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Acute Phase Proteins in Healthy and Sick Dairy and Beef Calves and Their Association with Growth

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ACUTE PHASE PROTEINS IN HEALTHY AND SICK DAIRY AND BEEF CALVES AND THEIR ASSOCIATION WITH GROWTH

Leena Seppä-Lassila

ACADEMIC DISSERTATION

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ABSTRACT

The acute phase response is a component of the innate immune system, a general and non-specific response that limits the spread of infection and removes factors potentially harmful to the body. Infection, neoplasm or tissue trauma initiates an acute phase response, resulting in visible systemic signs of illness, fever, reduction of appetite and depression. Acute phase response also induces a variety of less discernible changes. One measurable sign of acute phase response is the increased synthesis of acute phase proteins by the liver, mediated by cytokines. Cytokines have short half-life, but the acute phase proteins can be measured for longer. In bovine medicine, increased concentrations of the acute phase proteins haptoglobin, serum amyloid A and fibrinogen have been associated with several diseases, including respiratory tract infections in calves, metritis, mastitis, and hoof diseases.

The aim of the work embodied in this thesis was to explore further the associations between acute phase proteins and common diseases in calves and assess the ability of acute phase proteins to differentiate between severities of disease during different stages. The studies also addressed the possibility of using the variation in acute phase protein concentrations in apparently healthy animals to predict the growth.

The thesis is based on four individual studies, performed as field studies on commercial Finnish farms. Acute phase protein measurements and collection of data for explanatory variables were got from dairy calves on dairy farms, beef calves in suckler herds and bull and heifer calves on a calf-rearing farm.

The reference values for haptoglobin, serum amyloid A and fibrinogen were established from the healthy population of dairy calves (Study I). The association between the diseases and acute phase proteins were assessed in different calf populations, and increased concentrations of haptoglobin, serum amyloid A and fibrinogen were recorded in calves with respiratory tract infection and umbilical inflammation (Studies I, II, III).

Fibrinogen, haptoglobin and serum amyloid A differed between surgically confirmed diagnoses of local umbilical inflammation and severe umbilical inflammation reaching the abdominal cavity (Study I). Decreased haptoglobin concentrations were associated with weak signs of eimeriosis (diarrhoea or low oocyst count or presence of non-pathogenic *Eimeria* spp.; Study II).

Increased albumin concentrations at around two weeks of age were associated with better growth rate over the first 30 days of life (Study III) in a population of beef calves. However, a longer-term effect was recorded for serum amyloid A concentrations around two to three weeks of age (Studies III and IV). A negative association between increased serum amyloid A and

growth rate was evident for beef calves over the period 30 to 200 days of age in (III). A similar association between serum amyloid A and growth was apparent in rearing dairy calves over two months, seven months and even at slaughter, at 15 to 18 months (Study IV).

The results reported in this thesis support the concept that acute phase proteins are suitable markers for inflammation and several calf diseases. The novel finding of an association between serum amyloid A and growth rate represents opportunities for purposes other than aiding clinical work. The increase in non-specific markers of inflammation can indicate general disturbance in animal health.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Seppä-Lassila, L., Orro, T., LePage, J.-P., Soveri, T., 2013. Reference values for acute phase proteins in calves and its clinical application. Veterinary Record 173: 319.

II Seppä-Lassila, L., Orro, T., Lassen, B., Lasonen, R., Autio, T., Pelkonen, S., Soveri, T., 2015. Intestinal pathogens, diarrhoea and acute phase proteins in naturally infected dairy calves. Comparative Immunology, Microbiology and Infectious Diseases 41: 10–16.

III Seppä-Lassila, L., Eerola, U., Orro, T., Härtel, H., Simojoki, H., Autio, T., Pelkonen, S., Soveri, T., 2017. Health and growth of Finnish beef calves and the relation to acute phase response. Livestock Science 196: 7–13.

IV Seppä-Lassila, L., Oksanen, J., Herva, T., Dorbek-Kolin, E., Kosunen, H., Parviainen, L., Soveri, T., Orro, T. Associations between two group sizes, serum protein levels, calf morbidity and growth in dairy beefs in a Finnish calf rearing unit. *Submitted to Preventive Veterinary Medicine.*

ABBREVIATIONS

ADG	average daily gain (g/day)
AGP	α-1-acid glycoprotein*
Alb	albumin
APP	acute phase protein
AUC	area under curve
AT	α1 antitrypsin
BRD	bovine respiratory disease
BVDV	bovine viral diarrhea virus
Cp	ceruloplasmin
EDTA	ethylenediamine tetraacetic acid
Fb	fibrinogen
Hp	haptoglobin
IL	interleukin
kDa	kilodalton
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
ROC	receiver-operating characteristic
SAA	serum amyloid A
Tf	transferrin
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α
WBC	white blood cell

* In earlier literature also termed seromucoid or orosomucoid (converted into AGP in the thesis)

1 INTRODUCTION

The acute phase response is an element of the innate immune system, which represents the first line defence in a body's battle against infectious agents or other harmful factors that threaten the homeostasis. The research on acute phase response has been extensive, but with the exception of fibrinogen, the acute phase proteins (APPs) are still little used in bovine medicine.

Diarrhoea, respiratory tract infections and umbilical disease are common in calves, and are usually easily diagnosed and treated. Therefore, verifying inflammation in a calf is usually not feasible and may not add information on the state of the animal. However, the quantitative nature of APPs may provide the means to assess the severity of inflammation, an important piece of information when the practitioner is contemplating treatment of the animal or possibly euthanasia. The non-specific nature of the APPs can open possibilities to make more general judgment on conditions at the herd or farm level.

Immunology has been a productive field of study for decades. New information on innate immunity is accumulating, providing a base for novel perspectives and generating new questions. Knowledge on long-term effects conferred by early life events in immunology, intestinal microbiota or functioning of the brain is increasing.

In this thesis, practical implementations for measuring APPs were sought. Defining the reference values for calves and exploring the capacity of the APPs to differentiate different severities of the disease were considered a good start. Subsequently, studies exploring more applied use of APPs in association with growth and medication undertaken.

2 REVIEW OF THE LITERATURE

2.1 THE ACUTE PHASE RESPONSE

Normal body function is at equilibrium, termed homeostasis, where the metabolism is under the control of feedback mechanisms involving hormones and enzymes. Infection, neoplasms or trauma disrupt this balance and the tissue injury inflicts a local inflammatory response (Kushner, 1993), including vasodilatation, coagulation, and migration of neutrophils, monocytes and lymphocytes (Stadnyk and Gauldie, 1991). At the site of injury vascular permeability increases and leukocytes are attracted (Kushner, 1982), resulting in the classical signs of inflammation: *rubor*, *tumor*, *calor* and *dolor* (redness, swelling, heat and pain). Macrophages, mast cells, dendritic cells and other immunocompetent cells recognise the situation, begin secreting cytokines and initiate a cascade of systemic changes termed the acute phase response, which includes rapid synthesis of APPs, mainly in the liver (Koj et al., 1984; Baumann and Gauldie, 1994).

The hepatic APP synthesis is initiated within hours of the infection or local trauma recognised by the immune system (Stadnyk and Gauldie, 1991; Horadagoda et al., 1994). The acute phase response is a part of the innate immune system, and includes activation of the complement and clotting system among other systemic changes (Koj, 1996). Activation of the complement also initiates the adaptive immune system during the acute phase response, providing more precise defence against the infection (Dunkelberger and Song, 2009). Characteristically, the acute phase response is not specific to the initiating agent, and in contrast to the adaptive immune system, previously resolved infections do not alter the acute phase response.

The changes in the acute phase response aim at re-establishing homeostasis, but when the inflammation is not resolved, chronic inflammation can develop (Jain et al., 2011). The acute phase response includes fever, leucocytosis, changes in the concentrations of plasma trace elements (copper, iron, zinc), and readjustment of the hepatic protein synthesis, poor appetite and depression, inflicted by an assortment of cytokines (Kushner, 1982; Stadnyk and Gauldie, 1991; Weingarten, 1996; Elmquist et al., 1997; Gabay and Kushner, 1999). The major systemic changes are summarized in Table 1.

Soluble signalling molecules, including endocrine hormones, neurotransmitters, eicosanoids and cytokines convey the intercellular communication (Kushner, 1993). Pro-inflammatory cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor α (TNF-α) are secreted from activated macrophages and generate a second wave of cytokines from the fibroblasts and endothelial cells, which essentially begins the acute phase response (Baumann and Gauldie, 1994).

Table 1 Systemic effects of the acute phase response according to Kushner (1982), Baumann and Gauldie (1994) and Gabay and Kushner (1999).

General signs	Fever
	Somnolence
	Anorexia
	Pain
Metabolic changes	Gluconeogenesis ↓
	Muscle catabolism
	Lipolysis in adipose tissue ↑
	Synthesis of acute phase proteins in the liver ↑
Changes in endocrinology	Adrenocorticotrophine and glucocorticoids ↑
	Insulin-like growth factor 1 ↓
	Thyroid stimulating hormone ↑
	Glucagon and insulin ↑
	Growth hormone ↑
Haematological changes	Leucocytosis
	Thrombocytosis
	Activation of the complement system
	Serum zinc↓
	Serum iron ↓
	Serum copper ↑

The effect of various cytokines on the synthesis of the APPs is well studied. IL-6 is considered to be one of the major acute phase response mediators because it affects the synthesis of a number of plasma proteins and its concentration in the blood correlates with changes in APP concentrations (Kushner, 1993). The up-regulating effect of the cytokines is amplified or inhibited by other cytokines, and the outcome of APP synthesis is the sum of these effects (Mackiewicz et al., 1991; Baumann and Gauldie, 1994). Overall, cells *in vivo* typically are not subjected to a single type of cytokine only, but to an assortment of molecules (Kushner, 1993). However, APPs have been divided into two groups, depending on whether their synthesis is primarily promoted by IL-1-like (Type I APPs) or IL-6-like (Type II APPs) cytokines, even though Type I APPs can also be induced by IL-6-like cytokines (Baumann and Gauldie, 1994; Moshage, 1997). In addition to cytokines, the synthesis of the APPs and the acute phase response is also modulated and modified by glucocorticoids, insulin and growth factors in various ways (Kushner, 1993; Moshage, 1997; Koj, 1998).

The regulation and termination of the acute phase response is at least as complex as the initiation, although, in simple terms, it is based on the short half-lives of pro-inflammatory cytokines, production of anti-inflammatory cytokines and removing excess pro-inflammatory cytokines by soluble cytokine receptors (Koj, 1998). Anti-inflammatory cytokines IL-10, IL-4, IL-

13 and transforming growth factor β (TGF-β), secreted from the T cells and macrophages, inhibit the production and action of pro-inflammatory cytokines (Koj, 1998; Burger and Dayer, 2002). IL-10 has a dominant role in inhibiting the synthesis of many cytokines (Baumann and Gauldie, 1994). Low level production of IL-10 from macrophages and monocytes seems also to prevent the acute inflammation from becoming a chronic inflammation (Brown et al., 1996). Soluble, antagonistic cytokine receptors are particularly efficient in inhibiting the actions of pro-inflammatory cytokines competing over the agonistic membrane-bound receptors (Koj, 1998).

2.2 BOVINE ACUTE PHASE PROTEINS

The bovine APPs include haptoglobin, serum amyloid A, fibrinogen, albumin, transferrin, lipopolysaccharide binding protein, α-1 acid glycoprotein and ceruloplasmin (Petersen et al., 2004; Ceciliani et al., 2012). APPs are broadly classified as major positive (10- to 100-fold increase in concentration), minor positive (2- to 10-fold increase) or negative (decrease at least of 25 %) depending on to the direction and magnitude of the changes in their concentrations during the acute phase response (Gabay and Kushner, 1999; Cerón et al., 2005). For cattle, haptoglobin and serum amylod-A are considered as major APP and fibrinogen, lipopolysaccharide binding protein, α-1 acid glycoprotein and ceruloplasmin as minor APPs (Petersen et al., 2004; Ceciliani et al., 2012). Other APPs in domestic animals include C reactive protein in dogs and pigs and the pig major acute phase protein (pig-MAP or inter-alpha-trypsin inhibitor heavy chain 4; ITIH4) (Petersen et al., 2004; Ceciliani et al., 2012; Schmidt and Eckersall, 2015). The latter has also been reported for cattle (Piñeiro et al., 2004). The negative APPs of cattle include albumin and transferrin (Petersen et al., 2004; Gruys et al., 2005).

Bovine haptoglobin (Hp) consists of two chains, an α chain of 20 kDa and a β chain of 35 kDa, but it also occurs in polymeric form of several α and β chains in electrophoresis (Morimatsu et al., 1991). During inflammation Hp binds haemoglobin before bacteria degrade the haemoglobin (Eaton et al., 1982), thus limiting access of bacteria to the free iron molecules that are essential for bacterial propagation (Skaar, 2010). The concentrations of Hp begin to increase within hours of an experimental inflammatory stimulus or experimental infection, accelerating after one day and peaking at three to four days after the challenge (Conner et al., 1988; Horadagoda et al., 1994).

Serum amyloid A (SAA) is an apolipoprotein of 14 kDa (Husebekk et al., 1988), that shows considerable similarity in structure among domestic species (Westermark et al., 1986). Bovine SAA has multiple isoforms; a varied combination of the isoforms occur in different types of diseases, and the SAA profiles of diseased cattle also differ from those of healthy animals (Alsemgeest et al., 1995a; Jacobsen et al., 2005; Takahashi et al., 2009). Extrahepatically produced SAA isoforms have been detected in mastitic milk

(Jacobsen et al., 2005). The main function of SAA remains unresolved but various functions have been recorded: binding and transporting cholesterol (Kisilevsky and Manley, 2012), acting as a chemoattractant of leucocytes (Badolato et al., 1994) and opsonisation of gram-negative bacteria (Hari-Dass et al., 2005; Shah et al., 2006). SAA concentration starts to increase almost immediately after an experimental bacterial infection, accelerating within hours, and peaks within two days (Boosman et al., 1989; Horadagoda et al., 1994).

Fibrinogen (Fb) is a very large glycoprotein of 340 kDa (Kent, 1967), mainly promoting blood coagulation or fibrin formation, but also considered to be an APP in cattle (Conner et al., 1988). Unlike most of the other APPs, Fb is present in the plasma of healthy animals at fairly high concentrations, 3–7 g/l (Schalm, 1976). Maybe partly because of this, the proportional increase during the acute phase response is moderate, amounting to a 2–3 fold increase (Ek, 1972; Liberg, 1978; Conner et al., 1988). The increased concentrations of Fb can be detected even three to four days after a momentary challenge (Conner et al., 1988). If the disease continues, Fb concentrations will remain elevated (Ek, 1972).

Lipopolysaccharide binding protein (LBP) is a 58–60 kDa glycoprotein (Tobias et al., 1986). The main function of LBP is to bind to lipopolysaccharides (LPS) of gram-negative bacteria, and the resulting LBP-LPS-bacteria complex further binds to monocytes and macrophages (Schumann et al., 1990). LBP enhances the synthesis of TNF-α by monocytes (Horadagoda et al., 1995) and can also bind to lipoteichic acids and glycolipids of gram-positive bacteria (Zweigner et al., 2006), an extension to their typical function. The concentration of LPB starts to increase 6–12 hours following intratracheal inoculation with the bacteria and peaks for 24 to 48 hours (Horadagoda et al., 1995; Schroedl et al., 2001). Even faster increase was achieved by intra-mammary infusion of LPS (Bannerman et al., 2003)

α_1-acid glycoprotein (AGP), in earlier literature referred to as seromucoid or orosomucoid (Eckersall and Conner, 1988), is a 42 kDa highly glycosylated glycoprotein (Tamura et al., 1989). Although the precise biological function of AGP is unclear (Hochepied et al., 2003), it belongs to a group of immunocalins that have multiple immunomodulating functions (Lögdberg and Wester, 2000); AGP can induce the secretion of pro-inflammatory and immunosuppressing cytokines by mononuclear cells (Hochepied et al., 2003), inhibit the activity and aggregation of leucocytes (Costello et al., 1984) and inhibit lymphocyte proliferation (Chiu et al., 1977). AGP concentrations in calves start to increase within hours after intravenous LPS stimulation, and reach peak concentration, twice the baseline value, at about 24 hours after the challenge (Hinds et al., 2014). Subcutaneous turpentine injection results in a slower response of AGP change: maximum increase in calves is observed only at four days post-challenge (Conner et al., 1988). Subcutaneous turpentine injection inflicts a local tissue damage, leading to acute phase response, while LPS mimics systemic bacterial infection.

A more extensive and detailed description of the functions of Hp, SAA, LBP and AGP was compiled by Ceciliani (2012).

Ceruloplasmin (Cp) and α_1-antitrypsin (AT) are minor positive APPs in cattle (Ceciliani et al., 2012). The size of bovine Cp is 125 kDA and it shows great similarity in amino acid composition with human Cp (Calabrese et al., 1981). Cp is involved in iron homeostasis (Hellman and Gitlin, 2002), but during inflammation, Cp scavenges the free oxygen radicals (Goldstein et al., 1982). The increase of Cp concentration after turpentine injection is slower than that of Hp, peaking only after 5 to 6 days after injection (Conner et al., 1988). AT is a 58 kDa protein, isolated from the bovine plasma (Sinha et al., 1994). AT is also termed α_1-proteinase inhibitor, its main function being inhibition of the action of proteinases (Beatty et al., 1980; Eckersall and Conner, 1988) The concentrations of AT increase slowly after turpentine injections, peaking around 5 to 6 days after injection, similarly to Cp, and show no response to increased turpentine doses (Conner et al., 1988).

Albumin and transferrin are negative APPs (Gruys et al., 2005). Albumin (Alb) is physiologically present in bovine plasma at concentrations of approximately 22–39 g/l (Chorfi et al., 2004; Alberghina et al., 2011). It is responsible for maintaining the colloid oncotic pressure of blood and binding and transporting various compounds in blood (Quinlan et al., 2005). Increased albumin concentrations occur in dehydrated animals, while decreased albumin concentrations have been associated with malnutrition (Kaneko, 1997). As the Alb is a constituent plasma protein, it is produced physiologically on a daily basis (Barle et al., 1997). During the acute phase response initiated by subcutaneous turpentine injection in anesthetised rats, the synthesis rate of Alb is decreased and the synthesis rate of positive APPs is increased (Milland et al., 1990). The regulation mechanisms of Alb is largely unknown, but decrease in Alb is most likely regulated by changes in IL-6 concentration (Morimoto et al., 1995). Transferrin (Tf) is a glycoprotein (Kent, 1967) that is classified as a negative APP similarly to Alb. The main role of Tf is to transport and sequester iron (Gomme et al., 2005).

2.3 KINETICS AND FEATURES OF THE BOVINE ACUTE PHASE PROTEINS

Hp, SAA, AGP and Fb are considered to be the major APPs of clinical interest in cattle (Petersen et al., 2004; Ceciliani et al., 2012). Strong association has been established between respiratory tract infection and increased Hp concentrations in the blood (Godson et al., 1996; Heegaard et al., 2000; Grell et al., 2005; Idoate et al., 2015), Hp being the most frequently used APP in this connection. Another major APP, SAA, is less used in research, but has been proven to be a valuable marker of inflammation (Alsemgeest et al., 1994; Werling et al., 1996; Heegaard et al., 2000; Orro et al., 2011). The ability of Fb to indicate inflammation in cattle was reported already in the

1970s (Ek, 1972). In humans SAA concentrations increase in hours, while considerable increase of Hp and Fb concentrations is observed after 24 hours (Van Leeuwen and Van Rijswijk, 1994). In cattle relative increases of SAA and Hp resemble the pattern observed in humans (Horadagoda et al., 1994).

After subcutaneous turpentine injection, Fb, AGP and Hp concentrations peak within 2 to 4 days, while AT and Cp peak after 5 and 6 days, respectively (Conner et al., 1988). SAA was not analysed in the study by Conner and colleagues, but in another study the SAA concentration started to increase almost immediately after the experimental infection, while any change in Hp concentrations takes several hours (Horadagoda et al., 1994; Werling et al., 1996). Although Hp is classified as a positive APP, a slight decrease in Hp concentration can occur in the early course of an inflammation or shortly after turpentine injection (Jacobsen et al., 2004; Suojala et al., 2008; Risalde et al., 2011; Lassen et al., 2015). This might be due to the increased binding of Hp to haemoglobin at the beginning of inflammation, before the hepatic Hp synthesis has begun. Although Fb peaks simultaneously with Hp, it appears to be less sensitive in identifying inflammation in cattle when compared with Hp or SAA (Nazifi et al., 2009a). Fb and Hp can also show some stability in concentrations when compared with SAA, remaining elevated when screening for herd health or assessing stress (Hickey et al., 2003; Gånheim et al., 2007) and representing situations where no clear injury or infection is perceivable. SAA is considered as an APP of high sensitivity and its concentration increases swiftly in cattle (Alsemgeest et al., 1995b; Werling et al., 1996; Horadagoda et al., 1999). When several APPs are measured simultaneously, the combined sensitivity of identifying sick animals increases (Alsemgeest et al., 1994; Humblet et al., 2004; Nazifi et al., 2009a). Eventually, the response is either resolved and the APP concentrations decrease or the condition remains unresolved or can become chronic (Jain et al., 2011).

2.3.1 APPS AND DISEASES

A vast amount of research on APPs has concentrated on exploring associations between APPs and various diseases and severity of the disease. In calves, respiratory tract infections are the most frequently diagnosed condition worldwide and present an economic burden to the farmer (Smith, 1998; Loneragan et al., 2001), and are thus the focus of research. Limited numbers of studies exist on APPs in diarrhoeic calves, sepsis and parasitic infections. An overview of the scope of APP research in calves is shown in Table 2.

The increase of APP concentrations is relative to the severity of the inflammation; increased doses of turpentine oil injected subcutaneously elicit a corresponding increase in Hp and AGP concentrations in calves as the amount of tissue damage increases (Conner et al., 1988). Similarly, intravenous administration of increasing doses of LPS results in

corresponding increases in SAA and Hp, although there seems to be a maximum dose of LPS after which the response ceases to increase (Gerros et al., 1993; Jacobsen et al., 2004). The dose response of the APPs varies from mild to suggestive or absent when acute phase response has been explored using an experimental infection of various doses of an infectious agent (Dowling et al., 2002; Gånheim et al., 2004; Ostermann et al., 2013; Lassen et al., 2015). However, in clinical studies APPs can differentiate between mild and severe conditions (Alsemgeest et al., 1994; Horadagoda et al., 1999).

Differences in APP changes exist between the types of infectious agent; bacterial infections typically elicit a larger increase of APP than viral infections (Godson et al., 1996; Gånheim et al., 2003), reflecting corresponding differences in clinical signs for bacterial and viral infections. Elevated concentrations of Hp, SAA and AGP have been recorded in bacterial infections of calves with BRD (Godson et al., 1996; Dowling et al., 2002; Gånheim et al., 2003), but respiratory syncytial virus (RSV) infection also results in an increase in both APPs and clinical signs (Heegaard et al., 2000; Orro et al., 2011). Infection with rotavirus or coronavirus also results in a lesser increase in APPs in diarrhoeic calves, compared with bacterial infection (Pourjafar et al., 2011; Balikci and Al, 2014), although the increases in APPs overall remain lower in diarrhoeic calves than in calves with BRD. While the acute phase response in diarrhoeic calves varies according to the aetiology, the cytokines TNF-α and INF-γ produce similar responses to all the aetiologies (Pourjafar et al., 2011). Combined experimental infection of bovine viral diarrhea virus (BVDV) and *Mannheimia haemolytica* or bovine herpesvirus-1 and BVDV resulted in greater APP increase and more severe clinical signs than infection with a single virus (Gånheim et al., 2003; Risalde et al., 2011), as more extensive tissue damage occurs.

The magnitude of the APP increase, especially of Hp, is correlated with clinical signs (Godson et al., 1996; Deignan et al., 2000; Heegaard et al., 2000), which has been applied to assessment of the severity of the diseases. Skinner et al. (1991) suggested cut off values of >0.2 g/l and >0.4 g/l for Hp for mild and significant infections in adult cattle, respectively. In diarrhoeic calves, mild clinical signs indicate no difference in APP response compared with that in healthy calves, and only severe clinical signs result in increased Hp and SAA concentrations (Hajimohammadi et al., 2013). In addition, an experimental re-infection of calves with respiratory syncytial virus resulted in no clinical disease and, similarly, no acute phase response (Grell et al., 2005), probably because the adaptive immunity restricted the propagation of the virus and resulting tissue damage. Fb is less sensitive to differences in the severity of the diseases, shown at least for mastitis in heifers (Hirvonen et al., 1996).

Correlation of the APPs with clinical signs is also utilised when evaluating the nature of the inflammation or differentiating between acute and chronic inflammation. SAA, Hp and AGP are able to differentiate between acute and chronic inflammation, although the range of concentrations of Hp and AGP

overlap under these conditions (Horadagoda et al., 1999), reducing their sensitivity. SAA has very good sensitivity for severe inflammation, while Hp seems to be better for screening the more chronic conditions in cows (Alsemgeest et al., 1994; Horadagoda et al., 1999). The Hp, SAA and Alb concentrations of the calves suffering from chronic respiratory tract diseases lie between the values for healthy calves and those for calves with acute respiratory disease (Tóthová et al., 2010; Csilla et al., 2013).

The ability of the APPs to predict approaching disease varies according to disease. Increased Hp and decreased Alb concentrations in clinically healthy cows predict uterine disease postpartum, and cows with postpartum uterine disease have decreased Alb already during the pre-partum period (Huzzey et al., 2009; Schneider et al., 2013). In calves, however, Hp measurement fails to differentiate between the animals catching the BRD from the animals that remain healthy (Wittum et al., 1996; Young et al., 1996). Hp also fails to predict initiation of the clinical signs even in experimental infection of BVDV, where infrared thermography shows early changes (Schaefer et al., 2004). Unfortunately the potential for predicting AGP, or the more sensitive APP, SAA, has not been assessed.

APPs in sick animals can predict the outcome of a disease during its early stages, as increased concentrations indicate more severe condition, which may not yet be clinically apparent. In experimental respiratory tract infections Hp concentrations were significantly elevated already after two days post infection in calves that eventually died from the infection (Godson et al., 1996). In freshly diseased calves, higher concentrations of Hp predicted that more antimicrobial treatments were needed for the recovery from BRD (Carter et al., 2002; Berry et al., 2004). A combination of early Hp and Fb measurements provides a more precise prediction for the severity of BRD, when sensitivity and specificity of Hp, Fb or Hp+Fb for recognising BRD is calculated (Humblet et al., 2004). Moreover, in calves with chronic respiratory disease, those with a deteriorating condition had higher initial Hp and SAA concentrations than calves that were eventually cured (Tóthová et al., 2010). The decrease of negative APP Alb contributes similarly to prognosis: in one study, the calves suffering from chronic respiratory disease (and eventually euthanized) had an initial Alb concentration <35 g/l, while over half of the calves that recovered had higher Alb concentrations (Tóthová et al., 2010). A recent study shows that even the course of diarrhoea, either recovery or prolonged diarrhoea, can be predicted by determining the IL-6 concentrations at the time of initial diagnosis (Fischer et al., 2016).

2.3.2 APPS AND STRESS

APPs have also been considered as potential markers of undefined poor well-being, which has led to several experiments aimed at associating stress with APP. In humans where purely psychological stress is more easily stated, the stress shows in increased concentrations of cytokines IL-1 and Il-6, mediated

through hypothalamic-pituitary-adrenal axis (Connor and Leonard, 1998). In calves the association between stress and the APPs has been explored by measuring APP concentrations after occurrence of various stressors, including transport, weaning, diet changes or commingling of the calves.

Transportation increases concentrations of Hp, SAA and Fb in cattle (Arthington et al., 2003; Fazio et al., 2015), similarly to weaning (Kim et al., 2011) when compared with initial values. Weaning stress seems also to amplify the increase of APPs when calves were additionally challenged by transport (Arthington et al., 2005) or LPS injection (Carroll et al., 2009). The acute phase response inflicted by the stressors can be modulated by diet preceding the experiment: heifers fed with concentrate supplemented with possibly immunomodulating polyunsaturated fatty acids, had lower acute phase response after transport than the controls (Araujo et al., 2010). Commingling of the calves with unfamiliar animals results in no change in APPs (Arthington et al., 2003), although commingling can cause the animals stress. Overall, the measurement of APPs could have some potential as indicators of stress. However, the measurements should be interpreted cautiously because even seemingly minor physical injuries can induce acute phase response.

2.3.3 APPS IN NEONATAL ANIMALS

Various biochemical and haematological parameters can show significant changes in concentrations in blood of newborn calves. Typically, the parameters that change either peak immediately after birth and decrease to normal levels within a week, or low concentrations are observed at birth but increase towards normal concentrations during the subsequent days or months (Knowles et al., 2000; Mohri et al., 2007).

The concentrations of Alb, Tf and Cp start increasing towards adult levels after birth, Alb at a slightly steeper rate than Tf and Cp (Knowles et al., 2000; Rocha et al., 2013; Tóthová et al., 2015). Fb and AGP show some fluctuation after birth with no consistent direction of change (Knowles et al., 2000; Rocha et al., 2013; Tóthová et al., 2015), although there is some evidence of peaking and thereafter decreasing AGP concentration after birth (Itoh et al., 1993; Orro et al., 2008). Hp shows some increase or fluctuation after birth that settles within two weeks (Knowles et al., 2000; Tóthová et al., 2015). The concentrations of SAA peak rapidly after birth and remain elevated for the first two or three weeks of the calf's life (Orro et al., 2008; Tóthová et al., 2015).

Increased SAA concentrations in newborn calves have been recorded after difficult delivery (Alsemgeest et al., 1995c), but more likely the increases in APPs in newborn animals are physiological and originate from the release of cytokines during the birth process (Marchini et al., 2000; Chiesa et al., 2001) or even in late gestation, before delivery (Itoh et al., 1993). The physiological

increase of some APPs should be accounted for when changes in APP concentrations are assessed in clinical settings.

2.4 CLINICAL USE OF APPS IN VETERINARY MEDICINE

The semi-quantitative glutaraldehyde test (brand names e.g. Glutal®, Glutavac®), originates from work by Sandholm (1974) and reflects the approximate amount of Fb and gamma globulins in blood. Exact or semi-quantitative determination of the Fb by the heat precipitation method (Millar et al., 1971) or cow side glutaraldehyde tests, respectively, is currently the only APP measurement commonly used in clinical bovine medicine. Although Alb can be easily determined using automated measurements, the determinations are not performed to assess the inflammatory status of the animal.

The use of SAA, together with Fb, is becoming more common in equine medicine, although consecutive measurements are currently recommended and more research is still warranted (Jacobsen and Andersen, 2007; Westerman et al., 2015). The use of C reactive protein is similarly expanding in canine medicine, providing additional information for treating patients in critical care and those with respiratory tract infections (Eckersall and Bell, 2010; Viitanen et al., 2014; Torrente et al., 2015). A recent review by Schmidt and Eckersall (2015) provides a clear framework of major APP concentrations for dogs and cats in common conditions of varying stages of severity. In human medicine, a single C reactive protein measurement is a valid method for detecting bacterial infection, but serial measurements are more useful in clinical practice and for monitoring the efficacy of treatment (Van Leeuwen and Van Rijswijk, 1994).

The different kinetics of various APPs might be a factor that has hindered their clinical use because assessing the severity of inflammation can be difficult if one APP concentration is clearly increased when other is yet not. However, this characteristic has been utilised in development of an acute phase index that combines fast and slow reacting APPs or negative and positive APPs (Toussaint et al., 1995). Toussaint et al. (1995) developed a general model of the acute phase index: (slow positive APP ∗ rapid positive APP)/(slow negative APP ∗ rapid negative APP), and an acute phase index specifically for cattle:

$$INDEX = \frac{(\text{Hp mg/ml}+0.1) * \text{SAA µg/ml}}{\text{Alb mg/ml} * \alpha\text{-2-macroglobulin U/ml}}$$

A more straightforward calculation of the Hp/SAA ratio aids in differentiating among acute, chronic and subacute inflammations (Alsemgeest et al., 1994). In acute infections, thresholds for Hp concentration in mild (>0.2 g/l) and significant (>0.4 g/l) infection for cattle

were presented decades ago (Skinner et al., 1991). More recently, the cut-off values of Hp (≥0.81 mg/ml) and LBP (≥0.33 μg/ml) were defined, particularly meant for detecting respiratory tract infection in feedlot calves (Idoate et al., 2015). Despite all these efforts, the clinical use of APP measurements in cattle has not yet began in earnest.

APPs have potential for screening subclinical diseases in herds, as veterinarians were unable to identify some cattle with sub-clinical inflammation defined by increased SAA concentrations (Karreman et al., 2000). However, well-defined cut-off values are needed before application. APP measurements can also be used to compare the health status of groups of animals: clinical health status of the calf groups correlates with the aggregate of the APPs and WBC count for the groups (Gånheim et al., 2007). Increased SAA concentrations in young, clinically healthy reindeer calves have been associated with decreased average daily gain for the following four months (Orro et al., 2006); whether this was because of subclinical infections or related to function of immature immune system, remained under discussion.

The use of APPs as an additional or substitutive tool in cattle meat inspection has been suggested (Saini et al., 1998; Tourlomoussis et al., 2004). Cow carcasses retained for further post-mortem inspection had higher blood Hp concentrations than cows and steers assumed healthy in antemortem inspection (Saini et al., 1998), as could be expected. When slaughter animals were classified into groups of cows with acute pathology, for cows with non-acute pathology, healthy cows and healthy beef cattle, Hp and SAA measurements decreased in mean values between the groups (except no significant difference in SAA concentrations between cows with non-acute pathology and healthy cows) (Tourlomoussis et al., 2004). Saini et al (1998) claimed that Hp measurement provides little more supporting evidence in animals clearly suffering a disease, but animals could be directed to traditional meat inspection or only visual meat inspection based on their Hp concentrations measured before slaughter (Tourlomoussis et al., 2004). If APP measurements were to be used concurrently with or additional to the traditional organoleptic meat inspection, samples are best taken before transport because it can induce increase in APP concentration.

Table 2 Examples of the studies on acute phase proteins in various diseases and study designs in calves.

Disease	Study design[1]	Pathogen	Results[2]	Reference
Respiratory tract disease	Exp.	*Mannheimia haemolytica*	Hp↑ SAA↑	Horadagoda et al. (1994)
	Exp.	*Mannheimia haemolytica*	LBP↑	Horadagoda et al. (1995)
	Exp.	*Mannheimia haemolytica*	Hp↑ SAA↑ Fb↑	Gånheim et al. (2003)
	Exp.	*Mannheimia haemolytica*	Hp↑ Cp↑ AGP↑ AT↑	Fagliari et al. (2003)
	Exp.	*Pasteurella multocida*	Hp↑ SAA↑ AGP↑	Dowling et al. (2002)
	Exp.	bovine respiratory syncytial virus	Hp↑ SAA↑	Heegaard et al. (2000)
	Exp.	bovine respiratory syncytial virus	Hp↑	Grell et al. (2005)
	Exp.	bovine viral diarrhoea virus	Hp↑ SAA↑ Fb↑	Gånheim et al. (2003)
	Exp.	*Dictyocaulus viviparus*	Hp↑ SAA↑ Fb↑	Gånheim et al. (2004)
	Obs.	multiple bacteria species	Hp↑ SAA↑ LBP↑ AGP -	Orro et al. (2011)
	Obs.	not specified/multiple viruses	Hp↑ SAA - Fb↑ AGP -	Carter et al. (2002)
	Obs.	bovine respiratory syncytial virus	Hp - SAA↑ LBP↑ AGP -	Orro et al. (2011)
	Obs.	not specified	Hp↑ LBP↑ Tf -	Idoate et al. (2015)
Diarrhoea	Exp.	*Salmonella dublin, S. enteriditis, S. heidelberg*	Hp↑	Deignan et al. (2000)
	Exp.	*Salmonella dublin*	Hp↑ Cp↑, AGP ↑ Alb↓ Tf -	da Silva et al. (2011)
	Exp.	*Escherichia coli*	Hp↑ SAA↑	Balikci and Al (2014)
	Exp.	bovine rotavirus, bovine coronavirus	Hp↑ SAA↑	Balikci and Al (2014)
	Exp.	*Ostertagia ostertagi*	Hp↑ SAA↑ Cp - AT - AGP -	Conner et al. (1989)
	Exp.	*Eimeria* spp.	Hp↑ SAA↑	Balikci and Al (2014)
	Exp.	*Eimeria zuernii*	Hp↑	Lassen et al. (2015)
	Obs.	*Escherichia coli*	Hp↑ SAA↑	Pourjafar et al. (2011)
	Obs.	bovine rotavirus, bovine coronavirus	Hp↑ SAA↑	Pourjafar et al. (2011)
	Obs.	*Cryptosporidium parvum*	Hp↑ SAA↑	Pourjafar et al. (2011)
	Obs.	not specified	Hp↑ SAA↑ Fb↑ Cp↑	Hajimohammadi et al. (2013)
	Obs.	not specified	Hp - SAA -	Tóthová et al. (2012)
Umbilical disease	Obs.	not specified	Hp - SAA - Fb -	Tóthová et al. (2012)
Sepsis	Obs.	not specified	Hp - SAA - Fb -	Tóthová et al. (2012)
No clinical signs	Obs.	GI tract[3] nematodes, *Dictyocaulus viviparus*	Hp↑ Alb -	de Cezaro et al. (2016)
Theileriosis	Exp.	*Theileria annulata*	Hp↑ SAA↑ AGP↑	Glass et al. (2003)

Hp = haptoglobin, SAA = serum amyloid A, Fb = fibrinogen, Alb = albumin, Cp = ceruloplasmin, AGP = α1 acid glycoprotein, LBP = lipopolysaccharide binding protein, AT = antitrypsin

[1] Study design: Exp. = experimental study or trial, Obs. = observational study, either cross-sectional or longitudinal

[2] Results: ↑ = increase and ↓ = decrease of an APP compared to basal level, control group or healthy animals. - = no change

[3] GI tract = gastrointestinal tract

3 AIMS OF THE STUDY

The general aim of the study was to explore the concentrations of acute phase proteins in dairy and beef calves using observational field studies. Most interest was directed to association with disease and severity of infection, but also aspects related to calf management, growth and beef production were dealt with. The specific aims were:

1) to determine concentrations of selected major APPs, Hp, SAA and Fb, in healthy calves and define a reference range for those (Study I).

2) to explore associations between diseases in calves and concentrations of APPs in an observational study and to assess the severity of infection using APP measurements (Studies I, II, III)

3) to explore for possible associations between growth and serum amyloid A in calves and assess relation of APP concentrations to antimicrobial treatment (Studies III, IV)

4 MATERIALS AND METHODS

The summary of study designs and the examinations performed on blood and faecal samples in the original studies are given in Table 3. The study designs, laboratory analyses and main statistical analyses are summarized here. The details of material and methods for the studies (I-IV) are available in the original articles included at the end of the thesis.

4.1 SAMPLING AND STUDY DESIGN

4.1.1 REFERENCE VALUES FOR ACUTE PHASE PROTEINS (I)

For reliably determining the reference values for Hp, SAA and Fb in dairy calves under two months of age, blood samples from 120 healthy dairy calves were obtained. The calves were living on farms around the University of Helsinki Production Animal Hospital in southern Finland. Samples were taken, when calves were to be disbudded by the veterinarian. Calves were clinically examined and blood samples were collected into plain serum tubes and EDTA tubes by puncture of the *vena jugularis* prior to administering the sedative intravenously.

4.1.2 CALVES WITH UMBILICAL DISEASE (I)

For examining the calves with umbilical diseases, calves from nearby farms diagnosed with an umbilical condition were submitted to the University of Helsinki Production Animal Hospital for the study. Calves underwent full clinical examination on arrival. When no co-morbidities existed, a blood sample was taken into a plain serum tube and an EDTA tube, and calves underwent surgery. During surgery the findings were recorded and retrospectively divided in three categories based on the severity and extent of the lesions: hernia, mild inflammation and extended inflammation. Mild inflammation was defined as an abscess or inflammation of umbilical stalk, restricted outside the abdominal cavity, while extended inflammation included omphalophlebitis, omphaloarteritis or patent urachus, inside the abdominal cavity.

4.1.3 CALVES WITH EIMERIOSIS (II)

In this study, a convenience sample of 100 calves from 20 farms was examined. Inclusion criteria were that farmers volunteered to participate in the study, and that no metaphylactic *Eimeria* treatment (toltrazuril) or

prophylactic rotavirus vaccinations were used on the farm. On the farms, all the calves under 60 days of age were included in the study. They were clinically examined and a blood sample was taken into a plain serum tube and an EDTA tube, a faecal sample was taken from the rectum and a deep nasotracheal swab was taken.

4.1.4 BEEF CATTLE CALVES AND GROWTH (III)

The study was carried out on six beef cattle farms participating in the beef cattle development project of a Finnish slaughterhouse. On each farm groups of 5–8 calves, born within 2–5 days of each other, were included in the study.

The farms were visited approximately at two week intervals, when calves were 3, 16 and 30 days old. At each visit calves were weighed (or measured with a measuring tape on one farm), clinically examined, and blood samples were taken into plain serum tubes and EDTA tubes, and faecal samples and deep nasopharyngeal swabs were obtained. The individual weaning weights of the calves at approximately 7 months of age were acquired from Faba, a national cattle breeding cooperative organisation. The dams' body condition score was assessed after parturition and on the third visit at approximately one month from parturition.

4.1.5 CALF GROWTH AND HEALTH IN CALF REARING UNIT (IV)

The study was performed in a single calf-rearing unit for about 1000 calves. In the experiment for assessing the effect of group size on diseases and growth, on arrival the 80 calves were allocated to large group (40 calves, one pen) or small groups (10 calves, four pens). The small groups were separated from each other and the large group by a 1.5 meters high solid fence, but the group shared the same air space. Calves in large and small groups had equal areas per calf. The experiment was repeated for six arrival batches so that overall 480 calves were included in the study.

All the calves were clinically examined and were blood sampled (serum tube) on days 0 or 1, 21 and 42 after arrival. Calves were inspected daily by farm workers for respiratory tract infection and medicated if the criteria for medication (respiratory rate >50/min or fever >40.0 °C) was fulfilled. The sick calves were not separated from the group in either experimental group or control group. Calves were weighed at the end of the milk feeding period of approximately 50 days, at about seven months of age and at slaughter (carcass weight) at around 15–18 months of age.

4.1.6 ETHICAL CONSIDERATIONS

In all the studies samples were collected inflicting minimal distress or harm on the calves. In Study I the blood samples were collected as part of the

clinical veterinary practice. In the eimeriosis and APP study (Study II) blood samples, faecal samples and deep nasotracheal swabs were collected as a part of the Finnish Food Safety Authority Evira's research project surveying respiratory tract infections in calves. Calves in Studies III and IV were living at the slaughterhouses' (HKScan and Atria Ltd., respectively) contract producer farms, and the samples were collected as a part of the slaughterhouses' development project or herd health investigations.

4.2 ANALYTICAL METHODS

4.2.1 ACUTE PHASE PROTEINS AND WHITE BLOOD CELL COUNT (I-IV)

Serum tubes were centrifuged in the laboratory on the day sample was taken or on the following day. After centrifugation, serum was frozen in cryotubes and stored at -20 °C until analyses for Hp and SAA. The EDTA tubes were stored refrigerated and analysed for WBC count and Fb on the day sample was taken or on the following day.

Hp concentrations were measured from serum using spectrophotometric measurement developed by Makimura and Suzuki (1982). The method was slightly modified, as reported in Alsemgeest et al. (1994), by using tetramethylbenzidine as the chromogen instead of potentially carcinogenic o-dianisidine. Control samples of low and high Hp concentrations were included in all analyses.

SAA concentrations were determined from serum using a commercial sandwich ELISA kit (Phase SAA essay, Tridelta Ltd., Maynooth, Ireland) according the manufacturer's instructions.

Fb concentration was determined by the heat precipitation method (Millar et al., 1971). The EDTA blood sample was drawn into haematocrit tubes, centrifuged, heated to 56 °C in order produce precipitate Fb and centrifuged again. The Fb concentration was calculated based on the lengths of fibrinogen column and plasma column measured with ocular micrometer.

Albumin measurements were carried out in an automatic chemistry analyser (KONE Pro, Thermo Fisher Scientific, Vantaa, Finland).

The white blood cell count (Study I-III) was performed with an automatic counter (Coulter-Counter Model T850, Coulter Electronics, Luton UK).

4.2.2 INTESTINAL PATHOGENS (II, III)

Faecal samples were collected from the rectum into plastic bags and analysed for the pathogens defined by the Evira calf diarrhoea examination panel (salmonella, enterotoxigenic *Escherichia coli*, rotavirus, coronavirus, *Cryptosporidium* spp., parasite egg counts and *Eimeria* spp. oocyst count) of

the Research and Laboratory Department, Veterinary Bacteriology Research Unit of Finnish Food Safety Authority Evira.

In Study II the faecal samples were also examined in the parasitology section in The Institute of Veterinary Medicine and Animal Sciences of the Estonian University of Life Sciences, where the species of *Eimeria* oocysts were identified and semi-quantified. Detailed description of all the examinations of the intestinal pathogens is included in the original articles (Study II and III).

Table 3 Summary of the populations examined, study designs and methods used in the original studies I–IV.

	I	II	III	IV
Number of calves	120[1)], 63[2)]	100	37	480
Number of farms	-	20	6	1
Study design[3)]	cross.	cross.	long.	long.
Acute phase proteins or WBC analysed	Hp, SAA, Fb, WBC	Hp, SAA, Fb, WBC	Hp, SAA, Fb, Alb, WBC	Hp, SAA, Alb
Clinical examination	x	x	x	x
Umbilical surgery	x[4)]			
Calf diarrhoea package (Evira)		x	x	
Eimeria opg + species		x		
Giardia spp., *Cryptosporidium* spp.		x		
Respiratory tract infection package (Evira)		x	x	
Measuring of the growth			x	x
Records of medication				x

1) The number of calves examined for the reference population

2) The number of calves with umbilical disease, undergoing surgery

3) Study design: cross. = cross-sectional study, long. = longitudinal study

4) For the calves suffering from umbilical disease

4.2.3 RESPIRATORY TRACT PATHOGENS (II, III)

The deep nasotracheal swab samples from Studies II and III were sent cooled overnight to the Research and Laboratory Department, Veterinary Bacteriology Research Unit of the Finnish Food Safety Authority Evira, where they were examined for bovine respiratory syncytial virus and bovine corona virus and cultured for aerobic and anaerobic bacteria and *Mycoplasma* spp.

4.3 STATISTICAL METHODS

Because the data for the examined variables failed to follow normal distributions, or group sizes were small, only non-parametric tests were used in group-wise comparisons. Dependent variables for the regression models were transformed when necessary to achieve normal distribution. All statistical analyses were carried out using Stata/IC 11.2 or Stata/MP 14.1 for Windows (StataCorp, College Station, Texas, USA).

Reference values (Study I) were defined as a mean ±1.96 SDs for Fb (two-sided reference value), and a mean +1.645 SDs for log-transformed Hp and SAA (one-sided reference values). Kruskal Wallis one-way analysis of variances, followed by Tukey post hoc tests, were used to examine differences for Hp, SAA, Fb and WBC between groups of umbilical disease and healthy calves (Study I) and a Wilcoxon Rank Sum test was used to compare APP concentrations between sexes and breed (Study I).

A receiver operating characteristic (ROC) analysis was used for examining the ability of Hp, SAA, Fb and WBC count to differentiate between local and extended umbilical inflammation (Study I).

Linear mixed regression models (Studies II, III, IV) were used to explore associations between dependent and independent variables. In Study II transformed APPs (1/Hp, 1/Fb and ln(SAA)) were dependent variables and in Study III and Study IV dependent variables were average daily gains over different time periods. In Study II and Study III, farm was included as a random effect. In Study IV, the arrival batch and the calf pen were included as random effects. A more sophisticated version of linear regression, a random intercept linear model, was used in Study III. In Study IV a multilevel mixed-effects parametric survival model was also used to explore the factors associated with time before the first and second antimicrobial treatment of the calves.

For examining associations between APPs and eimeriosis severity (Study II), an independent variable for eimeriosis severity categories was created by combining eimeriosis-related signs. The presence of diarrhoea, total oocyst count >500 opg in faecal sample and presence of pathogenic *Eimeria* species (*E. bovis* or *E. zuernii*) were recorded. If no criteria were met, no evidence of eimeriosis was recorded (category 1); if one of the criteria was met, the evidence of eimeriosis was considered weak (category 2); if two criteria were met, the evidence was considered moderate (category 3); and if all three criteria were met, the evidence were considered strong (category 4).

In all models with SAA as a dependent or independent variable, age was included as an independent variable for controlling its effect on SAA concentrations in young calves.

5 RESULTS

5.1 APPS IN HEALTHY ANIMALS (I, II, III)

Reference values were established for healthy dairy calves aged 0–60 days for Fb, Hp and SAA, 2.6–5.9 g/l, <196 mg/l and <178 mg/l, respectively (Study I).

No difference in APP concentration was recorded between sexes in Study I. In Study II, the regression model showed significantly higher Hp concentrations in bull calves than in heifer calves, when Hp was controlled for diseases, signs of eimeriosis and age, but no difference was observed for Fb concentrations. No differences between sexes were recorded in Hp, SAA or Fb concentrations in Study III.

For beef breeds, albumin concentration at three days of age was higher in Charolais calves (28.8 ± 0.37 g/l) than in Hereford calves (25.1 ± 0.37 g/l) (Study III), but no differences in Hp, SAA or Fb concentrations were evident among breeds (Study III). For dairy calves, Ayrshire calves had higher mean concentration of SAA (65.4 mg/l ± 37.8 mg/l) on arrival at the calf rearing unit than Holstein Friesian calves (55.6 mg/l ± 34.4 mg/l) (Study IV).

Young animals had higher SAA concentrations; median SAA concentration was significantly higher (83 mg/l, range 20–315 mg/l) in healthy dairy calves 0–14 days of age compared with older calves of age 15–60 days (32 mg/l, range 2–150 mg/l) (Study I). Increased SAA was associated with younger age in dairy calves of 15–60 days (Study II), and beef calves (Study III) displayed a decrease in SAA concentrations during the first month of life (Fig. 1). No significant associations between age and Hp concentration were established (Study I, II and III). Albumin increased and globulin decreased in concentration during the first month of life in beef calves (Fig. 1). Fb concentration showed some age-related changes in beef calves (Study III; Fig. 1), but was not associated with age in dairy calves (Study I and Study II).

5.2 APPS IN CALF DISEASES AND SEVERITY OF THE DISEASE (I-IV)

5.2.1 APPS IN DISEASES (I, II, III)

Study II showed associations between increased Hp and Fb concentrations and respiratory tract infection and umbilical inflammation (see original article for detailed results). Increased SAA concentrations were associated with a positive sampling result for respiratory tract bacteria culture from a deep nasopharyngeal swab; half of the calves with a positive

sample had SAA concentrations over the reference value of 178 mg/l (Study II). Elevated Hp and Fb concentrations were also associated with umbilical inflammations (Study II). In Study I, Fb and SAA concentrations were increased in calves suffering from local or extended umbilical inflammation compared to healthy calves, whereas severe inflammation only increased the Hp concentration significantly (Fig. 2).

In the first month of life, beef calves (Study III) had signs of clinical disease (respiratory tract infection, diarrhoea or umbilical inflammation) in 14.9% of the examinations. No associations were observed between any of the disease and increased APPs concentrations. The majority of the samples (81 %) had Hp concentrations below the detection limit of 60 mg/l.

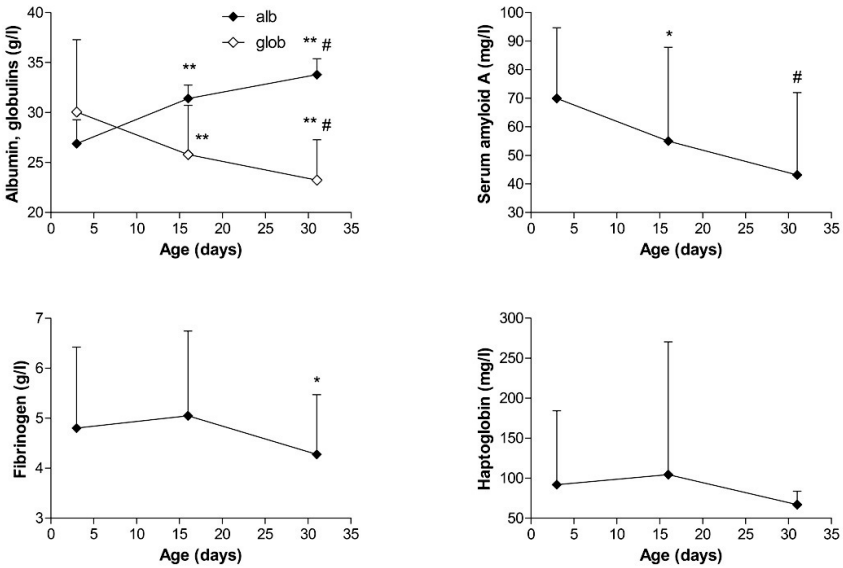

Figure 1 Mean (±SD) serum concentrations of albumin, globulins, serum amyloid A, fibrinogen and haptoglobin in beef calves sampled three times during first month of life (n = 37, n = 35 and n = 35 respectively). (Study III)
* Significant difference from previous sampling time (P < 0.05).
** Significant difference from previous sampling time (P < 0.001).
# Significant difference from first sampling time (P < 0.001).

5.2.2 APPS IN ASSESSING THE SEVERITY OF INFECTION (I)

The mean concentrations of Fb, SAA and Hp were higher in calves with extended umbilical inflammation than in calves with local inflammation (Fig. 2). The ROC analysis showed that Fb was the most efficient APP for differentiating between local and extended inflammation according to an area under the curve (AUC) value of 0.91.

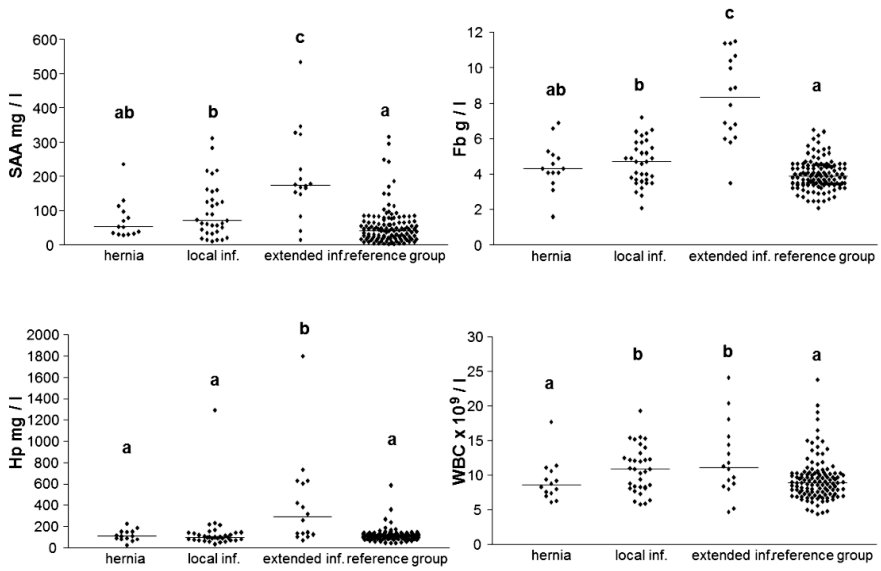

Figure 2 Concentrations of acute phase proteins serum amyloid A (SAA), fibrinogen (Fb), and haptoglobin (Hp), and whole blood cell count (WBC) in healthy calves (reference group) and in calves with various umbilical diseases, at 0–60 days of age. (Study I)

SAA and Hp performed reasonably well, with AUCs of 0.78 and 0.81, respectively, while the ability of WBCs to differentiate the umbilical conditions was poor, with an AUC of 0.55. Optimal cut-off values for Fb, Hp and SAA were 5.9 g/l, 235 mg/l and 133 mg/l, respectively (Table 4).

Table 4 Cut off points for Fb, Hp and SAA for differentiating local and extended umbilical inflammation (Study I)

	AUC	Cut-off point: optimal from ROC curve	Se	Sp	Cut-off point: reference value	Se	Sp
Fb	0.91	5.9 g/l	0.88	0.85	5.9 g/l	0.88	0.85
Hp	0.81	235 mg/l	0.56	0.97	196 mg/l	0.56	0.88
SAA	0.78	133 mg/l	0.81	0.76	178 mg/l	0.38	0.85

5.2.3 APPS IN SUBCLINICAL INFECTION (II)

Mild or subclinical eimeriosis was associated with decreased Hp concentrations and increased Fb concentrations. Concentrations of Hp in different eimeriosis categories are also shown in Fig. 3. SAA concentration was not associated with any eimeriosis category.

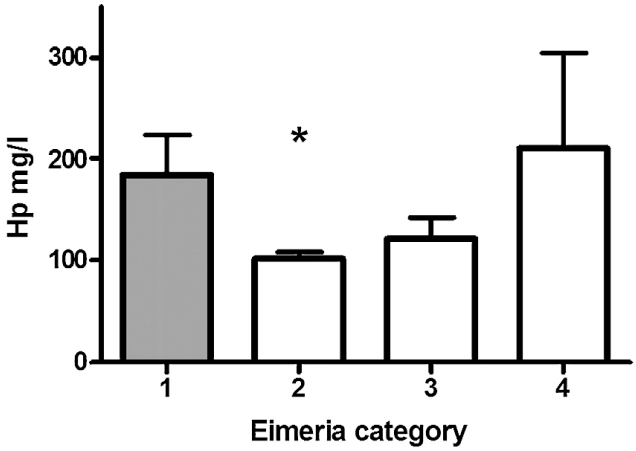

Figure 3 The mean haptoglobin (Hp) concentrations (+standard error of mean) for Finnish dairy calves aged 15–60 days with different *Eimeria* infection status. *Eimeria* category: 1 = no *Eimeria* or diarrhoea, 2 = weak signs of eimeriosis, 3 = moderate signs of eimeriosis, 4 = strong signs of eimeriosis. * Statistical difference as compared to category 1; P = 0.04.

5.2.4 APPS AND ANTIMICROBIAL TREATMENT NEEDS (IV)

An increased albumin concentration lengthened the time before the first antimicrobial treatment of a calf in the rearing unit, as shown in Tables 5A and B (Study IV).

5.3 APPS AS PREDICTORS OF GROWTH (III, IV)

The APPs and globulin concentration had several associations with the average daily gain of the calves (Studies III and IV). Higher SAA concentration in young calves of 16 days of age was associated with poorer growth rate over 30–200 days of age (Study III) or for the milk feed period of the following 50 days, to seven months of age and even until slaughter (Study IV) (Fig. 4). The result of the full models was reported in the original Studies III and IV. The SAA concentrations at these time points were mostly under 87 mg/l (mean + SD corresponding to 84.1 % of the samples in Study III, Fig.

1) or under the reference value of 178 mg/l (91.1 % of the samples, Study IV), indicating that no clinical inflammation was present. In contrast to SAA, increased Hp concentrations at the first month of life were associated with better growth rates until slaughter (Study IV). Higher globulin concentration at 3 days of age or 13 to 30 days of age indicated better growth rates for the next month or the next 50 days (Studies III and IV, respectively).

Table 5A, B Risk factors for dairy and mixed calves for the time before first antimicrobial treatment and relapse.

A. Risk factors for days to first ab treatment						
	n	Hazard ratio	[95% Conf.	interval]	*P* value	Wald test
IgG[1] (day 0) g/l	476	0.957	0.931	0.983	0.002	
Albumin (day 0) g/l	476	0.907	0.869	0.947	<0.001	
Age on arrival (days)	476	1.008	0.997	1.019	0.152	
Breed Ayrshire	178	1				0.067
Holstein Friesian	218	0.954	0.771	1.180	0.662	
Mixed	80	0.724	0.547	0.959	0.024	
Group size Small	238	1				
Large	238	0.979	0.740	1.297	0.152	
B. Risk factors for days to first relapse						
	n	Hazard ratio	[95% Conf.	interval]	*P* value	Wald test
IgG[1] (day 0) g/l	473	0.932	0.903	0.962	<0.001	
Albumin (day 0) g/l	473	0.938	0.894	0.983	0.008	
Age on arrival (days)	473	1.002	0.988	1.015	0.820	
Breed Ayrshire (n=177)	177	1				0.046
Holstein Friesian (n=218)	216	0.858	0.677	1.088	0.206	
Mixed (n=79)	20	0.659	0.473	0.919	0.014	
Group size Small	238	1				
Large	238	0.984	0.792	1.222	0.883	

[1] Immunoglobulin (IgG)

Albumin concentration at 16 days of age was positively associated with the growth rate during the first month of life (Study III). However, increased albumin concentration in the first month of life was negatively associated with growth rate until slaughter (Study IV).

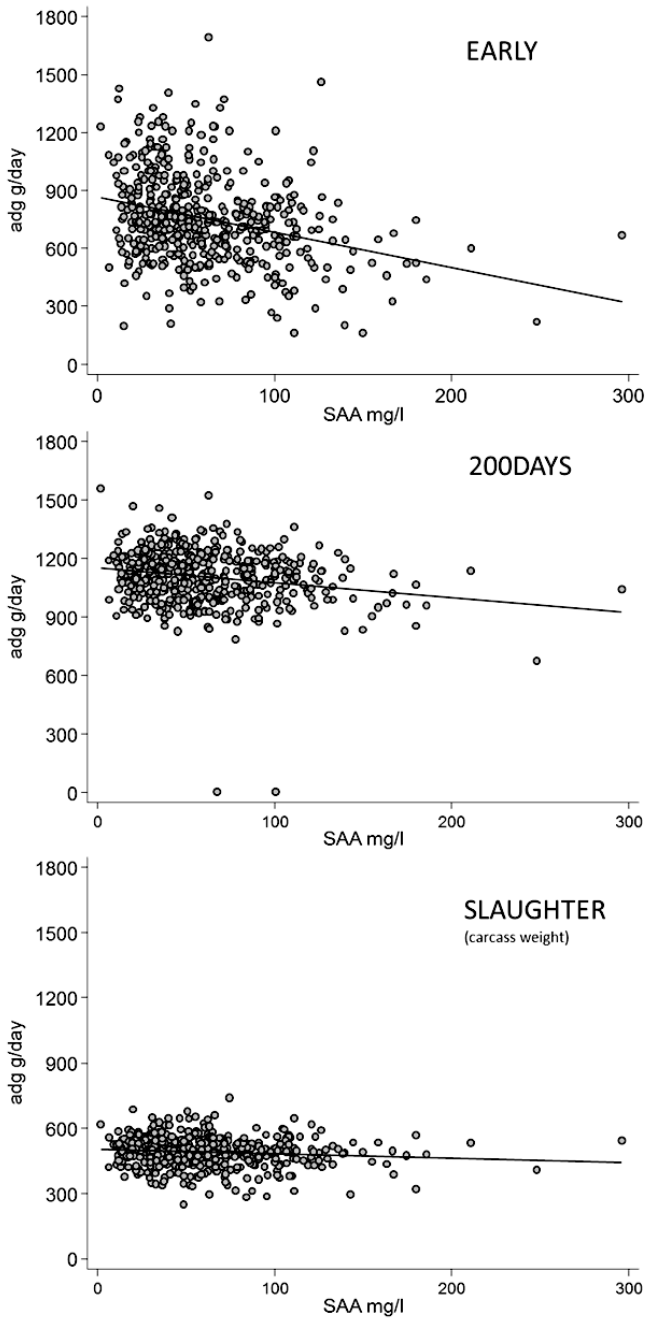

Figure 4 Serum amyloid A (SAA) concentration and average daily weight gains (g/day) at three different time period:
a) EARLY = average daily gain for 50 days following calf arrival
b) 200DAYS = average daily gain at approximately 200 days of age
c) SLAUGHTER = average daily gain at slaughter, calculated from the carcass weight

6 DISCUSSION

6.1 APPS IN HEALTHY ANIMALS (I, II, III)

Reference values for the calves were established in Study I. SAA and Hp concentrations of <200 mg/l were reported in healthy calves (Orro et al., 2008; Tóthová et al., 2012), similar to the reference values established in Study I. Gånheim et al. (2003) suggested basal levels, defined by the mean plus two standard deviations, of 24 healthy calves, resulting in lower values for Hp (130 mg/l) and especially SAA (25.6 mg/l), but a higher value for Fb (6.45 g/l) than in our study. Reference values are needed in order to define the normal and the deviation from normal. Currently the reference values for major APPs of common production and companion animals have been reported for growing pigs, sows, boars and mini pigs (Piñeiro et al., 2009; Diack et al., 2011; Christoffersen et al., 2015), goats of various ages (Heller and Johns, 2015), horses (Nunokawa et al., 1993; Cray and Belgrave, 2014), puppies and adult dogs (Yuki et al., 2010) and cats (Kann et al., 2012). The reference range of APPs for adult cattle does not currently exist; an anecdotal Hp concentration of <20 mg/l is mentioned by Eckersall and Bell (2010).

For the reference values, blood samples were collected from animals zero to two months of age. During the sample collection, we had not yet established the nature of the association between age and SAA concentrations. Although results of increased SAA concentrations in healthy, young calves (Orro et al., 2008) and reindeer (Orro et al., 2006) were published before the analysis stage of the Study I, animals under two weeks of age were still included in formation of the reference ranges. The reference value for SAA, 178 mg/l, might be considered high because the concentrations of SAA decrease fast during the first two to four weeks of life (Studies I, II and III). Re-calculation of reference value for 97 healthy calves over 14 days of age in Study I produces SAA concentration <125 mg/l (a mean of for log-transformed SAA+1.645 SDs), clearly smaller value than what was reported in Study I. For a proper reference range of older calves, at least 120 calves should be included.

High SAA concentrations in young animals were nonetheless confirmed in Studies I, II and III. Because the calves with increased SAA concentrations were clinically healthy, at least in the population sampled for reference values, the increase probably does not originate from an infection. Due to the cotyledonary synepitheliochorial structure of the bovine placenta (Peter, 2013), few immunoglobulins or any proteins are transferred to the foetal calf. Post-natal administration of colostrum provides energy and immunoglobulins for the newborn (Quigley III and Drewry, 1998). Colostrum contains also inflammatory cytokines IL-1β, IL-6 and TNF-α (Hagiwara et al., 2000), but whether these can initiate hepatic APP

production in neonatal calves is unclear. A similar phenomenon of increased APP in young calves was recorded with AGP, the concentrations of which decrease from a high 1400 mg/l in neonatal calves to normal levels of 180 mg/l in the three weeks after birth (Itoh et al., 1993). SAA and AGP belong to Type I APPs, induced by IL-1 whereas (human) Hp and Fb belong to Type II APPs, induced by IL-6 like cytokines (Moshage, 1997). This might explain why increased concentrations of SAA and AGP in neonatal calves are observed but not increases in concentrations of Hp and Fb.

Establishing differences in APP concentrations between the sexes was not a specific aim of the studies, but as both sexes were in the studies, some comparisons were done. A difference between the sexes was noticed only in Study II, where bull calves had lower Hp concentrations than heifer calves. As the diseases where controlled in the model, the difference reflects a true difference between the sexes. In Studies I and III no differences between the sexes were observed, and in Study IV this was not examined.

Similarly to the sexes, the effect of breeds was not explored in detail in the studies, but in the study involving beef calves (Study III), the concentrations of SAA, and particularly Hp, were very low. Differences in APP concentrations between beef breeds were reported after LPS administration (Carroll et al., 2011) and after weaning or transportation (Qiu et al., 2007), but the associations between breed and APPs were not detected in the dairy breed studies.

In addition to age, sex and breed, APP concentrations have a certain amount of variability between and within individuals. Intravenous administration of LPS induced changes of Hp, SAA and Alb, but individual variability accounted for approximately 25% of the change (Jacobsen et al., 2004). Between-animal and within-animal variability was also observed in APP concentrations of healthy dogs (Kjelgaard-Hansen et al., 2003). Reference ranges account for this variability in healthy animals to some extent, when established properly and based on sufficient number of individuals. Still, when assessing the relevance of change in the APP concentrations, the best basal value would be a collection of samples from the animal in question, achieved when it was healthy. This concept of critical difference has been introduced and explored for example for acute phase proteins in dogs and humans (Clark and Fraser, 1993; Kjelgaard-Hansen et al., 2003) and for enzymes and other blood analytes in cattle (Jensen et al., 1992). Currently, using critical difference is not feasible in production animal medicine, as numerous samples would be needed obtained in advance.

6.2 APPS IN DISEASES AND THE SEVERITY OF DISEASES (I-IV)

6.2.1 APPS IN DISEASES (I, II, III)

Respiratory tract infection was associated with increased Hp and Fb concentrations, and was also evident for deep nasal swab results and increased SAA concentration in Study II. Numerous experimental studies (Horadagoda et al., 1994; Godson et al., 1996; Dowling et al., 2002; Gånheim et al., 2003; Grell et al., 2005; Risalde et al., 2011) detected increased APPs in calves with respiratory tract disease. The elevations in APPs in those experimental studies were evident, and they leave no ambiguity regarding differences between healthy and diseased animals. In observational studies, the difference between healthy and diseased calves is not as clear. The aetiology of the disease varies and the exact state of the disease is difficult to define by clinical examination in a cross-sectional study, resulting in large variation in APPs in diseased animals. The number of studies with an observational study design on respiratory tract disease and APPs in calves is limited. Gånheim et al. (2007), Orro et al. (2011) and Idoate et al. (2015) presented information on natural changes in APPs in apparently healthy and sick calves related to the course of disease and provided a cut-off for differentiating sick calves from healthy. However, a meta-analysis of Abdallah et al. (2016) concluded that studies of the APPs in natural occurring respiratory tract diseases did not provide sufficient evidence to draw any conclusions about the usefulness of APPs in setting the diagnosis of BRD.

Unlike Hp and Fb, SAA was not associated with any of the diseases in Study II, although in experimental studies SAA has been described as the most sensitive and most rapidly increasing bovine APP (Werling et al., 1996; Plessers et al., 2015). In the studies concerning natural infections, the stage of disease at sampling time is undefined, which leads to large variation in SAA concentrations in sick animals and also the rapid increase of SAA concentration cannot be fully employed. Attempting to exert some control on the variation in SAA in young animals, age was always included in the regression model when SAA was included either as an outcome or as an independent variable.

Our studies showed an increase for Hp, Fb (Study I and II) and SAA (Study I) in umbilical diseases in calves, especially in severe umbilical inflammation. Previously, only one study reported increases of Hp, SAA and Fb in calves with umbilical disease, although they were not statistically significant (Tóthová et al., 2012). An approximate diagnosis of umbilical disease is fairly easily achieved by palpating the umbilicus (Virtala et al., 1996) and is maybe of reduced economic interest, lessening the relevance in APP research. However, our study (Study I) recognised and separated the milder cases from the more severe umbilical inflammations in the abdominal cavity accurately through use of Fb, Hp and SAA measurements. In a study of

surgically managed calves, most of the calves diagnosed with extended inflammation were presented with leucocytosis but less than half with fever (Marchionatti et al., 2016). Measuring the APPs of calves with initial umbilical diagnosis could help more precisely differentiate those animals that benefit from umbilical surgery from those ones where abscess draining and local treatment is sufficient.

Diarrhoea can be regarded as a sign of disease rather than a definite diagnosis, and common mechanisms of diarrhoea (hypersecretory or malabsorption) do not necessarily involve the initiation of the inflammatory pathway. Diarrhoea is thus seldom included in APP studies. In our studies (Study II and III) no association between measured APPs and diarrhoea was established. In experimental infection with bacterial, viral or parasitic intestinal pathogens an increase in SAA has been recorded, while changes in the Hp and Fb concentrations have been more inconsistent (Deignan et al., 2000; Risalde et al., 2011; Balikci and Al, 2014; Molina et al., 2014). However, a strong association with Hp and haemorrhagic diarrhoea was evident in heavy experimental *E. zuernii* infection with the dose of 250 000 oocysts (Lassen et al., 2015). In natural infections, where the infective agent can remain unidentified, some changes in SAA, Fb or Hp concentrations have been reported (Pourjafar et al., 2011; Tóthová et al., 2012; Hajimohammadi et al., 2013). In our studies, the intensity of diarrhoea might have been too low to elicit an acute phase response, or the small number of diarrhoea cases meant limited statistical power to detect significant differences.

In beef calves (Study III) no association between APPs and disease was apparent, although diagnoses including umbilical disease, respiratory tract disease and diarrhoea were made. The concentrations of Hp were commonly below the detection limit in beef calves, so that variation between healthy and diseased animals was difficult to gauge.

Based on our studies and previous studies, the concentrations of several APPs (Hp, SAA, Fb, AGP, LBP) increase evidently in BRD and Hp, SAA and Fb also quite likely in severe umbilical conditions and experimentally induced diarrhoea. However, as previously mentioned, the grounds of using APPs is not solid even in setting diagnosis for BRD (Abdallah et al., 2016). As a mere indicators of clinical disease, increased APP concentrations add very little certainty compared to thorough clinical examination.

6.2.2 APPS IN ASSESSING SEVERITY OF INFECTION (II)

The three APPs studied, Hp, SAA and Fb, performed well in differentiating local and extended, more severe umbilical inflammation in calves (Study I). Although Hp and SAA showed some capacity for separating extended and less severe umbilical inflammation from each other, Fb performed best (Study I). In the ROC analysis, a curve describes the sensitivity plotted against 1 - specificity at each measured concentration. Mathematical comparison of the different variables is made by comparing the AUCs.

Increasing the cut-off value improves specificity and correspondingly decreases the sensitivity; the optimal combination of sensitivity and specificity is calculated from the curve.

In our study (Study I), optimal cut-off value for Fb in differentiating the two stages of umbilical disease was similar to the upper limit of the reference value. Interestingly, the mean Fb concentration of the calves with local umbilical inflammation differs from the mean of the reference population, and naturally, from the Fb concentration in those calves with extended inflammation. For Hp and SAA, the optimal cut-off values were higher and lower than the reference values, respectively: increasing the cut-off value of Hp increased the specificity, while decreasing the cut-off value of SAA improved sensitivity, as the numbers of false positive and false negative decrease accordingly.

The measurements of APPs combined with the ROC analysis were used to detect diseased animal (Svensson et al., 2007; Nazifi et al., 2009a; Nazifi et al., 2009b; Gutiérrez et al., 2015). Fewer studies exist on exploiting the association of changing concentration and stages of clinical disease. For colic horses, a model including clinical parameters and SAA concentration correctly differentiated horses managing with conservative care from horses needing surgery (Pihl et al., 2016). IL-6, the cytokine preceding APP production, was able to differentiate groups of calves with covering or non-recovering diarrhoea (Fischer et al., 2016). Based on those studies and our results, measuring of the APPs could assist in determining disease severity.

In Study II lower Hp and higher Fb concentrations were recorded for calves with weak signs of eimeriosis, compared with calves exhibiting no signs of eimeriosis. Calves with stronger signs of eimeriosis were not different regarding Hp or Fb concentrations when compared to healthy calves. The category “weak signs of eimeriosis” can signify subclinical disease or early infection. Parasite invasion activates acute phase response signalling pathways (Li and Gasbarre, 2009) and the acute phase response at cellular level was reported for heavy experimental *E. bovis* infection (Taubert et al., 2009), explaining the observed increase in Fb concentration in our study.

Classically, Hp concentration increases during the acute phase response. However, in an experimental study a minor increase and subsequent decrease of Hp concentration were observed during the prepatent period of mild *E. zuernii* infection, before increase in patent period again (Lassen et al., 2015). The decline in Hp concentrations at the beginning of experimentally-induced *E. coli* mastitis was reported also from longitudinal study of Suojala et al. (2008). Such a decrease in Hp at the beginning of infections can reflect the role of Hp in haemoglobin binding (Eaton et al., 1982), when small lesions on the intestine wall result in small haemorrhages and release of haemoglobin and its binding by Hp before the acute phase response is initiated. The low Hp concentration, with slight signs of eimeriosis, can also signify a mild or later phase of infection, rather than

early infection. The category “weak signs of eimeriosis” includes also some calves with diarrhoea of unknown aetiology; although *Eimeria* spp. are common cause of calf diarrhoea, also non-infectious cases of diarrhoea are frequent. In any case, a low Hp value alone cannot be used to define early or subclinical eimeriosis (or any other disease) in an individual animal because low concentrations fall within the normal range of values. However, it is an interesting phenomenon that calls attention to an intriguing characteristic of APPs.

6.2.3 APPS AND ANTIMICROBIAL TREATMENT NEEDS (IV)

Of the APPs measured, only Alb was associated with the use of antimicrobials in rearing calves; the higher concentration of Alb lengthened the time before the first antimicrobial treatment and similarly the time before the first relapse. Alb is a negative APP, the concentrations of which decrease during the acute phase response (Petersen et al., 2004). This, however, cannot lead to the conclusion that a higher Alb concentration indicates less inflammation or a more healthy calf. The concentration of albumin increases over time after birth for calves (Knowles et al., 2000; Tóthová et al., 2016), and older calves can be more resistant to infections. However, the effect of age was accounted for in the model, thus there must be an alternative explanation.

6.3 APPS AS PREDICTORS OF GROWTH (III, IV)

Our studies showed association between increased SAA in young calves and decreased growth rate in beef calves from birth to weaning at 7 months of age (Study III) and with dairy or mixed dairy beef calves during the milk feed period, at 7 months of age and at slaughter at 15–18 months of age (Study IV). Similar observations were made also for reindeer calves (Orro et al., 2006) and lambs (Peetsalu et al., 2013), where increased SAA concentrations at 1–2 weeks of age were associated with decreased growth rates at 3–4 months of age, denoting the end of the study.

APPs have been used to predict the severity of infection (Godson et al., 1996; Deignan et al., 2000; Heegaard et al., 2000; Hooijberg et al., 2014) or the outcome of an infection (Godson et al., 1996; Carter et al., 2002; Berry et al., 2004; Humblet et al., 2004; Tóthová et al., 2010). Prediction during the acute stages is undoubtedly connected with the acute phase response and the function of the immune system itself. The SAA concentrations in animals with poorer growth performance (Study III and IV) are not as elevated as for cases of infection; they are only increased in relation to other animals in the groups. The APP concentrations of such animals are not suggestive of disease, which reduces growth rate, at least temporarily (Virtala et al., 1996; Windeyer et al., 2014). A simple, but maybe inadequate, explanation would be that these animals show only the acute phase response for subclinical or

chronic disease, described by Heidarpour et al. (2012) and Horadagoda et al. (1999), which could reduce their growth rate. The acute phase response reduces growth rate via reduced feed intake, increased protein catabolism and decreased production of insulin-like growth factor-1 (Gabay and Kushner, 1999), but it is unclear if the acute phase response was initiated in the slower growing animals in our studies. Characteristic increase of SAA in young calves (Orro et al., 2008) adds to complexity of this phenomenon.

The innate immune system is a complex, intertwined and dynamic net of reactions, feedback, inhibition and amplification (Gardy et al., 2009). The synthesis of APPs is only a minor component of the acute phase reaction, as presented in the literature review. During the acute phase response, the inhibition of insulin-like growth factor-1 (IGF-1) by Il-1 and IL-6 (Borghetti et al., 2009), links the acute phase response directly to growth; the increase of IGF-1 is associated with higher growth rates in beef calves (Suda et al., 2003). However, these changes initiated by cytokines are transient and the growth rate should return to normal when the acute phase response ceases.

It is possible that the poorer growth rate is not a reaction or consequence of immune modulation after an external stimulus, but some inherent characteristic, revealed by the relatively increased SAA concentration in early age. A study of two commercial lines of pigs revealed a negative association between the proportion of antigen-specific (CD8+) and non-antigen-specific (CD2+/CD16+) lymphocytes in lymphocyte subsets at 6–7 weeks of age and average daily gain from birth to slaughter (Galina-Pantoja et al., 2006). The proportion of the lymphocyte types can be considered to be an inherent trait, and Galina-Pantoja et al. (2006) even suggested that these immune biomarkers could be used in early identification of fast-growing pigs. Similar identification of possible biomarkers for long-term growth in calves would improve efficiency of beef production, if the genetic background for this inherent characteristic was identified and could be included in breeding programs.

6.4 LIMITATIONS AND STRENGTHS OF THE STUDIES

All the studies included in this thesis were observational field studies. The hypotheses for observational studies differ from those for randomised controlled trials; observational studies are more hypothesis generating than hypothesis proving. Observational studies can also be more susceptible to bias. The value of observational studies is in generating large datasets from the environment where applications will eventually be used, thus providing an opportunity to assess feasibility. In addition, a controlled study is sometimes impossible to conduct.

Both observational and experimental studies should be of optimal sample size with as few animals as possible, but still produce relevant results. In addition, the procedures performed on the animals should be justifiable and

necessary. Samples from 120 healthy individuals are considered to represent a minimum when defining the reference range (Horn and Pesce, 2003): this was the guideline in Study I. In Study II the sample size was estimated on the basis of estimated differences in prevalence of *Eimeria* spp. on different farms, resulting in 20 farms and 100–200 calves being the smallest number needed to detect a statistically significant difference. In Study III the number of farms and calves (six farms and 3–8) was based on practical reasoning, with some estimation of the differences in weight gains on farms. In Study IV one compartment of 80 animals was chosen as an experimental unit for practical reasons, and the study was repeated six times to increase the accuracy of the results. In Study III, the increased number of animals and farms might have produced more statistically significant differences between the farms but in Study IV fewer animals might have been sufficient to produce the same result that was achieved using the reported number of animals. However, the number of repetitions can be considered sufficient. In all the studies included in this thesis minimal stress and restraint were used for calves when collecting samples and performing clinical examinations. The sample-taking methods were as little invasive as possible (blood sampled using venapuncture, faecal samples manually extracted from the rectum, and deep nasal swab sample taken instead of more invasive procedures).

In relation to APP research, problems arise when reliable treatment history of animals included is difficult to document. In our studies, calves were classified healthy or sick based on the results of the clinical examination. Defining healthy is difficult overall; health as absence of disease is quite unsatisfying. Even if the medical records with strictly defined diagnoses were available, subclinical diseases remain unnoticed but can affect APP concentrations. Causal inference is not justified based on results of observational studies but based on biology and knowledge of the innate immune reaction, some deduction regarding the order of the events is possible.

Exploring the effect of natural infections, although setting them in a time frame is difficult, provides valid information on the kinetics of APPs. In experimental studies the infectious agent is often “overdosed”, meaning dosed in amounts rarely observed in natural infections, providing strong evidence for the effect but creating doubt as to it truly reflecting the course of natural infection that could result in much smaller changes.

The long follow-up time in Studies III and IV support the association of increased SAA with poorer growth. However, with the limited number of variables assessed, it is not possible to confirm the importance of SAA in this measurement. Some additional factor, currently unmeasured or unknown, might strengthen the association.

6.5 FUTURE STUDIES

In order to establish the use of APPs in veterinary medicine, research on both APPs and more rapid analysis methods for APPs are needed. Automated methods are already used in equine and canine medicine, facilitating everyday use of APPs. For cattle, automated measurements are still being developed. Cattle differ from horses and dogs in that their diagnosis and treatment must be economically attractive to farmers that have narrow profit margins.

As herd sizes continue to grow, any automated measurements for monitoring health and very small changes in it can save money and effort on farms. In dairy cattle one easily obtainable body fluid is milk, which can be collected noninvasively on a daily basis. The determination of APPs from many other body fluids is possible; APPs in saliva have been detected in swine with increasing interest (Gómez-Laguna et al., 2010, Gutiérrez et al., 2013, Soler et al., 2013). For the present, the reference ranges for APPs in other fluids than serum are lacking. Continuous monitoring of calves might be possible by collecting saliva samples, but this also requires some basic research to be done.

The exploration of the mechanisms behind the relationship between SAA and growth prediction would be at least of academic interest, but also may provide basic knowledge on functions of SAA. However, using SAA measurements as a means to reduce the burden of beef production by increasing the production efficiency by rearing the optimally growing animals in the herd, might be welcomed by farmers, environmentalists and economists.

The possibility of using APP measurement during beef inspection was discussed in the literature review. Carcasses identified not healthy by APP measurement before slaughter could be rejected, improving the efficiency and precision of beef process. The sensitivity of APPs would be an asset in this application because very few false negative carcasses (infected carcasses not detected) would pass into the food chain.

7 CONCLUSIONS

Based on the studies included in this thesis, and the existing literature, the following conclusions can be reached:

1) Sensible reference ranges were established for haptoglobin (<196 mg/l) and fibrinogen (2.6–5.9 g/l) in calves from 0 to 63 days of age. For serum amyloid A the established reference range (<178 mg/l) might be slightly overestimated because healthy young calves have increased SAA concentrations for the first two weeks of life.

2) Increased haptoglobin, serum amyloid A and fibrinogen concentrations are associated with two major calf diseases, respiratory tract infections and umbilical inflammations. An association between the acute phase proteins and diarrhoea was not evident in these studies. All the measured APPs – haptoglobin, serum amyloid A and fibrinogen – differentiated local umbilical inflammation from more severe, extended umbilical inflammation.

3) The increase of serum amyloid A in calves aged about two weeks was associated with poorer growth rate for time periods of 50 days to 18 months. No positive APP was associated with the risk of antimicrobial treatment of the calves, but an increase in albumin concentration (a negative APP) lengthened the time before the first antimicrobial treatment for the calf.

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