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Paediatric reference intervals for plasma anti-Müllerian hormone: comparison of data from the Roche Elecsys assay and the Beckman Coulter Access assay using the same cohort of samples

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Abstract

Background: Autoanalyser methods for the measurement of anti-Müllerian hormone have been introduced into clinical laboratories but few reports of paediatric reference intervals using these new assays have been published.

Methods: After prior evaluation of the Roche Elecsys anti-Müllerian hormone assay against the Beckman Coulter modified second generation anti-Müllerian Hormone enzyme-linked immunosorbent assay using samples from adult females, a cohort of paediatric samples which had previously been assessed using the Beckman Coulter Access anti-Müllerian hormone assay was analysed using the Roche Elecsys anti-Müllerian hormone assay.

Results: The Roche Elecsys anti-Müllerian hormone assay measured significantly lower than the Beckman Coulter modified second generation anti-Müllerian Hormone enzyme-linked immunosorbent assay. In the paediatric cohort measured with the Roche Elecsys assay, male levels are very high from birth to puberty after which they fall towards postpubertal female levels. Male results were similar to those previously obtained using the Beckman Coulter Access anti-Müllerian hormone assay on the same cohort. Roche Elecsys anti-Müllerian hormone in the females was very low in the neonatal and prepubertal years and the postpubertal trend, with a steady rise from 15 years, was smoother than previously modelled using the Beckman Coulter Access anti-Müllerian hormone assay.

Conclusion: Anti-Müllerian hormone levels measured with the Roche Elecsys assay were significantly lower than the Beckman Coulter modified second generation enzyme-linked immunosorbent assay suggesting the need for new reference ranges. In the paediatric cohort, Roche Elecsys anti-Müllerian hormone levels between boys and girls showed good prepubertal delineation and small but statistically significant differences to previously measured levels using the Beckman Coulter Access anti-Müllerian hormone assay on the same sample cohort.

Keywords

Anti-Müllerian hormone, paediatrics, assay, reference range

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Introduction

Measurement of anti-Müllerian hormone

The bioactive form of anti-Müllerian Hormone (AMH) termed $AMH_{N.C}$ is a homodimer of two identical chains formed by the cleavage and subsequent non-covalent linkage of its precursor pro-AMH.[1] Pro-AMH and $AMH_{N.C}$ circulate in approximately equal amounts and are both detected by the antibodies used in most AMH assays, which thus report this so-called total AMH. Commercial assays for the measurement of AMH have been available since the late 1990s. Two of the most popular assays were the Beckman Coulter Immunotech (IOT) AMH enzyme-linked immunoassay (ELISA) and the Diagnostic Systems Laboratories (DSL) AMH ELISA. Due to different reagent components, these assays reported significantly different results and to remedy this Beckman Coulter acquired the DSL assay and then merged it with the IOT assay to form a second generation (Gen II) AMH ELISA, henceforth termed the Beckman Coulter Gen II AMH ELISA.[1] Shortly after its release it became apparent that the Gen II assay was reporting lower than expected AMH concentrations, thought to be a problem with interference in capture antibody binding by complement in the sample.[1,2] This was solved by introducing a predilution step (with assay diluent) in the protocol.[3–5] The modified assay is henceforth called the Beckman Coulter *modified* Gen II AMH ELISA.

More recently diagnostics companies Roche and Beckman Coulter have developed electrochemiluminescence immunoassay (ECLIA) methods for the measurement of AMH, namely the Roche Elecsys AMH (Roche Diagnostics, UK) and the Beckman Coulter Access AMH (Beckman Coulter United Kingdom, UK) appropriate for their respective autoanalyser platforms.[6,7] These have larger scale sample throughput, quicker turnaround times, increased sensitivity and broader measuring ranges than previous commercial AMH assays. The dynamic measuring ranges for these assays (i.e. from limit of quantitation [LOQ] to top calibrator) are as follows: Beckman Coulter Access 0.04–181.0 pmol/L and Roche Elecsys 0.2–164.1 pmol/L. (NB: the quoted top calibrator for the Access method is 171.0; however, at the time of the present study this was stated by the manufacturer to be 181.0 pmol/L). These new assays utilize the capture and detection antibody pair from the previous Beckman Coulter modified Gen II ELISA AMH assay and have been evaluated by several laboratories.[7–11] Methodological adjustments in these autoanalyser methods prevent interference by complement[1,11] and they show very good correlation with the Beckman Coulter modified Gen II ELISA method.[8] However, several authors have reported a negative bias relative to the Beckman Coulter modified Gen II ELISA method for both the Beckman Coulter Access assay[7,9,10] and the Roche Elecsys assay.[7,9–11] Most evaluations have been made using serum from adult females as the assessment of ovarian reserve is the most common clinical reason for an AMH request. Consequently, recent reports have detailed reference ranges for adult females that are either unchanged from previous assays[8] or are lower[9,10] using either of these new methods.

Paediatric AMH

The clinical biochemistry department at Manchester University Hospitals NHS Foundation Trust (MFT) serves a women's hospital and a children's hospital on the same site and while the measurement of AMH in adult women is most common, there is an important clinical role for AMH measurement in paediatrics.

In males AMH, produced by Sertoli cells from the seventh week of gestation, plays an important role in fetal sexual development, triggering regression of the Mullerian ducts and progressing testicular development.[12] Female AMH is produced by granulosa cells in pre-antral and small antral follicles and is not expressed until 36 weeks' gestation. Healthy male AMH concentrations are very high from birth to puberty whereupon they decrease significantly but in general remain higher than those seen in females. In healthy females, AMH is low at birth and rises slowly through infancy. Levels increase further at puberty and then remain relatively stable until the mid to late 20s when they begin to fall, becoming undetectable at menopause. AMH measurement is useful in the investigation of disorders of sexual development (DSD)[13] and premature ovarian failure, polycystic ovary syndrome and granulosa cell tumours in girls and anorchia/cryptorchidism, hypogonadotropic hypogonadism and Klinefelter's syndrome in boys.[12,14] It is also used to monitor reproductive potential in girls undergoing cancer treatment with gonadotoxic chemotherapy.[15]

There are few reports of paediatric AMH reference ranges using the new Elecsys and Access assays. Recently Demirdjian et al.[16] reported partial paediatric reference intervals using the Beckman Coulter Access AMH assay and we have recently reported comprehensive reference range data on a paediatric cohort of samples using the Beckman Coulter Access AMH with the Beckman Coulter DxI 800 autoanalyser.[17]

Aim of the present study

Following our validation of the Roche Elecsys AMH assay against our previous service assay the Beckman Coulter modified Gen II AMH assay using samples

from adult females, the primary aim of this study was to derive full paediatric age- and gender-related reference ranges for AMH using the Roche Elecsys assay on the Cobas e602 autoanalyser and to compare these AMH values to those obtained previously on the same cohort of paediatric samples using the Beckman Coulter Access assay.[17] Finally, paediatric male and female AMH using values generated on samples which had both a valid Roche Elecsys and previously measured Beckman Coulter Access AMH value were directly compared.

Materials and methods

Validation of the Roche Elecsys automated assay

Prior to analysis of the paediatric samples a comparison of the Roche Elecsys assay was made against our previous AMH assay, the Beckman Coulter modified Gen II AMH ELISA. Fifty-eight routine adult female samples, previously assayed using the Beckman Coulter modified Gen II AMH ELISA and stored for up to six months at −80°C, were thawed and measured using the Roche Elecsys AMH assay. Deming regression plots of Roche Elecsys AMH versus the Beckman Coulter modified Gen II AMH were constructed (Supplemental Figure 1).

In our initial evaluation of the Roche Elecsys method, serum from 64 adult females was measured fresh on the day of collection and then re-assayed after three months storage at −20°C and the paired fresh/frozen results were compared. No significant difference in AMH concentration was found (Wilcoxon matched pair test, $p = 0.147$). This in-house study alongside manufacturers' data confirmed that there was less than a 4% change in AMH after one freeze/thaw cycle, regardless of the assay used (Supplemental Table 1). Performance parameters for the above two

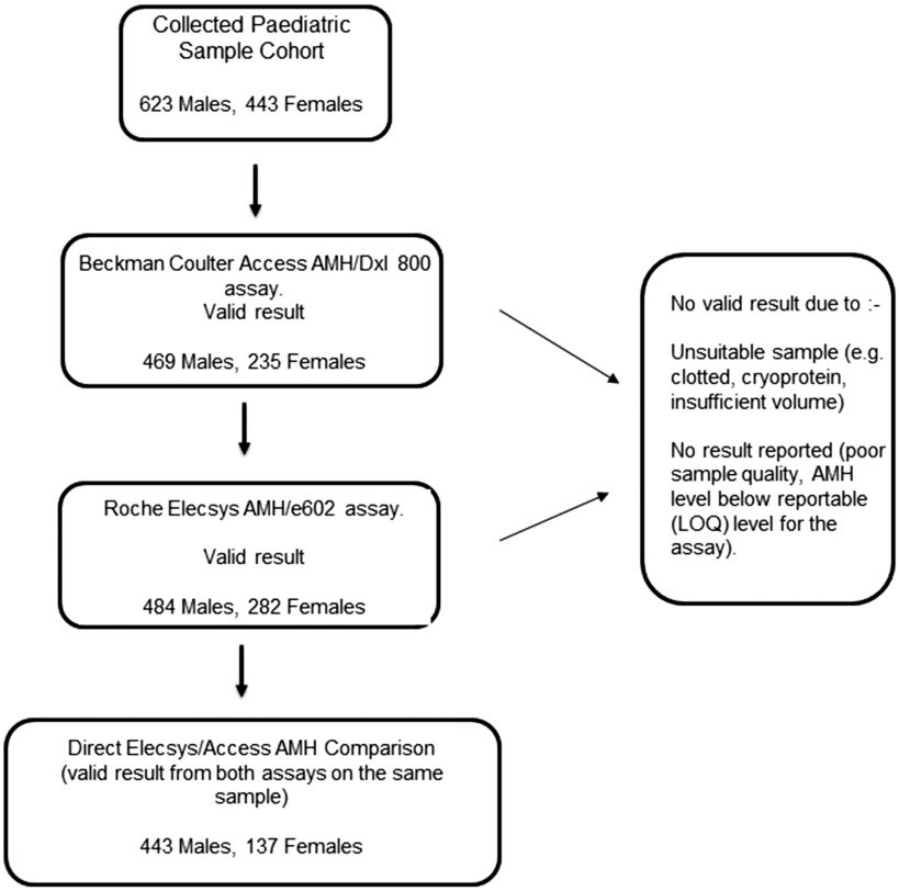

Figure 1. Chronological sample flow chart. Chronological flow chart showing the sample numbers collected in the paediatric cohort, how many were previously assayed using the Beckman Coulter Access AMH assay,[17] how many were available for the Roche Elecsys AMH assay and how many of these samples had both a valid (i.e. a reportable value above the LOQ) Roche Elecsys result and Beckman Coulter Access result to enable direct comparison. The attrition of the cohort after each assay is noted on the right with some samples not being suitable (insufficient remaining volume, sample quality, etc.) and some reported as 'sample error' or 'insufficient sample'. AMH: anti-Müllerian Hormone.

assays, along with manufacturer's published data for the Beckman Coulter Access AMH assay are shown in Supplemental Table 1.

Paediatric plasma samples

A total of 1066 unselected lithium heparin plasma samples (623 phenotypically male and 443 phenotypically female) were collected from patients aged between 1 day and 18 years over a six-month period from surplus routine samples sent to the biochemistry department at MFT as described previously.[17] As this was a method development study utilizing anonymized surplus acellular plasma marked for disposal from routinely requested samples explicit consent was not required (further information is available at https://www.hta.gov.uk/). Samples were allocated a study number and frozen at −80°C for up to two years. The interval between the Beckman Coulter Access analysis and the Roche Elecsys analysis was 7–9 months with only one freeze/thaw between assays. Only age and phenotypic sex were recorded after patients undergoing investigation for any DSD or other endocrinopathy were excluded from the study.

Roche Elecsys AMH assay

Paediatric samples analysed in the current study were from the same cohort as those measured previously using the Beckman Coulter Access assay.[17] Specimens were assayed using the Roche Elecsys AMH assay on the Cobas e602 autoanalyser (Roche Diagnostics, Burgess Hill, West Sussex, UK). An adequate sample cup volume which did not cause an 'insufficient sample' error on this analyser was 80 μL. As paediatric male AMH concentrations are normally very high, these samples were always assayed after prior offline 1 in 10 dilutions in manufacturer's diluent. Thus, 80 μL of neat female sample and only 20 μL of neat male sample (diluted up to 200 μL) were required for analysis. There were 484 male and 282 female samples that were suitable for the current study. The Elecsys assay showed an intermediate precision of 4.7 and 3.6% for two levels of quality control material (6.64 and 37.0 pmol/L, respectively, see Supplemental Table 1. NB: 7.14 pmol/L AMH = 1.0 µg/L) measured over the same time period as the samples. Samples from this cohort were previously analysed using the Beckman Coulter Access AMH assay[17] and in some cases the volume required for that assay (180 μL) reduced the remaining sample volume such that subsequent analysis with the Roche Elecsys AMH assay was not possible This occurred more frequently among the neonate female groups where original sample volume was relatively low and they had to be analysed undiluted. Thus, not all of the samples in the cohort (a) had sufficient volume to be measured by the first Access analysis (180 μL) or (b) had sufficient remaining volume for the Elecsys assay. A chronological flow chart outlining sample numbers from the original cohort assayed by each of the methods is shown in Figure 1, along with the samples which were of a suitable quality and volume (i.e. with valid results on both the Beckman Coulter Access and the Roche Elecsys assays) to enable a direct comparison between the two assays.

Statistical methods

Deming regression plots on the adult female values from the Roche Elecsys AMH validation against the Beckman Coulter modified Gen II AMH ELISA and the direct comparison of Roche Elecsys and Beckman Coulter Access derived paediatric AMH values was performed using Analyse-it software (Version no. 4.65.2, Analyse-it Software, Leeds, UK), as was the Wilcoxon matched pair test for difference on the paired Roche Elecsys AMH versus Beckman Coulter modified Gen II AMH data, the direct comparison of paired Roche Elecsys AMH versus Beckman Coulter Access AMH data and the freeze/thaw data between fresh and frozen samples in the preliminary evaluation of the Elecsys assay using adult female samples.

Paediatric Roche Elecsys AMH values were analysed as described previously for the Beckman Coulter Access AMH data.[17] Individual $\log_{10}$-transformed Roche Elecsys AMH values were plotted against age for males and females. Next quantile regression analysis was carried out using the R package Quantreg with taus of 0.05, 0.5 and 0.95. Models for the males and females were constructed and compared to those obtained previously using the Beckman Coulter Access AMH values.[17] Regression lines were fitted to the data with Bezier splines where the number of inflection points was minimized through Akaike Information Criterion optimization. This diminished the influence of outliers, which were not removed. Robust statistical analysis was then used to determine the reference intervals. After confirmation that there was no significant difference between the neonatal female groups (classes 1–5, birth to one month) using Tukey's studentized range test in ANOVA analysis, results from these groups were combined enabling a direct comparison to the same reference intervals from the previous Beckman Coulter Access AMH analysis. Then, for the whole cohort, 95% quantiles were generated using a robust fit to a gamma distribution with 95% confidence intervals derived using a bootstrap sampling method with 1000 iterations.[17]

Results

Validation of the Roche Elecsys AMH assay against the Beckman Coulter modified Gen II AMH assay

The Deming regression plot of the adult female Roche Elecsys AMH values against the paired Beckman Coulter modified Gen II values (Supplemental Figure 1) showed a high degree of correlation between methods (Spearman rank correlation r_s 0.98); however, absolute values from the Roche Elecsys AMH assay were significantly lower (Wilcoxon matched pair test for paired data $p < 0.005$) than those reported with the Beckman Coulter modified Gen II AMH assay. Median Roche Elecsys AMH was 23% lower than the Beckman Coulter modified Gen II assay.

Comparison of male and female Roche Elecsys AMH results

Log-transformed male and female Roche Elecsys AMH values were plotted along a continuous age axis (Figure 2). Male AMH ranged from 17.6 to 1609 pmol/L across the whole group whereas female AMH concentrations ranged from 0.2 to 71.3 pmol/L across the whole group. It is important to note that male and female levels show no overlap in the prepubertal years. There is a small degree of overlap in the older children.

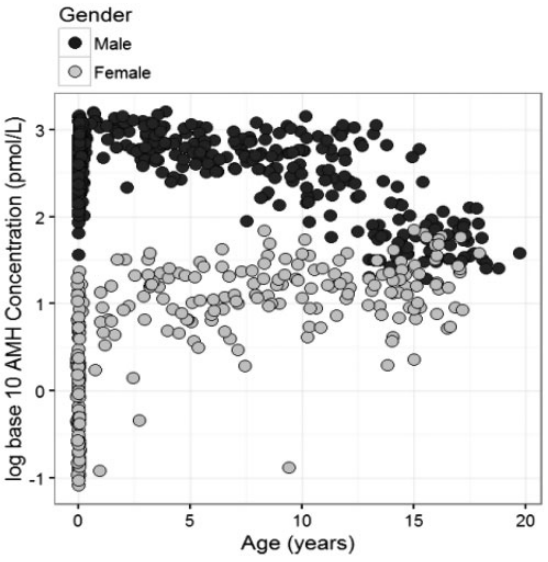

Figure 2. AMH concentration by gender and age. AMH was measured in 484 males and 282 females using the Elecsys assay and Cobas e602 analyser and results were segregated according to age and gender. AMH (pmol/L) was plotted on a logarithmic scale to enable direct comparison of male and female levels. NB: Values below the LOQ of the Roche Elecsys AMH assay (0.2 pmol/L) were not recorded. AMH: anti-Müllerian Hormone.

General comparison of Roche Elecsys AMH results with Beckman Coulter Access AMH results

The quantile regression model for the male cohort of samples analysed using the Roche Elecsys AMH assay was very similar to the model obtained previously[17] using the Beckman Coulter Access AMH data; there was a high concentration of AMH at birth, increasing to a peak within the first year of life before falling gradually, plateauing during infancy and then decreasing further from age 10 to 12 years. The model in females using the Roche Elecsys data was smoother than that previously modelled with the Beckman Coulter Access AMH values. The Roche Elecsys female data showed a rise from very low AMH concentrations around birth to 2–3 years of age which then flattened. There was a further 'pulse' of AMH rise around 11 years and then a steady rise from 15 years up to 18 years. In contrast, the female data modelled using the Beckman Coulter Access values showed AMH pulses at 2–3 years and around 9 years but then showed a fall in AMH from 15 years on. These previously modelled Beckman Coulter Access data are included in Figure 3 for comparison. Roche Elecsys data may be more robust due to the greater number of samples suitable for analysis.

Direct comparison of paired Roche Elecsys and Beckman Coulter Access AMH values

A direct comparison using data from 443 male and 137 female samples which had generated both a valid measurable Roche Elecsys AMH value (i.e. between the LOQ and the upper limit of the assay) and a previous valid measurable Beckman Coulter Access AMH value was undertaken. Deming regression plots of AMH from each of these assays for each gender are shown in Figure 4. The assays were strongly correlated (Spearman rank correlation r_s was 0.98 for the males and 0.96 for the females) with small but statistically significant differences (see below) between them within each gender. Male Roche Elecsys AMH values were significantly higher than the previous Beckman Coulter Access AMH value obtained on the same sample (Wilcoxon matched pair test, $p < 0.0001$). Median male AMH was approximately 5% higher on the Roche Elecsys assay. In contrast, the Deming regression plot of female Roche Elecsys AMH concentrations was significantly lower than the previous Beckman Coulter Access plot obtained on the same samples (Wilcoxon matched pair test for paired data,

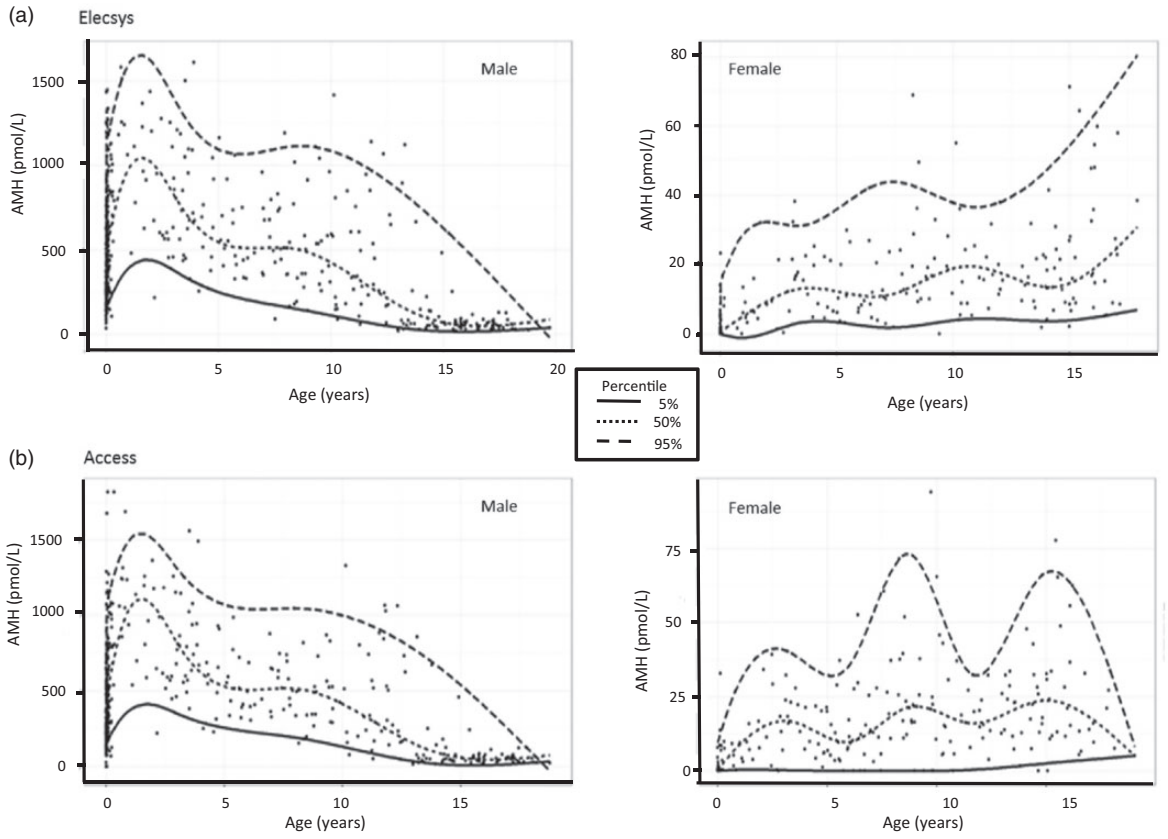

Figure 3. Male and female age-related quantile regression with smoothed splines. AMH results from both the Elecsys (484 males, 282 females) and Access (469 males, 237 females) assays were analysed using quantile regression with smoothing splines using the R package Quantreg with taus of 0.05, 0.5 and 0.95. (Adapted from Access assay-only data previously published by the same authors[17] under a creative commons agreement). NB: Values below the LOQ of the Roche Elecsys AMH assay (0.2 pmol/L) and the Beckman Coulter Access AMH assay (0.04 pmol/L) were not recorded. The zero point on the AMH axis is included for aesthetic appearance and ease of construction. AMH: anti-Müllerian Hormone.

$p < 0.0001$). Median female AMH was approximately 4% lower on the Roche Elecsys assay when compared to the Beckman Coulter Access assay.

Age-stratified reference intervals using the Roche Elecsys assay

Data from the Roche Elecsys analysis were compartmentalized into the 11 male age groups and 7 female groups as outlined in Tables 1 and 2, respectively. It is of note that the female age class 5 (0–28 days) in the Roche Elecsys group contained 87 results; however, these were combined after statistical analysis to enable a direct comparison to the 24 results previously obtained for the equivalent Beckman Coulter Access female class 5. Roche Elecsys results, and the previous Beckman Coulter Access results for comparison, are shown in Tables 1 and 2. There is a very clear delineation in median AMH between the two genders across the paediatric age range, irrespective of which assay was used. Importantly, the AMH values for the 2.5th percentile in the youngest two female age classes are extremely low. These values obviously fall below the LOQ of each assay so they are not 'real' values, they are calculated characteristics of the statistical data distribution and as such are included for completeness.

Discussion

The Roche Elecsys and Beckman Coulter Access AMH assays show increased sensitivity with a wider measuring range compared to the previous Beckman Coulter modified Gen II ELISA assay (Supplemental Table 1) and are not susceptible to complement interference. Requests can be analysed in real time thus improving turnaround times compared to previous protocols involving batch analysis using manual ELISAs. Supplemental Table 1 also shows that the one freeze/thaw of samples between the Beckman Coulter Access

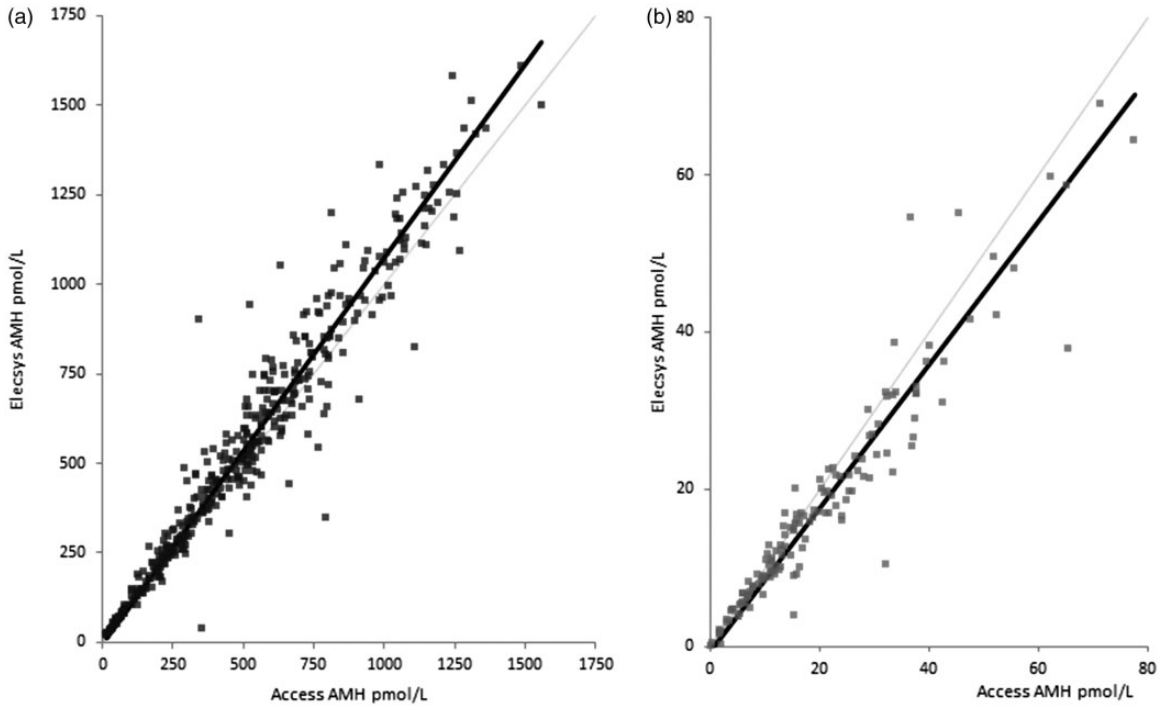

Figure 4. Direct comparison of male (a) and female (b) Elecsys AMH versus Beckman Coulter Access AMH measured by Deming regression. Equations were as follows: Males (n=443) Elecsys AMH = 0.709 + 1.079 (Access AMH) with a Spearman Rank correlation coefficient rs = 0.98. Females (n=137) Elecsys AMH = −0.055 + 0.896 (Access AMH), rs = 0.96. NB: Values below the LOQ of the Roche Elecsys AMH assay (0.2 pmol/L) and the Beckman Coulter Access AMH assay (0.04 pmol/L) were not recorded. The zero point on each AMH axis is included for aesthetic appearance and ease of construction. AMH: anti-Müllerian Hormone.

and the Roche Elecsys analysis does not cause a clinically significant change in values.[2,16]

The evaluation of the Roche Elecsys AMH method outlined here included the comparison of values obtained from adult female patients attending our routine reproductive medicine clinic. The Roche Elecsys method showed excellent correlation with our previous service assay, the Beckman Coulter modified Gen II AMH ELISA, but had a significant proportional negative bias. Median AMH was 23% lower for the Roche Elecsys assay compared to the Beckman Coulter modified Gen II assay. This is in keeping with most previous evaluations of the Roche Elecsys assay against the Beckman Coulter modified Gen II method.[18–20] Similar findings have been reported between the Beckman Coulter Access AMH assay and the Beckman Coulter modified Gen II assay.[9,16] The difference between the Roche Elecsys and Beckman Coulter modified Gen II assays may reflect differences in their respective calibrations; the Roche Elecsys assay is calibrated to the unmodified version of the Beckman Coulter Gen II ELISA, which underestimated AMH compared to the modified protocol of the Gen II ELISA,[1,3–5] and it is being compared to the modified Gen II method here. It should be noted that traceability of the Roche Elecsys assay is to the unmodified Beckman Coulter Gen II-referenced set of standards and not to a higher metrological method. Furthermore, lab-to-lab differences in assay evaluation could also be due to local variability with the Beckman Coulter modified Gen II assay itself, which in most laboratories is only semi-automated at best. The unmodified Beckman Coulter Gen II ELISA was prone to interference which was eventually corrected by modification of the assay protocol[1,3–5] whereas ECLIA protocols do not suffer complement interference.[1,11] Quantitative differences with the Roche Elecsys and Beckman Coulter Access methods to the previous modified Gen II AMH ELISA method reinforce the need for assay-specific reference ranges[4] and emphasizes the pressing need for development of an international reference material for AMH which has recently shown progress.[21]

We believe this study is the first to generate Roche Elecsys AMH reference ranges on a complete male and female paediatric cohort. Log-transformed data show that there is a clear delineation between normal male and female AMH values. Male AMH is very high compared to females in the neonatal and prepubertal age groups (0–10 years) after which male AMH falls towards, with some small overlap of, female levels

Table 1. Paediatric male AMH reference intervals. Roche Elecsys vs. Beckman Coulter Access.

			AMH (pmol/L)		
Age group	Class	N	2.5th percentile (90% CI)	Median	97.5th percentile (90% CI)
0–2 d	1	**42**	**78.11 (58.89–108.94)**	**258.8**	**606.46 (409.51–699.86)**
		51	72.73 (50.25–80.37)	258.1	628.54 (502.06–691.89)
3–7 d	2	**44**	**159.60 (57.93–199.81)**	**554.3**	**1186.20 (982.69–1495.78)**
		45	119.30 (69.53–128.08)	486.1	1112.11 (1088.47–1417.21)
8–10 d	3	**15**	**225.52 (0–330.32)**	**703.0**	**1391.71 (1113.36–1962.28)**
		14	193.19 (0–287.76)	563.4	1074.24 (636.36–1531.02)
11–20 d	4	**43**	**161.70 (59.38–203.13)**	**537.0**	**1310.46 (1138.87–1641.31)**
		37	211.15 (179.28–298.88)	522.3	987.52 (739.18–1051.61)
21–28 d	5	**25**	**244.98 (142.28–359.27)**	**567.0**	**1102.37 (777.43–1284.35)**
		26	201.06 (166.23–287.95)	504.7	1055.39 (845.16–1238.07)
29–364 d	6	**66**	**235.52 (155.15–323.18)**	**551.2**	**1125.86 (706.65–1358.86)**
		66	287.97 (251.82–388.01)	662.9	1242.42 (904.46–1372.25)
1–4.9 y	7	**60**	**310.70 (246.56–422.97)**	**692.7**	**1425.29 (1089.83–1577.96)**
		58	282.08 (214.91–355.71)	690.9	1525.92 (1364.20–1739.17)
5–7.9 y	8	**39**	**238.33 (187.26–301.84)**	**511.5**	**1108.32 (868.53–1296.48)**
		39	221.18 (171.59–244.59)	517.3	1062.66 (868.91–1368.39)
8–11.9 y	9	**60**	**96.58 (0–100.14)**	**426.3**	**1131.44 (1045.31–1497.03)**
		49	84.25 (0–73.35)	380.9	1109.54 (1122.89–1480.67)
12–14.9 y	10	**39**	**9.43 (0–15.48)**	**71.65**	**331.80 (144.80–464.85)**
		36	3.57 (0–3.20)	64.66	444.49 (383.94–592.82)
15–18.9 y	11	**51**	**16.76 (7.83–22.03)**	**58.2**	**130.04 (95.39–153.18)**
		48	16.13 (14.68–19.62)	55.61	120.35 (99.43–132.26)

Male paediatric AMH age-specific reference ranges. The 95% confidence intervals at 2.5th and 97.5th centiles were generated using a robust fit to a gamma distribution. Table adapted from Access-only data previously published by the same authors (17) under a creative commons agreement. Roche Elecsys values in bold, Beckman Coulter Access values in roman. The 2.5th percentile values and the corresponding Confidence intervals are calculated characteristics of the statistical distribution. They obviously fall well below the LOQ of either of the two assays but are included for completeness. AMH: anti-Müllerian Hormone.

(Figure 2). The age-related distribution of male and female log-transformed Roche Elecsys AMH values is very similar to that previously obtained using the Beckman Coulter Access AMH assay.[17] This study confirms the Roche Elecsys AMH assay as an ideal marker for assessing normal sexual development in both neonatal and prepubertal children of both genders[12,22] and for the investigation of reproductive disorders such as hypogonadotropic hypogonadism and Klinefelter's syndrome during the pubertal transition.[12]

Quantile regression modelling of male AMH data using the Roche Elecsys assay is very similar to the modelled male AMH data obtained previously using the Beckman Coulter Access values; however, the female data show more variation between these two autoanalyser assays (Figure 3). In females the modelled data using the Roche Elecsys values show a rise after 15 years, in keeping with previous studies[23] whereas the modelled female data using the Beckman Coulter Access values did not.[17] This difference is less apparent in the compartmentalized data (Table 2) and it should be acknowledged that the former is a modelled day-to-day change whereas the latter analysis represents a whole age interval covering three years subjected to rigorous statistical analysis. Direct comparison of Roche Elecsys and Beckman Coulter Access AMH values on the same sample (Figure 4) showed a statistically significant difference in male and female AMH values for each assay; however, these differences may not be clinically relevant in the paediatric setting.

The data presented here qualitatively concur with the relatively few studies which have looked at paediatric AMH reference ranges. We recently reported complete paediatric reference ranges on the same cohort of samples using the Beckman Coulter Access AMH method,[17] that data are included here for direct comparison. Another recent study using the Beckman Coulter Access AMH assay reported reference intervals for adult females, for males aged 8–19 years and for newborn (<60 days) males and females.[16] Lindhardt Johansen et al.[12] mapped male and female age-related AMH ranges using the earlier IOT ELISA. These authors included extra axes on their plots to enable

Table 2. Paediatric female AMH reference intervals. Roche Elecsys vs. Beckman Coulter Access.

			AMH (pmol/L)		
Age group	Class	N	2.5th percentile (90% CI)	Median	97.5th percentile (90% CI)
0–28 d	5	**87**	**0.000863 (0–0.00148)**	**0.42**	**6.73 (2.49–9.93)**
	24	0.002 (0–0.0038)	0.366	4.08 (0.66–7.47)	
29–364 d	6	**28**	**0.000333 (0–0.00066)**	**1.39**	**31.23 (7.77– 57.05)**
	17	0.05 (0–0.10)	7.36	38.58 (28.12–50.99)	
1–4.99 y	7	**37**	**1.25 (0–2.06)**	**11.55**	**43.72 (33.20–58.55)**
	42	1.28 (0–1.75)	14.56	50.77 (46.4–61.17)	
5–7.99 y	8	**29**	**1.36 (0–2.06)**	**10.81**	**39.51 (24.21–58.55)**
	42	0.80 (0–1.25)	11.44	51.35 (35.05–64.32)	
8–11.99 y	9	**40**	**2.92 (0.11–4.63)**	**16.99**	**52.82 (32.16–68.88)**
	47	2.29 (0–3.88)	20.42	68.16 (62.40–94.51)	
12–14.99 y	10	**31**	**2.97 (0–4.97)**	**15.75**	**46.56 (28.19–61.91)**
	33	3.18 (0–4.35)	17.87	55.38 (47.61–82.74)	
15–18.99 y	11	**30**	**2.05 (0–2.82)**	**19.76**	**84.09 (60.86–121.49)**
	30	0.53 (3.33–74.21)	21.14	74.21 (64.80–84.23)	

Female paediatric AMH age-specific reference ranges. Data was analysed as per Table 1. Results from the neonatal period (0-28d) were grouped together due to the limited number of results using the Access assay. Elecsys assay results were also grouped to enable direct comparison. (Table adapted from Access-only data previously published by the same authors (17) under a creative commons agreement). Roche Elecsys values in bold, Beckman Coulter Access values in roman. The 2.5th percentile values and the corresponding Confidence intervals are calculated characteristics of the statistical distribution. They obviously fall well below the LOQ of either of the two assays but are included for completeness.
AMH: anti-Müllerian Hormone.

conversion to other assay calibrations, namely DSL and the unmodified version of the Beckman Coulter Gen II assay. The IOT ELISA was also used by Hagen et al.[23] to generate complete age-related female AMH ranges and Jeffery et al.[24] published detailed AMH concentrations in children between the ages of 5 and 14 years using the Beckman Coulter Gen II assay. Where included,[12,16,17] these studies showed high AMH concentrations in young boys which fall after the onset of puberty to lower adult levels. In contrast, female AMH concentrations are low at birth, rise slowly with plateaus around 2–4 years, rising further to another plateau around 8–11 years depending on the assay used.[23,25] In general, postmenarche AMH concentrations rise to a peak in the mid-20s whereupon they decline, becoming undetectable after menopause. This closely mimics the results seen in this study for subjects up to the age of 18 years using the Roche Elecsys assay.

While important conclusions can be taken from this work certain limitations in the study should also be clarified as follows:

- The wide spread in AMH concentrations, especially in males, probably reflects different stages of development within each chronological age group. As this was a retrospective analysis of stored samples, developmental markers such as Tanner stage, which may have confirmed this, were not recorded. Our previous study using the Beckman Coulter Access AMH analysis also measured testosterone in the male children.[17] AMH concentrations were low in samples with high testosterone levels, almost exclusively in boys over 12 years of age, probably signalling increased Leydig cell function coupled with the post-pubertal inhibition of AMH by testosterone via the expression of the Sertoli cell androgen receptor.[26]
- Only samples from patients that were being investigated for known DSDs or endocrinopathies were excluded from this study. Apart from this the patients were unselected so the possibility exists that our data could have been skewed by the inclusion of children with as yet undiagnosed endocrinopathies or reproductive disorders, contributing outliers which may have augmented the wide spread in values mentioned above. It should be borne in mind that the robust statistics used in this study does limit the influence of outliers but does not exclude them.
- The upper measuring ranges of all commercial AMH assays fall well below the levels seen in young males, necessitating relatively high dilution with assay diluent. Dilution studies performed in our laboratory on adult female samples of >100 pmol/L gave coefficients of variation of less than 10% at 1 in 10 dilutions for both the Elecsys and Access assays, which is acceptable. It is assumed

but not proven that 1 in 10 dilutions of very high (>1500 pmol/L) samples from young males would therefore exhibit similar linearity on dilution. Linearity problems seen with the unmodified Beckman Coulter Gen II AMH ELISA assay[2,27] have not been reported with the Elecsys and Access methods. Dilution with different diluents may introduce matrix effects which may be responsible for the observed differences between Roche Elecsys and Beckman Coulter Access AMH assays in the boys compared to the girls. Male samples were assayed after prior dilution whereas female samples were not diluted prior to analysis. In order to preserve specimen volume, male samples were manually diluted. This was necessary as the auto-dilute options on autoanalysers generally require much more sample. Most AMH assays using chemiluminescent immunoassay technology will have an auto-dilute option on the analyser and to our knowledge all commercial AMH assays, automated or otherwise, are provided with diluent for this purpose. AMH is mostly used in fertility investigations in adult females, where levels are normally low, whereas the investigation of paediatric males necessitates dilution in order to achieve a readable value from the assay.

- High-dose hook effects should always be considered with any non-competitive "sandwich" immunoassay but are not reported for the Roche Elecsys and Beckman Coulter Access AMH assays until much higher values than the highest (male) value seen in our cohort (1600 pmol/L) are reached.[2,7,28]
- We are not aware of any recent studies looking at day-to-day variation in the paediatric population. Daily variation in AMH has been investigated in females during the menstrual cycle with mixed results and a recent study in adult men showed a modest (<5%) variation in AMH between the 6:00 and the 19:00h samples.[29] Although these factors may have a small influence on postpubertal, postmenarche AMH concentrations, given the relatively much wider inter-individual variation within each age group then these effects will in our view be minimal. Long-term storage and freeze/thaws between analyses may have also contributed to variability in values between the two assays. Some samples were kept at −80°C for up to two years. However, the gap between the Access and Elecsys analyses was only 7–9 months. Our results with adult female samples showed that there was no significant difference between AMH in the same sample when assayed fresh or after three months storage at −20°C. Grassner and Jung[6] saw no effect of freeze/thaw cycles or nine months storage at −80°C on AMH concentrations and Johansen et al.[30] assayed AMH in paediatric samples that had been stored at −20°C for up to five years.
- It should also be noted that the problems associated with the collection of sufficient samples and construction of reference intervals in very young children are well described.[31,32] In terms of biological variability when group inter-individual variability is much larger than intra-individual variability then reference intervals lose their power for judging individual patients.[33] Utility can be restored by partitioning patients into smaller, similar groups. The constantly changing physiology in the paediatric population requires that large numbers of age and sex-matched partitions be constructed – hence the very large numbers of partitions in this study. The Clinical and Laboratory Standards Institute guideline for reference intervals (CLSI C28-A3) is acknowledged to be extremely difficult to achieve in paediatrics[32,33] and the recommended recruitment of a minimum partition size of 120 would be an impossible task for one centre measuring a relatively specialized analyte. The age partitions outlined in this study were assigned by the lead investigator to encompass realistic intervals which incorporate major developmental phases in the paediatric period. For example, in the male neonates there is an increasing trend to a peak of AMH activity at one month old which we felt was important to highlight (i.e. to partition). This occurs during the commencement of the so-called mini-puberty period.[30] This is the postnatal activation of the hypothalamic-pituitary-gonadal axis lasting from approximately the first week postpartum to three months. It is characterized in boys by high AMH, testosterone and LH (with a high LH/FSH ratio). Clinically this is an important period in the investigation of DSDs and for detecting palpable gonads or ambiguous genitalia. This increasing trend in AMH was absent in the age-matched females. Similarly, the observed 'pulse' of AMH in the female age group 9 (8–12 years) may represent hormonal changes at the onset of puberty (Figure 3 and Table 2). It should be noted that large national and global projects to establish paediatric reference intervals, such as the Canadian Laboratory Initiative on Pediatric Reference Intervals[32] have yet to incorporate some of the less common analytes such as AMH.

Notwithstanding these points measurement of AMH using the automated Roche Elecsys assay in a paediatric cohort gives values which are statistically different from values from our previous analysis using the Beckman Coulter Access AMH assay on the same cohort of samples[17] and in the case of females exhibits a different pattern of change in relation to age.

The Roche Elecsys assay gives quantitatively lower values than our previous routine service AMH assay, the Beckman Coulter modified Gen II ELISA, necessitating in our view new reference ranges applicable to the new Roche Elecsys and Beckman Coulter Access autoanalyser assays.[9] The sample cup volume requirement for the Roche Elecsys assay on the e602 analyser (80 μL) enabled many more neonatal female samples to be assayed than the higher sample cup volume requirement for the Access assay (180 μL) measured on the DxI 800 analyser with the set-up we used in our previous study,[17] making the Roche Elecsys assay on the Cobas e602 analyser more suited to the assay of neonatal female samples. The obvious delineation between normal male and female levels using the Roche Elecsys and Beckman Coulter Access autoanalyser AMH assays and their improved parameters make them valuable clinical tools for the monitoring of paediatric sexual and reproductive development from early childhood through the pubertal transition into adulthood.

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Not applicable. This was a method development study for patient benefit performed on anonymized surplus acellular plasma marked for disposal.

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LT.

Contributorship

LT and CMC conceived the study and LT, CMC, KH, APY and HMJ were involved in the study design. APY and HMJ were responsible for the analysis of AMH. NJB and APY were responsible for statistical analysis, with input from CMC, HMJ and LT. APY wrote the first draft and all authors were responsible for data interpretation and subsequent draft revisions through to approval of the submitted manuscript.

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