analysis was calculated using software R version 3.0.1. A one-way analysis of variance (ANOVA) F-test was applied to analyze the mean differences in continuous variables (age, AFC, AMH, duration of stimulation, number of follicles and number of oocytes) between response classes. Body mass index (BMI) was tested by a non-parametric Kruskal-Wallis test. Differences in proportion were compared by χ^2 -test for smoking status and by Fisher's exact test for race and occurrence of OHSS. A post-hoc test (Tukey's honest significance test) was performed on the transformed AMH values to compare the mean difference between each pair of the three response groups. Significance was determined when $p < 0.05$.

Clinical performance of AMH for prediction of hyper-responders was defined in two ways: classification accuracy was assessed by receiver operating characteristic (ROC) analyses and the corresponding area under the curve (AUC) [21], and clinical performance was evaluated by applying a pre-specified cut-off of 15.0 pmol/L (2.10 ng/mL) [16,17]. Sensitivity for detecting hyper-

response; specificity for detecting non-hyper-response (poor and/or normal response), poor response and normal response; positive predictive values; and negative predictive values were also calculated. Clinical performance of AFC was also evaluated by ROC analyses and corresponding AUC.

Results

In total, 149 patients met the eligibility criteria for the study: 16 had a hyper-response (11%), 88 had a normal response (59%) and 45 had a poor response (30%) (Table 1). Among those who developed a hyper-response, five experienced OHSS (two mild, three moderate; Table 2). The mean (standard deviation [SD]) age was 33.9 ± 4.5 years and mean (SD) BMI was 24.0 ± 4.0 kg/m². The majority of patients were white (66.4%) and non-smokers (82.6%). AMH concentrations showed a non-Gaussian distribution with a median value of 12.4 pmol/L (inter-quartile range: 8–19.6 pmol/L) and a mean (SD) value of 14.4 ± 9.9 pmol/L. AMH values differed significantly between the three response classes, ($p < 0.001$; Fig. 1; Table 1) with highest levels seen in hyper-responders. Poor responders were older than hyper-responders, with slightly higher BMI, lower AFC and lower mean AMH values.

The association between response class and outcome is shown in Table 2. No significant differences were noted between the response classes for the duration of stimulation. However, significant differences were seen for the number of follicles prior to follicle puncture ($p < 0.001$ for follicles >12 mm, 12–16 mm and >16 mm), the number of retrieved oocytes ($p < 0.001$) and the occurrence of OHSS ($p < 0.001$), which were highest in hyper-responders.

Clinical performance of AMH for prediction of hyper-responders was assessed by ROC analysis, based on hyper-responders ($n = 16$) and non-hyper-responders (poor/normal, $n = 133$). The AUC of the ROC was 82.1% (95% confidence interval [CI]: 72.5–91.7; Fig. 1). Similarly, for prediction of poor response, based on poor-responders ($n = 45$) and normal-/hyper-responders ($n = 104$), the AUC of the ROC was 85.5% (95%CI: 77.8–93.2).

Applying the AMH cut-off of 15.0 pmol/L, a sensitivity of 81.3% for predicting hyper-response (95%CI: 54.4–96.0) and a specificity of 64.7% (95%CI: 55.9–72.8) for identifying poor/normal responders was reached (Table 3). The probability that patients with a positive test (AMH > 15.0 pmol/L) were true hyper-responders

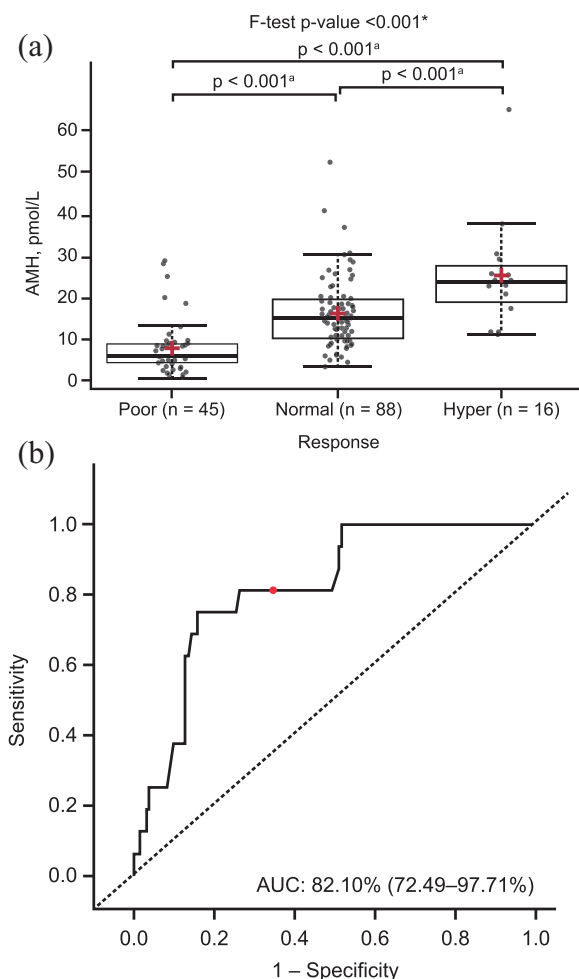


Fig. 1. Distribution of AMH per response class and clinical performance of AMH for prediction of hyper-response.

AUC = area under the curve; AMH = anti-Müllerian hormone; ANOVA = analysis of variance; ROC = receiver operating characteristic.

Upper panel shows the distribution of AMH by response groups. Red crosses are the mean values of AMH in each group. *A one-way ANOVA F-test and ^aTukey's honest significance test were performed on the transformed AMH values to compare the mean difference between the three response groups. Tukey's test corrects the p values for multiple comparisons.

Bottom panel shows the ROC curve of AMH for prediction of hyper-responders and the clinical performance at the cut-off. Data from n = 16 hyper-responders and n = 133 poor and/or normal responders. Cut-off point marked in red on ROC curve.

(positive predictive values) was 21.7% (95%CI: 12.1–34.2). The probability that patients with a negative test result ($\text{AMH} \leq 15.0$ pmol/L) were non-hyper-responders (poor or normal responders) was 96.6% (95%CI: 90.5–99.3; Table 2). When considering poor and normal responder groups separately, true negative rate of the AMH cut-off of 15.0 pmol/L was 88.9% (95%CI: 76.0–96.3) in the poor responder group and 52.3% (95%CI: 41.4–63.0) in the normal responder group, respectively. Therefore, 89% of poor responders and around half of normal responders had AMH values of 15.0 pmol/L or below (Table 3).

Clinical performance of AFC was also assessed. The area under the ROC curves was 79.8% (95%CI: 71.5–88.1) for the prediction of a poor response and 91.9% (95%CI: 87.0–96.7) for the prediction of hyper-response.

Comment

Using the Elecsys[®] AMH assay, we demonstrate that the prespecified AMH cut-off of 15 pmol/L is strongly correlated with

ovarian response categories in women undergoing COS with a GnRH antagonist protocol and showed good performance for prediction of hyper-response. Sensitivity for the detection of hyper-responders was 81.3%, and the negative predictive value for ruling out hyper-response was 96.6%. Furthermore, the 15 pmol/L cut-off correctly identified 88.9% of patients with a poor response. Therefore, approximately 10% of patients with a poor response would be incorrectly identified as hyper-responders (i.e. false positives); however, this must be balanced against the high proportion of true hyper-responders who could be prevented from risk of OHSS by dose adjustment. Additionally, we note that a false-positive rate of approximately 10% is consistent with that deemed acceptable in other clinical settings; for example, first trimester screening for pre-eclampsia [22].

Previous studies assessing the role of the Elecsys[®] AMH assay in this therapy area have contributed evidence supporting AMH as the preferred biomarker for prediction of response to COS. Anderson et al. reported that the Elecsys[®] AMH assay revealed good correlation with age and AFC in women of reproductive age, and provided a reproducible measure of the growing follicle pool [23]. In addition, Anckaert et al. noted the excellent analytical performance of the assay under routine clinical conditions [22]. The ultimate goal of facilitating patient-tailored stimulation protocols requires that AMH cut-offs are defined to support clinicians in identification of patients who are at risk of poor- or hyper-response. Given the small sample size of the OPTIMIST antagonist subgroup analyzed, it was difficult to determine antagonist-specific cut-offs, resulting in the need to use a previously reported cut-off to evaluate clinical performance [16,17]. This may be considered a limitation of the current study.

Use of an AMH cut-off to predict response to a GnRH antagonist protocol has been evaluated in four previous studies. A randomized, phase II trial evaluated 265 women aged <37 years who received COS with recombinant human FSH or follitropin alfa in a GnRH antagonist cycle [16]. Randomization was stratified according to serum AMH at screening (low: 5.0–14.9 pmol/L; high: 15.0–44.9 pmol/L). The number of oocytes retrieved increased in a recombinant human FSH dose-dependent manner. Across all doses of recombinant human FSH, a higher number of oocytes were retrieved in patients in the high compared with the low AMH stratum. A large prospective cohort study in 487 women receiving GnRH antagonist treatment reported that AMH levels could accurately predict the number of retrieved oocytes, as well as identify high and low ovarian response. Notably, AMH had greater accuracy for predicting high vs. low response (AUC: 0.87 vs. 0.79) [9]. As expected, poor responders were older than hyper-responders in our study, with lower mean AMH values. However, we also noted a significant difference between the three BMI groups ($p = 0.015$, Kruskal Wallis test); poor responders had higher BMI than hyper-responders ($p = 0.005 < 0.017$, Mann Whitney test, Bonferroni correction). The $0.8 \mu\text{g/L}$ (5.7 pmol/L) cut-off proposed was not optimal, only identifying 50% of poor-responders. A secondary analysis of data from 749 women aged 21–34 years enrolled in a randomized study categorized patients according to AMH concentration and showed that the 25th percentile ($\text{AMH} < 13 \text{ pmol/L}$) had significantly fewer oocytes retrieved compared with the other percentiles [15].

These four previous studies measured AMH using the AMH Gen 2 ELISA [9,15,16,24]. As differences in analytical performance have been demonstrated between commercially available AMH immunoassays, cut-offs proposed for one assay may not be directly transferable to other assays [18,19]. Specifically, the AMH Gen 2 ELISA systematically measures 10% higher than the Elecsys[®] AMH assay, which would potentially lead to misclassification of 29% of women [25]. Furthermore, poor assay reproducibility was observed with the AMH Gen 2 ELISA assay [26]. Clinical

Table 3

Response prediction performance measures and accuracy of AMH for prediction of response.

	Estimate	
	%	95%CI
AMH cut-off	15 pmol/L [2.10 ng/mL]	
Performance measures, hyper-response		
Sensitivity (hyper-response) ^a	81.3	54.4–96.0
Specificity (normal and poor response) ^b	64.7	55.9–72.7
PPV (hyper-response) ^c	21.7	12.1–34.2
NPV (normal and poor response) ^d	96.6	90.5–99.3
Performance measures, other		
True negative rate (poor response) ^e	88.9	75.9–96.3
True negative rate (normal response) ^f	52.3	41.4–63.0
False negative rate (hyper-response) ^g	18.8	4.0–45.6
False positive rate (poor response) ^h	11.1	3.7–24.1
False positive rate (normal response) ⁱ	47.7	37.0–58.6

AMH = anti-Müllerian hormone; CI = confidence interval; NPV = negative predictive value; PPV = positive predictive value.

^a The proportion of patients with a hyper-response correctly identified by AMH > 15 pmol/L.

^b The proportion of patients with a poor or normal response (i.e. without a hyper-response) correctly identified by AMH ≤ 15 pmol/L.

^c The proportion of patients with AMH > 15 pmol/L that are true positive (a hyper-response).

^d The proportion of patients with AMH ≤ 15 pmol/L that are true negative (a poor or normal response).

^e The proportion of true negatives (AMH ≤ 15 pmol/L) in poor responders.

^f The proportion of true negatives (AMH ≤ 15 pmol/L) in normal responders.

^g The proportion of false negatives (AMH ≤ 15 pmol/L) in hyper-responders.

^h The proportion of false positives (AMH > 15 pmol/L) in poor responders.

ⁱ The proportion of false positives (AMH > 15 pmol/L) in normal responders.

implications of potentially using imprecise AMH cut-offs necessitate that they are validated for specific assays and protocols. We report for the first time a validation of the 15.0 pmol/L cut-off with the Elecsys[®] AMH assay for patients receiving a GnRH antagonist protocol. In addition, a recent study presented at ASRM 2018 investigating the Elecsys[®] AMH immunoassay for ovarian response prediction in a large cohort of 1248 women in GnRH antagonist cycles reported an optimal AMH cut-off of 14.2 pmol/L for predicting excessive response (>15 oocytes retrieved) [24]. This is very close to the cut-off of 15.0 pmol/L we have validated in this study, providing support for our findings.

In the present study, the area under the ROC curve for the prediction of a hyper-response was greater for AFC than for AMH; however, interpretation is limited by the small sample size. In previous single-center studies and a large meta-analysis of individual patient data, AMH and AFC were comparable predictors of ovarian response to gonadotropin therapy [4,27]. In contrast, data from large randomized multicenter trials have shown AMH is a more accurate predictor than AFC [13–15,28]. For example, AMH was a stronger predictor of ovarian response to gonadotropin therapy than AFC at the study center level in randomized trials using GnRH-antagonist and GnRH-agonist protocols; AFC provided no added predictive value beyond AMH in these studies [13]. Furthermore, AMH appears to be a more robust marker due to the significant intra- and inter-center operator variability observed for AFC [13,23].

Recently, a randomized, multicenter, assessor-blinded, non-inferiority trial (ESTHER-1) examined the efficacy and safety of follitropin delta with individualized dosing based on serum AMH and body weight, in comparison with conventional follitropin alfa dosing for COS in women undergoing IVF. Individualized dosing of FSH follitropin delta based on AMH serum levels (reduced FSH if AMH ≥ 15 pmol/L) using the Elecsys[®] AMH immunoassay had similar efficacy (rates of pregnancy and live birth) and improved safety (fewer measures taken to prevent OHSS) compared with conventional COS [29].

In conclusion, our findings add to growing evidence of the reliability of the Elecsys[®] AMH immunoassay for assessing ovarian reserve and predicting hyper-response to COS. Specifically, we demonstrate that the Elecsys[®] AMH immunoassay, and a 15 pmol/L cut-off, provides reliable prediction of hyper-response to COS in women who undergo a GnRH antagonist treatment protocol. If hyper-responsive patients can be identified with confidence using AMH levels, this approach can be used to inform clinical decision-making and may provide steps towards personalized, optimized COS with FSH to potentially improve patient safety [8,29].

Contribution to authorship

All authors contributed to the conception and design of this study, were involved in the interpretation of the data and the development of the manuscript. All authors have approved the final article.

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Conflicts of interest

EA and HLT have no conflicts of interest to disclose. BD and MH are employees of Roche and own stocks/shares in Roche. YH is an employee of Roche. FB reports personal fees from advisory board Ferring, personal fees from advisory board Merck Serono, personal fees from consultancy work for Gedeon Richter, personal fees from educational activities for Ferring, personal fees from strategic cooperation Roche, personal fees from research cooperation Ansh Labs, outside the submitted work.

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