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Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows

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Mastitis is the most frequent and costly disease in dairy herds, as it negatively affects yield and milk quality. The presence of clinical mastitis is quite easy to asses, whereas the diagnosis of the subclinical form can be more difficult and requires laboratory assays. Somatic cell count (SCC) is widely used as a rapid and low-cost indicator of mastitis, even if is not useful in discriminating between the clinical and subclinical form. As amyloid A has been investigated as a marker of mastitis, the aim of this study was to assess the potential value of measuring amyloid A in serum and milk and the correlation with SCC in the diagnosis of subclinical mastitis. The reliability of two different ELISA kits for the measurement of amyloid A in milk was also tested. During a 1-month trial period, 21 cows were assigned to three experimental groups according to their health status: 6 cows with clinical mastitis (CM), 10 cows with subclinical mastitis (SM) and 5 healthy cows (HE). Amyloid A was measured both in serum (SAA) and in quarter milk samples (mAA) with a serum ELISA kit, and in quarter milk samples (MAA) with a milk ELISA kit. SCC, total microbial count (TMC) and bacterial examination of the milk were also carried out. After a log transformation, the data were submitted to ANOVA and linear regression. TMC was significantly higher in cows with clinical mastitis, while no differences were observed between the other two experimental groups. SCC and MAA levels were significantly different among the three groups. mAA concentrations were similar between cows with subclinical and clinical mastitis, and SAA was not affected by mastitis. A significant correlation between SCC and MAA or mAA was detected, while no correlation was recorded between SAA and mAA. A close relationship between MAA and mAA was noticeable even at low concentrations, suggesting MAA as a potential physiological marker of subclinical mastitis.

Keywords: Subclinical mastitis, serum amyloid A, milk amyloid A.

Mastitis is considered the most frequent and costly disease in dairy herds, owing to its negative effects on the yield and quality of the milk (Kossaibati et al. 1998). In addition, mastitis alters the technological properties of milk, especially in the cheese-making process. Somatic cell count (SCC) is widely used for evaluating milk quality, even in the absence of clear scientific evidence linking SCC levels in bulk tank milk with changes in cheese-making quality (Le Roux et al. 2003).

Mastitis can be caused by a large number of infectious agents, which are commonly divided into three categories depending on the way the infection is spread. The first division includes *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma bovis* and *Corynebacterium bovis*, which are all spread from infected quarters to the following quarter, or directly to other cows. The second division are the *coagulase-negative Staphylococcus* spp., which cause opportunistic mastitis and are normally found on teat skin. The third division includes *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp., which cause environmental mastitis. This last group of bacteria reach the teat from the environment around the cows, where they are usually present (Bramley et al. 1996; Hillerton & Berry, 2003; Hoe & Ruegg, 2005; Radostits et al. 2007; Pyörälä & Taponen, 2009).

Cows with clinical signs of mastitis are easily spotted by inspection and palpation of the teats and udder, and are

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thus usually properly treated. In contrast, the diagnosis of subclinical infections requires laboratory analysis and the disease may not be observed, so remaining untreated. As is well known, the subclinical form of mastitis can negatively affect the technological properties of milk (Hamann, 2002). Since the majority of the milk produced in several EU countries (e.g. Italy) is used to produce cheese, there is a need to identify and evaluate specific and sensitive biomarkers that can be used to assess both subclinical mastitis and unfavourable changes in milk quality (Åkerstedt et al. 2008). SCC is one index that is widely used as a rapid and low-cost indicator to assess subclinical mastitis either in milk from individual quarter or cow composite milk (O'Mahony et al. 2006; Åkerstedt et al. 2007). Thus, SCC is routinely monitored with various automated tools based on fluoro-opto electronic or near infrared spectroscopy technologies, but these devices are not yet in use at the farm level (Tsenkova et al. 2001; Kawasaki et al. 2008). In addition, the increasing diffusion of robotic milking systems seems to limit the use of SCC as biomarker of subclinical mastitis (Åkerstedt et al. 2007). Thus, there is a need for other recognizable and rapidly assessable markers of udder and cow health, by monitoring milk and blood serum, respectively.

Eckersall et al. (2001) showed that acute phase proteins (APP) change significantly in bovine milk as a consequence of early clinical mastitis. Serum amyloid A (SAA) has been suggested as a more sensitive marker than a bacteriological examination or SCC determination, since the SCC is more weakly correlated with the physiological stage of the cow (Biggadike et al. 2002). SAA is an apolipoprotein (Malle et al. 1993; Uhlar & Whitehead, 1999) with a molecular weight of approximately 180 kDa in its native form (Pepys & Baltz, 1983). The concentration of SAA can increase over 100-times during infections (Boosman et al. 1989; Eckersall et al. 2001; Murata et al. 2004). Two isoforms of SAA have been described in dairy cows (McDonald et al. 2001), in which the presence of a milk-specific form of this protein shows the synthesis of SAA in the mammary gland induced by mastitis (Eckersall et al. 2001; McDonald et al. 2001). Another study (Jacobsen, 2005) detected four hepatically derived SAA isoforms and another three isoforms, which are probably produced locally in the udder. The aim of this transverse study was to assess the potential value of measuring amyloid A in serum and milk and the correlation with SCC in the diagnosis of subclinical mastitis. Furthermore, we investigated the reliability of two different ELISA kits used for the measurement of amyloid A in milk, in comparison with SCC.

Materials and Methods

Animals and milk samples

The study was carried out in 2007 in a dairy farm located in the Po Valley of the Veneto Region (Italy). The farm reared 122 lactating Italian Holstein cows kept in an open-sided barn with cubicle beds in the lying area, and equipped with self-locking stanchions. The cows were in lactation numbers 1–7 (median 2), lactation months 1–14 (median 5) and had daily milk yields of 18·7–53·4 kg (median 28·9 kg). Cows were milked twice a day in a double-seven herringbone milking parlour. On three different days of a 1-month experimental period, 6 cows were detected with clinical mastitis (CM) according to clinical signs such as observable inflammation of the infected quarter (i.e. redness or swelling, heat, pain, presence of clots in milk); the clinical mastitis status was confirmed by positive bacteriological examination and high average composite SCC (>1 000 000 cells/ml). On the same three experimental days, 10 cows were detected with subclinical mastitis (SM) based on the presence of clots in milk and the absence of abnormalities in the udder on inspection or palpation; the subclinical mastitis status was confirmed by positive/negative bacteriological examination and moderate average composite SCC (>300 000 cells/ml). The cows with CM showed the following productive and reproductive traits on average: 119±59 days in milk (DIM), 30·5±7·1 kg daily milk yield and 2·3±1·3 parity; and in the case of SM cows, 154±124 DIM, 31·2±10·9 kg daily milk yield and 2·6±1·0 parity. According to the parameters of the CM and SM experimental groups, 5 healthy cows (HE thesis) were selected that did not show any clinical signs of mastitis, nor abnormalities in the udder or milk. In addition, a complete clinical examination along with a gynaecological exam was performed in order to confirm the cows were healthy (i.e. absence of chronic uterine infection). The healthy status was confirmed by negative bacteriological examination and low average composite SCC (<150 000 cells/ml). Moreover, the HE group had the following productive and reproductive traits: 142±89 DIM, 32·3±11·2 kg daily milk yield and 2·3±0·8 parity. As the clinical status of every cow was detected (healthy *v.* mastitis), a single milk sampling was performed in each quarter by using sterile plastic vials at the end of the morning milking. Milk samples were immediately analysed for bacteriological examination, total microbial count (TMC) and SCC. At the same time, a single blood sample from a jugular vein was taken, using vacutainer tubes, before the morning meal. Blood samples were left to clot for 2 h at 37 °C and then centrifuged at 2500 ***g*** for 15 min; serum was separated and stored. Milk and serum samples were immediately refrigerated and then stored at −24 °C until they were analysed for amyloid A.

Bacteriological examination, TMC and SCC

Bacteriological examination of the quarter milk samples was carried out in accordance with standards of the National Mastitis Council (Harmon et al. 1990; Hogan et al. 1999). In each culture, the number of colony-forming units (cfu) per ml for each bacterial species was counted.

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The cut-off to categorize a milk sample as positive was set to ⩾1 cfu/ml for contagious bacteria, and to ⩾100 cfu/ml for opportunistic and environmental ones. TMC was determined in fresh milk by an automated analyser (BactoScan, Foss Electric, Hillerød, Denmark). SCC was assessed in fresh milk by fluoro-opto-electronic cell counting (Fossomatic, Foss electric, Hillerød, Denmark).

Amyloid A in serum and quarter milk samples

Concentrations of amyloid A were determined both in serum (SAA) and in quarter milk samples (mAA) with a serum ELISA kit (PHASE™ RANGE Serum Amyloid A Assay; Tridelta Development Ltd, Wicklow, Ireland; cat. TP-802) as first described by McDonald et al. (1991). A modified version of the kit TP-802 was used to detect amyloid A in milk (MAA) (Mast ID RANGE Milk Amyloid A Assay; cat. TP-807). This milk ELISA kit is based on different dilutions both of the calibration standards and of streptavidin peroxidase, as reported from the manufacturer. Serum and milk samples were initially diluted 1 : 500 and 1 : 50, respectively, and all the samples, including the standards, were tested in duplicate. Samples with an optical density outside the range of the standard curve were diluted further and re-analysed. Optical densities were read on an automatic plate reader (model Sunrise; Tecan, Salzberg, Austria) at 450 nm using 630 nm as reference. The limit of detection (LOD) of the ELISAs was 0·3 mg/l for serum (SAA) and milk (mAA) samples analysed with ELISA kit cat. TP-802, and 0·1 mg/l for milk (MAA) samples analysed with ELISA kit cat. TP-807, according to the manufacturer.

Statistical analysis

After verifying the absence of normality and variance homogeneity (PROC UNIVARIATE and Shapiro-Wilk test) data were transformed to a log-scale in order to balance the distribution. SCC (cells/ml of milk) was transformed as [log2 (SCC/100)+3]; SAA, mAA and MAA (mg/l of milk) as natural log (ln). TMC data were not normally distributed also after transformation; therefore, the average and median values were tested by using the non-parametric Kruskal-Willis criteria (PROC NPAR1WAY) in order to discriminate among the three experimental groups (PDIFF Bonferroni adjusted). After log transformation, SCC, mAA and MAA data were submitted to ANOVA (PROC GLM) according to the following linear model:

$$Y_{ijk} = \mu + M_i + C_{ij} + U_k + e_{ijk},$$

where Y_{ijk} = dependent variable; μ = general mean; M_i = effect of Mastitis (i = Healthy, Subclinical Mastitis, Clinical Mastitis); C_{ij} = effect of Cow, random factor nested in Mastitis (j = 1, …, 21); U_k = effect of Udder (k = 1, …, 4); e_{ijk} = random residual effect. Interaction Mastitis per Udder was not included in the model because it was never statistically significant. When Mastitis was significant ($P<0·05$) differences among treatments, means were determined using the PDIFF option along with the BONFERRONI adjust. In the case of SAA, the data (ln SAA) were submitted to a one-way ANOVA that considered the fixed effect Mastitis. Correlations between SCC and SAA, mAA or MAA concentrations were tested by linear regression analyses (PROC CORR). All the statistical analyses were carried out by using the software SAS (2002).

Table 1. Mean, median and ranges of total microbial count (TMC) in quarter milk according to health status of cows

	Samples detectable†	TMC ($\times 10^{-3}$) cfu/ml		
		Mean	Median	Range
Healthy	1/20	11	NA‡	—
Subclinical mastitis	15/39	48	10 ($P>0·10$)§	10–1433
Clinical mastitis	14/23	140	25 ($P<0·05$)§	10–1314

† Number of samples with contamination of over 10^3 colony-forming units per ml of milk/total number of samples
‡ NA: not applicable owing to the small number with detectable levels of contamination (over 10^3 cfu/ml)
§ *P* value of the comparison with healthy cows by the Kruskal-Willis test adjusted by Bonferroni

Results

Bacteriological examination, TMC and SCC

A total of 84 quarter milk samples were examined from the 21 cows considered in the trial. In the milk samples from the six cows with clinical mastitis (CM), the following pathogens were detected: *Str. uberis*, *coagulase-negative Staphylococcus*, *Staph. simulans*, *Enterobacter*, *Corynebacterium* spp, coliforms and polymicrobism. Three cows had bacteriologically positive samples from 3 quarters, one cow from 2 quarters, and two cows from 1 quarter only. In the milk samples from the ten cows with subclinical mastitis (SM), the following pathogens were detected: *Str. uberis*, *Str. D*, *coagulase-negative Staphylococcus*, *Corynebacterium* spp, coliforms and polymicrobism. Three cows had bacteriologically positive samples from 4 quarters, two cows from 3 quarters, and five cows from 2 quarters. In the milk samples from the five healthy (HE) cows, *coagulase-negative Staphylococcus* (<100 cfu/ml) was detected from 1 quarter in two cows, and polymicrobism was detected from 3 quarters in two cows and from 2 quarters in three cows.

Table 1 summarizes the mean (ranges) and median of TMC in quarter milk samples of the three experimental groups. In the case of HE cows, TMC was always below the normal contamination level (10^3 cfu/ml) except in one sample. Comparison of the median of TMC among the experimental groups showed a significant difference ($P<0·05$) only between CM and HE cows.

SCC levels as mean of quarter milk samples for CM, SM and HE cows are reported in Table 2. However, as

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Table 2. Means (SE) and medians of somatic cell count (SCC) and amyloid A (mAA and MAA)† in milk from udder quarter and amyloid A (SAA) in serum according to health status of cows

	SCC‡ ($\times 10^{-3}$) cells/ml	mAA‡, mg/l	MAA‡, mg/l	SAA§, mg/l
Healthy (n=20)				
mean (SE)	58^{b} (289)	0·5^{c} (2·7)	0·1^{b} (1·4)	50^{b} (52)
median	28	0·4	0·1	21
Subclinical mastitis (n=40)				
mean (SE)	618^{a} (204)	9·8^{b} (1·9)	5·5^{a} (1·0)	104^{b} (37)
median	163	2·3	0·5	118
Clinical mastitis (n=24)				
mean (SE)	2704^{a} (311)	16·1^{a} (2·9)	6·9^{a} (1·5)	245^{a} (56)
median	1120	8·0	3·8	344

† mAA was measured with ELISA kit TP-802 and MAA with ELISA kit TP-807
‡ Average of quarter milk samples
§ Average of serum samples
Means within the same column followed by letters are statistically different ($^{a–c}$, P<0·05). These variables are characterized by the absence of normality and variance homogeneity; therefore, the data were transformed to a log scale and submitted again to ANOVA.

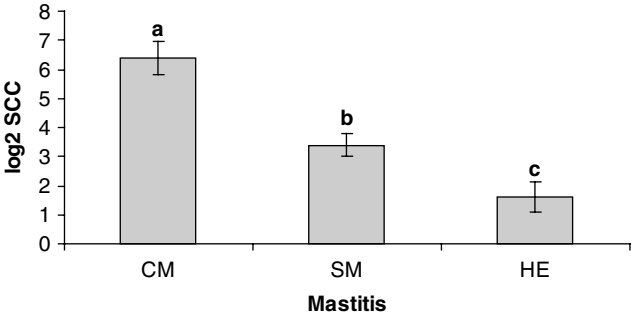

Fig. 1. [log2 (SCC/100)+3] of somatic cell count (SCC) in quarter milk (mean ± SEM) of dairy cows with clinical mastitis (CM), subclinical mastitis (SM) or healthy (HE). Means with different letters differ significantly (P<0·05).

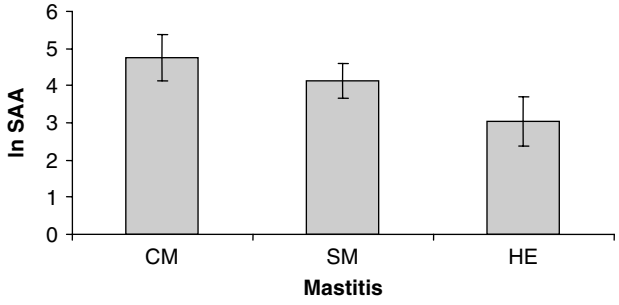

Fig. 2. Natural logarithm (ln) of serum amyloid A (SAA) (mean ± SEM) of dairy cows with clinical mastitis (CM), subclinical mastitis (SM) or healthy (HE). Means are not significantly different.

described in Materials and Methods, SCC was not normally distributed, and a log transformation was therefore considered in order to perform the analysis of variance (ANOVA). The linear score of SCC, which was calculated as [(log2 of SCC/100)+3], showed a significantly difference among experimental groups (Fig. 1).

Amyloid A in serum and quarter milk samples

SAA concentrations were affected by the health status of cows (Table 2). As already described for SCC, a log transformation was necessary in order to normalize the distribution of data before carrying out the ANOVA. After the log transformation, SAA was not affected by mastitis status (Fig. 2). As expected, mAA concentrations seemed to be different among groups, and a similar trend was observed for MAA (Table 2). mAA and MAA data were also not normally distributed, thus they were transformed by the same log transformation applied to SAA. Ln mAA data only showed a detectable difference in content in the comparison between mastitic (CM and SM) and HE cows; there was no significant difference between the two mastitis groups (Fig. 3). MAA results, on the other hand, were significantly (P<0·05) different among experimental groups, with the CM thesis having the highest level (Fig. 4).

Correlation

For the quarter milk samples (n=84) linear regression analysis revealed significant correlations between SCC and mAA (R^2=0·59; P<0·001) or MAA (R^2=0·67; P<0·001). MAA was significantly correlated (R^2=0·72; P<0·001) with mAA, especially in the range of positive values. Using as data the average for each cow (n=21) no significant relationship between SAA and mAA (R^2=0·10; P>0·10) was detected.

Discussion

Consistent with the results of Hillerton & Berry (2003) and Hoe & Ruegg (2005), in this study the presence of

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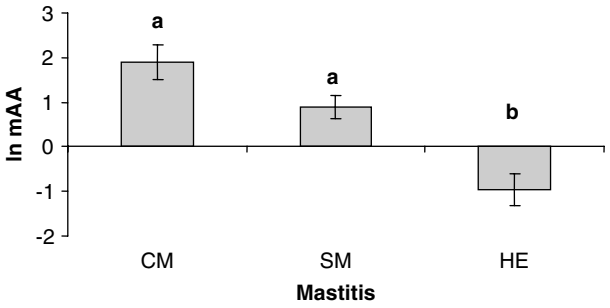

Fig. 3. Natural logarithm (ln) of milk amyloid A (mAA; ELISA kit TP 802) in quarter milk (mean ± SEM) of dairy cows with clinical mastitis (CM), subclinical mastitis (SM) or healthy (HE). Means with different letters differ significantly ($P<0{\cdot}05$).

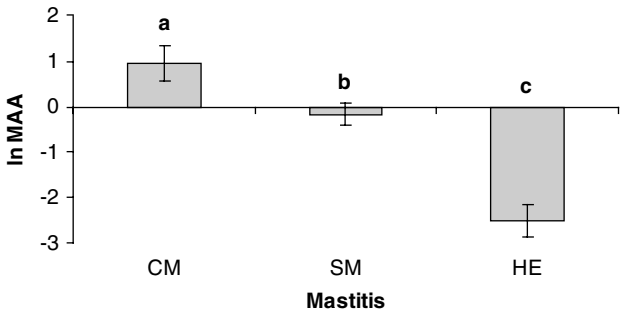

Fig. 4. Natural logarithm (ln) of milk amyloid A (MAA; ELISA kit TP 807) in quarter milk (mean ± SEM) of dairy cows with clinical mastitis (CM), subclinical mastitis (SM) or healthy (HE). Means with different letters differ significantly ($P<0{\cdot}05$).

environmental pathogens such as *Str. uberis*, *Esch. coli*, *Klebsiella* spp. and *coagulase-negative Staphylococcus* spp. were the prevalent pathogens isolated from the milk of cows with clinical and subclinical mastitis. Furthermore, it is important to emphasize that more than one udder quarter showed bacteriologically positive results, as found by others (Adkinson et al. 1993; Barkema et al. 1997). In the healthy cows, the presence of *coagulase-negative Staphylococcus* (<100 cfu/ml) in two cows, in one udder quarter each, and the presence of polymicrobism in some quarters could be related to cutaneous contamination (Bramley et al. 1996; Radostits et al. 2007).

TMC was different in the three experimental groups of cows, although a significant difference was observed only between cows with clinical mastitis and healthy cows. A variation in TMC at different time points during udder infection is possible, particularly if the quarters are only examined once (Sears et al. 1990).

SCC was significantly different in the three experimental groups of cows, confirming that cell counts can be used routinely for the diagnosis of subclinical mastitis, although the SCC recorded in this study were higher than those normally found during milk analysis (Eckersall et al. 2001). Milk samples were collected at the end of milking, thus, the SCC levels were probably higher than normal as the cells are concentrated in the milk remaining in the glands (Brolund, 1985). SCC is considered as a standard indicator for subclinical mastitis (Schepers et al. 1997); however, it may not be a specific sign of the inflammatory status of the udder owing to its high variability, especially in cows with moderate mastitis (Eckersall et al. 2001). In addition, in the case of subclinical mastitis, the presence of some clots and flakes in the milk could affect the automated counting procedure.

The present study showed a tendency for higher SAA concentration in cows with clinical mastitis, even though this difference was not significant ($P=0{\cdot}20$). This finding is partly in accordance with the report of Eckersall et al. (2001), who observed significantly different SAA levels in cows with both mild and moderate mastitis compared with healthy cows, but not significant differences between the two mastitic groups. This result confirms that SAA concentrations are not useful to discriminate between cows with mild or moderate mastitis. Several studies have shown that healthy cows have very low serum levels of SAA (Alsemgeest et al. 1994; Eckersall et al. 2001; Grönlund et al. 2003) and that concentrations of SAA increase during either spontaneous or induced mastitis (Hirvonen et al. 1999; Eckersall et al. 2001; Pedersen et al. 2003). Finally, Nielsen et al. (2004) reported significantly different SAA levels in cows with mastitis compared with healthy or with extramammary inflammatory conditions (interdigital phlegmon, purulent metritis, periarthritis, bursitis) cows. In this study, the lack of significant differences among the experimental groups could be related both to the limited number of serum samples analysed and to the presence of hidden inflammatory diseases, which could have contributed to raising the values in cows with subclinical mastitis.

mAA (serum ELISA kit) concentrations in milk were lower in healthy cows than in cows with mastitis, but no significant difference was observed between subclinical and clinical. These results are consistent with those of Grönlund et al. (2003; 2005), who observed a significant difference of amyloid A in milk concentrations from the different udder quarters of cows with chronic subclinical mastitis, compared with healthy control cows. Additionally, these authors considered levels below the detection limit (0·9 mg/l) to be a good indicator of healthy udder quarters. Similarly, previous studies reported elevated amyloid A in milk in clinically inflamed mammary quarters compared with non-infected quarters (Nielsen et al. 2004), and Eckersall et al. (2001) even showed significant differences in the values of this marker between cows with mild and moderate mastitis.

In the present study, MAA (milk ELISA kit) concentrations were significantly different between cows with subclinical and clinical mastitis. This may be one of the first studies to report the use of a more sensitive assay to determine MAA. In fact, the milk ELISA kit TP-807 (MAA) has a smaller reference range (0–7·5 μg/ml) which allows it to detect lower quantities of amyloid A than the serum ELISA

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kit TP-802 (mAA). According to these results, the use of MAA as a marker of the acute phase seems to give a statistically better response than mAA for distinguishing subclinical from clinical mastitis. Moreover, this assertion is strengthened by the slightly higher correlation between MAA and SCC, than between mAA and SCC.

A significant correlation was observed between SCC and mAA or MAA in quarter milk samples. For mAA, the correlation seems to be consistent at lower SCC levels, in apparent contrast with results in the literature (Nielsen et al. 2004; Lindmark-Månsson et al. 2006; O'Mahony et al. 2006; Åkerstedt et al. 2007). Furthermore, Hogarth et al. (2002) reported high levels of milk amyloid A in cows with clinical mastitis prior to a significant increase in SCC. The loose relationship between SCC and amyloid A in milk, reported by Grönlund et al. (2005), is probably related to their mechanisms of entry into the mammary gland; in fact, leucocytes migrate actively through adhesion molecules, while SAA migrates passively from blood to milk owing to the increased permeability of the inflamed tissue (Eckersall et al. 2001), and/or is produced locally in the udder (McDonald et al. 2001). Moreover, O'Mahony et al. (2006) reported a reduced correlation between milk amyloid A and SCC in cows with raised SAA; this finding is probably due to the amyloid A ingress into the mammary gland from the systemic circulation, and could be an expression of subclinical mastitis. Moreover, in the case of both clinical signs of mastitis and high SCC, the moderate correlation between SCC and mAA at the lowest SCC values is probably due to differences in the length or severity of the udder infection, as reported by Nielsen et al. (2004). In this trial, both the good correlation between MAA and SCC, and the close relationship between MAA and mAA, suggest it is possible to consider MAA as a physiological marker of subclinical mastitis. The absence of a significant relationship between SAA and milk amyloid A concentrations, as observed by Eckersall et al. (2001), Nielsen et al. (2004) and O'Mahony et al. (2006), is probably related to the presence both of a serum amyloid protein homologue in the udder (Molenaar et al. 2002) and of a milk-specific form of bovine SAA (McDonald et al. 2001). These findings show that a form of SAA is produced locally in the mammary gland. In addition, using the average mAA concentration without normalizing for the differences in milk production among the quarters, could contribute to differences between the SAA and mAA levels.

In conclusion, the present results confirm the importance of APP as diagnostic markers of subclinical mastitis, even relative to the use of the MAA assay as a biochemical modification of the SAA assay. Although MAA and SAA determinations require an ELISA method that is carried out routinely only in reference laboratories, MAA could be considered a more reliable marker than SCC. The random measurement of this APP in milk samples could allow the identification of subclinical mastitis in equal or higher measure than currently obtained with SCC. Moreover, systematic control of MAA and SAA on dairy farms could reduce both the laboratory costs and the time required for milk analysis. Lastly, as the actual methods for MAA and SAA analysis are not suitable for online measurements during milking, it is necessary to develop and implement biosensors to be used with automated milking systems, to enable early detection of subclinical mastitis, to reduce both the treatment time and the economic impact, and to improve the health and welfare of dairy cows.

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