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Association of Anogenital Distance, circulating AMH levels, miR-138 and miR-199a-5 Expression with Pregnancy Following First Timed Artificial Insemination in Primiparous Holstein Cows

Ayman E. Taha
King Faisal University
Rabie Abdel Aziz
rabea.ramadan@vet.bsu.edu.eg
Beni-Suef University Faculty of Arts https://orcid.org/0000-0003-2582-3567
Marwa A. Ibrahim
Cairo University
Shaimaa Kamel
Cairo University Faculty of Veterinary Medicine
Hassan A. Rudayni
Imam Muhammad Ibn Saud Islamic University
Haifa E. Alfassam
Princess Nourah bint Abdulrahman University
Ahmed A. Allam
Imam Muhammad Ibn Saud Islamic University
Mohamed M. A. Zeinhom
Beni-Suef University

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Abstract

The objective of this study was to evaluate the associations of anogenital distance (AGD), circulating concentrations of anti-Müllerian hormone (AMH), and miR-138, and miR-199a-5 expression with pregnancy following first timed artificial insemination (TAI) in primiparous Holstein cows. Thirty clinically healthy primiparous Holstein cows were evaluated at the time of first TAI. Anogenital distance, body and production traits, progesterone concentration, and circulating concentrations of AMH, in addition to miR-138, and miR-199a-5 expressions were determined. Pregnancy was diagnosed 30 days after TAI. Associations among variables were evaluated using Pearson correlation analysis, and exploratory receiver operating characteristic (ROC) analysis was performed to assess the discriminatory performance of the measured variables for pregnancy. Nine cows (30.0%) became pregnant. AGD ranged from 13.5 to 16.0 cm and was not significantly different between pregnant and open cows (15.00 ± 0.56 vs. 14.67 ± 0.84 cm, respectively; P = 0.216). In contrast, pregnant cows had markedly greater circulating AMH (0.786 ± 0.127 vs. 0.046 ± 0.018 ng/ml), higher miR-138 (0.712 ± 0.311 vs. 0.092 ± 0.127), and miR-199a-5 (0.733 ± 0.210 vs. 0.178 ± 0.163; all P < 0.001) expression levels. Strong positive correlations were observed among AMH, miR-138, and miR-199a-5 (r = 0.769–0.863; P < 0.001), whereas AGD was not significantly correlated with these biomarkers. Exploratory ROC analysis showed high discriminatory performance for AMH (AUC = 1.000), miR-138 (AUC = 0.971), and miR-199a-5 (AUC = 0.968), while AGD showed limited discrimination (AUC = 0.614). These findings indicate that circulating reproductive molecular biomarkers may be more closely associated with pregnancy following first TAI than AGD in primiparous Holstein cows. Larger prospective studies are warranted to validate their potential utility for fertility assessment.

Introduction

Reproductive efficiency is a major determinant of the biological and economic sustainability of dairy production systems (Inchaisri et al. 2010). Despite substantial advances in genetic selection, reproductive management, estrus synchronization, and timed artificial insemination (TAI), suboptimal conception at first insemination remains a persistent challenge in high-producing dairy cows (Fricke et al. 2018). Fertility is a complex phenotype resulting from interactions among ovarian function, oocyte competence (Kussano et al. 2024), endocrine regulation, uterine receptivity, embryo development, metabolic status, and environmental factors. Consequently, identification of practical biomarkers that can characterize reproductive potential before or around the time of insemination remains an important objective in bovine reproductive medicine. Recent studies have highlighted the potential of circulating and molecular biomarkers to complement conventional reproductive and phenotypic measurements and improve the identification of animals with divergent fertility potential (Abeysinghe et al. 2025; MacLeay et al. 2025).

Anogenital distance (AGD), defined in cattle as the distance between the anus and the base of the clitoris, has emerged as a readily measurable developmental phenotype associated with reproductive performance. AGD is thought to reflect aspects of prenatal androgen exposure and sexual

differentiation, providing a potential link between early developmental endocrine conditions and subsequent reproductive function (Salazar-Martinez et al. 2004; Sathyanarayana et al. 2010). In Holstein cattle, several large observational studies have reported an inverse association between AGD and fertility. In 921 lactating Holstein cows, increasing AGD was associated with reduced pregnancy to artificial insemination in first- and second-parity cows, whereas the relationship was not evident in older cows (Gobikrushanth et al. 2017). A subsequent validation study involving 4,709 North American Holstein cows confirmed a linear inverse association between AGD and pregnancy to first artificial insemination, although the discriminatory performance of AGD was modest (Carrelli et al. 2022). More recently, large-scale genetic analyses have reinforced interest in AGD as a potentially heritable fertility-related trait, suggesting that it may have value as an indicator phenotype for selection and reproductive management (Rajesh et al. 2022; Stephen et al. 2023). Nevertheless, the association between AGD and fertility is not entirely consistent across populations and reproductive stages. For example, a recent study of Holstein heifers reported earlier reproductive milestones in animals with shorter AGD but found no significant relationship between AGD and circulating anti-Müllerian hormone (AMH), emphasizing that phenotypic developmental markers and ovarian biomarkers may capture different dimensions of reproductive potential (Machado et al. 2025). In another study, Akbarinejad et al. (2019) reported that cows with longer AGD had inferior fertility but greater concentrations of anti-Müllerian hormone (AMH).

Anti-Müllerian hormone (AMH) provides a complementary biological measure because circulating AMH concentrations are closely related to the population of growing ovarian follicles and are therefore widely investigated as an indicator of ovarian reserve in cattle. Unlike many reproductive hormones that fluctuate substantially during the estrous cycle, AMH exhibits comparatively stable concentrations and has consequently attracted considerable interest as a candidate biomarker of ovarian follicular status (Mossa and Ireland 2019). Recent work in Holstein cattle demonstrated positive relationships between AMH concentrations and ovarian response to superovulation (Abdel Aziz et al. 2017; Koca et al. 2024), including corpus luteum number and embryo production, supporting its biological relevance to ovarian reproductive capacity. More recent evidence from lactating dairy cows also suggests that higher AMH concentrations are associated with greater antral follicle counts and may be related to first-service conception, although the strength and consistency of these relationships remain to be established. Thus, AMH may provide information that is complementary to morphological traits such as AGD, particularly when reproductive potential is evaluated at the individual-animal level.

Beyond circulating protein biomarkers, increasing attention has been directed toward microRNAs (miRNAs) as regulators of post-transcriptional gene expression and potential indicators of reproductive competence (De Bem et al. 2017; Putz et al. 2019; Lee et al. 2020). miRNAs participate in pathways controlling follicular development, steroidogenesis, oocyte maturation, embryo development, corpus luteum function, and uterine–conceptus communication. Their stability in biological fluids and their ability to reflect physiological or pathological states make them attractive candidates for minimally invasive reproductive biomarker development. Research work has demonstrated differential circulating miRNA profiles associated with pregnancy and fertility status in cattle (Kasimanickam and Kastelic 2016). For example, circulating miRNAs have been investigated as potential biomarkers of early

pregnancy (Ninomiya et al. 2024), while plasma small extracellular-vesicle miRNAs have recently been associated with divergent fertility phenotypes in Holstein-Friesian dairy cows (Abeysinghe et al. 2025). In addition, bovine miR-138 has been directly associated with sperm fertility potential, providing evidence that this miRNA may participate in reproductive processes extending from gamete function to fertilization and early embryogenesis (Salas- Huetos et al. 2023). The miR-199 family is also biologically relevant to bovine follicular physiology; miR-199a-5p and related family members have been detected in bovine granulosa cells and associated with differences between dominant and subordinate follicles, suggesting a potential role in follicular development and cellular regulation (Donadeu et al. 2017).

Importantly, most previous investigations have examined AGD, AMH, or individual molecular markers independently. Far less is known about whether a developmental phenotype such as AGD is related to circulating reproductive molecular markers and whether these complementary measures jointly characterize pregnancy success following first TAI. This question is particularly relevant in primiparous cows, in which reproductive performance is influenced by the transition from growth and first lactation to establishment of sustained reproductive competence. Furthermore, evaluating biomarkers specifically at the time of first TAI may provide greater practical relevance than measurements obtained at later reproductive stages.

Therefore, the objective of the present study was to evaluate the associations of AGD, AMH, miR-138, and miR-199a-5 expression with pregnancy following first TAI in primiparous Holstein cows. We hypothesized that these phenotypic and molecular measurements would differ according to pregnancy outcome and that the molecular biomarkers would show stronger associations with pregnancy than AGD alone. We further explored the discriminatory performance of these variables using receiver operating characteristic analysis. By integrating a readily measurable developmental phenotype with endocrine and molecular indicators of reproductive biology, this study provides preliminary evidence for a multidimensional approach to characterizing fertility in primiparous dairy cows.

Material and Methods

Animals and general management

The study was conducted on 30 clinically healthy, primiparous Holstein cows maintained in a well-managed dairy herd in Beni-Suef Governorate, Egypt. All cows had completed a normal postpartum period and were free from apparent metabolic, productive, and reproductive disorders at enrollment. Animals were selected to minimize variation in age, days in milk (DIM), milk production, and body weight. The study population comprised cows undergoing their first timed artificial insemination (TAI) during the study period.

The experimental procedures were reviewed and approved by the Beni-Suef University Institutional Animal Care and Use Committee (BSU-IACUC; approval No. 022–305). All procedures were performed in accordance with institutional requirements for the care and use of animals in research.

Cows were housed in an open-yard system with approximately 25 m² of space available per animal. The feed bunk provided approximately 70 cm of feeding space per cow, and automatic waterers were continuously available. Animals were offered a total mixed ration based primarily on corn silage and formulated according to the nutritional requirements of dairy cattle (National Research Council (NRC), 2001). Cows were milked three times daily in an automatic milking parlor. Individual milk yield and reproductive records were maintained as part of the routine herd-management system.

Reproductive management and timed artificial insemination

Cows were managed under a voluntary waiting period of 45 days postpartum. A presynchronization–Ovsynch protocol was used to synchronize ovulation and facilitate TAI, following the general approach described by Machado et al. (2017).

Briefly, two presynchronization treatments with prostaglandin F2α (PGF2α) were administered 14 days apart. Each treatment consisted of 500 µg cloprostenol sodium (2 mL Estrumate; MSD Animal Health, Egypt), administered intramuscularly. Twelve days after the second PGF2α treatment, the Ovsynch protocol was initiated.

The first gonadotropin-releasing hormone (GnRH) treatment consisted of 12 µg buserelin (3 mL Receptal; MSD Animal Health, Egypt), administered intramuscularly at approximately 70 days postpartum. Seven days later, cows received 500 µg cloprostenol sodium (2 mL Estrumate; MSD Animal Health, Egypt) intramuscularly. A second GnRH treatment consisting of 12 µg buserelin (3 mL Receptal; MSD Animal Health, Egypt) was administered approximately 48 h after PGF2α. Timed artificial insemination was performed approximately 16 h after the second GnRH treatment, corresponding to approximately 80 days postpartum.

All cows included in the study therefore received the same reproductive management protocol and were evaluated at the time of their first TAI.

Body condition score and morphometric measurements

Body condition score (BCS) was assessed on the day of TAI using the five-point scoring system described by Edmonson et al. (1989). The cows included in the study had BCS values ranging from 2.75 to 3.0 at the time of insemination.

Morphometric measurements were obtained on the day of TAI. Body height was measured as the vertical distance from the ground to the highest point of the withers. Thorax circumference was measured around the thorax immediately caudal to the forelimbs.

Anogenital distance (AGD) was measured on the day of TAI using a 20-cm graduated stainless-steel caliper according to the procedure described by Gobikrushanth et al. (2017). AGD was defined as the

linear distance between the center of the anus and the base of the clitoris. Measurements were obtained externally without causing injury or discomfort to the animals. Cows with apparent external genital lesions were excluded.

AGD was treated as a continuous quantitative variable in all analyses. No categorization of cows into short- and long-AGD groups was applied. The observed AGD values in the study population ranged from 13.5 to 16.0 cm.

Daily milk production was recorded at the time of first TAI. Milk yield was expressed as kilograms per day. These production variables were included in the overall characterization of the study population.

Blood collection and serum preparation

Blood samples were collected from the coccygeal vein on the day of first TAI. Samples intended for molecular analyses were collected into sterile Vacutainer tubes and allowed to clot before centrifugation. Samples were subsequently centrifuged at 3500 rpm for 15 min. The resulting serum was carefully separated into labeled tubes and stored at − 80°C until molecular analysis. Serum samples were used for quantification of circulating miRNA expression, an approach previously described for the analysis of circulating cell-free mature miRNAs in cattle (Kasimanickam and Kastelic 2016). A separate aliquot of serum was used to quantify AMH concentration.

A separate blood sample was collected for determination of serum progesterone concentration. Serum was obtained using the same centrifugation procedure and stored under frozen conditions until hormonal analysis.

Blood sampling was performed at the time of first TAI to ensure that hormonal and molecular measurements represented the physiological status of the cows immediately before or around insemination

Determination of serum progesterone concentration

Serum progesterone concentration was determined using a commercially available automated immunoassay (Progesterone Cobas, Elecsys; Roche, Germany) according to the manufacturer's instructions. Measurements were performed using the corresponding Elecsys analyzer system. The analytical detection limit of the assay was 0.1 ng/mL.

Progesterone concentrations were expressed as ng/mL and were subsequently evaluated in relation to pregnancy outcome and the other reproductive variables (Colazo et al. 2008).

Isolation and quantification of circulating miRNAs

Circulating microRNAs were isolated from serum using the mirVana miRNA Isolation Kit (Ambion, Cambridge, UK; Cat. No. AM1560) according to the manufacturer's instructions. Following extraction, RNA concentration and quality were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Extracted RNA was diluted to a working concentration of 20 ng/μL before reverse-transcription analysis.

The relative expression of mature miR-138 and miR-199a-5 was determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Small nucleolar RNA U87 was used as the endogenous reference for normalization. Reverse transcription was performed using miRNA-specific primers, followed by quantitative amplification using TaqMan MicroRNA Assays (Applied Biosystems; Cat. No. 4427975) containing sequence-specific probes. The procedure followed the methodology described by Ali et al. (2019). Quantitative PCR amplification was performed for 40 cycles, with denaturation at 95°C for 15 s followed by annealing/extension at 60°C for 1 min. Each sample was analyzed in triplicate, and a reverse-transcription negative control was included in each analytical run. Relative expression levels were calculated using the 2^−ΔΔCt method.

Measurement of AMH

Anti-Mullerian hormone was measured with the Active MIS/ AMH ELISA® kit (Cloud-Clone Corp, Houston, TX, USA) with a sensitivity of 177 pg/mL. The AMH was measured in 50 microliter undiluted plasma samples following manufacturer protocol. Intra and interassay coefficients of variation were less than 10% and 12%, respectively (Abdel Aziz et al. 2017).

Pregnancy diagnosis and definition of reproductive outcome

Pregnancy diagnosis was performed 30 days after TAI by transrectal ultrasonography using an Easi-Scan ultrasound system (BCF Technology) equipped with a 6-MHz linear transducer. Pregnancy was confirmed by visualization of an embryo proper together with characteristic anechoic amniotic fluid and an echogenic amnion (Romano et al. 2006).

Pregnancy following first TAI (P/1stAI) was calculated as the proportion of cows diagnosed pregnant 30 days after their first TAI relative to the total number of cows inseminated at the first service. Of the 30 cows included in the study, 9 were diagnosed pregnant and 21 were classified as open, corresponding to a pregnancy rate of 30.0%.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Continuous variables included BCS, height, AGD, thorax circumference, milk yield, progesterone concentration, and the circulating levels of AMH in addition to the relative expression levels of miR-138, and miR-199a-5.

Descriptive statistics were calculated for the entire study population (n = 30). Continuous variables are presented as mean ± standard deviation (SD), median, and range. Pregnancy outcome was expressed as the number and percentage of pregnant and open cows.

For comparison according to pregnancy outcome, cows were classified as pregnant (n = 9) or open (n = 21). Continuous variables were compared between the two groups using the appropriate independent-group statistical test based on the distributional characteristics of the data. Results were expressed as mean ± SD, and two-sided P-values were reported. A P-value < 0.05 was considered statistically significant.

Pearson correlation analysis was used to evaluate associations among AGD, progesterone, AMH, miR-138, and miR-199a-5. Correlation coefficients (r) were calculated using observations from all 30 cows. The strength and direction of associations were assessed from the corresponding Pearson correlation coefficients, with $P < 0.05$ considered statistically significant (Mukaka 2012). Particular attention was given to the relationships among AMH, miR-138, and miR-199a-5 and to the potential relationships between AGD and the molecular biomarkers.

Exploratory receiver operating characteristic (ROC) analysis was performed to assess the discriminatory performance of AGD, progesterone, AMH, miR-138, and miR-199a-5 for pregnancy following first TAI. The area under the ROC curve (AUC) was calculated for each variable. Exploratory cutoffs were derived from the present dataset, and the corresponding sensitivity and specificity were calculated. Because the ROC analysis was exploratory and based on a relatively small sample, the resulting cutoff values and diagnostic performance estimates were interpreted as preliminary rather than externally validated measures (Zou et al. 2007).

Results

Characteristics of the study population

The characteristics of the 30 primiparous Holstein cows included in the study are summarized in Table 1. Nine cows (30.0%) became pregnant following first timed artificial insemination (TAI), whereas 21 (70.0%) remained open. The mean body condition score (BCS) was 2.93 ± 0.23, and mean anogenital distance (AGD) was 14.77 ± 0.77 cm, ranging from 13.50 to 16.00 cm. Mean milk yield was 26.53 ± 1.07 kg/day, and mean serum progesterone concentration was 0.93 ± 0.75 ng/mL.

Table 1
Characteristics of the study population and measured reproductive biomarkers in 30 primiparous Holstein cows

Variable	n	Mean ± SD	Median	Range
Pregnancy outcome	30	—	—	—
└ Pregnant, n (%)	9	—	—	30.0%
└ Open, n (%)	21	—	—	70.0%
Body condition score	30	2.93 ± 0.23	3.00	2.40–3.30
Height (cm)	30	140.30 ± 3.09	140.00	133–149
Anogenital distance (AGD, cm)	30	14.77 ± 0.77	15.00	13.50–16.00
Thorax circumference (cm)	30	200.37 ± 6.67	200.00	189–217
Milk yield (kg/day)	30	26.53 ± 1.07	26.50	25–28
Progesterone (ng/mL)	30	0.93 ± 0.75	0.85	0.05–4.00
AMH (ng/mL)	30	0.268 ± 0.351	0.051	0.026–0.920
miR-138 relative expression	30	0.278 ± 0.349	0.067	0.025–0.990
miR-199a-5 relative expression	30	0.345 ± 0.312	0.235	0.027–0.950

AGD, anogenital distance; AMH, anti-Müllerian hormone; SD, standard deviation. **Note**: Pregnancy was diagnosed 30 days after first timed artificial insemination. The molecular variables represent relative expression levels determined by quantitative reverse-transcription PCR. AGD was analyzed as a continuous variable; no short- versus long-AGD categorization was applied.

The measured molecular biomarkers showed considerable variation among cows. Mean circulating anti-Müllerian hormone (AMH) concentration was 0.268 ± 0.351 ng/mL, while mean relative expression levels were 0.278 ± 0.349 for miR-138 and 0.345 ± 0.312 for miR-199a-5. The overall characteristics of the study population and the distributions of the measured reproductive biomarkers are presented in Table 1.

Comparison of reproductive biomarkers according to pregnancy outcome

Comparisons of phenotypic, hormonal, and molecular variables between pregnant and open cows are presented in Table 2. AGD did not differ significantly between pregnant and open cows (15.00 ± 0.56 vs. 14.67 ± 0.84 cm, respectively; P = 0.216). Similarly, serum progesterone concentration was not significantly different between the two groups (0.79 ± 0.50 vs. 1.00 ± 0.83 ng/mL, respectively; P = 0.401).

Table 2
Phenotypic, production, hormonal and molecular characteristics according to pregnancy outcome

Variable	Pregnant n = 9	Open n = 21	P
AGD (cm)	15.00 ± 0.56	14.67 ± 0.84	0.216
Progesterone (ng/mL)	0.79 ± 0.50	1.00 ± 0.83	0.401
AMH (ng/mL)	0.786 ± 0.127	0.046 ± 0.018	< 0.001
miR-138	0.712 ± 0.311	0.092 ± 0.127	< 0.001
miR-199a-5	0.733 ± 0.210	0.178 ± 0.163	< 0.001

Values are presented as mean ± SD. AGD, anogenital distance; AMH, anti-Müllerian hormone. Pregnancy was diagnosed 30 days after first timed artificial insemination. $P < 0.05$ was considered statistically significant; $P < 0.01$ was considered highly significant.

In contrast, pregnant cows had substantially higher circulating AMH concentrations than open cows (0.786 ± 0.127 vs. 0.046 ± 0.018 ng/mL; P < 0.001). Relative expression levels of miR-138 were also higher in pregnant cows than in open cows (0.712 ± 0.311 vs. 0.092 ± 0.127; P < 0.001). A similar difference was observed for miR-199a-5 expression (0.733 ± 0.210 vs. 0.178 ± 0.163; P < 0.001). Thus, AMH, miR-138, and miR-199a-5 differed significantly according to subsequent pregnancy outcome, whereas AGD and progesterone did not.

Exploratory discriminatory performance of reproductive biomarkers

The exploratory receiver operating characteristic (ROC) analysis results are summarized in Table 3. AMH showed an area under the curve (AUC) of 1.000, with an exploratory cutoff of 0.51 ng/mL, yielding 100.0% sensitivity and 100.0% specificity in this dataset. MiR-138 also demonstrated high discriminatory performance (AUC = 0.971), with a cutoff of 0.11, sensitivity of 100.0%, and specificity of 85.7%. For miR-199a-5, the AUC was 0.968, with an exploratory cutoff of 0.48, sensitivity of 100.0%, and specificity of 90.5%.

Table 3
Exploratory receiver operating characteristic analysis of AGD, progesterone, AMH and molecular biomarkers for pregnancy following first TAI

Biomarker	AUC	Exploratory cutoff	Sensitivity	Specificity
AMH	1.000	0.51 ng/mL	100.0%	100.0%
miR-138	0.971	0.11	100.0%	85.7%
miR-199a-5	0.968	0.48	100.0%	90.5%
AGD	0.614	15.0 cm	77.8%	52.4%
Progesterone	0.450	1.10 ng/mL	44.4%	61.9%

AUC, area under the receiver operating characteristic curve; AGD, anogenital distance; AMH, anti-Müllerian hormone. TAI, timed artificial insemination

In comparison, AGD showed limited discriminatory performance (AUC = 0.614), with a cutoff of 15.0 cm, sensitivity of 77.8%, and specificity of 52.4%. Progesterone showed poor discrimination (AUC = 0.450), with a cutoff of 1.10 ng/mL, sensitivity of 44.4%, and specificity of 61.9%. These exploratory results indicate that AMH and the two miRNAs showed greater apparent discrimination between pregnant and open cows than AGD or progesterone in the study population. However, the estimates, particularly the perfect discrimination observed for AMH, should be interpreted cautiously because of the small sample size and the absence of independent validation.

Correlations among phenotypic, hormonal, and molecular variables

Pearson correlation analysis revealed strong positive correlations among AMH, miR-138, and miR-199a-5 (Table 4). AMH was strongly correlated with miR-138 ($r = 0.863$; $P < 0.001$) and miR-199a-5 ($r = 0.786$; $P < 0.001$). A strong positive correlation was also observed between miR-138 and miR-199a-5 ($r = 0.769$; $P < 0.001$).

Table 4
Pearson correlations among AGD, progesterone, AMH, miR-138 and miR-199a-5

Variable	AGD	Progesterone	AMH	miR-138	miR-199a-5
AGD	1.000	0.007	0.235	0.033	0.030
Progesterone	0.007	1.000	−0.084	−0.088	−0.209
AMH	0.235	−0.084	1.000	0.863*	0.786*
miR-138	0.033	−0.088	0.863*	1.000	0.769*
miR-199a-5	0.030	−0.209	0.786*	0.769*	1.000

Values are Pearson correlation coefficients (*r*). **P* < 0.001. AGD, anogenital distance; AMH, anti-Müllerian hormone.

AGD was not significantly correlated with progesterone, AMH, miR-138, or miR-199a-5 ($r = 0.007$, 0.235, 0.033, and 0.030, respectively; all $P > 0.05$). Similarly, progesterone showed no significant correlations with AMH ($r = -0.084$), miR-138 ($r = -0.088$), or miR-199a-5 ($r = -0.209$; all $P > 0.05$). Overall, the correlation analysis demonstrated strong associations among the three molecular biomarkers but no statistically significant relationships between AGD or progesterone and the investigated molecular variables.

Discussion

The present study identified strong associations between circulating reproductive biomarkers and pregnancy following first timed artificial insemination (TAI) in primiparous Holstein cows. Pregnant cows exhibited substantially greater circulating AMH, greater relative expression levels of miR-138, and miR-199a-5 than cows that remained open, and these three biomarkers were strongly and positively correlated with one another. In contrast, AGD was not significantly associated with pregnancy or with circulating AMH or molecular biomarkers in the present dataset.

The association between AMH expression and pregnancy is biologically plausible given the established role of AMH as a marker of ovarian follicular reserve and antral follicle count in cattle since AMH is produced by granulosa cells in proportion to the total number of ovarian follicles and is therefore used to estimate functional ovarian reserve. Circulating AMH has previously been linked to pregnancy maintenance and subsequent conception in dairy cows bred by estrus detection or natural service (Ribeiro et al. 2014), and low-AMH cows have shown a greater incidence of pregnancy loss between days 30 and 65 of gestation (Alward et al. 2025). However, the relationship between AMH and fertility appears to depend heavily on breeding protocol: first-service conception rate under strict TAI was not affected by AMH category in at least one recent report (Alward et al. 2025), and in Bos indicus heifers bred by TAI, greater AMH was actually associated with a smaller preovulatory follicle and a lower ovulation response (Batista et al. 2020).

This protocol-dependence should temper any causal interpretation of the present AMH findings and points to the need for standardized sampling relative to the TAI protocol in future work. The parallel elevation of miR-138 and miR-199a-5 in pregnant cows, together with their strong correlation with AMH, is consistent with mechanistic evidence implicating both miRNAs in reproductive tissue function. miR-138-5p has been shown to regulate GPR124 and NLRP3-inflammasome signaling in decidual tissue, with reduced expression associated with miscarriage rather than normal pregnancy in other species, supporting a role in supporting embryo–maternal cross-talk during implantation (Wu et al. 2022). Similarly, miR-199a has been shown to mediate mucin-1 expression in the uterine epithelium during the implantation window, indicating a plausible endometrial mechanism through which this miRNA could influence receptivity (Inyawilert et al. 2014). Because AMH and the two molecular biomarkers were sampled at TAI and pregnancy was diagnosed 30 days later, however, the design cannot distinguish whether these molecules causally promote conception or instead mark a more favorable ovarian and uterine physiological state already present at the time of insemination.

The absence of a significant relationship between AGD and pregnancy contrasts with several prior reports describing an inverse association between AGD and fertility in Holstein cattle, including in lactating cows (Gobikrushanth et al. 2017), nulliparous heifers (Vidal et al. 2024), and larger validation cohorts (Carrelli et al. 2022). Notably, however, not every study has found this relationship, and at least one recent report found no association between AMH and AGD and only a modest link between AGD and time to conception, underscoring that findings are not fully consistent across populations (Machado et al. 2025). The lack of association in the present cohort may reflect the smaller sample size, a narrower range of AGD values, or differences in population and management characteristics relative to the larger validation studies. These results should not be interpreted as evidence against AGD as a reproductive phenotype, but rather indicate that its association with fertility was not demonstrable in this particular cohort.

Serum progesterone at TAI was similarly not associated with pregnancy in the present dataset. Prior work indicates that the relationship between circulating progesterone and fertility around TAI is highly time-dependent and can even be curvilinear: elevated progesterone at TAI itself, often reflecting incomplete luteolysis, has been associated with reduced pregnancy per AI (Colazo et al. 2017), whereas greater progesterone before and after TAI has been associated with improved outcomes (Madureira et al. 2021). A single measurement at insemination may therefore be insufficiently informative unless timed precisely relative to luteolysis and ovulation.

Because AMH, miR-138, and miR-199a-5 were all independently associated with pregnancy, their intercorrelation may partly reflect a shared relationship with pregnancy status rather than direct molecular regulation between them. Experimental studies involving ovarian and endometrial tissue, functional manipulation of the candidate miRNAs, and longitudinal sampling are needed to establish causal mechanisms. Overall, the present data support the potential value of AMH, miR-138, and miR-199a-5 as candidate biomarkers of pregnancy following first TAI, while larger prospective studies are needed to validate their predictive performance and determine whether combining them with phenotypic measures such as AGD can improve reproductive decision-making in dairy cattle.

Conclusions

This study demonstrated that circulating AMH, miR-138, and miR-199a-5 at the time of first timed artificial insemination were strongly and consistently elevated in primiparous Holstein cows that subsequently became pregnant, and that these three biomarkers were closely correlated with one another. These findings support their potential as candidate molecular indicators of a reproductive state favorable to conception, consistent with their established or plausible roles in ovarian follicular reserve, embryo implantation, and endometrial receptivity. By contrast, AGD and serum progesterone at insemination were not significantly associated with pregnancy outcome in this cohort, a result that should be interpreted as a limitation of the present dataset rather than as evidence refuting their biological relevance, given that both have shown associations with fertility in other studies and populations.

Because AMH measurement and biomarker expression was assessed at a single time point and pregnancy was confirmed only 30 days later, the associations reported here are correlative rather than causal. Larger, prospective studies incorporating longitudinal sampling, functional validation of the candidate miRNAs, and combined phenotypic-molecular models are needed before these biomarkers can be recommended as practical tools for predicting fertility outcomes. Nonetheless, the present findings provide a foundation for future work aimed at improving early, non-invasive prediction of pregnancy success in dairy cattle reproductive management.

Declarations

Ethics Declaration

All experimental work on live animals was approved by the Institutional Animal Care and Use Committee, Beni-Suef University, Egypt (BSU-IACUC: 022–305)

Competing interests

The authors declare no competing interests

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Author contributions

Ayman E. Taha: Conceptualization, Methodology, Funding acquisition, Writing – original draft. Rabie Lotfy Abdel Aziz: Conceptualization, Investigation, Methodology, Data curation, Writing – review & editing. Marwa A. Ibrahim: Conceptualization, Data curation, Formal analysis, Writing – original draft. Shaimaa Kamel: Methodology, Data curation, Writing – original draft. Hassan A. Rudayni: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. Haifa E. Alfassam: Conceptualization, Investigation, Data curation, Funding acquisition, Writing – review & editing.. Ahmed A. Allam: Investigation, Data curation, Resources, Supervision, Writing – review & editing. Mohamed M. A. Zeinhom: Validation, Methodology, Investigation, Formal analysis, Writing – review & editing All authors have read and agreed to the published version of the manuscript.

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Data Availability

All data will be available on request

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