Anti-Mullerian Hormone (AMH) Levels Are Reduced by Age, But Do Not Correlate With Pregnancy In Timed-Mated Rhesus Monkey Females (Macaca mulatta).

By

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I understand that my project will become part of the permanent collection of the Oregon State University Library, and will become part of the Scholars Archive collection for BioResource Research. My signature below authorizes release of my project and thesis to any reader upon request.
1. Detection of menses with continued hormonal monitoring to predict ovulation

Female rhesus macaques (Macaca mulatta) (n=47)

 Estradiol and Progesterone measuring

2. Timed mated breeding and blood serum collection

Daily blood draws beginning -7 days post menses

Ovulation

3. Anti-Mullerian Hormone (AMH) and activin A assay measurement

Sandwich ELISA

4. Analysis

No significant correlation between AMH, activin A and pregnancy; significance between AMH, activin A with age and weight

Male and female paired
ABSTRACT

Infertility is an issue affecting millions of women across the globe, yet the causes are not fully understood, and treatment options are often ineffective. The evaluation of reproductive hormones has been a common tool used by fertility clinics as a way to predict the ovarian response to treatment and gain a better understanding of a patient’s prognosis. Specifically, Anti-mullerian hormone (AMH) and activin A are two ovarian hormones which contribute to folliculogenesis and may be associated with follicle quality, yet their predictive ability to determine pregnancy outcomes are still not well characterized in primates. The objective of this study was to use the nonhuman primate as a well-characterized primate model to assess the predictive power of AMH and activin A to determine pregnancy outcomes. Female rhesus macaques (n=47) were monitored during timed breeding protocols, where daily samples were collected beginning around day seven post menses until estradiol levels indicated ovulation was imminent, and 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 sample (days range 5-15) was utilized for analysis of serum levels of AMH and activin A in each TMB cycle (n=90). Pregnancy status was determined via ultrasound (Pregnant cycles n=28, non-pregnant cycles n=62). Enzyme linked immunosorbent assays (ELISA) were utilized that were previously validated in nonhuman primates (Ansh Labs). 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). There was no significant difference between AMH levels by pregnancy status (p=0.46). Similarly, there was no significant difference between activin A levels by pregnancy status (p=0.25). However, similar to data in women, there was a negative correlation between age and AMH (Spearman Bias-adjusted R=-0.32, p=0.002), and between weight and AMH (Spearman Bias-adjusted R= -0.22, p=0.036). There was also a significant negative correlation between activin A and weight in the subjects that became pregnant (R= -0.38, p=0.047); however, this appears to be driven by 2 outliers.
with very high levels of activin A. While there are limitations to the present study, these data demonstrate that the predictive ability of AMH to determine reproductive success in primates may need to be reconsidered in a “natural” or unstimulated mating scenario. In addition, activin A is also not a reliable predictor of pregnancy success. Further investigation into other ovarian markers is needed to reliably predict mating success in primates not undergoing assisted reproductive therapies. Funded by ONPRC Core Grant (ONPRC ART Core, C. Hanna).
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.

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 produces [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 Assisted Reproductive Technology (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 [4]. The two have complex interactions with each other as well as with the corresponding follicles of the ovary. AMH is a gonadal-specific glycoprotein 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 assisted reproductive technology, and AMH had a higher predictive value than antral follicle count [11]. The importance of AMH in subjects with no known reproductive disabilities 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 AMH concentration had smaller 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]. 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.
While infertility is extremely common, the various causes are yet to be fully understood. From idiopathic origins to cancer inducing infertility, ART is often the course of treatment. In 2019, 2.1% of all infants born in the United States were conceived with the use of ART. 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 model makes this possible.

The nonhuman primate (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 NHP shares striking similarities to female 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 associated pregnancy outcomes. The objective of this study was to use the nonhuman primate 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 pregnancy in naturally cycling nonhuman primate models, 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 nonhuman primates 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 nonhuman primates 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.

MATERIALS AND METHODS

Ethics: All animal procedures were approved by the Oregon Health & Science University, Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee (IACUC), and comply with the Animal Welfare Act and the APA Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research.
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 The Oregon Health & Science University (OHSU)’s Oregon National Primate Research Center (ONPRC), Beaverton, OR and maintained by the ONPRC Division of Comparative Medicine. The female nonhuman primates (NHPs) 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. The study was performed as a retrospective, non-randomized, cohort analysis and was a population-based study. These were reproductive aged females selected from the ONPRC colony as part of the Timed-Mated Breeding Resource run in collaboration between the ONPRC Department of Comparative Medicine and the ONPRC Assisted Reproductive Technologies (ART) Core [21].

Behavioral management of all nonhuman primates 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. 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. Daily blood draws continued until ovulation was imminent. The whole blood samples were centrifuged (1,000 x g for 15 minutes at 4ºC) to retrieve serum. Sera were assayed by the Endocrine Service Core at ONPRC, to measure Estradiol (E2) and Progesterone (P4) by use of an automated radioimmunoassay platform validated for NHP serum [22]. Estradiol and Progesterone 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 Timed-Mated Breeding (TMB) protocol (Figure 1). Blood collections continued in order to monitor a rapid surge and then decline in E2 blood 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 two and a half weeks post pairing and 3 and a half 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. 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 degree F freezer or -80 degree F 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 Biorender.com)

ELISA assays

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 nonhuman primates were used to measure both AMH and activin A. 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. Both ELISA kits used are Sandwich ELISAs, in which the antigen (hormone of interest) is sandwiched between a capture and detection antibody while following the kits’ procedures. 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.

The procedure from both manuals were followed exactly as described; however, only half of a plate (48 wells) rather than the entire plate (96 wells) was used at a time for all assay procedures completed. 500 microliters of the kit calibrators were aliquoted into plastic tubes after they were reconstituted with 1ml of milli-pure water and placed in the -20 degree F freezer, along with the other half of the well plate that was not used, to be used later. 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 x g, Legend Micro 21R, for 1 minute at 4°C).

Assays were completed to quantify AMH concentrations as suggested by manufacturer’s protocols (Ansh Labs, Webster, TX). All calibrators and quality controls were provided with known concentrations of the hormone of interest and were duplicated on the plates. The Ultra-Sensitive AMH/MIS ELISA is a three-step sandwich type immunoassay in which calibrators, controls, and samples are added to microtiter wells pre-coated with antibodies specific to the antigen of interest, AMH. This was followed by the washing of the wells with an AMH/MIS Assay Buffer to prevent detection of non-specific interactions. The plates were incubated at room temperature for 90 minutes. During all incubations, the plates were covered to prevent drying. After incubation, the wells were aspirated and washed as outlined in the manual, and a biotinylated AMH antibody solution was added. This solution detects the bound antigen and amplifies the signal, as well as binds the preceding Streptavidin bound enzyme. The plates were then incubated for 30 minutes at room temperature. AMH/MIS streptavidin horseradish peroxidase conjugate (SHRP) solution was then added and incubated for 30 minutes at room temperature, acting as the conjugated secondary antibody that will chemically react with the substrate solution for detection of the antigen. Finally, Tetramethylbenzidine (TMB) Chromogen solution was added to
the wells, acting as the substrate for the antibody-antigen-biotin conjugate-SHRP complex, initiating an enzymatic color reaction indicating how much antigen is bound by the antibodies present in our unknown samples. This occurred throughout a 10-12 minute incubation at room temperature and stop solution was added to stabilize the reaction. During this final incubation, the plates were covered with foil in addition to the Sani wrap.

Similarly, the Activin A ELISA is a quantitative three-step immunoassay sandwich ELISA, and the procedure was followed as suggested by manufacturer’s protocols. The calibrators, controls, and samples are added to microtiter wells pre-coated with antibodies specific to our antigen of interest, activin A. Two Activin A Assay Buffers, A and B, were added to prevent detection of non-specific interactions, and the plates were incubated at room temperature for two hours. This was followed by the addition of a biotinylated activin A antibody solution for detection of the bound antigen. The plates were incubated at room temperature for one hour. In the same way that the Ultra-Sensitive AMH/MIS ELISA was completed, streptavidin horseradish peroxidase conjugate (SHRP) solution was added, followed by Tetramethylbenzidine (TMB) Chromogen solution, allowing for enzymatic turnover and detection of antibody of interest. 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.

The Versamax Tunable Microplate Reader provided an electronic copy of data, reading the optical density for each well of the microplate. This data was manually transferred to an Excel spreadsheet and 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. After analysis, samples reading higher than the highest calibrator and whose concentrations
were undetectable were diluted in half and re-assayed. This was done to measure AMH concentrations only. 15 microliters of the AMH/MIS Calibrator A/Sample Diluent was added to 15 microliters of the intended sample. 25 microliters of this solution were then added to the well. The Ultrasensitive AMH/MIS ELISA was then completed exactly as stated in the manual and previously done above.

STATISTICAL ANALYSIS

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. Additionally, the Linear Regression Analysis of SAS with Spearman Bias Adjustment for multiple correlations was used to detect correlations between AMH vs E2, Age, and Weight; activin A vs E2, Age and 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).

RESULTS

The lowest amount of AMH that could be detected in serum through the Ultrasensitive AMH/MIS ELISA 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 lowest amount of activin A that could be detected in serum through the activin A ELISA 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%.

Throughout this study we sought to further characterize the impact of ovarian hormones AMH and activin A on pregnancy outcomes in the nonhuman primate model.
Of the 90 samples that were collected, 62 cycles did not result in a pregnancy and 28 cycles did result in a pregnancy (Supplemental Figure 1). 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 (Supplemental Figure 2).

Supplemental Figure 1. A representation of the pregnancy outcomes in the cohort of cycles that were used in the study. Frequency of pregnant or not pregnant is seen on the x-axis, and the resulting pregnancy outcome is on the y-axis. Out of 92 cycles from 47 females, 20 resulted in pregnancy, as seen in red, and 62 did not result in pregnancy, as seen in blue.
Supplemental Figure 2. 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. The day of the cycle (x-axis) ranges from day 5 to day 15 post menses. The majority of the frequency of the cycle day out of the 90 cycles used (y-axis) was day 7 post menses, as seen in turquoise.

There was no significant difference in AMH by pregnancy status for all monkeys (P=0.4609; Figure 2). Analysis of only young monkeys (15 years and younger) also showed no significant difference of AMH by pregnancy status (P=0.2455; Figure 3). Similarly, there was no significant impact of activin A on pregnancy status for all monkeys; however, both groups (pregnant and nonpregnant) 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 with a 95% confidence interval (R= -0.32; P=0.002; Figure 5). There was also a significant negative correlation between weight and AMH with a 95% confidence interval (R= -0.22; P=0.036; Figure 6). Lastly, there was a significant negative correlation between activin A and weight for only
pregnant animals, however, this data also included two extreme outliers (R=-0.38;P=0.047;Figure 7)

**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=0.4609.
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=2455.
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=0.3836.
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. 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. 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.

**DISCUSSION**

Medicine has evolved to alleviate the burdens of infertility and ovarian aging through assisted reproductive technology; 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 Anti Mullerian Hormone [23]. 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 blood serum of naturally cycling nonhuman primates of various ages with no known reproductive health issues. We identified that neither AMH nor activin A levels have a significant impact on pregnancy outcomes. Both hormones are naturally occurring ovarian hormones secreted early during folliculogenesis. AMH has been previously characterized to reflect the number of preantral 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 [24]. Yet, previous studies consistent with our findings have found no difference in follicular production of AMH in infertile patients compared to the fertile control group [25], and neither higher nor lower AMH levels were associated with fecundability [26]. 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 [27]. 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 [28]. 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 [29], as well as greater follicular growth and ovarian response to IVF treatment in patients with a higher AMH at initial screening [30].

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 Inhibin B, oestradiol, and progesterone, yet it is not
known to be associated with the gene expression of important granulosa cell hormone receptors [31]. 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 nonhuman primate model of women’s reproductive health. In this pilot study, it was found that that activin A had no impact on pregnancy outcomes; however, additional investigation is needed to further establish this in the literature.

In the present study a negative correlation between age and weight with AMH exists, which is consistent with the literature in humans [1,32,33,34] and other model species [35,36]. 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 [37]. Yet, it is known that obesity has a profound impact on hormone secretion and metabolism, which leads to impaired folliculogenesis and follicular atresia [37]. 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. 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 [38], but prior to late gestation, activin A levels are unchanged. Therefore, the cause of this elevation in our outliers is unknown, since these data were obtained much earlier in gestation, but there is a possibility that it may be related to 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 the limited 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 assisted reproductive therapies such as ovarian stimulation and embryo transfer.

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 interesting to further investigate the relationship between weight and activin A, and the significance that occurred in only the pregnant monkeys. It has the potential to further elucidate the impact of obesity on reproductive hormones and folliculogenesis.

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. Using AMH as a snapshot of a woman’s ovarian status may be producing inaccurate predictions. 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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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

C.V.B was involved in all aspects of the study including the experimental design, data collection, data analysis, and completion of the manuscript.

M.R. was involved in data collection, data analysis, writing and editing manuscript.

C.H. and J.X. were involved in experimental design, sample preparation, data interpretation, and manuscript approval.

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