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Richard Fleming[1,2,*] and Scott M. Nelson[2]
[1]*Glasgow Centre for Reproductive Medicine, Glasgow G51 4FD, UK*
[2]*School of Medicine, University of Glasgow, Glasgow G11 6NT, UK*

*Correspondence address. GCRM Ltd, 21 Fifty Pitches Way, Glasgow G51 4FD, UK. E-mail: richard.fleming@gcrm.co.uk

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Reply: Reproducibility of AMH

Sir,

We are grateful for the opportunity to respond to the comments in the letter from Drs Fleming and Nelson concerning our recent paper (Rustamov *et al.*, 2012). It was our intention in publishing to stimulate discussion and further research in this area; however, we consider that they appear to have misunderstood several points about our study.

The authors adopt a very narrow definition of assay reliability and we would not dispute that this assay performs adequately on the sample in which these tests have been performed. Fleming and Nelson point out that the correct way to validate a new assay is to simultaneously compare results in the same sample—this is exactly what we did when the new Gen II assay was introduced and we obtained results which were in good agreement with other published comparisons. The issue is not about how two assays may agree in measuring AMH in a single sample, but about what has happened to the AMH in that sample prior to analysis. We presented evidence that, in routine clinical samples processed strictly within the protocols specified by the manufacturer, more variability is found. We speculated that this may be due to some aspects of sample preparation, about which Fleming and Nelson are in agreement. It does seem that the various AMH assays differ in their sensitivity to these factors in ways which are not understood. Moreover, we have demonstrated in a series of real clinical samples (which again we stress were prepared strictly in accordance with the assay manufacturers protocols) that a basic property of any assay, linearity to dilution, is violated.

These differences may well be due to pre-analytical aspects of the sampling process. The work described by Fleming and Nelson appears to have been carried out in stored samples while our own work was performed either on fresh samples or serum which had usually been frozen for only a few days, the assays being performed in the same lab by the same staff as previous assays. An earlier publication (Rey *et al.*, 1993) showed that long-term storage of serum at −20°C caused an approximate doubling of the AMH concentration compared with fresh samples, an effect that could be prevented by the addition of proteolytic inhibitors. Our work is consistent with an interpretation that proteolytic or conformational change in the AMH dimer occurs prior to analysis. Differences in measured AMH levels seen in samples stored under different conditions may reflect the extent of this change prior to analysis; if the process has gone to completion, the samples will appear to be stable; if 'activation' has not occurred at all, then results may appear to double under assay conditions or dilution. Most samples will lie somewhere between these extremes as we found in the 7-day room temperature storage experiment.

We have been unable to find comprehensive reports in the literature concerning AMH assay validation and details of the exact methodology employed when handling blood samples from patients are lacking. For example Wallace *et al.*, 2011, quoted by Fleming and Scott, made no mention whatsoever of how serum samples were processed or stored. Linearity studies were performed using serum pools containing known concentrations of AMH (by definition already assayed for AMH, i.e. not fresh serum) which had been diluted using serum from post-menopausal women which had no detectable AMH (presumably also confirmed by assay). None of this material is 'fresh' serum, so presumably the 'phenomena' which lead to a higher AMH reading will already have occurred and the measurable AMH levels will be stable in these pools if the process has gone to completion. If the change has already occurred in their pooled samples, they will not be able to reproduce our results.

Fleming and Nelson provide additional data, which reassuringly confirm our observations and which we look forward to seeing fully published. They show a significant 27% increase in measured AMH over 7 days at 4°C, compared with our 58% in separated serum samples stored at room temperature for the same period. Their dilution data also shows an average increase in recovery of 20–30% on dilution with diluent or serum which although not as dramatic at the 57% seen in our samples is nonetheless consistent with the effects we observed. It will be interesting to see more detailed data here, as we noted that the non-linearity on dilution does seem to be sample dependent. Larger numbers of better-characterized samples are needed to understand this phenomenon.

We hope that this evidence might help to identify further experiments, which will lead to an understanding of the cause of this variability and ultimately lead to an assay which fulfils the promise that AMH measurement offers.

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Oybek Rustamov[1,*], Alexander Smith[2], Stephen A. Roberts[3], Allen P. Yates[2], Cheryl Fitzgerald[1], Monica Krishnan[4], Luciano G. Nardo[1,5] and Philip W. Pemberton[2]

[1]*Department of Reproductive Medicine, St Mary's Hospital, Central Manchester University Hospitals NHS Foundation Trust, Manchester M13 9WL, UK*
[2]*Department of Clinical Biochemistry, Central Manchester University Hospitals NHS Foundation Trust, Manchester M13 9WL, UK*
[3]*Health Sciences – Methodology, Manchester Academic Health Science Centre (MAHSC), University of Manchester, Manchester M13 9PL, UK*
[4]*School of Medicine, University of Manchester, Manchester M13 9WL, UK*
[5]*Present address: Reproductive Medicine and Gynaecology Unit, GyneHealth, Manchester M3 4DN, UK*

*Correspondence address. E-mail: oybek.rustamov@cmft.nhs.uk; oybek_rustamov@yahoo.co.uk

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