

Anti-Mullerian Hormone (AMH) Levels are Reduced by Age, but do not Correlate with Pregnancy in Timed-Mated Rhesus Monkey Females (*Macaca mulatta*)

Maggie Roth ¹, Jared Jensen ², Jing Xu ³, Carol B. Hanna ², Cecily V. Bishop ^{1,2,*}

¹. Department of Animal and Rangeland Sciences, College of Agricultural Sciences, Oregon State University, Corvallis, OR 97331, USA

². Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Oregon Health & Sciences University, Portland, OR, 97239-3098, USA

³. Department of Biology and Chemistry, School of Health Sciences, Liberty University, Lynchburg, VA, 24515, USA

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* Correspondence

Cecily V. Bishop

Department of Animal and Rangeland Sciences,
College of Agricultural Sciences, Oregon State
University, Withycombe Hall rm 112, 2901 SW
Campus Way, Corvallis, OR 97331, USA

Division of Reproductive & Developmental
Sciences, Oregon National Primate Research
Center, Oregon Health & Sciences University,
Portland, OR, 97239-3098, USA

E-mail: Cecily.bishop@oregonstate.edu

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Abstract

Background: Evaluation of reproductive hormones is a common tool used to predict a patient's prognosis for fertility therapies. Specifically, anti-mullerian hormone (AMH) and activin A are two ovarian hormones which may be associated with follicle quality, yet their predictive ability to determine pregnancy outcomes are still not well characterized in primates.

Objective: To use rhesus macaque as a well-characterized nonhuman primate model to assess the predictive power of AMH and activin A to determine pregnancy outcomes. **Methods:** Female rhesus macaques (n = 47) were monitored during timed breeding protocols beginning around day seven post menses until serum estradiol levels indicated ovulation was imminent. Females were then pair-housed with fertile male monkeys (Timed-Mated Breeding; TMB). Females were re-paired with males until the end of the breeding season if they failed to become pregnant. The first blood sample collected (days range 5-15) was analyzed for serum levels of AMH and activin A (n = 90). Pregnancy status was determined via ultrasound (Pregnant cycles n = 28, non-pregnant cycles n = 62). Mean age of females was 13 years (range: 6.3-17.7 years old), and mean weight was 7.35 kg (range: 5.35-10 kg). **Results:** There was no significant difference between AMH levels by pregnancy status ($p > 0.4$). Similarly, there was no significant difference between activin A levels by pregnancy status ($p > 0.2$). However, similar to data in women, negative correlations between age and AMH ($R = -0.32$, $p = 0.002$), and between weight and AMH ($R = -0.22$, $p = 0.036$) were detected. **Conclusions:** These data demonstrate that the predictive ability of AMH to determine reproductive success in primates is poor in a "natural" or unstimulated mating scenario. In addition, serum levels of activin A are not a reliable predictor of pregnancy success.



1 Introduction

Reproductive health is a basic component of a woman's physiological well-being, yet fertility is often taken for granted, and its complexities often overlooked. Infertility affects millions of women worldwide and is a condition that reproductive medicine has sought to resolve for decades. It is a complex issue that can be composed of a network of ailments within the reproductive tract, many of which are not understood. Infertility can be idiopathic, arising spontaneously, or iatrogenic, a result of a different medical treatment [1]. Further, environmental factors can affect the functionality of the reproductive tract [1]. Not only that, in our current modern western society women are choosing to have children later in life and they are faced with the realities of their reproductive timeline dictated by their ovaries. Therefore, it is important to be able to accurately assess a woman's ovarian fertility status and treat her accordingly. While infertility is extremely common, the various causes are yet to be fully understood. From idiopathic origins to cancer inducing infertility, Assisted Reproductive Technologies (ART) are often chosen during the course of treatment. According to the 2021 National ART Summary by the United States CDC, in the US 97, 128 live infants were born as a result of ART cycles began in 2021. Yet this option is costly, time consuming, and often not effective. Thus, further research is imperative in addressing infertility and advancing the field of medicine, and the nonhuman primate (NHP) model makes this possible.

The ovary is a highly studied reproductive organ as the producer of follicles and the source of the mature ovulated oocyte for fertilization. For this reason, the ovary plays an important role in infertility. A woman's reproductive potential is limited by her ovarian reserve, or the quantity and quality of oocytes that the ovary contains [2]. Reproductive aging is a natural and

inevitable process resulting in a decline in the number of follicles present in the ovarian cortex throughout a woman's lifetime. However, it is also extremely variable amongst individuals, and a woman's ovarian reserve is highly influenced by many factors such as genetic, mitochondrial, cytoplasmic, and environmental factors [3]. Historically, determining a person's ovarian reserve has been used as an indicator of their reproductive abilities and has been an important component to clinical decision making, especially in the realm of ART [4]. In fact, the ovarian reserve assessment is often more important than assessing a woman's age, due to it being one of the most important independent factors in female reproductive capability [5].

A woman's ovarian reserve is estimated based upon endocrine status evaluation and ultrasound ovarian assessment to determine antral follicle count [5]. Currently, follicle stimulating hormone (FSH) and anti-Mullerian hormone (AMH) are the two biochemical markers most often used clinically to measure ovarian reserve [3]. The two have complex interactions with each other as well as with the corresponding follicles of the ovary. AMH is currently known as a gonadal-specific glycoprotein in adult females that belongs to the transforming growth factor beta (TGF- β) superfamily [6]. AMH's physiological role in the ovary has been previously studied in mice; Salmon, Handyside, and Joyce confirmed that oocytes of primary and preantral follicles upregulated the expression of AMH mRNA levels in the granulosa cells of the ovary [7]. Another study done by Durlinger and colleagues determined that the secretion of AMH from growing follicles acts to inhibit primordial follicle initiation and growth [8]. Therefore, AMH serum levels of growing follicles are thought to regulate the primordial follicle pool, and therefore reflect the ovarian reserve.

Understanding the role of AMH in the ovary revealed

the potential clinical value of AMH as a biochemical marker of a woman's ovarian status, with value in predicting future fertility outcomes in women. In women with various degrees of premature ovarian failure, AMH has been validated as a reliable marker of the extent of follicular pool depletion and predictor of ovarian senescence [9,10]. Additionally, AMH is particularly relevant in determining ART fertility outcomes. Nelson et al. reported that AMH levels were determined to be an accurate and reliable predictor of ovarian response and thus live birth outcomes, in those undergoing ART treatment, and AMH had a higher predictive value than antral follicle count [11]. The importance of AMH in subjects with no known reproductive disorders has also been investigated. In a study involving bovine models, Ribeiro and associates reported a positive association between AMH and fertility in dairy cows; cows with low plasma AMH concentration had lower pregnancy rates and greater incidence of pregnancy loss [12]. Additionally, cows that failed to become pregnant by the end of the breeding season had a lower concentration of AMH in plasma than cows that became pregnant [12]. However, the reliability of AMH serum levels and its predictive ability are still in question, and its direct correlation to pregnancy outcomes needs further investigation. In a study that looked at AMH levels in women attempting to conceive naturally with no history of infertility, low AMH levels were not associated with a lower probability of conception [13]. Thus, while AMH's physiological role in the ovary is relatively well understood, how it can effectively be used needs to be carefully considered.

As previously noted, reproductive function and follicle development is largely regulated by the complicated interactions of intraovarian hormones. While significant research has been conducted around the physiological role of AMH, reproductive biology still lacks a complete understanding of all ovarian hormones and their various roles in reproduction. Of

specific interest is activin A, an ovarian hormone produced by the granulosa cells of primary and preantral follicles. Previous research has illustrated the importance of activins in folliculogenesis and reproductive biology [14]. Activins are a subfamily of the superfamily TGF- β and have a unique role regulating FSH, a highly important hormone in folliculogenesis [15], although this protein can also be found during gestation in primates, produced by the placenta. Activin receptor type II is the major activin receptor on the anterior pituitary [15]. A study that knocked out this receptor in mice caused a defect later in life, with few antral follicles and a higher incidence of follicular atresia, elucidating the potential impact of the presence of activins in the ovary [15]. A different study that modeled early folliculogenesis through an in vitro organ culture system in mice, established activin A's anti-apoptotic properties; primary and preantral follicles that were treated with activin A alone had an increase in healthy versus atretic follicles [16]. Therefore, activin A is thought to play an important role in early follicle development; however, the use of this hormone as a predictive measure of ovarian status has not been characterized. The relationship between activin A levels and the quality and quantity of follicles is unclear and needs further investigation in order to be able to incorporate this hormone into a clinical assessment of a woman's ovarian status.

The NHP model is an ideal candidate for reproductive research because of its direct translation and relevance to the human model. The rhesus macaque (*Macaca mulatta*), an Old World monkey originating in Asia, is a NHP species that has been used in biomedical research for decades across multiple disciplines, due to its phylogenetic proximity and genetic similarities to humans [17]. Furthermore, the female NHPs and women shares striking similarities in reproductive biology, from hormonal and neural control to local control of reproduction, offering research insights that other laboratory or agricultural models cannot [18].

Firstly, NHPs are unique in that they have a cyclical menstrual cycle similar to that of humans, dictated by the hormonal control of estrogen and progesterone, with subsequent uterine proliferation, differentiation, and sloughing [18]. The ovarian cycle is also similar in that ovarian follicle maturation is under the control of FSH and LH, resulting in follicular atresia or ovulation. This is followed by the formation of the corpus luteum, which lasts fourteen to sixteen days, until luteal cell apoptosis is complete [19]. NHPs have also been characterized as experiencing reproductive senescence and undergoing ovarian and neuroendocrine changes in the same way that humans do, resulting in a loss of menstruation later in life [20]. Further comparisons involve pregnancy and placentation in NHPs. NHPs display a hemochorial placenta known to be villous in structure within cotyledons, resulting in a direct contact of maternal blood with fetal tissue, comparable to humans [18]. Thus, these similarities allow novel findings that can be directly translated to humans. The NHP model provides opportunities that other animal models cannot, and has been utilized to accelerate the understanding of human reproductive biology, reiterating the continued importance today.

Based upon previous work and knowledge of AMH and activin A, this study sought to determine the relationship between hormone levels and establishment of early pregnancy. The objective of this study was to use the NHP model as a well-characterized animal model to assess the predictive power of AMH and activin A in naturally cycling individuals, and their impact on the resulting pregnancy status. We hypothesized that AMH and activin A measurements taken early in the ovulation cycle can be used to predict establishment of pregnancy in naturally cycling rhesus macaques, and therefore, there will be higher levels of AMH and activin A in the animals that became pregnant compared to those that did not. The reproductive

cycles and follicle characteristics of NHPs are highly understood and extremely relevant to the human model, making this study not only relevant to future primate research, but also for clinical application in humans. A greater understanding of how to best utilize these ovarian hormone levels in NHPs may improve future research projects, offering a screening of candidates before beginning costly protocols. Additionally, this study could improve clinical decision making, screening, and treatment options for women seeking fertility consultation by offering a more accurate snapshot of their ovarian status and further cutting down potential costs and time spent for treatment.

2 Materials and methods

2.1 Animals

All animal procedures were approved by the Oregon Health & Science University (OHSU), Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee (IACUC) and comply with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (The Guide; <https://doi.org/10.17226/12910>).

Forty-seven female rhesus macaques (*Macaca mulatta*) from the Rhesus Macaque SPF4 breeding colony were primarily housed outdoors in seven one-acre corrals, sheltered housing units, and indoor/outdoor corn cribs at OHSU's ONPRC, Beaverton, OR and maintained by the ONPRC Division of Comparative Medicine (DCM). The female macaques were all naturally cycling animals with no known reproductive health issues. The female NHPs were of various ages, ranging from 6.3 to 17.7 years old, with an average age of 13 years old. The female NHPs were of various weights, ranging from 5.35-10 kg, with an average weight of 7.35 kg. All animals are weighed on a monthly basis in the colony, and the last weight before onset of mating was used for evaluation. The study was performed as a retrospective,

non-randomized, cohort analysis and was a population-based study in a colony of managed rhesus monkeys at a National Primate Center. These were reproductive aged females selected from the ONPRC colony as part of the Timed-Mated Breeding (TMB) Resource run in collaboration between the ONPRC DCM and the ONPRC ART Core [21]. Samples were obtained during the breeding season until females either became pregnant, or were released from the program (as described below). In all, data from 90 menstrual cycles were collected from this cohort.

Behavioral management of all NHPs is overseen by the Behavioral Services Unit (BSU) at ONPRC, including the training and conditioning of the animals for awake blood sample collections, reducing stress associated with the event. The animals were monitored by DCM staff daily for the detection of menses. Onset of menses were determined by visual inspection of perianal area daily, and in some females with daily vaginal swabbing for presence of menstrual bleeding, similar to previous studies in rhesus macaques within this colony [22]. After menses had been detected, daily blood collections began approximately 7 days post-menses using a bleed box to secure the animal and allow for a safe collection. 1 mL of blood was collected, beginning at around day 7 post-menses; however, this varied from day 5 post-menses to day 15 post-menses, depending on the animal's menstrual

cycle history and were timed to allow for detection of the rise of estradiol (E2), indicating pending ovulation. Daily blood draws continued until ovulation was imminent. The whole blood samples were centrifuged ($1,000\times g$ for 15 minutes at 4 °C) to retrieve serum. Sera were assayed by the Endocrine Service Core at ONPRC, to measure E2 and progesterone (P4) by use of an automated radioimmunoassay platform validated for NHP serum [23]. E2 and P4 levels were utilized as an indicator of ovulation and luteinization in the female NHP, indicating a cycle had a high probability of fertility. When daily E2 > 100 pg/mL, indicating ovulation was likely, the female was then pair-housed with a fertile male monkey in a TMB protocol (Figure 1). Blood collections continued in order to monitor a rapid surge and then decline in E2 serum levels with P4 levels on the rise. As P4 levels approached 1.0 ng/mL, the animals were unpaired from one another. Daily bleeds were discontinued. After 2.5 and 3.5 weeks post pairing, 1 mL of blood was collected and submitted to the Endocrine Service Core to measure P4 levels as an indicator of pregnancy. Pregnancy status was determined via ultrasound around embryo day 30, for visualization of gestational sac [22]. Females were re-paired with males if they failed to become pregnant until the end of the breeding season. Serum samples were placed in either a -20 °C freezer or -80 °C freezer to be safely stored and used later for analysis.

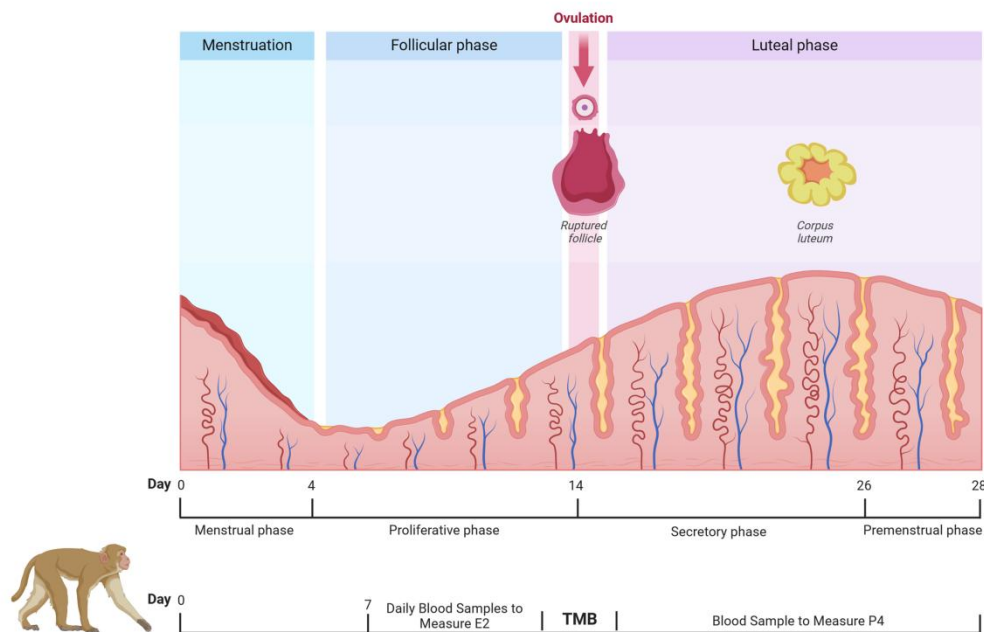


Figure 1 Summary of timed-mated breeding (TMB) timeline in conjunction with the hormonal monitoring of the animals at the corresponding time in the ovarian cycle. Daily blood sample collections began around day 7 post menses to measure estradiol (E2) and progesterone (P4) and predict time of ovulation. At the time of ovulation, a female was paired with a male (TMB), followed by the continued measuring of P4 to determine unpairing time and pregnancy. (Created with <https://www.biorender.com/>).

2.2 Hormone Analyses

The first sample taken from the daily blood draws was utilized for analysis of serum levels of AMH and activin A in each TMB cycle. Enzyme linked immunosorbent assays (ELISA) that were previously validated in NHPs were used to measure both AMH and activin A [24]. The Ultra-Sensitive AMH/MIS ELISA RUO AL-110 (Ansh Labs, Webster, TX, USA) kit was used to measure AMH and the Activin A ELISA AL-110 (Ansh Labs, Webster, TX, USA) kit was used to measure activin A. All assays were conducted by a single person that was blinded to all concentrations of other hormones and pregnancy outcomes. The researcher only knew the animal ID and sample date of the serum collected while conducting the assays.

All serum samples to be used were thawed on ice before beginning the ELISA procedure. In the instance that a large clot was detected in the samples that would interfere with retrieval of the serum, the

samples were centrifuged for pure serum retrieval ($8,000\times g$, Legend Micro 21R, for 1 minute at 4°C). Assays were completed to quantify AMH and Activin A concentrations as suggested by manufacturer's protocols (Ansh Labs, Webster, TX). Following the completion of both ELISA procedures, the optical density absorbance wavelength of each well was determined immediately using a Versamax Tunable Microplate Reader (Molecular Devices, LLC, San Jose, CA, USA) at 450 nm, and data analyzed using OriginPro 2019 (OriginLab Corporation, Northampton, MA, USA). Using the known concentrations of the calibrators plotted against the corresponding optical densities, a standard curve was generated using a cubic regression curve fit function. The unknown concentrations of the hormone of interest were then extrapolated from the optical density data. The lower limit of sensitivity for AMH was 0.023 ng/mL. The resulting intra-assay coefficient of variation of all AMH/MIS ELISAs completed was 7.9%, while the

inter-assay coefficient of variation was 8.9. The lower limit of sensitivity for activin A was 0.065 ng/mL. The resulting intra-assay coefficient of variation for all activin A ELISAs completed was 5.97%, while the inter-assay coefficient of variation was 7.3%.

2.3 Statistical Analyses

The concentrations of AMH and activin A were analyzed using linear models function of SAS (SAS Enterprise Guide version 8.2; Cary, NC, USA) including AMH by pregnancy status and activin A by pregnancy status, as well as age and weight by pregnancy status. Additionally, the Linear Regression Analysis of SAS with Spearman Bias Adjustment for multiple correlations was used to detect correlations between AMH vs E2, AMH vs age, and AMH vs body weight; activin A vs E2, activin A vs age and activin A vs body weight. A sub-analysis of each correlation within all females who became pregnant and all females who failed to become pregnant was used. Data were considered statistically significant if $p < 0.05$, and trends were noted for $p = 0.051$ - 0.10 . A linear models sub analysis was conducted including only younger females (15 and younger), as rhesus females > 15 years are considered nearing the end of their reproductive lifespan and follicle cohort declines significantly above 15 years of age [25] and chances of successful pregnancy declines above 15 years of age [26].

3 Results

Of the 90 samples that were collected from 47 females, 62 cycles did not result in a pregnancy, while 28 cycles did result in a pregnancy (Figure S1). There was variation in the day of the cycle in which the sample was collected, as there was variation in ovulation times depending on the individual. Over half of the samples were collected on day 7 after menses;

however, samples from days 5-11 post-menses, as well as day 15 post-menses were also included (Figure S2). As expected, there were no effects of body weight, nor age on pregnancy success rates (Figures S3 and S4), as all females were at a healthy body weight and had evidence of regular menstrual cycles which were criteria for inclusion in a rhesus breeding colony.

There was no significant difference in serum AMH by pregnancy status for all monkeys ($p = 0.46$; Figure 2). Analysis of only young monkeys (15 years and younger) also showed no significant difference of AMH by pregnancy status ($p = 0.25$; Figure 3). Similarly, there was no significant impact of activin A on pregnancy status for all monkeys; however, both groups (pregnant and nonpregnant cycles) had extreme outliers present ($p = 0.3836$; Figure 4). The age and weight of the monkeys were of importance to us in this study and taken into consideration. The impact of these additional factors on levels of AMH and activin A was evaluated. Linear Regression analyses revealed a significant negative correlation between age and AMH ($R = -0.32$; $p = 0.002$; Figure 5). When analyzed by TMB results for that cycle (pregnant cycles vs non-pregnant cycles), a similar negative correlation between age and AMH was detected when sample data from the fewer pregnant cycles analyzed were removed (Figure S5). There was also a significant negative correlation between weight and AMH with a 95% confidence interval ($R = -0.22$; $p = 0.036$; Figure 6 and Figure S5). There was a significant negative correlation between activin A and weight for only pregnant cycles, however, this data also included two extreme outliers ($R = -0.38$; $p = 0.047$; Figure 7 and Figure S6). Finally, no significant correlations were detected between serum E2 and AMH ($R = -0.06$; $p = 0.56$) or between E2 and activin A ($R = -0.07$; $p = 0.54$).

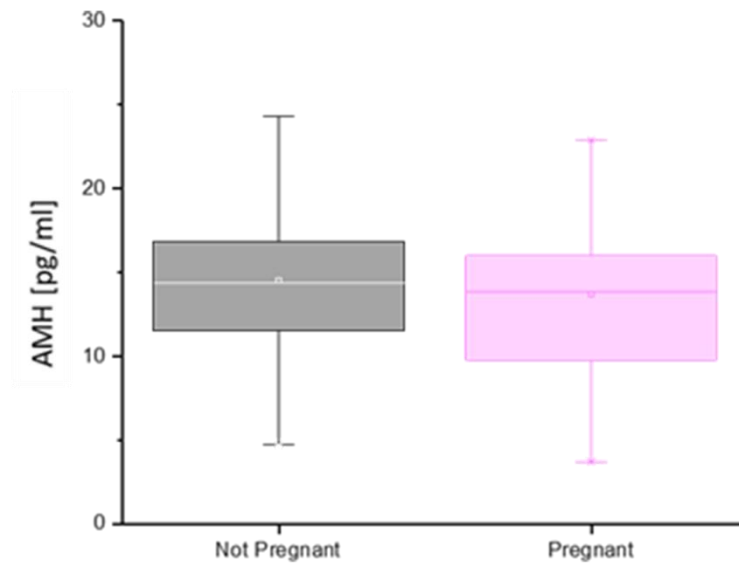


Figure 2 Anti Mullerian hormone concentrations (pg/mL) (y-axis) of initial blood sample collected relative to the pregnancy outcome (x-axis). No significant difference in AMH concentration based on pregnant (pink) versus non pregnant (gray) outcome, $p = 0.46$.

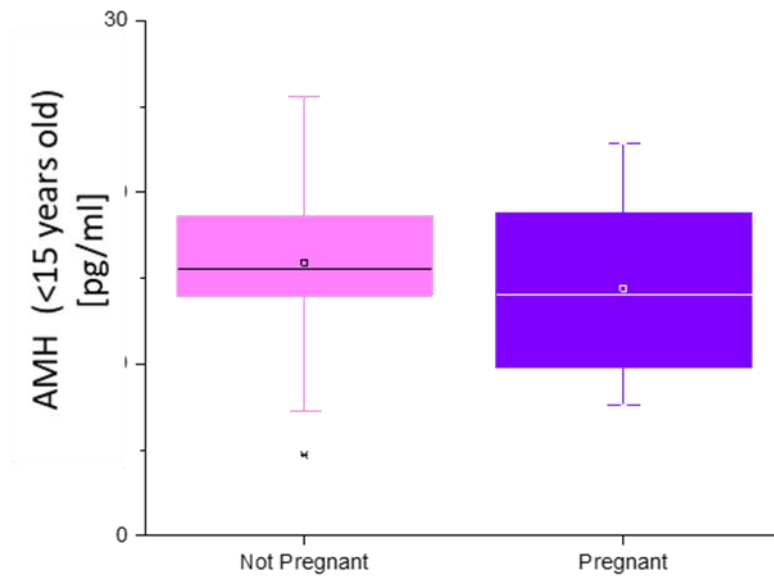


Figure 3 Anti Mullerian Hormone concentrations (pg/mL) of females ages fifteen and younger (y-axis) of initial blood sample collected relative to the pregnancy outcome (x-axis). No significant difference in AMH concentration based on pregnant (purple) versus non pregnant (pink) outcome, $p = 0.25$.

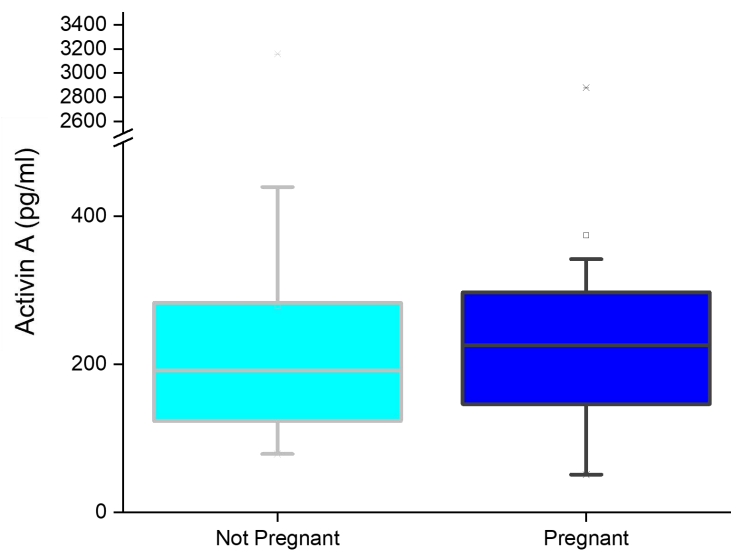


Figure 4 Activin A concentrations (pg/mL) (y-axis) of initial blood sample collected relative to the pregnancy outcome (x-axis). No significant difference in activin A concentration based on pregnant (dark blue) versus non pregnant (light blue) outcome, $p = 0.38$.

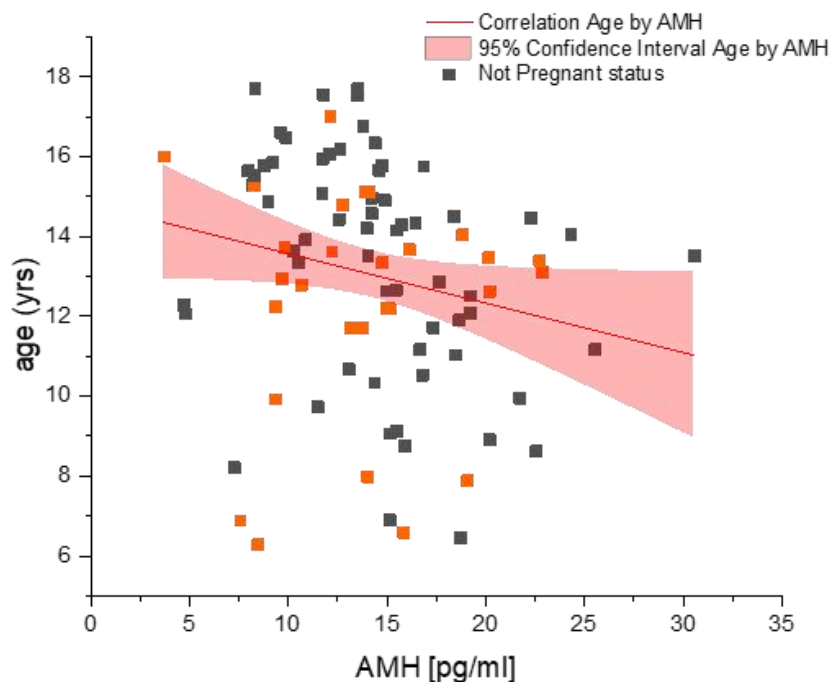


Figure 5 Evaluation of anti-Mullerian hormone concentration (AMH) (x-axis) and correlation to age of rhesus macaque females used in study ($n = 47$) (y-axis). Not pregnant females are represented in dark gray squares and pregnant females are depicted by orange squares. As a clarification, these were pre-mating samples from both groups combined. The linear correlation is shown by the red line. Significant correlation exists ($p = 0.002$) between AMH and age with 95% confidence, as seen within the light red region on the graph. Correlation with Spearman bias adjustment gives a negative slope of -0.32 .

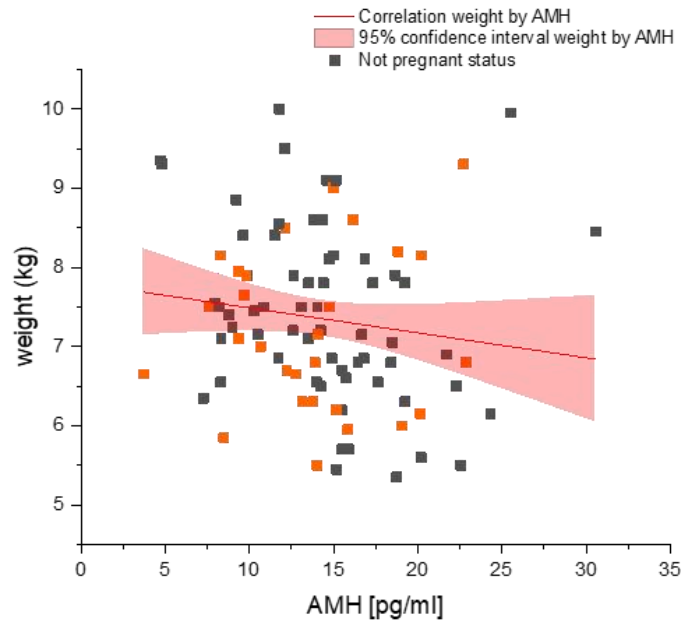


Figure 6 Evaluation of anti-Mullerian hormone concentration (AMH) (x-axis) and correlation to weight of rhesus macaque females used in study ($n = 47$) (y-axis). Not pregnant females are represented in dark gray squares and pregnant females are depicted by orange squares. As a clarification, these were pre-mating samples from both groups combined. The linear correlation is shown by the red line. Significant correlation exists ($p = 0.036$) between AMH and weight with 95% confidence, as seen within the light red region on the graph. Correlation with Spearman bias adjustment gives a negative slope of -0.22.

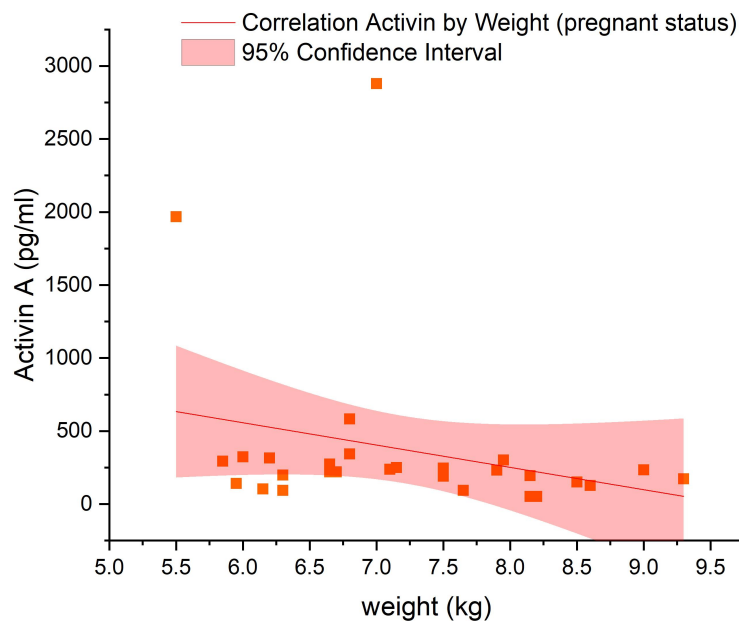


Figure 7 Evaluation of activin A concentration (pg/mL) (x-axis) and correlation to weight of rhesus macaque females used in study ($n = 47$) (y-axis) for only cycles that resulted in pregnancy. Pregnant females are depicted by orange squares. The linear correlation is shown by the red line. Significant correlation exists ($p = 0.047$) between activin A and weight in pregnant females only with 95% confidence, as seen within the light red region on the graph. Correlation with Spearman bias adjustment gives a negative slope of -0.38.

4 Discussion

Medicine has evolved to alleviate the burdens of infertility and ovarian aging through ART; however, with this costly and time-consuming process, successful pregnancy rates have been less than 50% in most fertile women over the last decade. Before proceeding with ART, prediction models are often created to assess various components of a women's reproductive history, including ovarian hormone levels, specifically AMH [27]. However, the predictive abilities and accuracy of AMH and other ovarian hormones is still unclear in fertile women. The direct impact of these hormones on pregnancy outcomes has yet to be characterized. A greater understanding of AMH and activin A and their role in fertility will facilitate more efficient and effective reproductive decisions.

In this study, we measured AMH and activin A levels in the serum of naturally cycling NHPs of various ages with no known reproductive health issues. We identified that neither AMH nor activin A levels are significantly predictive for establishment of early pregnancy. Both hormones are naturally occurring ovarian hormones secreted early during folliculogenesis. AMH has been previously characterized to reflect the number of growing follicles present in the ovary and is considered the best available measure of the ovarian reserve; a poor ovarian reserve is associated with reduced pregnancy rates [28]. Yet, previous studies consistent with our findings have found no difference in the overall follicular production of AMH in infertile patients compared to the fertile control group [29], and neither higher nor lower serum AMH levels were associated with fecundability [30]. Additionally, a study similar to this study examined the effects of AMH on natural pregnancy, suggesting a weak predictive value for AMH, and stating that decreased AMH values do not represent decreased natural fertility [31]. Yet, there may be an important distinction between the

utilization of AMH for ART treatments compared to natural fertility decisions. In a study measuring low AMH levels due to severe endometriosis and the resulting oocyte yield, embryo quality, and pregnancy outcomes during IVF, there was no reduction in embryo quality or pregnancy outcomes, however, there was a reduction in oocyte yield [32]. This could show the value of AMH to predict the quantity of eggs retrieved if relevant to the ART treatment desired. Furthermore, previous studies in women report high follicular fluid AMH levels associated with successful oocyte fertilization in IVF cycles [33], as well as greater follicular growth and ovarian response to IVF treatment in patients with a higher AMH at initial screening [34].

Activin A has been previously characterized as having a unique regulating role in folliculogenesis; however, knowledge is limited. It is known to stimulate FSH secretion and has inverse associations with serum Inhibin B, estradiol, and P4, yet it is not known to be associated with the gene expression of important granulosa cell hormone receptors [35]. Our study aimed to establish any predictive ability that activin A may have in fecundability based on what is known of its role in the ovary. This is the first study to our knowledge to directly study the correlation of activin A to pregnancy outcomes in rhesus monkeys, a valuable NHP model of women's reproductive health. In this pilot study, it was found that that activin A had no impact on early pregnancy success; however, additional investigation is needed to further establish this in the literature.

In the present study a negative correlation between age and body weight with AMH exists, which is consistent with the literature in humans [1,36-38] and other model species [39,40]. As a female ages, AMH decreases until menopause is reached and AMH production is absent. The impact of obesity on female reproduction is widely acknowledged, albeit a complex

process that has not been fully elucidated [41]. Yet, it is known that obesity has a profound impact on hormone secretion and metabolism, which leads to impaired folliculogenesis and follicular atresia [41]. Therefore, it is expected that these two hormones of interest would decrease as weight increased. As with AMH, there was a negative correlation between weight and activin A; however, this was only significant in the animals that became pregnant. This was an unexpected finding, since the data were not significant in the entire cohort nor in females who failed to become pregnant in that cycle (Figure S6). It is worth noting that there are two extreme outliers present in this data set; if they are excluded, the data lose significance. There is some emerging evidence suggesting activin A is elevated in late pregnancy in women just before a diagnosis of preeclampsia [42], but prior to late gestation, activin A levels are unchanged. However, this previous study reported data from samples obtained later in gestation and were likely of placental and not ovarian origin, whereas levels reported in this study were measured in monkeys prior to mating, before placentation. There is a possibility, however, that activin A production may be regulated by metabolic processes, and these outliers were associated with differences in body weight as both were on the lower end of the weight spectrum.

As previously noted, both AMH and activin A are secreted early during folliculogenesis. In our study design, we collected the blood samples at approximately day 7 post-menses. This is a potential limitation to our study, as the collection may have occurred too late in the cycle period, affecting the data we observed. At day 7 in a cycle, both AMH and activin A would begin to decline [7,16]. Samples should be collected around days 1 to 3 post menses to obtain hormone levels when they are at their peak. However, this collection time was a result of the TMB protocol, and this study was limited by the amount of blood

allowed to be drawn from the animals. Further limitations could be a result of the sample size used, which may limit the ability to detect significant predictive power for AMH and activin A. Unfortunately, we were constrained by the number of breeding females in the colony; other studies in cattle and women have needed several hundred individuals to reach statistical significance in AMH concentrations between free-breeding or infertile populations not subjected to ART such as ovarian stimulation and embryo transfer.

In this research colony-based study design we did not track live birth rates. This is due to the fact that these animals were assigned to various research studies, some of which involved treatments later in gestation. Because those treatments could impact ongoing live birth rate, we did not analyze AMH or activin A levels later during gestation or correlate with live birth rate. Additional studies in a dedicated cohort of females would be necessary to generate these types of data for further analyses.

Based upon these findings, future work should involve measuring AMH levels on animals undergoing ovarian stimulation specifically, to determine the predictive ability of AMH solely for infertility treatment purposes. Samples should be taken around days 1 to 3 post menses. Another area of future exploration is to supplement animals undergoing ovarian stimulation with AMH to evaluate the impacts of this on folliculogenesis and if oocyte production increases. It would also be of interest to further investigate the relationship between weight and activin A, and the significance that occurred in only the cycles which resulted in a pregnancy. These studies have the potential to further elucidate the impact of obesity on reproductive hormones and folliculogenesis.

5 Conclusion

This study intended to reveal the potential clinical and investigative uses of AMH and activin A, to effectively

develop treatments and inform reproductive protocols. While AMH is commonly used clinically, it may not be as reliable as is believed. According to this study, both AMH and activin A are unable to predict the ability to conceive naturally in rhesus monkeys undergoing timed mating protocols. These data are in agreement with a human observational study, where AMH values were not correlated with samples collected early in pregnant cycles [43]. Using AMH as a single snapshot of a woman's ovarian status may produce inaccurate predictions, unless ovarian stimulation and oocyte retrieval are performed. Further investigation is required to assess its ability and use when developing a treatment plan for those undergoing ART treatments; however, caution must be used. Additionally, assessing these reproductive hormones may not be helpful to improve research techniques in reproductive studies.

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Conflicts of Interest

C. Bishop was a paid consultant for Oviva Therapeutics, and collaborates on projects supported by 21st Century Medicine and Expanse Bio, LLC. All other authors declare no conflicts of interest.

Author Contributions

M.R. was involved in data collection, performed data analyses, as well as writing and editing manuscript. J.J. was involved in sample preparation and identification, and manuscript approval. C.B.H. and J.X. were involved in experimental design, sample

preparation, data interpretation, and manuscript approval. C.V.B. was involved in all aspects of the study including the experimental design, data collection, data analysis, and data interpretation as well as writing and editing of the manuscript.

Ethics Approval and Consent to Participate

All animal procedures were approved by the Oregon Health & Science University (OHSU), Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee (IACUC) and comply with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (The Guide; <https://doi.org/10.17226/12910>).

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Availability of Data and Materials

All summarized data supporting this study are included within the article and supporting materials. Full datasets are available upon reasonable request from the corresponding author.

Supplementary Materials

The following supporting information can be downloaded at: <https://ojs.exploverpub.com/index.php/jhre/article/view/215/sup>. Figure S1. A representation of the pregnancy outcomes in the cohort of cycles that were used in the study. Figure S2. Representation of the day of the cycle in which the first blood sample was taken that was utilized in hormone measuring of AMH and

activin A. Figure S3. Box plot analyses of impact of age (years) on cycle outcomes (pregnant vs not pregnant, $p > 0.12$). Figure S4. Box plot analyses of impact of weight (kg) on cycle outcomes (pregnant vs not pregnant, $p > 0.36$). Figure S5. Correlation analyses between serum AMH concentration prior to breeding (pg/mL) versus either age (years) or weight (kg) as separated by cycles resulting in a Pregnancy (Panel A, top graphs) or not (Non-Pregnant Cycle; Panel B, bottom graphs). Figure S6. Correlation analyses between serum Activin A (pg/mL) concentration prior to breeding (pg/mL) versus either age (years) or weight (kg) as separated by cycles resulting in a Pregnancy or not (Non-Pregnant Cycles), as compared with combining analyses from all cycles.

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