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ORIGINAL ARTICLE

Serum anti-Müllerian hormone levels in women are unstable in the postpartum period but return to normal within 5 months: a longitudinal study

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Abstract

Purpose Anti-Müllerian hormone (AMH) levels fall during pregnancy but the amount of time required for AMH levels to return to normal has not been accurately determined. We have previously shown that AMH levels have yet to return to normal in some women at 3-months postpartum. In this study, AMH levels were examined at 1- and 5-months postpartum to examine whether AMH levels had returned to normal within this interval.

Methods Longitudinal study involving 38 pregnant women, with serum samples taken in the first trimester, third trimester, 1-month postpartum, 5-months postpartum and 4–6 years postpartum. Participants were recruited from a tertiary maternity clinic (single centre). All women in the study were intending to breastfeed exclusively for at least 5 months, with all 38 participants achieving this at 1-month postpartum and 36/38 after 5 months.

Results Serum AMH concentrations had not returned to expected non-pregnant levels by 1-month postpartum. At 5-months postpartum, mean AMH concentrations were similar to expected non-pregnant levels but the rank order of AMH concentrations was still dissimilar to the non-pregnant state.

Conclusions The regulation of AMH secretion appears to be distinctly different in non-pregnant, pregnant and postpartum populations. This may affect the conclusions that can be drawn from AMH measurements in women during pregnancy and the postpartum period.

Keywords AMH · Postpartum · Pregnancy · Longitudinal study

Introduction

Anti-Müllerian hormone (AMH) is produced by the granulosa cells of nonatretic developing ovarian follicles in adult women [1]. AMH production is first observed in primary follicles shortly after the transition from the primordial state and production continues in the preantral, and small antral follicle stages [1]. Small antral follicles are thought to produce the majority of the AMH secreted into blood because production declines in more-advanced follicle stages [2]. The use of serum AMH to estimate antral follicle numbers has applications in patient assessment for assisted reproduction [3]. AMH levels have also been used to estimate ovarian reserve (remaining number of primordial follicles), as this parameter is correlated with antral follicle counts [4].

Serum AMH levels decline slowly throughout life, as menopause approaches and the number of developing antral follicles decline. However, AMH levels within an individual are relatively stable from year to year [5] and across diurnal [6] or menstrual cycles [7]. Pregnancy represents an exception, as AMH levels decline by 30–80% between the first and third trimester [8–11]. Pre-pregnancy samples are often difficult to obtain but the results from two studies

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suggest that pre-pregnant and first trimester AMH levels are similar [9, 12]. In the postpartum period, AMH levels remain low for at least 4 days [10] and population means appear to return to first trimester levels within 3–4 months [8, 13]. However, at 3-months postpartum, some individuals exhibit AMH levels that are substantially higher or lower than first trimester concentrations [8]. This suggests that for these individuals, AMH levels are still unstable or fluctuating in the first 3-months postpartum, despite the finding that the population mean had returned to normal.

The objective of this study was to extend the characterisation of the postpartum recovery in AMH synthesis, as longitudinal data are currently only available at 1–4 days [10], and 3-months [8] postpartum. Serum samples from a previous longitudinal study of pregnancy and postpartum were used to investigate postpartum changes in AMH levels [14]. To compare pregnant and non-pregnant AMH levels, the women from the original study were re-recruited to provide a non-pregnant blood sample. Changes in mean AMH levels and changes in the rank order of AMH levels were assessed in the postpartum period to determine when AMH levels had returned to the levels expected for non-pregnant women.

Materials and methods

Participants

Study participants ($N = 129$) were initially recruited as part of a prior study into vitamin D levels during pregnancy and the postpartum period between June 2011 and January 2013 [14]. The women were recruited from September 2011 to June 2013 through the Queen Mary Maternity Centre, Dunedin Hospital, Dunedin, New Zealand. The inclusion criterion for the original study was, first trimester pregnancy, with intention to breastfeed exclusively for at least 5-months postpartum. Exclusion criteria included preterm delivery at <37 weeks of gestation, taking or intending to take vitamin D supplements in the postnatal period, a history of disorders known to affect calcium and/or vitamin D metabolism, including abnormal calcium concentrations or urinary calcium-to-creatinine ratio at study baseline, planned travel outside of New Zealand or Dunedin over the study period. None of the patients were taking oral contraceptives during the pregnant and postpartum phase of the study. A smaller cohort was approached for re-recruitment between June and October of 2017. Women were eligible if the biobank still contained at least one aliquot of serum from their first trimester, late pregnancy, 1-month postpartum and 5-months postpartum. The exclusion criteria were pregnancy within the last 12 months, reproductive endocrine disorders, radiotherapy, chemotherapy, full or partial oophorectomy or onset of menopause or perimenopausal symptoms. Of the eligible women, 38 were recruited into the present study and provided an additional final blood sample and information about whether they were currently using oral contraception. All participants were breastfeeding at 1-month postpartum and 36 of 38 women had continued to breastfeed at 5-months postpartum. This project was approved by the University of Otago Human Ethics Committee (Health) and was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants provided written informed consent prior to participation in the original study and again during the re-recruitment phase.

Sampling

The first blood sample was obtained in early pregnancy, usually in the first trimester but in four individuals the sample was taken in the early second trimester. This timepoint is referred to as the "first trimester" sample below. The second sample was obtained in late pregnancy, from gestational week 27 onwards. The third sample was obtained at 1-month postpartum and the fourth sample was collected at 5-months postpartum. To obtain a (5th) non-pregnant, non-postpartum sample, participants were re-sampled at 4–6 years after the pregnancy from the original study. Samples were allowed 1–2 h to clot at room temperature, were centrifuged to obtain serum and were stored at −80 °C.

Assays

AMH levels were assayed using the picoAMH ELISA (ANSHLabs, Cat# AL-124). Samples were diluted by a factor of ten in sample diluent prior to assay. Eighteen samples (all from the 5-months postpartum timepoint) were above the upper detection limit and were assayed a second time with a 20-fold dilution. The assay was conducted according to the Manufacturer's instructions. Standard curves were fitted with a quadratic equation to interpolate sample values using Prism v7.04 (Graphpad Software). Mean inter-assay variability was 4.9% CV and mean intra-assay variability was 2.8% CV.

Retrospective modelling of AMH levels

Two hypotheses were investigated to explain the declines in AMH levels between the 5-months timepoint and the non-pregnant sample taken 4–6 years later. The first hypothesis was that AMH levels return to normal at 1-month postpartum followed by an over-compensatory increase at 5-months and subsequent restoration to normal levels. A second, alternate hypothesis was that mean AMH levels

have returned to normal by 5-months postpartum and that the AMH declines in the intervening 4–6 years are caused by age-related reductions in the number of antral follicles. Modelling was used to estimate what AMH levels would have been during the postpartum period, if the participants had not been pregnant. The model consisted of non-linear natural splines that were fitted to AMH levels with a mixed model generated in a prior longitudinal study investigating the decline in AMH levels with ageing in 3362 women [5]. In this longitudinal model, age was included as the time axis in order to enable comparison of age-independent AMH levels. Four participants reported using oral contraceptives after the postpartum period. Oral contraceptive use was included as a variable in the model for these four participants to account for the slight reduction of circulating AMH concentrations caused by oral contraceptive use [5, 15]. Thus taking into account oral contraceptive use, the population-based average AMH was calculated for each participant based on her age at the 1-month postpartum timepoint.

Statistical analysis

Statistical analysis was conducted with SPSS v25.0 (IBM corporation). Longitudinal changes in AMH concentrations were analysed with mixed models followed by Sidak post hoc test. Changes in the rank order of AMH levels at each timepoint were assessed with Spearman rank correlation. There were two samples missing from the 1-month postpartum timepoint and two missing from the 5-months postpartum timepoint. Rank orders were recalculated at these timepoints to exclude individuals with missing data. Mixed model analysis can accommodate longitudinal series with missing data. Correlation analyses were conducted with Prism 8 (GraphPad Software).

Results

Participant characteristics are shown in Table 1. None of the participants were smokers. Mean AMH levels declined between the first trimester to the perinatal period (Fig. 1a) in a manner consistent with prior studies [8–11]. AMH levels had increased significantly between the perinatal period and 1-month postpartum but not to the levels observed in the first trimester samples. At 5-months postpartum, AMH levels had increased significantly from 1-month postpartum levels but were no longer significantly different from first trimester levels. AMH levels declined in the non-pregnant sample (taken 4–6 years after the end of pregnancy), relative to the 5-months postpartum levels. Individual longitudinal plots (Fig. 1b) indicate that the rate of the postpartum restoration of AMH levels was variable between participants. Some women had large increases in AMH between the perinatal phase and 1-month postpartum but others did not exhibit substantial increases until the period between 1 and 5-months postpartum.

Table 1 Participant characteristics

	Mean	SD	Range
Age at delivery (years)	34.3	4.6	24.7–42.5
Gestation-length at 1st sample (weeks)	7.4	2.7	0.4–13.3
Gestation-length at 2nd sample (weeks)	35.0	3.0	26.6–41.3
Time postpartum at 3rd sample (months)	0.9	0.07	0.9–1.1
Time postpartum at 4th sample (months)	4.7	0.3	3.7–5.5
Age at non-pregnant 5th sample (years)	39.1	4.7	29.7–48.2
Age difference at delivery vs. non-pregnant sample (years)	4.8	0.5	4.0–5.7
Parity prior to study-pregnancy	1.26	0.97	0–4

AMH concentrations are highly variable between individuals, hence the rate of change in AMH levels for each participant was examined as a percentage of that individual's first trimester level. AMH levels declined by 64.9% on average (range: 21.5–92.6%) between the first trimester and the perinatal sample (Fig. 1c). The majority of individuals (30/36) had a rebound that was less than first trimester values at 1-month postpartum but at 5-months postpartum, only 12 of the 36 individuals had an AMH level lower than in their first trimester (Fig. 1d). In the non-pregnant sample, at 4–6 years postpartum, AMH levels were reduced relative to first trimester levels in 30 of the 36 participants. Unlike our previous study [8], there was no correlation between age and the percent decline at the perinatal timepoint ($r = -0.124$, $p = 0.459$, data not shown). Collectively, these data demonstrate that AMH levels continue to increase in the 1–5 months postpartum and that restoration to basal AMH levels has a variable rate between women.

Serum AMH levels are correlated with the number of antral follicles currently present in the ovary [4]. Over the course of 9 months, AMH and antral follicle counts would not be expected to change substantially in a healthy, non-pregnant woman. Subtle variations in AMH levels might be expected within individuals, but in a group of women, the rank order should remain largely conserved. During the progression from the first trimester to the postpartum period, the rank order of AMH values undergoes substantial rearrangement, particularly at the at the 1- and 5-months postpartum timepoints (Fig. 2 and Table 2). The Spearman rank correlation coefficients determined that the first trimester and non-pregnant timepoints were the most similar. The non-pregnant rank order is less correlated with timepoints that lie closer to parturition, particularly the postpartum timepoints (Table 2).

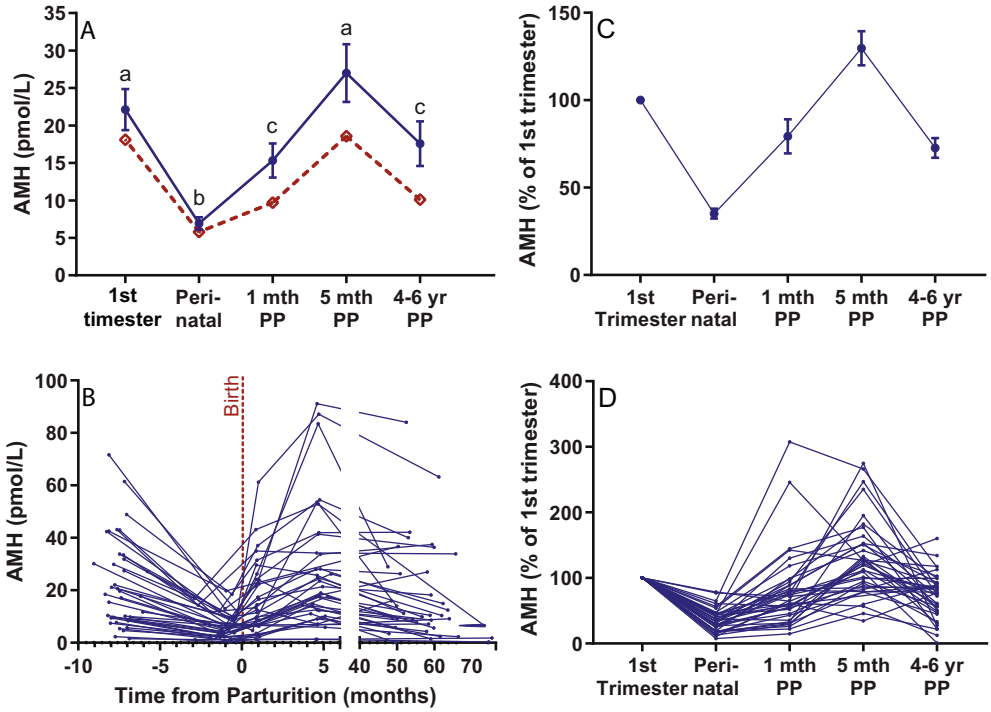

Fig. 1 AMH levels in women during pregnancy and the postpartum (PP) period. **a** Mean (blue circles, continuous lines) and median (red diamonds, dashed line) AMH levels classified under the blood sampling categories; first trimester ($n = 39$), perinatal ($n = 39$), 1-month postpartum ($n = 37$), 5-months postpartum ($n = 37$) and 4–6 years postpartum ($n = 39$). **b** Longitudinal series of AMH concentrations for each participant relative to the time of birth. **c** Mean changes in AMH expressed as a percentage of the participant's first trimester value. **d** Longitudinal plots of AMH values expressed as a percentage of first trimester values. Error bars = SEM, $n = 37$–39, $p < 0.001$ (mixed models with Sidak post hoc analysis), timepoints that share the same letter are not significantly different from each other

Mathematical modelling was used to estimate what AMH levels would have been at the 1-month timepoint, if the participant had not recently been pregnant. The estimated non-pregnant AMH level was significantly larger than the levels observed at 1-month postpartum but was not significantly different to the 5-months postpartum timepoint (Fig. 3). This supports the hypothesis that AMH levels take ~5 months to return to normal, rather than the hypothesis that AMH levels return to normal at 1-month with an overcompensatory increase at 5 months.

Serum follicle-stimulating hormone (FSH) levels were low or undetectable in most participants in late pregnancy but were increased in all participants by 1-month postpartum (Fig. 4). Serum FSH was not significantly correlated with serum AMH concentrations or the percentage recovery of AMH relative to first trimester levels at 1- and 5-months postpartum.

Discussion

AMH levels are unstable in the postpartum period particularly at the 1-month timepoint but mean AMH levels had returned to normal levels 5-months postpartum. The fluctuations in AMH levels at 1 and 3 [8] months postpartum suggest that the rate of restoration of normal AMH levels is variable between women. The rank order of postpartum AMH levels remains dissimilar to non-pregnant and first trimester levels at 5 months but the mean levels appear normal. The Spearman rank correlation coefficients in the pregnant-postpartum comparisons from this study appear to be lower than in other, non-pregnant cohorts (see below). This suggests that the non-pregnant, pregnant and postpartum phases each exhibit different patterns of AMH expression across the population. In the non-pregnant phase, antral follicle numbers are the primary determinant of AMH levels but the determinants of AMH expression in pregnant and postpartum states have not been elucidated.

Plasma volume increases have been proposed as a mechanism of AMH decline during pregnancy [16] but this can only explain part of the decline [8]. Pregnancy suppresses FSH secretion leading to the loss of large antral follicles, but small antral follicles are the primary producers of AMH and their numbers remain in high-abundance throughout pregnancy [17–20]. Thus, if the AMH-producing follicles are not absent, then it is likely that there is an inhibitory factor that limits AMH production.

It is a common misconception that lactation suppresses gonadotropin secretion but within 30–40 days of parturition, serum levels of LH and FSH both return to the normal

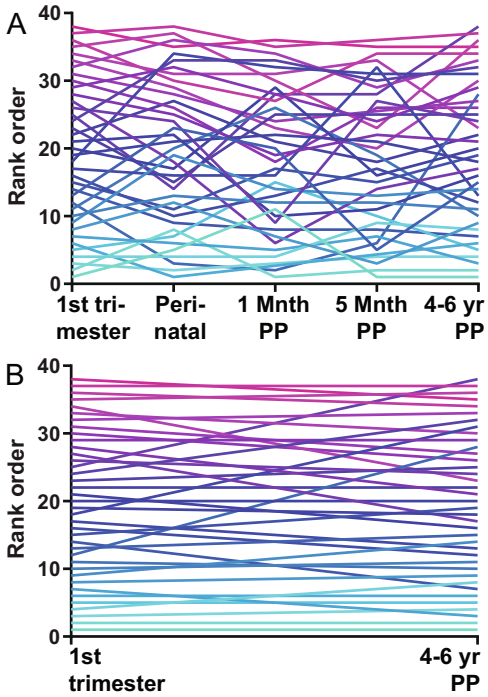

Fig. 2 Longitudinal changes in the rank order of serum AMH for each participant during pregnancy and postpartum. **a** Rank order changes across all timepoints; first trimester ($n = 39$), perinatal ($n = 39$), 1-month postpartum ($n = 37$), 5-months postpartum ($n = 37$) and 4–6 years postpartum ($n = 39$). **b** Rank order changes from first trimester to 4–6 years postpartum (non-pregnant)

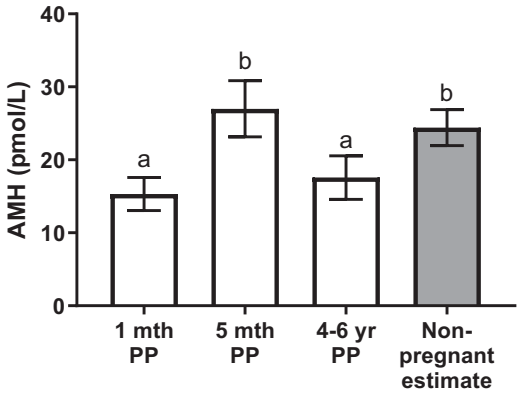

Fig. 3 Retrospective modelling of expected non-pregnant AMH levels. Actual AMH concentrations (white bars) were compared to a non-pregnant estimate of AMH levels (grey bar). The non-pregnant estimate was modelled to determine the expected AMH level at the 1-month postpartum (PP) timepoint, if the study participants had not been recently pregnant. Error bars = SEM, $n = 37$–39, $p < 0.001$ (mixed models with Sidak post hoc analysis), timepoints that share the same letter are not significantly different from each other

range observed during the follicular phase of the ovarian cycle [21]. The only aspect of gonadotropin secretion that is inhibited during lactation is the preovulatory LH surge, which is inhibited via neural signals induced by the suckling-stimulus during breastfeeding [22]. Granulosa cells in preantral and small antral follicles produce the majority of AMH in circulation but do not yet express LH receptors [2, 23]. Granulosa cells begin to express LH receptors at the preovulatory stage but produce very little AMH at this stage [1, 23], hence LH is not considered to be a determinant of serum AMH levels. Resumption of FSH secretion was shown at both the 1- and 5-months postpartum timepoints in the present study. Therefore, lactation is not expected to greatly influence AMH expression via gonadotropin suppression in the 1–5 months postpartum period.

Lactation-related hormones have elevated secretion in pregnancy and the postpartum period and are therefore, suitable candidate regulators of AMH levels during pregnancy. However, there may be other hormones with similar expression patterns in late pregnancy and parturition that could serve as suitable candidate regulators of AMH production. Steroidal hormones such as estrogens and progesterone are less suitable candidates, as their levels plummet within hours of parturition, while AMH secretion remains suppressed for months [9].

Breastfeeding rates were 100% at 1-month postpartum, 95% at 5-months postpartum, and 84% in our prior study, which investigated 3-months postpartum [8]. This nearly eliminates the potential hormonal changes in non-lactating women to act as a confounding factor in the experiment. Despite this, high variability in the rate of restoration of AMH production was still observed. It is possible that higher variation may occur in the general population because breastfeeding rates at 6-months postpartum tend to be substantially lower [24]. However, the effect of cessation of lactation on AMH production has not been determined. The date of first menstrual period and cessation of breastfeeding was not recorded in this study which raises questions about whether prolonged lactation could have affected the AMH level in the subsequent non-pregnant sample taken 4–6 years later. However, the mean breastfeeding interval in New Zealand is 7 months suggesting most, if not all of the women would have ceased lactation well before the non-pregnant sample was taken. Furthermore, the endocrine changes that accompany lactation (e.g. elevated prolactin, suppression of ovulation) usually return to normal in 6–12 months even if lactation and breastfeeding continues beyond this period [22, 25]. While the factor(s) that influence AMH suppression during pregnancy and postpartum have not been identified, current evidence suggests antral follicle numbers are not the only determinant of AMH levels in pregnancy.

An increasing number of studies involve AMH measurements taken during pregnancy, including studies investigating associations with oocyte aneuploidy, repeated miscarriage, preterm birth rates, gestational diabetes and ovarian reserve [26–30]. This is valid in some instances, such as a recent study investigating whether changes in

Table 2 Spearman's rank correlation coefficient matrix for AMH levels at each timepoint

	1st Trimester	Perinatal	1-month PP	5-months PP	4–6 year PP
1st trimester	1.000	0.855*	0.712*	0.827*	0.871*
Perinatal		1.000	0.813*	0.778*	0.792*
1-month PP			1.000	0.804*	0.750*
5-months PP				1.000	0.797*
4–6 years PP					1.000

*$p < 0.001$. NB p values have not undergone correction for multiple testing but all p values remain below a Bonferroni-corrected p value cut-off for ten analytical tests ($p < 0.005$)

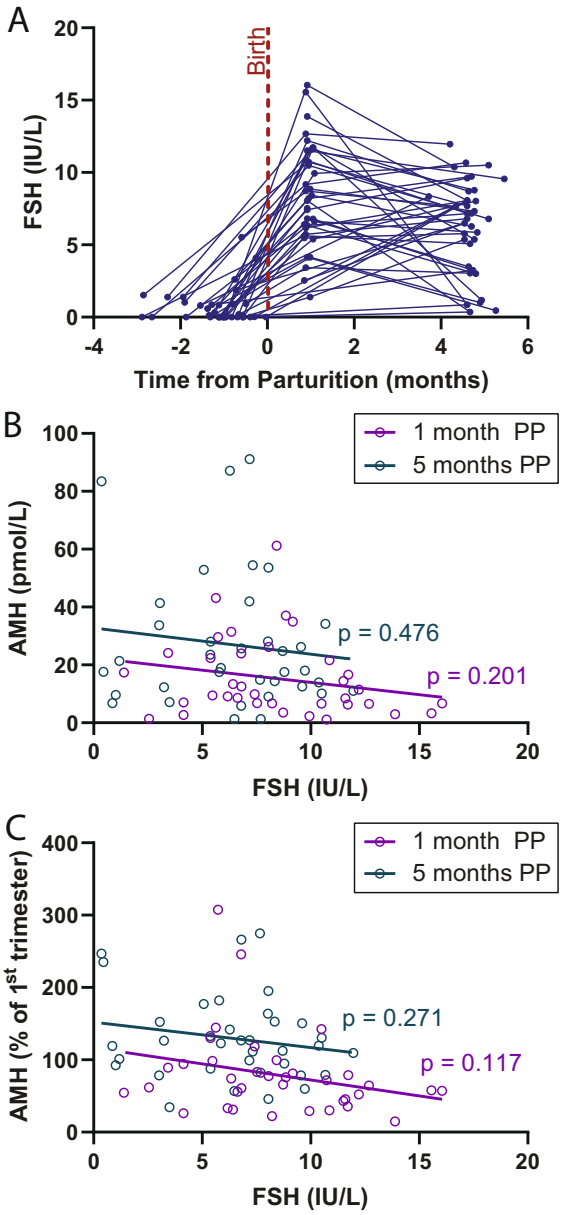

Fig. 4 The postpartum FSH levels do not correlate with the recovery of AMH expression. **a** Serum FSH levels for individual participants between the late pregnancy, 1-month postpartum and 5-months postpartum timepoints. **b** Scatter plot of serum FSH levels and serum AMH concentrations at 1-month (purple circles) and 5-months (green circles) postpartum (PP). **c** Scatter plot of serum FSH levels and the recovery of serum AMH levels expressed as a percentage of first trimester level for each patient 1-month (purple circles) and 5-months (green circles) postpartum (PP)

gravid AMH levels relate to the incidence of preterm birth [31]. However, many of these studies have measured AMH as an indicator of ovarian reserve. Women with higher parity tend to experience later age of menopausal onset than nulliparous women [32], indicating that pregnancy is unlikely to cause a reduction in the ovarian reserve. Furthermore, the current consensus is that the ovarian reserve cannot be replaced in adulthood in humans. Therefore, the postpartum increase in AMH levels is not consistent with a postpartum increase in the size of the ovarian reserve. This strongly suggests that AMH is not an indicator of the ovarian reserve during pregnancy. However, it is plausible that the pregnant, and postpartum changes in serum AMH levels could arise from changes in the number of preantral and small antral follicles or changes in rates of protein expression from the *AMH* gene.

The present study was intended to investigate whether a slow restoration of AMH levels after pregnancy could affect further research or clinical practice. The use of longitudinal data enabled analysis of changes in rank order and the comparison of pregnant and non-pregnant states. A key strength of the present study was the high proportion of women breastfeeding at the 1- and 5-months postpartum timepoints, as there was no need to account for the withdrawal of lactation-related hormones. The primary limitation was that the non-pregnant sample was taken 4–6 years after the original pregnancy. Many of the women in the study population were at an age where AMH levels are declining at a rate of ~3–6 pmol/l per year [5]. Using an algorithm to estimate mean levels of AMH if the study population had not been pregnant during the original study, it was determined that AMH levels at 5-months postpartum were similar to expected non-pregnant levels. The ideal study would incorporate a pre-pregnancy blood sample to compare but recruitment for this study design carries considerable challenges, particularly to recruit naturally conceiving fertile couples, as there is no common community catchment point for these individuals.

The results of the present study are sufficient to advise caution when measuring AMH in the pregnant or postpartum periods in research or clinical contexts. The primary use of clinical AMH assays is for patient assessment in fertility clinics, which usually occurs prior to pregnancy but other applications are being investigated. It has been

recognised that the potential use of AMH as a predictor of time to menopause [33] and for diagnosis of polycystic ovary syndrome [34] both require further refinement. Ensuring that patients have not been pregnant in the prior 5 months may be necessary to ensure that the AMH value is a true reflection of the condition being assessed. The use of AMH to assess fertility in women with recurrent miscarriage or recent late miscarriage is another area of concern, as it is possible that the restoration of AMH levels after miscarriage also occurs over a long period. Unfortunately, data relating to changes in AMH levels post-miscarriage have not been collected.

Repeated measures of AMH in a single cohort would be expected to retain some level of correlation between time-points but it is not clear how much the correlation coefficient would need to deviate from 1.0 to consider the rank order "altered". For context, rank order correlation was conducted on two cohorts of non-pregnant women from previously published studies (Supplementary Tables 1 and 2). The first consisted of 16 women with blood samples taken every 4–6 days during the ovarian cycle [35] and the second from a study involving 42 women receiving vitamin D or placebo, with four AMH measurements over 7 days [36]. The Spearman correlation coefficients ranged from 0.874 to 0.974 in the ovarian cycle study and between 0.956–0.987 for the vitamin D study. The rank order correlation coefficients in the present study were all below the ranges observed in the non-pregnant cohorts indicating that the changes in the rank order arrangement occurring during pregnancy are unlikely to be solely explained by the processes that cause normal fluctuations in AMH levels.

The present study demonstrates that the restoration of AMH levels after pregnancy occurs over several months. The correlation between antral follicle counts and serum AMH has been extensively characterised in non-pregnant women. However, caution is advised for the use of serum AMH assays where an estimate of antral follicle counts is sought in women who have recently been pregnant.

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Compliance with ethical standards

Conflict of interest M.W.P., A.C.K., S.J. and B.J.W. have nothing to disclose. F.J.M.B. discloses personal fees as a member of the external advisory boards for Ferring BV, Merck Serono and Gedeon Richter and personal fees from educational activities for Ferring BV during the conduct of the study.

Ethical approval This project was approved by the University of Otago Human Ethics Committee (Health) and was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Informed consent All participants provided written informed consent prior to participation in the original study and again during the re-recruitment phase.

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