CHARACTERIZATION OF PREGNANCY-ASSOCIATED GLYCOPROTEINS AND PROGESTERONE AS A PREDICTOR OF TWINS AND CONCEPTUS LOSS IN HIGH-RISK PREGNANCY HOLSTEIN COWS

BY

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THESIS

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ABSTRACT

Pregnancy loss is a multifactorial condition that compromises reproductive performance in dairy operations. Despite the high oocyte fertilization rate in dairy cows, only 28 % of those maintain a pregnancy to term. Pregnancy loss is estimated to cost U$600.00 per case. Identification of cows losing the pregnancy as early as possible can be helpful in providing timely opportunities for rebreeding, thus potentially minimizing economic losses. Traditionally, early pregnancy diagnosis is performed via ultrasonography, starting at 30 days, which provides information regarding embryo viability, uterine health, and ovarian structures. In addition, this technique allows the diagnosis of twin pregnancy that is three times more likely to be lost than a singleton. Despite its benefits ultrasonography requires well-trained personnel and incurs additional costs involving equipment purchase and maintenance. The use of biomarkers has been studied throughout the years, based on a demand for an easier, less costly, and more accurate test. Pregnancy-associated glycoproteins (PAG) is the most common biomarker marker to assess pregnancy status in cows. Produced and secreted on the maternal circulation by binucleate giant cells. Measurement of PAG in the blood has high sensitivity when performed between 25 32 days of gestation, however, the specificity can be as low as 83%. One of the major components that affect test accuracy is pregnancy loss. It has been reported that cows experiencing early pregnancy loss, present lower plasma concentrations of PAG. Another indirect biomarker to detect pregnancy in cows is progesterone. Cows experiencing pregnancy loss showed lower concentrations of this hormone, in comparison to cows keeping the pregnancy. The development of a threshold for PAG and progesterone that can predict pregnancy loss may aid in management decisions to provide earlier rebreeding opportunities. It was hypothesized that the plasma concentration of PAG and

progesterone is reduced and can predict pregnancy loss in cows experiencing a high-risk pregnancy. Additionally, it was hypothesized that the concentration of PAG and progesterone are increased and can predict twins. High-risk pregnancy (HR) were characterized using transrectal ultrasonography 37 days post-AI based on the following criteria: small embryo size (SE, embryo < 15 mm, n=10), slow heartbeat (SH, <60 beats per minute, n = 11), extra amniotic membrane (EM, additional amniotic membrane, n=3). A cohort of twins (TW, n = 41) diagnosed at day 37 post-AI was also enrolled. Twins were also subgroups in unilateral (UT, n=17) and bilateral (BT, n=24). Each HR and TW cow was paired with the same parity cow carrying a normal singleton at d 37 post-AI (CON, n = 65). Blood samples were collected to measure PAG and progesterone at 37, 44, and 51 post-AI. Statistical analysis was performed using ANOVA, logistic regression and receiver operation characteristics (ROC) with JMP. Pregnancy loss at day 51 post-AI was greater ($P < 0.01$) in HR than CON and TW (CON=1.5%; HR=87.5%; TW=12.2%). Concentration of PAG at day 37 post-AI did not differ ($P = 0.75$) among groups (CON = 5.3 ± 0.7; HR = 4.8 ± 1.2; TW = 4.0 ± 0.9 ng/ml). The subgroup SE showed a statistical difference regarding the concentration of PAG at day 51 post-AI ($P < 0.05$), EM showed a tendency ($P < 0.10$) whereas SH, UT and BT did not when compared to CON. Concentration of progesterone at day 37 post-AI was greater in TW than HR and CON, and lower ($P < 0.01$) in HR than CON cows (CON = 7.0 ± 0.3; HR = 5.9 ± 0.4; TW = 8.4 ± 0.3 ng/ml). Regression and ROC analysis for PAG at day 37 post-AI did not find a threshold to predict pregnancy loss ($P = 0.24$) or twins ($P = 0.30$). Regression and ROC analysis for progesterone at day 37 post-AI found that a threshold of 6.5 ng/ml predicted ($P < 0.01$) pregnancy loss with an area under the curve (AUC) of 0.65, and threshold of 7.2 ng/ml predicted ($P < 0.01$) twins with AUC of 0.70. In summary, pregnancy loss and twins were predicted with only moderate accuracy by progesterone concentration at day 37

post-AI and the variability in PAG concentrations at day 37 post-AI was insufficient to generate a threshold to predict pregnancy loss and twins in Holstein lactating cows.

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CHAPTER 1: LITERATURE REVIEW

1.1.Pregnancy loss in the first trimester

Pregnancy loss is one of the major factors affecting reproductive efficiency in dairy operations. Despite high oocyte fertilization rates of, about 80-90%, only approximately 30 % of high producing dairy cows have pregnancies reaching term after timed artificial insemination (TAI) [1]. The incidence of pregnancy loss in cattle is the highest in the first trimester of gestation [1-4]. Wiltbank et al.[5] proposed a chronological subdivision for the pregnancy loss on the first trimester in pivotal periods: (1) First week, fertilization failure (2) second week to 27 days, maternal recognition (3) 28 to 60 days, placentome development (4) 60-90 days, placentome and fetal growth and twins. Based on this subdivision, important essential aspects of each period are detailed.

Fertilization failure occurs in 10 to 20 % of cows living under cool weather conditions, inseminated with semen from fertile bulls [6-9]. The inadequate gamete transport can prevent the sperm acrosomal reaction in the zona pellucida, precluding the sperm-oocyte fusion, ultimately leading to fertilization failure [10]. This poor transportation can be related to inadequate AI technique [11], elevated polymorphonuclear cells in the uterine environment [12] and a slight increase in progesterone concentrations during timed AI [13,14]. Heat stress also plays a role in fertilization failure, since poor oocyte quality had been reported in cows kept under warm weather [6.12]. The oviduct has an important physiological role in this process, supplying an important combination of ions, such as phosphate, sulfate, and magnesium [15]. Amino acids such as glycine, glucose and lactate are energy-rich substrate present in the oviduct [16,17]. Additionally, spermatozoa are optimally capacitated by the isthmus cells improving the efficacy of fertilization [18,19]. Another challenging step in this early pregnancy process is embryonic development,

which initially consists of a series of cell divisions within the zona pellucida. The first stages of embryo development depend on maternal mRNA and proteins that are stored in the oocyte [20]. To develop to the blastocyst stage, the maternal-to-embryonic genome transition is necessary [21-23]. This activation of the embryonic genome is the fundamental step that allows specific cell differentiation, leading to the development beyond the 16-cell stage division [24]. The preovulatory follicle condition is also involved in embryo development. Follicles exposed to reduced progesterone levels during the luteal phase, may extend their follicle dominance period, and become persistent, with an ovulatory size of more than 15 mm [25]. Persistent follicles are associated with poor embryo development to the 16-cell stage, even though the fertilization rate was similar compared to normal follicles [26]. Lactating dairy cows reported with persistent follicle during TAI, showed lower pregnancy rate per AI at day 31 than cows ovulating younger follicles [14]. Also, lower plasma levels of progesterone during the preovulatory follicle development, yield fewer embryos grade 1 and 2 at day 7 compared to cows exposed to higher concentrations of progesterone [13]. It is believed that increased duration of follicle growth can expose the follicle to more LH pulses, as during low plasma progesterone concentrations, which could ultimately result in the ovulation of larger follicles [5]. It is possible that oocytes could be experiencing premature germinal breakdown [27]. Another issue is related to the nutrition offered to the cow. Lactating cows experiencing profound weight loss, measured by the reduction in body condition score (BCS) between parturition and first AI, are also associated with poor fertility [28-30]. According to a study [30], cows that experienced a considerable bodyweight loss presented a few good quality embryos at seven days post-AI.

Between the second week to 27 days of gestation entails the second pivotal period of pregnancy loss, involving three important events: (1) the embryonic growth and elongation, (2)

maternal recognition of pregnancy and (3) maternal immune down-regulation [5]. The embryo hatches the zona pellucida and initiates the free-floating state. The trophoblast/embryo expands from a 0.15 mm in diameter blastocyst to an elongated form of approximately 40 cm in length, extending to both uterine horns by day 25 [31], supported by nutrients from the histotroph to the embryonic cells. The elongation process involves complex crosstalk between the conceptus and maternal system [32]. Endometrium cells are regulated by factors produced by the conceptus, most notably interferon-tau (IFNT), that ultimately stimulate these cells to produce and transport substrates, optimizing the histotroph for embryonic nutrition and elongation [33]. Another important aspect of this relationship between uterine cells and histotroph is the circulating concentration of progesterone. Inadequate levels of progesterone, during the early luteal phase, can alter gene expression in endometrial cells, embryo growth, and pregnancy success [34,35]. The second important event during this period is the maternal recognition. The process consists of the conceptus signaling its presence in the uterus by producing IFNT, a key factor that prevents luteolysis and supports the pregnancy [36]. Although the luteolysis does not initiate until day 18 or 19 post-ovulation in cows, signals from the embryo need to be present by day 16 to alter the endometrium cells' gene expression to induce an extended estrous cycle [37,38]. The mechanism involved in this process still unclear. One of the models postulates that the presence of an elongating conceptus produces IFNT, which reduces expression of specific proteins within the uterine endometrial cells, such as E2 and oxytocin receptors, preventing oxytocin pulses from stimulating the synthesis of prostaglandin F2α (PGF2α) resulting in lack of luteolytic signals [39-41]. The third process during this period is the relationship between the newly generated conceptus and the maternal immune response. Under normal circumstances, the foreign antigenic peptides are presented to cytotoxic T lymphocytes via MHC molecules, ultimately removing the foreign

cell [42]. The trophoblast down-regulates the expression of MHC class I molecules, preventing the embryo from being rejected [43]. Additionally, IFNT is involved in the silencing of MHC class I in the endometrium lumen [42]. Another factor in this scenario is the presence of T regulatory cells in the uterus, signaling other maternal immune cells to an immune tolerance status, impeding the rejection of the semi allograft conceptus [44]. Therefore, the interaction among MHC expression, IFNT, progesterone, maternal T regulatory cells, coordinates immunological downregulation and protection of the embryo and placenta in pregnant cows [45,46]. Therefore, IFNT has a fundamental role in all events that entails this pivotal period, the embryonic elongation, maternal recognition of pregnancy, and maternal immune regulation against the conceptus.

The third pivotal period comprises the second month of gestation. This stage is marked by the attachment between endometrium and the allantoic membranes, and subsequent development of placentomes. The complex embryo/amnion weight approximately five grams with no cotyledons between days 20 to 25 of gestation but grows substantially in the next five days reaching approximately 30 grams in weight. Alongside with this dramatic growth, the first distinguishable cotyledons are also observed around day 30 [47]. The placentome development was initiated around day 18-19 with the apposition between trophoblast and uterine epithelial cells microvilli, followed by adhesion of the membranes two days later, and subsequently, weak attachments linking the maternal and fetal tissues in the next week [48]. Evidence of interdigitation between maternal and trophoblastic tissues by Day 30 of pregnancy is noticed [49,50]. Also, a significant number of binucleate giant cells are observed during the placentome formation [50].

A switch on embryonic nutrition from histotroph to choriovitelline is reported with the development of the yolk sac as early as day 18 [31]. The yolk sac remains as the primary source of the embryonic substrate until gradually replaced (up to day 30) by the chorioallantoic

placentomes, providing optimal diffusing of nutrients and gas exchange between maternal/fetal circulatory system [31]. A cotyledonary epitheliochorial, placenta is formed between 30 to 60 days of gestation in cattle [51]. Towards the end of the second month of gestation several cotyledons are distinguishable in both uterine horns, firm connections between maternal and fetal tissues, and nutrition from a chorioallantoic placental origin [31].

The factors causing pregnancy loss in this period are related to developmental issues, such as improper placentation [52,53], inadequate shifting from amniotic to allantoic nutrition [54], altered vascularization of the placenta [55] and suboptimal development of embryo/fetus [56]. A few risk factors have been linked as increasing the likelihood for pregnancy loss at this period; reduced concentrations of progesterone during follicle growth [13], parity (multiparous present higher pregnancy loss percentage) [29], change in BCS [28], uterine [57,58] and non-uterine diseases, such as mastitis [59]. Also, cows reported with a lack of expression of estrus in AI programs have been reported with a higher percentage of pregnancy loss [60]. It is s believed that this non-estrus behavior may be linked to inadequate levels of estradiol [60]. Independent of the cause, the losses during this period are approximately 12 %, but it ranges between 3 to 26.3 % depending upon farm, synchronization protocol, health status, etc. [5].

In the last pivotal period, dramatic growth in fetal and total membrane mass is noticeable. Fetus weights approximately 10 grams alone at 60 days and up to 166 g at 90 days in gestation [60] and membranes total weight from approximately 70 g to 240 g in the same period [47,61]. Additionally, placentomes increase their volume and vasculature [60]. Limited information is available regarding pregnancy loss in this period. It has been estimated that the losses are approximately 2% [62-65]. The increase in placentome volume and vasculature during the first trimester of pregnancy are critical to support the nutrient uptake demand and fetal growth that will

occur during this gestational period. However, the major contributor to pregnancy loss in this period is twin pregnancy [61] Carrying twins is a non-infectious factor that could compromise the pregnancy maintenance in cows [62-66]. Cows experiencing twin parturition are associated with a higher incidence of dystocia, retained placenta, and calf mortality [67-70], consequently longer calving to conception interval and shorter mean production lifespan of 200 days have been reported for cows delivering twins compared to cows delivering singletons [70-73]. Lopez-Gatius et al. [62], reported 75% of this loss in twins between 68 to 90 days in gestation. In ipsilateral twins, the scenario is even more worrisome, with pregnancy loss being 3.45 times more likely than in bilateral twins [62]. Infectious diseases caused by viral, bacterial and protozoal agents are also responsible for a share of fertility issues, leading to pregnancy losses throughout the whole pregnancy. However, the incidence of pregnancy loss caused by some of those agents is significantly diminished with the implementation of vaccination and preventive measures [74-78].

1.2. Pregnancy diagnosis methods.

Determination of pregnancy status in dairy herds is a routine practice. Sooner the non-pregnant cows are found, a management strategy can be implemented aiming to re-inseminate those animals, diminishing the interval from calving to conception [79-80]. The ideal pregnancy test should have high sensitivity and specificity, at a low cost, simple to perform and present real-time results [81]. Unfortunately, none of the tests available cover all the mentioned requirements.

The easiest and least costly method to individually determine the non-pregnant animal is through observation of estrus return, from 18 to 32 days post-AI. However, a few factors affect this method's efficacy. Estrus detection efficiency in the United States is estimated to be less than 50% [82]. Estrous cycle duration varies widely with a high degree of variability among individual

cows [83]. New techniques and technologies allowed a direct method of pregnancy diagnosis. The direct method consists of the detection of tissue, fluids, or the conceptus itself, either manually via transrectal palpation or using ultrasonography [81]. Efficacy of these methods is affected by operator ability and stage of gestation, however, practitioners with proficiency can achieve high sensitivity and specificity with both methods. [84,85,79].

Transrectal palpation of the uterus to diagnose pregnancy in cattle is the oldest and most common direct method to perform early pregnancy diagnosis in dairy cows [86]. This technique can be performed by detecting the presence of the amniotic vesicle on the gravid uterine horn and /or slipping the chorioallantoic membranes between the thumb and forefinger, on about 30 days of gestation [84]. Considering that pregnancy can be terminated by disrupting the amniotic vesicle [87,88], studies have investigated the extent of iatrogenic pregnancy loss via transrectal palpation. Conflicting results have been reported about the impact of transrectal palpation with few studies reporting higher pregnancy loss in cows after submitted to the technique [89,90], while others reported no effect [91,92]. This technique supplies high accuracy at a low cost per cow. Transrectal palpation is the standard method of choice for many practitioners worldwide [81].

Another common direct method is the use of transrectal ultrasonography. Not only it allows the diagnosis of early pregnancy, but it also provides information about ovarian structures and the determination of sex [79,93]. Additionally, this technique is less invasive when compared to transrectal palpation [94,95]. The use of transrectal ultrasonography allows accurate and rapid results to evaluate the pregnancy status of cows [79,81]. Well-trained personnel can accurately diagnose pregnancy as early as 35 days after insemination using transrectal palpation, but with transrectal ultrasonography pregnancy the range used is 28 to 34 days after insemination, which reduces the interval from insemination to pregnancy diagnosis by a few days [81]. Although

ultrasound conducted at 45 or more days after breeding did not increase the accuracy of pregnancy diagnosis for an experienced palpator, it may improve the diagnostic accuracy of a less experienced one [85]. For instance, treatment of cows without a CL at the first GnRH treatment of an OvSynch protocol with exogenous progesterone, increased fertility in lactating cows [96,97]. Also, the treatment of cows presenting a CL of 20 mm, upon non-pregnant diagnosis, with PGF 2α increased the proportion of cows inseminated after a detected estrus [98].

Many bovine practitioners attempted to perform pregnancy diagnosis earlier than 30 days of gestation using the transrectal ultrasonography, based on a study that pushed this lower date threshold [51]. The investigation was conducted using a high-quality transducer and reported an embryonic heartbeat as early as 21 days in gestation under experimental conditions [51]. Other studies reported correct diagnosis using ultrasound as early as 26 days after AI [99]. However, there is evidence that conducting pregnancy diagnosis using transrectal ultrasonography before 30 days post-AI under field conditions, negatively affect the accuracy of pregnancy diagnosis outcomes [68]. The criteria to assign a cow as pregnant below 30 days post-AI is often based on the uterine fluid presence and aspects, since embryo identification is not always possible on the ultrasound. Giordano and Fricke [81], reported that cows classified pregnant based on uterine fluid alone 29 days after timed AI were 3.8 times more likely to be classified as not pregnant 74 days after timed AI than cows diagnosed pregnant based on visualization of an embryo with a heartbeat.

Transrectal palpation and ultrasonography technology remains as the standard technique of many practitioners worldwide to accurately diagnose cow's reproductive status. However, with the limited availability of skilled practitioners, and the urge for easier and more accurate diagnosis, indirect detection in cattle has become common practice [100-102].

The indirect methods for pregnancy detection consist of the use of hormones or fetal substances in maternal body fluids as indirect indicators of pregnancy [81]. Milk progesterone and blood levels of pregnancy-associated glycoproteins (PAG) are the most common indirect test available commercially. Progesterone is a steroidal hormonal produced by the corpus luteum during the estrous cycle and placenta during pregnancy. On-farm qualitative tests for assessing progesterone levels in milk were commercialized for pregnancy diagnosis in dairy cows in the early 80s [103]. Based on a study, the use of plasma progesterone presented a high accuracy on detecting non-pregnant cows, but not for detecting early pregnant cows [104]. The poor sensitivity (ability to correctly identify pregnant cows) is associated with a few factors. Cows undergoing pregnancy loss can present lower concentrations of progesterone and extended luteal phase [104], in which the maternal recognition of pregnancy occurs, supporting the CL, but later on experience pregnancy loss [80]. In conclusion, the use of a milk progesterone assay should be considered as a tool to monitor non-pregnant cows instead of pregnant ones between 18 to 24 days post-breeding.

The monitoring of progesterone levels was traditionally performed using immunoassays. The demand for a more practical, less costly and more accurate method led to studies involving an automated system to monitor the progesterone in dairy cows' milk at the parlor [105,106]. Despite presenting high accuracy, new techniques such as the use of immune sensor still request a high investment [105]. Others are relatively less costly, such as the apoenzyme reactivation immunological system (ARIS), but showed accuracy issues [105]. The ARIS has an analyte conjugate flavin adenine dinucleotide (FAD), that will bind to the analyte (in this case progesterone) and interact with a glucose oxidase (apoGOx) that will ultimately produce hydrogen peroxide, that can be quantified using HRP and specific chromogen [107]. The response can be determined using a reflectometer and is directly correlated with the progesterone concentration

[107]. However, a limitation of the ARIS is the presence of free FAD in raw bovine milk [108,109]. This free FAD competes with the progesterone for the apoGOx binding, interfering with the HRP reaction, hence compromising the assay efficacy [110]. Thus, a challenge remains to implement these methods into a workable fashion that can be used by farmers in the milking parlor.

1.3.Pregnancy-associated glycoprotein

Placental proteins have been used as an indirect method to diagnose pregnancy for many years in multiple species. Human chorionic gonadotropin was discovered in 1927 [111] and can be assayed in urine or blood as early as 8 to 10 days after conception [112]. One of the first placental origin proteins discovered in animals was the equine chorionic gonadotropin, [113] which became a useful marker of pregnancy diagnosis in this species [113].

In ruminants, a morphologically distinct cell type named binucleate cells can first be identified during the cell-to-cell attachment between the trophoblast and the uterine wall [114]. Sheep and cattle experience the fusion of binucleate cells with uterine epithelial cells, leading to a syncytium [115]. Binucleate cells correspond to 20% of the trophectoderm layer and are constantly being replaced as migration to the uterine epithelium proceeds. These fused cells secrete their granules, via exocytosis, towards the underlying maternal capillary beds, allowing trophoblast cell products to reach the maternal circulation [115]. One of these products secreted by the binucleate cells is the pregnancy-associated glycoprotein (PAG) [116,117] also known as pregnancy-specific protein B [118]. The PAG are members of the family of inactive aspartic proteinases and are produced by the binucleate giant cells [119]. In cattle, the PAG gene family comprises at least 22 transcribed genes and some variants [120]. Their physiological role remains unclear. It has been suggested that PAG may be immunomodulatory, protective for the corpus luteum or both, based

on their ability to induce chemokines, alter neutrophils activity and prostaglandin levels in reproductive tissues [121-123]. The PAG secretion was serologically detected, via radioimmunoassay, in the maternal circulation as early as 15 days post-breeding [124]. The maternal circulation of PAG fluctuates according to the day of gestation [118,124,125]. During the first trimester it reaches its highest concentration approximately 25 days post-breeding and a nadir approximately 60 days [64,118,125]. From week 8 to week 10 of gestation plasma concentration of PAG remain stable, about 10 ng/ml, but on week 12 PAG concentrations present a steady increase until parturition [125]. The peak of plasma PAG is approximately five days to parturition, with concentrations ranging from 588 ng/ml to 2462 ng/ml [118,125]. Plasma concentration of PAG has a steady decrease from 24 – 48h post-partum, reaching undetectable or baseline concentrations between 60 - 100 days postpartum, depending on the assay implemented [64,118,125]. Due to PAG expression into the maternal circulation in ruminants, these markers have been studied throughout the years as an indirect pregnancy diagnosis method.

Several studies were performed to test the effectiveness of ELISA using plasma and milk PAG to diagnose pregnancy in cattle. Fricke et al [81], presented a detailed table having the results among authors that investigated the accuracy of the use of plasma and milk PAG in specific days post-breeding in dairy cows and heifers. The sensitivity and specificity percentage for blood ranged from 81–100 at 22 to 35 post-AI and 57-100 % respectively [100] [101,118,126], and 98-100 and 83-100 % respectively for milk PAG, although only two researches compared for the milk tests [64,127]. Results from the animals tested using plasma PAG ELISA, should be interpreted cautiously. Positive results are uncertain in the first 30-35 days post-breeding, especially for dairy cows, since the antigen concentrations in the maternal circulation are low, and somewhat variable [118,124,128].

Maternal circulation of PAG is influenced by days post-partum [64,124,125], twin pregnancies [129], sire and parity [130], which in turn, all factors, affects the assay precision. Additionally, it was reported that milk production negatively correlates with the maternal circulation of PAG [64,131,132]. The relationship between high milk production and decreased PAG concentrations in the plasma is not fully understood. It is speculated that the increased metabolization rate of progesterone in high-producing dairy cows [133] may reflect in slower-growing embryos during early development, consequently leading to lower maternal circulation of PAG [64].

Another contributor to the assay precision is pregnancy loss. Cows experiencing late embryonic and early fetal loss have lower concentrations of PAG when compared to cows that maintain the pregnancy [104,128,130,132]. It has been speculated that once the maternal–placental interface is disrupted, such as during pregnancy loss, the binucleate cells (the primary source of PAG) migration would cease, preventing protein secretion by these cells from reaching the maternal circulation and leading to a decreased plasma level of PAG [104]. The onset of the reduction of PAG in the event of pregnancy the loss was reported to be rapid (between 1 to 3 days) under experimentally induced pregnancy loss [104,134]. However, the plasma levels of PAG may persist on detectable levels for about seven days after a cow experience pregnancy loss [104,134].

Szenci et al [126], reported that cows facing embryonic mortality between day 29 to 38 post-AI, had a steady decrease in PAG plasma, but it only reached non-pregnant levels up to day 53 to 58 post-AI. Another investigation reported that plasma PAG levels reached nonpregnant levels between 7 to 14 days after pregnancy loss [64]. Under experimentally induced abortions, the plasma concentration of PAG presented an imminent decrease (after cows were treated with PGF 2α intramuscularly and intrauterine hypertonic saline) one day after treatment but remained

detectable for up 7 days after treatment [104]. A threshold of below 1.4 ng/ml for plasma PAG was reported [135], to predict pregnancy loss in cows between days 31 and 59 post-AI with 95 % accuracy. However, a study reported pregnancy loss in cows with plasma levels of PAG above the mentioned threshold, using a similar assay [132]. A recent study in beef cattle was able to establish a threshold to determine the pregnancy in cows (≥ 0.33 ng/mL) and heifers (≥ 0.54 ng/ml) at 24 days post-breeding, however, no statistical difference was found between cows that maintained or lost the pregnancy at the same point [130].

Pregnancy loss is a routine situation faced in cattle operations, with losses ranging up from 3 to 40 % depending on cow type, location and seasonality [1,2,5]. This issue plays an important essential role in the use of PAG as an early pregnancy indicator. Still, limited information is available regarding temporal changes in plasma concentration of PAG in cows identified with a potential conceptus loss.

1.4. References

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CHAPTER 2: CHARACTERIZATION OF PREGNANCY-ASSOCIATED GLYCOPROTEINS AND PROGESTERONE AS A PREDICTOR OF TWINS AND CONCEPTUS LOSS IN HIGH-RISK PREGNANCY HOLSTEIN COWS[†]

2.1.Abstract

The objective of this study was characterizing plasma concentration of pregnancy-associated glycoprotein (PAG) and progesterone as predictors of twins and pregnancy loss in high-risk pregnancy Holstein cows. High-risk pregnancy (HR) were characterized using transrectal ultrasonography 37 days post-AI based on the following criteria: small embryo size embryo < 15 mm, n=10), slow heartbeat (<60 beats per minute, n=11), extra amniotic membrane (additional amniotic membrane, n=3). A cohort of twins (TW, n=41) diagnosed at day 37 post-AI was also enrolled. Each HR and TW cow was paired with a cow of the same parity carrying a normal singleton at d 37 post-AI (CON, n = 65). Blood samples were collected to measure PAG and progesterone at 37, 44, and 51 post-AI. Statistical analysis was performed using ANOVA, logistic regression and receiver operation characteristics (ROC) with JMP. Pregnancy loss at day 51 post-AI was greater (P < 0.01) in HR than CON and TW (CON=1.5%; HR=87.5%; TW=12.2%).

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Concentration of PAG at day 37 post-AI did not differ (P = 0.75) among groups (CON = 5.3 ± 0.7; HR = 4.8 ± 1.2; TW = 4.0 ± 0.9 ng/ml). Concentration of progesterone at day 37 post-AI was greater in TW than HR and CON, and lower (P < 0.01) in HR than CON cows (CON = 7.0 ± 0.3; HR = 5.9 ± 0.4; TW = 8.4 ± 0.3 ng/ml). Regression and ROC analysis for PAG at day 37 post-AI did not find a threshold to predict pregnancy loss (P = 0.24) or twins (P = 0.30). Regression and ROC analysis for progesterone at day 37 post-AI found that a threshold of 6.5 ng/ml predicted (P < 0.01) pregnancy loss with an area under the curve (AUC) of 0.65, and threshold of 7.2 ng/ml predicted (P < 0.01) twins with AUC of 0.70. In summary, pregnancy loss and twins were predicted with only moderate accuracy by progesterone concentration at day 37 post-AI and the variability in PAG concentrations at day 37 post-AI was not sufficient to generate a threshold to predict pregnancy loss and twins in Holstein lactating cows.

Keywords: Pregnancy loss, pregnancy-associated glycoprotein, progesterone, high-risk pregnancy, twins

2.2.Introduction

Suboptimal fertility is a multifaceted issue [1] that negatively impacts the profitability and sustainability of dairy herds [2,3]. Pregnancy loss is a major contributor to compromised reproductive performance, with an average cost per case ranging from U$555 to 640 per case [4,5]. It has been reported that most of the pregnancy losses in dairy cows occur in the first trimester with approximately 12% occurring between 28 and 60 days of gestation [6]. Identification of cows losing the pregnancy as early as possible can be helpful providing timely opportunities for rebreeding minimizing reproductive losses [3,4]. The transrectal palpation technique emerged in

the last century and remained the most common method to diagnose pregnancy in cattle [7,8]. The procedure allows immediate results, no investment in equipment or laboratory facilities, and presents accurate outcomes when performed after 35 days post-breeding [7,8]. The earlier diagnosis was feasible when the transrectal ultrasonography technology was implemented in the 1980s [9,10]. The pregnancy diagnosis with the aid of ultrasound can be performed as early as 25 days after AI [11] and allows identification of suggestive signs of high-risk pregnancy, such as reduced fetal heart rate, abnormal conformation, and amniotic membrane integrity [9,12]. Additionally, transrectal ultrasonography allows the diagnosis of twins' pregnancy that are three times more likely to be lost than singletons. Despite its benefits, ultrasonography requires well-trained personnel and incurs additional costs with equipment purchase and maintenance [13]. A third alternative that became available was the use of biochemical markers in plasma. The most common biomarker used as a pregnancy test in cows is pregnancy-associated glycoproteins (PAG). The PAG are members of the family of inactive aspartic proteinases produced by the binucleate giant cells of the placenta in ruminants [14,15]. Studies reported PAG secretions in cattle maternal circulation as early as 15 days after conception [16,17], but the highest sensitivity and specificity to use PAG for pregnancy diagnosis occur between 26 to 32 days post-AI [16 -21]. It has been reported that plasma PAG concentrations present a high sensitivity, between 94-100% [20-24], but some authors registered specificity as low as 83% when compared to ultrasound in dairy cows between 28 to 32 days after AI [24]. Studies also revealed that dairy cows experiencing early pregnancy loss have lower concentrations of circulating PAG [25,26]. Pohler et al. [19] reported a threshold of 1.4 ng/ml for plasma PAG, to predict pregnancy loss in cows between days 31 and 59 post-AI. However, pregnancy loss was still reported in dairy cows presenting plasma levels of PAG above 1.4 ng/ml, using similar assay and days post-AI [26]. The maternal circulation of PAG

is also influenced by cows bearing twins since it presents a greater concentration of this marker compared to singletons [27]. A threshold to predict twins using PAG is not well established and configures necessary since cows bearing twins are at higher risk of suffering pregnancy loss compared to singletons, especially in unilateral twins [28]. Progesterone is another biomarker used to diagnose pregnancy in cattle at 18-24 days post-breeding [29,30]. As a point in time test, measuring progesterone is not a viable test for pregnancy because sensitivity is only 75% [31,32]. Serial, automated testing in advanced parlor systems improves the accuracy of progesterone as an indicator of pregnancy [33]. Additionally, progesterone can be used to predict pregnancy loss in dairy cows. There is a positive relationship between plasma progesterone concentration and maintenance of pregnancy at week five of gestation [34]. The biomarkers could be a useful tool to assess the likelihood of a cow to experience pregnancy loss and advance opportunity for resynchronization. Additionally, pregnancy diagnoses have been traditionally performed via transrectal ultrasonography, but based on a demand for an easier, last costly and more accurate test, indirect methods are being investigated throughout the years [17-20,26] as an alternative technique. However, establishing a precise threshold for PAG and progesterone to predict pregnancy loss and twins is challenging, since those markers can present a broad range of concentration in the plasma [23-28]. Perhaps, the relationship between biomarkers and pregnancy loss can be evaluated with more accuracy by enrolling cows identified on ultrasound carrying a high-risk and twin pregnancy. In this manner, the interval from enrollment to pregnancy loss may be shortened, allowing the simultaneous assessment of PAG and progesterone closely before and after pregnancy loss, which could provide more efficacy to generate a threshold. Therefore, the objective of this study was characterizing the plasma temporal concentrations of PAG in Holstein dairy cows presenting high-risk pregnancy and determine a threshold value of PAG and

progesterone that can be used as a predictor of twins and pregnancy loss. We hypothesized that the plasma concentration of PAG and progesterone is reduced and can predict pregnancy loss in cows experiencing a high-risk pregnancy. We also hypothesized that the concentration of PAG and progesterone are increased and can predict cows carrying twins.

2.3.Material and methods

2.3.1.Animals and husbandry

The study was conducted between October 2018 and November 2019, in a commercial dairy farm Illinois. The herd contained approximately 3,300 lactating Holstein cows, milked three times daily, with an average milk yield of 48.30 ± 5.87 kg/cow/day. Cows were housed in free-stall barns with feedline headlocks, fed a TMR ad libitum, formulated for high-producing dairy cows. A modified Double-OvSynch was used as an estrus synchronization protocol for the first service. Cows enrolled in the study received their first service timed AI at 66 ± 3 DIM. Briefly, the Double-OvSynch protocol began with a modified Ovsynch (Pre-Ovsynch: GnRH - 7 d - $PGF_{2\alpha}$ -3 d- GnRH) to pre-synchronize the estrous cycle followed 7 d later with a CoSynch 72, GnRH- 7 d - $PGF_{2\alpha}$ – 72 h GnRH with concurrent timed AI and an additional AI 24h later. Pregnancy diagnosis was performed 37 days post-AI using a portable ultrasound scanner (Easi-Scan, BCF Technology Ltd., Livingston, UK). All animals were enrolled in the resynchronization program at day 33 post-AI receiving GnRH. Then cows diagnosed open at day 37 post-AI cows received the other steps of resynchronization that included $PGF_{2\alpha}$ at day 40 post previous AI and GnRH concurrent new insemination at 42 after the previous AI. The injections for both GnRH (2 ml containing 50 µg of gonadorelin hydrochloride per ml; Factrel, Zoetis Inc.) and $PGF_{2\alpha}$ (2 ml containing 12.5 mg of dinoprost tromethamine per ml; Lutalyse HighCon, Zoetis Inc., Madison NJ) were given intramuscularly.

2.3.2.Study design

Cows were enrolled in the study based on three group categories as follow: high-risk pregnancy (HR n= 24), twins (TW n = 41) and control (CON n =65), according to pregnancy diagnosis at day 37 post-AI, via transrectal ultrasonography. Among HR, TW and CON, a total of 130 cows (n = 48 primiparous and n = 82 multiparous) were enrolled (Fig. 1). Cows from HR and TW were paired with herd mates (CON) from the same pen, at the same day of pregnancy diagnosis, and same parity that had a viable pregnancy (singleton, embryo size more than 15 mm and heartbeat more than 60 bpm) (Fig. 2). The HR cows were enrolled based on three subgroup criteria: small embryo size (n = 10), largest width of amniotic vesicle less than 15 mm of diameter (Fig. 3A); slow heartbeat (n =1 1), during ultrasonography the embryo's heart rate were less than 60 beats per minute; extra membrane (n =3), uterine horn identified with an amniotic vesicle without embryo despite a viable pregnancy in the same or different horn (Fig. 3B). Cows enrolled in TW were divided into two subgroups: bilateral twins (n = 24), characterized by the presence of one viable embryo in each uterine horn (Fig. 4); and unilateral twins (n =17): two embryos identified at the same uterine horn (Fig. 5). Cows had their embryo sizes recorded and a blood sample collected at enrollment. and then again on 44 and 51days post-AI. Pregnancy loss (Fig. 6) was denoted when the fetus was absent or dead (had no heartbeat). In twins, pregnancy loss was only reported when both fetuses were non-viable. If only one viable embryo was present in cows previously diagnosed with twins, the animal was reported as fetus number reduction. Plasma PAG concentrations of HR and TW, were compared to CON at day 37, 44 and 51. The interaction with pregnancy loss on day 51 was also evaluated. Additionally, cows that eventually lose their pregnancy post 51 days were reported by the farm personnel.

2.3.3. Determination of plasma progesterone and pregnancy-associated glycoprotein concentrations.

Blood samples from all cow were collected via venipuncture from the coccygeal vein using evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K2 EDTA for plasma separation. Samples were placed immediately on ice and kept refrigerated until arrival to the laboratory. Blood tubes were centrifuged at 3000 x g for 15 min for plasma separation. Two aliquots of 2 ml of plasma were transferred into polypropylene vials and stored at - 20^{o} C until further analyses. Progesterone concentrations were analyzed using a chemiluminescence assay (Immulite 2000 XPi platform; Siemens Medical Solutions USA, Inc.). Serum concentrations of PAG-I were measured using a sandwich ELISA similar to that described by Green et al. [16] and modified using a polyclonal antibody (Ab 63) as previously described by Reese et al. [35,36]. The intraassay coefficient of variation for the progesterone assay was 2.44 %, whereas the intraassay coefficient of variation for PAG was 10%.

2.3.4.Statistical analysis

All responses were analyzed using JMP®, Version 14.2.0 (SAS Institute Inc., Cary, NC). Categorical data such as the proportion of pregnancy loss from days 37 to 44 and from day 37 to 51 were conducted using logistic regression considering a binary distribution. The model included the effect of group, parity, and their interaction. Continuous data such as the concentration of PAG and progesterone were evaluated using ANOVA with models including the effect of group, parity, time and their interactions. The PAG concentration by the pregnancy loss criteria was assessed via linear regression, including their time interaction. Linear regression and receiver operating characteristics were used to generate a threshold to predict pregnancy loss at day 51 and twins based on PAG and progesterone plasma concentration. A significant difference was considered

when $P < 0.05$, whereas differences between $P \geq 0.05$ and $P \leq 0.10$ were considered a statistical tendency.

2.4.Results

2.4.1.Descriptive data

Average milk yield, number of services and days in milk (DIM) at enrollment, are presented in Table 1. No statistical difference was revealed in milk yield among groups. Cows that experienced pregnancy loss at day 51 were compared to cows that maintained the pregnancy ($P = 0.28$). Retained placenta, stillbirth and mastitis cases among all animals in the study are also presented in Table 1. Twin group had a greater number of mastitis cases compared to the other groups ($P < 0.05$).

2.4.2.Pregnancy Loss

A total of 24 cows were diagnosed as HR and 41 cows were diagnosed as TW at day 37 post-AI. The overall percentage of pregnancy loss during the data points in the study for all groups and subgroups is represented in Table 2. The HR group had greater pregnancy loss ($P < 0.01$) than CON and TW (Table 2). Subgroups pregnancy loss are presented in (Table 3). Cows carrying small embryos and extra membrane had 100% of pregnancy loss, whereas slow heartbeat (8/11) 72.2%. Bilateral and Unilateral twins had 8 and 12 % of pregnancy loss respectively. Pregnancy reduction was observed in approximately 29 % of cows carrying twins (12/41) until day 51. Only four cows experienced fetal loss between the last data collection and the end of the first trimester.

2.4.3.Plasma concentration of pregnancy-associated glycoproteins

Primiparous cows had higher ($P < 0.01$) plasma concentration of PAG than multiparous cows (4.72 ± 0.45 and 2.83 ± 0.36, respectively). The concentration of PAG was not different

when pregnant cows were compared to non-pregnant cows (Fig. 7) at day 44 post-AI ($P = 0.41$) but were higher ($P < 0.05$) in pregnant than non-pregnant cows at day 51 post-AI (Fig. 8). However, it did not differ among groups at any day of gestation (Fig. 9). No difference was found among groups regarding PAG plasma concentrations ($P = 0.18$) and their interaction with time ($P = 0.79$). In order to evaluate the effectiveness of using plasma PAG at d 37 to predict pregnancy loss and twins at d 51, regression and ROC analysis were conducted (Fig. 10). The tests were unable to generate a threshold to predict pregnancy loss ($P = 0.24$) or twins ($P = 0.30$) using plasma PAG. A comparison between the subgroup categories and their respective controls (Fig. 11, Fig 12), for each data collection day, were presented for PAG. The plasma levels of PAG between small embryo subgroup and their controls at d 51 post-AI were different ($P < 0.05$). Extra membrane subgroups tended to present lower plasma concentration of PAG than control ($P < 0.1$). Plasma concentration of PAG was also compared regarding the pregnancy loss criteria (Fig. 13) and their time interaction for the high-risk subgroups, but no statistical difference was between subgroups and controls (P = 0.14)

2.4.4.Plasma concentration of progesterone

The plasma concentration of progesterone ($P < 0.01$) was higher in TW than CON and HR (Fig. 14). In HR cows, progesterone was lower ($P<0.01$) than in CON cows (Fig. 14). Regression and ROC analysis were also conducted to assess plasma progesterone effectiveness at d 37 to predict pregnancy loss and twins at d 51. A threshold of 6.5 ng/ml predicted ($P < 0.01$) pregnancy loss with an area under the curve (AUC) of 0.65, and a threshold of 7.2 ng/ml predicted ($P < 0.01$) twins with AUC of 0.70 using progesterone as a predictor (Fig. 15). Additionally, the progesterone plasma levels differed among all subgroups and their respective controls ($P < 0.05$). The combination of PAG and progesterone concentrations at day 37 post-AI did not generate a

threshold to predict loss at day 51 (P =0.47). However, the use of PAG and progesterone at day 44 post-AI generated a threshold, 3.70 and 6.60 ng/ml respectively for PAG and progesterone, to predict pregnancy loss at day 51 post-AI (P < 0.01) with AUC of 0.78 (Fig. 18). The statistical analysis to predict twin pregnancy using the combination PAG and P4 concentrations at day 37 (P = 0.70) and 44 post-AI (P = 0.62) did not generate a threshold to predict twins at day 51 post-AI.

2.5.Discussion

The present study was the first designed to identify cows carrying a high-risk pregnancy via ultrasonography and attempt to establish a threshold to predict conceptus loss and twins using plasma concentrations of PAG and progesterone.

Approximately 20% of the cows enrolled in this study experienced pregnancy loss, 87.5 % of those were enrolled in the HR group and 12.2% in the TW group. Traditionally, the embryonic viability is monitored via ultrasonography mostly based on the presence of a heartbeat [12,22,37]. However, in the current study, the adoption of criteria such as extra membrane and small embryo also revealed that 100% of animals experienced subsequent pregnancy loss, whereas slow heartbeat 72.2%. The percentage of pregnancy loss in twins was similar to the results reported by Silva-del-Río et al. [38]. The current study design allowed a comparison between the secretion pattern of progesterone and PAG before, during and after the occurrence of pregnancy loss. This timeline relationship involving PAG, progesterone and pregnancy loss could be the key contributor to establish a threshold to predict in high-risk and twin gestations.

A statistical difference between pregnant and non-pregnant cows at day 51 was found for the plasma concentration of PAG which agrees with previous reports [19,26]. No statistical difference was observed between HR in comparison to CON (P = 0.44). Also, the regression and ROC analysis were not able to generate a threshold to predict pregnancy loss using plasma PAG.

A Lack of statistical difference among groups might be associated with two factors. First, a broad range of plasma concentrations of PAG has been reported in cattle [16-22,39]. In the present study, pregnant cows at day 51 ranged from 0.52 – 25.07 ng/ml and the non-pregnant ones 0.01 to 13.23 ng/ml at day 37 post-AI. Since there was an overlap between groups, it made difficult to use PAG as a predictor for pregnancy loss. Second, plasma concertation of PAG peaks between 29 to 32 days of gestation; however, a steady reduction is noticed between 32 to 50 days [16,19,27,40], which comprises approximately the days in gestation investigated in this study. Therefore, this secretion pattern might make identification of differences in plasma concentration of PAG in cows with impending pregnancy loss difficult to discern.

The maternal circulation of PAG was greater in CON compared to TW, contrary to our hypothesis that cows carrying twins would present greater circulation of this marker compared to herd mates bearing singletons. Although, no statistical difference was found between TW and CON regarding plasma levels of PAG (P = 0.75). Conflicting results were found on previous reports comparing cows bearing twins versus singletons. One study reported no statistical difference between 35 to 49 days of gestation [27], whereas another study reported it at the same time point [41]. A few design differences may play a part in these differences. First, in the present study 12.2% of cows carrying twins experienced pregnancy loss and 41 % pregnancy reduction between 37 to 51 days of gestation, whereas in the other studies that information is not available. Second, milk production is reported to negatively impact the plasma levels of PAG [36], the herds in those studies had a lower milk yield compared to the herds in the current study, of about 8-10 kg per cow [27,41]. Therefore, it is reasonable to assume that part of these conflicting results among studies involving the maternal circulation of PAG in twins, could be associated with embryonic/fetal death or reduction, milk yield, seasonality and management. Although, limited

information is available about PAG in cows bearing twins and the exact reason behind these differences still unknown. Twin pregnancies also presented a wide range of PAG, 0.52 to 18.07 ng/ml at day 37 which may also negatively affect the ROC analysis to generate a threshold to predict twins.

The subgroup's plasma levels of PAG revealed a statistical difference for the small embryo and a tendency in the extra membrane when compared to their respective controls on day 51. As expected, those subgroups suffered most pregnancy losses between day 37 and 44, but their plasma levels of PAG statistically differed only at day 51 of gestation. The gradual reduction in plasma PAG found in our study resembles the temporal changes reported under experimentally induced abortions, using intramuscular $PGF_{2\alpha}$ and intrauterine infusion of hypertonic saline [40]. Both treatments registered a steady decrease in PAG concentration at one-day post-injections, but only at about 8 to 9 days post-treatment, this marker reached baseline levels in the cow's blood [40]. Another study investigated the PAG secretion pattern after inducing intrauterine infection, injecting a solution containing *Actinomyces pyogenes* [42]. The author reported that PAG plasma concentrations presented a half-life of about seven days post-treatment until reaching non-detectable concentrations [42]. Regardless of the treatment, it caused deleterious effects on the fetal membranes. In our study, pregnancy was ceased without any intervention, but the precise mechanism that impairs the binucleate giant cells, leading to the reduction in plasma concentration of PAG, is not clear. It has been suggested that a maternal-placental disruption could cease the binucleate cell migration, preventing proteins secreted by these cells from reaching the maternal circulation, hence decreasing plasma levels of PAG [40]. The subgroup slow heartbeat showed a lower concentration of plasma PAG compared to the CON but was the only subgroup within the HR group that did not reveal a statistical difference when compared to the respective CON. Cows

that lost the pregnancy were reported based on two ultrasonography evidence: conceptus absence or death, which could reflect on the concentration of PAG. Most cows carrying small embryos were reported as non-pregnant by the absence of the conceptus. Four of the cows carrying slow heartbeat had a dead conceptus (so as extraembryonic tissues) within the uterus and were also classified as non-pregnant. Perhaps the maternal-placental disruption model suggested [40], happened slowly and/or in less extent in the slow heartbeat subgroup compared to small embryos, explaining the non-statistical difference for the PAG plasma levels compared to CON. Although, the plasma concentration of PAG showed no difference when pregnancy loss per fetal absence or fetal death were compared ($P = 0.14$). This distinct secretion pattern among subgroups and the fact that 28 % of slow heartbeat cows maintained the pregnancy, could also be involved in the lack of statistical difference between HR and CON groups.

Progesterone is a key hormone to maintain pregnancy in ruminants [34]. Circulating progesterone is involved in regulating the uterine environment and histotroph secretion [43-45]. Additionally, progesterone is partially involved in the down-regulation of the complex maternal immune system, preventing immune cells from identifying the embryo as a semi allograft [46,47]. It's been reported that cows with low plasma concentrations of progesterone between late embryonic/early fetal stage have greater odds of experiencing pregnancy loss [34,48-50]. In the present study, the plasma concentration of progesterone was higher in cows that maintained the pregnancy ($P < 0.05$). The CON had a greater concentration of progesterone compared to HR and their interaction group by day also differed ($P < 0.05$). These findings agree with our hypothesis that HR cows would present a lower concentration of progesterone than CON. The lower concentration of progesterone in the HR group compared to CON is perhaps associated with a conceptus death and subsequent luteolysis of the corpus luteum regression led to the conceptus

death, terminating the pregnancy [40]. A threshold to predict pregnancy loss using progesterone was established at 6.5 ng/ml with 65% accuracy. Another study also investigated the circulation levels of progesterone in cows experiencing pregnancy loss and a threshold of < 3.76 ng/ml of progesterone was associated with greater odds of losing the pregnancy, but about 80 % of the cows in this lower concentration category were still pregnant [34]. Thus, more investigation is necessary to find a more accurate cutoff point to predict conceptus loss. Twin pregnancies had a greater concentration of progesterone compared to singletons, which is in agreement with a previous report [27]. Additionally, a plasma concentration to predict twins using progesterone found a threshold of 7.2 ng/ml, although with only moderate accuracy of 70%. The use of both PAG and progesterone concentrations at day 44 was able to generate a threshold to predict pregnancy loss ($P < 0.01$), however with moderate accuracy (AUC = 0.78). Limited literature is available regarding plasma levels of progesterone in twins, so more studies are necessary to find a more accurate threshold to predict twins.

In conclusion, this data suggests that cows experiencing pregnancy loss have lower concentrations of PAG, but the wide range of PAG concentrations did not allow the identification of pregnancy in high-risk cows nor twin pregnancy. Progesterone was able to predict pregnancy loss and twins with only moderate accuracy indicating more research is necessary to determine its importance as a biochemical marker to predict pregnancy loss and twins.

Acknowledgments

The study was funded by the Department of Veterinary Clinical Medicine. Stoneridge dairy farm personnel is acknowledged for their help during data collection.

Disclosure Statement

None.

2.6. Figures and tables

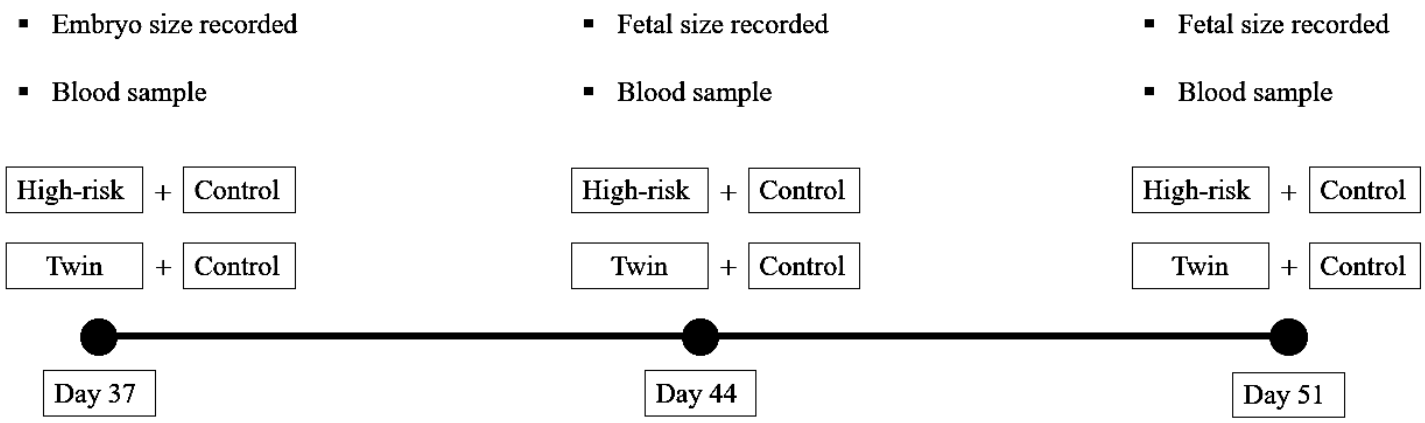

Fig. 1. Representation of the study design. Cows identified bearing a high-risk pregnancy or twins were enrolled in the study at day 37. A control cow from the same pen, parity and pregnancy diagnosis day was immediately paired with the cow carrying a high risk or twin pregnancy. Conceptus size was recorded, and a blood sample collected at day 37, 44 and 51 post-AI.

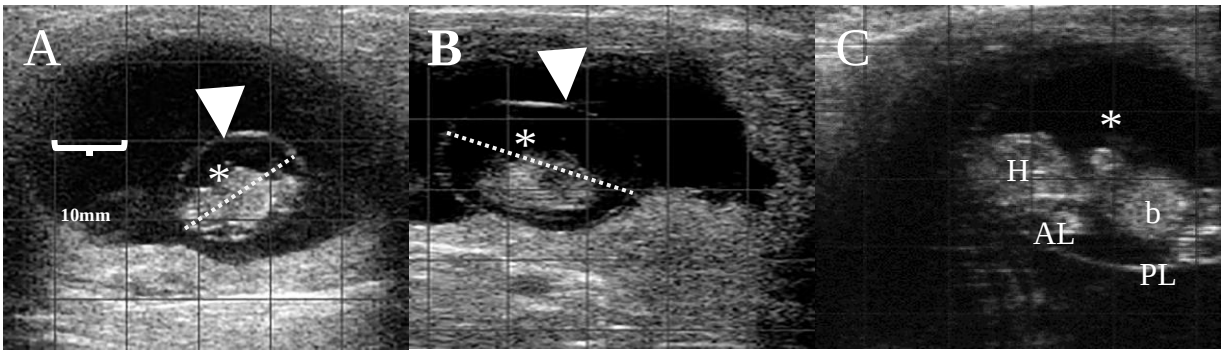

Fig. 2. Representative ultrasonography images of the uterus of Holstein cows carrying a normal singleton pregnancy at days (A) 37, (B) 44, (C) 51 post insemination. (*) Denotes the conceptus inside the amniotic vesicle (white arrowhead). On (C) the fetus head is denoted by (H), the anterior and posterior limbs (AL and PL respectively) and body (b). The dotted line across the widest portion of the amniotic vesicle crosses the fetus.

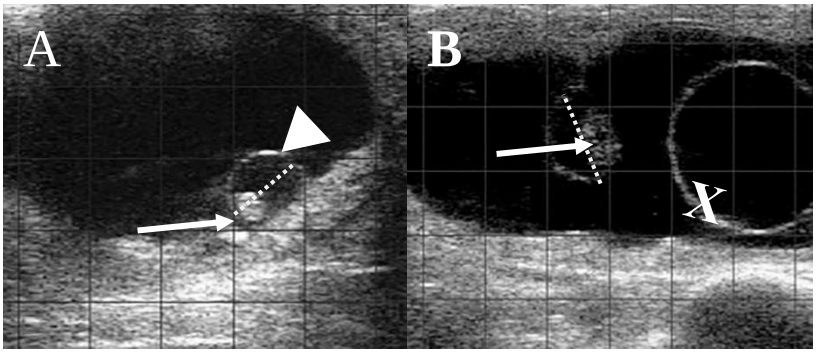

Fig. 3. Representative ultrasonography images of the uterus of Holstein cows diagnosed with a (A) small embryo size (less than 15 mm) at 37 days. (B) A normal pregnancy at 37 days with an extra amniotic membrane identified without a viable embryo. The arrow is indicating the embryo and the arrowhead the amniotic vesicle (A), while the (x) denotes the empty amniotic vesicle. The dotted line across the widest portion of the amniotic vesicle crosses containing the embryo.

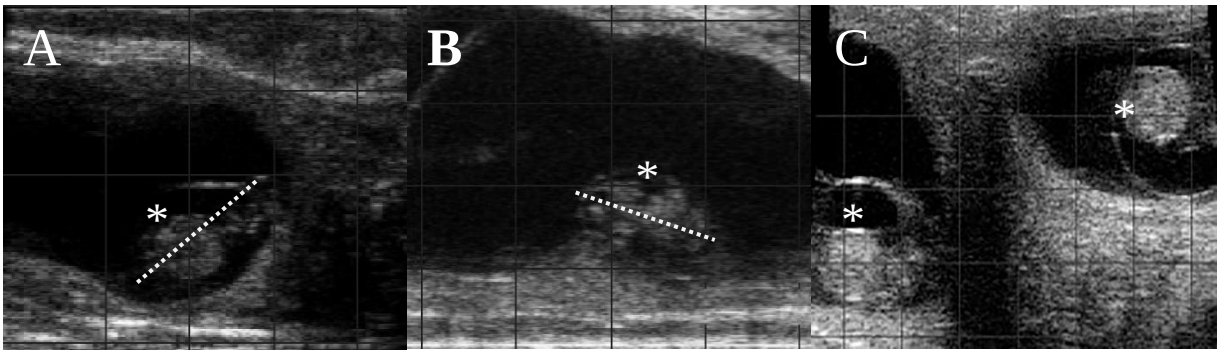

Fig. 4. Ultrasonography image of a Holstein cow uterus carrying a bilateral twin pregnancy at 37 days (A, B) and 44 days (C). Denotes the embryo (*). The dotted line across the widest portion of the amniotic vesicle crosses the conceptus.

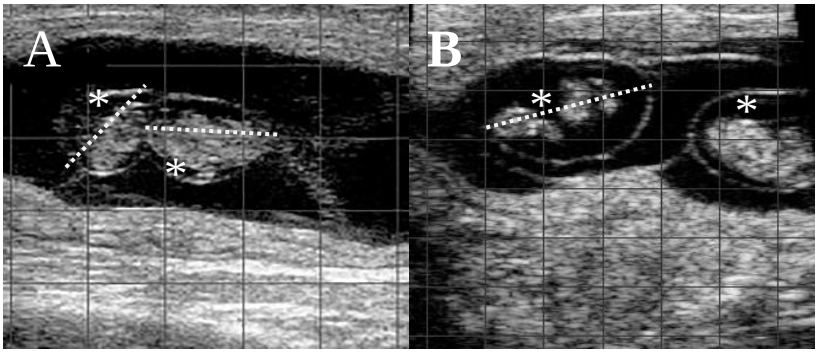

Fig. 5. Ultrasonography image of a Holstein cow uterus carrying a unilateral twin pregnancy at (A) 37 and at (B) 44 days. (*) Denotes an embryo. The dotted line across the widest portion of the amniotic vesicle containing the fetus.

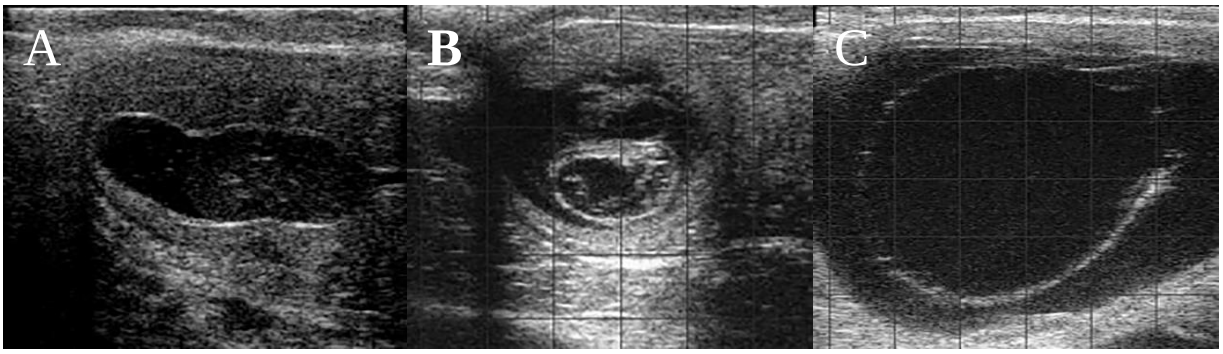

Fig. 6. Representative image of cows experiencing pregnancy loss at (A) 37, (B) 44, (C) 51 days respectively. Excessive echogenicity on the uterine fluids (A), floating structures (A) (B), compromised membranes (B), empty amniotic membrane (C) were clinical signs of pregnancy loss reported with ultrasound.

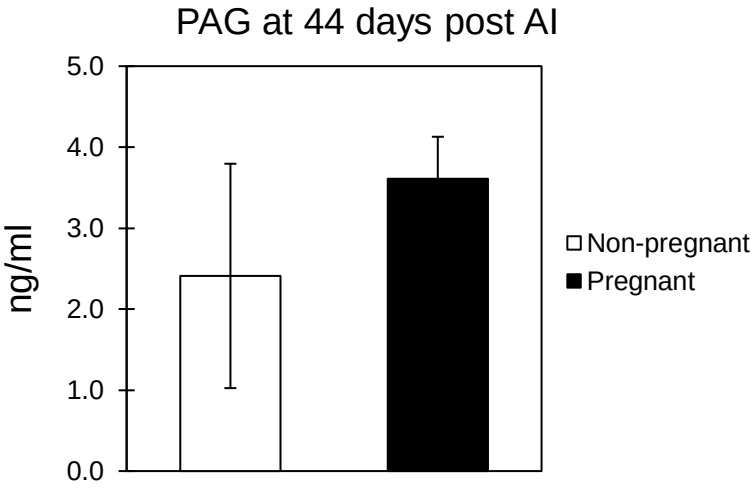

Fig. 7. Plasma concentration of PAG in pregnant and non-pregnant cows. Mean ± SE plasma PAG-I in cows reported as pregnant and non-pregnant via transrectal ultrasonography at day 44 post-AI. No statistical difference was present between pregnant and non-pregnant cows ($P = 0.41$).

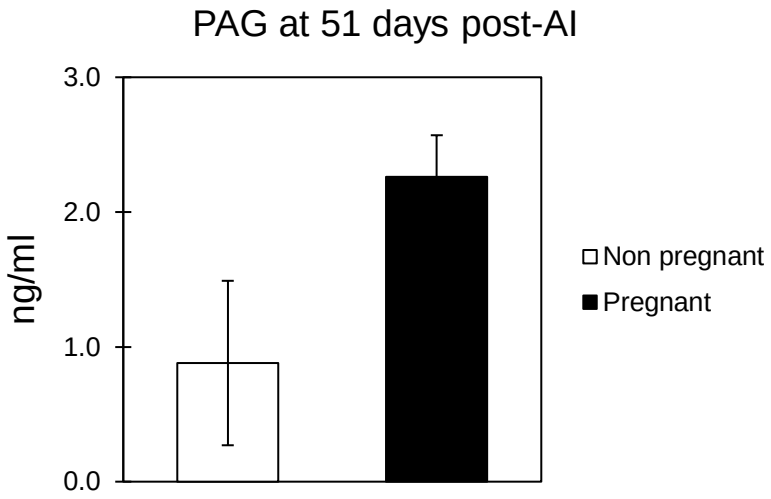

Fig. 8. Plasma concentration of PAG in pregnant and non-pregnant cows. Mean ± SE plasma PAG-I in cows reported as pregnant and non-pregnant via transrectal ultrasonography at day 51 post-AI. Cows deemed as bearing a conceptus presented statistically lower concentration of PAG ($P < 0.05$) at this specific data point.

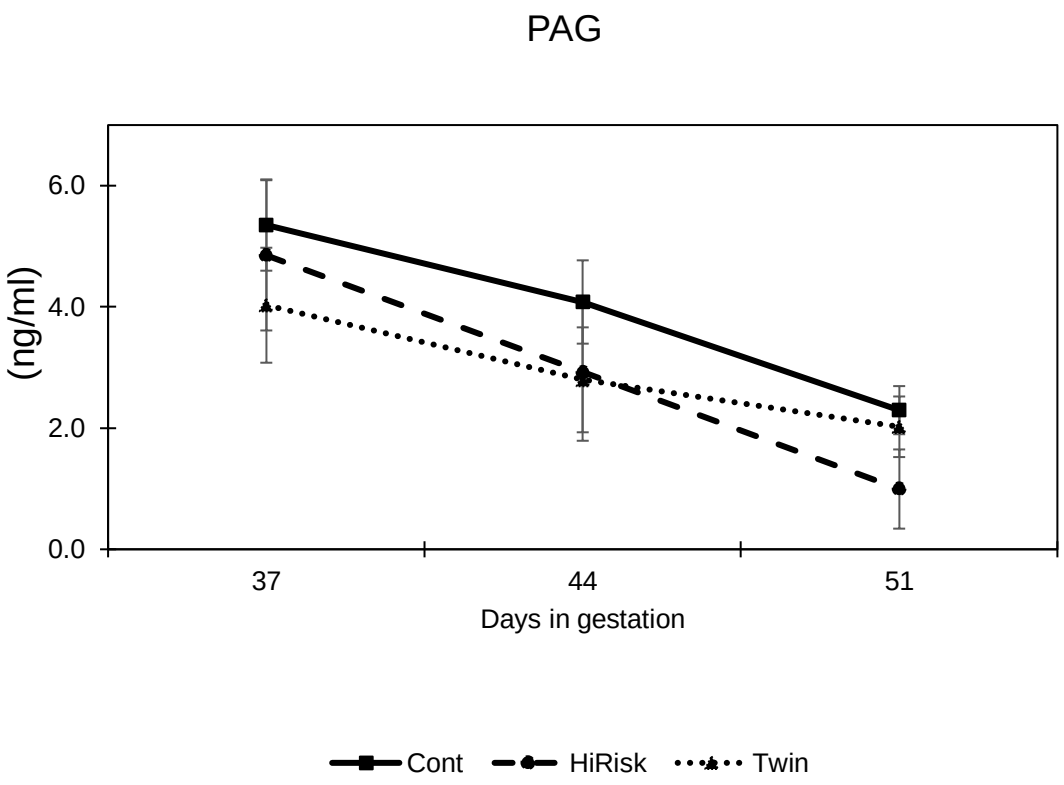

Fig. 9. Plasma concentration of PAG among groups at day 37, 44 and 51 post-AI. No statistical difference was observed among groups ($P = 0.18$) and their time interaction ($P = 0.79$). The overall mean ± S.D for control, high-risk and twin pregnancy was 3.90 ± 0.37 vs. 2.92 ± 0.61 vs. 2.98 ± 0.46 ng/ml, respectively.

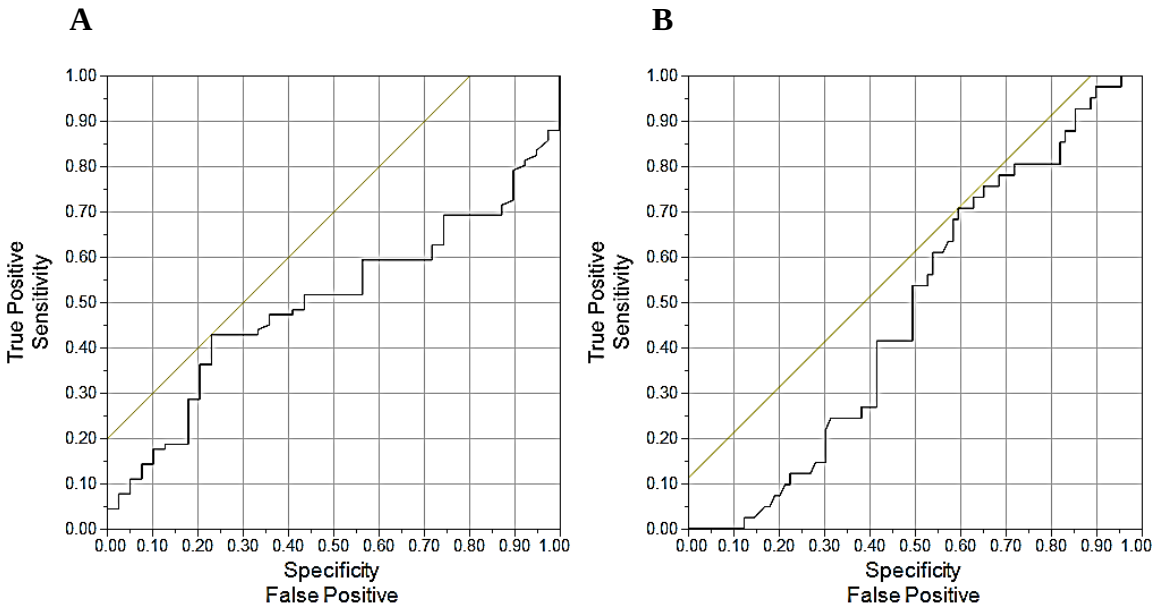

Fig.10. Day 37 post-AI ROC curve to predict pregnancy loss and twins at day 51 post-AI using PAG. Receiver operating characteristics (ROC) was used to predict pregnancy loss (A) and twins (B) at day 51 using values of PAG at 37 d post-AI. Threshold for PAG to predict pregnancy loss (P = 0.24) and twins (P = 0.30) were not generated.

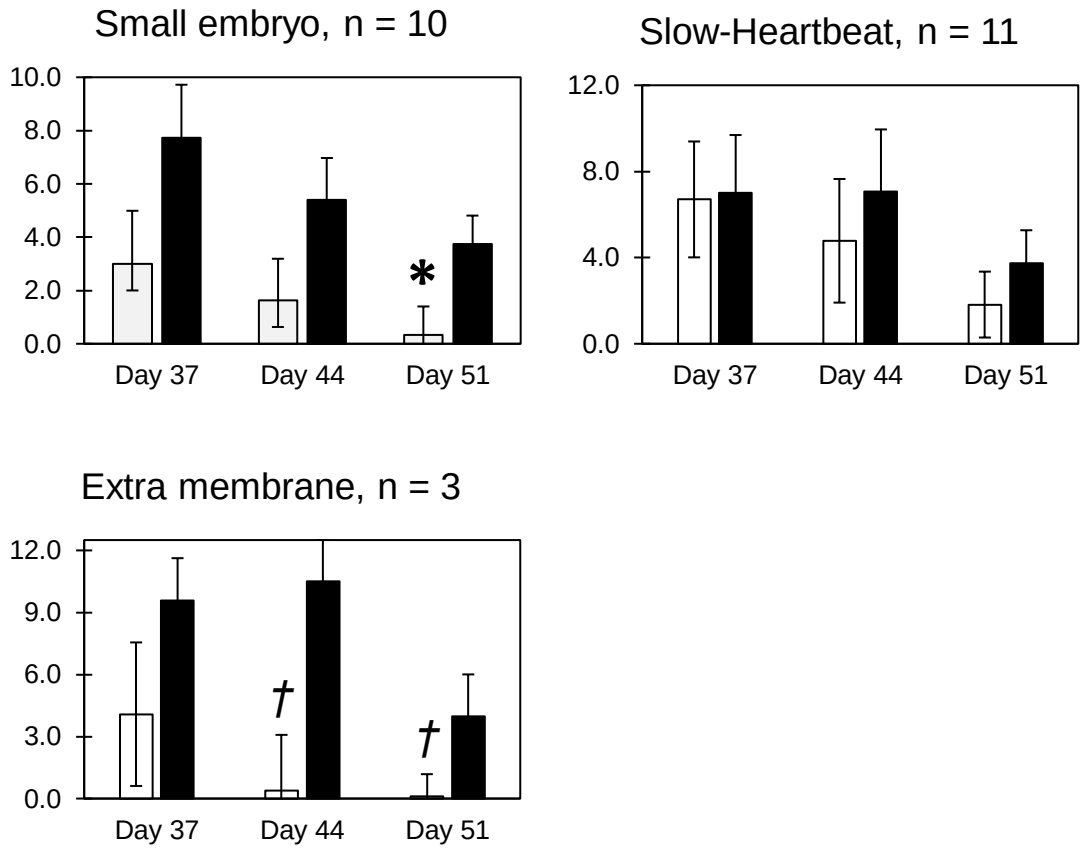

Fig.11. Subgroup (small embryo, slow- heartbeat and extramembrane) comparison with controls regarding plasma concentrations of PAG in ng/ml. A statistical difference and tendency were found for cows carrying small embryos (*$P < 0.05$) and extra membrane (†$P < 0.1$) pregnancies respectively, at day 51 post-AI compared to control. Additionally, a tendency was also observed at day 44 post-AI for the extra membrane subgroup. No statistical difference was found between slow-heartbeat compared to control (P = 0.44) and their time interaction (P = 0.93)

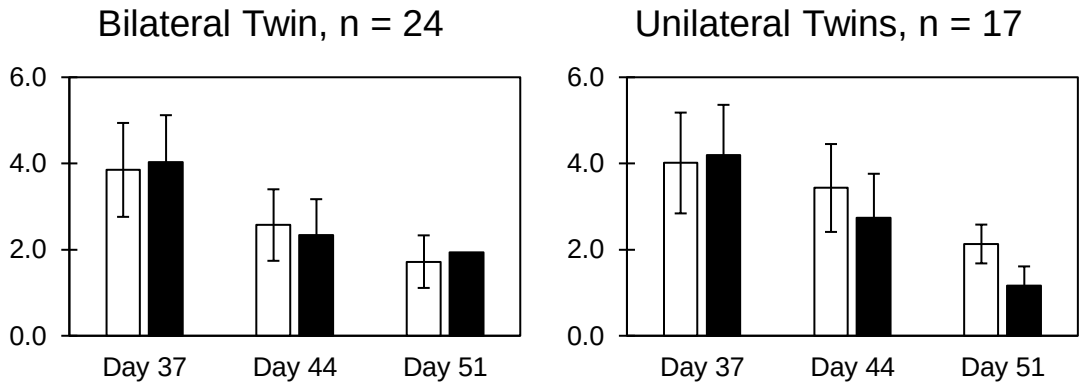

Fig. 12. Subgroup (bilateral and unilateral twins) comparison with controls regarding plasma concentrations of PAG in ng/ml. No statistical difference was found among unilateral (P = 0.61) and bilateral (P = 0.95) compared to controls. The subgroup time interaction, respectively for unilateral (P = 0.78) and bilateral (P = 0.96) was not statistically significant.

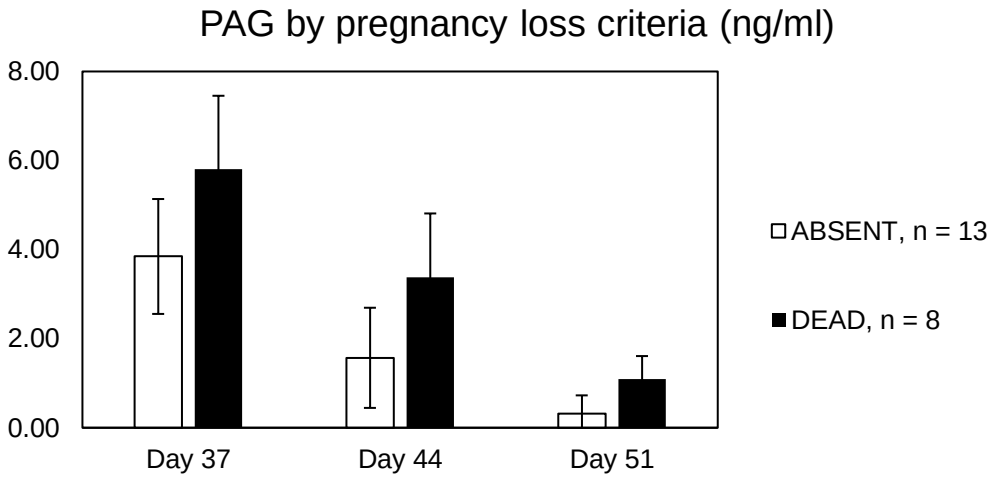

Fig. 13. Plasma concentration of PAG regarding the pregnancy loss criteria (fetal absence, n = 13 and fetal death, n = 8), for the high-risk subgroups (small embryo, slow-heartbeat and extra membrane). No statistical difference was found among the two criteria (P = 0.14) and their time interaction (P = 0.85). The overall mean ± S.D for fetal absence and death were 1.91 ± 0.62 vs. 3.42 ± 0.79 ng/ml, respectively.

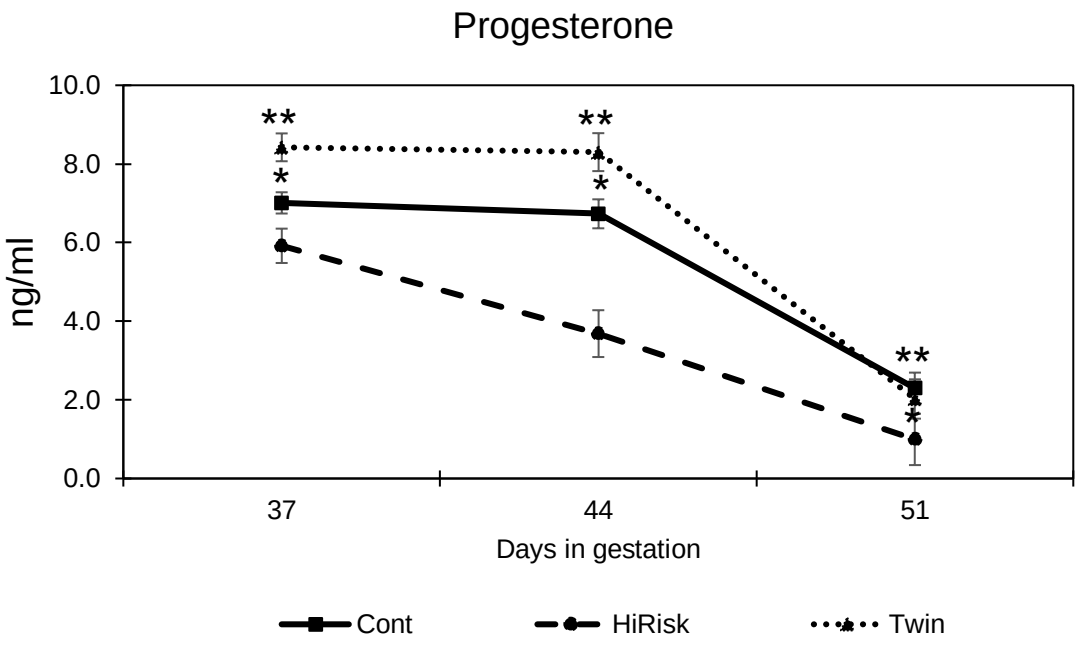

Fig. 14. Plasma concentration of progesterone among groups at days 37, 44 and 51 post-AI. A statistical difference was present among groups ($P < 0.01$). Additionally, the group and time interaction was greater ($P < 0.01$) in TW (**) than CON and HR. In HR cows, progesterone was lower ($P < 0.01$) than in CON (*) cows. in twins. The overall mean ± S.D for control, high-risk and twin pregnancy was 6.81 ± 0.20 vs. 8.13 ± 0.26 vs. 3.96 ± 0.32 ng/ml, respectively.

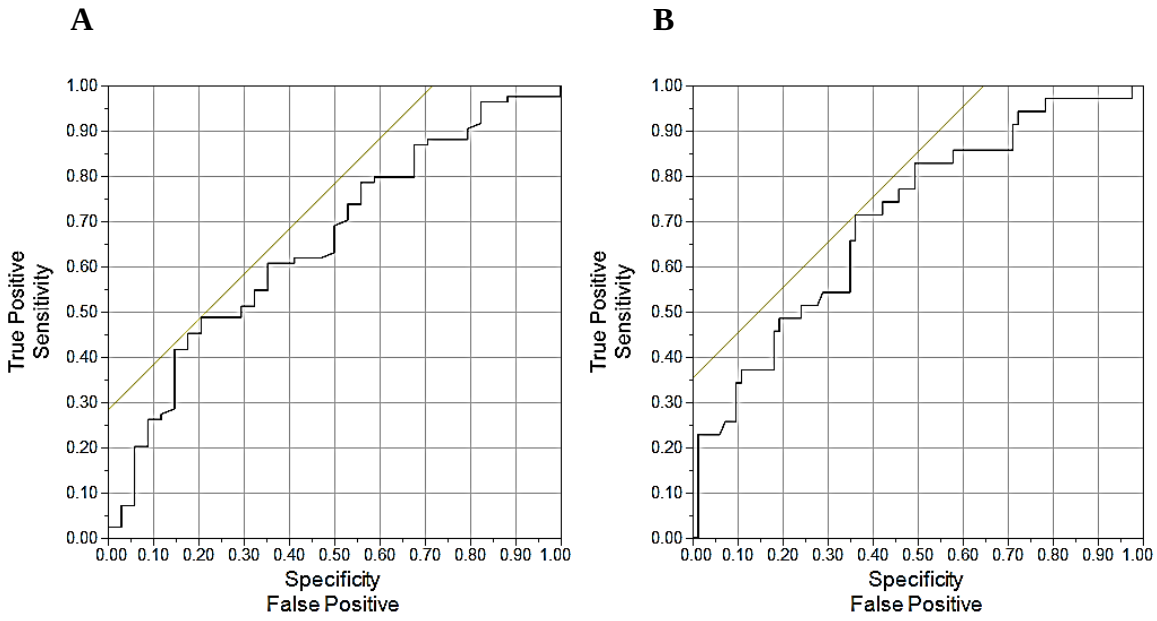

Fig 15. Day 37 post-AI ROC curve to predict pregnancy loss and twins at day 51 post-AI using progesterone. Receiver operating characteristics (ROC) was used to predict pregnancy loss (A) and twins (B) at day 51, using values of progesterone at day 37 post-AI. The test generated a threshold of 6.5 ng/ml for progesterone to predict pregnancy loss ($P < 0.01$) with an area under the curve of 0.65 and 7.2 ng/ml for progesterone to predict twin pregnancy ($P < 0.01$) with an area under the curve of 0.70.

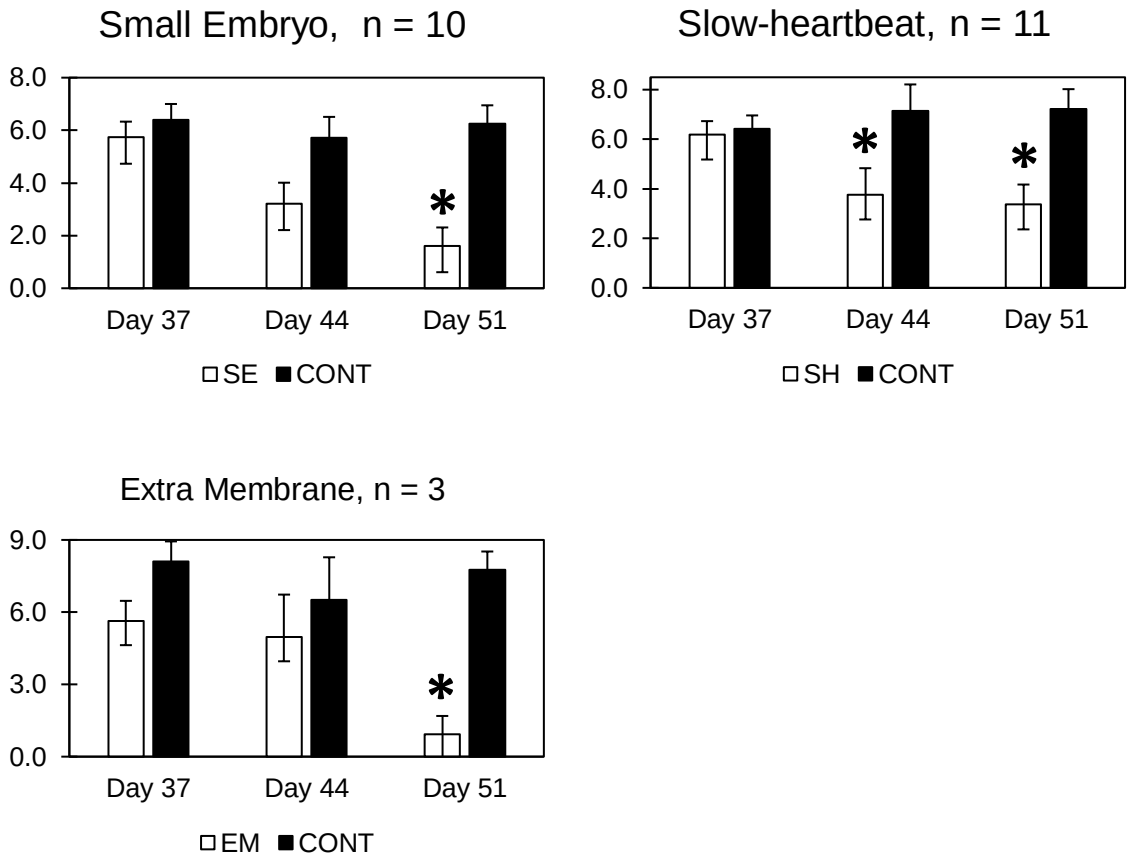

Fig.16. Subgroup (small embryo, slow- heartbeat and extramembrane) comparison with controls regarding plasma concentrations of progesterone in ng/ml. A statistical difference was found among all subgroups and their time interaction at day 51 post-AI compared to control ($P < 0.05$). Additionally, a difference was also observed at day 44 post-AI for the slow-heartbeat subgroup.

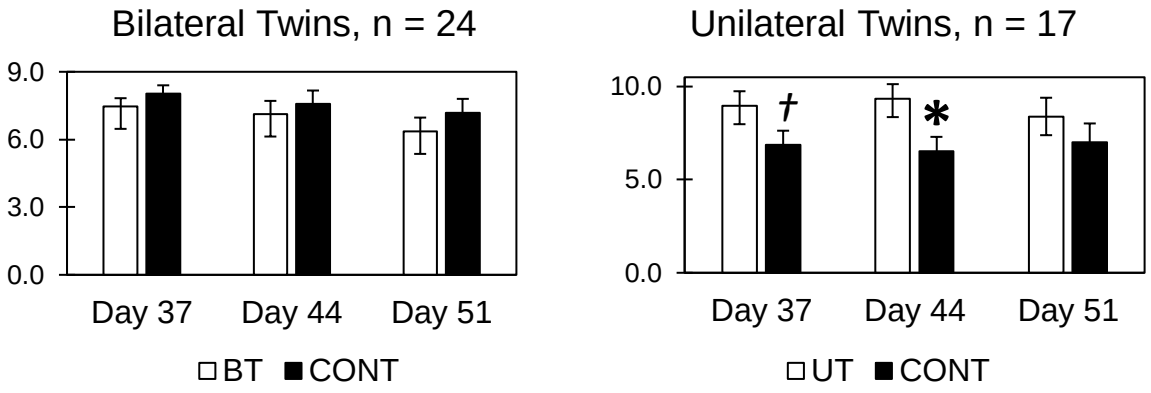

Fig.17. Subgroup (bilateral and unilateral twins) comparison with controls regarding plasma concentrations of progesterone in ng/ml. Group and time interaction differences were found between unilateral twins and control at day 44 post-AI compared to control. Additionally, a tendency for lower plasma progesterone was noticed at day 37 post-AI for the unilateral twin. No statistical difference was found between bilateral twins and control (P = 0.15) and their time interaction (P = 0.93).

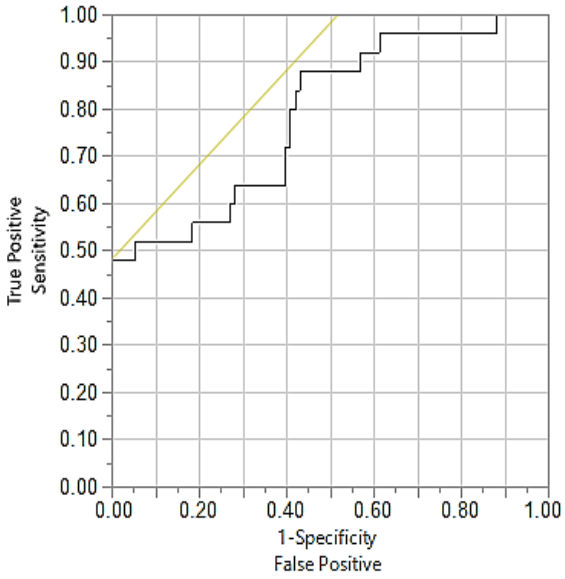

Fig 18. Day 44 post-AI ROC curve using a combination of plasma PAG and P4 concentrations to predict pregnancy loss at day 51 post-AI. Receiver operating characteristics (ROC) was used to predict pregnancy at 51 d, using values of progesterone at 37 d post-AI. The test generated a threshold of 3.70 and 6.60 ng/ml for PAG and progesterone respectively, to predict pregnancy loss ($P < 0.01$) with an area under the curve of 0.78.

Table 1. Descriptive data of each group (CON, n = 65; HR, n =24; TW, n = 41), containing milk yield, days in milk (DIM) at enrollment and number of services[1].

Traits	Groups			*P-value*
	CON	HR	TWIN	
No. of cows	65	24	41	-
Parity[1]	1.93 ± 0.13	2.04 ± 0.21	2.17 ± 0.16	0.53
Milk yield, kg[2]	49.2 ± 1.89	47.8 ± 2.17	49.7 ± 1.83	0.28
Enroll, DIM[1]	142.2 ± 23.7	140.3 ± 12.8	151.1 ± 11.2	0.81
No. of services[1]	2.01 ± 0.59	1.95 ± 0.32	2.21 ± 0.28	0.86
Stillbirth, n	1	2	3	0.21
Retained placenta, n	2	0	0	0.24
Mastitis, n	3	0	7	< 0.05

[1]Results shown as mean ± S.D

[2] Milk yield at pregnancy diagnosis in kg

Table 2. The overall percentage of pregnancy loss of groups among d 37 – d 44, d 44 – d51 and the total percentage at d 37 – d 51.

Item	Groups			
	CON	HR	TW	*P- value*
No. of cows, n	65	24	41	
Preg. Loss: d 37 - 44 (%)	1.5 (1/65)	66.6 (16/24)	4 (2/41)	<0.01
Preg. Loss: d 44 - 51 (%)	0 (0/64)	62.5 (5/8)	7 (3/39)	<0.01
Total: d 37- 51 (%)	1.5 (1/65)	87.5 (21/24)	12.2 (5/41)	<0.01

Table 3. The overall percentage of pregnancy loss of groups among d 37 – d 44, d 44 – d51 and the total percentage at d 37 – d 51.

Item	Subgroups				
	SE	SH	EM	BT	UT
No. of cows, n	10	11	3	24	17
Preg. Loss: d 37 – 44 % (n/n)	80 (8/10)	54 (6/11)	66 (2/3)	8 (2/24)	0 (0/17)
Preg. Loss: d 44 - 51 % (n/n)	100 (2/2)	40 (2/5)	100 (1/1)	0 (0/22)	17 (3/17)
Total: d 37- 51 % (n/n)	100 (10/10)	72 (8/11)	100 (3/3)	8 (2/24)	17 (3/17)

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CHAPTER 3: CONCLUSION AND FUTURE DIRECTIONS

The present study investigated the secretion pattern of PAG and progesterone in cows carrying a high risk and twin pregnancy. The study design adopted proved its efficacy since most of the cows enrolled in the high-risk group experienced a subsequent pregnancy loss. The variability in the secretory pattern of plasma concentration of PAG influenced the attempt to establish a cutoff to predict high-risk and twin pregnancy. Regardless of the study, these variabilities revealed as one inevitable challenge involving PAG as a predictor of conceptus loss in dairy cows. Another issue in predicting high-risk pregnancy is that the concentration of PAG in cow's blood was gradually reduced in cows experiencing pregnancy loss. Cows diagnosed as non-pregnant at 44 days post-breeding were still presenting high concentrations of plasma PAG 7 days later. A maternal-placental disruption is suspected to preclude the PAG secretory activity via binucleate giant cell, but the exact mechanism involving the reduction of plasma levels of PAG needs more investigation. Perhaps an additional plasma sample alongside an ultrasonography evaluation on day 30 post-AI could provide information regarding the high-risk and twin pregnancy cows on day 37. The use of plasma progesterone revealed thresholds to predict high-risk pregnancy and twins, but only with moderate accuracy. Reduced plasma levels of progesterone in the HR group compared to CON is perhaps associated with a conceptus death and subsequent luteolysis, or, the corpus luteum (CL) regression led to the conceptus death, terminating the pregnancy. Perhaps collecting data on the cow’s CL could provide additional information to understand the nature of the pregnancy loss. The combination of PAG and P4 revealed useful to predict pregnancy, although the use of those two biomarkers together still needs more investigation. The findings in this study involving the relationship of the biochemical markers before, during, and after pregnancy loss could be used as a model for future studies.