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Serum Amyloid A in ruminants:

diagnostic value and food contamination assessment

Vet/03 - Patologia Generale e Anatomia Patologica Veterinaria

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Dubium sapientiae initium

(René Descartes)

Contents

Abstract

The aims of the work presented in this thesis were to investigate the bovine acute phase protein Serum Amyloid A, focussing on its value as safety marker in farm animals.

SAA can be considered as a natural anti-inflammatory and immunomodulatory agent and local expression of SAA, at the site of the initial acute phase reaction, could protect against the deleterious effects of inflammation.

In this study whether SAA can be isolated from tissues of bovine with clinical amyloidosis was investigated. We also investigated if AA fibrils present in milk can be then found in cheese after caseification, i.e. if the process of ripeining can degrade the AA fibrils.

In bovine, SAA was identified as potential marker of mastitis, and SAA milk concentration in milk increases before the raising of somatic cells.

In this thesis two aspects of the involvement of SAA in food safety were explored:
a) the acute phase reaction strongly focused on the mammary gland. The animal model chosen was water buffalo, since no information is available so far about the acute phase reaction in this species. The acute phase proteins sequences are unknown, and also their concentration in physiological and pathological conditions are not established.
b) The possibility that high concentration of SAA in milk induce the formation of amyloid fibrils, which are considered to be potentially dangerous for human safety.

Results presented in this thesis advanced the knowledge of the acute phase reaction in water buffalo: the five APPs included in this investigation, namely Serum amyloid A, Haptoglobin, Ceruloplasmin, α1-acid glycoprotein and Lipolysaccaride Binding Protein were sequenced for the first time, and two of them were quantified.

In the second part of the thesis, we purified amyloid fibryls from amyloidosis-affected cows, and added purified fibrils at a given concentration in milk before ripening.

Results demonstrated the presence of insoluble fibrils in cheese added with amyloid proteins, even if a lower amount of precipitated insoluble SAA could be detected also in negative control cheese.

Chapter 1

Introduction to the thesis

The systemic reaction of inflammation and the acute phase proteins
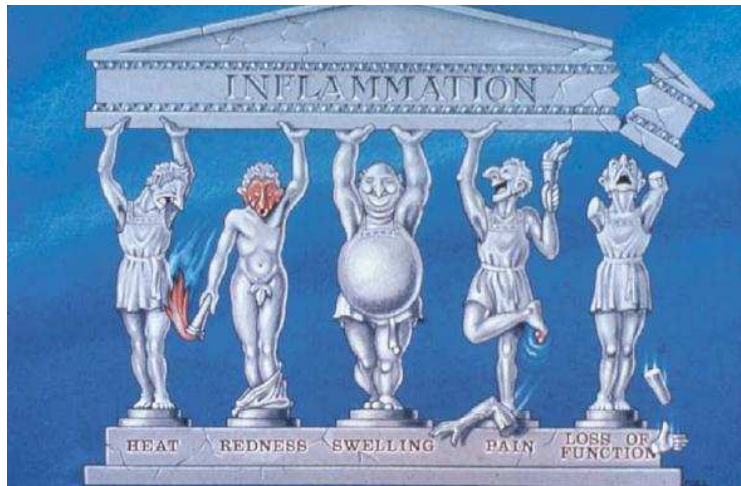
1.1 Inflammation

Inflammation is a defensive response that enables survival during infection or injury and maintains tissue homeostasis under a variety of noxious conditions.

Inflammation has been known to humankind for thousand years, in part because it accompanied two major scourges of the past, wounds and infections, and in part because it is rather conspicuous. Although references to inflammation can be found in ancient medical texts, apparently the first to define its clinical symptoms was the Roman doctor Cornelius Celsus in the 1ˢᵗ century AD. These symptoms came to be known as the four cardinal signs of inflammation: "*Notae vero inflammationis sunt quattuor: rubor et tumor cum calore et dolore*". Celsus mentions these signs in his treatise *De medicina*, while describing procedures for treating chest pain (Majno, 1975). The fifth cardinal sign, *functio laesa,* was added by Galen in 3ʳᵈ century AD and restates by Rudolph Virchow in 1858 in his book *Cellularpathologie* a collection of lectures published in 1858, which was instantly accepted as the cornerstone of modern pathology: *"Nobody would expect a muscle which is inflamed, to perform is function normally...Now there can be no doubt that to the four characteristics symptoms of inflammation functio laesa must be added"* (Majno, 1975).

Advances in microscopy and cell biology in the 19th century gave rise to cell based definitions of inflammation. This represented a completely novel way of understanding and defining inflammation. By the end of the 19th century it was acknowledged that changing cell populations arising from both the blood and local proliferation were a key feature of many models of inflammation (Cotran, 1999). With the advent of the microscope, such a complexity of events underlying inflammatory reactions was revealed that researchers began to question whether inflammation was indeed a single process (Table 1.1).

AUTHOR, YEAR	QUOTATION	MODERN SIGNIFICANCE
Celsus, 1st century AD	*Rubor et tumor cum calore et dolore*	Emphasised the importance of clinical observations rather than philosophy based medicine
Galen, 3rd century AD	*pus bonus et laudabilis – functio lesa*	Inflammation was seen as an expression of humoral theory well into the 19th century
Virchow, 1858	The inflammatory reaction is a consequence of an excessive intake by interstitial cells, of food…filtering through the vessel wall	Recognised cellular nature of inflammatory response
Cohnheim, 1873	Finally…there lies outside the vessel…a colorless blood corpuscle	First description of diapedesis
Metchnikoff, 1908	The *primum movens* of the inflammatory reaction is a digestive action…toward the noxious agent	First to express the view that phagocytes were protective, not pathological
Lewis, 1927	Triple response to injury	First recognition of neurogenic inflammation; first physiological characterisation of vascular events
Rocha & Silva, 1974	Multi-mediated phenomenon, of a pattern type in which all mediators would come and go at the appropriate moment…increasing vascular permeability, attracting leucocytes, producing pain, local edema and necrosis	Biochemical definition of inflammation

Table 1.1. Key advances in developing a definition of inflammation between 1st and 20th centuries AD (Scott 2004)

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury (Majno et al 2004; Kumar et al 2003). Inflammation is fundamentally a protective response, the ultimate goal of which is to rid the organism of both initial cause of cell injury and the consequences of such injury. At the site of invasion by a microorganism and the place of tissue injury a number of responses of the tissue itself are initiated. During repair the injured tissue is replaced by regeneration of native parenchymal cells, by filling of the defect with fibroblastic tissue or by a combination of these two processes. The inflammatory response occurs in the vascularised connective tissue, including

plasma, circulating cells, blood vessels, cellular and extra-cellular constituents of connective tissue. If this delicate balance between inflammation and resolution becomes deregulated, inflammation can lead to disease.

Local inflammation is divided into acute (angioflogosis) and chronic (histoflogosis). Systemic inflammation, also called acute phase response (APR), can also occur. It is defined as "*a prominent systemic reaction of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma or surgery, neoplastic growth or immunological disorders*" (Gordon & Koy, 1985; Gruys et al., 1999).

At a local level, the acute inflammatory response triggered by infection or tissue injury involves the coordinated delivery of blood components (plasma and leukocytes) to the site of infection or injury (Majno, 2004; Kumar, 2003). This response has been characterized best for microbial infections (particularly bacterial infections), in which it is triggered by receptors of the innate immune system, such as Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) (Barton, 2008). This initial recognition of infection is mediated by tissue resident macrophages and mast cells, leading to the production of a variety of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades.

The main and most immediate effect of these mediators is to elicit a local inflammatory exudate: plasma proteins and leukocytes (mainly neutrophils) that are normally restricted to the blood vessels now gain access to the extravascular tissues at the site of infection. The activated endothelium of the blood vessels allows selective extravasation of neutrophils while preventing the exit of erythrocytes. This selectivity is afforded by the inducible ligation of endothelial-cell selectins with integrins and chemokine receptors on leukocytes (Pober & Sessa, 2007). When they reach the afflicted tissue site, neutrophils become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells. Moreover, the neutrophils try to kill the invading agents by releasing the toxic contents of their granules, which include reactive oxygen species (ROS) and reactive nitrogen species, proteinase 3, cathepsin G and elastase (Nathan, 2006). These highly potent effectors do not discriminate between microbial and host targets. Therefore, collateral damages to host tissues are unavoidable (Nathan, 2002). A successful acute inflammatory response results in the elimination of the infectious agents followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages (Serhan & Savill, 2005). The switch in lipid mediators from pro-inflammatory prostaglandins to lipoxins, which are anti-inflammatory, is crucial for the transition from inflammation to resolution. Lipoxins inhibit the

recruitment of neutrophils and, instead, promote the recruitment of monocytes, which remove dead cells and initiate tissue remodelling (Serhan & Savill, 2005). Resolvins and protectins, which constitute another class of lipid mediator, as well as transforming growth factor-β and growth factors produced by macrophages, also have a crucial role in the resolution of inflammation, including the initiation of tissue repair (Serhan & Savill, 2005; Serhan, 2007).
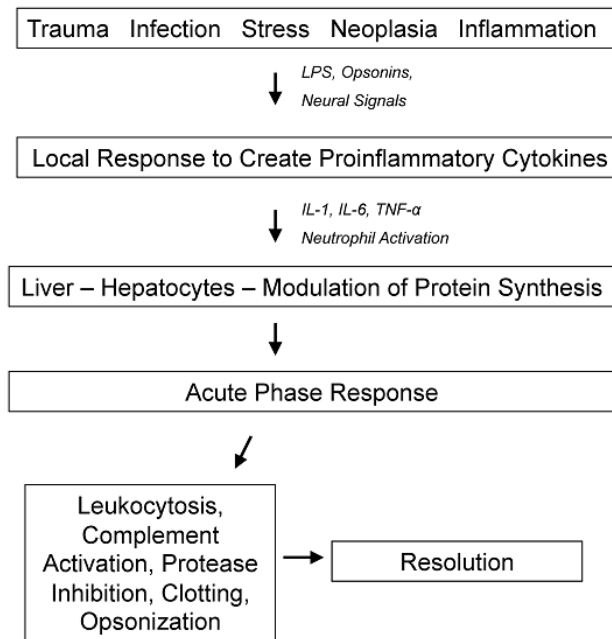
Figure 1.1. The acute phase response. (Cray, 2009).

If the injurious agent cannot be quickly eliminated, the result may be chronic inflammation. Chronic inflammation is a prolonged process where active inflammation, tissue destruction and attempt at healing may all proceeding simultaneously. Considerable progress has been made in understanding the cellular and molecular events that are involved in the acute inflammatory response. On the contrary, the events that lead to local chronic inflammation, particularly in chronic infections and autoimmune diseases, are still partially understood. Much less is known, however, about the causes and mechanisms of systemic chronic inflammation, which occurs in a wide variety of diseases, such as, for example, type 2 diabetes and cardiovascular diseases. Therefore, given that inflammation not only is beneficial but also can be damaging if prolonged, it is not surprising that the entire process is tightly regulated (Fig.1.2)
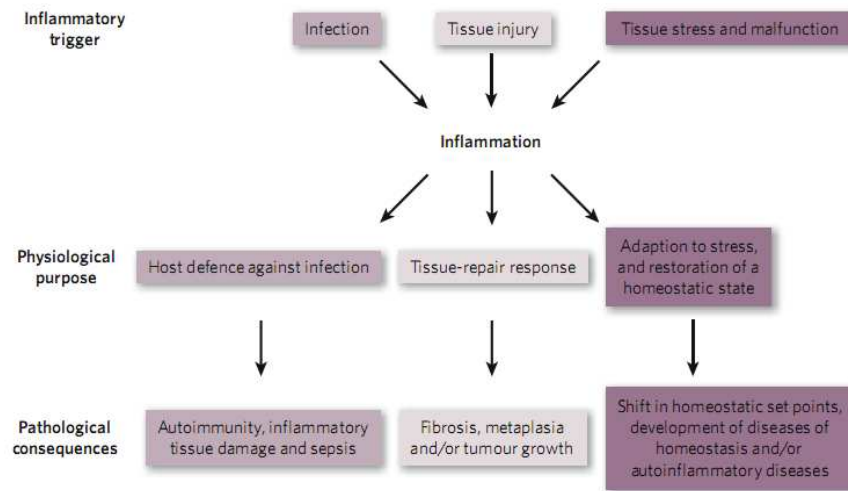
Figure 1.2. Causes, and physiological and pathological outcomes of inflammation. Depending on the trigger, the inflammatory response has a different physiological purpose and pathological consequences. Of the three possible initiating stimuli, only infection-induced inflammation is coupled with the induction of an immune response (Medzhitov, 2008).

The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks. They activate specialized sensors, which then elicit the production of specific sets of mediators. The mediators, in turn, alter the functional states of tissues and organs in a way that allows them to adapt to the conditions indicated by the particular inducer of inflammation. Thus, a generic inflammatory pathway consists of inducers, sensors, mediators and effectors, with each component determining the type of inflammatory response (Fig. 1.3).
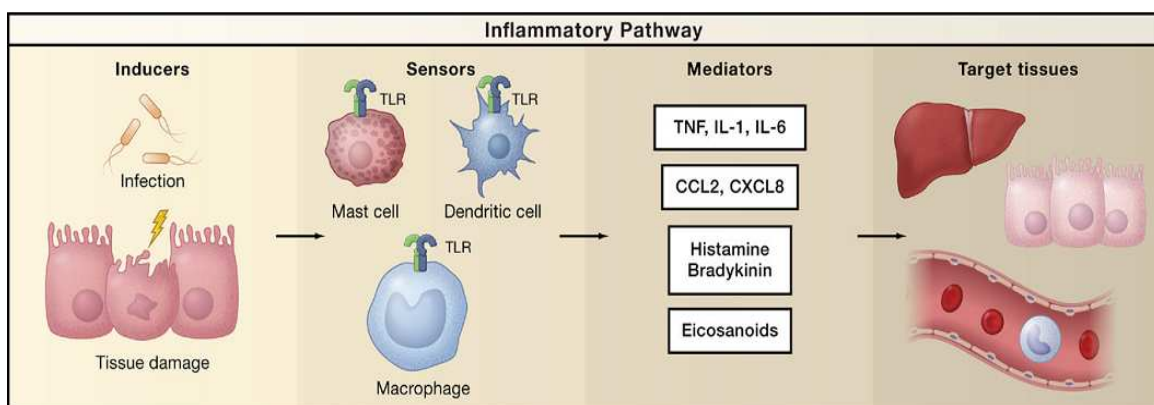
Figure 1.3. The inflammatory pathway consists of inducers, sensors, mediators, and target tissues. Inducers initiate the inflammatory response and are detected by sensors. Sensors are expressed on specialized sentinel cells and they induce the production of mediators. These inflammatory mediators act on various target tissues to elicit changes in their functional states that optimize adaptation to the noxious condition associated with the particular inducers that elicited the inflammatory response. (Medzhitov, 2010).

1.1.1 Acute Phase Proteins and other systemic responses to inflammation

A large number of changes, distant from the site or sites of inflammation and involving many organ systems, may accompany inflammation. These systemic changes have previously been referred to as the acute-phase response, even though they accompany both acute and chronic inflammatory disorders (Kushner, 1993).

Acute-phase changes may be divided into changes in the concentrations of many plasma proteins, known as the acute-phase proteins, and a large number of behavioural, physiologic, biochemical, and nutritional changes (Table 1.2).

NEUROENDOCRINE CHANGES	HEMATOPOIETIC CHANGES	METABOLIC CHANGES	HEPATIC CHANGES	CHANGES IN NONPROTEIN PLASMA CONSTITUENTS
Fever, somnolence, and anorexia	Anemia of chronic disease	Loss of muscle and negative nitrogen balance	Increased metallothionein, inducible nitric oxide synthase, heme oxygenase, manganese superoxide dismutase, and tissue inhibitor of metalloproteinase1	Hypozincemia, hypoferremia, and hypercupremia
Decreased production of insulin-like growth factor I	Leukocytosis	Osteoporosis	Decreased phosphoenolpyruvate carboxykinase activity	Increased plasma retinol and glutathione concentrations
Increased secretion of corticotropin-releasing hormone, corticotropin, and cortisol	Thrombocytosis	Increased hepatic lipogenesis Increased lipolysis in adipose tissue		
Increased secretion of arginine vasopressin		Decreased lipoprotein lipase activity in muscle and adipose tissue		
Increased adrenal secretion of catecholamines		Decreased gluconeogenesis Cachexia		

Table 1.2. Acute Phase responses (modified from Gabay & Kushner 1999).

The acute phase proteins (APPs) are a group of blood proteins considered to be non-specific innate immune components involved in the restoration of homeostasis and the restraint of microbial growth before animals develop acquired immunity to a challenge. APPs plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25% during inflammatory disorders. In any given species, particular APPs

demonstrate major, moderate or minor responses. A major APP "responder" has a low serum concentration (<1 mg/l) in healthy animals that rises dramatically by 100–1000-fold on stimulation, peaking at 24–48 hours and then declining rapidly during the recovery phase. Moderate responders increase some 5–10-fold on activation, peak after 2–3 days, and decrease more slowly than major APP "responder". A minor APP responder gradually increases by between 50% and 100% of its resting level. "Negative" APP biomarkers, which fall in concentration during the inflammatory response, have also been identified, although except for albumin, their use in veterinary clinical pathology is not common (Eckersaal & Bell, 2010). The positive APP are glycoproteins synthesized mainly by hepatocytes upon stimulation by pro-inflammatory cytokines and released into the bloodstream. The positive APP include haptoglobin (Hp), C-reactive protein (CRP), serum amyloid A (SAA), caeruloplasmin (Cp), fibrinogen (Fb) and alpha 1-acid glycoprotein (AGP). The negative APP include albumin, the most abundant constitutive plasma protein, and transferrin.

Conditions that commonly lead to substantial changes in the plasma concentrations of acute-phase proteins include infection, trauma, surgery, burns, tissue infraction, various immunologically mediated and crystal-induced inflammatory conditions, and advanced cancer. Moderate changes occur after strenuous exercise, heatstroke, and childbirth. Small changes occur after psychological stress and in several psychiatric illnesses (Gabay & Kushner, 1999) (Fig. 1.4).
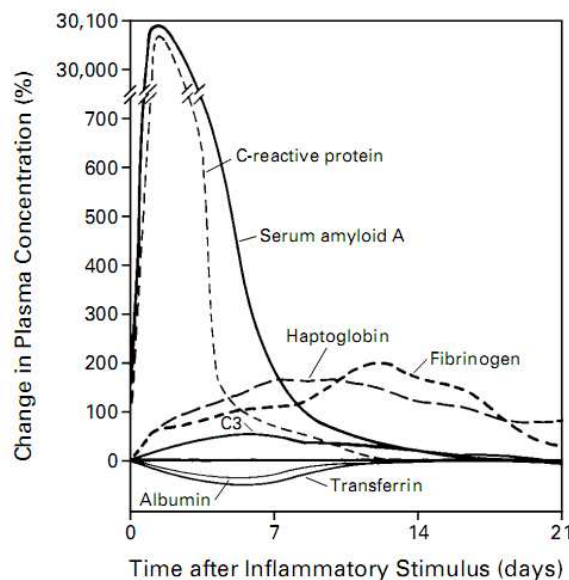

Figure 1.4. Characteristic Patterns of Change in Plasma Concentrations of Some Acute-Phase Proteins after a Moderate Inflammatory Stimulus (Gabay et al. 1999)

Extrahepatic productions of APP have also been described in most of the mammalian species that have been studied so far (Uhlar and Whitehead, 1999; Vreugdenhil et al., 1999). Among the proinflammatory cytokines, interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-alpha) and interleukin-1-beta (IL-1-β) are the major mediators of APP synthesis in the liver (Nakagawa-Tosa et al., 1995; Alsemgeest et al., 1996; Yoshioka et al., 2002). These cytokines are released mainly by macrophages but also by other cells in response to various external or internal stimuli. For example, IL-6 can be synthesized by Kupffer cells and keratinocytes (Heinrich et al., 1990), in the pituitary (Abraham & Minton, 1997) or in the mucosal epithelium (Pritts et al., 2002). Inflammation, infection or tissue injury triggers cytokine release by defence-oriented cells, thereby inducing APP synthesis. The induction is associated with a decrease in synthesis of negative APP such as albumin (Gruys et al., 1994). It is widely accepted that, in humans and experimental animals, physical and psychological stress elevates plasma IL-6 and APP levels (Deak et al., 1997; Nukina et al., 2001). There is also evidence in cattle that physical stress can induce APP (Murata & Miyamoto, 1993; Alsemgeest et al., 1995). Although the mechanism of APP induction in response to stress is yet to be elucidated, activation of the hypothalamic–pituitary–adrenal (HPA) axis by stress signals may be a trigger of systemic or local (intra-pituitary) cytokine production, thereby augmenting hepatic APP synthesis and release into the bloodstream. APP levels also increase in healthy cows, mares or dogs at parturition (Yamashita et al., 1991; Taira et al., 1992; Alsemgeest et al., 1993; Uchida et al., 1993; Vannucchi et al., 2002), in cows with hepatic lipidosis (Yoshino et al., 1992; Nakagawa et al., 1997), or in fasted cows following dexamethasone administration (Yoshino et al., 1993), possibly as a result of hepatic APP production in response to glucocorticoids or oestradiol (Higuchi et al., 1994). Glucocorticoids, which are synthesized through the HPA-axis, act to suppress pro-inflammatory cytokines and up-regulate anti-inflammatory cytokines (Bethin et al., 2000). Thus the HPA-axis contributes to the modulation of APP synthesis. Conversely, cytokines modulate glucocorticoid production by the HPA-axis. For example, IL-6 stimulates the HPA-axis and induces augmented adrenal function, which in turn suppresses cytokine production by negative feedback (Bethin et al., 2000). This network of induction and regulation of APP synthesis is shown in Fig. 1.5. In such a complicated but precise regulation network, the APP play an important role in a variety of defence-related activities such as killing infectious microbes, repair of tissue damage and restoration of the healthy state.
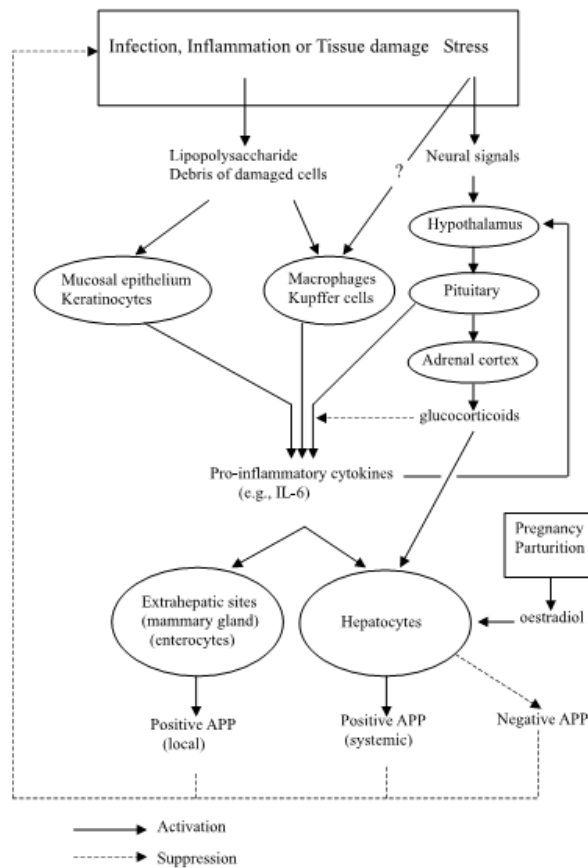
Figure 1.5. Induction and regulation network of acute phase protein (APP) synthesis in animals subjected to internal or external challenge (Murata et al., 2004).

1.2 Biological functions of APPs

The assumption that the changes in plasma concentrations of acute-phase proteins are beneficial is based largely on the known functional capabilities of the proteins and on logical speculation as to how these might serve useful purposes in inflammation, healing, or adaptation to a noxious stimulus. The function of most APPs has not been definitely elucidated. The positive APPs are regarded as having general functions in opsonization and trapping of microrganisms and their products, in activating complement, in binding cellular remnants like nuclear debris, in neutralizing enzymes, scavenging free haemoglobin and radicals, and in modulating the host's immune response (Uhlar & Whitehead, 1999; Vreugdenhil et al., 1999, Lecchi et al., 2008).

C-reactive protein (CRP) plays important roles in protection against infection, clearance of damaged tissue, prevention of autoimmunization and regulation of the inflammatory response (Mold et al., 2002). Is structurally conformed as a ring consisting of five 23KDa units (Volanakis, 2001) and is the first described APP (Tillett & Francis, 1930). It was discovered due to its

binding to the C-polysaccharide of pneumococci. It binds directly to several microorganisms, degenerating cells and cell remnants, activates complement by the classical C1q pathway and acts as opsonin. CRP interacts also with specific receptors on phagocytic cells to mediate phagocytosis and induces the production of anti-inflammatory cytokines, linking non-specific innate immunity with specific adaptive immunity (Du Clos & Mold, 2001).

Serum Amyloid A (SAA): see below.

Alpha 1-acid glycoprotein (AGP) is a sialo-glycoprotein synthesized and secreted mainly by hepatocytes. Extrahepatic AGP production and gene expression have also been confirmed (Fournier et al., 2000; Lecchi et al., 2009). Local AGP may contribute to the maintenance of homeostasis by reducing the tissue damage associated with the inflammatory process taking place in extrahepatic cell types, notably epithelial and endothelial cells. Systemic AGP has two major physiological functions: drug binding and immunomodulation. concentration. AGP is a natural anti-inflammatory agent, in that AGP inhibits neutrophil activation and increases the secretion of IL-1 receptor antagonist by macrophages (Fournier et al., 2000). AGP may help to enhance the clearance of lipopolysaccharide (LPS) by binding directly with LPS and neutralizing its toxicity (Moore et al., 1997). AGP also inhibits mitogen-induced lymphocyte proliferation (Itoh et al., 1989) and natural killer cell activity (Okumura et al., 1985).

Haptoglobin (Hp) strongly binds haemoglobin, has anti-inflammatory capabilities and binds to CD11b/CD18 integrines (El Ghmati et al., 1996) which are major receptors on the cell membranes of leukocytes. Although representing a positive APP, its concentration may decrease on massive erythrolysis and when blood is haemolytic, therefore the determination by haemoglobin binding assays may give unreliable results.

Ceruloplasmin (Cp) is a copper-containing ferroxidase that oxidizes toxic ferrous iron to its non-toxic ferric form. Cp promotes iron loading onto transferrin, which only binds the ferric form of the metal. In addition, Cp is an effective antioxidant, because of its ability to oxidize highly toxic ferrous iron to the relatively nontoxic ferric form and thus help prevent oxidative damage to proteins, lipids, and DNA (Inoue et al., 1999; Patel et al., 2002). Cp may act as an anti-inflammatory agent by reducing the number of neutrophils attaching to the endothelium and by acting as an extracellular scavenger of peroxide (Broadley & Hoover, 1989; Segelmark et al., 1997).

Collectin family includes lung surfactant proteins, conglutinin (a mammalian lectin) and mannan-binding lectin (MBL). MBL has been reported in chickens as a minor acute phase reactant and an opsonin of the innate immune defence against various microorganisms (Nielsen et al., 1999). The family as a whole is considered to mediate the alternative pathway of complement, a primitive immune mechanism related to the innate immune system, in an antibody-independent manner, thereby contributing to the capacity of the pathway to distinguish between self and non-self (Tabel, 1996).

Fibrinogen (Fb) is involved in homeostasis after tissues injuries by providing a substrate for fibrin formation, and in tissue repair and a matrix for the migration of inflammatory related cells. Fb specifically binds to CD11/CD18 integrins on the cell surface of migrated phagocytes, thereby triggering a cascade of intracellular signals that lead to enhancement of degranulation, phagocytosis, antibody-dependent cellular cytotoxicity and delay of apoptosis (Sitrin et al., 1998; Rubel et al., 2001).

1.3 APPs in veterinary medicine

Acute phase proteins may provide an alternative means of monitoring animal health. An increased focus on the application of APP for this purpose has recently been developed (Skinner et al., 1991). Due to a relatively short half life in serum and high response in diseased animals (Mackiewicz, 1997), APP serum responses constitute a valid measure of a systemic response to an initiating stimulus at the time of blood sampling. Like rectal temperature, APP levels are not suitable for establishing a specific diagnosis but can provide objective information about the extent of ongoing lesions in individual animals. At the herd level, APP might be useful for determining where the spread of the disease is taking place (age group, part of the production system), by providing information about the prevalence of ongoing clinical and subclinical infections indicated by the high serum concentration of selected APP (Petersen et al., 2002) and by serving as a prognostic tool, with the magnitude and duration of the acute phase response reflecting the severity of infection (Hirvonen et al., 1999; Hultén et al., 2002; Peltola, 1982; Skinner et al., 1991). Distinct positive APPs from some species do not behave in the same way in other species; for example Serum Amyloid P-component (SAP) is an APP in the mouse, but not in man, and CRP reacts as APP in several monogastric species, but not very well in ruminants (Gruys et al., 1994) (Table 1.3). Transferrin, which is a negative APP of most mammalian species, reacts as positive APP in chicken (Hallquist & Klasing, 1994; Tohjo

et al., 1995). CRP, SAA, Hp and other APPs, have been described as useful for assessing health in human patients (Blackburn, 1994; Ferard et al., 2002) and in various animals (Gruys et al., 1994; Petersen et al., 2004; Toussaint, 2000; Toussaint et al., 1997), including also marine mammalians (Duffy et al., 1996; Funke et al., 1997).

APP	CHICKEN	DOG	HORSE	PIG	RUMINANTS
AGP	▲	▲	▲-•	▲	▲
Cp	▲	▲	•	•	•
CRP	NC	■	▲	▲	▲
Fb	•	▲	▲-•	▲-•	▲
Hp	NC	▲	▲	▲	■
SAA	▲	▲	■	▲	▲-■

Table 1.3. Classification of APP in domestic animals: ■, major (10–100-fold increase in response to stimuli); ▲, moderate (2–10-fold increase); •, minor (<2-fold increase); and NC, not confirmed (Murata et al., 2004).

1.4 Serum Amyloid A (SAA)

The SAA family was originally considered to comprise only a single circulating precursor of the amyloid A protein from where its name is derived from. The amyloid A protein is the principal component of the secondary amyloid plaques that may be deposited in major organs as an occasional consequence of chronic inflammatory disease (Husby et al. 1994). Serum Amyloid A (SAA) is associated with the serum high-density lipoprotein (HDL) and is a non-glycosylated apolipoprotein with a molecular weight ranging between 11 and 14 KDa, depending on species (Benditt & Eriksen, 1977; Nakayama et al., 1993; Gruys et al., 1994). HDL is a sphere of 7-10 nm in diameter, and has an apparent molecular weight of 200 KDa (van der Westhuyzen et al., 1986). The particle consists of a core of esterified cholesterol and triglycerides surrounded by apolipoproteins, unesterified cholesterol, and phospholipids (Rosenthal et al., 1976). HDL also contains the enzymes lecithin cholesterol acyl transferase (LCAT), which esterifies cholesterol, and cholesterol ester transfer protein, which transfers cholesterol esters between lipoproteins (Mahley & Innerarity, 1983; Jiao et al.,1980). One major role proposed for HDL is to reverse cholesterol transport, which is removing cholesterol from peripheral tissues to the liver for catabolism and secretion. This is vital, since the liver is the major site of cholesterol degradation and removal. HDL's role in reverse cholesterol transport is supported indirectly by the observation that HDL concentrations are negatively associated with the risk of coronary heart disease from atherosclerosis (Lacko, 1994). Under non-inflammatory conditions, HDL contains very low levels of SAA (Rosenthal et al., 1976). Once inflammation occurs, SAA levels increase dramatically. Most of the SAA is present in the denser HDL3 subclass, and accounts

for 20% to 50% of HDL's total apolipoprotein (van der Westhuyzen et al., 1986); only 5-10% of total plasma SAA is associated with the other lipoproteins. The remainder is free or associated with albumin (Husby et al., 1994). In tissues it attracts inflammatory cells and inhibits the respiratory burst of leukocytes (Linke et al., 1991) and modulates the immune response (Gruys et al., 1994). It has been reported to bind lipopolysaccharides (LPS), comparable to LPS binding protein (LBP) (Schroedl et al., 2001).

The SAA family is now known to contain a number of differentially expressed apolipoproteins which are synthesized primarily by the liver and can be divided into two main classes based on their responsiveness to inflammatory stimuli "Acute phase" SAA (A-SAA) and "Constitutive" SAA (C-SAA). The A-SAA, which increases in concentration in the plasma during the acute phase response, is mainly synthesized by the hepatocytes, whereas the C-SAA, which is found to be associated with HDL under normal conditions and which is not significantly increased during the acute phase response, is produced both in the liver and in other organs (Whitehead et al., 1992; Steel et al., 1993, 1996; de Beer et al., 1994). In birds, only one SAA type, corresponding to mammalian A-SAA, has been described (Guo et al., 1996; Ovelgönne et al., 2001; Kovacs et al., 2005). There is not only a response difference between A-SAA and C-SAA, but various publications also describe different SAA genes that code for A-SAA and its isotypes in many species including man.

1.5 SAA structure and expression

1.5.1 Gene

Multiple SAA genes and proteins have been described for several mammalian species including human, mouse, hamster, rabbit, dog, mink, cow, sheep and horse. The high degree of conservation of the SAA genes and proteins that has been maintained through the evolution of eutherian mammals (Uhlar et al., 1994) extends to other vertebrates including marsupials (Uhlar et al., 1996) and fish (Jensen et al., 1997) thereby providing further evidence that they are likely to have important biological functions. All of the SAA genes described to date share a four-exon three-intron organization which is characteristic of many other apolipoproteins (Fig 1.6) (Steel & Whitehead, 1994). The SAA genes of human and mouse, which comprise four and five members, respectively, are those that have been subjected to the most comprehensive analyses. In other mammalian species the SAA family members are less well defined. There are at least three transcribed A-SAA genes in dog (Sellar et al., 1991), mink (Marhaug et al., 1990; Rygg et al., 1993) and rabbit (Ray & Ray, 1991; Mitchell et al., 1991). In the case of horse, three

SAA isoforms have been found in acute-phase serum (Sletten et al., 1989; Hulten et al., 1997). There are also at least two A-SAAs in hamster (Webb et al., 1989), and one each in cow (Rossevant et al., 1992; Yamamoto eet al., 1998), sheep (Syversen et al., 1994) and wallaby (Uhlar et al., 1996). A-SAA isoforms have also been detected in fox, goat sera (Foyn Brunn et al., 1995) and in arctic char (a salmonid closely related to trout) (Jensen et al., 1997).
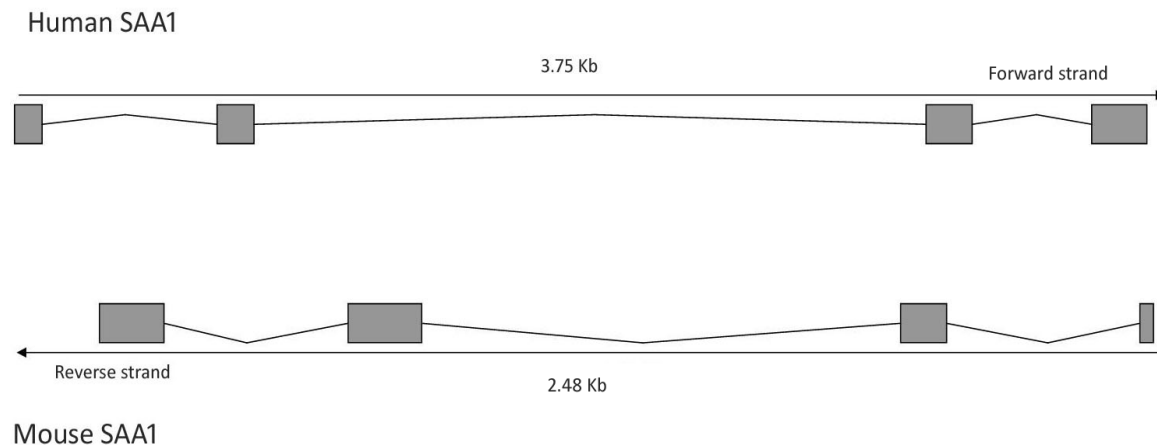
Figure 1.6. Human and Mouse SAA gene structure. Each block represents an exon (Ensembl).

The SAA genes of both the human and mouse lineages have remained in close physical linkage (Fig. 1.7) and it seems likely that the SAA genes will be similarly clustered in the genomes of other species (Uhlar et al., 1996). The human SAA1, 2, 3 and 4 genes are within 150 kb of each other on chromosome 11p15.1 (Sellar et al., 1994a; 1994b). This region of the human genome is syntenic with proximal mouse chromosome 7 (Junien & Heyningen, 1990) to which the mouse SAA genes were originally mapped (Taylor & Rowe, 1984) and within which the position of the cluster, containing Saa1, 2, 3, 4 and 5 and spanning only 45 kb, has been refined (Butler et al.,1995; Butler & Whitehead, 1996). The human SAA1 and SAA2 and the mouse Saa1 and Saa2 genes all encode A-SAAs. It is impossible to determine the interspecies evolutionary relationships of these individual SAA family members based on nucleotide or protein sequence comparisons alone (Uhlar et al., 1996). However, their relative map positions and transcriptional orientations (Fig. 1.5.2) provide strong evidence that human SAA1 and mouse Saa1 (formerly Saa2) are evolutionary homologs, as are human SAA2 and mouse Saa2 [Saa1] (Sellar et al., 1994; Butler & Whitehead, 1996). Within each species these two genes have almost identical sequences and organizations, suggesting that the A-SAA genes have been subjected to recent gene conversion events within each evolutionary lineage (Uhlar et al., 1996). They are co-ordinately induced during the acute-phase response.

Human SAA gene Family
chromosome: 11; Location: 11p15.1
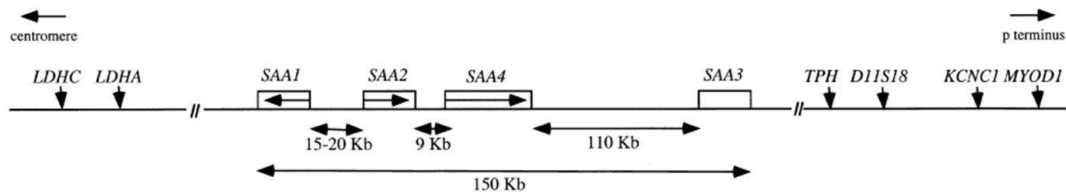
Mouse SAA gene Family
chromosome: 7; Location: 7p
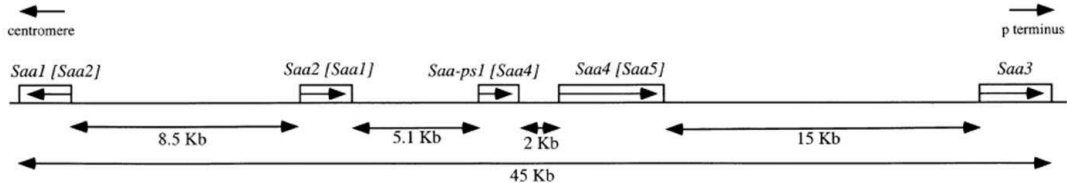
Figure 1.7. A comparative map of the human and mouse SAA gene families. The human family spans 150 kb on chromosome 11p15.1 while the mouse family spans 45 kb on chromosome 7p; these regions of the human and mouse genome are syntenic. Arrows within SAA genes represent 5'→3' orientation of the gene. (Modified from Uhlar et al. 1999)

1.5.2 The protein structure

Works based on predictive methods suggested that A-SAA is likely to contain two regions of α-helix in addition to β-sheet regions (Turnell et al., 1986), the latter being common to all amyloid proteins, including amyloid A protein (Glenner, 1980). Several regions of mammalian A-SAA proteins that may be important in facilitating the beneficial role(s) SAA may play during inflammation, and/or its pathogenic behavior after over-expression during chronic inflammation have been identified. It has clearly been established that A-SAA is the serum precursor of the amyloid A protein that is found in secondary amyloid deposits by tracking the fate of human A-SAA-HDL introduced into mouse in vivo models of amyloidosis (Husebekk et al., 1985; Tape et al., 1988).

A-SAA and C-SAA both associate with HDL, the former being the major HDL-associated apolipoprotein during the acute-phase response and the latter being the predominant, perhaps only, HDL-associated SAA under normal physiological conditions. The early proposition that the lipid-binding, as well as the amyloidogenic, region of A-SAA resides within the first 11 N-terminal amino acids (Turnell et al., 1986) is consistent with subsequent findings that amyloid A protein which lacks the C-terminal 28 residues, can associate with HDL (Husebekk et al., 1987), and that epitopes defined by antibodies raised against N-terminal A-SAA peptides (residues 1-30) are masked when A-SAA is complexed with HDL (Malle et al., 1995). The region between residues 29 and 42 in human A-SAA contains two elements, YIGSD

and RGN, which are very similar to the distinct cell-binding domains of the two extracellular matrix (ECM) cell adhesive glycoproteins, laminin (YIGSR) and fibronectin (RGD), respectively. Synthetic peptides of these elements specifically inhibit the receptor-mediated adhesion of human T lymphocytes and mouse M-4 melanoma cells to laminin and fibronectin, respectively. The above suggests that A-SAA may be able inhibit immune cell migration towards inflammatory sites and, perhaps, metastatic processes *in vivo* (Preciado-Patt et al., 1994).

The RGD sequence of the ECM adhesive proteins is also the target that facilitates the binding of some of the mediators released from activated platelets, e.g. fibrinogen, to the platelet integrin glycoprotein IIb-IIIa receptor which is essential in platelet aggregation. It has been suggested that the inhibition of platelet aggregation that can be achieved using A-SAA, residues 25-76 of A-SAA, or amyloid A protein may occur through the conserved RGN sequence (Syversen et al., 1994). Further evidence of A-SAA involvement in platelet aggregation is provided by its modulation of the induction of prostaglandin $I_2\alpha$ potent antiaggregation agent; this is mediated by the first 14 N-terminal amino acids of A-SAA (Shainkin-Kestenbaum et al., 1996). A putative calcium-binding sequence, GPGG, between residues 48 and 51 (Turnell et al., 1986), is conserved in all SAA sequences identified to date except for mouse Saa4, in which the corresponding tetrapeptide is GSGG. Early reports that amyloid deposits are rich in calcium and are sites at which Ca^{2+}-dependent protein binding interactions occur (Pepys et al., 1979) suggested that this peptide may be important in amyloidogenesis. Although relatively few functional studies of the C-terminus of A-SAA have been performed, there is evidence that it may facilitate binding to neutrophils. A peptide corresponding to residues 77-104, but not peptides spanning other regions, competitively inhibits such binding (Preciado-Patt et al., 1996). Interestingly residues 77-104 are usually not present in amyloid A deposits, raising the possibility that they are specifically released after proteolysis of A-SAA and that they have an immune-related proteolysis of A-SAA and that they have an immune-related biological function. Recently A-SAA has also been shown to have glycosaminoglycan, i.e. heparin sulfate and heparin, binding activity at its C-terminus between residues 77 and 103 (Ancsin & Kisilevsky, 1999) (Fig 1.8).
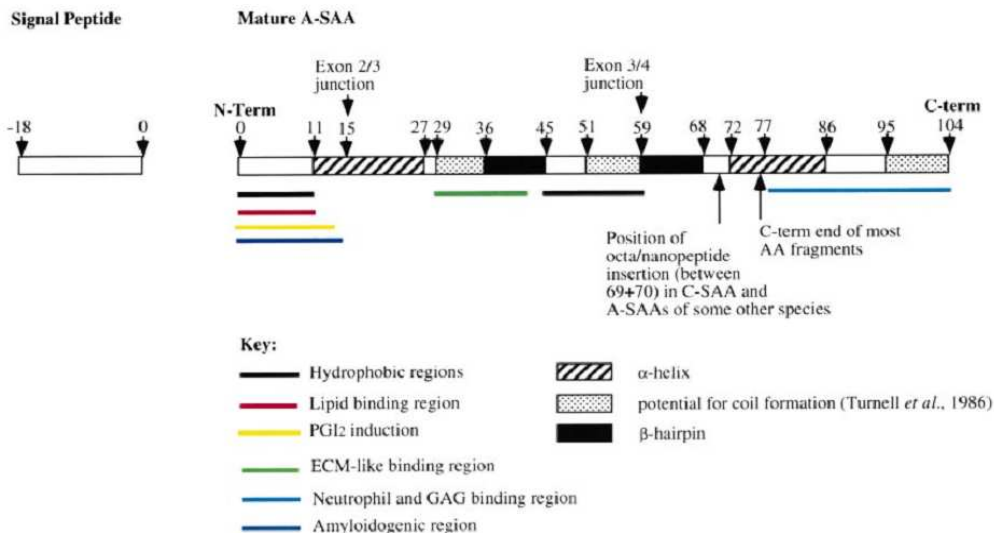
Fig. 1.8. The structure of human A-SAA protein. The 18-amino acid signal peptide is shown (-18 to 0) together with the 104 mature protein (1-104). The most frequently observed C-terminus of the amyloid A protein is indicated at residue 76. Regions of potential functional importance are indicated by underlining in color, while structurally important regions are indicated by shading/hatching. Sites delimiting sequences encoded by exons 2/3 and 3/4 are indicated (exon 1 contains only 50 untranslated region). (modified from Uhlar & Whitehead, 1999).

1.5.3 Hepatic and extrahepatic production of SAA

The hepatic acute phase response is induced by a wide range of mediators, including IL-1, IL-6, TNF-α, leukaemia inhibitory factor (LIF), transforming growth factor β (TGF β), interferon γ (INF γ), IL-11, oncostatin, ciliary neurotrophic factor (CNF) and retinoic acid, whereas others such as insulin and okadaic acid have inhibitory effects (Steel & Whitehead, 1993; 1994). Glucocorticoids have dual actions: they enhance the effects of other mediators on hepatocytes (Steel & Whitehead, 1993; 1994) and at the same time downregulate the production of, for example, TNF-α and IL-1 by macrophages and polymorphonuclear cells (PMN). The above mediators act by binding to their respective receptors on the cell surface. After binding they are internalized and/or transmit signals into the cells to effect a number of different intracellular regulatory mechanisms, activation and de novo synthesis of transcription factors and changes in processing and stability of RNA, translation efficiency and protein secretion.

A-SAA mRNA and protein synthesis are induced *in vivo* during the inflammatory response to challenges such as tissue injury, infection and trauma in all vertebrate species. These challenges, which can be experimentally produced using agents such as bacterial lipopolysaccharide (LPS), casein, turpentine, $AgNO_3$ and surgery, induce the pro-inflammatory cytokine cascade. The principal cytokines involved in the induction of A-SAA are IL-1, TNF-α and IL-6. Other cytokines that may be involved either directly or indirectly in A-SAA induction include IL-2, interferon-γ (IFN-γ) and ciliary neutrophic factor (Numerof et al., 1992; Fantuzzi et

al., 1995). In addition to IL-6 and ciliary neutrophic factor, four other IL-6 type cytokines, i.e. IL-11, leukemia inhibitory factor, oncostatin M and cardiotrophin-1, induce A-SAA when administered to mice. Glucocorticoids, which are also released during inflammation, have been shown to enhance cytokine-induced A-SAA expression.

Although the liver is the major site of APP synthesis, the extrahepatic tissue/cellular expression of a wide range of APPs has been documented (Colten, 1992). In most mammalian species, SAA1 and SAA2 are predominantly synthesized in the liver while SAA3 is the main isoform expressed at extrahepatic sites (Husby et al., 1994).

SPECIES	ORGANS OR CELL TYPES	SAA TYPE	MRNA	PROTEIN	REFERENCES
Human	Brain	Ns	+	+	Urieli-Shoval et al. 1998
	Pituitary gland	Ns	+	+	
	Breast	SAA1, SAA2, SAA4	+	+	
	Kidney	SAA1, SAA2, SAA4	+	+	
	Placenta	Ns	+	+	
	Pancreas	Ns	+	+	
	Prostate	Ns	+	+	
	Skin	Ns	+	+	
	Spleen	SAA1, SAA2, SAA4	+	+	
	Thyroid	Ns	+	+	
	Tonsil	Ns	+	+	
	Esophagus	SAA1, SAA2, SAA4	+	Nd	
	Stomach	Ns	+	Nd	
	Large intestine	SAA1, SAA2, SAA4	+	+	
	Small intestine	Ns	+	+	
	Synovial tissue	SAA1, SAA2, SAA4	+	+	Kumon et al. 1999; O'Hara et al. 2000
	Cartilage	A-SAA	Nd	+	Vallon et al. 2001
Rabbit	Brain	SAA3	+	Nd	Rygg et al. 1993
	Heart	SAA3	+	Nd	Marhaug et al. 1997
	Lung	SAA3	+	Nd	
	Spleen	SAA3	+	Nd	
	Kidney	SAA3	+	Nd	
	Small and large intestine	SAA3	+	Nd	
	Ovary	SAA3	+	Nd	
	Synovium	SAA3	+	+	Vallon et al. 2001
	Cartilage	SAA3	++	+	
Mink	Convoluted tubules of kidney	SAA1, SAA2	+	Nd	Marhaug et al. 1997
	Endometrium	SAA1, SAA2	+	Nd	
Chicken	Amyloidotic synovium	SAA	+	Nd	Ovelgonne et al. 2001
Duck	Lung	SAA	+	Nd	Stepanets et al. 2005
	Bursa di Fabrizio	SAA	+	Nd	
Cow	Colostrum	M-SAA3	Nd	+	McDonald et al. 2001
	Mammary epithelial cells	M-SAA3	+	Nd	
Sheep	Colostrum	M-SAA3			
Horse	Colostrum	M-SAA3			
Pig	Colostrum	M-SAA3			

Table 1.4. Extrahepatic sites of SAA production. Ns: no specific type of SAA; Nd: not done.

1.6. SAA functions and disease association

The A-SAA protein structure has been highly conserved through evolution and this, together with the dramatic induction of A-SAA expression in response to potentially life threatening physiological challenges, suggests a critical role in the acute-phase response. The concentration of SAA is usually low in normal plasma (1-5 mg/L) and its half life time is short (40-60 minutes). The mechanism and the site of its clearance are not known. The best documented clinical condition associated with sustained high expression of A-SAA is amyloid A amyloidosis. However, the range of clinically important functions that have been proposed for the SAA family members include some that may also have other negative consequences during chronic inflammation. The increase in SAA concentration during inflammation and its persistence despite short survival implies a specific role for SAA during inflammation or chronic infection. Indeed, several functions were attributed to SAA. Individually, none is significantly dominant, but when integrated, they seem to counteract the side effects of inflammation.

1.6.1. Immune-related functions

There are some reported immune-related functions of A-SAA. It can induce extracellular matrix (ECM) degrading enzymes, such as collagenase, stromelysin, matrix metalloproteinases 2 and 3, which are important for repair processes after tissue damage. It was also reported the capability to induce pro-inflammatory cytokines such as IL-1β and TNF-α. *In vitro* studies evidenced that A-SAA can act as a chemoattractant for immune cells as monocytes, polimorphonuclear leukocytes, mast cells and T lymphocytes.

1.6.2. Lipid-related functions

When A-SAA is released into the circulation it is incorporated into HDL, the class of lipoprotein particles that play an important role in the prevention of atherosclerosis by both mediating reverse cholesterol trasport and inhibiting the lipid (LDL) oxidation that promotes foam cells formation. There are two main hypotheses on the role that A-SAA plays in modulating cholesterol transport during inflammation. It alters reverse cholesterol transport to allow delivery of lipid, particularly cholesterol, via HDL to periperal cells that may have an increased requirement for cholesterol to facilitate tissue regeneration at inflammatory sites. The other is

that A-SAA facilitates removal of the large quantities of cholesterol liberated at sites of tissue damage during inflammation. In addition to its association with HDL, a number of lines of evidence implicate A-SAA in lipid metabolism/transport. A-SAA binds cholesterol and promotes its cellular uptake (Liang & Sipe, 1995; Liang et al., 1996) and A-SAA-HDL particles have a higher affinity for macrophages and a lower affinity for hepatocytes than HDL (Kisilevsky & Subrahmanyan, 1992). Furthermore, several enzymes involved in cholesterol metabolism, including lecithin-cholesterol acyltransferase, group-IIA non-pancreatic secretory phospolipase A_2 (sPLA$_2$) and neutral cholesterol ester hydrolase, are affected by induction of A-SAA during the acute-phase response.

1.6.3. Anti-inflammatory roles

Almost two decades ago, A-SAA was demonstrated to be implicated in the suppression of *in vitro* immune responses to antigens by affecting T cell-macrophage interactions and helper T lymphocyte function (Aldo-Benson & Benson, 1982). Human A-SAA was subsequently found to be a potent inhibitor of lymphocyte, HeLa and MRC5 cell function (Peristeris et al., 1989). A potential feedback relationship between SAA and immunoregulatory cytokines was proposed based on the observation that A-SAA inhibits IL-1-induced and TNF-induced fever in mice (Shainkin-Kestenbaum et al., 1991). IL-1 and TNF cause fever by inducing prostaglandin E_2 synthesis in the hypothalamus; prostaglandin E_2 production correlates directly with the magnitude of the fever (Dinarello et al., 1988). Platelet aggregation has also been reported to be inhibited by A-SAA, and A-SAA modestly induces prostaglandin I_2 which is also an antiaggregation agent (Syversen et al., 1990; Shainkin-Kestenbaum et al., 1996). As both platelets and the range of mediators released by them upon activation are involved in inflammatory and thrombotic processes, these findings suggest that A-SAA may act to down-regulate such pro-inflammatory events during the acute-phase response.

A-SAA has also been reported to bind to neutrophils and, like other apolipoproteins such as ApoA-I (Blackburn et al., 1991), inhibit the oxidative burst response, suggesting that it may help prevent oxidative tissue damage during inflammation (Linke et al., 1991). However, this effect may be concentration-dependent; in a recent study, acute phase concentrations of rhA-SAA could inhibit both directed neutrophil migration and degranulation (Gatt et al., 1998), whereas the inhibition of respiratory burst was restricted to lower concentrations. These results suggest that A-SAA may produce quite different effects according to local concentration

and that the anti-inflammatory effects intrinsic to this APP may be selective and specific rather than systemic.

1.6.4. SAA and Amyloidosis

A-SAA is the serum precursor of amyloid A protein which is the principal component of the amyloid deposits found in the heterogeneous group of disorders, the amyloid A amyloidoses (Husby, 1994). One of these, the reactive amyloidosis, is a well-documented occasional clinical consequence of chronic inflammation (e.g. rheumatoid arthritis) and recurrent acute inflammatory episodes (e.g. tuberculosis).

The predominant amyloid A protein type found in amyloidotic tissues corresponds to the N-terminal two thirds of A-SAA, i.e. the first 76 residues of mature human A-SAA (Husebekk et al., 1985). However, both smaller and larger amyloid A protein types, 45-95 residues, have also been found (Husby et al., 1994; Liepnieks et al., 1995; Westermark et al., 1990; Parmelee et al., 1982). Multiple proteolytic cleavage events may be involved in the processing of A-SAA as it appears to be degraded first into an intermediate product with the same size and antigenic properties as amyloid A protein and is subsequently processed further. Amyloid A amyloidosis may therefore be the result of the incomplete digestion, and consequent accumulation, of amyloidogenic intermediate peptides of A-SAA.

A large number of cell-associated and serum proteases have been implicated in the degradation of A-SAA (Table 1.5).

PROTEASES
Cell-associated activities
Serum serine proteases (thrombin, kallikrein and plasmin)
Elastase, collagenase and stromelysin
Cathepsin B
Aspartate proteases and Cathepsin D
Cathepsin G

Table 1.5. proteases involved in the degradation of A-SAA

A-SAA is probably degraded after its disassociation from HDL, as full-length A-SAA can be found in amyloid fibrils (Westermark et al., 1982; Arai et al., 1994). Furthermore, lipid-free A-SAA can be degraded *in vitro* to form fibrils (Yamada et al., 1994). In addition, A-SAA degradation *in vivo* is inhibited by lipoproteins, in particular HDL, and differences between the

plasma clearance rates of A-SAA and ApoA-I also suggest that the former is not associated with HDL when it is degraded (Bausserman & Hebert, 1984).

Mouse A-SAA can bind to two of the major components of the basement membrane, i.e. laminin and type-IV collagen, with high and low affinity, respectively. Its binding to laminin is inhibited by entactin, a protein that normally binds laminin. As the basement membrane matrix appears to be disrupted in the vicinity of amyloid deposits, these interactions further support the involvement of A-SAA as an active, rather than a passive, participant in the process of amyloidosis (Ancsin & Kisilevsky, 1997). Inflammatory macrophages and reticuloendothelial cells have both been implicated in the formation of amyloid A protein and amyloid fibrils from A-SAA: both processes can occur intracellularly (Takahashi et al., 1989). Mouse peritoneal macrophages can bind either HDL or A-SAA-HDL, which undergo receptor-mediated endocytosis and subsequent retro-endocytosis (Rocken & Kisilevsky, 1997). The binding of the latter probably involves heparin sulfate for which A-SAA has a binding site. Mouse peritoneal macrophages can also endocytose exogenous mouse Saa1 and Saa2, which are transported to endosome-lysosomes and are partially degraded to products similar in mass to amyloid A. Although derivatives of SAA1 and SAA2 proteins are both found in human and mouse amyloid A deposits, those from SAA1 predominate (Bell et al., 1996; Meek et al., 1986). This bias in favor of SAA1 deposition is supported by *in vitro* studies in which rhSAA1 had greater amyloid fibril-forming potential than either rhSAA2 or rhSAA4 (Yamada et al., 1994).
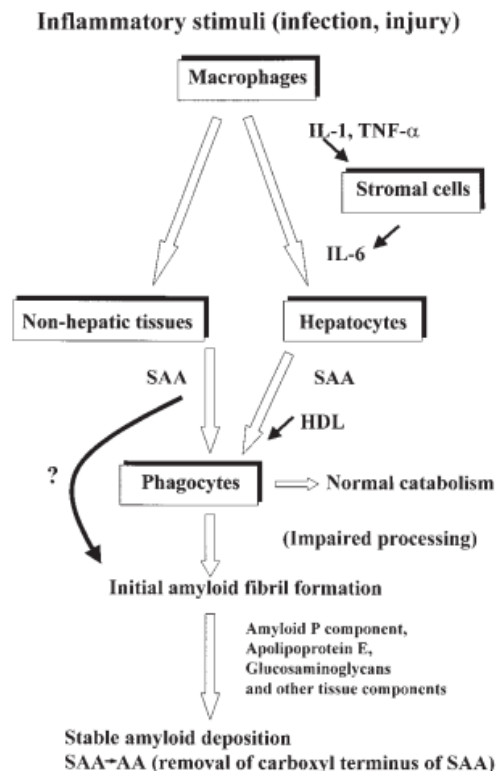
Fig. 1.9. Synthesis of SAA and catabolic pathway leading to amyloidogenesis (Yamada et al.,1999).

1.7. Bovine SAA

Measurements of serum concentration have demonstrated that SAA is a major acute phase protein in cattle, but its diagnostic value as an indicator of inflammation in clinical investigation has not been determined.

In cattle, an increased SAA serum and plasma concentration has been found following experimentally induced and naturally occurring inflammation as well as experimental and natural infection. SAA has been shown to reflect the severity of mastitis; cows with clinical mastitis had almost 100-fold levels of SAA in serum and milk compared with healthy cows (Eckersall et al., 2001).

A total of seven different SAA isoforms were characterized in the serum of cows with chronic inflammation or amyloidosis and in healthy cows. The isoforms are distinguishable using the isoelectric point (pI) in 2-dimensional electrophoresis. Isoforms of pI 5.2 and 8.6 are the only two identified in amyloidotic sample (Takahashi et al., 2009). SAA1 and SAA2 are expressed constitutively by liver and SAA3 is expressed locally and also present in milk. This last isoform is also called Milk Amylod A (MAA) because it was found in milk from dairy cows (Eckersall et al., 2001; McDonald et al., 2001).

The sequence data obtained from genomic walking revealed a four-exon, three-intron organization for bovine MAA, typical of the other sequenced SAA genes (Ulhar & Whitehead, 1999). Exon 1 contained a 53bp 5'-untraslated region (UTR), downstream and adjacent to this first exon was a 556bp intron. The second exon (92bp) contains the beginning of the coding sequence for the MAA gene precursor protein and exon 3 (139bp) encoded the next 46 aminoacids. Exon 4 encoded the last 55 aminoacids (Fig. 1.10). The splice junction for all the three introns conformed to the highly conserved 5'-GT/AG-3' consensus sequence for splice donor/acceptor sites at the two ends of an intron. A polyadenilation signal (AATAAA) was located 97 nt downstream to the STOP codon and was 26 nt upstream of a GT-rich region, typically found 11 to 30 nt after this site (Larson et al., 2006).

1.8. Aims of the thesis

The aim of the thesis is to go insight the expression and some functions of SAA.

From a clinical perspective the availably of a quick, and reliable, diagnosis marker specific for inflammatory diseases of cattle is of great importance. Acute phase proteins are good candidates. Unfortunately, few information are available about their functions, as well as their localization or local expression. This information are very important given the very different behavior of acute phase proteins among different species.

Therefore, the first part of the thesis analyze the possible relationship of Water Buffalo SAA with an important defensive function during natural occurring bacterial intramammary infection, was assessed, with the aims to evaluate if high concentrations of SAA, similar to those found during systemic reaction, may influence the resolution of inflammatory response.

The second part of the thesis was devoted to analyze the possible local, extra-hepatic, expression of Bovine SAA, in order to assess the origin of the protein and the possibility of a local concentration in milk and dairy product as fibrils. This concentration should lead the possibility of transmission of fibrils between species and induced the amyloidotic degeneration.

Chapter 2

Acute Phase Response in Water Buffaloes

2.1 INTRODUCTION

2.1.1 Water buffalo in Italy

In Europe, Italy is the main producer of buffalo milk. Reasons for increasing interest in buffalo breeding during recent years are the popularity of buffalo Mozzarella cheese, and the absence of quotas in the European Community for this production. In addition, buffalo milk is more valuable; farmers typically receive at least twice the price relative to bovine milk. Italian water buffalo (*Bubalus bubalis*), typically reared in central middle and southern Italy, also have been recently introduced in northern Italy. Italian buffaloes produce small quantities of milk; the average production in a standardized lactation length (270 days) is about 2100 kg (Associazione Italiana Allevatori, 1999; Catillo et al., 2002).

Milk from buffaloes differs from that from cattle. The biggest difference is with respect to fat. In cattle, the milk contains between 3 to 5 %, depending on feed and breed. In buffalo milk the average fat content is usually 7 to 8% but may be as high as 13% in some breeds. Buffalo milk fat has a higher melting point than that of cattle, due to its higher proportion of saturated fatty acids. Phospholipids and cholesterol are lower in buffalo milk. It is also more resistant against oxidative changes. The content of protein, lactose and ash is somewhat higher in buffalo milk than in cattle milk. Buffalo milk lacks or only contains traces of β-carotene, this makes the milk look very white, as opposed to cattle milk which has a slight yellow shade. In buffalo milk, vitamin A is present instead of its precursor. The different types of casein found in bovine milk are found in buffalo milk, although in slightly different proportions. (Table 2.1).

MILK COMPOSITION	BOVINE	BUFFALO
Kcal/kg	740	1258
Proteins (g/kg)	31	45
Lipids (g/kg)	40	87
Lactose (g/kg)	48	48
Ca (g/kg)	1.2	2.0
P (g/kg)	0.9	1.2

Table 2.1. Composition of bovine and buffalo milk.

2.1.2 What is mastitis?

Mastitis (*mast* = udder; *itis* = inflammation) results from the inflammation of the mammary gland, due to a wide spectrum of bacterial strains, algae and fungi (Pyorala, 2003). The severity of the inflammation can be classified into sub-clinical, clinical and chronic forms, and its degree is dependent on the nature of the causative pathogen and on the age, breed, immunological status and stage of lactation of the animal.

Sub-clinical mastitis is difficult to detect due to the absence of any visible indications, and it has major cost implications. Chronic mastitis is an evolutive form of clinical or sub-clinical mastitis and results in persistent inflammation of the mammary gland.

Normally, the teat canal is tightly closed by sphincter muscles, preventing the entry of pathogens. It is lined with keratin, a waxy material derived from stratified squamous epithelium that obstructs the migration of bacteria and contains antimicrobial agents, such as long-chain fatty acids, that assist in combating the infection. However, the efficiency of keratin is restricted (Capuco et al., 1992; Paulrud, 2005). Fluid accumulates within the mammary gland as parturition approaches, resulting in increased intramammary pressure and mammary gland vulnerability caused by the dilation of the teat canal and leakage of mammary secretions (Sordillo & Streicher, 2002). Additionally, during milking, the keratin is flushed out and there is distension of the teat canal (Rainard & Riollet, 2006). The sphincter requires about 2 hours returning back to the contracted position (Capuco et al., 1992). Once inside the teat, bacteria must also elude the cellular and humoral defence mechanisms of the udder (Sordillo & Streicher, 2002). If they are not eliminated, they start multiplying in the mammary gland (Fig. 2.1). They liberate toxins and induce leukocytes and epithelial cells to release chemoattractants, including cytokines such as tumour necrosis factor-α (TNFα), interleukin (IL)-8, IL-1, eicosanoids (like prostaglandin F2α [PGF2α]), oxygen radicals and acute phase proteins (APPs) (e.g. α_1-acid glycoprotein [AGP], haptoglobin [Hp], serum amyloid A [SAA]). This attracts circulating immune effector cells, mainly polymorphonuclear neutrophils (PMNs), to the site of infection (Paape et al., 2003; Zhao & Lacasse, 2008). PMNs act by engulfing and destroying the invading bacteria via oxygen-dependent and oxygen-independent systems. They contain intracellular granules that store bactericidal peptides, proteins, enzymes (such as myeloperoxidase) and neutral and acidic proteases (such as elastase, cathepsin G, cathepsin B and cathepsin D). The released oxidants and proteases destroy the bacteria and some of the epithelial cells, resulting in decreased milk production and release of enzymes, such as N-acetyl-β-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH) (Fig. 2.1). Destruction of most of the PMNs takes place by apoptosis once their task is fulfilled. Subsequently,

macrophages engulf and ingest the remaining PMNs (Paape et al., 2002; Paape et al., 2003). The dead and sloughed off mammary epithelial cells, in addition to the dead leukocytes, are secreted into the milk, resulting in high milk SCCs. If the infection persists, internal swelling within the mammary epithelium, not normally detectable by an external examination, can occur. The mammary gland alveoli become damaged and start losing anatomical integrity (Fig. 2.1). The blood–milk barrier is breached, causing extracellular fluid components, such as chloride, sodium, hydrogen, potassium and hydroxide ions, to enter the gland and mix with the milk (Zhao & Lacasse, 2008). When extensive damage to the blood–milk barrier has occurred, blood might be detected in the milk. This leads to visible changes on the udder, such as enhanced external swelling and reddening of the gland. Changes also occur in the milk, including increased conductivity, increased pH, raised water content and the presence of visible clots and flakes (Kitchen, 1981; Milner et al., 1996; Zhao & Lacasse, 2008). This marks the initial stage of clinical symptoms, and the most severe infections might ultimately result in the death of the animal.
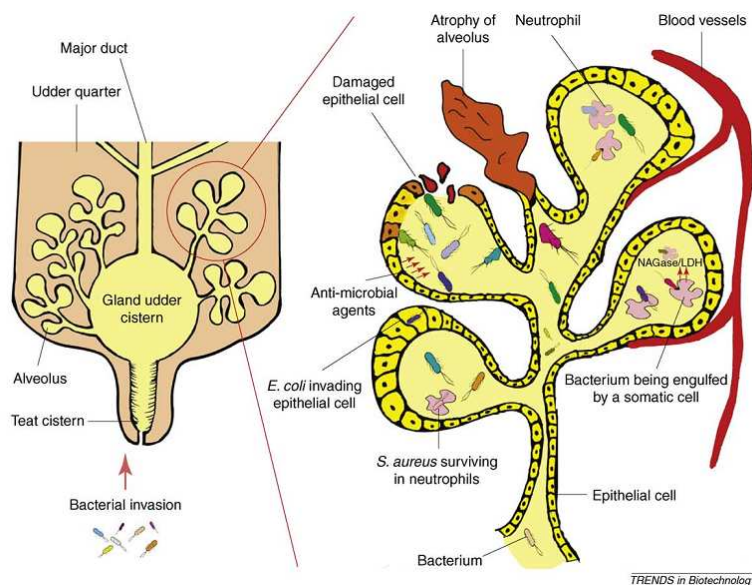
Figure 2.1. Schematic representation of mastitis development in an infected udder. Environmental and contagious microorganisms invade the udder through the teat cistern. They then multiply in the udder where they are attacked by neutrophils. The epithelial cells lining the alveoli release enzymes like NAGase and LDH and also secrete anti-microbial compounds (modified from Viguier et al., 2009).

2.1.3 Bovine and Water buffalo mastitis

Bovine mastitis is a disease with high incidence worldwide, even in herds with mastitis control programs. It causes considerable economic losses due to decreases in the quality and quantity of milk production, increases in the cost of treatment and veterinary services, and animal

waste. Mastitis is inflammation of the mammary gland that develops as a response to pathogenic microorganisms entering through the teat canal and multiplying inside the gland (Bannermann et al., 2004). The risk of acquiring the disease may be increased by chemical, physical, or traumatic factors. For this reason, mastitis can be considered as a multifactor disease that includes: 1) the bovine as host; 2) the microorganisms as causal agents; and 3) the environment, which affects both the cow and the causal microorganism. In a dairy herd, it is possible to find healthy cows, cows with one affected quarter, and cows with four quarters affected (Barkema et al., 1997).

This variability suggests that the incidence and prevalence of mastitis depends on differences in the mammary gland susceptibility to intramammary infection (IMI). Among factors that influence bovine IMI susceptibility are parity, nutrition, stage of lactation, milk production, and breed (Smith et al., 1997; Barkema et al., 1999). For example, cows with the highest productive capacity during partum and early lactation are the most sensitive to infection by environmental pathogens (Wagter et al., 2000; Burvenich et al., 2003). It is possible that during the peripartum period, cows experience certain alterations in defence mechanisms associated with either changes in hormonal profile or metabolic and physiologic stresses (Mallard et al., 1998) (Fig. 2.2).
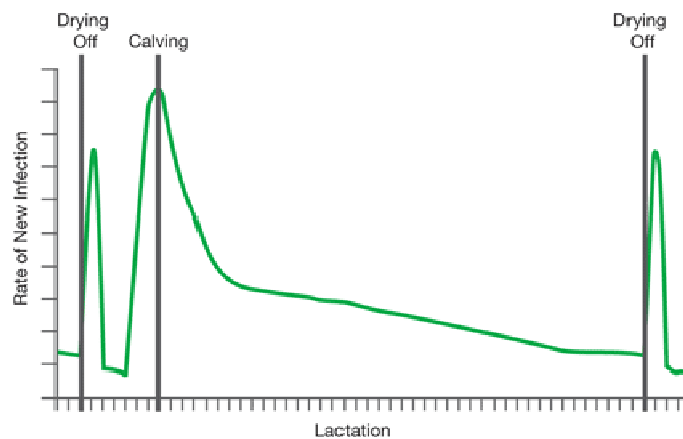
Figure 2.2. Frequency of intramammary infections during the lactation cycle.

There are many different contagious and environmental bacteria that cause mastitis (Table 2.2). Contagious bacteria live and multiply in infected mammary gland and can be spread from cow to cow or among quarters in the same animal. This group of bacteria includes *Staphylococcus aureus*, *Streptococcus agalatiae,* and several *Mycoplasma* and *Arcanobacterium* spp. (Dego et al., 2002; Zadoks et al., 2001; Bradley, 2002). Environmental pathogenic bacteria are present in the animal's environment and their presence in mammary

gland mainly occurs by teat contamination. This group includes *Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis, Klebsiella pneumoniae*, and *Bacillus* spp.

Staphylococcus aureus	*Mycoplasma bovis*
Streptococcus agalactiae	*Mycoplasma californicum*
Streptococcus dysgalactiae	*Mycoplasma canadense*
Streptococcus uberis	*Klebsiella pneumonie*
Corynebacterium pyogenes	*Enterobacter* spp
Bacillus cereus	
Escherichia coli	Prototheca
Proteus spp	Fungi
Pseudomonas aeruginosa	

Table 2.2. Micro-organisms frequently isolated from clinical bovine mastitis.

As said above, mastitis is one of the most costly diseases in dairy production, and even though the buffalo has been traditionally considered less susceptible to mastitis than cattle, some researchers have shown similar mastitis frequencies for the two species (Kalra & Dhanda, 1964; Badran, 1985; Bansal et al., 1995; Moroni et al., 2006). Buffaloes have some characteristics that may contribute to greater risk of mastitis. For example, the udder is more pendulous and teats are longer in comparison with cattle. Conversely, the buffaloes have a long narrow teat canal, which may be expected to prevent the invasion of microorganisms. The type of bacteria most frequently isolated in milk samples of buffaloes, with mastitis in previous studies, has been coagulase-negative staphylococci (CNS) followed by *Corynebacterium* spp. and *Streptococcus* spp. (Chander & Baxi, 1975; Paranjabe & Das, 1986; Saini et al., 1994; Naiknaware et al., 1998; Moroni et al., 2006). *Staphylococcus aureus* was the most important micro-organism responsible for mastitis in buffaloes in one study (Jaffery & Rizvi, 1975).

2.1.4   Mammary gland immunology

In general, the immune system is characterized by its capacity to recognize and discriminate between foreign invading agents and molecules produced by the organism (Janeway & Medzhitov, 2002). The mammary gland performs a variety of immunological functions conferring protection; during pre-partum, antibodies secreted in colostrum are produced to protect the newborn against infectious agents (Sordillo et al., 1997).

The mammary gland tissue is protected by two forms of immune defence mechanisms: innate immunity and acquired immunity. The innate and acquired immune systems interact closely in

an attempt to provide protection against mastitis micro-organisms (Burvenich et al. 2003; Sordillo et al., 1997; Rivas et al., 2002).

First, the innate immune response stimulates the acquired immune response and influences its nature. Second, the acquired immune response uses many innate immune effector mechanisms to eliminate micro-organisms, and its action frequently increases innate immune response antimicrobial activity. The efficiency of these responses determines mammary gland susceptibility or resistance to infection.

Innate immunity predominates in the early stage of infection and is mediated by macrophages, neutrophils, natural killer cells (NK) and cytokines. It recognizes and responds to different pathogens, even if they are invading the mammary gland for the first time. In particular, bacteria have different cell wall structures that are recognized by specific plasma membrane receptors. These structures are lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA), which constitute the pathogen-associated molecular patterns (PAMPs) (Han et al., 2003; Bannerman et al., 2004). These PAMPs are recognized by Toll-like receptors (TLRs), which induces production of cytokines and other endogenous mediators that are essential in protection against pathogenic microorganisms (Lembo et al., 2003).

The teat canal is the first line of defence, because bacteria must penetrate through it to cause an IMI. The function of the teat sphincter muscle is to keep the orifice closed and thereby isolate the interior of the mammary gland. This means that any damage of this structure is related to an increase in the incidence of mastitis (Myllys & Honkanen-Buzalski, 1994). The teat canal is lined with keratin, which provides an additional physical barrier, preventing bacterial migration towards the cistern (Sordillo & Streicher, 2002; Capuco et al., 1994).

The second line of defence consists of neutrophils, macrophages, and lymphocytes. These cells regulate both innate and acquired immune responses (Sordillo et al., 1997; Sordillo et al., 2002; Soltys & Quinn, 1999). In mammary gland defence against bacterial infection, tissue or milk macrophages recognize the invading pathogen and initiate the inflammatory response. In this response, pro-inflammatory cytokines induce neutrophil recruitment to the mammary gland (Zhang & Issekutz, 2002; Rainard, 2003). When macrophages recognize bacteria, they release pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, stimulating the bactericidal activity of neutrophils and also producing prostaglandins and leukotrienes, which increase the local inflammatory reaction (Bannerman et al., 2004; Boulanger et al., 2003; Stein et al., 2003). IL-6 produced by macrophages is involved in acute septic shock during mastitis caused by coliforms or *S. aureus*. This cytokine facilitates the exchange of neutrophils for monocytes in the mammary gland, which is necessary for reduction of the deleterious effects of neutrophils. Also, IL-6 is one of the main regulatory cytokines of acute phase protein synthesis in

hepatocytes (Slebodzinski et al., 2002; Ohtsuka et al., 2001), and also a local production in the mammary gland (Eckersall et al., 2001).

2.1.5   Current approaches for diagnoses of mastitis

Early diagnosis is of the utmost importance due to the high costs of mastitis. European Union legislation (Regulation 853/2004) stresses that milk selected for human consumption must originate from healthy animals. Diagnostic methods have been developed to check the quality of the milk through detection of mammary gland inflammation and diagnosis of the infection and its causative pathogens. Currently, assays usually include measurement of SCCs, enzymatic analysis and the California milk clotting tests (Pyorala, 2003). In Europe, elevated SCCs above 200.000 cells/mL are widely used as an indicator of mastitis (Schukken et al., 2003) and are determined using haemocytometers or cell counters. Colorimetric and fluorometric assays have been developed for measuring the concentrations of enzymes elevated in milk during mastitis (e.g. NAGase or LDH). Use of culturing techniques for the detection of mastitis-causing micro-organisms is still the gold standard, although it is very labour-intensive and therefore expensive.

Mastitis can also be detected using "cow-side" or "on-site" tests, which can be used by both farmers and veterinarians and which require relatively little training. One of the oldest and best known is the California mastitis test (CMT) (Schalm & Noorlander, 1957). It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and lead to the formation of a "gel-like" matrix consistency. However, the interpretation can be subjective, and this might result in false positives and negatives. Mastitis can also be detected via changes in conductivity or pH. Although these effects are easy to monitor, they are relatively insensitive. Thus, there is a major need for new biomarkers that are specific for mastitis, easy to detect and occur at a very early stage.

Technological advances, together with increased proteomic and genomic information, have resulted in improvements in the sensitivity of assays used for the detection of mastitis. Immunoassays, such as enzyme-linked immunosorbent assay (ELISA), can provide a reliable and inexpensive approach provided that suitable antibodies are available against specific inflammation-related biomarkers or the causative microorganisms. Numerous immunoassays have been developed for the detection of pathogens in milk (Barbuddhe et al., 2002; Arimi et al., 2005; Arora et al., 2006) and are used for monitoring milk quality. However, very few

studies have been undertaken for the development of immunoassays that detect pathogens in milk as definitive causative agents of mastitis. Such assays might also be useful for mastitis detection. However, studies to validate this assumption are required. Immunoassays can also be used to detect inflammation-related biomarkers present in the milk at different stages of sub-clinical mastitis. For example, single radial immunodiffusion was developed for the quantitative measurement of bovine α_1-acid glycoprotein (AGP) in serum. The mean serum value of AGP in healthy cattle was 283.2 μg/ml. Elevated values were observed in cattle with traumatic pericarditis, arthritis, mastitis, pneumonia and mesenteric liponecrosis (Tamura et al., 1989). AGP in cattle with experimentally induced abscesses, increased in concentration for 7 to 10 days after *F.necrophorum* inoculation and high concentration was also found in naturally affected cattle (Motoi et al., 1992). A second acute phase protein suggested as a potential marker for diagnosis is Haptoglobin (Hp). Its concentrations have been reported to increase significantly in plasma, as well as in milk, during mastitis and (Gronlund et al., 2003). Hiss and co-workers (2004) developed an ELISA for its detection, with a detection limit of 0.07 μg/mL in both milk and serum. Serum amyloid A (SAA) is another example of an APP marker that shows elevated levels in mastitic milk (Eckersall, 2007; Akerstedt et al., 2007; Molenaar et al., 2009). Szczubial and colleagues (2008) were able to detect elevated concentrations of SAA of up to 322.26 μg/mL in mastitic milk (compared with normal levels of 11.67 μg/mL) using a commercially available solid-phase-sandwich ELISA (Tridelta Phase[TM] range SAA kit, Tridelta Development Ltd, Ireland).

There have also been significant developments in nucleic-acid-based testing for the identification of the pathogens. The genome sequences of many of the major mastitis-causing pathogens are now available and can be utilized to develop nucleic acid-based testing methods, such as PCR. Such tests are generally more expensive than, for example, immunoassays. However, they are highly sensitive and specific, can be performed rapidly and can overcome the sensitivity and time-constraints sometimes encountered with culture-based tests (Studer et al., 2008) and thus could complement or replace them in the long-term. PCRs allow the identification of closely related organisms within a few hours. Multiplex PCR and real-time PCR assays that can simultaneously detect different mastitis-causing organisms in milk samples have been described (Cremonesi et al., 2005; Gillespie et al., 2005; Cai et al., 2005), and the most recently developed assay is capable of detecting 11 of the major mastitis-associated pathogens, including *Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae* and *Streptococcus uberis* (Koskinenen et al., 2009).

2.1.6 Acute Phase Proteins and mastitis

In bovine, SAA was identified as potential marker of mastitis (Hirvonen et al., 1999; Pedersen et al., 2003), even if there are no evidence of a correlation between SAA levels and severity of the pathology (Eckersall et al., 2001). In bovine with experimentally induced mastitis, milk concentration of SAA increases before the increase of somatic cells (Pedersen et al., 2003). SAA levels in milk from an infected udder can increase to 110 μg/ml (Eckersall et al., 2001; 2006; Gronlund et al., 2003; Nielsen et al., 2004). The changing in concentration can be used as an early marker of mastitis.

During mastitis the protein are produced by liver and it can reach the site of inflammation by blood. It is known that exist a local production of acute phase protein, and in particular the milk SAA (MAA) is produced by the mammary gland (Eckersall et al., 2001; 2006; Larson et al., 2005; McDonald et al., 2001; Molenaar et al., 2009; Weber et al., 2006). The production of SAA mRNA by cells in extrahepatic tissues is an interesting feature, in that these cells can provide a local source of SAA proteins. The locally expressed proteins may be produced under conditions that do not evoke the systemic acute-phase response and may play a role related to the site of expression. The properties and the physiological significance of the SAA family of proteins in normal and in disease states are poorly understood. In addition to SAA release to the circulation by the liver during an acute-phase response, one or more SAA proteins produced in tissues may be constitutively expressed to serve as a first line of defence or in proper maintenance of tissues (Urieli-Shoval et al., 1998).

In water buffalo there are no evidence of a possible correlation between somatic cell counts (SCC), the most common diagnostic tool for diagnosis of mastitis, and the presence of SAA. For this reason it should be useful to identify complementary or alternative tools for diagnoses and between them the measurement of acute phase proteins.

All these information fail in buffalo specie. The acute phase proteins sequences are unknown, and also their concentration in physiological and pathological conditions are not established.

2.2   MATERIALS AND METHODS

2.2.1 Samples

Samples were collected from healthy water buffaloes and buffaloes with intramammary infections (IMI).

Blood specimens were collected by venipuncture into serum collection tubes. Samples were kept for up to 4 hours at room temperature and centrifuged at 2500g for 15 minutes to remove serum from the clot. Serum was stored frozen at –20°C until use.

Milk samples were collected monthly before each milking and during all lactating period. A stream of milk was discarded prior to disinfection of the teat surface using pledges of cotton soaked in chlorhexidine. A sample of 10ml was collected in a sterile container. Whey was stored frozen at –20°C until use.

2.2.2   Bacteriological procedures

Bacteriological culturing of milk samples was performed according to standards of the National Mastitis Council (NMC, 1999). Ten µl of each milk sample was spread on blood agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37°C and examined after 24 hours. Colonies were provisionally identified on the basis of Gram stain, morphology, and hemolysis patterns, and the numbers of each colony type were recorded. Representative colonies were then subcultured on blood agar plates and incubated aerobically at 37°C for 24 hours to obtain pure cultures. Catalase and coagulase production was tested for Gram-positive cocci. Specific identification of staphylococci and streptococci were made using commercial micromethods (API Staph and API 20 Strep, BioMérieux, Italy). Gram-negative isolates were identified by using colony morphology, gram-staining characteristics, oxidase, and biochemical reactions on MacConkey's agar and API 20E (BioMérieux). Contagious pathogens *Staphilococcus aureus* and *Streptococcus agalactiae* were considered to cause IMI if at least one colony (≥100cfu/ml) was isolated. For other microorganisms, IMI was defined by the isolation of ≥500cfu/ml and 1 to 3 colony types. Milk samples from which >3 colony types or <500cfu/ml colonies of any micro-organism were isolated were regarded as contaminated or uninfected, respectively. No contaminated samples, however, were found.

2.2.3   Determination of Somatic cells counts (SCC)

For each milk sample, the SCC was determined by an automated fluorescent microscopic somatic cell counter (Bentley Somacount 150, Bentley Instrument, Italy). The instrument utilizes a laser based flow cytometry. The laser based counting section uses the fluorescence characteristics of the dye to count the cells one by one. Ethidium bromide dye was used for specific binding to DNA in cell nuclei. When cells are exposed to light of a specific wavelength, they emit red-orange fluorescent light. The light of cell is detected and counted by a laser scan.

2.2.4   Testing the cross-reactivity of the commercially available assays in water buffalo

Serum SAA and AGP concentrations were determined by using immunoenzymatic assays. The cross-reactivity of the antibodies included in the commercially available tests with buffalo proteins had never been tested before in this species. Therefore, a preliminary step to validate the possible utilization of the commercially available assays with buffalo serum was carried out. These experiments were performed by testing 1 µl of buffalo serum with SDS-PAGE on a 12% (for AGP detection) and 15% (for SAA detection) polyacrylamide gel, and Western blotting onto nitrocellulose. The membranes were then incubated with different concentrations of anti-boAGP and anti-SAA primary antibodies (1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10000, 1:20000 and 1:40000) using a multiscreen apparatus (Bio-Rad, Italy) and incubated for 1, 2 or 3 hours at room temperature. Both antibodies were those included in the commercial kits, and were made available by the courtesy of Dr Martin Gallagher, from Tridelta Company. Serum AGP positive bands were visualized by immunostaining using an anti-bovine AGP polyclonal antibody which was, after preliminary experiments, eventually utilized at a concentration of 1:40000 for 45 minutes of incubation at room temperature. Detection was carried out by enhanced chemiluminescence (ECL) using Immobilon™ Western Chemiluminescence HRP Substrate (Millipore, Italy). Bovine purified AGP (Ceciliani et al., 2007) was used as positive control (50 ng each lane). Serum SAA positive bands were visualized by immunostaining using anti-SAA biotinylated monoclonal antibody (1:200 dilution from the original antibody included in the kit, incubated overnight at 4°C in order to reduce the background), followed by a streptavidin–biotin peroxidase-conjugated complex, using the Vectastain ABC kit (Vector Laboratories, UK) and developed using ECL as previously described.

2.2.5 Quantification of SAA in serum and milk

Serum Amyloid A was quantified using a commercial solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) kit (Phase Range Serum Amyloid A Assay Tridelta Dev. Ltd, Ireland) used for quantification of serum SAA. Each sample was diluted in Dilution Buffer 1X (1:500) in a final volume of 50µl and was added to 50µl of a SAA specific biotinilated monoclonal antibody (1:100) for 1 hour at 37°C. Each sample was plated in duplicate. The plate was then washed three times with Washing Buffer, and then incubated with 100µl of Streptavidin-Peroxidase (HRP) (1:4000) for 30 minutes, at room temperature in the dark. The plate was washed three times and then incubated with 100µl of 3,3',5,5' tetramethyl-Benzidine (TMB) for 30 minutes at room temperature. Finally the reaction was stopped adding 50µl of Stop solution and the absorbance was read in a spectrophotometer at 450nm.

The mean absorbance for each sample, standard was calculated. The absorbance of the standards was plot against the standard concentration and the best smooth curve through these points was drawn to construct the standard curve. The concentrations of the test samples and controls were calculated from the standard curve by multiplying the interpolated value by the appropriate dilution factor.

The same protocol was used for SAA quantification in milk. Raw milk was centrifuged at 4000g for 20 minutes at 17°C and the supernatant rich of fats were discarded. The whey samples were diluted 1:50 in Diluent Buffer.

2.2.6 Quantification of AGP in serum

Once the cross-reactivity of commercially available assay in buffalo is assessed, the concentration of AGP was determined by a radial immunodiffusion assay commercial kit (Bovine α1-AGPlate, Tridelta Development Ltd., Kildare, Ireland). Five µl of each test sample thought to contain AGP is placed in an individual test well and incubated at 37°C for 48–72 hours. As the sample diffuses radially from the well into the agar gel plate, a specific precipitin reaction occurs between AGP and the specific antiserum to incorporated in the gel. A visible precipitin ring is formed. Since the area within this ring is directly proportional to the concentration of AGP in the test sample, measurement of the ring's diameter allows calculation of that AGP concentration, as compared to the two known standards solutions. Diameters were plot on the vertical axis of a semi-logarithmic graph and the AGP

concentration on the horizontal axis. From the reference curve, the AGP concentration of each test sample may be calculated.

2.2.7 Statistical analysis

All statistical analyses were computed using PASW 18.0 for Windows (SPSS Inc, USA). The comparisons between concentration in serum and milk of SAA between healthy and IMI animals were performed using ANOVA test. Significance was assessed for P<0.05 (*) and P<0.001 (**).

Comparison between SCC in healthy and in IMI buffaloes was performed using Mann-Whitney test, significance was assessed for P<0.05 (*), P<0.001 (**) and P<0.0001 (***).

Statistical analysis were also performed to evaluate the correlation between SAA and SCC in milk and serum. Descriptive statistics are expressed as the mean ± SD. Normality of data distribution was assessed by a Shapiro-Wilk test. Since data was not normally distributed a non-parametric Spearman correlation was tested. The threshold for statistical significance was considered to be P<0.05 (*).

2.2.8 Determination of primary structure of Ceruloplasmin, Haptoglobin, α1 acid glycoprotein, Serum amyloid A and Lipopolysaccharide binding protein

Total RNA was extracted from Water Buffalo liver using the RNeasy MiniKit (Qiagen, Italy) according to the manufacturer's protocol. The reverse transcription (RT) reaction was carried out on 1mg RNA using iSCRIPT cDNA SYNTESIS Kit (Bio-Rad, Italy). The cDNA was used as the template for the PCR (Eppendorf Mastercycler 1) (Eppendorf, Italy). PCR reactions were performed in 10 µl final volume under the following condition: 1x buffer Eppendorf, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1mM of each primer and 0.5 unit of Taq Polymerase (Eppendorf, Italy). The primers used to amplify the coding sequences of buffalo AGP, SAA, Hp and Cp are listed in Table 2.3. PCR was carried out in an Eppendorf thermal cycler (Eppendorf, Italy), using the following thermal profile: 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds, 59-60°C for 30 seconds and 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. PCR products were applied on a 1.5% agarose gel electrophoresis and the segments of predicted molecular weight obtained were gel-purified using the QIAquick gel extraction kit (Qiagen, Italy) and then were quantified by NanoDrop 1 ND-1000. The fragments were sequenced in both directions.

The predicted aminoacid sequence was obtained using the ExPASy proteomic server (www.expasy.ch). Interproscan and prosite (www.ebi.ac.uk) analysis was carried out to detect post-translational modifications of the five proteins.

APP	Primers	Sequences 5'-3'	Annealing temperature
AGP	AGP_mono_F1	CCAACCTGATGACAGYGGC	59 °C
	AGP_end	GCACCGAAACAAACTTTATTGATGC	
SAA	SAA_F1	GGCAGCTCAGCTTCACCAG	58 °C
	SAA_R1	GCTGCCTTCTGAGGACAGAG	
Hp	Apto_F1	GCTCTTCCAGAGCCAGACAC	58 °C
	Apto_R1	CGAGATGAGGTTATGGTGAG	
	Apto_F2	GCAGCTTTCCCTGGCAGG	58 °C
	Apto_R2	CCAGACACATAACCCACACG	
	Apto_F3	CCTTCGAAAGACTATGTGGC	58 °C
	Apto_R3	CCACTCCTGTGCAGCTTTCC	
Cp	Ceru_F1	GCTGTCGGGGCTCCAAGA	60 °C
	Ceru_R1	GCTGTCTTCCTCACCAGG	
	Ceru_F2	GTGCAGCCAGGTGAACAG	58 °C
	Ceru_R2	CCAGAAAAAGGCCTGCAAACC	
	Ceru_F3	GCTCAGAACCCTGGGCAATG	60 °C
	Ceru_R3	GGGACAGTCCACTCATAGG	
	Ceru_F4	CCTTCAGGCTCCCATGTGGC	58 °C
	Ceru_R4	CCACGTCAAAAGTCCCTGC	
	Ceru_F5	CCTGTCAAGAGGAGAAAGG	58 °C
	Ceru_R6	GCCATTCCAAAGTAAGCCTTC	
LBP	LBP_F1	GCGACAGATCCAGCCCAG	58 °C
	LBP_R1	CTCCTGATATGTGCACCTCC	
	LBP_F2	GGCTGTCTCCAGCTGCAG	58 °C
	LBP_R2	GGGTACAGTCTGGCTATCC	
	LBP_F3	CTTCCGCGCCTTCGTTCC	58 °C
	LBP_R3	CCAGAATCTCGAGGAAAGTGC	

Table 2.3. primers pairs sequences and thermal profiles used for APPs sequencing.

2.3 RESULTS

2.3.1 Bacteriological procedures

Data from the 34 buffalo with an intramammary infection showed that in 73.5% of cases the *Coagulase-negative staphylococci* were present in the udder, followed by other *Streptococcus spp* (8.8%), *Aeromonas viridians* (5.9%), *Streptococcus uberis* (5.9%) and *Corynebacterium spp* (5.9%).

2.3.2 Determination of Somatic cells counts (SCC)

The comparison between SCC in healthy buffalo and during intramammary infection was assessed. The number of somatic cells increase significantly (P<0.0001) in buffaloes with an intramammary infection (Fig. 2.3).
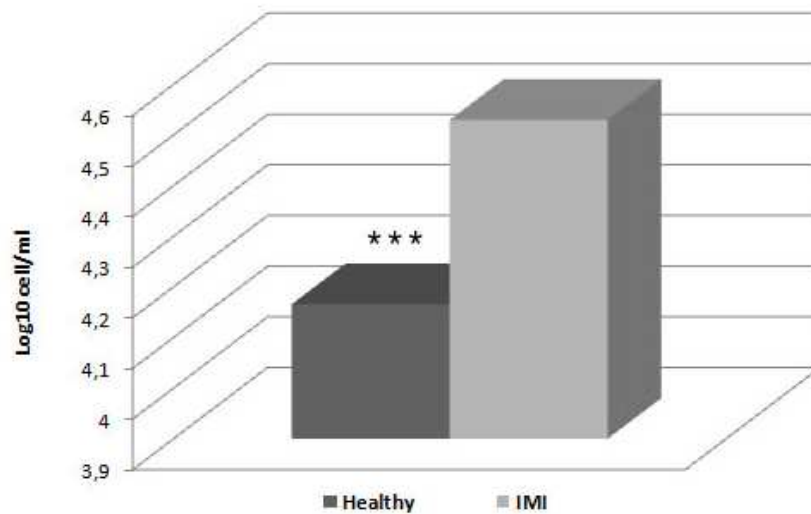
Figure 2.3. Graph showing the correlation between Somatic cells and pathological condition in buffalos.
*** P<0.0001

The interaction between the concentrations of SAA in milk (MAA) and in serum with the Somatic cell count was not significant. The number of somatic cells is very variable, but in general they increase with the lower levels of Serum amyloid A in milk (Fig. 2.4) and in serum (Fig. 2.5).

$$y = 0{,}0111x + 10{,}283$$
$$R^2 = 0{,}0003$$
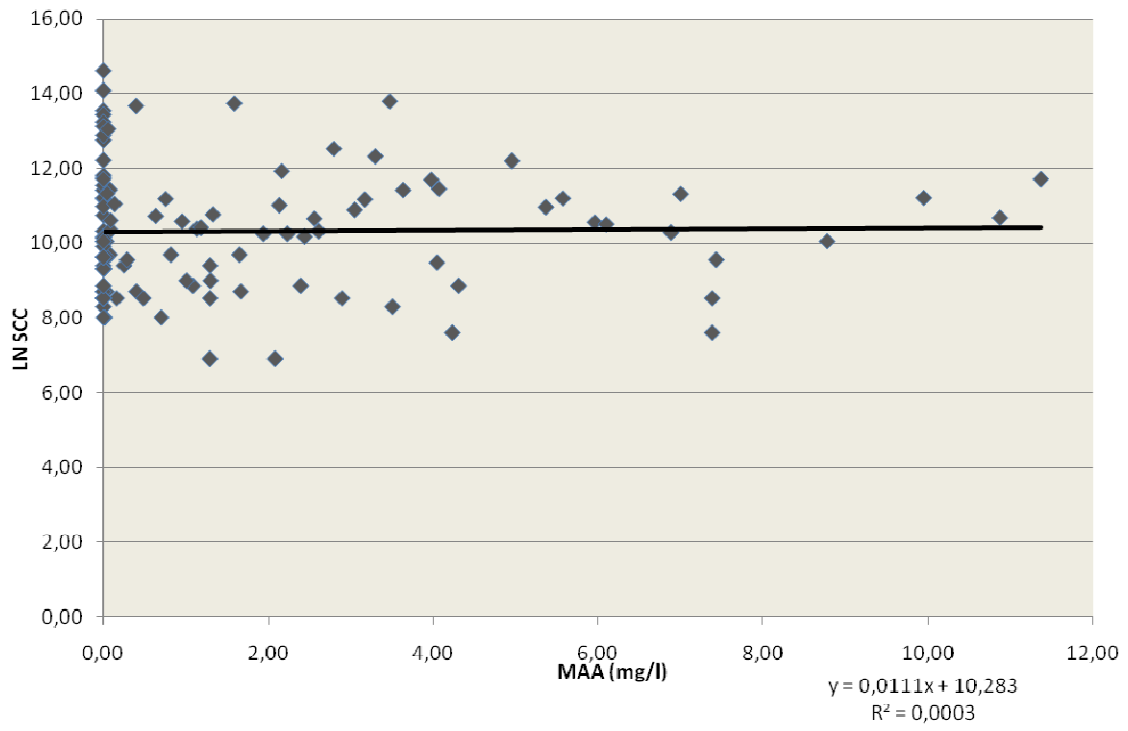
Figure 2.4. Graph showing the correlation between Somatic cells and Serum amyloid A in milk.

$$y = -0{,}001x + 10{,}082$$
$$R^2 = 0{,}0631$$
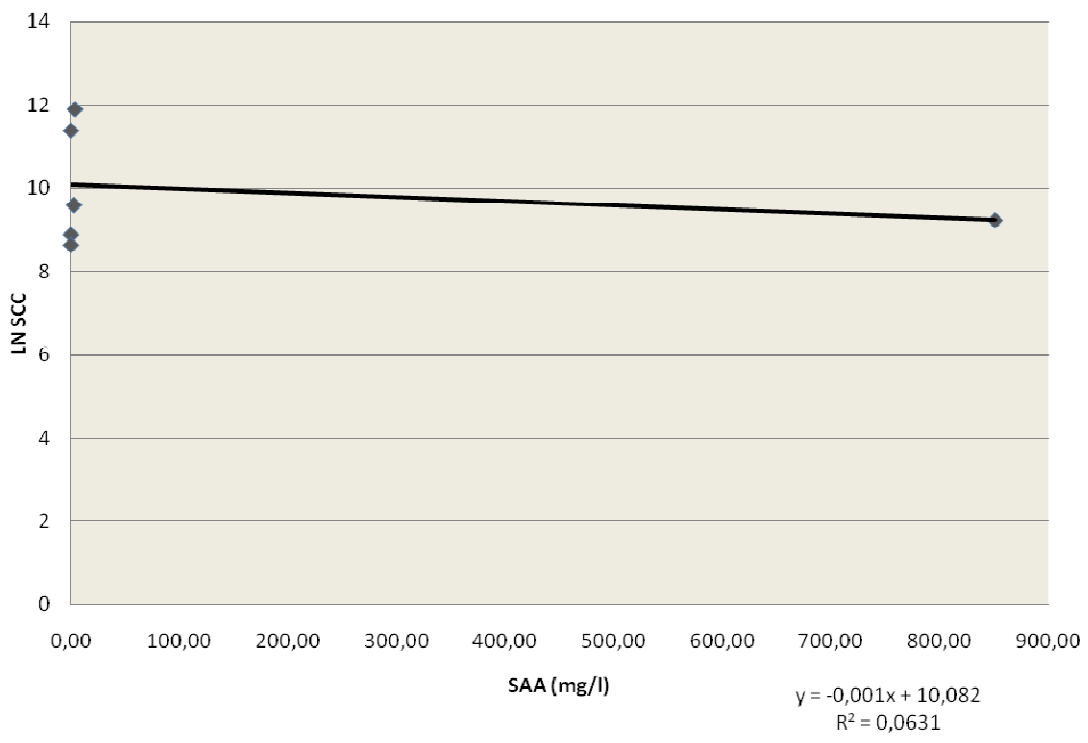
Figure 2.5. Graph showing the correlation between Somatic cells and Serum amyloid A in serum.

2.3.3 Assessment of the cross-reactivity of immunological assays for acute phase proteins

The assays used to determine the concentration of SAA and AGP in buffalo serum were based on immunological methods, such as ELISA and Radial Immuno Diffusion (RID). As a prerequisite for their utilization in buffalo, both of them had to be tested for the cross-reactivity of the anti-bovine SAA and AGP antibodies included in the kit with buffalo SAA and AGP, respectively. Western blotting experiments were therefore carried out to determine whether the specific antibody included in the kit cross-reacted with the respective protein and if the antibodies did not cross-react with serum proteins other than those targeted by this experiments. The anti-bovine AGP antibody included in the kit cross-reacts with a band with a molecular weight of 42–45 KDa, with an electrophoretical pattern (molecular weight and presence of high molecular weight glycoform) consistent with that of bovine AGP (Fig.2.6).
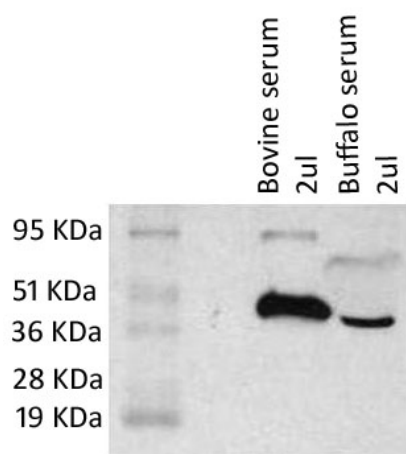
Figure 2.6. Western blot shows the presence of AGP in Bovine serum (Lane 1) and in Buffalo serum (Lane 2).

The same experiment was carried out using anti-bovine SAA antibody, using bovine serum as positive reference control. Results indicate that also the anti-bovine SAA is specific for buffalo SAA, which bands at a molecular weight of 14 KDa (Fig. 2.7).
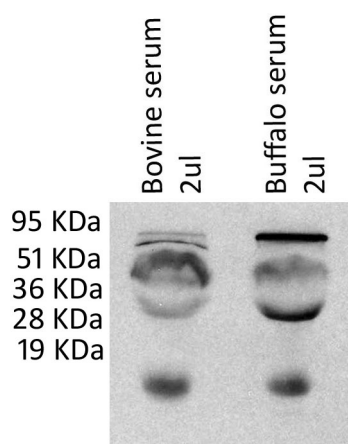
Figure 2.7. Western blot shows the presence of SAA in Bovine serum and in Buffalo serum.

In conclusion, this series of preliminary experiments demonstrated that the two kits that were utilized to determine the concentration of AGP and SAA in buffalo serum can cross-react with their respective antigens in buffalo serum.

2.3.4 Acute Phase Protein concentration measurement in serum and milk

The concentration of AGP and SAA in serum (34 samples) or milk (134 samples) were analyzed by established immunologic methods utilized for bovine, after validation of the cross-reactivity of the antibodies included in the kit with the buffalo proteins.

The concentration of serum and milk AGP was under the detection limit of the kit, and was therefore undetectable in both healthy and IMI buffaloes.

SAA cut-off values were assumed to be 4.1 mg/l in serum and 1.4 mg/l in milk as in bovine (Gronlund et al., 2003).

Data collected showed that the concentration of serum SAA was under the detection limit in healthy buffalo serum. On the contrary, SAA concentration was raised in serum from all of the buffaloes with IMI (17.8 mg/l ± 9.5 s.e., P<0.001) (Table 2.4, Fig. 2.8).

Group	N	Median	25%	75%
Healthy	26	-14,102	-19,028	-8,734
IMI	8	45,604	9,060	847,883

Table 2.4. SAA concentration (mg/l) in 26 serum from healthy buffaloes and 8 IMI buffaloes.
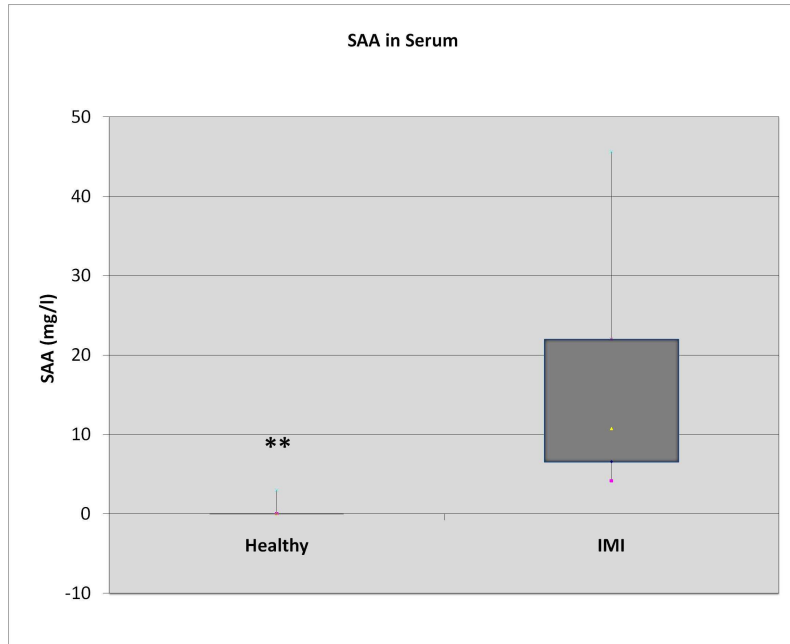
Figure 2.8. SAA concentration (mg/l) in serum samples. ■ Minimum value, ◆ 25% value, ▲ Median value, ✳ 75% value and ✖ Maximum value. ** P<0.001

Results in milk showed that in healthy buffaloes the protein was not detectable, but when IMI is present SAA was detectable in all samples with mean concentration of 4.1 mg/l ± 0.37 s.e. (p<0.001) (Table 2.5, Fig. 2.9).

Group	N	Median	25%	75%
Healthy	91	-0,0750	-0,260	0,250
IMI	43	3,386	2,231	5,369

Table 2.5. SAA concentration (mg/l) in 91 milk samples from healthy buffaloes and 43 from IMI buffaloes.
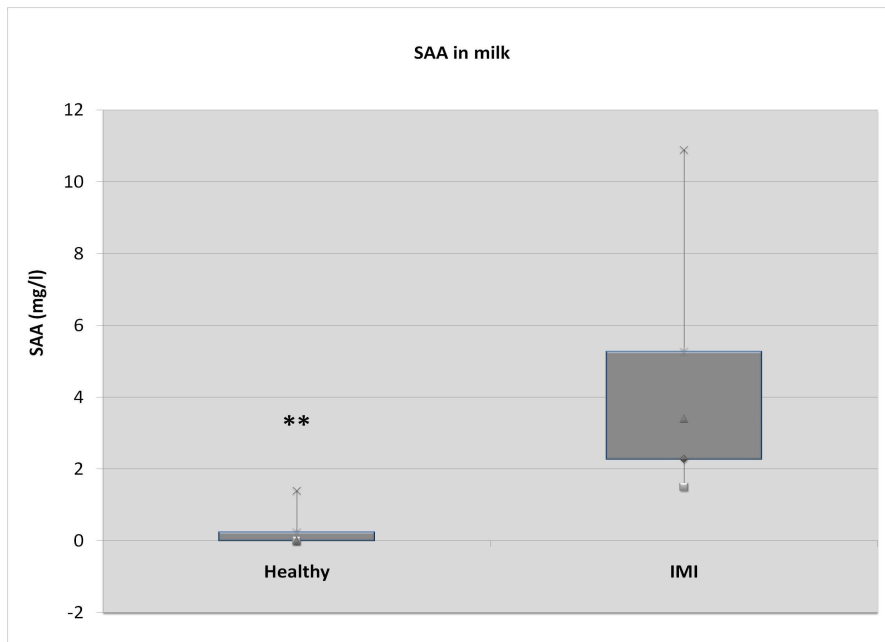
Figure 2.9. SAA concentration (mg/l) in milk samples. ■ Minimum value, ◆ 25% value, ▲ Median value, ✳ 75% value and ✖ Maximum value. ** P<0.001

2.3.5 Acute Phase Protein sequencing

In this study we determined the sequences of Haptoglobin (Hp), Serum amyloid A (SAA), α_1-acid glicoprotein (AGP), Ceruloplasim (Cp) and Lypolisaccaride binding protein (LBP) in water buffaloes (*Bubalus bubalis*). The nucleotide sequences of Hp, SAA, AGP, Cp and LBP were determined by cDNA sequencing and showed homology of 96%, 91%, 95%, 98% and 97% respectively with bovine one.

The cDNA sequences of Hp, SAA, AGP, Cp and LBP obtained from buffalo liver have been deposited in NCBI GenBank under the accession number of FN600415, FN599527, FN645647, FN649762 and FN645646, respectively.

Their corresponding polypeptide backbones are shown in figure 2.10 in which also presents homology comparisons between buffalo and bovine sequenced so far, as well as the description of post-translational modifications.

A
```
HpBuffalo   MSALQAVVTLLLCWQLLAVETGSEATADSCPKAPEIANSHVEYSVRYQCDKYYKLRAGNGVYTFNNKQWINKDIGQQLPECEEDDSCPEP 90
HpBovine    MSALQAVVTLLLCGQLLAVETGSEATADSCPKAPEIANSHVEYSVRYQCDKYYKLHAGNGVYTFNNKQWINKDIGQQLPECEEDDSCPEP 90
            ************* ***********************************************:*****************************

HpBuffalo   PKIENGYVEYSVRYQCKTYYKLRTSGDGVYTFNSKKQWINKDVGQQLPECEAVCGKPKHPVDQVQRIIGGFLDAKGSFPWQAKIVSHHNL 180
HpBovine    PKIENGYVEYLVRYQCKPYYTLRTCGDGVYTFNSKKQWINKNIGQKLPECEAVCGKPKHPVDQVQRIIGGSLDAKGSFPWQAKMVSQHNL 180
            *********  ******:**.***.*****************::**:********************** *************:**:***

HpBuffalo   ISGATLINERWLLTTAKNLYLGHTSDKKAKDITPTLRLYVGKNQLVEVEKVVLHPDHSKVDIGLIKLRQKVPVNDKVMPICLPSKDYVKV 270
HpBovine    ISGATLINERWLLTTAKNLYLGHSSDKKAKDITPTLRLYVGKNQLVEVEKVVLHPDHSKVDIGLIKLRQKVPVNDKVMPICLPSKDYVKV 270
            ***********************:*****************************************************************

HpBuffalo   DRVGYVSGWGRNENFNFTQHLKYVMLPVADQDKCVKHYEGVDAPKNKTAKSPVGVQPILNENTFCVGLSKYQEDTCYGDAGSAFVVHDKE 360
HpBovine    GRVGYVSGWGRNENFNFTEHLKYVMLPVADQDKCVKHYEGVDAPKNKTAKSPVGVQPILNENTFCVGLSKYQDDTCYGDAGSAFVVHDKE 360
            .*************:*****************************************************************:**********

HpBuffalo   DDTWYAAGILSFDKSCAVAEYGVYVKMTSIVERVWKTMGDNQERAGQEQMPRSTRGKLHRS 421
HpBovine    DDTWYAAGILSFDKSCAVAEYGVYVKVTSILDWVRKTIANN-------------------- 401
            **************************:***:: * **:..*

B
SAABuffalo  MNLSTGIIFCFLILGVSSQGWGTFLKEAGQGAKDMWKAYSDMKEANYKDADKYFHARGNYDAAQRGPGGAWAAKVISNARETIQGITDPL 90
SAABovine   MNLSTGIIFCFLILGVSSQRWGTFLKEAGQGAKDMWRAYQDMKEANYRGADKYFHARGNYDAARRGPGGAWAAKVISNARETIQGITDPL 90
            ******************* ****************:**.*******:.**************:**************************

SAABuffalo  LKGMTRDQVREDSKADQFANEWGRSGKDPNHFRPAGLPDKY 131
SAABovine   FKGMTRDQVREDSKADQFANEWGRSGKDPNHFRPAGLPDKY 131
            :****************************************

C
AGPBuffalo  MDLLSGKWFYIGSAFRNPEYNESARAIQAAFFYFEPRHAEDKLIAREYQTIADKCVYNCSFIKIYRQNGTLSTIESDREHFADLLLSKHF 90
AGPBovine   MDLLSGKWFYIGSAFRNPEYNKSARAIQAAFFYLEPRHAEDKLITREYQTIEDKCVYNCSFIKIYRQNGTLSKVESDREHFVDLLLSKHF 90
            *******************:************:**********:******:*****************.:*******.********

AGPBuffalo  RTFMLAASWNGTKNVGVSFYADKPEVTQEQKKEFLDVIKCIGIQESEITYTDEKKDACGPLEKQHEEERKKETEAS 166
AGPBovine   RTFMLAASWNGTKNVGVSFYADKPEVTQEQKKEFLDVIKCIGIQESEIIYTDEKKDACGPLEKQHEEERKKETEAS 166
            *********************************************** ***************************

D
CpBuffalo   MKIFLLCIFLILCGTSAWAKDKHYYIGIIETAWNYASGHGEKKLISVDTEHSNIYLQNGPNRIGSVYKKAVYLQYTDENFRTVIEKPVWL 90
CpBovine    MKIFLLCIFLILCGTSAWAKDKHYYIGIIETAWNYASDYGEKKLISVDTEHSNIYLQNGPNRIGSVYKKAVYLQYTDENFRTVIEKPVWL 90
            *************************************.:*************************************************

CpBuffalo   GFLGPIIKAETGDKVYVHLKNFASRPYTFHAHGMTYYKEHEGAIYPDNTTGFQKADDKVQPGEQCMYILHANPEQGPGQEDSNCVTRIYH 180
CpBovine    GFLGPIIKAETGDKVYVHLKNFASRPYTFHAHGMTYYKEHEGAIYPDNTTDFQKADDKVQPGEQCMYILHANPEQGPGQEDSNCVTRIYH 180
            *************************************************** .*************************************

CpBuffalo   SHIDAPKDIASGLIGPLIHCKKDSLDEEKEKNIDKEFVVMFSVVDENLSWYLEENIKTYCSEPEKVEKDNEDFQESNRMYSVNGYAFGSL 270
CpBovine    SHIDAPKDIASGLIGPLIHCKKDSLDEEKEKNIDKEFVVMFSVVDENLSWYLEENIKTYCSEPEKVEKDNEDFQESNRMYSVNGYAFGSL 270
            ********************************************************************************************

CpBuffalo   PGLSMCAKDRVKWYLFGMGNEIDVHAAFFHGQVLTSKNYRVDTINLFPATLFDALMVAQNPGQWMLSCQNLNHLKAGLQAFFRVQDCKKS 360
CpBovine    PGLSMCAKDRVKWYLFGMGNEIDVHAAFFHGQVLTSKNYRVDTINLFPATLFDALMVAQNPGQWMLSCQNLNHLKAGLQAFFWVQDCKKS 360
            *********************************************************************************** *******

CpBuffalo   SSEDNIHGKNVRHYYIAAEEVVWNYAPSGIDAFTKENLRTPGSASEAFFEQGPTRIGGSYKKLVYREYTDASFSNQKERGPEEEHLGILG 450
CpBovine    SSKDNIHGK-IRHYYIAAEEVIWNYAPSGIDAFTKENLRAPGSASEAFFEQGPTRIGGSYKKLVYREYTDASFSNQKQRGPEEEHLGILG 450
            **:******  :*********:*****************:***********************************:************

CpBuffalo   PVISAEVGDTIRVTFHNKAAHPLSIEPIGVRVDKKNEGTYYSPSGSGPPPSGSHVAPKGTFTYEWTVPREVGPTYKDPVCLAKMYYSAVD 540
CpBovine    PVISAEVGDTIRVTFHNKAAHPLSIEPIGVRVDKKNEGTYYSPSGSGPPPSGSHVAPKGTFTYEWTVPREVGPTYKDPVCLAKMYYSAVD 540
            ********************************************************************************************

CpBuffalo   PTKDIFTGLIGPMKICRNGTLLANGRLKGVDKEFYLFPTVFDENESLLLDDNIKMFTTAPDQVDKENEDFQESNKMHSMNGFMYGNQPGL 630
CpBovine    PTKDIFTGLIGPMKICRNGTLLANGRLKDVNKEFYLFPTVFDENESLLLDDNIKMFTTAPDQVDKENEDFQESNKMHSMNGFMYGNQPGL 630
            *******************************.*:***************************************************

CpBuffalo   SMCQGDSVMWYLFSAGNEVDIHGIYFSGNTFLSRGERRDTANLFPQTSLSLFMKPDIAGTFDV 693
CpBovine    SMCQGDSVMWYLFSAGNEVDIHGIYFSGNTFLSRGERRDTANLFPQTSLSLFMKPDTAGTFDV 693
            ******************************************************* ******

E
LBPBuffalo  RQIQPSPLGIQDGDSTGTLPSLLLGTLLTFTSRALGANPGLVVRITDQGLEYVAQEELLALQSKLHKVTLPNFDGDVRIKHFGSVDYRFH 90
LBPBovine   -----------MVTSTGTLPSLLLGTLLTFTSGALGANPGLVVRITDQGLEYVAQEELLALQSKLHKVTLPDFNGDVRIKHFGSVDYRFH 79
                       *************************.*****************************************:*:**************

LBPBuffalo  SLNIQSCKLLGSALKLLPNRGLHFSISDSFIQVTGDWKVRKRILRLDGSFDVKVKGITISVNLLLDSEPSGRPKVAVSSCSSHIRDVEVH 180
LBPBovine   SLNIQSCKLLGSALKLLPNQGLHFSISDSFIQVTGDWKVRKRILRLDGSFDVKVKGITISVNLLLDSEPSGRPKVAVSSCSSHIRDVEVH 169
            *******************:**********************************************************************

LBPBuffalo  ISGDLGWLLNLFHNQIESRFRRVLESKICEIIEDSVTSELQPYLQTLPVTTGIDHLAGLDYSLMGAPQATAQMLDVMFKGEIFSRDDRSP 270
LBPBovine   ISGDLGWLLNLFHNQIESRFRRVLESKICEIIEDSVTSELQPYLQTLPVTTEIDHLAGLDYSLMGAPQATAQMLDVMFKGEIFSRDDRFP 259
            ***************************************************  ************************************ *

LBPBuffalo  VSFLAPVMNLPEEHSRMVYFAISDYAFNTASLVYHKAGFLNFTITDDMIPPDSNIRLNTKSFRAFVPRIARLYPITNLELQGAVISAPCL 360
LBPBovine   VAFLAPVMNLPEEHSRMVYFAISDYAFNTASLVYHKAGFLNFTITDDVIPPDSSIRQNTKSFRAFVPRIARLYPNTNLELQGAVISAPCL 349
            *:*****************************************************.** .** ***************** **********

LBPBuffalo  NFSPGNLSTAAQMEIEAFVLLPNSIKEPVFRLGVATNVSAVLTFNTSKITGFLEPGKIQVELKESKVGLFNVELLEALLNYYLLNNFYPK 450
LBPBovine   NFSPGNLSTAAQMEIEAFVLLPNSVKEPVFRLSVATNVSAMLTFNTSKITGFLEPGKIQVELKESKVGRFNVELLEALLNYYLLNNFYPK 439
            ***********************:*******.*******:*************************** *****************

LBPBuffalo  VNDKLAEGFPLPLLRKIQLYDPILQIHKDFLFLGTNVRYLRIRGQGERQGLEATAGSPSCISRCAALSSRFW 522
LBPBovine   VNDKLAEGFPLPLLRKIQLYDPILQIHKDFLFLGTNVRYLRV----------------------------- 481
            *****************************************:
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Figure 2.10. alignment of APPs primary structure, in red site of glycosilation.

2.4 DISCUSSION

The acute phase proteins are non-specific markers of inflammation, their serum concentration increasing in response to infections and inflammatory conditions. For them to be used as specific indicators of mastitis it is therefore essential that they accumulate in milk only during episodes of mammary inflammation. The results of this study showed that the concentration of serum amyloid A in serum and in milk in buffalo with intramammary infection were higher than in healthy animals. An inflammatory stimulus thus seems to be required for the concentration of SAA to be increased. On the contrary, the concentration of α1-acid glycoprotein was not apparently modified, since the concentration of the protein was under the limit of detection of the Radial Immuno Diffusion (RID) kit in both healthy and IMI buffaloes. While the concentration in milk of AGP could be assessed only after a concentration step relying on an anionic exchange cromathography enrichment (Ceciliani et al., 2005), its measurement in milk is challenging. In blood serum on the contray, AGP concentration can be easily evaluated following RID assays, since its amount ranged from 100 to 300 µg/ml. Previous studies have reported increases in levels of AGP in a variety of inflammatory conditions (Sheldon et al., 2001; Motoi et al., 1992) and also AGP is a clinically important acute phase protein in cattle and has been used to monitor the inflammatory process (Conner et al., 1988; Eckersall et al., 2001, Itoh et al., 1990) including water buffalo diseases (Horadagoda et al., 2001; 2002) .

The higher concentration of acute phase proteins in the milk from infected quarters could be due to either to their production within the gland or to their leakage across the blood-milk barrier as a result of its disruption by the inflammation, or to a combination of these mechanisms. It has been shown that the permeability of the blood-milk barrier increases during episodes of mastitis allowing serum proteins to gain access to the milk (Shuster et al., 1997). However, the demonstration of serum amyloid A gene expression at several extra-hepatic sites and of several cell types that can synthesise the protein (Marhaug et al., 1997; Vreugdenhil et al., 1999), together with the recent identification of a unique isoform of the protein in normal bovine colostrums (McDonald et al., 2001), suggests that serum amyloid A may be synthesised locally during episodes of mastitis. In this study the correlation between the presence of an inflammatory condition in the mammary gland and the raised levels of milk and serum SAA have been reported in water buffalo for the first time.

The finding of SAA as a possible marker of mastitis in water buffalo is remarkable. Few information is available for what concern water buffalo mastitis diagnosis. While the commons

parameters utilized for bovine mastitis diagnosis are also applied to water buffalo milk quality definition, in fact it is well known that the relationshipe between Somatic Cell Count and the presence of an intrammary infection is regarded as poor in water buffaloes.

In fact, the data presented in this thesis revealed that the correlation between the SCC and the presence of pathogens is poor, whereas the high concentration of SAA can better define the presence of IMI.

In conclusion, the present results confirm the importance of APP as diagnostic markers of mastitis, even relative to the use of the MAA assay as a biochemical modification of the SAA assay. Although MAA and SAA determinations require an ELISA method that is carried out routinely only in reference laboratories, MAA could be considered a more reliable marker than SCC. The random measurement of this APP in milk samples could allow the identification of subclinical mastitis in equal or higher measure than currently obtained with SCC. The use of MAA/SAA as markers of the acute phase seems to give a statistically better response than the loss of correlation between acute phase proteins and SCC, correlation present in cattle where can be used as an indicator of clinical and subclinical mastistis (Gerardi et al., 2009; O'Mahony et al., 2006).

Moreover, systematic control of MAA and SAA on farms could reduce both the laboratory costs and the time required for milk analysis. Lastly, as the actual methods for MAA and SAA analysis are not suitable for online measurements during milking, it is necessary to develop and implement biosensors to be used with automated milking systems, to enable early detection of mastitis, to reduce both the treatment time and the economic impact, and to improve the health and welfare of animals.

Chapter 3

Study on the possible presence of amyloid (AA) in dairy products

3.1 INTRODUCTION

3.1.1 Conformational diseases and Amyloidosis

The amyloidoses constitute a large group of diseases where misfolding of extracellular protein fulfils a prominent role. This dynamic process, which occurs in parallel with or as an alternative to physiologic folding, generates insoluble, toxic protein aggregates that are deposited in tissues in bundles of β-sheet fibrillar protein. Isolation of the protein components of natural amyloid and the chemical characterization of these components are indispensable investigative tools. To date, at least 21 different proteins have been recognized as causative agents of amyloid diseases (Table 3.1). The essence of amyloidosis lies in the capacity of these proteins to acquire more than one conformation, a feature that has earned them the sobriquet of chameleon proteins.

AMYLOID PROTEIN	PRECURSOR	DISTRIBUTION	TYPE	SYNDROME OR INVOLVED TISSUES
Aβ	Aβ protein precursor	Localized Localized	Acquired Hereditary	Sporadic Alzheimer's disease, aging Prototypical hereditary cerebral amyloid angiopathy
APrP	Prion protein	Localized Localized	Acquired Hereditary	Sporadic CJD, new variant CJD Familial CJD, GSSD, FFI
ABri	ABri protein precursor	Localized or systemic?	Hereditary	British familial dementia
ACys	Cystatin C	Systemic	Hereditary	Icelanding hereditary cerebral amyloid angiopathy
Aβ2M	Beta$_2$-microglobulin	Systemic	Acquired	Chronic hemodialysis
AL	Immunoglobulin light chain	Systemic or localized	Acquired	Primary Amyloidosis, myeloma-associated
AA	Serum amyloid A	Systemic	Acquired	Secondary Amyloidosis, reactive to chronic infection or inflammation including hereditary periodic fever
ATTR	Transthyretin	Systemic Systemic	Hereditary Acquired	Prototypical FAP Senile heart, vessels
AApoAI	Apolipoprotein A-I	Systemic	Hereditary	Liver, kidney, heart
AApoAII	Apolipoprotein A-II	Systemic	Hereditary	Kidney, heart
AGel	Gelsonin	Systemic	Hereditary	Finnish hereditary amyloidosis
ALys	Lysozyme	Systemic	Hereditary	Liver, kidney, spleen
AFib	Fibrinogen Aα chain	Systemic	Hereditary	Kidney

Table 3.1. Amyloid proteins and their precursors. CJD Creutzfeldt-Jakob disease, GSSD Gerstmann-Sträussler-Scheinker disease, FFI Fatal familial insomnia, FAP Familial amyloidotic polyneuropathy (modified from Westermark et al. 2002).

Virchow (1854) coined the name "amyloid" for the pathological extracellular material he found during histopathological examination of human patients, since it was thought to be cellulose. He used the word, amyloid, because of iodine staining similarities with extracellular plant material as wood and starch. In 1859 Friedreich and Kekulé described the proteic nature of amyloid. Chemical investigations in the second half of the twentieth century revealed a role for glycosaminoglycans and heparan sulphate (Snow et al., 1987; 1995) and chondroitin sulphate-containing proteoglycans in amyloid (Niewold et al., 1991). A burst in amyloid research developed after the electron microscopical discovery of amyloid fibrils in 1959 by Cohen (Cohen & Calkins, 1959) and subsequent description by others (Spiro, 1959). It offered new possibilities for isolation (Pras et al., 1968), peptide separation and analysis of a large series of different chemical amyloid types corresponding to the known variations in clinicopathological patterns. Amyloidosis is associated with a wide range of medical disorders, including cancer, rheumatoid arthritis, Alzheimer's disease, chronic renal dialysis, familial amyloid polyneuropathy and diabetes, but it can also occur on the absence of pathologies (Sipe, 2004). Amyloidosis is characterized by deposition of insoluble fibrils with the β-sheet conformation in the extracellular spaces of organs. These insoluble amyloid fibrils, derived from polymerization of normally soluble protein precursors, are rigid, fine, nonbranching, 8 to 10 nm in width and indeterminate in length (Merlini & Bellotti, 2003). Amyloid fibril deposition can be systemic, involving multiple organs of the body or localized to a single organ such as the pancreas in diabetes or the brain in Alzheimer's disease. In general, amyloid fibril precursors (monomers) are small polyanionic proteins, ranging from 3 to 30 KDa, with a high content of glutamic and aspartic acid residues and serine. Once deposited in tissues, the fibril precursor proteins are resistant to proteolytic digestion (Husebekk et al., 1991) and are insoluble under physiologic conditions (Glenner et al., 1968). In the fibrillar form, amyloid proteins are invariably associated with extracellular matrix constituents, including amyloid-P component, glycosaminoglycans, fibronectin and other basement membrane or connective tissue components.

The conversion of the structure of the native protein into a predominantly antiparallel β-sheet secondary structure (where the N- and C-terminals are oriented in opposite directions) is a pathologic process closely related to physiologic protein folding. The folding of a newly synthesized polypeptide occurs in a rapid sequence of conformational modifications in the cytoplasm. According to the "folding energy landscape theory," the process follows a funnel-like pathway in which the conformational intermediates progressively merge into a final species (Dobson et al., 1999). In addition, at a minimum of energy similar to that reached by

the native protein, the polypeptide can acquire an alternative and relatively stable "misfolded state" (Schultz, 2000), which is prone to aggregation. Once the folding process has been completed and the native protein secreted (Fig 3.1), many proteins are in dynamic equilibrium with a partially folded conformation, and in this state, they retrace the final part of the folding pathway, ultimately forming either a native or misfolded protein. In amyloid disease, potentially pathogenic misfolded proteins can form in different ways. The protein may have an intrinsic propensity to assume a pathologic conformation, which becomes evident with aging (e.g., normal transthyretin in patients with senile systemic amyloidosis) (Saraiva, 2001) or at persistently high concentrations in serum (e.g., β2-micro-globulin in patients undergoing long-term haemodialysis) (Verdone et al., 2002). Another mechanism is the replacement of a single amino acid in the protein, as occurs in hereditary amyloidosis (Buxbaum et al., 2000). A third mechanism is proteolytic remodelling of the protein precursor, as in the case of β-amyloid precursor protein (APP) in Alzheimer's disease (Hardy & Selkoe, 2002). These mechanisms can act independently or in association with one another. In addition to the intrinsic amyloidogenic potential of the pathogenic protein, other factors may act synergistically in amyloid deposition. For example, the protein precursor must reach a critical local concentration to trigger fibril formation, a process enhanced by local environmental factors and by interactions with extracellular matrixes (McLaurin et al., 2000).
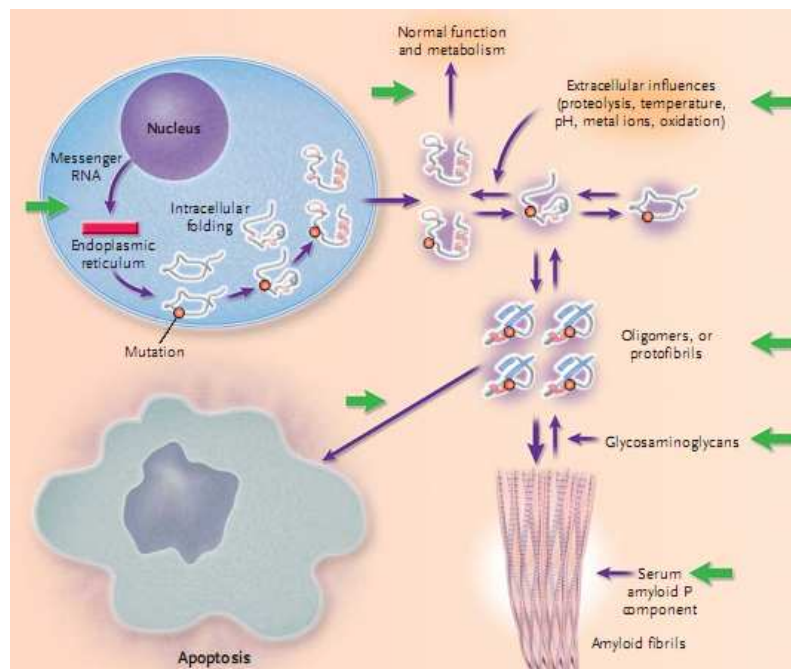

Figure 3.1. The chains undergo synthesis in the endoplasmic reticulum, fold, pass through the cellular quality-control mechanism, and are secreted. In the extracellular environment, the mutants may change from a fully folded to a partially folded state and then retrace the final part of the folding pathway. Normal proteins are functionally active and are normally metabolized. The partially folded polypeptides can generate misfolded molecules, which have a high propensity to self-aggregate. Environmental conditions, chemical modifications, and common constituents favour the pathologic pathway. Oligomers, or protofibrils, may mediate cellular toxicity through a mechanism that activates apoptosis in cells of the target tissues. (Merlini & Bellotti, 2003).

Microscopically, routine stains reveal amorphous, acellular, hyaline, eosinophlic extracellular material. With special stains as Congo Red, amyloid is salmon-pink, and characteristic yellow-green birefringence may be seen using polarized light. The organs commonly affected are:

a. Kidneys

Kidneys are classically enlarged, pale grey, waxy and firm. In advanced disease, chronic vascular occlusion may result in a shrunken, contracted organ. Amyloid deposit in several area such as glomeruli, peritubular regions and blood vessels.

b. Spleen

spleen may be enlarged and amyloid deposits begin between cells. With time two patters emerge, sago spleen, in which deposits are limit to splenic follicles, giving rise to tapioca-similar granules, or lardaceous spleen where amyloid spares also in red pulp.

c. Liver

Amyloid induces hepatomegaly with a pale, waxy gray, firm appearance. Amyloid deposit first in the space of Disse, gradually encroaching on parenchyma and sinusoids to produce atrophy with massive hepatic replacement.

d. Heart

Focal amyloid accumulations are minute and typically atrial, vascular or subepicardial localized as pink-gray subendocardial droplets. Microscopically there are interstitial and perimyocyte deposits progressively leading to pressure atrophy.

3.1.2 Systemic amyloidosis

a. Immunoglobulin light chain (AL)

The first amyloid fibril proteins to be characterized by amino acid sequence analysis were from patients with AL amyloidosis, an uncommon disease in which plasma cell dyscrasias result in monoclonal light chain production (Glenner et al., 1970). Both intact and amino-terminal

fragments of monoclonal immunoglobulin light chains have been found to infiltrate heart, kidneys, liver, and spleen and other organs of AL patients (Glenner et al., 1980; Cohen, 1967). AL fibrils are usually deposited systemically, but localized deposits are occasionally observed in lung and skin (Page et al., 1972; Husby et al., 1981). AL fibrils are usually considered to be degradation products of intact light chains. However, it is also possible that AL fibril proteins originate as independently synthesized light chain fragment (Buxbaum et al., 1991). The underlying causes of AL amyloidosis apparently involve, in addition to the formation of a monoclonal population of fragmented or truncated immunoglobulin chains, specific amino acid sequences in the light chain variable region that predispose adoption of secondary and tertiary structures necessary for polymerization into fibrils (Solomon et al., 1982).

b. Serum amyloid A (AA)

Two main mechanisms of AA amyloidogenesis are commonly suggested. Both propose that AA protein is a split product of SAA. According to one, SAA is cleaved enzymatically to form AA, which in turn is deposited in tissues. Alternatively, intact SAA is first precipitated in tissues and then is processed enzymatically to form AA. These hypotheses are supported by the following evidence: amyloidosis is often correlated with substantial increase in SAA level (DeBeer et al., 1982). Incubation of SAA proteins with monocytes, macrophages, and even erythrocytes led to the production of AA protein, probably by proteolytic enzymes released from these cells or by membrane-associated proteases (Shainkin-Kestenbaum et al., 1982; Kedar et al., 1982). Administration of human or mouse labelled SAA to mice resulted in deposition of the labelled SAA or their intermediates in amyloid fibrils (Husebekk et al., 1985). The immunochemical and immunohistochemical studies point to the presence of intact or partially degraded SAA along with protein AA in amyloid deposits (Yakar et al., 1994). These studies suggest that AA protein is an intermediate fragment in the catabolism of SAA. However, the proteolytic cleavage hypotheses are still not certain. Despite extensive efforts, proteolysis of SAA to AA has not been demonstrated directly by *in vivo* study. In addition, amyloidosis usually occurs only in low incidence among patients suffering from amyloid-inducing diseases, despite the presence of chronically elevated SAA concentrations in most patients (McAdam et al., 1982; Husby, 1985). In mice, despite similar solubility or accessibility of SAA1 and SAA2 to proteolytic enzymes, only SAA2 is structurally related to AA protein (Hoffman et al., 1984). Therefore, additional factors or different mechanisms must be operating in the formation of amyloid. Some of the answers to the question of amyloidogenesis raised above may lie in the interaction between SAA and other constituents of the amyloid fibrils.

In summary, the SAAs are a family of apolipoproteins, increasing to substantial quantities during inflammation, chronic infection, and certain malignancies. This elevation may be followed by the development of AA amyloidosis. The AA protein is thought to be a split product of SAA. However, until the precise metabolic pathway leading to AA formation is disclosed, current concepts of AA amyloidogenesis should be considered hypothetical.

c. Transthyretin (ATTR)

Transthyretin is a 55 KDa non-glycosilated plasma protein originally called Prealbumin because of its electrophoretic migration ahead of albumin; the molecule was later renamed transthyretin because of its function as a carrier for the thyroid hormones (Blake, 1981; Cornwell et al., 1987; Saraiva et al., 1993). TTR also serves as a carrier for the retinol binding protein complex (Smith et al., 1971). More than 35 TTR variants have been identified by protein and DNA screening studies and they exhibit clinically heterogeneous amyloidosis (Saraiva et al., 1992; Jacobson et al., 1991; Cohen et al., 1994). X-ray diffraction studies of TTR amyloid (ATTR) fibrils showed a mutation in val → met at 30 TTR monomers that permit the formation of intersubunits disulfide bonds between the single cysteine facilitating polymerization into fibrils (Wahlquist et al., 1991; MIgita et al., 1991). Fibrils are made up of both full-length TTR and fragments of TTR monomers and of variable quantities of both mutant and normal TTRs (Felding et al., 1985). The pathogenesis of ATTR differs from AL and AA amyloidosis in that the ATTR fibril precursor is a constitutive protein present in plasma at concentrations of about 100-400µg/ml from birth, whereas both AL and AA require specific pathological events to stimulate precursors synthesis.

d. Apolipoprotein AI (AApoAI)

Apolipoprotein AI-derived (AApoAI) amyloidosis can be present as a non-hereditary form with wild-type protein deposits in atherosclerotic plaques, or as a hereditary form with the variant protein depositing more systemically. The clinical manifestations of hereditary AApoAI amyloidosis frequently involve liver, kidney, larynx, skin, and myocardium. In rarer cases, amyloid is also found in the testes and adrenal glands (Obici et al., 2006). ApoAI is a plasma protein of 28 KDa synthesized by the liver and the small intestine. It is the main protein of high-density lipoprotein particles and important for the formation and metabolism of high-density lipoprotein cholesterol esters (Sorci-Thomas et al., 2002). Mature apoAI consist of 243 amino acids encoded by exons 3 and 4 of the *APOA1* gene (Karathanasis et al., 1983). More

than 50 apoAI variants have been described (Obici et al., 2006), and about half of them are associated with a decreased plasma level of high-density lipoprotein-apoAI.

3.1.3 Amyloidosis in cattle

Bovine Amyloidosis belongs to the same group of diseases as human systemic reactive AA Amyloidosis and is characterized by persistent and intractable diarrhea and systemic edema; a rectal examination often confirms nephromegaly. Clinicopathological features include nephrotic syndrome-like symptoms, such as severe hypoproteinemia and proteinuria (Johnson et al., 1984; Konishi et al., 1975).

Histopathological analysis reveals severe amyloid deposition in various organs, especially the kidney and liver (Murray et al., 1972; Yamada et al., 2006). A kidney biopsy is needed to confirm the diagnosis, but as it is difficult to perform this procedure in a clinical setting, some cows may die without a diagnosis being made. Moreover, it can be clinically diagnosed only at the terminal stage. Epidemiologically the disorder is more common in aged cows or associated with chronic inflammatory diseases of the mammary gland, joints, and respiratory system (Gruys, 2004; Yakar et al., 1995). Bacterial infections usually lead to a strong systemic acute phase response (APR) (Alsemgeest et al., 1994); because of the strong reaction of mononuclear/phagocytic system cells to viral infections, APR is generally milder in viral infections (Alsemgeest et al., 1995; Nakayama et al., 1993).

3.1.4 Transmissibility of systemic amyloidosis

Certain aspects of animal models of the Amyloidosis caused by serum amyloid A (Lundmark et al., 2002) and apolipoprotein A-II (Higuchi et al., 1999) have introduced the possibility that amyloidosis is transmissible. In mice, amyloid protein A amyloidosis (AA) is caused by an inflammatory reaction that results in overproduction of the acute phase protein serum amyloid A. Injection or oral administration of amyloid-enhancing factor, a crude homogenate of natural amyloid fibrils, accelerates the deposition of amyloid during the inflammatory process (Lundmark et al., 2002; Elliott-Bryant et al., 1998). These findings are consistent with the capacity of fibrillar seeds to catalyze conformational changes in the soluble protein. The capacity of preformed fibrils to trigger fibrillogenesis has been demonstrated *in vitro* for amyloid β (Aβ) peptides (Jarrett et al., 1993), lysozyme (Morozova-Roche et al., 2000), and beta2-microglobulin (Naiki et al., 1997) (Fig. 3.2).
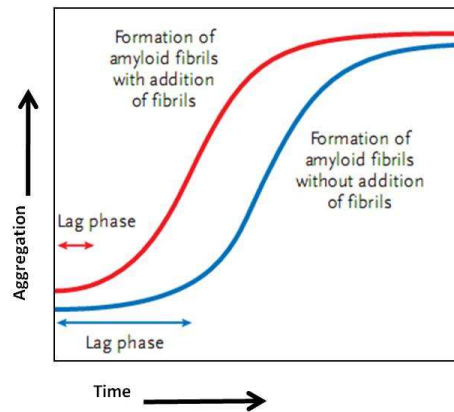
Figure 3.2. Kinetics of Fibril Formation. The blue line indicates the formation of amyloid fibrils, beginning from a solution of monomeric proteins, which may temporarily assume an amyloidogenic conformation. Initially, conditions do not favour aggregation, and this period corresponds to the lag phase that precedes the formation of fibrils. Once a critical nucleus has been generated, the conditions change to favour aggregation with very fast kinetics. Any available monomers in the amyloidogenic conformation quickly become entrapped in the fibril. The red line represents a similar condition in which preformed fibrils are added, thus making the lag phase much shorter.

With the exception of prion diseases, there is no evidence that amyloidosis is transmissible in humans. However, the formation of amyloid can be accelerated by the presence of fibril nuclei in tissues. In 1957 a strange phenomenon was described in experimental mouse AA amyloidosis, in fact many strains of mice develop a form of systemic amyloidosis when given a chronic inflammatory challenge. The finding that formation of amyloid fibrils is a nucleation dependent event is of fundamental importance and this seems to be principally the same for all forms of amyloid including that of the prion protein (Jarrett & Lansbury, 1993). In a solution of an amyloid fibril protein above the critical concentration a nucleus forms after a lag phase which can be quite long. Seeding such a solution with preformed fibrils of the same nature more or less abolishes this lag phase (Jarrett & Lansbury, 1993). In some yet unknown way, the misfolded and aggregated protein induces a rapid misfolding and aggregation of the protein in solution (Fig. 3.3).
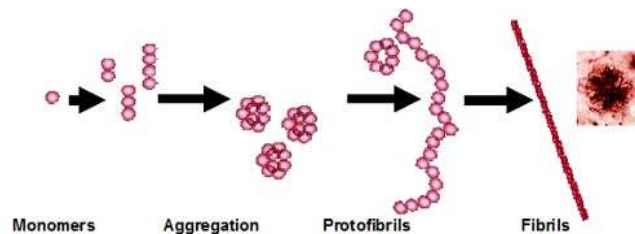

Figure 3.3. Mechanism of fibrils formation by nucleation.

Prion disorders are transmissible by different routes, including the oral way, most dramatically shown by the example of kuru (Gajdusek et al., 1966; Collinge et al., 2006). In most experiments, the infectious material has been given intravenously or intraperitoneally. However, addition of a small amount (about 1 mg/l; calculated total dose per mouse 0.2 mg)

of a fibril preparation to the drinking water for 5 weeks before induction of inflammation accelerated the development of AA-amyloidosis (Lundmark et al., 2002). Where and how the seeding AA-amyloid fibril or particle is taken up from the gastrointestinal tract is completely unknown. Also the route for the infectious AA-material to the spleen has not yet been clarified (Johan et al., 1998; Cohen & Cathcart, 1972). Further spreading can take place by the blood since in murine AA-amyloidosis, some circulating monocytes were found to carry amyloid fibrils and had the capability to accelerate amyloidosis in recipient animals (Sponarova et al., 2008). Prion diseases can be transmitted between species, a well-known fact since the work by Gajdusek et al. (1966) but particularly since the occurrence of human variant spongiform encephalopathy (variant Creutzfeldt-Jakob disease) (Collinge, 1999) originating from cattle and transmissible to mice (Wadsworth et al., 2008). Given the resemblance of prion protein with other amyloid fibril proteins' behaviour *in vitro* (Rochet & Lansbury, 2000), it is natural to question if AA-amyloidosis is transmissible between species. In 1969, Shirahama et al. reported transfer of experimental amyloidosis by human splenic amyloid homogenate to mice (Shirahama et al., 1969). In more extensive studies, AA-amyloid fibrils from several different mammalian (mouse, cow, cheetah and cat) and one avian species were found to accelerate experimental murine amyloidosis (Liu et al., 2007; Cui et al., 2008). Westermark et al. (2009) found a considerable difference in "infectivity" between donor species and while AA-fibrils extracted from donkey were efficient as amyloid enhancers, human AA-fibrils were not. Cui et al. (2008) found that bovine AA-fibrils have the ability to induce AA-amyloidosis in mice. However, fibrils from none of the other tested species were as efficient as those of mice in agreement of a species barrier, similar to that described for prions (Scott et al., 2008).

Another possible route of transmission of AA-amyloidosis could be by heterologous seeding. Systemic amyloidosis is common in many mammalian and avian species and may enter our food chain. Tojo et al. found a high incidence of AA-amyloidosis in slaughtered Japanese cows (Tojo et al., 2005). This form of amyloidosis is particularly common in several species of birds and is a problem in duck and goose industry. AA-amyloid was found in commercially available duck liver and in paté de foie gras and fibrils from such material accelerated amyloidosis in mice over-expressing IL-6, both when administered intravenously and given orally (Solomon et al., 2007). Similar to the situation with bovine spongiform encephalopathy where a large human population is believed to have ingested infected meat from such animals, it is very likely that many, perhaps a majority of individuals in many countries have eaten food containing AA-amyloid fibrils which may, if the conditions are the right, induce amyloidosis in the recipient.

Korenaga et al. (2006) demonstrated a vertical transmission of AA-amyloidosis from female mice inoculated with amyloid fibrils to their offspring in which the amyloid deposition in intestine is significantly accelerated. It was also shown that the transmission via milk from mice to their offspring is possible. As shown by Eckersall et al. (2006) SAA is an acute phase protein which increases its levels in cattle serum and also in milk during inflammation. The presence of a pathological status in the bovine mammary gland should facilitate the induction of unfolding of protein which led to the starting of a process of conformational shifting into β-sheet. One of the characteristics of fibrils is the difficult inactivation by physical or chemical methods (Zhang et al., 2006), for this reason the purpose of this first study is the evaluation of the possible degradation of fibrils by proteolysis during processing of milk in cheese making procedures.

The aim of this study was to explore the possibility that, give the high concentration of SAA in milk, there is a possibility that

a) the milk SAA may be converted in AA fibrils by the ripening procedure utilized in cheese making and

b) the same ripening procedure may induce the formation of amyloid fibrils, which are considered to be potentially dangerous for human safety.

Models for neither *in vivo* nor *in vitro* amyloid fibril formation in milk is available so far. It is therefore not possible to verify the spontaneous or induced precipitation of SAA in AA fibrils in milk.

Therefore, we decided to purify amyloid fibrils from spleen and to add them to crude milk, at a concentration similar to that found in bovine mastitis.

The experimental hypothesis that we verified was that serum amyloid A fibrils added to crude milk before ripening are not destroyed by the enzymes commonly utilized for cheese making, and therefore they may possibly represent a possible treath for human safety, on the background of the oral transmission of amyloidosis in mice.

In order to verify this hypothesis, we

a) set a protocol of extraction of serum amyloid A fibrils from bovine tissues, and

b) apply a commercial ELISA kit used for SAA quantification in serum to quantify the fibrils obtained from tissues.

Then we

c) added amyloid fibrils to crude milk at a concentration similar to that of SAA during mastitis and induced an experimental caseification

d) finally, the possible presence of amyloid fibrils in cheese was determined by Western Blotting using specific anti-SAA bovine antibodies.

3.2 MATERIALS AND METHODS

3.2.1 Samples and histological diagnoses of amyloidosis

Spleen and kidney from adult cows were collected in a local abattoir and stored at -80°C until use. Macroscopically amyloidotic organs were selected using the following criteria: increased dimensions and pale gray colour. Diagnosis for amyloidosis was definitely confirmed by histology. Fragment of tissues were directly frozen in liquid nitrogen and then cryosections of 5μm were prepared on positive charged slides. Section were fixed with frozen acetone for 3 minutes and stored at -20°C until use. Before staining sections were warmed at room temperature, then were re-hydrated using a sequence of decrescent ethanol concentration (100%, 90%, 70% 50%) and distilled water. The slides were stained in Hematoxilin for 10 minutes and after washing in distilled water, they were incubated in Congo Red for 1 hour. Finally sections were dehydrated rapidly in absolute alcohol, cleared in xylene and mounted.

3.2.2 AA fibrils purification

Fibrils were isolated from 14g of spleen tissue by the classical water extraction procedure (Pras et al. 1968; Mangione et al. 2001) in the presence of 1.5 mM phenylmethylsulfonyl fluoride (PMSF) after repeated homogenization in TE Buffer pH8 containing 10mM Tris/EDTA, 140mM NaCl, 0.1%NaN$_3$, 1.5mM PMSF in ice and centrifugation at 40000 rpm in ultracentrifuge for 35 minutes at 4°C. When the supernantant became clear the TE Buffer was substitute with distilled water containing 1.5mM PMSF and the cycles of homogenization and centrifugation were repeated 9 times. All the water extraction fractions were lyophilized and used for further studies. Part of the pellet and aliquot of extraction fraction were stained with Congo Red.

3.2.3 Quantification of fibrils

The extraction fractions were resuspended in 4ml of distilled water and quantified using a commercial solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) kit (Phase Range Serum Amyloid A Assay Tridelta Dev. Ltd, Ireland) commonly used for quantification of serum SAA. Each sample was diluted in Dilution Buffer 1X (1:500) in a final volume of 50μl and was added to 50μl of a SAA specific biotinilated monoclonal antibody (1:100) for 1 hour at

37°C. Each sample was analyzed in duplicate. The plate was then washed three times with Washing Buffer, and then incubated with 100µl of Streptavidin-Peroxidase (HRP) (1:4000) for 30 minutes, at room temperature in the dark. The plate was washed three times and then incubated with 100µl of 3,3',5,5' tetramethyl-Benzidine (TMB) for 30 minutes at room temperature. Finally the reaction was stopped adding 50µl of Stop solution and the absorbance was read in a spectrophotometer at 450nm.

The mean absorbance for each sample, or standard was calculated. The absorbance of the standards was plot against the standard concentration and the best smooth curve through these points was drawn to construct the standard curve. The concentrations of the test samples and controls were calculated from the standard curve by multiplying the interpolated value by the appropriate dilution factor.

3.2.4 SDS-PAGE, Western Blotting and Coomassie Brilliant Blue Staining

The extraction fractions were separated by SDS-PAGE electrophoresis on 12% acrylamide gel. The homogeneity of purified fibrils was confirmed by Congo red stain of aliquots of pellet resuspended in distilled water and supernatants. Fibrils were then Western-blotted onto a nitrocellulose membrane. Membrane was blocked for 1 hour at room temperature with Phosphate Buffered Saline (PBS) with 2% Bovine Serum Albumin (BSA). After three wash with PBS with 0.2% Tween-20 (PBST) the membrane was incubated with SAA specific biotinilated monoclonal antibody (1:200) (Tridelta Dev. Ltd, Ireland) for 14 hours at 4°C. The membrane was then washed three times in PBST and then incubated with Vectastain ABC Elite standard kit (VectorLab, UK) for 5 minutes with a final wash in PBST.

Using the same conditions another SDS-PAGE electrophoresis has been performed. The gel was stained for 20 minutes with a solution of Coomassie Brilliant Blue (40% methanol, 10% acetic acid, 0.025% Coomassie Brilliant Blue R-250). Then it was destained with a solution containing 40% methanol and 10% glacial acetic acid until background of the gel is fully destained.

3.2.5 Experimental cheese making

The experimental cheeses were made from whole, raw milk (un-pasteurized milk). The milk was purchased from a local market. Raw milk (500ml) was heated up to 35°C in agitation. To one of the milk amyloid fibrils of bovine origin previously extracted in concentration of

20µg/ml were added. Liquid rennet extract of bovine origin (1-3 ml/l) was used and left resting for 1 hour. The coagulum was cut with miniature stainless-steel tools and was placed in sterile boxes fitted with a grid to facilitate whey draining. One of the cheeses was inoculated with amyloid fibrils of bovine origin previously extracted in concentration of 20µg/ml. The two kind of cheese were stored at 4°C for 10 days. At the end of seasoning the cheese was cut in pieces and store at -80°C for the following analysis. Cheese without fibrils was called "control" and cheese with fibrils was called "treated".

3.2.6 Extraction of fibrils from cheese

Fibrils were isolated from 20g of control and treated cheese by the classical water extraction procedure used for isolation from tissues and described above. All the water extraction fractions were lyophilized and used for further studies. Part of the pellets was stained with Congo Red.

3.2.7 SDS-PAGE and Western Blotting

The extraction fractions were separated by SDS-PAGE electrophoresis on 12% acrylamide gel. Fibrils were then transferred to a nitrocellulose membrane. Membrane was blocked for 1 hour at room temperature with Phosphate Buffered Saline (PBS) with 2% Bovine Serum Albumin (BSA). After three wash with PBS with 0.2% Tween-20 (PBST) the membrane was incubated with a mouse anti-bovine SAA specific policlonal primary antibody (1:100) (kindly gifted by dr.McDonald) for 1 hour at room temperature. The membrane was then washed three times in PBST and incubated 45 minutes with a goat anti-mouse IgG peroxidase conjugated secondary antibody (1:2000). Detection was carried out by enhanced chemiluminescence (ECL) using Immobilon^{TM} Western Chemiluminescence HRP Substrate (Millipore, Italy).

3.3 RESULTS

3.3.1 Morphology and histology of bovine spleen and kidney

The macroscopical analysis of the organs collected at slaughterhouse confirm the presence of amyloid deposition, kidneys and spleen are enlarged, pale grey, waxy and firm. Microscopically amyloid deposition is clearly present in spleen around blood vessels (Fig. 3.4a) and in kidney in glomeruli and blood vessels (Fig. 3.4b).
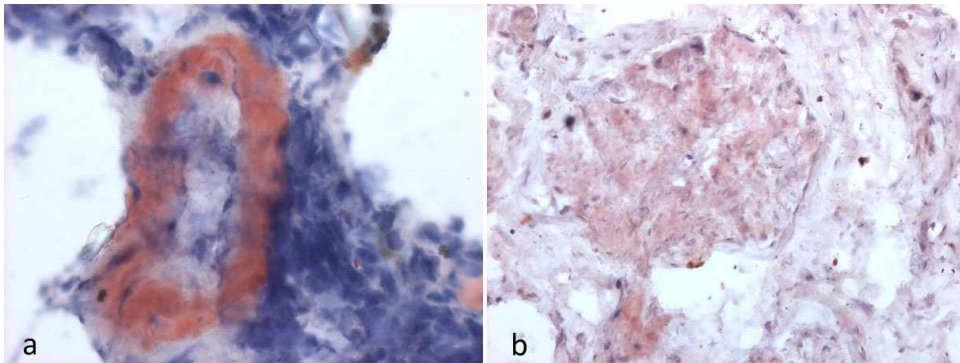

Figure 3.4. Picture showing sections stained with Congo Red and Hematoxilin which shown the amyloid deposits in and in the endothelial cells of spleen (a) and in the glomerulus of kidney (b).

3.3.2 Purification, quantification and analysis of fibrils extracted from tissue

The extraction protocol commonly used for amyloid purification in human tissues was applied in bovine and it permits to isolate a good quantity of fibrils from spleen. The presence of amyloid fibrils in the purified fraction was assessed by Congo Red staining. These amyloid deposits present an orange-red stain in light microscopy (Fig 3.5a) and are identified on the basis of their apple-green birefringence under a polarized light microscopy (Fig 3.5b).
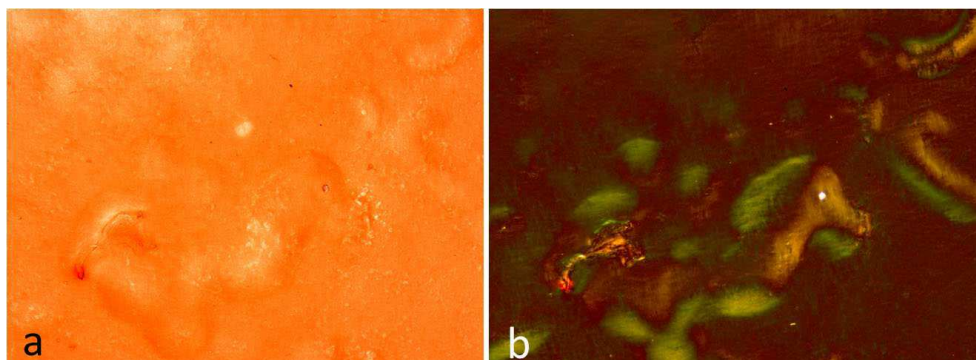

Figure 3.5. Representative picture of fibrils from water extraction fraction 3. Under light microscope the fibrils appear as orange-red aggregates (a) and under polarized light they are characterized by apple-green birefringence color (b).

The quantification of fibrils from the extraction fraction performed using an ELSIA kit used for quantification of SAA in serum showed good results also starting from a suspension. The fibrils mean concentration in aqueous fraction is 1.041 μg/ml.

SDS-PAGE gel electrophoresis and Coomassie Brilliant Blue showed the presence of fibrils aggregates of different molecular weight (Fig. 3.6).
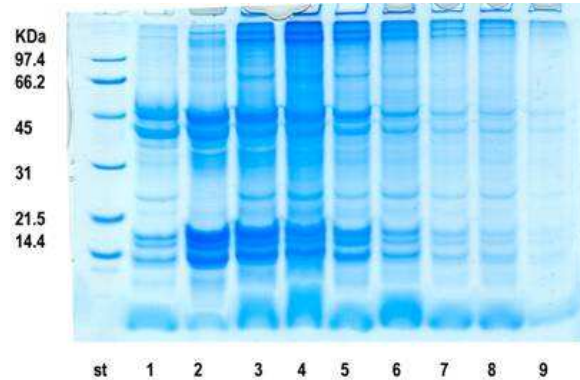

Figure 3.6. Coomassie Brilliant Blue staining of fibrils from water extraction fractions. The staining shows the distribution of fibrils in different molecular weight aggregates. St: standard molecular weight; lane from 1 to 9: fibrils from water extraction fractions.

The presence of aggregates of SAA fibrils was confirmed by western blot using an antibody specific for this protein (Fig. 3.7).
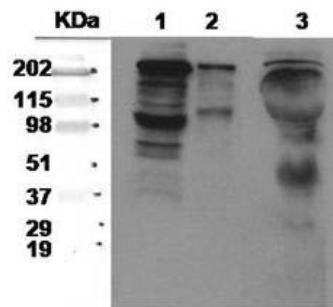

Figure 3.7. Western blot of fibrils purified in water extraction fraction 3 (Lane1) and 4 (Lane2). Bovine serum was used as positive control (Lane3).

3.3.3 Experimental cheese

The cheese making starting from 500ml of raw milk led to the creation of two different kinds of cheeses. As shown in figure 3.8 the two cheeses showed a different colour, the control one is white (a) instead of the treated one which showed a yellowish colour (b).
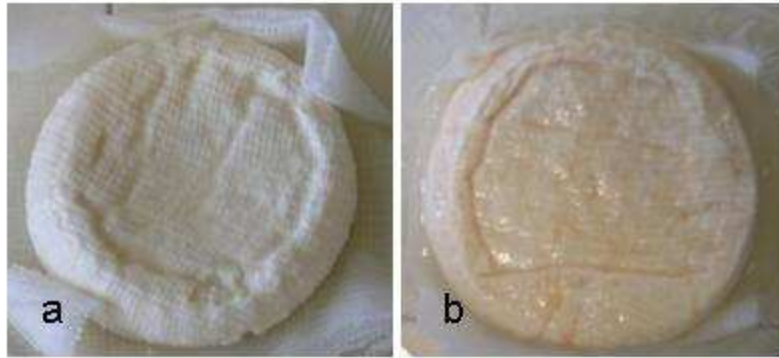

Figure 3.8. Experimental cheeses prepared from raw milk. The two kinds of cheeses showed a different colouring, the one without fibrils is white (a) instead of the one with addition of fibrils is yellowish (b).

3.3.4 Purification and analysis of fibrils extracted from cheese

The extraction protocol used for amyloid purification in tissues was applied in cheese and it permits to isolate a good quantity of fibrils from both control and treated cheeses. The presence of amyloid fibrils in the purified fraction was assessed by Congo Red staining. These amyloid deposits present an orange-red stain in light microscopy (Fig 3.9a) and identified on the basis of their apple-green birefringence under a polarized light microscope (Fig 3.9b).
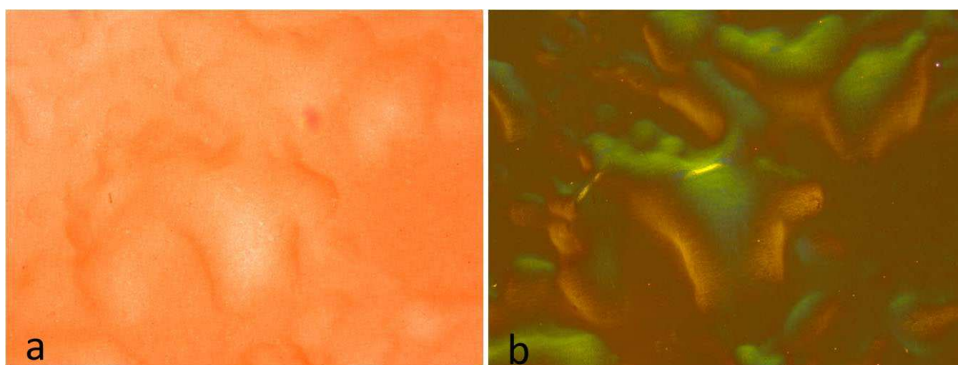

Figure 3.9. Amyloid aggregates preset in pellets from treated cheese showed by orange-red stain in light microscope (a) and apple-green birefringence under a polarized light microscope (b).

The presence of bovine amyloid fibrils, as well as their electrophoretic pattern, was determined by SDS-PAGE followed by Western blotting and immunostaining using an antibody specific for this protein in both kinds of cheese (Fig. 3.10).
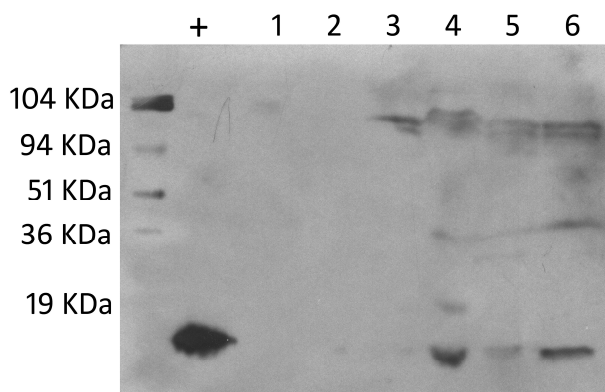

Figure 3.10. Western blot performed on fibrils purified from experimental cheeses. SAA fibrils are present in control and in treated extraction fractions. Bovine liver was used as positive control (+) and SAA protein is identified as expected at 14KDa. Lane 1-3: blot of extraction fraction of control cheese. Lanes 4-6: blot of extraction fraction from treated cheese.

The figure cleary showed that no amyloid protein was present in lanes 1, 2 and 3, where samples of cheese prepared with milk without previous adding of fibrils were loaded. On the contrary, lanes 4, 5 and 6, which include samples of cheese previously added with amyloid fibrils, clearly showed a positive staining at a molecular weight correspondent to SAA, as shown in lanes + (positive control form liver). High molecular weight immunoreactive bands were also present.

The results clearly showed that the enzymatic processes triggered for cheese making were not able to destroy amyloid fibrils added to milk before ripening.

3.4 DISCUSSION

All of diagnostic tools and research that concern amyloidosis start from the characterization of the fibrils isolated from tissues of affected patients. For these reasons, we showed in this study the isolation of amyloidogenic fibrils from bovine tissues, after the confirmation of the pathology by morphological and histological analysis of the considered tissues, i.e. kidney and spleen. The purification was obtained by the application of the aqueous extraction protocol used in humans (Pras et al., 1968). The same extraction protocol was also applied to isolate fibrils from experimental cheese.

Furthermore, our study demonstrated that a commercial kit commonly used for quantification of SAA in serum could be also applied for the quantification of a suspension of fibrils previously purified from tissue and from cheese.

The antibody used in ELISA and in Western Blot confirm the presence of AA in the extraction fraction from tissue and cheese. The data collected from Western Blot compared to the Coomassie Brilliant Blue staining showed the presence of aggregates of fibrils at different molecular weight.

As previously reported by some authors amyloid fibrils can resists to physical and chemical methods (Zhang et al., 2006), our purpose was to evaluate if the proteolitic activity of rennet during cheese making should destroy the AA fibrils experimentally added to raw milk. The fibrils are only in part degraded but we could show that some of them are present after 10 days of resting. Therefore, our results clearly show that the normal process of ripening is not capable to destroy amyloidotic fibrils.

Some reports showed that amyloid fibrils can be transmitted from a species to another via milk (Korenaga et al., 2006), raw tissue homogenates (Zhang et al., 2006), via feces (Zhang et al., 2008) and also via food, for example in paté de foie gras (Solomon et al., 2007). All these data and the confirmation of the presence of fibrils in cheese let us to suggest that cheese may, at least theoretically, be included in the aliments "at risk" for oral transmission of amyloidosis.

Similar to the situation with bovine spongiform encephalopathy, where a large human population is believed to have ingested infected meat from such animals, it is very likely that many, perhaps a majority of individuals in many countries have eaten food containing AA-amyloid fibrils which may, if the conditions are the right, induce amyloidosis in the recipient. One interesting phenomenon, noted in the mouse, is that there may be a long delay between the intake of amyloid enhancing material and the development of disease. When amyloid fibrils were given intravenously into animals without an inflammatory challenge, the mice stayed healthy. However, if inflammation was induced amyloidosis developed almost immediately. That means the individuals may be primed for the amyloid disease in the case that a chronic inflammatory disease is acquired later. A similar primed state has been described in murine AA-amyloidosis after resolution of the deposits (Hawkins et al., 1990). It will be very difficult to prove an association of ingestion of amyloid and development of AA-amyloidosis if this kind of delayed effect is true also in human.

Several issues remain to be addressed: for example, it is not known if the increase of concentration of SAA in milk can induce the precipitation of SAA in AA fibrils.

The fibrils were added at a concentration similar to that found in milk during the systemic response to inflammation after acute mastitis. From a theoretical point of view, the biochemical conditions of the mammary gland environment during acute inflammation, including lowering of pH, the presence of high concentration of activated proteases and the production of reactive oxygen species, may induce the destabilization of the secondary structure of serum amyloid A, and the development of an extracellular unfolded protein response. This phenomenom, in turn, may eventually drive the soluble SAA to precipitate in un-soluble fibrils.

It has to be demonstrated whether these pathologic conditions are sufficient to induce the generation of AA.

Chapter 4

Conclusions & Final Remarks

Amyloidosis in cattle is a disease characterized by Amyloid A protein precipitation in tissues such as kidney and spleen as unsoluble fibrils. We demonstrated in our study that AA-fibrils can be isolated from tissues using a protocol commonly applied to humans and based on aqueous extraction. We also found that the addiction of these fibrils, in a concentration that resemble the SAA levels during an acute phase response, in raw milk that undergoes cheese making procedures, should make us able to find them after purification from cheese.

We validated the capability of a commercial ELISA kit, commonly used for serum quantification of SAA, to identify and quantify an aqueous suspension of purified fibrils properly diluted.

It would be interesting to compare the potential transmissibility of fibrils from normal cheese and cheese with fibrils added. Future development include the feeding of mice with "infected" cheese, in order to verify if this should be potentially pathological and can be the seed of a process of endogenous fibrils accumulation. Further study can be done to understand this process of transmission.

In water buffalos five of the major bovine acute phase proteins have been sequenced to verify if in this species their primary structures are conserved. Our study demonstrated an homology of more than 95% of all of this proteins. For this reason we decided to evaluate the potential biological role during an intramammary infection. We have chosen two of the main bovine acute phase proteins, including AGP and SAA. AGP, remarkably, i.e. the protein that increases its concentration during an acute phase response in cattle, is under the detection limit in healthy animals but also in buffaloes that showed a intramammary infection, whereas SAA concentration in serum and in milk are increased in infected animals in comparison with healthy buffaloes.

For all these reasons we could say that SAA can be used as a marker of pathology in buffaloes. Further study should be done to understand if SAA serum concentration can be helpful to determine the severity of a mastitis and how its production is modulated during the course of the disease.

Future investigations will verify the possible role of Hp, Cp and LBP as other markers of pathology in water buffaloes.

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Publications

I spent my PhD programme in other studies and part of the results have been published

- **LF Pisani**, S Antonini, P Pocar, S Ferrari, TAL Brevini, SM Rhind and F Gandolfi. "Effect of premating nutrition on mRNA levels of developmentally-relevant genes in sheep oocytes and granulose cells". *Reproduction 2008 136: 303-312*.

- S Violini, P Ramelli, **LF Pisani**, C Gorni, P Mariani. "Horse bone marrow mesenchymal stem cells express embryo stem cells markers and show the ability for tenogenic differentiation by *in vitro* exposure to BMP12". *BMC Cell Biology 2009 10:29*

- **LF Pisani**, C Lecchi, G Invernizzi, F Ceciliani, P Sartorelli, G Savoini. "*In vitro* modulatory effect of n-3 polyunsaturated fatty acid (EPA and DHA) on phagocytosis and ROS production of caprine neutrophils". *Veterinary Immunology and Immunopathology 2009 15;131(1-2):79-85*

- **LF Pisani**, P Ramelli, B Lazzari, S Braglia, F Ceciliani, P Mariani. "Characterization of Maternal Antigen That Embryos Require (Mater/NLRP5) gene and protein in pig somatic tissues and germ cells". *Journal of Reproduction and Development 2010 56: 41-48*

- **LF Pisani**, G Pennarossa, E Papasso Brambilla, MM Rahman, G Lazzari, A Zecconi, TAL Brevini, F Gandolfi. "Newborn pig ovarian tissue xenografted into Severe Combined Immunodeficient (SCID) mice acquires limited responsiveness to gonadotropins". *Theriogenology. 2010 Sep 1;74(4):557-62.*

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