Matrix Metalloproteinases as Plasma Indicators of Bovine Cystic Ovarian Disease

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Abstract:

Cystic ovarian disease (COD) is one of the main causes of infertility in dairy cattle and has a high economic impact on farmers. COD is caused by an endocrine imbalance within the hypothalamic-pituitary-ovarian axis which prevents the mature Graafian follicle from ovulating. The cause at the molecular level is not well understood. Our research investigated the possibility of using plasma concentrations of matrix metalloproteinases (MMP) -2 and -9 and their natural tissue inhibitors (TIMP) -1 and -2 as prognostic indicators of COD. Plasma samples from cystic and non-cystic dairy cows were analyzed using ELISA. Although plasma concentrations of MMP-2 and -9 were greater and TIMP-2 was lower in non-cystic compared to cystic cows, no significant differences were observed in MMP-2 and -9 and TIMP-1 and -2 due to cyst status. However, the TIMP-1:MMP-9 and TIMP-2:MMP-2 molar ratios were greater, (P=0.099) and (P=0.038), respectively, in cystic compared to non-cystic cows, suggesting a proteolytic insufficiency in cows with COD that may be a contributing factor to the anovulatory pathology. These data may

provide the groundwork for future research and development of tools for dairy farmers to selectively choose replacement heifers less likely to develop COD.

Introduction:

Cystic ovarian disease (COD) is a reproductive pathology affecting dairy cattle in which the mature Graafian follicle fails to ovulate. Because the Graafian follicle does not ovulate in a timely fashion, the postpartum interval to first estrus is prolonged and estrous cyclicity is irregular. This is a concern for dairymen because it decreases the reproductive efficiency of cows thereby increasing culling rates and costs of production. Over a lifetime, COD is estimated to affect 10% to 30% of high producing dairy cows (Kesler & Garverick 1982). Currently, there are no prognostic indicators of COD that can be used as a tool for dairy farmers to select replacement heifers less likely to develop COD. Our research investigated potential plasma indicators of the disease.

The Estrous Cycle

The estrous cycle of a cow is 21 days in duration and includes two distinct phases; the follicular phase and the luteal phase. The follicular phase accounts for about 25% of the estrous cycle, during which estradiol, secreted from the ovarian follicles, is the primary hormone. The follicular phase consists of proestrus and estrus. The three steps of follicular development are

recruitment, selection and dominance. During the first stage, follicle stimulating hormone (FSH) and luteinizing hormone (LH) stimulate recruitment of follicles. Follicles that do not undergo atresia during recruitment enter the selection phase and increase in size while producing estradiol. The follicle that becomes dominant produces inhibin which suppresses FSH needed by other follicles to continue growing, causing them to undergo atresia. The luteal phase accounts for the remaining 75% of the estrous cycle and consists of metestrus and diestrus. During the luteal phase, the corpus luteum (CL) is producing progesterone. The estrous cycle consists of a hormonal cascade, eventually leading to the release of an oocyte. On day 0 of the estrous cycle the follicle is producing estradiol, which triggers gonadotropin-releasing hormone (GnRH) to be released from the hypothalamus. GnRH triggers a surge in FSH and LH from the pituitary gland. LH begins the proteolytic cascade resulting in the follicle wall degrading, rupture of the preovulatory follicle, and release of an oocyte. From approximately day 1 to day 5 the ruptured follicle luteinizes. This causes the CL to form and begin producing progesterone. The hormonal cascade from approximately day 16 to day 18 depends on whether the cow becomes pregnant or remains open. If the cow is pregnant, the newly formed embryo blocks uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthesis, allowing the CL of pregnancy to form, and the pregnancy is maintained. If the cow does not become pregnant, $PGF_{2\alpha}$ regresses the CL, causing progesterone to decrease and allowing FSH and LH to increase, which begins formation of a new wave

of follicles. During this process, the follicle wall undergoes significant remodeling to transform into the CL. Ovulation depends on proliferation and differentiation of the follicles on the ovary. There are many follicles developing at once in cattle, but one must become the dominant follicle. The remaining follicles undergo atresia, an apoptotic process, and then regress. The dominant follicle continues to grow and eventually becomes the mature follicle containing the oocyte. In cows with COD, there is a failure of the hypothalamus to trigger the preovulatory surge of LH (Silvia *et al.* 2002). Once the follicle reaches 2.5 cm in diameter, it is considered a cystic follicle.

Cystic Ovarian Disease

There are two different types of ovarian cysts: follicular and luteal. Ovarian cysts are defined as ovarian structures that are over 2.5 cm which have persisted for longer than 10 days without a CL present (Roberts 1971). Follicular cysts are single or multiple thin-walled structures, while luteal cysts are generally single structures with a thicker wall of luteal tissue (Kesler & Garverick 1982). Of the two, follicular cysts account for approximately 70% of COD cases (Kesler & Garverick 1982). Luteal cysts are often associated with high plasma progesterone while follicular cysts are associated with lower progesterone concentrations (Dobson *et al.* 1977). Luteal cysts are often follicular cysts that have persisted and formed a thicker layer of luteal tissue around the edges of the cyst (Garverick 1997).

The only practical method of detection of COD for dairy farmers is rectal palpation. COD can also be detected by ultrasound, but this is not an economically feasible option for many dairy farmers. Due to the large size of cystic follicles, a trained professional can easily identify them by rectal palpation.

COD in cattle can be treated with an intramuscular injection of GnRH. This injection causes the pituitary gland to release LH which luteinizes the ovarian cyst wall. To cause regression of the luteinized ovarian cyst, an injection of $PGF_{2\alpha}$ is given approximately one week after the GnRH injection. This treatment causes the luteal tissue to regress and progesterone to decrease allowing the estrous cycle to restart in approximately 2 to 3 days. After this treatment, 80% of cows reestablish their ovarian cycles and 50% become pregnant on their first estrus (Kesler & Garverick 1982). Other treatment options for COD are LH and human chorionic gonadotropin (hCG). GnRH is currently the most frequently used treatment because cows are less likely to form antigens against these products due to the small molecular size.

If a cow is open more than 85 days postpartum there is an estimated loss of $2.50 to $3.00/cow/day. Cows with cystic ovaries have calving intervals that are 22-64 days longer than herd-mates with no cystic history (Hatler *et al.* 2003). Thus, each case of COD costs a farmer approximately $150-$180 in decreased milk production, breeding and medical costs, as well as increased culling rates (Coleman, Dairy Integrated Reproductive Management, West

Virginia University). According to the USDA, as of 2015, there are approximately 125,000 dairy cows in the state of Oregon. At $150 per case and a 10% incidence rate, Oregon farmers are losing approximately $1.875 million annually due to COD. Nationally, farmers are losing $139.5 million each year to COD ($150, 9.3 million cows, 10%). COD has a high economic impact for dairy farmers due to increased postpartum and calving intervals, breeding and treatment costs, and culling rates and decreased milk yield. Research is necessary to help decrease the economic impact of COD on dairy farmers.

COD is associated with a dysfunction of the hypothalamic-pituitary-ovarian axis, but there is no consensus on a specific intra-ovarian dysfunction that causes COD (Silvia *et al.* 2002). Stangaferro *et al.* (2014) found that expression of components in the activin-inhibin-follistatin system were altered. This system could be responsible for the endocrine alterations and follicular persistence seen in COD. Other factors can be stress, herd management, nutritional status, body condition, and infectious disease (Silvia *et al.* 2002). Seventy-one percent of ovarian cysts develop within 45 days postpartum (Whitmore *et al.* 1974). This means that most cysts develop before the first ovulation after calving. There is also an association between high milk production and the incidence of ovarian cysts (Marion & Bier 1968). It is possible that the increased milk production is a result of the hormonal imbalance, and not the actual cause of COD (Kesler & Garverick 1982). Some research supports the idea of ovarian cysts having a genetic link. Casida &

Chapman (1951) found that 26.8% of daughters from cows who had a history of ovarian cysts also have a history of cysts, while those from cows with no history of ovarian cysts only have an occurrence rate of 9.2%. A genetic link was also shown in a study completed on a Swedish dairy farm from 1954 to 1977, where the occurrence of COD dropped from 10.8% to 3.0% over the course of 23 years. This reduction in COD was accomplished by culling bulls whose daughters had high rates of COD (Bane 1968; Swedish Agriculture 1978). Other possible predisposing factors may be nutrition, seasonal changes, age or genetics (Roberts 1955; Dawson 1957; Peter 1997). While many researchers do not agree on a specific cause of COD, many agree that it is due to some type of endocrine imbalance, which affects the mechanism for extracellular matrix remodeling.

Currently, the cause of COD at the molecular level is not fully understood. Animals with COD have decreased cell proliferation, apoptosis and expression of pro-apoptotic proteins in comparison to anti-apoptotic proteins in ovarian tissue (Salvetti *et al.* 2010). This suggests these important systems are altered in cows with COD. Gonadotropins in granulosa and theca cells are responsible for steroidogenesis and folliculogenesis. Recent research found that gonadotropin receptor mechanisms are altered in cows with COD. This affects proliferation and apoptosis and could contribute to the cause of COD (Ortega *et al.* 2015). Steroids, such as estrogens, androgens and

progesterone, play a critical role in follicle and ovarian development. Steroid receptors in the ovaries of cows with COD are disrupted (Ortega *et al.* 2015).

Extracellular Matrix Degrading Proteinases

Ovulation is dependent on breakdown of the extracellular matrix (ECM), which allows for the release of the oocyte. The breakdown of the ECM is dependent on the matrix metalloproteinase (MMP) and plasminogen activator (PA) families.

The MMP family consists of 26 different proteins, all of which are proteolytic enzymes. MMP are zinc-dependent proteinases known for reorganizing the ECM. MMP-2 and -9 are in the gelatinase family of MMP. MMP are important for many cellular behaviors, including ECM remodeling during the estrous cycle (Matthew *et al.* 1995, Curry & Osteen 2003) and ovulation (Smith *et al.* 1999). Other reproductive processes MMP play an important role in include placentation and development of the embryo (Schatz *et al.* 1999), spermatogenesis (Siu & Cheng 2004) and menstruation in women (Dong *et al.* 2002). They are critical participants in the proteolytic cascade of the estrous cycle. The role of MMP in ovulation is not fully understood at this time, but it is known that they play an important role in degrading the follicle wall to allow for release of the oocyte (McIntush & Smith 1998; Ny *et al.* 2002).

Follicles on bovine ovaries greater than 2.5 cm, had more proMMP -2 and -9 activity in the follicular fluid than follicles smaller than the threshold for

cystic follicles. These cystic follicles also had significantly lower inhibin concentrations (Imai *et al.* 2003). Inhibin inhibits FSH secretion. Although these results conflict with the traditional role of MMP, other studies have found similar results, in which both circulating and follicular concentrations of MMP-2 and -9 were higher in women with polycystic ovarian syndrome (PCOS), which is a condition similar to COD (Lewandowski *et al.* 2006; Baka *et al.* 2010). MMP-2 and -9 expression are also linked to ovarian cancer (Davidson *et al.* 1999).

Another proteolytic system associated with ovulation, which is similar to the MMP family, is the PA system. This system converts the zymogen plasminogen into the active protease, plasmin (Dano *et al.* 1985). Plasmin is responsible for degrading components of the ECM and activating pro-MMP, which go on to further degrade the follicle wall. Plasmin generation is dependent on tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). In some processes, the PA and MMP systems are regulated together (Ny *et al.* 2002).

Our laboratory conducted a study which investigated the PA system in cows with cystic follicles (McNeel & Menino 2011). In this study, no significant differences were found in plasma concentrations of tPA or PA inhibitor-1 (PAI-1) in cystic and non-cystic cows. Ratios of tPA and PAI-1 were not different between the two groups. This study also looked at gene expression of the PA family at the ovarian level using relative quantitative RT-PCR. tPA and PAI-1

198 expression did not differ (P>0.10) between preovulatory follicles and follicular
199 cysts. However, more uPA (P=0.08) and less uPA receptor (uPAR) (P<0.05)
200 expression was observed in preovulatory follicles compared to cystic follicles.
201 The ratio of PAI-1 to uPA was also greater (P<0.10) in follicular cysts
202 compared to preovulatory follicles. These results suggest gene expression is
203 altered in follicular cysts, leading to a cow having impaired proteolysis. This
204 could explain the molecular mechanism behind COD where a greater protease
205 inhibitor to protease ratio impairs follicle wall breakdown leading to the cyst
206 pathology. McNeel (2010) also collected blood samples for PCR to determine
207 if a single nucleotide polymorphism (SNP) was present in the promoter region
208 of the PAI-1 gene (serine protease inhibitor E1; SERPINE1), like there is with
209 PCOS. A four base pair insertion/deletion was detected upstream from the
210 transcriptional start site in 27 of the 78 cows tested. Jersey cows had a
211 deletion rate of 56.4%, while Holsteins had the deletion at a rate of 12.8%. A
212 greater proportion of Jerseys with COD had this deletion compared to Jerseys
213 without COD (P=0.07). Similar results were not observed in Holsteins
214 (P>0.10). However, plasma concentrations of PAI-1 were not affected in either
215 breed in the presence or absence of the insertion/deletion. These data
216 suggest that although the SERPINE1 deletion polymorphism found in Jersey
217 cattle was associated with COD it had no effect on plasma PAI-1
218 concentrations.

MMP activity is inhibited by four tissue inhibitors of matrix metalloproteinases (TIMP-1, -2, -3 and -4) which form non-covalent bonds with MMP. Increased TIMP-1 expression is often seen in conditions with excess ECM components present, which then leads to fibrosis (Arpino *et al.* 2015). Hence, TIMP-1 may have a role in limiting ECM proteolysis. TIMP-2 and MMP-2 imbalance is associated with Dupuytren's disease, which is a condition where ECM components are deposited excessively on the joints of the hand to the point where the patient can no longer flex the joints. In this condition TIMP-2 is found excessively, which suggests that TIMP-2 is associated with ECM component buildup (Ulrich *et al.* 2009). A similar association is found in some cases of heart disease, where ECM components are excessively deposited. TIMP-2 directly inhibits MMP-2, although TIMP-2 is also required for activation of MMP-2. Arpino *et al.* (2015) found that in some cases, TIMP-2 may indirectly control ECM buildup, through activation of MMP-2. This finding was contrary to the traditional role of TIMP inhibiting MMP, leading to buildup of the ECM. TIMP also inhibit a disintegrin and metalloproteinases (ADAM) and ADAMs with thrombospondin motifs (ADAMTS), which are both closely related to MMP. In summary, high concentrations of TIMP are associated with the buildup of ECM components, while ratios that are higher in MMP support proteolytic activity. Ovulation depends on a proper balance between MMP and TIMP. If an imbalance exists between MMP and TIMP, ovulation may be disabled.

241

242 ***Polycystic Ovarian Syndrome***

243 Polycystic ovarian syndrome (PCOS) is a condition somewhat similar to
244 COD that affects 10-20% of women in developed countries (Russell *et al.*
245 2015). It is often associated with infertility and frequent abortions (Shalev *et al.*
246 2001). The physiologic pathway of PCOS has been associated with a SNP in
247 the promoter region of the SERPINE1 gene. It is possible that abnormalities in
248 MMP and TIMP concentrations in women with PCOS could be similar to cows
249 with COD. Circulating serum concentration of MMP-2 and -9 and one of their
250 tissue inhibitors, TIMP-1, was found to be significantly higher in women with
251 PCOS compared to healthy control women. The same study found no
252 significant difference in the other tissue inhibitor of MMP-2 and -9, TIMP-2,
253 between the two groups (Lewandowski *et al*. 2006). Another similar study
254 found women with PCOS have significantly higher concentrations of MMP-2
255 and -9 as well as TIMP-1 and -2 in their follicular fluid while going through in
256 vitro fertilization procedures (Baka *et al*. 2010). Shalev *et al.* (2001) also
257 researched MMP-2 and -9 concentrations in women with PCOS and normal
258 ovulatory women. In this study they found MMP-2 and MMP-9 concentrations
259 were 1.6 and 1.7 fold higher, respectively, in follicular fluid of women with
260 PCOS. In the same study, MMP-2 and MMP-9 secretion in granulosa cells
261 was quantified. Cultured granulosa cells from women with PCOS have higher
262 concentrations of MMP-2 and -9, but similar concentrations of TIMP-1

compared to normal ovulatory women. Due to the similar nature of PCOS and COD, MMP and TIMP concentrations in cows with COD could have comparable ratios to women with PCOS.

If differences in plasma concentrations between normal and cystic cows correlate with follicular expression of MMP-2 and -9, then plasma MMP could emerge as an indicator of COD in dairy cattle. This could assist with providing dairy producers tools in selecting replacement heifers that are less likely to develop COD. The ramifications of this could be great for the dairy industry, providing a reduction in veterinary, breeding and culling costs. We expect that cows with COD will have lower plasma concentrations of MMP-2 and MMP-9, and higher concentrations of TIMP-1 and TIMP-2 compared to cows that have no history of follicular cysts.

Materials and Methods

Animals

A total of 65 lactating cows were used in this study. Four of the animals were Jersey cows housed at the Oregon State University Dairy Center in Corvallis, Oregon. The remaining 61 cows used in this study were Holsteins housed at a cooperating dairy in Coburg, Oregon. This dairy had approximately 3,000 cows with 1,500 milking. At both sites, cows were provided with free-choice water and a total mixed ration consisting of corn

285 silage, grass silage, alfalfa, corn, cotton seed and soy bean meal. Cows were
286 divided into two groups based on ovarian palpation during herd health
287 evaluations by a licensed veterinarian at 14-day intervals. Cows diagnosed
288 with an ovarian follicular cyst, a follicle > 2.5 cm in diameter, were assigned to
289 the cyst group and a blood collection was performed. Cows selected to serve
290 as the control, or non-cystic group, were cows observed in estrus the day of
291 the blood collection by the herdsmen. Signs of estrus are standing to be
292 mounted, increased step count and decreased milk production. For the non-
293 cystic cows, medical records were inspected to verify no history of follicular
294 cysts. All work was performed in accordance with the Oregon State University
295 Institutional Animal Care and Use Committee.
296 Age and parity data were collected for the 61 cows housed at the
297 cooperating dairy. For cystic cows, the number of lactations in which a cow
298 had been diagnosed with a cyst was recorded. Health records for the four
299 cows housed at the Oregon State University Dairy were not available.
300
301 ***Blood Collection***
302 Blood samples were collected via coccygeal venipuncture using 10 mL
303 Vacutainer (Becton Dickinson) blood-collection tubes. Vacutainers for plasma
304 collection were preserved with heparin as the anticoagulant. Blood samples
305 were transported back to the laboratory within approximately one hour of
306 collection. Tubes were centrifuged at 5,000 X g for 10 minutes to separate

plasma from whole blood. Three 500-ml aliquots of plasma were collected from the top half of each vacutainer, and each tube was labeled with “A”, “B” or “C” according to the order in which the sample was taken, date of blood draw, and cow number. Aliquots were stored at -20° C until analysis for MMP-2 and -9 and TIMP-1 and -2.

Following blood collection, all cystic cows were injected intramuscularly with 2 mL of Factrel® (Zoetis, Florham Park, NJ), a synthetic GnRH, and 7 days later with 5 mL of Lutalyse® (Pfizer, New York, NY), a synthetic $PGF_{2\alpha}$. Cows were artificially inseminated at their next estrus.

ELISA

Plasma MMP-2 and -9 concentrations were quantified using Genorise (Berwyn, PA) ELISA kits. One-hundred microliters of standard or sample plasma in duplicate were incubated in a pre-coated antibody plate for one hour at RT. Any targeted MMP present in the sample were bound to the antibody on the plate. After the initial incubation, each well was washed with 200 µl of wash buffer 4 times. One hundred microliters of detection antibody specific for bovine MMP-2 or -9 were added to each well in the plate and the plate was incubated for one hour at RT. Another wash was completed to remove unbound antibody reagent. One hundred microliters of detection reagent were added to each well and incubated for 20 minutes. Another wash cycle was completed and 100 µl of substrate were added, which causes color formation

during the 20-minute incubation at RT. Fifty microliters of stop solution were added to the wells to halt color development. The optical density (OD) of each well in the plate was immediately quantified at 450 nm and 550 nm using a BIOTEK EL800 plate reader. The amount of color formation is directly proportional to the amount of MMP-2 or -9 bound to the antibody during the first incubation period.

To calculate MMP-2 and -9 plasma concentrations in the samples, OD measurements at 550 nm were subtracted from OD at 450 nm as a correction factor for imperfections in the plate. Corrected OD measurements were transformed into plasma concentrations using Excel and equation of the line calculations. A standard curve was created using the OD readings of the standards provided in the Genorise ELISA kit. Plasma concentrations of MMP-2 and -9 in the samples were determined by entering OD readings into the standard curve equation.

TIMP-1 concentrations were quantified using a MyBioSource (San Diego, CA) ELISA kit. All reagents were brought to RT prior to starting the assay. One hundred microliters of standard or sample were added to each well in the assay plate. All standards were run in duplicate. A closure membrane was then placed on top of the plate while the tray incubated for two hours at 37°C. After the first incubation, all liquid was removed from the wells and 100 µl of detection reagent A were added to each well. The plate was covered again and incubated for one hour at 37°C. Each well was aspirated

and washed four times using 400 µl of wash buffer. One hundred microliters of detection reagent B were added to each well and the plate was covered again for 1 hour at 37°C. Each well was washed five times. After all liquid was removed from the wash steps, 90 µl of substrate solution were added to each well. The plate was sealed and incubated at 37°C for 15 minutes, while being protected from light. Fifty microliters of stop solution were added to each well and the OD of each well was quantified within 5 minutes using a BIOTEK EL800 plate reader. Blank wells served as the correction factor.

TIMP-2 concentrations were also quantified using a MyBioSource (San Diego, CA) ELISA kit. All samples and reagents were brought to RT 30 minutes before starting assay procedures. Fifty microliters of sample, standard or sample diluent were added to each well in the assay plate. All standards were run in duplicate. Sample diluent was used as a blank control sample in duplicate. One hundred microliters of HRP-conjugate were added to each well. A closure membrane was placed on top of the plate and the tray was incubated at 37°C for 60 minutes. The plate was washed with approximately 400 µl of wash buffer four times. Fifty microliters of Chromogen solution A and B were added successively to each well. The tray was protected from light, covered with a membrane, and incubated at 37°C for 15 minutes. Fifty microliters of stop solution were added to each well, changing the color from blue to yellow. The OD of each well was measured at 450 nm within 15

minutes of adding the stop solution in a BIOTEK EL800 plate reader. The blank wells served as the correction factor.

To calculate plasma TIMP-1 and -2 concentrations in the samples, the average OD of the blank wells were subtracted from the OD of each standard as a correction factor. Corrected OD measurements were transformed into plasma concentrations using Excel and equation of the line calculations. A standard curve was created using the OD readings of the standards provided in the MyBioSource ELISA kit. Plasma concentrations of TIMP-1 and -2 in the samples were determined by entering corrected OD readings into the standard curve equation.

Statistical Analyses

Differences in plasma concentrations of MMP-2 and -9, TIMP-1 and -2 and the TIMP-1:MMP-9 and TIMP-2:MMP-2 molar ratios due to breed (Jersey vs. Holstein) and cyst status (cystic vs. non-cystic) were determined by two-way ANOVA. Differences in plasma concentrations of MMP-2 and -9, TIMP-1 and -2 and the TIMP-1:MMP-9 and TIMP-2:MMP-2 molar ratios due to cyst number were determined by one-way ANOVA. If significant effects were observed in the ANOVA, differences between means were evaluated by Fisher's least significant differences procedures. Correlation-regression analyses were conducted to determine the degree of relationship in the TIMP-1:MMP-9 and TIMP-2:MMP-2 molar ratios with cow age and parity. All

analyses were performed using the NCSS statistical software program (Number Cruncher Statistical System; 2007, Jerry Hintze, Kaysville, UT).

Results

For MMP-2 and -9 quantification, each group, cystic and non-cystic, consisted of 32 cows, which included three Jerseys, two cystic and one non-cystic. For TIMP-1 and -2, 33 cystic and 32 non-cystic cows were sampled, which included three cystic and one non-cystic Jersey. Two-way ANOVA conducted to detect differences due to breed and cyst status revealed no differences due to breed ($P>0.10$), hence all cows were pooled for subsequent analyses by one-way ANOVA. Average ages and parities of cystic and non-cystic cows were 44.4 ± 3.3 and 37.8 ± 2.2 months and 2.0 ± 0.3 and 1.8 ± 0.2 parities, respectively.

MMP-2

Although MMP-2 concentrations were greater in plasma recovered from non-cystic compared to cystic cows, no differences were observed ($P=0.33$). Mean plasma concentrations of MMP-2 in cystic and non-cystic cows were 228.8 ± 49.8 and 311.5 ± 68.2 pg/ml, respectively (Figure 1). Intra-assay coefficients of variation for MMP-2 assays 1 and 2 were 15.3% and 15.5%, respectively.

MMP-9

Similar to MMP-2, MMP-9 concentrations were greater in plasma recovered from non-cystic compared to cystic cows, however, no differences were observed (P=0.76). Mean plasma concentrations of MMP-9 in cystic and non-cystic cows were 89.5 ± 14.6 and 95.4 ± 12.3 pg/ml, respectively (Figure 2). Intra-assay coefficients of variation for MMP-9 assays 1 and 2 were 11.0% and 3.9%, respectively.

TIMP-1

No differences (P=0.86) in plasma TIMP-1 concentrations were detected between cows diagnosed with a cystic follicle and normal cows with no history of COD. Mean plasma concentrations of TIMP-1 in cystic and non-cystic cows were 4.71 ± 0.8 and 4.96 ± 1.2 ng/ml, respectively (Figure 3). Intra-assay coefficient of variation for the TIMP-1 assay was 9.0%.

TIMP-2

Although TIMP-2 concentrations were lower in plasma recovered from non-cystic cows compared to cystic cows, no differences were observed (P=0.15). Mean plasma concentrations of TIMP-2 in cystic and non-cystic cows were 39.7 ± 1.4 and 36.9 ± 1.3 ng/ml, respectively (Figure 4). Intra-assay coefficient of variation for the TIMP-2 assay was 3.7%.

MMP-2 and -9 Concentrations Relative to Cyst Number

MMP-2 and -9 plasma concentrations were analyzed relative to the number of lactations a cow had been diagnosed with a cyst as number of cysts. MMP-2 plasma concentrations decreased as number of cysts increased, except in the 3 cyst group, however no differences (P=0.79) were observed (Figure 5). MMP-9 plasma concentrations remained similar (P=0.98) in all groups (Figure 6).

TIMP-1 and -2 Concentrations Relative to Cyst Number

Likewise, TIMP-1 and -2 plasma concentrations were analyzed relative to the number of lactations a cow had been diagnosed with a cyst as number of cysts. Although TIMP-1 plasma concentrations decreased as number of cysts increased, no differences (P=0.90) were observed (Figure 7). No differences (P=0.46) were observed due to cyst number in plasma concentrations of TIMP-2, however the cow with a history of 3 cysts had the highest plasma concentration (Figure 8).

TIMP-1:MMP-9 and TIMP-2:MMP-2 Molar Ratios

TIMP-1:MMP-9 molar ratio was greater (P=0.099) in cystic compared to non-cystic cows (421.0 ± 108.0 vs. 224.1 ± 47.1, respectively; Figure 9). Similarly, TIMP-2:MMP-2 molar ratio was greater (P=0.038) in cystic

compared to non-cystic cows (3510.6 ± 1177.1 vs. 958.9 ± 134.0, respectively; Figure 10). Cystic cows with one cyst in their production record had a TIMP-1:MMP-9 molar ratio twofold greater ($P<0.05$) than non-cystic cows (Figure 11). The TIMP-2:MMP-2 molar ratio was four times ($P<0.05$) greater In cystic cows with one cyst compared to non-cystic cows (Figure 11).

Correlations for TIMP-1:MMP-9 and TIMP-2:MMP-2 molar ratios with age and parity were negative for cystic cows and only the TIMP-1:MMP-9 molar ratios with age and parity approached statistical significance (Table 1). No significant correlations were observed for TIMP-1:MMP-9 and TIMP-2:MMP-2 molar ratios with age and parity for non-cystic cows (Table 2).

Discussion

These data suggest cows diagnosed with COD do not have significantly altered plasma concentrations of MMP-2, MMP-9, TIMP-1 or TIMP-2 compared to cows with no history of cystic follicles. Although no statistical differences in plasma MMP-2 and -9 concentrations were observed between normal and cystic cows, both MMP-2 and -9 were higher in cows with no cystic history compared to cystic cows. In fact, MMP-2 plasma concentrations were approximately 36% higher in cows with no history of follicular cysts. However, noticeable between animal variation was observed in MMP-2 plasma concentrations, as evidenced by the SE associated with the means. Perhaps with a larger sample size significant differences may have been realized in

MMP-2. Sample size calculated from a Power analysis with Power similar to that for the TIMP-2:MMP-2 analysis for a one-sided test would require a sample size of 112 cows per group. Results from the present study differ from those of Imai *et al.* (2003) who observed more proMMP-9 activity, albeit in follicular fluid, in bovine cystic follicles compared to follicles below the threshold diameter for cysts. Our results also differ from several studies reporting elevated concentrations of MMP-2 and -9 in plasma and follicular fluid in women with PCOS (Shalev *et al.* 2001; Lewandowski *et al.* 2006; Baka *et al.* 2010).

Concentrations of TIMP-1 were very similar in both groups and as TIMP-1 is often regarded as being constitutively expressed this observation was not surprising. TIMP-2 plasma concentrations were elevated in cystic compared to non-cystic cows and the difference approached significance with a P-value of 0.15. Similar to MMP-2, a larger sample size may have been able to detect significant differences in plasma concentrations of TIMP-2. Sample size calculated from a Power analysis with Power similar to that for the TIMP-2:MMP-2 analysis for a one-sided test would require a sample size of 52 cows per group. TIMP-2 is tightly tied to regulation of MMP-2 and MMP-9 is regulated by multiple TIMP. Interestingly, serum TIMP-1, but not TIMP-2, was higher in women with PCOS compared to healthy women (Lewandowski *et al.* 2006). However Baka *et al.* (2010) reported higher concentrations of both

503 TIMP-1 and -2 in follicular fluid recovered from women with PCOS compared
504 to women without the pathology.
505 Molar ratios of TIMP-1:MMP-9 and TIMP-2:MMP-2 were at least 100
506 and 1000-fold, respectively, greater in favor of TIMP compared to MMP. Both
507 TIMP-1:MMP-9 and especially TIMP-2:MMP-2 molar ratios were greater in
508 cystic cows compared to non-cystic cows suggesting an imbalance in the
509 protease inhibitor to protease ratio in favor of reduced proteolysis in cows with
510 COD. Whether this difference translates to impaired proteolysis at the follicular
511 level is not known however it suggests a plausible explanation for the follicular
512 cyst pathology where reduced follicular wall proteolysis could lead to the
513 anovulatory condition.
514 Age and parity have been suggested to be factors associated with COD
515 (Peter 1997; Silvia *et al.* 2002). In the current study the TIMP-1:MMP-9 molar
516 ratio approached significance for cystic cows where as age and parity
517 increased the ratio decreased. However relationships with the TIMP-2:MMP-2
518 molar ratio and age and parity were decidedly nonsignificant. Meaningful
519 relationships with either molar ratio with age and parity for non-cystic cows
520 were not observed.
521 Multiple ECM degrading proteinase systems play a role in the
522 regulation of ovulation. Having an imbalance in one of these multiple systems
523 could play a role in COD. In the present study, molar ratios of TIMP-1:MMP-9
524 and TIMP-2:MMP-2 in plasma were greater in cystic cows compared to non-

cystic cows and favored reduced proteolysis in cows with COD. If this difference translates to the ovarian level the impaired proteolysis may predicate development of the follicular cyst pathology. Whether the plasma TIMP-2:MMP-2 molar ratio can be used as a marker for heifers with a predilection to develop COD remains to be determined. However, given the economic losses suffered by dairy producers due to COD, evaluation of such a relationship bears merit for future research.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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688

List of Tables

697 Table 1. Correlation coefficients (r) for TIMP-1:MMP-9 and TIMP-2:MMP-2

698 molar ratios by parity and age in cystic cows.

Y-variable	X-variable	N	R	P-value
TIMP-1:MMP-9	Age (mos)	29	-0.353	0.06
TIMP-1:MMP-9	Parity	29	-0.293	0.12
TIMP-2:MMP-2	Age (mos)	30	-0.102	0.59
TIMP-2:MMP-2	Parity	30	-0.141	0.46

699

700

701 Table 2. Correlation coefficients (r) for TIMP-1:MMP-9 and TIMP-2:MMP-2

702 molar ratios by age and parity in non-cystic cows.

Y-variable	X-variable	N	r	P-value
TIMP-1:MMP-9	Age (mos)	30	-0.036	0.85
TIMP-1:MMP-9	Parity	30	-0.028	0.88
TIMP-2:MMP-2	Age (mos)	30	0.017	0.93
TIMP-2:MMP-2	Parity	30	-0.026	0.89

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704

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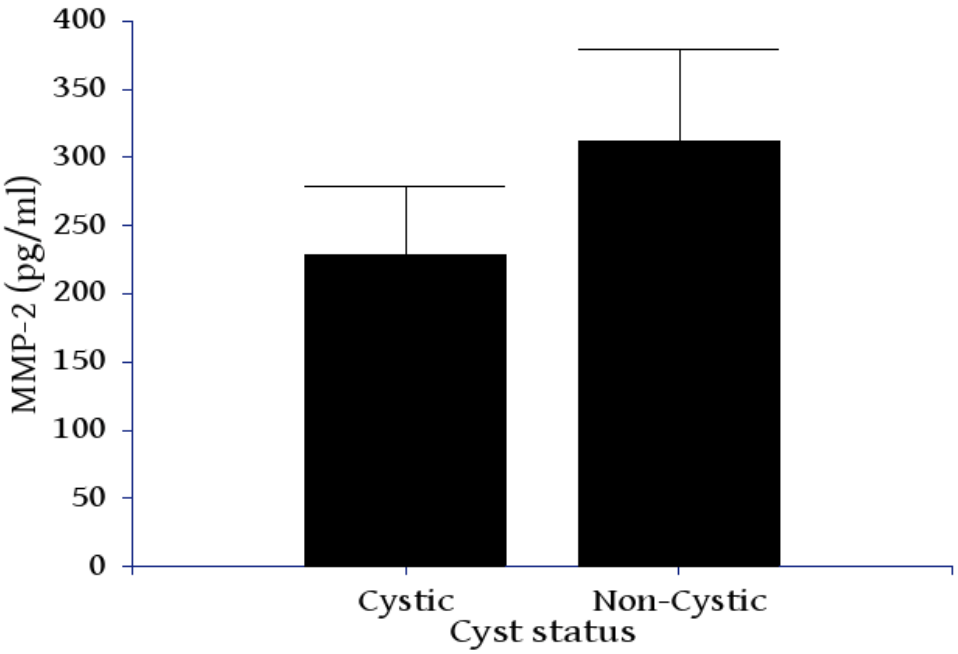
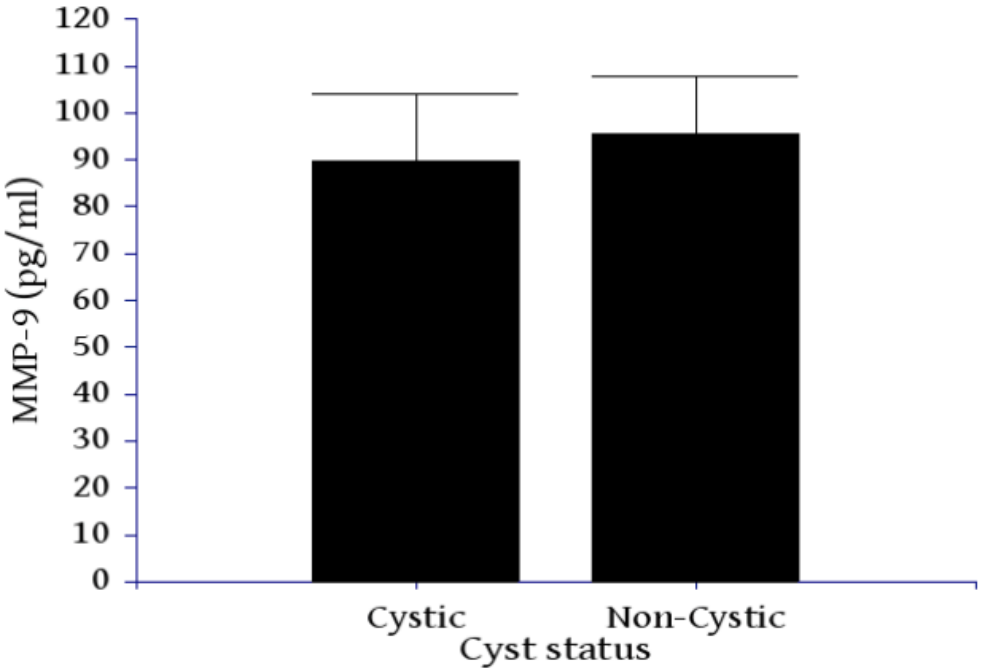
731



732

733 Figure 1. Plasma concentrations of MMP-2 (mean ± SE) in cows diagnosed

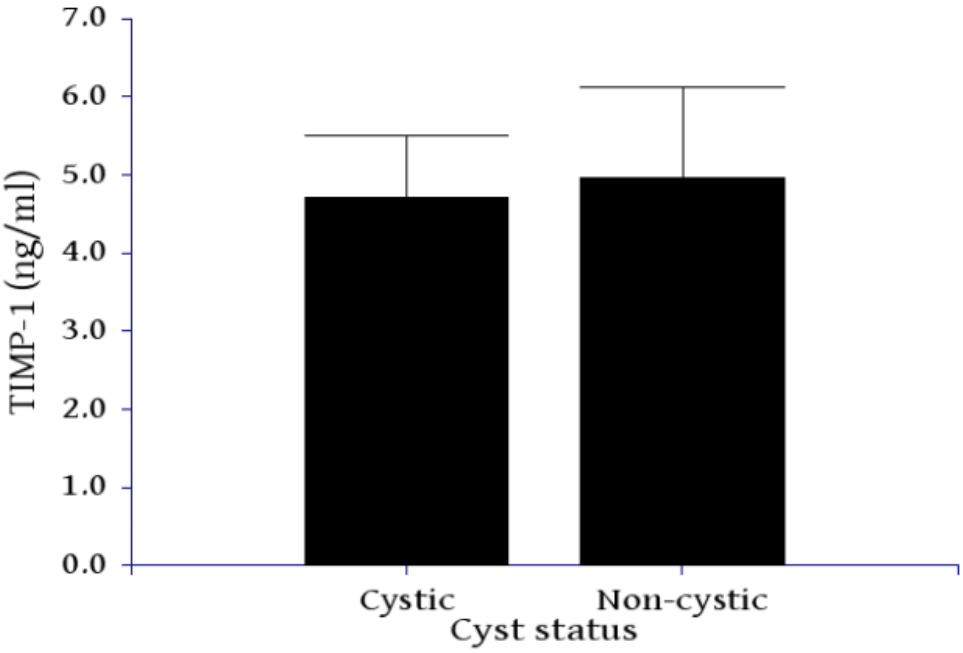
734 with a follicular cyst (n=32) or non-cystic cows (n=32).



735

736 Figure 2. Plasma concentrations of MMP-9 (mean ± SE) in cows diagnosed

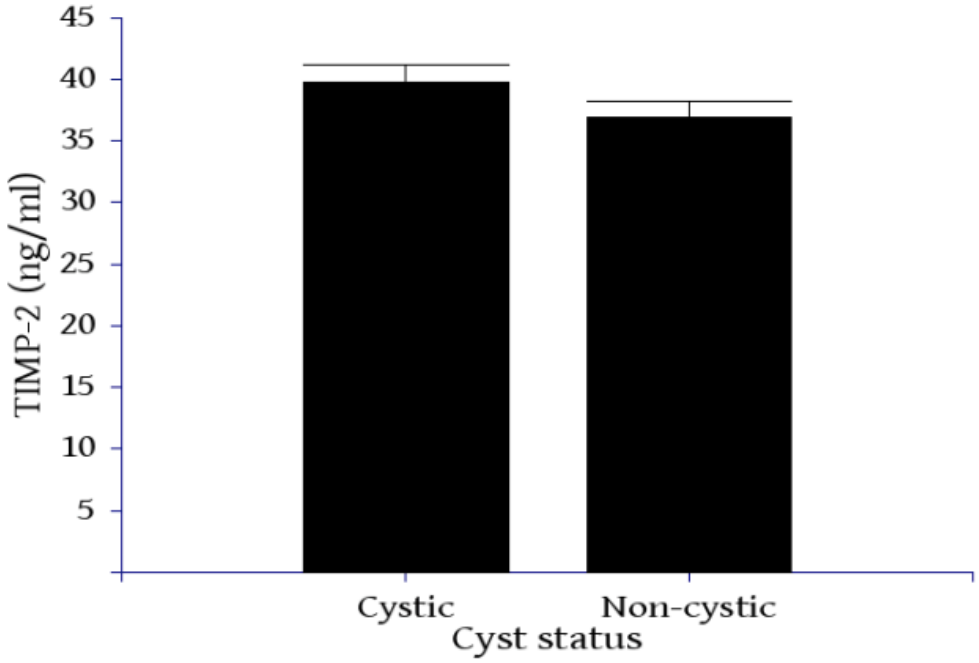
737 with a follicular cyst (n=32) or non-cystic cows (n=32).



738

739 Figure 3. Plasma concentrations of TIMP-1 (mean ± SE) in cows diagnosed

740 with a follicular cyst (n=33) or non-cystic cows (n=32).



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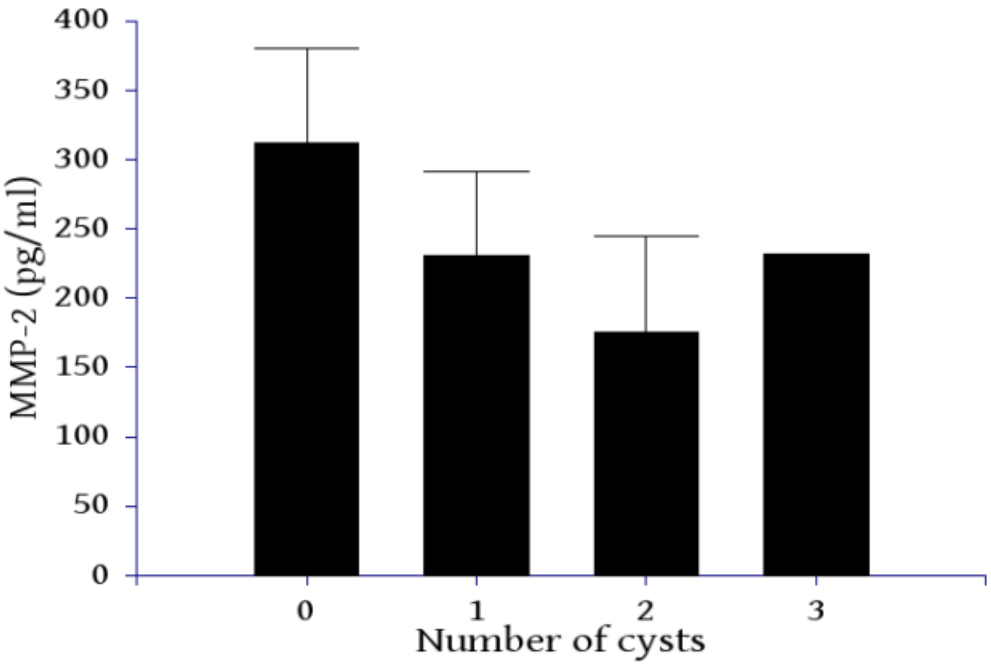
742 Figure 4. Plasma concentrations of TIMP-2 (mean ± SE) in cows diagnosed

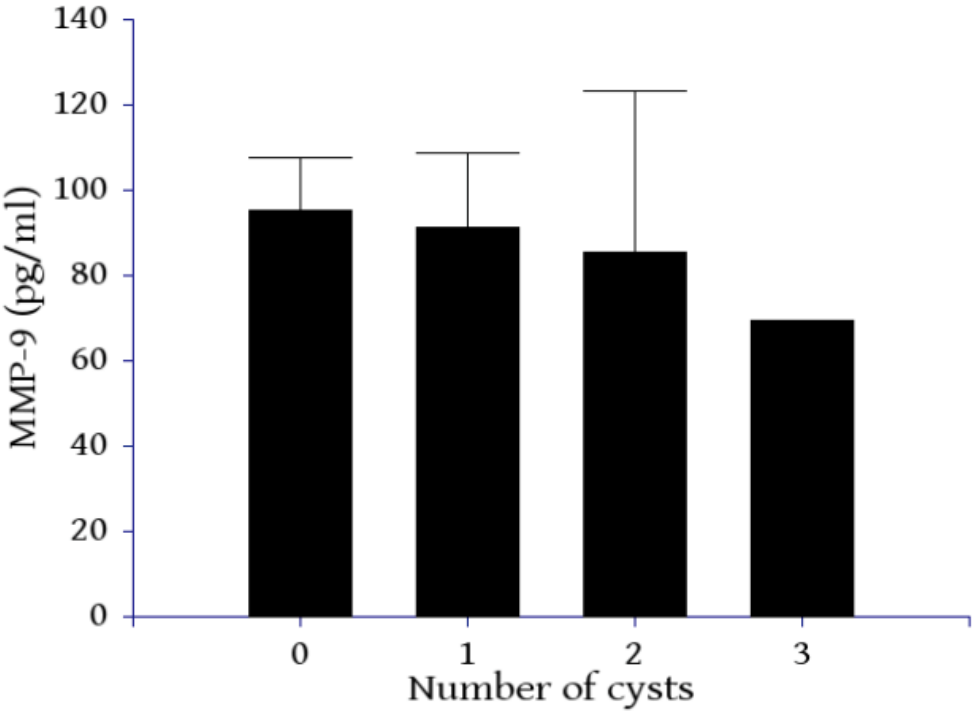
743 with a follicular cyst (n=33) or non-cystic cows (n=32).



744

745 Figure 5. Plasma concentrations of MMP-2 (mean ± SE) in cows with 0

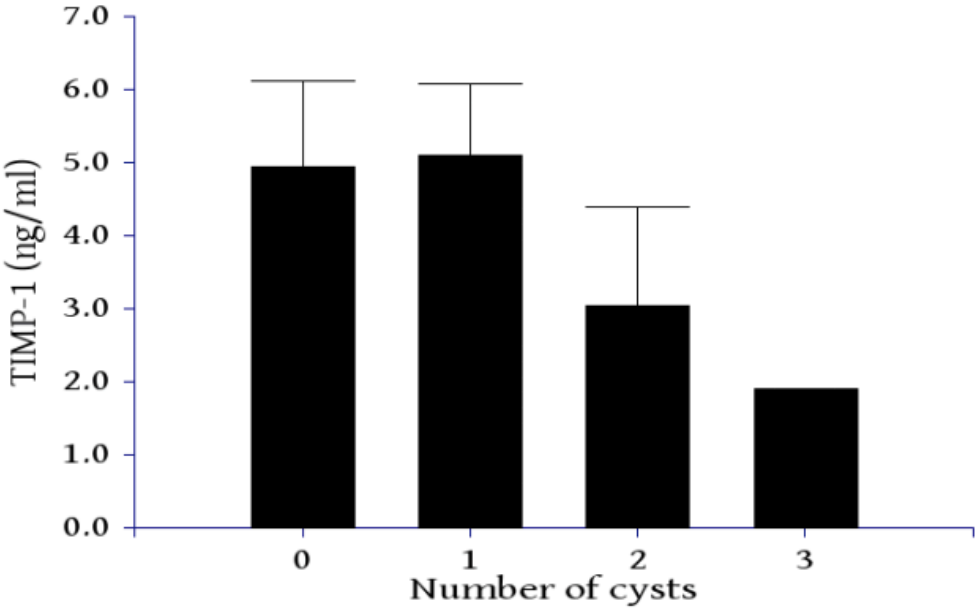
746 (n=31), 1 (n=26), 2 (n=3) or 3 cysts (n=1).



747

748 Figure 6. Plasma concentrations of MMP-9 (mean ± SE) in cows with 0

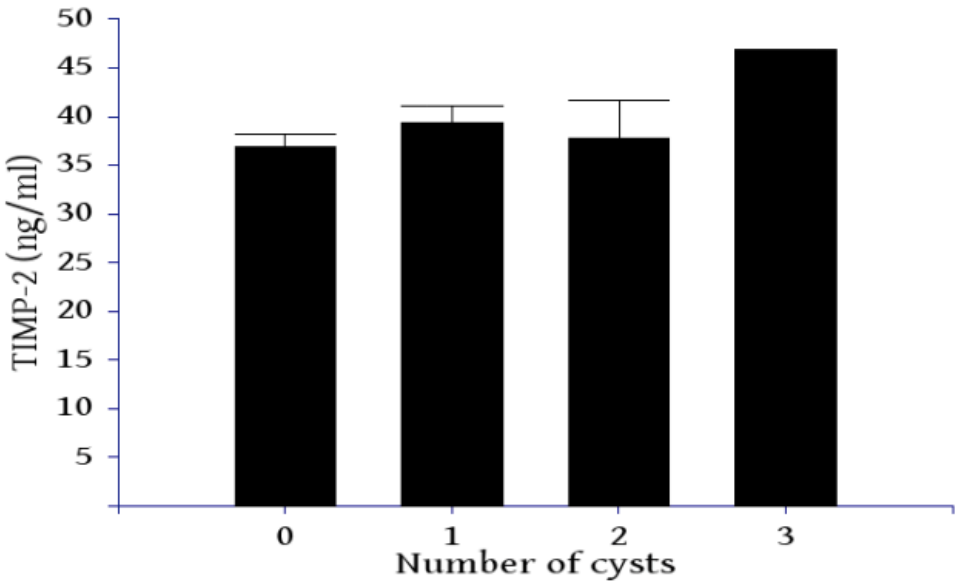
749 (n=31), 1 (n=26), 2 (n=3) or 3 cysts (n=1).



750

751 Figure 7. Plasma concentrations of TIMP-1 (mean ± SE) in cows with 0

752 (n=32), 1 (n=26), 2 (n=3) or 3 cysts (n=1).



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754 Figure 8. Plasma concentrations of TIMP-2 (mean ± SE) in cows with 0

755 (n=32), 1 (n=26), 2 (n=3) or 3 cysts (n=1).

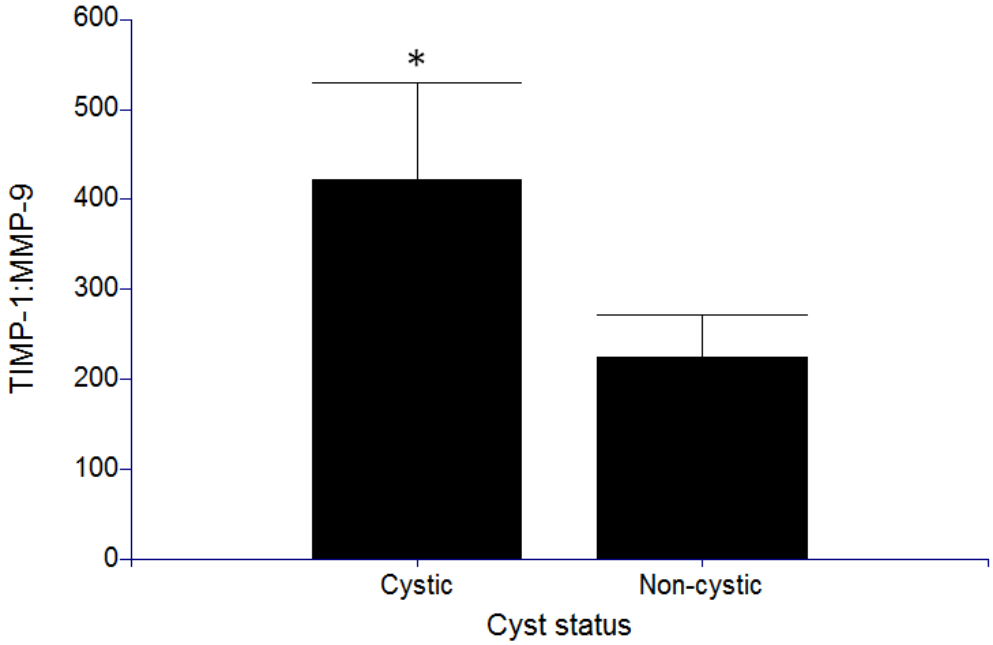
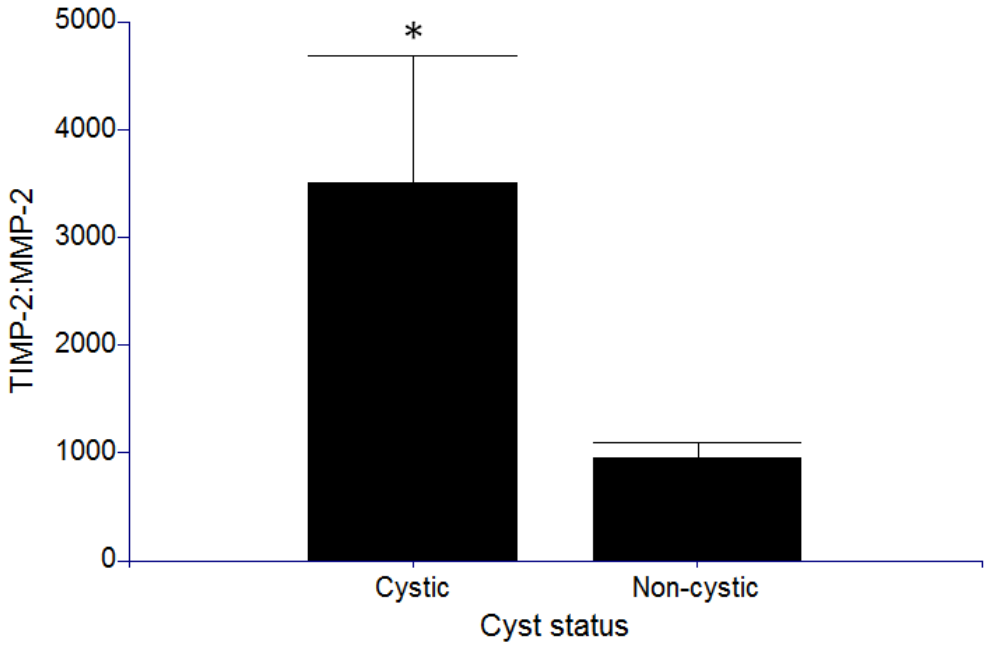


Figure 9. Molar ratios of TIMP-1:MMP-9 (mean ± SE) in cows diagnosed with a follicular cyst (n=31) or non-cystic cows (n=31).

***** Different from non-cystic cows (P=0.099).

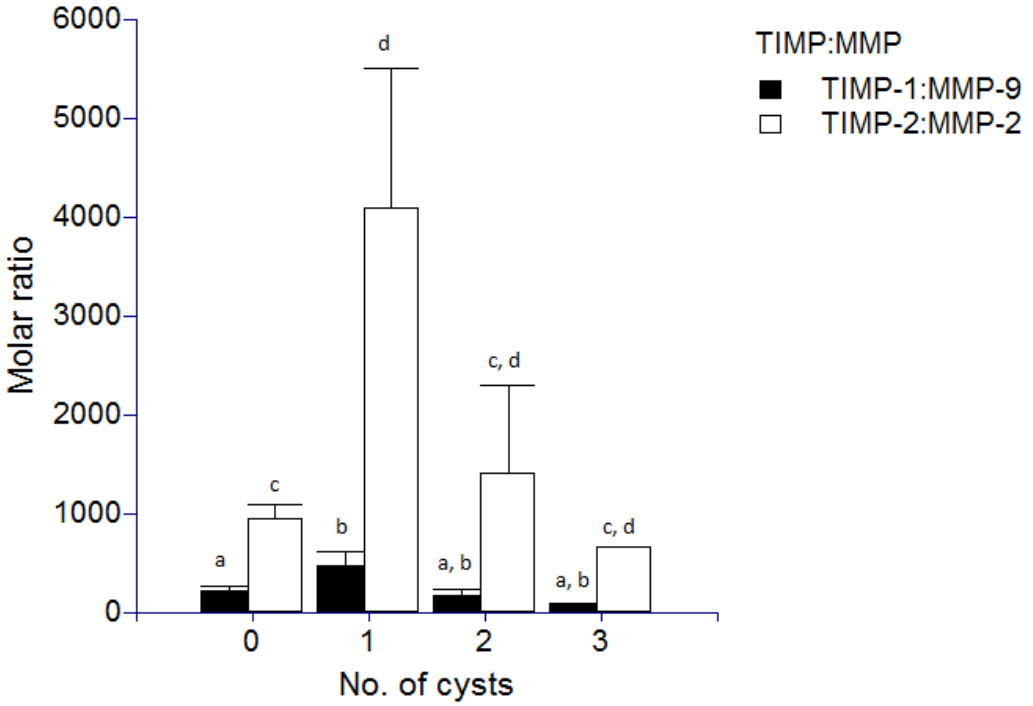


760

761 Figure 10. Molar ratios of TIMP-2:MMP-2 (mean ± SE) in cows diagnosed with

762 a follicular cyst (n=32) or non-cystic cows (n=31).

763 ***** Different from non-cystic cows (P=0.038).



764

765 Figure 11. Molar ratios of TIMP-1:MMP-9 (mean ± SE) in cows diagnosed with

766 0 (n=31), 1 (n=25), 2 (n=3) or 3 cysts (n=1) and TIMP-2:MMP-2 (mean ± SE)

767 in cows diagnosed with 0 (n=31), 1 (n=26), 2 (n=3) or 3 cysts (n=1).

768 [a,b] Means without common superscripts for TIMP-1:MMP-9 molar ratios differ

769 ($P<0.05$)

770 [c,d] Means without common superscripts for TIMP-2:MMP-2 molar ratios differ

771 ($P<0.05$)

772

773