Effects of a Single Prolonged Milking Interval in Cows

Study of Indicators and Mediators of Inflammation, Milk Composition and Yield

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Abstract

A single prolonged milking interval (PMI), such as might be associated with technical failures in automatic milking systems, leads to a subsequent somatic cell count (SCC) peak in bulk tank milk. Increased SCC indicates mastitis in cows. It is generally correlated with reduced milk yield and quality, and is often used as a criterion for milk payment. Whether the transient SCC peak after a single PMI affects yield and quality is not known. The short duration of the inflammatory reaction after a PMI and its non-pathological history raise questions about the underlying immunological mechanisms and possible role of immunocompetent natural milk components. It is important to learn more about this kind of physiological inflammation to improve the interpretation of SCC and the general understanding of udder immunology. This thesis investigated the inflammatory reaction after a single PMI of 24 h at both the cow and the quarter level, and how it influences milk yield and quality.

The PMI appeared to induce temporarily impaired epithelial integrity but no epithelial damage. Even so,milk yield per cow was reduced with 0.75 kg/day after the PMI, notably for up to the 10 days studied. Thus, based on the long-lasting reduction in yield and a possibly reduced milk payment due to the SCC peak, a PMI is of significant economic concern for the farmer although milk quality itself was shown not to be afflicted.

The PMI caused a two-fold increase in the SCC, which then remained elevated for 2 days and was associated with an increased proportion of polymorphonuclear leukocytes (PMN) in milk. The most pronounced SCC reaction after the PMI was not seen until the udder had been emptied once. The initiation of inflammation occurred first during the PMI and elicited a systemic acute phase response of serum amyloid A (SAA), observed in blood prior to its appearance in milk. The milk showed consistently high chemotactic activity *in vitro*, although no increased content of the cytokines IL-1β and IL-8 was detected. The PMI induced significant alterations in the content of α-lactalbumin (ALA) and prolactin (PRL), in relation to the PMN reaction. ALA inhibited and PRL stimulated PMN migration, when tested *in vitro.* Based on the findings in this thesis, it is probable that SAA played a significant role in the inflammatory reaction after a PMI but it cannot be excluded that ALA and PRL might also have been contributing factors.

Keywords: milking interval, SCC, PMN, yield, cytokine, acute phase reaction, α-lactalbumin, prolactin, chemotaxis

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Dedication

To My Brother Nenad and My Family

“The cure for boredom is curiosity. There is no cure for curiosity.”
Ellen Parr

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lakic B, Wredle E, Svennersten-Sjaunja K, & Östensson K. (2009). Is there a special mechanism behind the changes in somatic cell and polymorphonuclear leukocyte counts, and composition of milk after a single prolonged milking interval in cows? *Acta. Vet. Scand.* 51-4.

II Lakic B, Svennersten Sjaunja K, Norell L, Dernfalk J, & Östensson, K. (2011). The effect of a single prolonged milking interval on inflammatory parameters, milk composition and yield in dairy cows. *Vet. Immunol. Immunopathol.* 140(1-2), 110-118.

III Lakic B, Svennersten-Sjaunja K, Bruckmaier RM, Norell L, & Östensson K. Prolactin and cortisol in bovine milk and blood during the peak in somatic cell count and neutrophils after a prolonged milking interval and the chemotactic effect of prolactin, *in vitro, Submitted to J. Dairy Res.*

IV Lakic B, Svennersten Sjaunja K, Bruchmaier RM, Lundeheim N, Knight CH, & Östensson K. The inflammatory reaction in the bovine udder during and after a prolonged milking interval, and the in vitro effect of milk, alpha lactalbumin and prolactin on neutrophil migration. *Submitted to Vet. Immunol. Immunopathol.*

Abbreviations

ALA	α-lactalbumin
AM	Automatic milking
AMS	Automatic milking system
ATP	Adenosine triphosphat
APP	Acute phase proteins
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
FFA	Free fatty acid
FIL	Feedback inhibitor of lactation
FM	Frequent milking
IL	Interleukin
LDH	Lactate dehydrogenase
MAA	Serum amyloyd A in milk
MFG	Milk fat globule
NAGase	N-acetyl-β-D-glucosaminidase
ODM	Once daily milking
PMI	Prolonged milking interval
PMN	Polymorphonuclrear leukocytes
PRL	Prolactin
PRLb	Prolactin in blood
PRLm	Prolactin in milk
SAA	Serum amyloyd A
SCC	Somatic cell count
TJ	Tight junctions

1 Introduction

1.1 Dairy sector development in Sweden

During the last decades, modern dairy production has been characterized by rapid development towards larger herd sizes with more technology-based and automatic management. In the 1990s automatic milking systems (AMS) were introduced on the market which enabled milking of cows with significantly reduced labour hours compared to conventional milking. This trend has also been seen in Sweden, as shown in Table 1, although the number of cows and producers has been dramatically reduced since the 1970s and the dairy sector as a whole has diminished. The average herd size of 62 cows is modest in comparison to other countries, probably reflecting the family farm-based nature of Swedish dairy production, although the number of AMS has increased rapidly. Today a notably high proportion of Swedish cows are milked with AMS.

Table 1. Development of the Swedish dairy production 1980-2010 (Swedish Board of Agriculture).

Year	1980	2010
Number of Dairy Cows	655 738	348 095
Number of Dairy Herds	44 143	5619
Herd Size (cows)	15	62
Milk Yield/Cow (kg ECM) *	6044	9468
Number of AMS*	-	755

* ECM: Energy Corrected Milk; Data from the Swedish Dairy Association;

Good udder health is a necessary precondition for optimal milk production. Mastitis is one the most important causes of losses in milk yield (Hagnestam-Nielsen *et al*., 2009). It leads to increased milk somatic cell counts (SCC) (Prescott & Breed, 1910) and impaired milk quality (Auldist *et al*., 1998). Therefore, dairy plants in many countries, such as Sweden, give a price reduction for milk with increased SCC whereas extra good quality milk may get a premium payment. Even mild SCC reactions may affect yield and quality (Hamann *et al*., 2002). SCC has a dynamic pattern over time and may be influenced to some extent by management factors, such as milking interval (for review, see Davis *et al.,* 1999). Thus, elevated SCC may appear without a pathological background. However, the economic consequences for the dairy farmer may be the same, reduced yield and milk payments. Milking interval is an important aspect of automatic milking (AM). AMS enables frequent milking which, per se, has been shown to increase the cow's daily milk yield (Wagner-Storch & Palmer, 2003). In herds with AM, the SCC at cow level is generally higher than in herds with conventional milking (Rasmussen *et al*., 2001). This has partly been ascribed to an increased spread of mastitis pathogens due to lack of milking order but, in part, also to irregular milking intervals and long intervals due to incomplete milkings (Bach & Busto, 2005). A standstill due to technical failure in an AMS may lead to a severely prolonged milking interval for many cows because only one cow at a time can be milked when milking is resumed. Such an interruption is often associated with a subsequent increase in the tank milk (Pettersson *et al*., 2002).

The economy of the modern industrialized dairy sector is dependent on cost-effective milk production. To optimize profitability and animal welfare, registrations for the recording and monitoring of animal health and production have become increasingly fine-tuned. Several milking systems enable registration on-line and in the AMS even at the level of the individual quarter. More frequent and detailed registration of milk parameters at farm level requires improved knowledge of physiological variations to interpret when deviations indicate a pathological process. False alarms increase the risk of over-treatment and cause the farmer unnecessary work, while correct early warning and action, e.g. in case of mastitis, are beneficial. Hence, it is important to map and understand in detail, also subnormal processes occurring in the mammary gland. One such process is the reaction after a single prolonged milking interval, being described and discussed in this PhD thesis. First, some information about the healthy udder and milk, and how it is influenced by inflammation, is presented.

1.2 The healthy udder

1.2.1 Physiology of lactation

Lactation denotes the dynamic process of milk synthesis and secretion. In addition to hormonal regulation, maintenance and intensity of milk synthesis and secretion is, to a high degree, regulated through factors such as milking and suckling (Mepham, 1987).

Hormones involved in milk synthesis and secretion

The activity of several lactation-related hormones is necessary for initiation and maintenance of milk synthesis and secretion (Mepham, 1987; for review see Svennersten-Sjaunja & Olsson, 2005). The initiation of lactation in connection to parturition is a result of the interaction of hormones such as oestrogen, progesterone and prolactin (PRL). A surge in plasma PRL is observed shortly prior to calving (Mepham, 1987).

Prolactin (PRL) is a polypeptide that exerts a number of biologically active roles (Mepham, 1987). It is mainly synthesized in the pituitary gland. In addition, the local synthesis and secretion of PRL by mammary epithelial cells have been shown (Le Provost *et al*., 1994; Lkhider *et al*., 1997). Prolactin strongly influences cell differentiation and proliferation (Akers *et al*., 1981). In mammary epithelial cells, PRL enhances metabolism through the maintenance of high concentrations of mRNA, which influence the intensity of milk protein synthesis. Thus, PRL is frequently reported as an anabolic hormone. Prolactin also plays a role in the maintenance of tight junctions (TJ) (Linzell *et al*., 1975, Cowie *et al*., 1969). This is important since it is known that increased TJ permeability is associated with decreased milk secretion rate (Allen, 1990). More recent research indicates that the PRL effect on TJ is mainly exerted through the maintenance of mammary epithelium by keeping the DNA content of the cells constant (Flint & Gardner, 1994). The plasma PRL levels are significantly enhanced by suckling and milking but also by the stress (Bole-Feysot *et al*., 1998; Dorshkind & Horseman 2001; do Amaral *et al*., 2010). A surge of PRL was observed upon teat stimulation and it remained increased up to one hour subsequent to milking (Gorewit *et al*., 1992). Additionally, factors not related to stimulation of the udder, such as day length, have been shown to have a profound effect on PRL levels in plasma (Rius *et al*., 2005; Auchtung *et al*., 2005).

Oxytocin is a hormone required for successful milk ejection (Mepham; 1987). It is a pituitary hormone synthesized in the supra-optic and

paraventricular nuclei of the hypothalamus (Akers, 2002) that is released when the cow sees the calf and in response to teat stimulation. The teat has a well developed sensory innervation. Signals from the stimulated receptors are transmitted to the brain; thereafter oxytocin is transported to the neurohypophysis from where it is released to the blood circulation, and the milk ejection reflex is triggered. The main effect of oxytocin is expressed through its activity on the myoepithelial cells surrounding the alveolei, the milk synthesizing units, squeezing the milk into the duct system and further to the udder cistern (Knight *et al*., 1994; Pfeilsticker *et al*., 1996; Bruckmaier *et al.,* 1994; Bruckmaier & Hilger, 2001). The main part of the milk yield is stored in the alveolar region between milkings; thus and without the proper concentration and activity of oxytocin, milk ejection does not work properly. The lag time from the start of teat stimulation until onset of milk ejection ranges from 40 s to more than 2 min and depends on the degree of udder filling (Bruckmaier *et al.,* 1994; Bruckmaier & Hilger, 2001). A supraphysiological concentration, on the other hand, may be harmful because it has been shown to cause opening of the tight junctions (TJ), which in turn may result in reduced milk yield (Allen, 1990; Linzel & Peaker, 1971).

Maintenance of lactation is, additionally, influenced by *corticoids* (Topper & Freeman, 1980). Cortisol, as a primary glucocorticoid and stress hormone in cows, inevitably plays a role in differentiation of the mammary alveolar secretory cells in the final stages of lactogenesis. It also promotes transcription of the genes for caseins and α-lactalbumin (ALA; Akers, 2002). Cortisol has a general supporting effect on the metabolism of the lactating animal but also supports the secretory function of the epithelial cells and maintains an intact-coherent mammary epithelium (Zettl *et al*., 1992; Stelwagen *et al*., 1998).

1.2.2 Milk formation

Cow milk is a complex fluid that is a colloidal dispersion of fat globules and protein (casein and whey proteins) in an aqueous solution of lactose, minerals, vitamins and other minor constituents. The complex process of milk synthesis starts with recruiting the essential nutrients from the blood and thereafter transporting them into epithelial cells (Mepham, 1987). Most of the milk constituents are synthesized *de novo* in the mammary epithelial cells, while a few, e.g. ions, some proteins and immunoglobulins, pass unchanged from blood to the milk compartment.

Fat

The fat content in cow milk varies between 3.8 - 4.9% (Akers, 2002; Blowey & Edmondson, 2010). To date 440 different fatty acids have been indentified in

milk. Although the basolateral membrane is involved in absorption of invariably long fatty acids such as C16 and C18, the shorter fatty acids C4-C14 are synthesized *de novo* in the mammary gland (Jenness, 1986; Mepham, 1987). In ruminants fatty acid synthesis is also performed via acetate and b-hydroxibutyrate (Mepham, 1987). Triglycerides are synthesized in the smooth endoplasmic reticulum of epithelial cells, where the small fat droplets are formed. The microlipid droplets with a surface of protein and polar lipids are released in the cytoplasm (Mather & Keenan, 1998). Thereafter, numerous small droplets fuse and move towards the apical part of the membrane where they are secreted. The fused fat droplets are wrapped with an epithelial membrane which protects the fat from lipolysis. In this way a milk fat globule (MFG) is formed. Thus, the amount of membrane material is an important factor for the lipolytic resistance of MFG (Evers, 2004).

Proteins

The protein content in cow milk varies between 3.0-3.6% (Akers, 2002; Blowey & Edmondson, 2010). The proportion of the milk synthesized proteins and whey proteins is approximately 80 and 20%, respectively. Several amino acid transport systems are involved in the transfer of the different amino acids from one side of the basolateral membrane to the other for protein synthesis (Jenness, 1986; Mepham, 1987). Inside the cell, amino acids are covalently bound and form proteins in ribosomes and by the rough endoplasmatic reticulum (Mepham, 1987). Ribosomes are the major site of the synthesis of internal proteins (for instance most enzymes, proteins involved in cell-to-cell contacts and membrane bound enzymes), while proteins intended for secretion in the milk are synthesized by the endoplasmatic reticulum. For casein formation, proteins are further processed in the Golgi apparatus where the casein molecules, calcium and phosphorus form casein micelles. The major proteins that can be found in milk are several kinds of casein (milk proteins), ALA and β-lactglobulin (whey proteins). ALA is significantly important for the synthesis of lactose. Milk proteins and lactose are transported to the apical membrane of the cell via secretory vesicles that bud on the Golgi.

Lactose

Lactose represents the major carbohydrate-disaccharide in bovine milk with a content that varies between 4.6 and 4.8% (Akers, 2002; Blowey & Edmondson, 2010). Lactose is one of the major energy sources in bovine milk but is also a component that significantly regulates osmolarity and contributes to the iso-osmotic balance in the udder (Mepham, 1987). Thus, the volume of the synthesized and secreted milk is largely dependent on the amount of available

lactose. When lactose synthesis is impaired or the concentration is decreased, for instance due to leakage through impaired tight junctions, milk osmolarity is maintained by sodium and chloride ions (Kuhn *et al*., 1980; Mepham, 1987). Lactose is a milk parameter that is minimally subjected to changes under physiological conditions. It is synthesized in the Golgi apparatus from two molecules of glucose which are first transformed into galactose (Kuhn *et al*., 1980; Mepham, 1987). In general, the transformation of glucose into galactose occurs within the cell but, additionally, synthesized galactose may, to some extent, be absorbed by the epithelial cells. The enzyme lactose synthase plays an inevitable role as catalyst in lactose synthesis. It consists of galactosyltransferase and ALA. These enzymatic subunits, particularly ALA, have a strong impact on lactose synthesis. A restricted availability of ALA means a significant limiting factor for lactose synthesis. Exclusively in lactose synthesis, galactosyltransferase has a significant role during glycoprotein biosynthesis.

1.2.3 Cells in milk

The expression "somatic cell count" (SCC) denotes the concentration of body cells present in bovine milk. They mainly consist of leukocytes. (for review, see Burvenich *et al.,*1995). The mammary gland is unique compared to other organs in that leukocytes in fairly large numbers are also present in the normal secretion. Milk from a healthy bovine mammary gland may contain up to 100 x 10^3 cells/ml although some findings indicate that the upper threshold value should be less, such as 50 000 cells/ml (Hillerton, 1999; Hamann, 2002; Berglund *et al*., 2007; Forsbäck *et al*., 2010). The probability that a mammary gland with milk SCC below 100 000 cells/ml is harbouring an infection is low (Brolund *et al.,* 1985). In practice, mastitis control programmes often apply a higher upper threshold value of SCC in cow composite milk as "normal". Several physiological factors can, to some extent, influence the SCC, which must be considered when interpreting if a cow's SCC is normal or not.

Milk leukocyte populations consist of polymorphonuclear neutrophils (PMN), monocyte-macrophages and lymphocytes, and to a small extent, epithelial cells (for review see e.g. Burvenich *et al.*, 1995 and Sordillo *et al.,* 1997). No strict normal values have been established for the differential cell counts in milk, but most researchers who have studied milk from *healthy* glands, i.e. with a SCC < 100 x 10^3 cells/ml, report values of < 25% PMN and > 70% monocyte-macrophages, with a small proportion of lymphocytes (Fox & Schultz, 1985; Ostensson *et al.*, 1988; Ostensson, 1993b; Pillai *et al.*, 2001; Rivas *et al.*, 2001; Lindmark-Månsson *et al.*, 2006). Most studies show even lower values, < 20%, for the proportion of PMN. Milk lymphocytes are mainly

T lymphocytes, constituting up to 60 % of the lymphocyte population, and a smaller proportion of B cells of < 20% (Park *et al.,* 1992; Taylor *et al.,* 1994). The T cells consist mostly of CD 8+ cells, known as T cytotoxic cells and, to a lesser extent,CD4+cells or T helper cells (for review see Sordillo *et al.,* 1997).

1.3 Mastitis – a non-infectious or infectious condition

1.3.1 Definition

Mastitis means inflammation of the mammary gland. The inflammatory reaction is a response to an insult such as bacteria, toxins, chemical agents and physical trauma (Tizard, 2007). Thus, the terms infection and inflammation should be distinguished from each other. The inflammatory status of the udder can be evaluated by measuring the concentration of various factors in milk that change during the inflammatory reaction; i.e. inflammatory indicators (Sandholm, 1995a). Infection can only be diagnosed by bacteriological examination of the milk. Pronounced mastitis is usually a consequence of invasion by microorganisms. Milder mastitis reactions might occur as a result of management factors, for instance related to milking (Fernando & Spahr, 1983; Stelwagen & Lacy-Hulbert, 1996; Clark *et al*., 2006). Additionally, certain physiological conditions are known to be associated with an increased SCC, the most frequently used inflammatory indicator (for review see Harmon, 1994).

1.3.2 Forms and prevalence of mastitis

Mastitis is one of the most prevalent and costly diseases in dairy cows and a factor that severely affects milk production, health and welfare (Hortet & Seegers 1998; Seegers *et al*., 2003; Hagnestam *et al*., 2007; Hogeveen *et al*., 2011). The mastitis complex represents a huge economic problem, primarily attributable to decreased milk production but also due to early cow replacement, discarded milk and impaired milk composition (Østerås, 2000; Halasa *et al*., 2009). Mastitis can be divided in two forms according to the symptoms: clinical and subclinical. Clinical mastitis exhibits visible signs of inflammation while subclinical mastitis remains silent and can be diagnosed only with laboratory methods. The subclinical form is most prevalent and causes the greatest economic losses, primarily through reduced milk yield. Clinical mastitis cases can be detected easily and treated if necessary, while the silent subclinical form may remain undetected unless laboratory analysis of the milk is performed regularly, and in many cases becomes chronic. Elimination

of mastitis infections by treatment with antimicrobials often fails, particularly in subclinical mastitis (Barkema *et al*., 2006 ; van den Borne *et al*., 2010). The incidence of subclinical mastitis may vary from country to country but in Sweden around 2/3 of the cows are affected per lactation (Swedish Dairy Association, 2010). The incidence of clinical mastitis, based on veterinary treated cases, is between ca 15 – 20 %.

1.4 How inflammation and physiological factors affect milk SCC and cell populations

The cell concentration in milk has, for many decades, been the sovereign indicator of mammary gland inflammatory status in cows. In the beginning of the last century, Prescott & Breed (1910) microscopically observed so-called "body cells" in milk and found an increased concentration during mastitis. Some decades later, Paape *et al*., (1963) introduced the current term somatic cell count. Inflammation has a strong effect on SCC. In addition, the SCC can, to some extent, be influenced by physiological and management factors (for review see Harmon, 1994), or more correctly, physiological and management factors may also cause inflammatory conditions in the udder, as reflected in an increased SCC. Some inflammatory reactions in the udder that occur without pathological reasons are considered as physiological inflammation (Sandholm, 1995b; Manlongat *et al*., 1998).

1.4.1 Mastitis and its influence on SCC

Inflammation initiates an intensive and enhanced recruitment of leukocytes, especially PMN, from blood to the udder and milk, in order to eliminate or neutralize the insult (for review, see Sordillo *et al*., 1997). The SCC increases mainly due to an enhanced concentration of PMN which may rise to almost 100% of the total SCC during severe inflammations. During an intramammary infection, the SCC may rise very quickly and reach concentrations of several 10^6 cells/ml. An extremely high SCC is more regularly seen in clinical than in subclinical mastitis, in which the SCC elevation may vary more. The increased proportion of PMN during mastitis results in a decrease in the proportion of monocyte-macrophages while the relative contribution of lymphocytes to the SCC remains almost unaltered (Saad & Ostensson, 1990; Ostensson, 1993a). Among the milk lymphocytes there is a shift towards a predominance of CD4+T lymphocytes during mastitis, in contrast to non-mastitic milk where CD8+T lymphocytes prevail (for review, see Sordillo *et al.,* 1997).

1.4.2 Physiological factors affecting SCC

A number of physiological and management factors, such as the stage of lactation, lactation number, breed, season, milk yield and milking routines, have been shown to influence SCC (Brolund, 1985; for review, see Harmon, 1994). Thus, various physiological conditions should be considered when interpreting SCC. The SCC varies physiologically depending on the *stage of lactation* (Schepers *et al*., 1997; Piccinini *et al*., 2007). Increased SCC is observed for up to two weeks after calving and when lactation ceases (Miller *et al*., 1991; Manlogat *et al*., 1998). Additionally, an increased presence of PMN in milk at the onset and offset of the lactation has been reported (McDonald & Anderson, 1981a, b; Miller *et al*., 1991). Towards the offset of lactation SCC may also be influenced by milk yield; the lower the milk yield, the *more concentrated the milk* and the higher the SCC (Dohoo & Meek, 1982; Reneau, 1986; for review see Harmon, 1994). A concentration effect may also be a factor behind the proportional increase in SCC seen after *feed and water deprivation*, associated with decreased milk yield (Reneau, 1986; Kefford *et al*., 1995). Although an increased SCC is seen in cows of higher parity, it is more likely to be an effect of a higher prevalence of mastitis with parity than of parity per se (Emanuelson *et al.,* 1988). *Season* may also affect the SCC under certain conditions. In a study performed in Wisconsin, US, pronounced peaks in SCC were observed during periods of high temperatures in July and August, although the SCC was also elevated from April to October, compared with the winter season (Bodoh *et al.,* 1976). Therefore, it appears that the effect of season is not solely attributable to high temperature. During milking the SCC varies depending on the *fraction of milk*. It is higher in foremilk and strippings than in bulk milk, and highest in residual milk (Paape & Tucker, 1966; Ostensson *et al.*, 1988). Finally, *milking frequency* appears to have a strong influence on milk SCC (Fernando & Spahr, 1983; Stelwagen & Lacy-Hulbert, 1996; Clark *et al.,* 2006), having been shown to increase in response to prolonged milking intervals as well as very short milking intervals. Few studies have investigated the effect of single prolonged milking interval on SCC at cow level being described in this thesis but elevated SCC has been observed in herd *tank milk* after omitting one milking (Pettersson *et al*., 2002). Additionally, SCC shows a very varying and dynamic pattern over time, with short, transient periods of increased SCC which have been ascribed to *normal variation* and/or physiological inflammatory episodes.

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1.5 The inflammatory reaction and subsequent changes in milk

1.5.1 Inflammation – the key reaction of the innate immune system

The closing mechanism of the teat canal and its antibacterial keratin layer, soluble antibacterial factors present in normal milk, and the flushing out of milk, constitute important parts of the passive defence mechanisms of the mammary gland (Sandholm & Korhonen, 1995c). If invading pathogens have managed to overcome the passive defence mechanisms, the immune defence is activated as an ultimate line of defence (for review, see Sordillo *et al*., 1997; Tizard, 2007). Mastitis is primarily combated by the innate immune system. Inflammation stands for the key function of the innate immunity: to rapidly recruit humoral and cellular defence mechanisms to the site of injury or microbial invasion, to neutralize invaders, initiate the healing process and re-establish normal organ function. Although the innate immune system lacks memory, it promptly responds upon identification of a pathogen (Tizard, 2007) and the reaction is often sufficient to terminate an infection before clinical manifestation of the disease has occurred. Principally, the innate immune system is non-specific and based on recognition of pathogen-specific molecules that make them chemically diverse from normal body components (for review see Rainard & Riollet, 2003). The major leukocyte types involved in the innate immune system are PMN, monocytes-macrophages and lymphocytes, especially the population of natural killer (NK) cells. Besides alterations in cellular and humoral components, the vascular system is significantly affected during inflammation, as manifested by, for example, increased permeability.

1.5.2 Induction of the inflammatory reaction

Macrophages constitute the largest proportion of the cells in normal milk and play a role in early inflammatory stages, and in recognizing invaders and foreign factors (Sandholm, 1995b; for review see Sordillo *et al*., 1997). They are capable of sustainable phagocytosis but mainly act as scavengers at the end of the inflammatory process and, thereafter, aid in re-establishing physiological functions. Macrophages are also antigen processing cells and are important for antigen presentation to lymphocytes to initiate antibody production (Riollet *et al*., 2000). Macrophages are extremely potent producers of the cytokines (Tizard, 2007) necessary for initiating and mediating an inflammatory process, either alone or, often, interacting with other factors (Craven, 1983). The inflammatory reaction starts when macrophages identify the insulting factor (microorganisms or others) as foreign. This recognition process induces a pronounced production of cytokines in the macrophages, among which some

have an attractant effect on PMN. Cytokines may also be produced by tissue cells and leukocytes. Thus, they may increase in milk as a result of leakage from damaged cells or tissue fluid and/or blood in case of trauma and/or conditions of, for example, impaired TJ, and initiate inflammation. Even if these cytokines might act by stimulating macrophages and other cells to further cytokine production, they may also act directly, inducing an enhanced PMN recruitment to milk and initiation of other inflammatory responses.

1.5.3 PMN response

After recognition, the first phase of the inflammatory response is characterized by an intensive and rapid recruitment of PMNs to the site of inflammation, to neutralize microorganisms or other insulting factors (Tizard, 2007). To migrate from the blood compartment into the tissue the PMN must first be able to adhere to the endothelium. This is achieved through the expression of adhesion proteins on the endothelial cells and circulating leukocytes, upon inflammatory stimuli. After adhesion, the PMN migrate between the endothelial cells to the tissue, at the inflammatory site. Since milk exhibits chemoattractant properties, many PMN move further between the epithelial cells of the udder, into the milk (Sandholm, 1995b). PMN eliminate microorganisms through phagocytosis and intracellular killing by the oxygen burst (for review, see Burvenich *et al*., 1995; Paape *et al*., 2002). This phagocytic function is considered to be the most important defence of the udder, and PMN to be the most important actors to combat mastitis. The phagocytic capacity of the PMN becomes reduced during the time they spend in milk and the highest viability has been observed of PMN isolated from residual milk (Sarikaya *et al*., 2005). This phenomenon has been ascribed to loss of energy and exhaustion by phagocytosis of casein micelles and fat globules (Paape *et al*., 2002).

1.5.4 Cytokines

The inflammatory reaction aims to neutralize the insulting factor and restore normal function. This cannot be achieved solely through recruitment of PMN to the inflammatory site. The inflammatory process is a synchronized action of immune mediators, such as cytokines, with a wide variety of functions. Cytokines are small soluble proteins (<50 kDa) that act in low concentrations to initiate and mediate the inflammatory reaction in different ways, principally as communicators between leukocytes (Tizard, 2007). They influence cytokine secretion by other cells (paracrine action) and also act in a self-regulating manner (autocrine action; Tizard, 2007). Cytokines represent potent regulators of haematopoiesis, stress, inflammation, immunity and tissue repair (Belardelli & Ferrantini, 2002; Rouveix, 1997; Ebersole & Cappelli, 2000) and can be

divided into different groups according to their principal functions: chemokines, interferons, colony-stimulating factors, peptide growth factors and tumor necrosis factors (Nathan & Sporn, 1991; Sordillo *et al*., 1997; Ebersole & Cappelli, 2000; Alluwaimi, 2004).

Proinflammatory cytokines exert their main role through initiation of the host immune defence, e.g. by affecting the vascular system and priming of leukocytes, inducing chemotaxis and enhancing the phagocyting potential. Additionally, some cytokines have a down-regulatory role to ensure that the inflammatory reaction is terminated. Independently of the grade of an inflammatory stimulus, cytokine effects are expressed only after binding to special receptors (Akira *et al*., 2006). Cytokines can be synthesized and secreted by many different kinds of cells in the body, which are not necessarily organized in tissues, such as leukocytes. This makes the key distinction between hormones and cytokines although they act in similar ways (Okada *et al*., 1997; Paape *et al*., 2002; for review see Alluwaimi, 2004). The main cytokines that have been detected in bovine mastitis are interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12, TNF-α, colony stimulating factor (CSF) and interferon (IFN)-γ (for review, see Sordillo *et al*., 1997 and Alluwaimi, 2004). The mRNA for numerous cytokines has been identified in the healthy bovine mammary gland although the cytokine concentration in normal tissue and milk is negligible (Hagiwara *et al*., 2000; Alluwaimi *et al*., 2002).

The cytokines found to have the most profound effect on PMN chemotaxis during inflammatory reactions are IL-1, IL-8, tumor necrosis factor alpha (TNF-α) and Complement-5a (Nakagawa-Tosa *et al*., 1995; Alsemgeest *et al*., 1996). IL-1 and TNF-α are rapidly secreted in response to an inflammatory challenge. In the initial stages of inflammation, TNF-α regulates the innate immune response in unison with IL-1β and, in the later stages, in unison with IL-6, which facilitates the transition from innate to acquired immunity by antigen presentation and stimulation of T cells. There is a strong correlation between the blood concentrations of TNF-α and IL-6 in cows with mastitis (Sordillo & Peel, 1992; Hagiwara *et al*., 2001). Under the influence of IL-1β, arachidonic acid is converted to leukotriens, known to have a potent effect on PMN migration (Shuster *et al*., 1995; 1997; Riollet *et al*., 2000). IL-8 is a particularly potent cytokine for activation of PMN, enhancing their migration and respiratory burst (Tizard 2007).

The increased vascular permeability and other vascular events occurring during inflammation are mainly regulated by specific vasoactive cytokines, such as histamine, serotonin, kinins and prostaglandins. Cortisol, which is a stress related hormone, exerts a general profound effect on the immune defence through different inflammatory mediators (Tizard, 2007). Long-term stress is

considered to suppress immune function and increase susceptibility to infection, mainly due to the effect of cortisol (Dhabhar, 2009), while short-term stress may boost the immune response (Ortega *et al*., 1997). Increased serum cortisol levels in cows, e.g. in relation to transport-induced stress, have been associated with increased milk SCC (Yagi *et al*., 2004; Gygax *et al*., 2006).

1.5.5 Acute phase response

Acute phase proteins (APP) are serum proteins that increase shortly after exposition to an inflammatory stimulus. The synthesis and secretion of APP occurs primarily in hepatocytes upon stimulation by IL-1, IL-6 and TNF-α (Tizard 2007). The main APP in the bovine species are serum amyloid A (SAA) and haptoglobin (HP), while α1-acid glycoprotein, C reactive protein (CRP) and fibrinogen are less important (for review, see Petersen *et al*., 2004). Substantially increased concentrations of SAA and HP in serum have been observed in the acute phase of inflammation in the bovine. Additionally, the concentration of both proteins increases in milk during mastitis, and local production in the mammary gland has been shown (McDonald *et al*., 2001; Jacobsen *et al*., 2005). It has been found experimentally that in endotoxin-induced mastitis the up-regulation of the APP genes for SAA and HP occurs shortly after challenge, while increased concentrations of respective proteins appear later, at 8 and 12 h respectively, declining after 24-48 h (Vels *et al*., 2009).

Haptoglobin and SAA in both serum and milk have a high sensitivity and specificity for differentiating between healthy animals and those with clinical mastitis (Eckersall *et al*., 2001). They have also been found to be positively correlated with SCC in moderate mastitis reactions (Åkerstedt *et al*., 2007). Thus, quantification and monitoring of APP have been suggested as a means of diagnosing and mirroring inflammatory status in the mammary gland. SAA and HP have both been shown to exhibit immunomodulatory effects and SAA especially can enhance chemotaxis of PMN, monocytes and T-lymphocytes (for review, see Petersen *et al*., 2004).

1.5.6 Indirect measures of SCC and mammary gland permeability used as inflammatory indicators

The inflammatory reaction results in a number of changes in milk that can be used as indicators of mastitis (Sandholm, 1995a). Some are well correlated with SCC. Performing milk cell counts requires fresh milk, which is a limiting factor. The concentration of several enzymes and blood proteins increase in

milk during mastitis and can be used as indicators of mastitis, even after the milk has been stored frozen.

Enzymes

An indirect estimation of the SCC and PMN proportion in milk might be obtained through analysis of intracellular enzymes. The concentration of enzymes originating from PMNs increases exponentially with increased milk SCC (Sandholm, 1995a). Enzymes such N-acetyl-ß-D glucosaminidase (NAGase), lactate dehydrogenase (LDH), ß-glucuronidase and catalase are released from phagocytes as a result of phagocytosis and cell lysis, but they might also leak from damaged epithelial cells (Bogin *et al*., 1977; Emanuelson *et al*., 1987; Berning & Shook,1992). In mastitic milk their main source is considered to be the leukocytes. LDH and NAGase have been used in routine work with large sample quantities, and have shown a strong correlation with SCC (Kitchen *et al*., 1980; Zank & Schlatterer 1998). Concentrations of NAGase and LDH have also been shown to vary based on breed as well lactation stage. (Ostensson 1993b; Chagunda, 2006). It is considered that the intracellular concentration of these enzymes is fairly constant in healthy mammary glands. Similarly, the adenosine triphosphate (ATP) content of all living cells is approximately constant. Milk concentration of ATP can also be used as an indirect measure of SCC attributable to its positive correlation with SCC (Emanuelson *et al*., 1987). The disadvantage of ATP in comparison with SCC, LDH and NAGase is that ATP is unstable and rapidly degrades after the sample is taken if it is not stabilized, e.g. by EDTA. ATP has only been used to a small extent in practice as a mastitis indicator (Emanuelson *et al*., 1987).

Serum proteins

Apart from APP, blood-derived proteins such as bovine serum albumin (BSA) and anti-trypsins may be increased in milk during mastitis (Sandholm, 1995a). They indicate increased permeability in the endothelium and epithelium as an effect of inflammation. Thus, the content of the blood protein in milk adds information about the characteristics of the inflammatory process compared to the SCC. BSA and antitrypsins have a fairly good correlation with the SCC, although not as high as PMN, NAGase and ATP (Emanuelson *et al*., 1987). No causal relation exists between increased permeability and the enhanced recruitment of leukocytes to the milk during the inflammation. Analyses of serum proteins have mainly been used in research (Kitchen *et al*., 1980; Honkanen-Buzalski & Sandholm, 1981).

Lactose

The lactose content in milk is highly correlated with the inflammatory status of the mammary gland. The mastitis reaction causes tissue damage, resulting in disturbed synthesis of milk with depressed biosynthesis of lactose and consequently a lower lactose level in milk (Mepham, 1987). Lactose is a constant parameter in milk from healthy udder quarters and appears to be almost constant from one lactation to the next. The relation between SCC and lactose has been a subject of interest (Vangroenweghe *et al*., 2002; Berglund *et al*., 2007) in case lactose could be used as a reliable indicator of mastitis. Analysis of lactose is inexpensive and the handling of milk samples for analysis is easy. Lactose as an inflammatory indicator seems to be useful at the udder quarter level but is less reliable in tank milk (Berning & Shook, 1992; Berglund *et al*., 2007).

1.5.7 Influence of mastitis on milk yield and composition

The yield and composition of milk is influenced by the health status of the udder. Clinical mastitis results in a pronounced increase in SCC and intracellular enzymes, increase of serum proteins, ions and proteolytic and lypolytic enzymes derived from blood, and a decreased lactose content (see e.g. Sandholm, 1995a). The deleterious effects of mastitis on milk constituents vary depending on the intensity of inflammation. The inflammatory reaction is rapidly reflected in elevated SCC which, through their proteolytic enzymatic activity, especially by the PMNs, negatively affect the milk quality when they are present in high concentration (Le Roux *et al*., 2003). The inflammatory reaction affects major (lactose, protein, casein, fat) and minor milk constituents (minerals and enzymes) to varying degrees, attributable to their different amounts, ways of synthesis and chemical composition (Miller *et al*., 1983; Randolph & Erwin, 1974). Thus, lactose and casein content decrease, while the concentrations of serum proteins, fat and minor components such as minerals and enzymes, increase. This leads to impaired milk quality and processing properties of the milk (Auldist *et al*., 1998). Mastitis, additionally, causes decreased milk synthesis and reduction in yield. The latter has been estimated to be as much as 5 % during a lactation affected with subclinical mastitis (Hagnestam-Nielsen *et al*., 2009). The changes in milk yield and composition have been observable at a SCC as low as 50 x 10^3/ml (Tolle *et al*., 1971; Korhonen & Kaartinen, 1995; Hamann *et al*., 2002).

The lactose content of cow milk with an elevated SCC has been observed to be decreased (Miller *et al*., 1983). The lactose concentration has been shown to be sensitive to inflammation and a significant decrease has been observed, not

only during clinical mastitis (Claesson, 1965; for review see Harmon, 1994) but also when the SCC is moderately increased (Berglund *et al*., 2007). In comparison with other milk components the lactose concentration in the healthy udder is, constant, most likely due to its osmoregulating function (Candek-Potokar *et al*., 2006; Forsbäck *et al*., 2010). The lowered lactose content during mastitis is considered to be partly due to depressed synthesis and increased enzymatic degradation of lactose but also partly to leakage of lactose from the alveolus to the circulating blood because of disturbed integrity of the tight junctions during inflammation (Stelwagen *et al*., 1997; Coulon *et al*., 2002; Bruckmaier *et al*., 2004). To maintain the osmolarity in the milk, sodium and chloride pass from blood to milk, resulting in an increase of these ions in milk during mastitis.

The total milk protein concentration increases parallel with the SCC (Auldist & Hubble, 1998). Clearly altered milk protein profiles have been observed at SCC levels of slightly more than 100 x 10^3/ml (Urech *et al*., 1999). During inflammation in the udder, total milk protein concentration is increased due to a higher content of whey (serum) proteins, while the casein content has been observed to be lowered. According to Korhonen & Kaartinen (1995), major whey proteins such as β-lactglobulin and ALA are negatively affected during mastitis, due to lower synthesis as well as proteolysis, although the elevated content of BSA is due to leakage from the blood into milk through impaired TJ. The decrease of the casein content is considered mainly to be attributable to epithelial cells damaged during the inflammatory process. The negative balance in the protein content during inflammation can be ascribed to a certain extent to the increased content of both proteolytic enzymes (e.g. plasmin, plasminogen and cathepsin) and lipolytic enzymes that is observed during the course of inflammation. It appears to be a result of leakage from the blood compartment through impaired TJ and leads to enhanced degradation of protein and fat in mastitic milk.

High milk SCC also negatively influences the content of fat in milk, apparently due to decreased fat synthesis in the epithelial cells (Randolph & Erwin1974). Mastitis is associated with increased concentrations of free fatty acids (FFA) in milk which is indicative of fat deterioration. Fat hydrolysis is catalyzed by lipoprotein lipase which originates in epithelial cells (Wiking *et al*., 2006).

1.6 Natural milk components with immunomodulatory properties

There is an emerging field of research on common milk constituents and factors naturally present in milk, which might have immunomodulatory properties and induce physiological inflammatory reactions.

1.6.1 Prolactin

In addition to its lactational role, PRL has been shown to have a significant involvement in immune functions, playing an important role in signalling between immune and neuro-endocrine systems (for review, see Yu-Lee, 2002). Prolactin has been found to trigger a pro-inflammatory immune response and stimulate PMN chemotaxis (Brand *et al*., 2004; Boutet *et al*., 2007). Upon PRL stimulation, bovine mammary epithelial cells significantly amplified mRNA expression for several proinflammatory cytokines, such as: IL-1, IL-6, IL-8, granulocyte macrophage colony stimulating factor (GMCSF; delays PMN apoptosis) and TNF-α (Boutet *et al*., 2007). In contrast to its indirect chemotactic role, PRL might also have a direct effect on human macrophage and PMN cells (Dogusan *et al*., 2001; Ortega *et al*., 1997).

1.6.2 Alpha lactalbumin and other whey proteins

Several whey proteins and casein have been shown to exhibit immunomodulatory properties (Epps *et al*., 1977; Wong *et al*., 1997a, b; Rusu *et al*., 2009, 2010). ALA constitutes a major fraction of the whey proteins. It is a 142 amino acid long protein that, apart from its role in lactose synthesis (Ramakrishnan *et al*., 2001) and indirectly in balancing osmotic pressure in the milk, is suggested to play an immunomodulatory role (Wong *et al*., 1997b). However, the results are somewhat contradictory in that some researchers have indicated an inhibitory effect of whey proteins on PMN migration (Wong *et al*., 1997a, b) whereas others suggest a stimulatory effect on chemotaxis, phagocytosis, oxidative burst and degranulation (Rusu *et al*., 2009, 2010). Whey proteins have also been found to enhance the accumulation of cytokines, such as IL-1α, IL-8, IL-6, macrophage inflammatory protein (MIP)-1alpha, MIP-1beta, and TNF- α.

1.7 The effect of milking frequency on SCC, PMN, milk yield and composition

1.7.1 SCC and PMN

The length of the milking interval has been observed to influence the milk SCC. Milking once a day increases the SCC (Clark *et al*., 2006; Stelwagen & Lacy-Hulbert, 1996). Once-daily milking (ODM) on a regular basis also results in an increased proportion of PMN along with the increased SCC (Stelwagen & Lacy-Hulbert, 1996), while one omitted milking seems not to influence the proportion of PMN (Fox & Schultz, 1985) but studies of a *single* prolonged milking interval are sparse. Herds with AM, generally have higher SCC in tank milk as well as at the cow level (Rasmussen *et al*., 2001, 2002). This might be attributable to the increased risk of spreading mastitis pathogens between cows but to some extent, it has also been ascribed to irregular and irregularly occurring long milking intervals due to incomplete milking (Bach & Busto, 2005). Observations on the influence of frequent milkings, i.e. more than two times daily, on udder health and SCC are contradictory. An increased SCC in cows milked with short intervals (3 h) has been observed (Fernando & Spahr, 1983), while other studies have shown significantly lower milk SCC in cows milked four or six times a day (Dahl *et al*,. 2004; Shields *et al*., 2011). High milking frequency has been found to increase mastitis susceptibility (Philpot & Nickerson, 2000).

1.7.2 Milk yield and composition

The length of the milking interval has a significant influence on milk composition and yield (for review, see Davis *et al*., 1999; Bernier-Dodier *et al*., 2010). *Frequent milking* (FM) is associated with increased daily *milk yield*, which has been ascribed to enhanced cell proliferation and differentiation as well as increased milk synthesis (Soberon *et al*., 2010). FM has been shown to be positively correlated not only with milk yield but also *protein content* (Sorensen *et al*., 2001; Dahl *et al*., 2004; Bernier-Dodier *et al*., 2010). The benefit of FM on protein content is in lower activity of the enzyme plasmin, and shorter storage in the udder, which lead to lower degradation of protein (Sorensen *et al.,* 2001). Low udder pressure due to lower milk volume stored in the udder between milkings during periods of frequent milking, may also enhance the stability of tight junctions and thereby diminish leakage between blood and milk. *Milk fat* content may be affected negatively (Klei *et al.,* 1997) or positively (Dahl *et al*., 2004) by FM. The negative influence of FM on the fat have been explained in different ways including increased air exposure due to frequent milking, raised enzymatic activity of fatty acid syntethase, and

higher production of short-chain fatty acids (Klei *et al.,* 1997). FM has been shown to give undesirable effects on milk fat in terms of increased content of free fatty acids (Svennersten-Sjaunja *et al.,* 2002), which may impart for a sour-flavour to the milk.

Regularly applied *long milking intervals* lead to reduced *milk yield*. The milking interval should be less than 18 hours to avoid adverse effects on milk yield and milk quality (Stelwagen *et al.*, 1997; Bach & Busto, 2005). The lower milk yield observed during once daily milking (ODM) could be ascribed to a decline in the number of secretory cells due to involution. This is less likely to occur after one single omitted milking and it appears to be rather an effect of reduced milk synthesis upon increased pressure of accumulated milk (Bach & Busto, 2005). The influence of feedback inhibitor of lactation (FIL) on milk synthesis during longer milking intervals has also been considered (Hillerton *et al*., 1990). Recent studies have indicated that the FIL could be serotonin. By blocking serotonin receptors, gene expression for milk proteins and α-albumin was increased, as well as milk yield (Hernandez *et al*., 2008).

Changes in *milk composition* due to longer milking intervals during ODM have been observed (Stelwagen et al., 1994a; Stelwagen & Lacy-Hulbert, 1996). ODM in comparison with two or more daily milkings resulted in significantly higher SCC, protein and fat content in the milk in addition to a decrease in milk volume. It is observed that during ODM mammary cells become leaky, so that movements from milk to blood compartment and vice versa are present to a higher degree. The changes in milk protein content when cows are milked with prolonged milking intervals may be due to increased content of serum protein, suggesting leakage through the tight junctions (Stelwagen & Lacy-Hulbert, 1996). Protease activity has been found to be increased in milk from udders exposed to ODM. The higher protein and fat content in milk has been ascribed to a positive energy balance due to the ODM (Knutson *et al.,* 1993). In general the casein content is not considered to be affected by milking frequency. However, increased casein content has been reported when applying ODM regularly (Claesson, 1965; Lacy-Hulbert *et al.,* 1999), which has been ascribed to the large size of the casein micelles, making them incapable of leaking out to the blood compartment through TJs.

The effects of regularly applied long milking intervals on various milk parameters have been thoroughly studied but little is known about how a *single* prolonged milking interval (PMI) influences SCC, milk yield and quality, as shown in the literature review in this introduction. A single PMI, such as might be associated with technical failures in automatic milking systems, leads to a subsequent SCC peak in bulk tank milk (Pettersson *et al*., 2002). Increased

SCC indicates mastitis and is generally correlated with reduced milk yield and quality, and often used as a criterion for milk payment. Whether the transient SCC peak after a single PMI affects yield and quality is not known. A single PMI may be of economic concern for the farmer if it adversely affects yield and, additionally the milk payment may be reduced due to the SCC peak in tank milk. The short duration of the inflammatory reaction after a PMI and its non-pathological history raise questions about the underlying immunological mechanisms. It is important to learn more about this kind of physiological inflammation to improve the interpretation of SCC in practice, and the general understanding of udder immunology. This thesis describes the inflammatory reaction after a single PMI of 24 h at both the cow and the quarter level, and how it influences milk yield and quality.

2 Aims

The overall aim of this thesis was to gain further knowledge about the characteristics, immunological background and effect of the physiological inflammatory reaction in the udder of cows that occurs after a single prolonged milking interval (PMI) of 24 h, as previously indicated by increased herd milk SCC.

The specific aims were to:

- Investigate the effect of a single PMI on milk yield, quality and composition.
- Examine if the single PMI leads to epithelial cell damage and/or impaired epithelial integrity.
- Map the kinetics and magnitude of the SCC reaction and the relative contribution of PMN.
- Determine at what time after the PMI the SCC peak occurs and when the inflammatory reaction is initiated.
- Map the characteristics of the inflammatory response in terms of other inflammatory indicators than SCC and PMN, such as acute phase proteins.
- Examine possible immunological factors that could induce the enhanced leukocyte migration reflected in the SCC peak after a PMI, with special attention to natural milk components, such as ALA and PRL.
- Examine *in vitro* the chemotactic activity of the milk during and after a PMI and of ALA and PRL.

3 Materials and methods

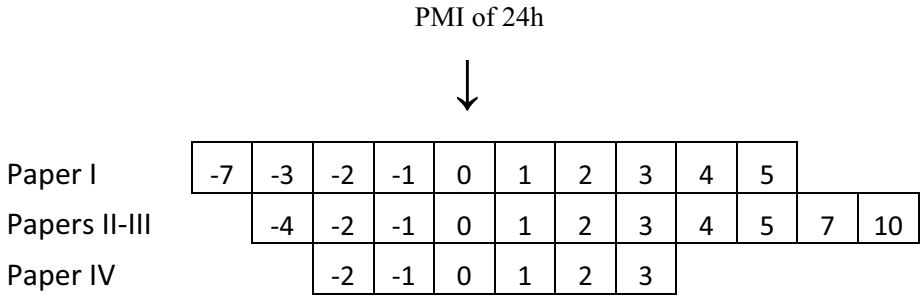
This section summarizes the materials and methods applied in the studies in this thesis. A more detailed description is given in each individual paper (I-IV).

3.1 Animals and management

All studies were conducted at Kungsängen Research Centre, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden on clinically healthy cows with low SCC. The cows were mostly in the middle of their first or second lactation. The selection criteria for cows to be included in the studies (papers I-IV) was that they should have a SCC<100 000/ml in cow composite milk before the start of the study. In papers II-IV, an additional selection criterion was that all udder quarter milk samples from the cows should be bacteriological negative. Composite milk samples of 29 and 27 Swedish Red cows were used for analyses in papers I and II-III, respectively. In paper IV, quarter milk samples of 9 mid-lactation Swedish Red cows were used. In all studies the cows were kept indoors in a tethered system, fed according to Swedish recommendations (Spörndly, 2003) and regularly milked twice daily at 6:30 and 15:30. In papers I-III, milking was performed with a Duovac milking machine system (DeLaval, Tumba, Sweden) and milk yield was recorded at each milking by True Test (Milk meter Cgm Tru-Tests Dk.2840 Italy, Denmark). In paper IV, the cows were milked twice daily with a monovac quarter milking machine (DeLaval, Tumba, Sweden; pulsation ratio 70/30; system vacuum 42 kPa) and milk yield was measured at each milking by weighing the bulk milk from each quarter. The average daily milk yield prior to the start of the study in paper I, II-III and IV was 24.8 kg, 22.6 kg and 26.2 kg, respectively. All studies were approved by the Uppsala Local Ethics Committee.

3.2 The study design

The study design had a similar concept in all papers. Routine milking was performed twice daily except for day 0 when the cows were exposed to a 24 h PMI by excluding the afternoon milking. The table shows the days when sampling was performed in each paper.



The major difference between the experimental design in paper I and that of paper II-III is the number of sampling days prior to, and subsequent to, the PMI. In paper IV, the main purpose was to study the reaction *during* the PMI, and the immediate subsequent days when the most pronounced SCC reaction had occurred, according to paper I and II-III. Besides the sampling at milking (M-samples), small volume quarter milk samples corresponding to the (non-strict) foremilk fraction, were additionally collected during the PMI (PMI-samples) and between the following milkings (inter-samples; I-samples), with the aim of studying the early phase of inflammation and the reaction in greater detail. *During the PMI*, half of each udder, randomly selected, was sampled at 16 h, 18 h, 20 h and 22 h after the start of the PMI. To evaluate if this sampling influenced the reaction in the gland during the PMI, the other half was used as control, with no PMI sampling. After the PMI the samples were collected from all quarters at 27 h, 30 h, 43 h, 52 h, and 57 h.

Blood samples were taken after milking on day -1, 0 and 1 in paper II and III. In paper IV the cows were fitted with a permanent catheter in the jugular vein and blood samples were collected in connection with each milk sampling.

3.3 Samples and sampling procedure

Blood samples were collected from the tail vein in papers II-III and from the jugular vein in paper IV in heparinized vacutainer tubes (Vacutainer, Terumo, Sweden). The blood sampling started approximately two and a half hours after milking was finished (papers II and III). Blood samples were put on ice

immediately after sampling, and subsequently centrifuged at 2200 x g for 15 minutes. The obtained plasma was split in aliquots and was stored in -20 °C until analysis. For paper IV, blood sample collection and handling was performed similarly but prior to each milk sampling instead of afterwards.

After milking, samples (200 ml) were taken from the bulk milk of each cow (papers I–III), or of each quarter (40 ml) in paper IV. The milk samples (15 ml) that were taken between the milkings in paper IV were collected manually ca 1 min after the start of the procedure of cleaning the teat ends and discarding the first 5 ml of milk. The intention was to obtain milk let-down to make the sample as representative of the total quarter milk as possible while minimizing the milk volume removed from the quarter. All milk samples were split in aliquots for the different analyses within 2 hours and milk smears intended for PMN counting were prepared within 6 hours of sampling. Milk intended for analysis of cytokines (papers II and IV) was transferred to 2 ml tubes immediately after collection, the tubes being kept on ice for a maximum of 2 h until they could be frozen (-20 °C). In samples for analysis of milk composition and SCC, bronopol, 2-bromo-nitropropane-1,3 diol (VWR International AB, Stockholm, Sweden) was added and the samples were kept at +4 °C until analyzed. The milk samples for all other analyses were stored at -20 °C until analyzed. The quarter level samples of 15-20 ml were similarly split in aliquots.

3.4 Sample analyses

3.4.1 Milk composition (papers I-II)

The milk composition was analyzed by spectroscopic mid infrared technique (MIR; MilcoScan FT 120 A/S N Foss Electric, Hillerød, Denmark). The casein proportion was calculated indirectly from the whey protein and total protein proportions, by the rennet casein method (Arla Foods analysis regulation 2000.004, 200001210) and as described by Forsbäck *et al*., (2009).

3.4.2 SCC and PMN (papers I-IV)

Milk SCC was analysed by fluorescence-based electronic cell counting (Fossomatic 5000, A/S N. Foss Electric, Hilleröd, Denmark) in a routine laboratory. PMN were counted in 20 µl of milk by light microscopy after Newman staining according to a modified version of the IDF standard (IDF 148-1/ ISO/DIS 13366-1).

3.4.3 Free fatty acids (paper I)

Free fatty acids (FFA) content in milk was analysed by the Auto analyzer II method (Lindqvist *et al*., 1975).

3.4.4 Lactate dehydrogenase (paper II)

LDH activity in milk (µmole product/min per liter, equivalent to conventional international units) was detected by a fluorometric, kinetic method according to the description by Larsen (2005), using a Biomek 2000©,USA Laboratory Automation Workstation, Beckman Coulter, and spectrophotometer /fluorometer, Fluostar ©, BMG Labtechnologies, USA.

3.4.5 Bovine Serum Albumin (paper II)

The BSA concentration in milk was determined using a commercial ELISA kit (Bovine Albumin Elisa Quantitation Kit, Bethyl Laboratories, Montgomery, TX., USA), according to the manufacturer's instructions. Optical densities were read using an automatic plate reader (Model ELx 800; Bio-tek Inc.,Winooski, VT, USA) at 450 nm with a reference at 630 nm.

3.4.6 Serum Amyloid A (papers II and IV)

Commercial ELISA kits (PHASE™ Milk Amyloid A [MAA] Assay; cat. TP-807 and PHASE™ Serum Amyloid A Assay [SAA] – Multispecies; cat. TP-802, Tridelta Development Ltd, Wicklow, Ireland) were applied to determine the amyloid A concentration in milk and blood plasma, respectively. Optical densities were read on an automatic plate reader (Model ELx 800; Bio-tek Inc.,Winooski, VT, USA in paper II and Multiscan EX, Thermo Labsystems , Altrincham, UK in paper IV) at 450 nm. The limit of detection of the ELISAs was 0.1 mg/l for milk (MAA) and 0.3 mg/l for serum (SAA), according to the manufacturer.

3.4.7 IL-1β (paper II) and IL-8 (paper IV)

To detect IL-1β, coupling of antibodies to microspheres and performance of xMAP assays were made as previously described by Dernfalk *et al*., (2007). Monoclonal antibodies against ovine IL-1β were coupled to the microspheres, rabbit anti-ovine IL-1β antibodies were used as reporters and recombinant ovine IL-1β was used in the standard curves. Milk samples were not centrifuged because earlier studies had shown a recovery of 60 % of added recombinant cytokine in such milk samples (Dernfalk *et al.,* 2004).

Quantification of IL-8 in milk was performed on undiluted whey using a commercially available human IL-8 ELISA kit (Quantikine™, R&D Systems,

Inc., Minneapolis, MN) according to the manufacturer´s instructions. The minimum detectable concentration of IL-8 given by the manufacturer was 1.5 pg/ml. The human IL-8 antibodies used in this kit have previously been shown to cross-react with bovine IL-8 (Shuster *et al*., 1995, 1997). The optical density was measured using a microplate reader as for ALA at 450 nm.

3.4.8 Alpha lactalbumin (papers II and IV)

ALA concentration in milk was detected by using a commercial ELISA kit (Bovine Alpha-Lactalbumin Elisa Quantitation Kit, Bethyl Laboratories, Montgomery, TX., USA), according to manufacturer's instructions. Optical densities were read in an automatic plate reader (Model ELx 800; Bio-tek Inc.,Winooski, VT, USA and Multiscan EX, Thermo Labsystems, Altrincham, UK) at 450 nm.

3.4.9 Lactose in blood (paper II)

Lactose in blood (paper II) was determined with a UV method using a commercial kit (Boehringer Mannheim/R-Biopharm, Lactose/D-galactose kit). Before determination of lactose the plasma samples were deproteinized according to Stelwagen *et al*., (1994b).

3.4.10 Cortisol and PRL (paper III) and (papers III-IV)

Cortisol and PRL concentrations in plasma and milk were analyzed by a radio immune assay technique. The cortisol analysis was performed as described by Blum *et al*., (1985), while analyses of PRL was performed as described by Bruckmaier *et al*., (1992).

3.5 Milk whey preparation intended for IL8 and chemotaxis analyses (paper IV)

For the preparation of whey, used in the IL-8 and PMN migration assays, the milk samples were centrifuged at 44,000 x g at 4 °C for 30 min and the fat layer was removed (Bannerman *et al*., 2003). The centrifugation was repeated once and the translucent supernatant was collected and stored at -70 °C until analyzed.

3.6 Isolation of PMN for the migration assay (paper III and IV)

Blood was collected from the jugular vein of healthy donor cows using Na-heparin as an anticoagulant. PMN were isolated (papers III and IV) as previously described (Barber & Yang, 1998). Fresh blood was diluted with an

equal volume of sterile phosphate buffered saline solution (PBSS) and centrifuged at 400 x g for 10 minutes. The plasma and buffy coat layer were removed. PBSS was added and the sample was centrifuged at 400 x g for 5 minutes, the supernatant was discarded and the pellet was resuspended in PBSS. The erythrocytes in the cell pellet were subjected to hypotonic lysis by exposing them to sterile water for 30 sec. Normal osmolarity was restored by adding 3.6% NaCl. The lysis procedure was repeated once. The cell pellet was washed twice with PBSS and finally resuspended in Geys' buffer. The PMN concentration in the cell suspension was determined in a Bürker chamber after staining with Türks dye.

3.7 PMN migration assay (paper III and IV)

The *in vitro* effect of the milk whey samples, PRL and ALA on PMN migration was examined by using a 48 well microchemotaxis chamber (Neuro Probe Inc, Cabin John,Md, USA; see e.g. Österlundh *et al*., 2001). In brief, the PMN were allowed to migrate through a cellulose nitrate filter with a pore size of 3 μm (Millipore Corp., Bedford, MA, USA) in the chamber, where the PMN suspension was applied in each of the top wells and the bottom wells were filled with the chemoattractant or control.

Ten percentage zymosan activated bovine serum (10 % ZAS) and sterile Geys' buffer were included in each assay as positive and negative controls, respectively. Migration towards the negative control was regarded as random migration. Sterile PBSS was used as a diluent for the milk samples and test substances. The samples tested in the chemotaxis assay were emanating only from udder quarters with a pronounced SCC and PMN reaction (paper IV). After testing different dilutions, the whey samples were finally diluted 1:7 in PBSS before analysis in the chemotaxis chamber.

For testing the chemoattractant properties of PRL (Prolactin, from sheep pituitary, Sigma-Aldrich Sweden AB, Stockholm, Sweden) on PMN, concentrations of 5, 10, 15 and 20 ng/ml PRL were used (paper III). This range of concentrations was similar to that observed in milk in the present study. Additionally, a possible priming effect of PRL on the PMN response to chemotactic stimulation by 10% ZAS was evaluated by pre-incubating PMN for 15 min in PRL concentrations of 10, 20, 30 and 40 ng/ml cell suspension, this range of concentrations being similar to that measured in blood plasma during the experiment. As a control, untreated PMN from the same batch were used. The migration of pre-incubated and untreated PMN towards Geys' buffer was also tested, as a negative control.

To test a possible indirect inhibiting effect of ALA on PMN chemotaxis (paper IV) through the influence of immunological systems present in serum, an ALA solution (α-lactalbumin from bovine milk, Sigma-Aldrich Sweden AB, Stockholm, Sweden) was added to ZAS (Wong *et al*., 1997b) to obtain final concentrations of 0,5 and 1,5 mg ALA, respectively, per ml of 10 % ZAS. Additionally, ALA only was tested as a chemoattractant in concentrations of 0.5 and 1.5 mg/ml. Furthermore, PMN were pre-incubated (Wong *et al*., 1997b) with ALA for 15 min in concentrations of 0.5 and 1.5 mg ALA per ml cell suspension to evaluate the effect on PMN migration towards 10 % ZAS.

The chamber was incubated for 1 h at 37 °C. The filter was removed, fixed, rinsed, stained and mounted on a glass slide. The migration distance of the PMN through the filter was examined by light microscopy and measured by a digital counter. The farthest distance from the level where the cells were initially applied at which 3 PMN were observed per vision field, constituted the migration distance (Österlundh *et al*., 2001). The part of the migration distance through the filter in the test wells that exceeds the average random migration in the same assay, is ascribed to the tested substance. The assay was performed in duplicate wells for each whey sample and the positive and negative control, and in 5 replicates for each concentration of PRL and ALA, respectively, that were tested for chemotactic or PMN-priming properties. Each well's filter was counted in 3 vision fields and a mean value from the replicates was calculated.

3.8 Statistical analysis

In all papers (I-IV) the statistical analyses were performed using the SAS Programme (Ver. 9.1 or 9.2, Cary, NC, USA). SCC values were transformed to 10-logarithmic values before the analyses, in order to obtain a more normal distribution of data. Besides calculation of means and SD (paper IV), the statistical analyses were performed using analysis of variance (PROC MIXED). Least squares means were calculated from the analyses of variance, and they were compared using t-test. For the analyses of the chemoattractant properties of the milk samples (paper IV), each value was expressed as the deviation (in percentage) from the average positive and negative control values, respectively, for each assay. In addition to the data of the parameters analysed in the study, the average output per unit time (output/h) since the previous milking was calculated for each parameter and tested (paper I-III) to obtain a measure that was not influenced by the different milking interval length or different milk volume (dilution/concentration effect) during the study.

4 Main Results & Discussion

This chapter summarizes and discusses the results from papers I-IV. More detailed information is given in each paper. I all papers, the values for each parameter were compared within morning and afternoon milk, respectively, with the baseline values before the PMI. In paper IV, the procedure for collection of M-samples at milking was not the same as that of the PMI- and I-samples which, per se, might have influenced the content of the factors analyzed. The concentration of PRL in *blood* might also differ between samples collected at milking and those collected between milkings. Therefore, in general, comparisons of the results in paper IV were performed separately for milking samples, the exception being SAA, for which it was considered more relevant to make comparisons among all blood samples, since SAA in blood serum is not expected to be affected by milking or the milk sampling procedure.

4.1 Milk yield and milk composition subsequent to a PMI

The results regarding milk yield and composition after a PMI of 24 h are mainly reported in papers I and II, and additionally for ALA in paper IV The results of the papers are consistent and show, in brief, that milk composition was altered but quality was not impaired and that the milk yield in afternoon milk was significantly reduced for more than a week.

4.1.1 Yield

At the first milking subsequent to the PMI, a significantly increased milk yield was observed due to the 24 h accumulation. When the output per hour was calculated, a measure that excluded the effect of different milking interval length on milk yield, it was shown that milk synthesis was actually significantly impaired during the PMI. On day 2, the morning milk yield returned to values that were not different from the baseline, while the afternoon

milk yield was reduced on day 1 compared to the baseline and, notably, remained significantly lower throughout the studies. The average reduction was 0.75 kg per afternoon milking during the 10 days studied after the PMI (paper II). Although the results on milk yield in paper IV have not been presented, the statistics indicated the same pattern as observed in papers I and II. The reduced yield in afternoon milking was confirmed by the calculated average output per unit time. It was shown, additionally, that the output rate in morning milk was also significantly reduced on day 1 when the major changes in milk composition were observed. A considerable reduction of milk yield has previously been observed in a study by Claesson *et al*., (1959) when one milking per week was omitted, i.e. a single PMI. When cows are subjected to once daily milking on a regular basis it has been found to result in reduced milk yield (Stelwagen & Lacy-Hulbert 1996; Davis *et al*., 1999; Stelwagen *et al*., 1997; O' Brien *et al*., 2002) which has been ascribed to a decline in the number of secretory cells due to involution and influence of FIL on milk synthesis (Stelwagen *et al*., 1994a; Hillerton *et al*., 1990). A continuous influence of longer milking intervals is not really comparable to the effect of a single PMI and it is more probable that the impaired milk synthesis during the PMI is mainly an effect of the increased intramammary pressure of accumulated milk and possibly also of FIL (Bach & Busto, 2005). In a study by Hernandez *et al*., (2008) it was shown that FIL or serotonin induces a significant reduction in ALA synthesis and it cannot be excluded that it might have been a factor behind the decrease in ALA concentration observed after the PMI. The drop in ALA, in turn, might be one of the reasons behind the lower concentration of lactose in milk observed after the PMI, and hence, indirectly responsible for decreased milk yield. It is known that ALA is a necessary part of the coenzyme galactosyl-transferase, which is responsible for lactose synthesis. On the other hand, a decrease in ALA as well as lactose, may not be just attributable to lower synthesis but also to increased leakage from milk to blood through impaired mammary epithelium (Mao *et al*., 1991; Stelwagen and Lacy-Hulbert, 1996). A plausible reason for the long duration of decreased afternoon milk yield observed after the PMI is difficult to find. A study by Stelwagen & Lacy-Hulbert (1996) suggested that a single PMI does not cause any damage to the mammary secretory epithelium. This is supported by the consistently low concentrations of LDH in milk found in paper II. A possible effect of FIL during the PMI is not a reasonable explanation as to why the yield was influenced up to 10 days thereafter, and only in afternoon milk. Whatever the reason, the reduced milk yield after a PMI is of economic concern for the farmer.

4.1.2 Composition

Milk composition was altered after the PMI . In brief, the concentrations of fat, total protein, casein and whey protein increased while lactose concentration decreased. Considering the results from both paper I and II, milk composition, was significantly changed mainly on the first day after the PMI. Subsequently the changes were not consistent except for lactose that steadily decreased throughout the study (paper I). Interestingly, increased concentrations of citric acid (paper II) and decreased concentrations of FFA (paper I) were observed. This strongly suggests that there was not a deteriorating enzymatic effect during and subsequent to the PMI. The concentrations of FFA/100 g fat decreased significantly after the PMI (paper I). Accumulation of FFA in the milk is related to higher hydrolysis of triglycerides catalyzed by lipoprotein lipase. It is known from previous studies that short and irregular milking intervals may result in elevated FFA content in milk (Klei *et al*., 1997; Wiking *et al*., 2006). Since the fat content increased while the FFA decreased after the PMI (paper I), there was, apparently, no effect of lipoprotein lipase originating from blood plasma.

In table 2, results from paper II for fat, protein, lactose, casein, citric acid and whey after the PMI are given. On the whole, similar results were obtained in paper I although the period of increased fat content was shorter. Furthermore, more consistently increased protein content in afternoon milk and consistent decreased lactose content in both morning and afternoon milk were observed throughout the study.

The results from papers I and II, suggest that, apart from lactose, synthesis of milk components was not impaired after the PMI, in contrast to Delamare & Guinard-Flament (2006) in a study of regularly applied longer milking intervals. The elevated protein content was apparently due to an increase in casein content while the content of serum proteins decreased. Claesson (1959) observed a higher concentration of casein during once-daily milking than twice-daily milking, as did Lacy Hulbert *et al*., (1999). The increase has been explained by the large size of the casein micelles, making them incapable of leaking out through TJs to the blood compartment. The elevated milk concentrations of casein observed in the present study were probably attributable to a concentration effect. In summary, the observed changes in milk composition, as well as in milk quality parameters, strongly suggest that PMI did not induce any deteriorating effect on milk quality.

Table 2. Alterations in concentration of different milk components after a prolonged milking interval (paper II)

	Milking	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 10
Fat (%)	M	4.35	4.64**	4.65**	4.28	4.07**	4.07**	4.27	4.33
	A	6.67	8.54***	6.47	6.22**	6.21**	6.17**	6.61	6.57
Protein (%)	M	3.67	3.82***	3.64	3.59***	3.70*	3.68	3.68	3.73***
	A	3.89	4.04***	3.80***	3.89	3.92	3.86	3.86	3.92
Lactose (%)	M	4.78	4.69***	4.72***	4.74**	4.76	4.76	4.77	4.76
	A	4.67	4.59***	4.68	4.68	4.69	4.7	4.65	4.70
Citric acid (%)	M	0.172	0.183***	0.177*	0.158***	0.158***	0.162**	0.177	0.177
	A	0.198	0.212***	0.196	0.189***	0.188***	0.186***	0.202	0.202
Casein (%)	M	2.70	2.80***	2.64***	2.60**	2.71	2.68		
	A	2.83	2.93***	2.73***	2.83	2.85	2.82		
Whey (%)	M	0.983	1.021***	1.000***	0.989	0.993*	0.996*		
	A	1.063	1.104***	1.068	1.058	1.060	1.044*		

4.2 The kinetics and magnitude of the SCC and PMN reaction after a PMI

The results regarding cell count reactions after a PMI of 24 h are reported in papers I, II and IV. Previously it had been found that cows regularly exposed to longer milking intervals have significantly increased SCC, (Stelwagen *et al*., 1994a; 1997) and PMN (Stelwagen & Lacy-Hulbert, 1996). It has been shown that herd tank milk SCC may be elevated after a single PMI (Pettersson *et al*., 2002) but reports on SCC at cow level and PMN proportion in milk after a single PMI are lacking. Results from the present studies show that SCC and PMN proportion, increase in cow composite milk, as well as quarter milk, after a PMI, and that the reaction starts during the PMI but that the most pronounced reactions do not occur until *after* the first milking subsequent to the PMI.

In paper I, the aim was to map the kinetics of the SCC and PMN reaction after a PMI. In paper II, which primarily aimed at investigating the possible influence on SCC and PMN of various factors, the cell reaction was mapped mainly to serve as a reference for the inflammatory reaction. In papers I and II, cow composite milk collected at milking was used, while in paper IV quarter

milk (M-samples) was analyzed. Additionally, in paper IV, sampling was performed during the PMI (PMI-samples) and between the two subsequent milkings (I-samples), in order to study the cell reaction during the early phase of the inflammation.

The *results* from all papers (I, II and IV), regarding SCC and PMN in samples collected at milking after the PMI, were generally consistent. The baseline SCC in morning and afternoon milk before the PMI in all studies were below 50 x 10^3 cells/ml and 100 x 10^3 cells/ml, respectively. The corresponding values of PMN proportion were 15 % and 17%. A pronounced peak in SCC and proportion of PMN were observed after the PMI. The SCC increased approximately three times and the PMN proportion was approximately doubled compared with the maximum value observed in afternoon milk (the baseline). In the first milking after the PMI there was a distinct increase in the proportion of PMN while the increase in SCC was small, although significant. The most pronounced changes in SCC (paper I, II and IV) and PMN (paper I and II) were not observed until the *second* milking after the PMI, in the afternoon of day 1. In morning milk, the peak SCC value was clearly lower than in afternoon milk and did not occur until day 2, notably, concomitantly with a *decreased* proportion of PMN. After morning milking on day 3 the SCC and PMN proportion had returned to values that were not significantly different from the baseline in paper II, whereas in paper I they remained significantly increased throughout the study, although at a low level.

In spite of the different study design that was used in paper IV, the results of SCC and PMN in samples collected at milking after the PMI, were largely in accordance with paper I and II, differing only in that the highest PMN value was seen at the *first* milking after the PMI. The PMN proportion tended to decrease more rapidly and was not significantly increased from the morning milking on day 2. In paper IV, it was shown that the PMN proportion started to increase during the PMI at 20 h after the start of the PMI while an increase in SCC was not noticed until 2 h later. The highest value of SCC and PMN was observed at 27 h, i.e. in samples collected shortly after the first morning milking subsequent to the PMI. There was no significant difference at any time of the study in SCC and PMN proportion in milk from quarters that had been sampled during the PMI, compared with control quarters that had not been sampled.

Thus, the inflammatory reaction, as shown in enhanced PMN migration, started *during* the PMI. The finding that an increase in PMN proportion could be noticed before an increase in SCC was observable agrees with the results of previous studies; i.e. that the proportion of PMN is a more sensitive inflammatory indicator than is the total cell count (Östensson *et al*., 1988;

Kelly *et al*., 2000; Rivas *et al*., 2001). The reason why the most pronounced increase in SCC and PMN does not occur until after the first milking subsequent to PMI is not obvious. It may be argued that increase in SCC at the first milking subsequent to the PMI was small due to the large accumulated milk volume and that the most pronounced increase in SCC at the second milking was due to the lower volume then. Volume is considered to influence the SCC due to the varying degree of dilution (for review, see Harmon, 1994). All changes in SCC during the study were, however, in accordance with the calculated average number of cells entering the milk *per unit time*, a measure which excludes the effect of dilution. Additionally the relative contribution of PMN to the SCC, i.e. the size of the PMN proportion, is not influenced by the milk volume. Thus, it is strongly indicated that the most enhanced PMN migration actually does not occur until the udder has been emptied after a PMI. That the highest PMN value was observed at the first milking after the PMI, in paper IV, indicates that the sampling during the PMI might have influenced the PMN reaction.

In morning milk, the SCC continued to increase from day 1 to day 2, although the PMN proportion was declining. This suggests that other leukocyte types than PMN contributed to the peak in SCC observed in morning milk on day 2 These findings are in contrast to those shown in numerous previous studies where changes in SCC and percentage of PMN are well correlated under both physiological and pathological conditions (see e.g. White & Rattray, 1965; Ostensson, 1993 a, b; Kelly *et al*., 2000).

The SCC reaction after a PMI and in ODM cows appears to be an inflammatory reaction of physiological character considering the obviously non-pathological history. Previous studies of ODM cows have discussed the observed increased SCC, although they have not aimed at identifying possible mechanisms behind it. Paper II and IV primarily aimed at investigating inflammatory parameters and possible chemotactic factors in milk related to the SCC and PMN reaction which will be discussed in the following sections.

4.3 Mammary gland permeability and non-cellular inflammatory indicators after a PMI

The reaction in the udder after a PMI was further studied in papers II, III and IV by examining other inflammatory parameters than SCC and PMN proportion in milk, and permeability in the mammary tissue. Additionally, some parameters were analysed in blood. The aim was to investigate further the character of the inflammatory reaction and to identify factors that could possibly have triggered the cell reaction. The results showed, that a PMI of 24

h leads to enhanced permeability in the tissue (indicated by the BSA concentration in milk) and triggers a response of the acute phase protein SAA, but that the milk concentration of the intracellular enzyme LDH was not affected.

4.3.1 Mammary gland permeability

It has been indicated previously that the increase in SCC and PMN migration in ODM cows is related with increased permeability in mammary tissue (Stelwagen & Lacy Hulbert, 1996; Stelwagen *et al*., 1997). To examine whether the effect of one *single* long milking interval could lead to enhanced permeability in the mammary tissue, the concentrations of BSA in milk and lactose in serum were examined after a PMI of 24 h (paper II). BSA is generally used as an indicator of increased permeability. The BSA concentration in milk increased from a baseline value of 0.14 mg/ml in both morning and afternoon milk to 0.16 mg/ml in morning milk and 0.17 mg/ml in afternoon milk on day 1 after PMI. The increase was small but highly significant. BSA remained significantly elevated until the morning on day 2, thereafter declining to concentrations that were not significantly increased during the rest of the study. Furthermore, the lactose concentration in blood increased significantly after the PMI, from a base line value of 0,06 mM in the morning and 0,04 mM in the afternoon to 0,12 mM and 0,05 mM, respectively, at day 1.

The indication of increased epithelial permeability that has previously been found when long milking intervals have been applied during a longer period of time (Linzell & Peaker, 1971; Stelwagen & Lacy Hulbert, 1996; Stelwagen *et al*., 1997) has been ascribed to exaggerated intramammary pressure from the large volume of milk accumulating in the udder. Enhanced permeability of the mammary epithelium itself is not directly responsible for increased PMN migration. However, indirectly, impaired TJ could lead to enhanced leakage of immunological factors from blood or tissue fluid to milk, thereby causing enhanced PMN migration. Increased epithelial permeability may also cause milk components to leave the milk compartment and enter the blood. The increase of lactose in blood was most pronounced at the first milking after PMI while the most prominent BSA concentration in milk was not seen until the subsequent milking, after the udder had been emptied once, when milking was resumed after the PMI. Thus, the TJ obviously became impaired during the PMI, leading to lactose being pressed out from the milk compartment when the intramammary pressure was exaggerated, while the high pressure hampered BSA from diffusing in the opposite direction at that time. When the pressure was lowered after the first milking subsequent to the PMI this barrier was

reduced, and the leakage of BSA through mammary epithelium into the milk compartment could escalate. It should, however, also be considered that increased milk concentration of BSA might also be a result of local synthesis (Shamay *et al*., 2005).

4.3.2 Acute phase proteins

One of the key bovine acute phase proteins is serum amyloid A. Serum amyloid A may also be present in milk and alters upon both local and systemic effects of cytokines (Jacobsen *et al*., 2005). In morning milkings (paper II), the concentration of serum amyloid A in milk (MAA; baseline: 0.57 mg/ml) was significantly increased after the PMI on day 1, 2 and 3 with the maximum value of 1.12 mg/ml recorded day 2. In afternoon milk, MAA (baseline 0.68 mg/ml) was significantly increased on day 1 and 2 (paper II) with the peak value (1.31 mg/ml) being recorded on day 1, thus showing a sharper peak pattern than in morning milk. In the subsequent milkings, MAA concentrations were not significantly increased compared to the baseline. Thus, MAA was increased at the first milking immediately after the PMI, indicating that the reaction started earlier.

In paper IV, MAA and SAA were analyzed during the PMI from 16 h to 22 h after the start of the PMI, to identify the initiation of the acute phase response, and additionally, at 27 h, after the first milking subsequent to the PMI. . In quarter composite milk in the morning at the start of the PMI (0 h) and immediately thereafter (24 h), MAA was 0.35 mg/ml and 0.71 mg/ml. At the first PMI-sample at 16 h, MAA was 0.54 mg/ml. Comparing PMI/I-samples, a significant increase in MAA was observed at 22 h, whereafter it continued increasing, with the highest value seen after morning milking on day 1 (27 h, 1.55 mg/ml). Moreover, the concentration observed at 22 h and thereafter in PMI-/I-samples, was significantly higher than that of composite quarter milk at 0 h.

SAA concentrations in blood plasma are not influenced by the milk sampling procedure and were therefore compared without considering if the blood sample was taken in connection with an M-sample or a PMI-/I-sample. Alterations in SAA concentrations followed a similar pattern to alterations in MAA concentrations but SAA started to increase 2 h earlier than MAA. From a baseline value of 60.1 mg/ml, SAA was significantly increased at 20 h and increased further up to 27 h, when the concentration was 146.3 mg/ml.

Increased MAA concentration was not seen until after the elevation in SCC was observed after the PMI. This is in contrast to the findings in experimental bacterial bovine mastitis, where the concentration of MAA was found to be increased *prior* to SCC (Pedersen *et al*., 2003). The MAA value in the M-

sample collected at 24 h was lower than that of each small volume sample collected immediately before and after. Serum amyloid A is an apolipoprotein (Malle *et al*., 1993) and it has been suggested that different milk fat content in various fractions of milk might influence the MAA concentration (Alsemgeest *et al.,* 1995; Westmark *et al*., 1986; Åkerstedt *et al*., 2007). The fat content of milk was not analyzed in paper IV, but it has been shown previously that the fat content of foremilk collected after milk ejection from healthy udder quarters is lower than that of composite milk (Bansal *et al*., 2005). In spite of the presumably lower fat content in the small volume foremilk samples higher values of MAA were observed than in the quarter bulk milk samples, in the present study. Since SAA started increasing earlier than MAA, the increase in MAA is more likely to be the result of leakage from the blood than local synthesis and secretion. The finding that SAA increased is interesting since it suggests that the inflammatory reaction observed subsequent to PMI involves not just a local, but also a systemic, effect.

It has been indicated that SAA might stimulate PMN migration (Tizard, 2007; Badolato *et al*., 1994), although it has not been shown if this is also true for MAA. In paper II, MAA reached the maximum concentration at the same time as the proportion of PMN, i.e. in the afternoon on day 1 after the PMI. Thereafter the PMN proportion declined more rapidly than the concentration of MAA, which lingered, remaining significantly increased for half a day longer than the PMN proportion. Based on the results from paper II it was not clearly indicated if MAA could have been an important player in the attraction of PMN to the milk. By investigating MAA and, additionally, SAA, at the time of initiation of the inflammatory reaction during the PMI (paper IV) it was shown that SAA started to increase simultaneously with the PMN proportion (at 20 h), that the maximum value was reached at the same time (27 h) and that the alterations showed, in general, a similar pattern. These findings indicate that the elevated SAA concentration could have contributed to stimulation of the PMN to enhanced migration towards the udder. The MAA concentration, which started to rise slightly later than SAA and the PMN proportion, continued rising until the PMN peaked and might, through a chemoattractant effect, have further enhanced the PMN migration to milk. The factors that might have triggered the synthesis and secretion of SAA remain to be identified.

4.3.3 LDH

Lactate dehydrogenase (LDH) is an inflammatory indicator, or indicator of cell damage, known to have a strong correlation with SCC (Kitchen *et al*., 1980). The LDH activity in mastitic milk originates in leukocytes (Bogin *et al*., 1977)

and epithelial cells (Kato *et al*., 1989). The baseline LDH activity (paper II) was 2.80 U/l in morning milk and was not altered in any of morning milk samples taken subsequent to the PMI except for an occasional decline in morning milk on day 4. In the afternoon milk samples, a significantly decreased LDH activity was observed from day 2 (baseline: 3.92 U/l; day 2: 3.55) and throughout the study. Thus, LDH was unaltered during the peak in SCC and PMN migration, and consequently a positive correlation between SCC and LDH could not be seen in paper II, in contrast to previous studies (Kitchen *et al*., 1980). The reason for this might be that the SCC, per se, was moderate on all sampling occasions in paper II and that LDH was not reacting to SCC alterations within this range. Still, the unaffected LDH activity in milk strongly suggests that there was no mammary epithelial damage subsequent to the PMI.

4.4 Proinflammatory cytokines and natural milk components that may express cytokine-like actions

An *in vitro* PMN chemotaxis assay was performed (paper IV) in order to examine whether the milk samples collected showed chemotactic properties. The composite quarter milk sample collected at 0 h and the foremilk samples taken during and after the PMI at 0, 16, 20, 22, 24 and 27 h were tested. The samples were diluted 1:7 to fit the level of PMN migratory response to the negative and positive control.

Proinflammatory cytokines such as IL-1β and IL-8 are known to express a potent chemotactic effect on PMN (Sordillo 1997, Shuster *et al*., 1997; Aluwaimi 2004). Thus, these cytokines were subjects of interest as possible factors behind the PMN response after a PMI and were investigated in paper II and IV, respectively. Cytokines in relation to a single PMI has not been investigated by others, to the best of my knowledge.

Additionally, ALA and PRL concentrations in milk and blood were examined (Paper II and IV), these factors being known to be able to induce stimulation of PMN migration (Wong *et al*., 1997b; Yu-Lee, 2002; Rusu *et al*., 2010).

4.4.1 Chemotactic properties of milk in vitro and IL-1β and IL-8 content of milk

The diluted milk samples showed chemotactic activity *in vitro* causing a mean PMN migration distance that was significantly above the random migration distance (ranging 137 % to 145 %) but with no significant difference over time. No detectable concentrations of IL-1β in milk were found after the PMI (paper

II). IL-8 could be observed in milk (paper IV) but in very low concentrations (the mean per sampling time ranged 1.2 pg/ml – 1.9 pg/ml) and it was not significantly altered over time.

Interestingly, the chemotaxis assays revealed a very strong chemotactic effect in milk from all sampling occasions tested. Considering the high dilution of the samples tested in the assay, the chemotactic effect on PMN migration *in vitro* of the undiluted milk ought to have been considerably higher. This result is in accordance with previous studies which have shown strong chemotactic properties of non-inflammatory milk (Rainard *et al*., 2008) but no difference over time was found that could explain the enhanced PMN migration starting in the end of the PMI.

No elevated concentrations of IL-1β or IL-8 during or after the PMI could be detected. Interleukin 1β is a pluripotent proinflammatory cytokine and initiator of acute phase protein production as well as PMN chemotaxis (Jensen &Whitehead, 1998; Sordillo *et al*., 1997; Alluwaimi, 2004). IL-1β and SAA can enhance the release of IL-8 which is also a potent chemoattractant for PMN (Badolato *et al*., 1994; He *et al*., 2003; Lee *et al*., 2009). The expressions of genes for these cytokines have been found in intact physiological mammary glands at various stages of lactation (Okada *et al*., 1997; Alluwaimi 2004) and IL-1β has also been detected in normal milk (Hagiwara *et al*., 2000). The inflammation seen during the PMI, with SCC and PMN values in such a low range, is a mild reaction. It can be assumed to be elicited by extremely small elevation of cytokine concentrations which could have remained undetected. It is also possible that cytokine concentrations in milk were elevated prior to the first sampling during the PMI. The results, though, makes it doubtful if IL-1β and IL-8 had a significant role in enhancing the PMN migration.

4.4.2 ALA and PRL in milk and blood

The results, indicating extremely low concentrations of pro-inflammatory cytokines in milk in relation to the PMI, raise questions whether other milk components may have expressed chemotactic activity, inducing the SCC and PMN reaction. Several natural milk constituents, such as ALA and PRL, have been shown to exhibit immunomodulatory properties (Epps *et al*., 1977; Wong *et al*., 1997a,b; Rusu *et al*., 2009, 2010) and might have played a pro-inflammatory role. The alteration in concentrations of ALA in milk and PRL in milk and blood (paper II and IV) after a PMI and their effect, respectively, on PMN migration *in vitro* were investigated (paper IV).

ALA

A possible immunomodulatory role of ALA, besides its significant role in lactose synthesis, has previously not been investigated inrelation to long milking intervals. From a baseline concentration (mg/ml) of 0.88 in morning and 0.86 in afternoon milk (paper II) a sharp and significant drop in ALA was observed after the PMI at day 1 in both morning and afternoon milk to 0.78 and 0.73, respectively. In morning milk, ALA remained at the same level on day 2. Thereafter the concentration in both morning and afternoon milk increased to values that were not significantly different from the baseline during the rest of the study (paper II). A corresponding pattern of the alterations in ALA was seen also in quarter composite milk (paper IV), but the reduction of ALA was not significant until the afternoon milking on day 1. The drop in ALA, as observed in composite milk, occurred simultaneously with the PMN peak.

In paper IV it was shown that an *increase* in ALA concentration occurred at the end of the PMI, prior to the decrease seen thereafter. ALA was significantly increased at 20 h (1.06 mg/ml) and 22 h (1.08 mg/ml) compared to the first PMI-value at 16 h (0.79 mg/ml), as well as compared to the morning milk (0 h) immediately before the start of the PMI. The increase in ALA during the PMI started concomitantly with the increase in PMN proportion. Thus, ALA was shown to increase during the PMI but was decreased thereafter.

Previous reports on the effect of ALA on PMN migration are contradictory. It has been suggested that ALA may enhance cytokine production and thereby indirectly stimulate PMN chemotaxis (Rusu *et al*., 2009, 2010). In the chemotaxis assay (paper IV), ALA, tested in concentrations of 0.5 mg/ml and 1.5 mg/ml, exerted no chemoattractant effect on PMN migration *in vitro*. The migration distance towards the different concentrations (72 µm and 65 µm, respectively) was not significantly different from random migration (58 µm). Thus, this finding does not support previous results (Rusu *et al*., 2009, 2010).

In contrast to the previously mentioned experiment, some studies have indicated that ALA may contribute to reduced PMN migration (Wong *et al*., 1997a; Yamaguchi *et al*., 2007). Such an effect could be accomplished by affecting different immune components present in serum and to a varying extent also in milk. An inhibiting effect of ALA on the chemotactic effect of ZAS was tested (paper IV) by adding ALA to ZAS with final concentrations of 0.5 mg/ml and 1.5 mg/ml ALA, respectively, in 10 % ZAS. The concentrations of ALA were chosen based on the range of concentration observed in relation to the PMI. It was found that ALA could significantly inhibit the chemotactic effect of ZAS, *in vitro.* PMN exposed to 1.5 mg/ml ALA + ZAS migrated a significantly shorter distance (86 µm) than the PMNs that were migrating

towards ZAS only (119 µm; control). The corresponding reduction of the PMN migration by 0.5 mg/ml ALA + ZAS was not significant.

The effect of ALA on PMN migration after incubation of PMN with ALA was also examined (paper IV). The migration towards ZAS of PMN pre-incubated in ALA concentrations of 0.5 mg/ml (87 µm) and 1.5 mg/ml (30 µm) was significantly reduced compared with the corresponding results for untreated PMN (109 µm) as was also their random migration. The corresponding values for migration towards Geys' buffer were for PMN pre-incubated in ALA 0.5 mg/ml, 28 µm; in ALA 1.5 mg/ml, 20 µm and for untreated PMN 40 µm.

The results from the chemotaxis assay are, to my knowledge, showing a direct inhibitory effect of ALA on PMN migration *in vitro* for the first time, Based on the effect *in vitro*, the decreased ALA observed after the PMI could have contributed to the enhanced PMN migration, attributable to a reduced inhibiting effect in accordance with Wong *et al.,* (1997 a; b).

However, this is contradictory to the finding that ALA concentration increased at the end of the PMI along with increased PMN migration; such findings would be in agreement with Rusu *et al*., (2009; 2010). The present findings during and after the PMI as well as previous indicatory results regarding the effect of ALA on PMN migration are contradictory to each other. The discrepancy between results could possibly be attributable to ALA exerting a different effect on PMN migration depending on the presence of other factors in milk that could compete with, or enhance, the effect of ALA.

PRL

Prolactin has been shown to influence and regulate the humoral and cellular immune response (for review see Yu-Lee, 2002). Several studies suggest that PRL triggers the pro-inflammatory immune response and stimulates chemotaxis, and that PRL within physiologically achievable concentrations may be selectively involved in PMN migration (Brand *et al*., 2004; Boutet *et al*., 2007). The PRL levels in milk (PRLm) and blood (PRLb) were subjects of interest in paper III and IV. Blood samples were collected in connection with the milk samplings.

In cow *composite milk* samples (paper III) the baseline PRLm concentration was 10.7 ng/ml in morning and 11.2 ng/ml in afternoon milk. After the PMI, a significant increase in PRLm was observed from day 1 in both morning and afternoon milk. In afternoon milk,the PRLm concentration was 13.1 ng/ml day 1. In morning milk, PRLm continued to increase until day 2 (12.7 ng/ml). Thereafter, the PRLm concentration in both morning and afternoon milk

remained significantly increased throughout the study except for an occasional drop in the afternoon on day 3, but the concentrations showed a somewhat fluctuating pattern. The PRLm concentrations in *quarter composite milk* samples were studied until the morning on day 3 after the PMI (paper IV). They were on a similar level and showed a similar pattern as in paper III but with significantly increased concentrations observed only from the afternoon on day 1 until the afternoon on day 2. *During the PMI*, the PRLm concentration was not altered. The first significant increase in PRLm levels occurred at 30 h after the start of the PMI.

The PRLb concentrations in *blood plasma* collected in connection with milking were significantly increased compared to the baseline (morning 23.3 ng/ml; afternoon 29.6 ng/ml) only in the morning milking samples on day 2 and 3 (31.1 ng/ml and 33.2 ng/ml, respectively). During the PMI, PRLb was not altered but the values observed at the first samplings during the PMI were significantly higher than at 0 h, immediately before the PMI.

Boutet *et al*., (2007), showed that upon exposure of mammary epithelial cells to PRL, the mRNA of several cytokines was significantly amplified. Although increased PRLm levels were observed subsequent to PMI (papers III and IV) in parallel with increased PMN migration, it is doubtful if increased PRLm levels could have induced such an effect considering the results regarding pro-inflammatory cytokines in milk after the PMI. IL-1β (paper II) could not be detected and IL-8 (paper IV) was consistently found in low concentrations.

However, PRLm could possibly have acted directly on PMN, as reported by Dogusan *et al*., (2001). A significant direct *chemotactic effect* of PRL in concentrations of 10-15 ng/ml on PMN was observed *in vitro* (paper III). According to Auchtung *et al*., (2003; 2005), PRL may down-regulate its receptors on immune cells over time. Thus, although the PRLm concentration remained increased long after the PMN migration had declined after the PMI, it could still have contributed to enhanced PMN migration initially while the remaining elevated PRLm concentrations could later have led to a downregulation of its receptors on PMN and consequently reduced migration. However, PRLm was found to be unaltered at the time when the PMN migration started to increase during the PMI. Thus it appears less likely that PRLm played a significant role in enhancing PMN chemotaxis.

In paper III, mainly PRLm concentration was investigated. In paper IV, the aim was to map in detail the PRLb concentrations in *plasma* during and after the PMI. The migration of PMN may be influenced by factors in blood, such as PRL, exerting a priming effect enhancing the PMN response to chemotactic stimuli from other sources, as indicated by Dogusan *et al*., (2001). PRL was

found to exhibit such a priming effect on PMN, when tested *in vitro* (paper IV). PMN were pre-incubated with PRL in concentrations of 10, 20, 30 and 40 ng/ml, i.e. comparable to the plasma concentrations observed in the current studies of a PMI (paper III and IV). The pre-incubated PMN responded to chemotactic stimulation of ZAS independently of the PRL concentration with similarly significantly enhanced migration distance (123, 123, 123, 120 μm), compared to untreated PMN (109 μm). PRL also enhanced random migration of the pre-stimulated PMN towards Geys' buffer. Although the PRLb concentration was not altered during the PMI, the concentration observed at the two first samplings (16 h and 18 h) was significantly elevated compared to that observed immediately before the PMI. During this time the proportion of PMN started rising and reached significantly increased values at 20 h. It should not be excluded that PRLb might have contributed to initiation of the enhanced PMN migration during the PMI, but it is less likely that it contributed to the peak in PMN, after the PMI. The increased PRLb levels that were found in the mornings on day 2 and day 3 (paper IV) can rather be considered to be a result of circardian and ultracardian oscillations of PRLb than an effect of the PMI.

Cortisol

Several previous studies have shown an association between milk cortisol concentrations and SCC (Gygax *et al*., 2006; Yagi *et al*., 2004) in relation to stress. Cows have been reported to exhibit signs of discomfort after exposure to long milking intervals (Stefanowska *et al*., 2000; Österman & Redbo, 2001). It has been suggested that the large milk volume during a PMI might cause stress, resulting in increased cortisol concentrations. In order to investigate if a single PMI may induce stress, levels of the milk and serum cortisol concentrations were investigated in paper III.

In blood plasma, the cortisol concentration was found to be unaltered. In morning milk, cortisol increased significantly from the baseline of 1.4 nmol/ml to 1.75 nmol/ml on day 1 after the PMI (paper III) and was occasionally significantly increased also on day 3. No significant alteration in cortisol concentration was observed in afternoon milk.The different patterns of PRLm and cortisol levels in milk (paper III) contradict the results of García-Ispierto *et al*., (2009), which showed a positive relation between these hormones in blood.

The increased milk cortisol concentration observed at the first morning milking after the PMI could, per se, have initiated the PMN migration, but the cortisol concentration dropped immediately and was low when the increase in PMN was most pronounced. The increase in PMN was confirmed, while that in cortisol was not, when total output/release per unit time was calculated. Thus, milk cortisol is un-likely to be a factor that contributed to the enhanced PMN

migration after a PMI. Moreover, no *plasma* cortisol alterations could be observed after the PMI. Enhanced plasma cortisol has been observed together with elevated milk SCC and PMN percentage in once- versus thrice-daily milked cows (Stelwagen and Lacy-Hulbert 1996; Keane *et al*., 2006), i.e. when long milking intervals are applied regularly. According to the results from the current study, a single PMI is not apparently inducing elevated cortisol concentrations in either blood plasma or milk.

5 Conclusions and issues for future research

The results presented in this thesis contribute new knowledge about the initiation and characteristics of the inflammatory reaction in the udder that follows a single PMI in cows and, furthermore, how this inflammatory reaction affects milk composition and yield.

The main findings and specific conclusions of the studies presented in the thesis are:

- Milk yield per cow was significantly reduced by 0.75 kg/day for up to 10 days, the composition was altered but the milk quality was not impaired.

- Epithelial integrity became impaired during the PMI and lasted for approximately one subsequent day as indicated by increased concentrations of lactose in blood and BSA in milk. Damage to the epithelial cells was not indicated, based on consistently low concentration of LDH in milk.

- The PMI caused a 3-fold increase in SCC in cow composite milk for approximately two subsequent days and the SCC peak was associated with an increased proportion of PMN in milk.

- The most pronounced SCC reaction after the PMI was not observed until the second milking, after the udder had been emptied once, but the peak in proportion of PMN was observed earlier, at the first milking. This confirms that inflammatory reactions are reflected in the PMN proportion before it is observable in SCC.

- Initiation of the inflammatory reaction occurred during the PMI and elicited a systemic acute phase response that was observed in blood prior to the reaction in the udder, as shown by the concentration of serum amyloid A in blood and milk.

- The milk consistently showed a significantly high chemotactic activity *in vitro*, but increased milk concentration of the PMN chemoattracting cytokines IL-1β and IL-8 could not be detected during or after the PMI.

- The PMI induced alterations in the concentration of ALA and PRL in milk and blood, in relation to the milk PMN reaction. A significant inhibitory effect of ALA and a stimulatory effect of PRL on PMN migration were shown, *in vitro,* supporting previous findings that these factors exhibit immunoregulatory properties.

- Based on the findings in this thesis it is probable that the acute phase protein serum amyloid A in blood and milk played a significant role in initiating and enhancing the inflammatory reaction after a PMI but it cannot be excluded that ALA and PRL might also have been contributing factors.

For the future:

- For the farmer it would be important to know if anything could be done to reduce the SCC reaction after a PMI has happened. The inflammatory reaction was most pronounced *after* the first milking subsequent to the PMI, suggesting that it was enhanced by the emptying of the udder. Thus, adjustments to milking immediately following a PMI should be investigated for their potential contribution to cutting the SCC peak.

- The results in the thesis are from low SCC cows. Based on previous findings it is reasonable to assume that the SCC reaction after a PMI will be more pronounced in mammary glands with higher SCC.

- To gain a better understanding of this kind of physiological inflammatory episodes in the udder that occur after a PMI, it would be important to investigate further the immunological mechanisms initiating the inflammation. Considering the absence of an obvious inflammatory challenge, an important question to answer is which factor(s) might have elicited a systemic acute phase response, as the initial observable sign of inflammation.

- The inflammatory reaction was reflected in an increased PMN proportion prior to observable alterations in SCC. This confirms results from several previous studies and once more suggests that, for the future, the proportion of PMN rather than SCC ought to be used for detection of inflammation in the udder.

6 Populärvetenskaplig sammanfattning

6.1 Bakgrund

Celltal i mjölken brukar förkortas SCC. Det kommer av engelskans ”somatic cell count” som betyder kroppsceller (till skillnad från t ex bakterieceller). De utgörs i komjölk nästan enbart av vita blodkroppar. Den mer specifika inflammationscell som står för den största delen av SCC-ökningen är neutrofilen.

Ett högt SCC i mjölken anses generellt vara förknippat med lägre kvalitet och nedsatt produktion. Ökat SCC i tankmjölk kan alltså innebära både reducerad volym levererad mjölk och anmärkningar eller missad premiumbetalning för producenten. Det har observerats att efter ett tekniskt stopp i automatiska mjölkningssystem ökar tillfälligt SCC i tankmjölken. Efter stoppet får många kor stå länge i kö innan de blir mjölkade och mjölkningsuppehållet för en enskild ko kan bli upp till ett dygn. Det är troligt att detta är orsaken till den observerade SCC-toppen. Det är känt sedan tidigare att olika långa mjölkningsintervall (MI), applicerade under längre tid, påverkar kornas SCC även om det inte är så uttalat som vid en infektiös mastit. Hur ett enda förlängt MI (FMI) påverkar SCC på konivå samt mjölkmängd och mjölksammansättning, har dock inte tidigare undersökts.

Ökad rekryteringen av celler/vita blodkroppar till juvret och mjölken utgör en väsentlig del av inflammationsreaktionen, som är en försvarsmekanism. Celltalen är således en indikator på inflammation i juvret men vid ett FMI finns inte något uppenbart inflammatoriskt stimuli, som t ex en infektion utgör. Så vad kan vara orsaken? Den ökade cellvandringen till mjölken skulle kunna bero på mikroskopiska vävnadsskador, på grund av att juvret spänns ut av den ackumulerade mjölkvolymen under FMI. Det kan resultera i läckage från vävnadsvätskan som omger cellerna, av faktorer (cytokiner) som verkar cellattraherande. Men orsaken kan också tänkas vara t ex en förändring i koncentrationen av vanliga mjölkkomponenter. Flera sådana, såsom

vassleproteinet alfalaktalbumin (ALA) och laktationshormonet prolaktin (PRL), har i olika sammanhang visats kunna ha en cytokinliknande effekt. Resultaten från tidigare undersökningar är dock delvis motsägelsefulla då det gäller de här ämnenas mera specifika effekt på cellvandringen. Vissa studier har visat att ALA respektive PRL kan hämma cellvandringen medan andra tvärtom har indikerat att den stimuleras av båda ämnena. Det kan tala för att de kan verka olika beroende på omständigheterna.

Att skaffa mer kunskap om den här typen av steril fysiologisk inflammation i juvret som inte har någon sjuklig historia är väsentligt, dels för att bättre kunna tolka SCC på både besättnings- och konivå, dels för att öka insikten i hur juvrets immunförsvar fungerar generellt.

6.2 Syftet med studierna

- Att undersöka SCC-reaktionen på konivå efter ett FMI på 24 tim och huruvida mjölksammansättning och mjölkmängd förändras. För att se om reaktionen liknar den man ser vid infektiös mastit undersöktes även andelen neutrofiler.
- Att undersöka den immunologiska bakgrunden till SCC-topparna efter ett FMI, genom att analysera förekomst i mjölken av vedertagna cytokiner samt även ALA och PRL. Några andra inflammationsindikatorer än SCC undersöktes också för att få en mera fullständig bild av reaktionen.
- Det långsiktiga målet var även att, baserat på resultaten från de aktuella studierna, analysera om det skulle kunna vara möjligt att kupera SCC-toppen genom åtgärder efter ett FMI.

6.3 Studiernas uppläggning

Kor med låga celltal som mjölkades 2 ggr dagligen, följdes före och efter ett FMI på 24 tim (genom att kvällsmjölkningen uteslöts vid ett tillfälle). Kornas celltal var < 50 000/ml i morgonmjölk och < 100 000/ml i kvällsmjölk vid början av samtliga studier. Vid varje mjölkning undersöktes samlingsmjölken per ko eller juverdel. Mindre mjölkprov togs även under FMI och mellan de efterföljande mjölkningarna för att mer i detalj undersöka den inflammatoriska reaktionen. Blodprov togs vid mjölkprovtagningarna.

6.4 Resultat och diskussion

6.4.1 Mjölkmängd och mjölksammansättning

Mjölkmängden var tydligt reducerad efter FMI - och under överraskande lång tid. Under de 10 dagar som korna följdes mjölkade varje ko i genomsnitt ca 0,75 kg mindre per kvällsmjölkning. Även i morgonmjölkningen sågs en reduktion men den var inte statistiskt säkerställd. Det är anmärkningsvärt att produktionen påverkades så starkt av en så kortvarig juverstörning. *Under* FMI var produktionen reducerad med hela 20 %. Mjölksammansättningen förändrades bara marginellt och påverkade inte kvaliteten negativt.

6.4.2 Celltal

Celltalstoppen kunde konstateras även på ko- och juverdelsnivå. Den största ökningen avseende både det totala celltalet och andelen neutrofiler inträffade dock inte *under* FMI utan först *efter* det att mjölkningen återupptagits. Förändringen var störst i kvällsmjölkning där toppen inträffade dag 1 (båda faktorerna ökade 2-3 gånger). Även om celltalet gick ned under dag 2 kunde det kvarstå lindrigt förhöjt både morgon och kväll under 5 dagar.

6.4.3 Liknar inflammationsreaktionen den som ses vid infektionsutlöst masitit?

Undersökningen både av SCC och andra inflammationsmarkörer visade att reaktionen efter ett FMI liknar den som ses vid infektiös mastit, men är mera ringgradig. Akutfasproteiner frisätts mycket tidigt under inflammation. Ett FMI framkallade, inte bara en lokal utan även en generell akutfasreaktion, vilken inträffade samtidigt med att neutrofilrekryteringen ökade till mjölken. Den generella reaktionen tyder på en central påverkan i kroppen och det är anmärkningsvärt att en så förhållandevis liten störning i juvret som ett FMI – jämfört med t ex infektiös mastit – ger en systemeffekt. Akutfasproteinet serum amyloid A kan stimulera de vita blodkropparnas förflyttning från blod genom vävnaden och sågs öka parallellt med ökningen av neutrofilerna i mjölken vilket gör det till en trolig faktor bakom neutrofilreaktionen.

Albumin som finns i vävnadsvätska och blod ökade i mjölken. Det skedde efter den första mjölkningen efter FMI, då mjölktrycket i juvret minskat. Det indikerar att det fanns ett visst läckage från vävnaden in mjölken då. Dock kunde inte ökade koncentrationer av cytokinerna IL-1β respektive IL-8 påvisas i mjölken. Det antyder att dessa cytokiner inte spelar en avgörande roll för SCC och neutrofilreaktionen efter ett FMI, även om det är välkänt att båda cytokinerna generellt har en stark effekt på neutrofilernas vandring.

6.4.4 Prolaktin och alfa-laktalbumin

Det förlängda mjölkningsintervallet framkalla de förändringar i koncentrationen av ALA (i mjölk) och PRL (i mjölk och blod) i anslutningen till ökningen av neutrofiler. I lab-tester visades ALA hämma och PRL stimulera neutrofilernas vandring. Baserat på dessa resultat kan det inte uteslutas att de båda faktorerna kan ha bidragit till SCC reaktionen efter ett FMI.

6.5 Sammanfattning och slutsatser

- Ett FMI leder till ca 3 ggr ökat mjölkcelltal under 1-2 dagar, mest uttalat efter det att mjölkning återupptagits samt dessutom till nedsatt mjölkproduktion per ko med > 0,75 kg/dag under 10 dagar eller mer.

- Mjölksammansättning påverkades men kvaliteten försämrades inte.

- Det är sannolikt att reaktionen efter ett FMI är större hos kor med högre SCC-nivåer.

- Akutfasproteinet serum amyloid A spelar sannolikt en betydande roll för att sätta igång cellreaktionen efter ett FMI men det kan inte uteslutas att även ALA och PRL kan vara bidragande aktörer. Vilka faktorer som initierar akutfasreaktionen har inte identifierats.

- Ett FMI ger väsentligt negativa ekonomiska konsekvenser för producenten, främst genom den långvarigt reducerade mjölkmängden men även genom eventuellt lägre betalning för mjölken på grund av SCC-toppen trots att mjölkkvaliteten inte tycks påverkas av ett FMI.

- Det är viktigt att förebygga mjölkstopp och om de inträffar, se till att de blir så kortvariga som möjligt.

- Att den största reaktionen inträffar först efter det att mjölkning återupptagits kan indikera att celltalstoppen möjligen skulle kunna kuperas, med anpassad mjölkning en eller några ggr efter ett FMI. Detta bör undersökas vidare.

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