Canine C-reactive protein

a study on the applicability of canine serum C-reactive protein

Kjelgaard-Hansen, M.

Publication date:
2004

Document version
Publisher's PDF, also known as Version of record

KVL
THE ROYAL VETERINARY AND AGRICULTURAL UNIVERSITY

CANINE C-REACTIVE PROTEIN

- a study on the applicability of canine serum C-reactive protein

Mads Kjelgaard-Hansen, DVM
Ph.D. Thesis

Veterinary Clinical Pathology
Department of Small Animal Clinical Sciences
The Royal Veterinary and Agricultural University
Frederiksberg, Denmark
2004

Canine C-reactive protein
- a study on the applicability of canine serum C-reactive protein

ISBN xx-xxxx-xxx-x
Printed by Samfundslitteratur Grafik, Frederiksberg, Denmark

Preface

Curiosity can be a very chaotic state of mind, but systemized and controlled to a certain degree and in a specific direction it is suddenly named a scientific skill (or at least a sign thereof). With three years of training within the field of simultaneously nursing and controlling curiosity and handling various chaotic states of the mind, I guess the process of a PhD is all about establishing and performing this exercise in a milieu of positive synergy.

I want to acknowledge the people who helped, nursed and guided me through this process.

Running high on the list of gratitude is my wife, Marianne, and my children Sara and Tobias who, without doubt, have experienced the widest range of chaotic states of my mind, throughout this process. Sometimes it takes a child for true objective reflection and in most cases it takes a caring spouse to make strenuous experiences worthwhile.

A sincere thank to my supervisors, Asger Lundorff Jensen and Annemarie T. Kristensen, who I suspect to have well-trained curiosities themselves and who I know to have cunning abilities to train that of others.

I have appreciated the skilful help from my colleagues at the Central Laboratory, Emma Thomsen and Natascha Errebo and the comprehensive introduction to the world of biochemical methodologies by Hilmer Sørensen, Jens Christian Sørensen and Kirsten Mortensen at the Department of Natural Sciences.

Finally, a warm thank to the co-authors on the various projects for exercising positive synergy (Inge Tarnow, Lisbeth Rem Jessen, Geoffrey A. Houser, Henrik Duelund Pedersen, Jakob Willesen, Jørgen Koch, Lars Friis Mikkelsen and Michael Luntang-Jensen) and to all the staff at the Department of Small Animal Clinical Sciences for a seemingly everlasting positive attitude to the various cravings of PhD-students.

Mads Kjelgaard-Hansen

Index

CHAPTER 3

CHAPTER 4

CHAPTER 5

SUMMARY

C-reactive protein (CRP) is a major acute phase protein in dogs characterized by low physiological levels, a marked and fast increase shortly after a systemic inflammatory stimulus, followed by quick normalization when no further stimulus is present. C-reactive protein is thought to possess high diagnostic specificity, as it is only induced by proinflammatory hypercytokinaemia. These properties should facilitate the use of canine CRP as a marker of systemic inflammatory activity for routine diagnostic, monitoring and screening purposes. The published information on canine CRP strongly indicate that CRP can be applied for routine purposes, but major impediments hindering a wide-spread application in canine medicine seemed to exist.

The objective of the thesis is to test the hypothesis that "Canine serum CRP measurements are applicable for routine purposes in canine medicine". This overall hypothesis was tested stepwise by addressing the following four phases:

PHASE I Analytical investigations	PHASE III Clinical investigations
PHASE II Overlap investigations	PHASE IV Outcome and utility investigations

The thesis consists of five chapters. In Chapter 1, the current knowledge of the properties of CRP as a molecule, an acute phase protein and as a possible useful parameter in canine medicine is reviewed. In Chapter 2, two assays for the determination of canine serum CRP are objectively evaluated, regarding analytical performance (Phase I). Chapter 3 assesses the apparent clinical performance of canine serum CRP measurements by means of overlap and clinical investigations (Phases II and III). In Chapter 4 an example of the possible positive effect of canine serum CRP measurements on the case management of an immune-mediated disease in dogs is reported (Phase IV). Finally, the results of the work performed in the four phases are discussed, concluded upon and perspectivated in Chapter 5.

The final conclusion of the thesis based on available published material and the work of the thesis (Chapters 2, 3 and 4) is that serum CRP measurements are applicable for routine purposes in canine medicine.

SAMMENDRAG (DANISH SUMMARY)

C-reaktivt protein (CRP) er et ”major” akut-fase protein hos hunde, kendetegnet ved et lavt normal-fysiologisk niveau, en markant stigning kort efter en systemisk inflammatorisk stimulus, efterfulgt af en hurtig normalisering når stimulationen er ophørt. C-reaktivt protein menes at have en høj diagnostisk specificitet, da det alene induceres af proinflammatorisk hypercytokinæmi. Disse egenskaber burde understøtte den rutinemæssige anvendelighed af CRP til diagnostiske, monitorerings- og screeningsmæssige formål. Den publicerede mængde af information vedrørende hundens CRP indikerer kraftigt at CRP kan anvendes til rutinemæssige formål, men større hindringer for dette og den generelle udbredelse af brugen af CRP, synes at eksistere.

Formålet med denne afhandling er at teste hypotesen at ”Målinger af hundens CRP i serum er anvendeligt til rutinemæssige kliniske formål”. Denne overordnede hypotese blev testet trinvist, ved at adressere de følgende fire faser:

FASE I Analytisk duelighed
FASE II Diagnostisk kapacitet
FASE III Klinisk anvendelighed
FASE IV Praktisk værdi

Afhandlingen består af fem kapitler. I Kapitel 1 bliver den tilgængelige viden omkring CRP’s egenskaber som molekyle, akut-fase protein og den mulige anvendelighed som para-klinisk parameter gennemgået. I Kapitel 2 bliver to tilgængelige målemetoder til bestemmelse af CRP i hundeserum evalueret objektivt mht. analytisk duelighed (Fase I). Kapitel 3 vurderer den tilsyneladende diagnostiske kapacitet og kliniske anvendelighed (Faserne II og III). I Kapitel 4 rapporteres et eksempel på den mulige positive effekt af CRP målinger på patient-håndteringen ved en immun-medieret lidelse hos hunde (Fase IV). Endelig bliver der i Kapitel 5 diskuteret, konkluderet og perspektiveret på baggrund af de opnåede resultater.

Afhandlingens endelige konklusion, som baseres på tilgængelig viden omkring hundens CRP og de opnåede resultater (Kapitlerne 2, 3 og 4) er at målinger af hundens CRP i serum har praktisk værdi i rutinemæssige kliniske henseender.

Chapter 1

INTRODUCTION

The acute phase proteins

The inflammatory reaction in a given disease process is a non-specific response to one or more inflammatory stimuli. The classical signs of local inflammation are: *tumor, rubor, calor, dolor* and *functio laesa* and they appear independent of the cause of inflammation. The systemic response of an inflammatory process extensive enough to cause such encompasses a wide range of phenomena designated the acute phase response (APR), which is mainly mediated through proinflammatory hypercytokinaemia (Figure1).

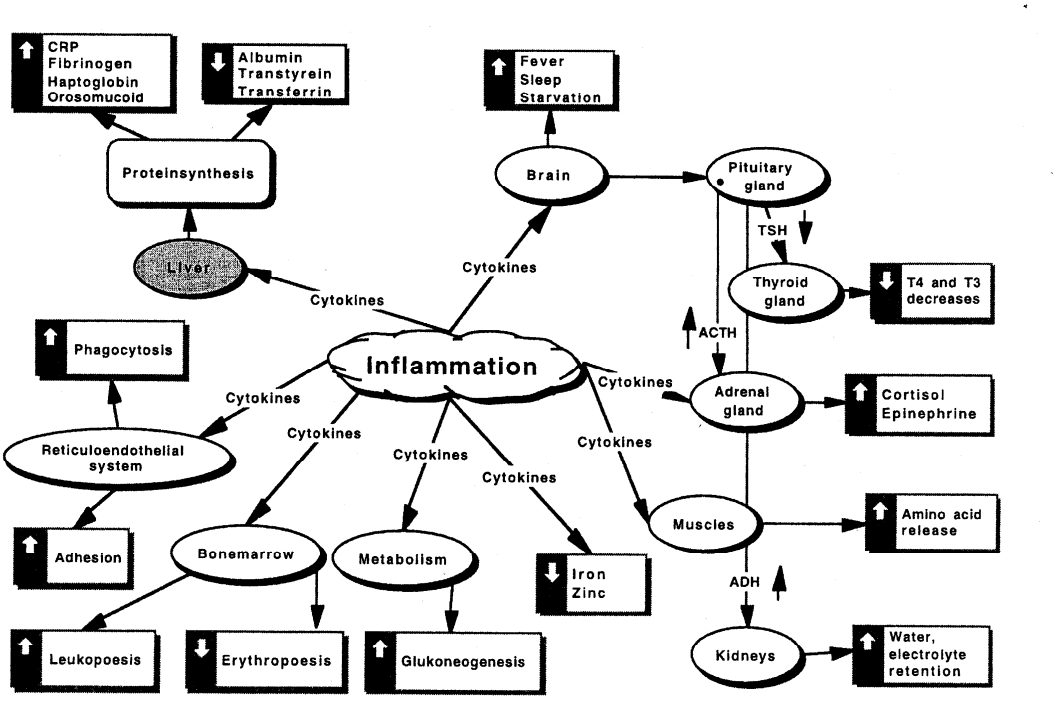

Figure 1. The local and general symptoms and the metabolic effects caused by systemic inflammation, mainly mediated through the proinflammatory cytokines Interleukin-1 (IL-1), IL-6 and tumor necrosis factor. CRP; C-reactive protein. TSH; Thyroid stimulating hormone. ACTH; Adrenocorticotrophic hormone. ADH; Anti-diuretic hormone. Modified from Hansson (1996).

A major property of the APR is the dramatic changes in several blood proteins known as acute phase proteins (APP) (Baumann and Gauldie, 1994), which are sub-grouped in 4 groups (major, intermediate, minor and negative) according to their kinetic properties during and after an acute phase response (Figure 2). In human and dogs, C-reactive protein is a major APP, characterized by low physiological levels, a marked

fast increase shortly after an inflammatory stimulus, followed by quick normalization in the absence of further inflammatory stimulus (Figure 2). The intermediate and minor APP have a more delayed response, whereas the negative APP are down-regulated during an acute phase response (Figure 2), excellently described in detail by others (Mackiewicz et al., 1993).

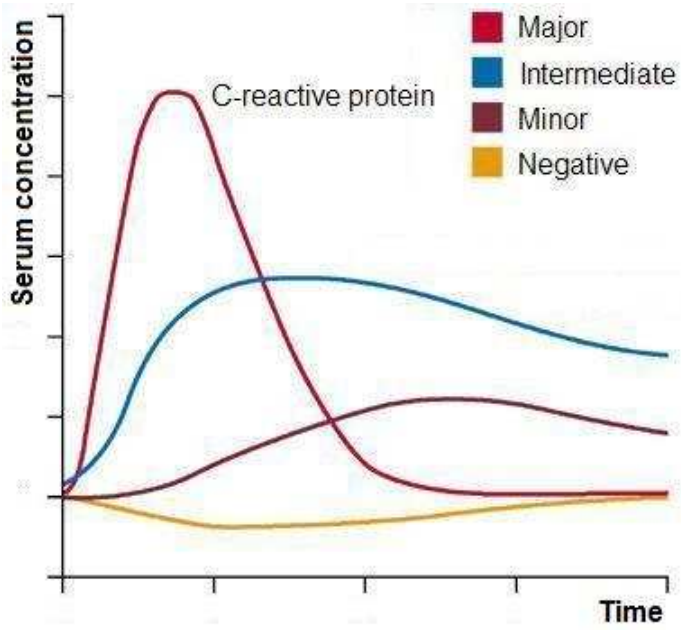

Figure 2. The relative kinetics of the serum concentration of major, intermediate, minor and negative acute phase proteins, respectively during an acute phase response. C-reactive protein is a major acute phase protein in human and dogs. Modified from Gabay and Kushner (1999).

The major APP have received most attention as bio-markers for the acute phase response due to the facilitating properties of low physiological levels, the fast incline, the marked rise in concentration during APR that eases detection and the fast decline after a ceased stimulus.

The history of C-reactive protein

C-reactive protein (CRP) was discovered in human acute phase serum as early as 1930 (Tillet and Francis, 1930) and has become to play a major role in human medicine. The discovery of CRP was based upon observations on human acute phase serum agglutinating certain members of *Pneumococcus spp.* by binding to a C-polysaccharide (the property that later gave CRP its name) (Tillet and Francis, 1930).

The agglutinating factor was identified as a protein in 1941 (Abernethy and Avery, 1941), where also antiserum to purified human CRP was produced in rabbits (MacLeod and Avery, 1941). Shortly thereafter human CRP was crystallized (McCarty, 1947) and in 1950 the first report of the medical applicability of CRP in monitoring rheumatic activity in rheumatic patients appeared (Anderson and McCarty, 1950).

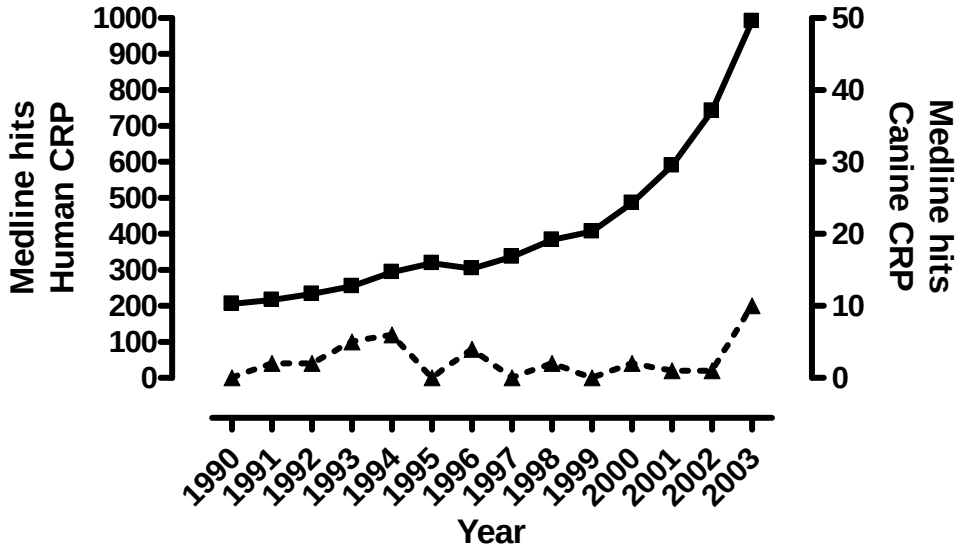

Figure 3. The annual distribution of Medline registered papers including human (squares) and canine (triangles) C-reactive protein (CRP), respectively. [Medline search strings: (YEAR[pdat] AND CRP AND Human) and (YEAR[pdat] AND CRP AND (dog or canine)), respectively].

A canine analogue to the human CRP was investigated in canine acute phase sera in the 1960's (Gotschlich, 1962; Dillman and Coles, 1966), canine CRP was isolated in 1970 (Riley and Coleman, 1970) and quantification made possible in 1972 by use of specific anti-canine CRP antibodies (Riley and Zontine, 1972). However, it was not really until the mid-1980's and early 1990's that canine CRP was studied more thoroughly. Dogs were found applicable as human acute phase models, due to observed similarities between the kinetics of human and canine CRP during an APR (Caspi et al., 1984; Conner et al., 1988; Eckersall and Conner, 1988; Yamamoto et al., 1992; Burton et al., 1994), for which they are also currently used (Higgins et al., 2003; Kumagai et al., 2004). Since the 1990's there has been a relative small, but ongoing research in the field of canine CRP (Figure 3), beneficial to the knowledge of both human and canine CRP. The field of research on human CRP is formerly exploding

with a nearby exponential increase in annually published material, reaching a mere 1000 Medline publications in 2003 (Figure 3).

Biochemical properties of CRP

C-reactive protein is a member of the pentraxin protein-family which is a phylogenetically ancient group of proteins found in both vertebrates and invertebrates (Robey and Liu, 1981; Baltz et al., 1982) with a conserved structure and a calcium dependent ligand function. All pentraxins consist of one type of subunit. For CRP five identical, non-covalently bound subunits are arranged in a planar pentagonal ring-structure that can be visualized by electron-microscopy (Osmand et al., 1977; Caspi et al., 1984). Also the three dimensional structure of human CRP was determined, where the grooves for calcium- and ligand-binding can be identified (Shrive et al., 1996) (Figure 4).

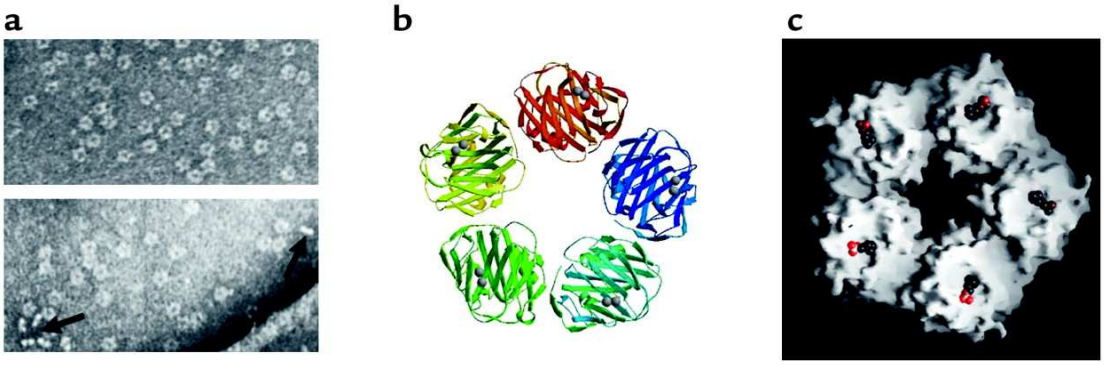

Figure 4. Molecular structure and morphology of human C-reactive protein (CRP). Negatively stained electron micrograph showing the typical pentameric disc-like structure face-on and side-on (arrows) [a]. Ribbon diagram of the crystal structure, showing the two calcium atoms (spheres) in the ligand-binding site of each subunit [b]. Space-filling model of the CRP molecule, showing a single phosphocoline molecule located in the ligand-binding site of each subunit [c]. Modified from Pepys and Hirschfield (2003).

The only major physico-chemical difference discovered between human and canine CRP consists of a glycosylation of two subunits of canine CRP, whereas human CRP is fully unglycosylated (Caspi et al., 1984). The molecular weight of canine CRP was estimated to be in the range of 100kDa (Caspi et al., 1984) and 155kDa (Yamamoto et al., 1992). Human CRP has been observed to have affinity to a wide range of molecules and interact with several types of cells, which are the basis for a wide range

of hypotheses of the physiological properties of CRP, commented in a following section. The ligand-affinity of CRP is either Ca^{2+}-dependent, Ca^{2+}-independent or dependent on the previous binding of another ligand (in part summarized in Figure 5 and Table 3). Several of these affinities have been confirmed for canine CRP also (Caspi et al., 1984; Fujise, 1992; Onishi et al., 1994) and the properties of canine and human CRP can be considered very much alike.

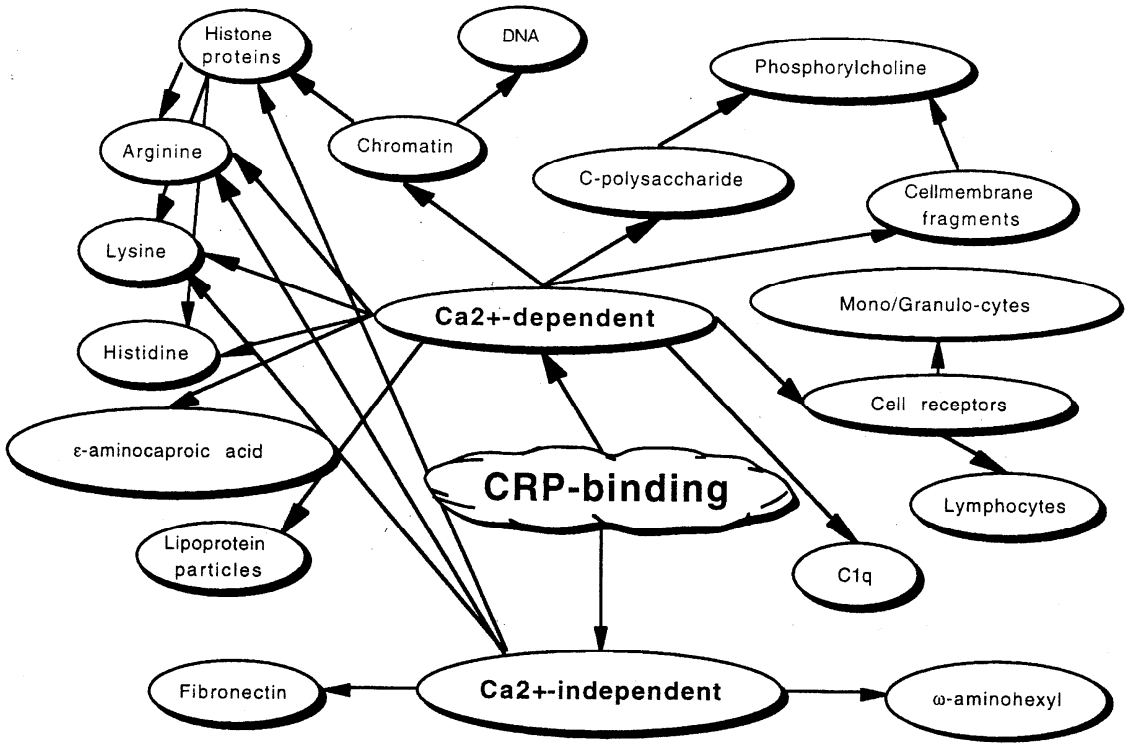

Figure 5. The Ca^{2+}-dependent and Ca^{2+}-independent ligand-affinity of C-reactive protein (CRP). C1q; Complement factor C1q. Modified from Hansson (1996).

Induction, release and regulation of CRP

The kinetic properties of canine CRP during an acute phase response are characterized by a low concentration at normal homeostasis (Yamamoto et al., 1992; Yamamoto et al., 1994b; Otabe et al., 1998), a short lag-phase after the inflammatory

stimulus of around 4 hours (Caspi et al., 1984; Conner et al., 1988; Higgins et al., 2003), reaching a peak in concentration after approximately 24 hours (Conner et al., 1988; Yamamoto et al., 1992), and a quick normalization after ended inflammatory stimulus (Conner et al., 1988; Yamamoto et al., 1992; Hayashi et al., 2001). Thus, canine CRP has the properties of an almost “real-time” marker of the inflammatory activity during an acute phase response.

The induction and regulation of the acute phase response is well studied in humans and seems to be a well orchestrated reaction, but so complex that many aspects still remain uncertain (Kushner et al., 1989; Suffredini et al., 1999).

However, the liver was identified as the major site for production and release of acute phase proteins (Hurlimann et al., 1966; Kampschmidt and Upchurch, 1974; Wannemacher, Jr. et al., 1975; Kushner and Feldmann, 1978; Kushner et al., 1980) and interleukin-6 is considered the major inducing cytokine of the hepatic production of CRP (Koj, 1985; Castell et al., 1989; Kishimoto, 1989; Yamashita et al., 1994). Interleukin-1 and TNF-alpha that is traditionally also considered as major proinflammatory cytokines seem not to be able to induce the hepatic synthesis of CRP alone or in combination, but should still be considered as major co-working modulators (Pepys and Baltz, 1983; Koj, 1985; Kushner et al., 1989; Taylor et al., 1989; Dinarello, 1997). A study on rabbits observed no presence of CRP in hepatocytes during normal homeostasis, followed by an increased expression and distribution of CRP-expressing hepatocytes over time after an appropriate inflammatory stimulus, concluding that CRP secretion was fully based on *de novo*-synthesis (Kushner and Feldmann, 1978).

Table 1. Inflammatory cells with capability to produce some of the proinflammatory cytokines Interleukin-6 (IL-6), IL-1 and tumor necrosis factor (TNF-alpha) Hansson (1996)

Monocytes-Macrophages	B- and T-lymphocytes	Nerve-cells	Synovial cells	Mast cells
Granular leukocytes	Endothelial cells	Astrocytes	Epithelial cells	Fibroblasts

The major source of systemic IL-6 during the acute phase response is the local release to the blood-stream from monocytes-macrophages and granular leukocytes at the site of inflammation, but a wide range of other cells have the capability to release proinflammatory cytokines and thus induce the acute phase response (Table 1).

There is also evidence for extrahepatic expression of C-reactive protein by various cell types (Table 2), but for none of them at levels of other than local significance and for

some, CRP is solely expressed on the cell surface (Kuta and Baum, 1986; Kolb-Bachofen et al., 1995). This could indicate that CRP is not merely acting as a systemic inflammatory factor but also as a part of the local immune-response. Thus, as the hepatocytes are the only significant systemic source of CRP, severe end-stage liver-disease could result in decreased release during an acute phase response, as it was observed for another canine APP, haptoglobin (Andersson et al., 1998).

Finally, an important observation made when reviewing the literature on CRP is that no non-proinflammatory inducers of CRP expression and release have been identified, that could impair a high diagnostic specificity, when using CRP as a marker of elevated proinflammatory activity (i.e. an acute phase response). Comprehensive reviews of the regulatory mechanisms of CRP and the APR at the cellular and transcriptional levels are available for readers interested in further details (Moshage, 1997; Suffredini et al., 1999; Volanakis, 2001).

Table 2. Examples of extra-hepatic cells capable of C-reactive protein expression

Cell-type	Species	References
Peripheral blood mononuclear cells	Human	(Murphy et al., 1991) (Kolb-Bachofen et al., 1995)
Peripheral blood lymphocytes	Human	(Kuta and Baum, 1986)
Renal cortical tubular epithelial cells	Human	(Jabs et al., 2003)
Alveolar macrophages	Human Dog	(Dong and Wright, 1996) (Casals et al., 1998)
Epithelial cells of the respiratory tract	Human	(Gould and Weiser, 2001)
Kupffer cells	Rat	(Egenhofer et al., 1993)

Physiological properties of CRP

C-reactive protein has been present and conserved through the evolution for at least 500 million years, as it is also found in the “living fossil” *Limulus polyphemus* (Robey and Liu, 1981), so it has undoubtedly some beneficial function. The major question, though, is: What is the major function of CRP? A regular screening-process in the search for the physiological properties of CRP has been going on, resulting in massive amounts of information on the binding, inductive and interactive capacities of CRP (exemplified by Figure 4 and 6). This has then been a source for further investigations

and especially for further speculations on the physiological properties of CRP, resulting in a very complex and unclear mixture of speculative and evidence-based statements.

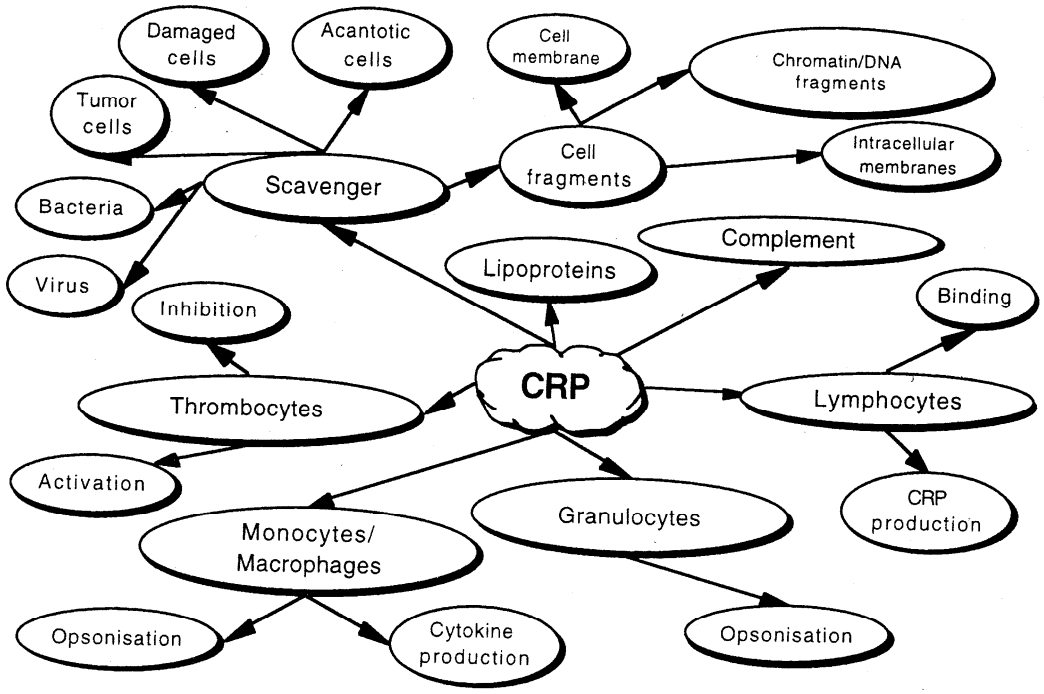

Figure 6. Different interactions of C-reactive protein (CRP). Modified from Hansson (1996).

Molecular interactions

One of the characteristic biochemical properties of pentraxins is the calcium-dependent ligand binding, but CRP also possess a calcium-independent affinity to certain molecules and affinities dependent on the previous binding to other ligands (ligand-dependent affinity) (Table 3).

Especially the ligand-dependent affinity to other key immunological molecules and receptors has been a source for further investigations on the role of CRP in the acquired cellular and humoral immune-response.

Cellular interactions

C-reactive protein modulates the function and activity of a wide range of cells (Table 4). The effect of CRP is mainly activation, but under some circumstances also inhibition.

Table 3. Examples of Ca^{2+}-dependent, Ca^{2+}-independent and ligand-dependent ligands for C-reactive protein

Type of ligand	References
Ca^{2+}-dependent ligands	
Phosphocholines	(Volanakis and Wirtz, 1979) (Gotschlich and Edelman, 1965)
Chromatin (Histones)	(Du Clos et al., 1988)
Lecithin	(Hokama et al., 1974)
Lipoproteins	(Mookerjea et al., 1994)
Ca^{2+}-independent ligands	
Polycations (poly-lysine and poly-arginine)	(Siegel et al., 1975) (Dougherty et al., 1991)
Fibronectin	(Salonen et al., 1984)
Galactose-rich glycoconjugates	(Uhlenbruck et al., 1981)
Ligand-dependent ligands	
Complement factor C1q (activation)	(Agrawal et al., 2001)
Fc-gamma receptors	(Bharadwaj et al., 1999)

Table 4. Examples of the modulatory effect of C-reactive protein on various cells

Cell type	Modulation	References
Monocytes-Macrophages	Activation by opsonization	(Pue et al., 1996)
	Promote chemotaxi	(Han et al., 2004)
	Increase endothelial adhesion	(Woollard et al., 2002)
Neutrophils	Inhibition of chemotaxi	(Shephard et al., 1990)
	Inhibition of degranulation	(Filep and Foldes-Filep, 1989)
	Inhibits endothelial adhesion	(Zouki et al., 1997)
Mast cells	Activation	(Fujimoto et al., 2003)
Platelets	Activation	(Fiedel et al., 1982)
	Inhibition	(Filep et al., 1991)
Vascular smooth muscle cells	Activation	(Hattori et al., 2003)

The ability of CRP to recognize pathogens on a molecular level and to mediate their elimination by recruiting the complement system and phagocytic cells makes it an

important constituent of the first line of innate host defence (Volanakis, 2001). Furthermore, the protein appears to play a role in the clearance of apoptotic, necrotic and injured host cells again by recognition on a molecular level, thus possibly contributing to restoration of normal structure and function, but also seemingly inhibiting induced autoimmunity by scavenging altered endogenous structures and molecules (Du Clos et al., 1994; Gershov et al., 2000).

Thus, the physiological function of CRP would seem to be: Provide innate unspecific protection by recognition of potentially harmful foreign molecules and altered endogenous structures and molecules.

Canine CRP as a clinical marker of systemic inflammation

The concentration of serum CRP is used for routine diagnostic, prognostic and screening purposes in a wide range of inflammatory diseases in human medicine, and investigations of new applications are ongoing. The recent issue of CRP being an early marker or maybe even a mediator of atherothrombosis (Jialal et al., 2004) and atherosclerosis (Manolov et al., 2003) appear to be one of the main reasons for the marked increase in published material on human CRP in the recent years (Figure 3). In canine medicine, a routine use of CRP measurements is not widespread, despite the rather comprehensive knowledge of important similarities to human CRP, regarding biochemical and physiological properties and not least, the kinetics during an acute phase response. These similarities suggest a potential for clinical applicability within canine medicine of similar magnitude as for CRP in human medicine.

CRP in healthy dogs

All previous investigations on the CRP level in non-diseased dogs report a low concentration compared to levels found in relation to inflammatory activity (Caspi et al., 1984; Caspi et al., 1987; Yamamoto et al., 1992; Yamamoto et al., 1993b; Burton et al., 1994; Yamamoto et al., 1994b; Börngen, 1998; Kuribayashi et al., 2003), although, a relative large variation between the observed ranges of CRP concentrations in healthy dogs are seen, most likely due to the use of different locally produced assays (Table 5). The effect of age and sex on the CRP concentrations in healthy dogs were investigated in the larger of the studies, where no correlation were observed (Yamamoto et al., 1994b; Kuribayashi et al., 2003). A single study on the circadian and long-term

variation of CRP could also not detect any systematic variation within individuals, but observed a difference in the homeostatic set-points of CRP between individuals (Otabe et al., 1998).

Table 5. The observed ranges of C-reactive protein (CRP) concentrations in non-diseased dogs

CRP range (mg/L)	Number of dogs	Assays	References
<5	20	ELISA local	(Caspi et al., 1984)
<5 – 67	42	EIA local	(Caspi et al., 1987)
0.2 – 0.8	20	ELISA local	(Yamamoto et al., 1992)
2.4 – 30.0	66	ELISA local	(Yamamoto et al., 1993b)
0.6 – 13.2	84	ELISA local	(Yamamoto et al., 1994b)
5.9 – 28.7	44	TIA local	(Burton et al., 1994)
0 – 70	50	ELISA local	(Börngen, 1998)
0.8 – 16.4	10	ELISA local	(Otabe et al., 1998)
1.5 – 18.9	144	ELISA local	(Kuribayashi et al., 2003)
0.2 – 4.0	21	ELISA[#]	(Martínez-Subiela et al., 2004b)

EIA; Electroimmunoassay. ELISA; Enzyme-linked immunosorbent assay. TIA; Turbidimetric immunoassay. [#]Phase-Range canine CRP, Tridelta, Kildare, Ireland.

A final interesting aspect of CRP in healthy dogs is that several studies have reported raised CRP concentrations in the normo-physiological state of pregnancy in dogs (Eckersall et al., 1993; Concannon et al., 1996; Kuribayashi et al., 2003). In pregnant dogs, a marked increase in CRP was observed in the period of day 21-55 after the ovulation compared to non-pregnant bitches in the same stage of the estrous-cyclus. The increase in CRP was equivalent to that seen in an acute phase response, but the specific source of the stimulus remains unclear (Eckersall et al., 1993), although, it is most likely due to an inflammatory reaction to the embryonic implantation.

Experimental studies

Several experimental studies on canine CRP have investigated the canine acute phase response to various stimuli and various features of CRP as inflammatory marker. Many of the studies have been conducted to assess the applicability of the dog as a model for the human acute phase and CRP response.

Aseptic stimuli

Experimental settings using aseptic induction of the acute phase response reported, as expected, that the acute phase response could be set-off from a wide variety of tissues using a wide variety of stimuli (Table 6). In addition, studies including various aseptic stimuli and various degrees of stimuli indicated that the relative CRP concentration reflected the relative degree of inflammatory activity (Otabe et al., 2000; Hayashi et al., 2001). Hayashi et al. (2001) also detected an interesting and important age-dependency of the increase in CRP after a variety of both aseptic and septic experimental inflammatory induction, where dogs under 3 months of age were observed to have a markedly lower response than dogs above 3 months of age after equivalent stimuli. This age-dependent aspect is important as it may impair the diagnostic sensitivity of CRP as an acute phase marker in very young dogs (Hayashi et al., 2001). Finally, a few of the studies compared the applicability of both CRP and complete blood cell count (CBC) concluding that a greater (Otabe et al., 2000) or equivalent (Yamashita et al., 1994; Burton et al., 1994) level of information on the inflammatory process could be derived from CRP compared to CBC in experimental aseptic inflammation.

Septic stimuli

A wide variety of studies on experimentally induced infections confirm the findings from the aseptic studies (Table 6). Furthermore, additional indications for CRP being a “real-time” marker of the relative inflammatory activity could be derived from infectious studies: Experimental infections with agents with different incubation period or immunogenic nature was clearly reflected by the observed kinetics of the CRP level, where increased levels of CRP were seen simultaneously with the expected active periods of the disease and the relative level corresponded to the observed disease activity. (Caspi et al., 1987; Ndung'u et al., 1991; Rikihisa et al., 1994; Yamamoto et al., 1994a; Martínez-Subiela et al., 2002).

Table 6. Examples of various stimuli and sites of application resulting in an elevated level of canine C-reactive protein in experimental studies

Stimulus	Application site	References
Aseptic		
Acetylic acid	Gastric mucosa	(Otabe et al., 2000)
Casein	SC	(Caspi et al., 1987)
Mineral oil	Intra-bronchial	(Dillman and Coles, 1966)
Talcum powder	IP	(Dillman and Coles, 1966)
Turpentine	IM SC	(Yamashita et al., 1994) (Hayashi et al., 2001)
- Surgical procedures		
	Gastrotomy	(Hayashi et al., 2001)
	Ovariohysterectomy	(Hayashi et al., 2001) (Burton et al., 1994) (Yamamoto et al., 1992) (Caspi et al., 1987) (Yamamoto et al., 1993b)
	Laparotomy	(Liu et al., 2000)
	Sterile pericarditis	(Kumagai et al., 2004)
	Elective orthopaedic surgery	(Caspi et al., 1987) (Yamamoto et al., 1993b)
	Aortic graft implant	(Conner et al., 1988)
Septic		
Bordetella bronchiseptica	Intra-bronchial	(Yamamoto et al., 1994a)
Ehrlichia canis	IV	(Rikihisa et al., 1994) (Shimada et al., 2002)
Endotoxin	IV	(Higgins et al., 2003)
Escherichia coli	IV	(Hulton et al., 1985)
Leptospira interrogans	IP	(Caspi et al., 1987)
Staphylococcus aureus	SC	(Hayashi et al., 2001)
Trypanosoma brucei	IV	(Ndung'u et al., 1991)

IM; intramuscular. IP; intraperitoneal. IV; intraveneous. SC; subcutaneous.

Studies on spontaneously diseased dogs

The clinical performance of a disease marker is best evaluated by controlled studies of patients recruited from the population where the marker is expected to be used (Bossuyt et al., 2003). Therefore, studies on spontaneously diseased dogs are

necessary to assess the applicability of CRP as a clinical useful marker of systemic inflammation in canine medicine. Several studies evaluating the clinical utility of canine CRP have been performed. The assessments of diagnostic accuracy may, although, be considered biased due to selection, as none of them are performed strictly as prospective randomized controlled studies.

A high diagnostic sensitivity of serum CRP was observed in detecting systemic inflammation as defined by a significant number of circulating band neutrophils (Burton et al., 1994). A high diagnostic sensitivity and specificity of CRP in combination with band neutrophil count in the differentiation of pyometra and endometrial hyperplasia was observed in a controlled study (Fransson et al., 2004). Eckersall et al. (1988) screened 155 dogs presented to their university hospital and found an increased CRP concentration in 67 dogs, all with identifiable inflammatory foci, indicating good predictive value of a positive result, as also reported by others (Riley and Zontine, 1972; Börngen, 1998). Furthermore, the magnitude of the response seemed to correlate to certain groups of causes, which they interpreted as an ability to graduate the inflammatory activity by means of CRP measurements (Eckersall et al., 1988), as it was also concluded by several of the experimental studies. A study on canine leishmaniasis reported CRP as a measure to detect subclinical states of the disease (Martínez-Subiela et al., 2002). However, CRP seemed not to possess that feature in the detection of subclinical ehrlichiosis (Rikihisa et al., 1994; Shimada et al., 2002). Then again, a CRP response was found in the chronic phase of naturally occurring canine ehrlichiosis (Rikihisa et al., 1994).

The relative CRP concentration seemed to correlate with the present disease activity and the severity or the prognosis of the diseased dogs in several studies; erosive vs. non-erosive arthritis (Caspi et al., 1987), metastatic vs. localized mammary neoplasia (Caspi et al., 1987), complicated vs. uncomplicated babesiosis (Matijatko and Kucer, 2002), oedematous vs. necrotizing pancreatitis (Spillmann et al., 2002), the relative disease activity in inflammatory bowel disease (Jergens et al., 2003) and correspond to clinical improvement in dogs with various disorders (Yamamoto et al., 1993b). Two studies also evaluated CRP as an end-point marker of treatment efficiency with positive results (Jergens et al., 2003; Martínez-Subiela et al., 2003a). However, in other diagnostic entities CRP was observed not to be clinically applicable as a prognostic or disease severity marker; glomerulonephritis (Caspi et al., 1987) and as an end-point

marker in the assessment of the anti-inflammatory effect of n-3 fatty acids in dogs with pruritus (Nesbitt et al., 2003).

Therapeutic modulation of the CRP acute phase response

The indication that CRP reflects inflammatory activity in an almost “real-time” manner during the state of disease facilitates applicability as a marker of therapy efficiency. However, this suggested use of CRP requires that the response is not modulated or induced by therapy itself. Therapeutic modulation and induction of the CRP response is also an important issue for the use of CRP as a diagnostic marker of inflammation, as it could impair diagnostic sensitivity and specificity. Only a few, but very important reports on this issue on canine CRP are available. The most important observations were that neither short-term nor long-term high-dose steroid therapy induced a CRP response in healthy dogs (Martínez-Subiela et al., 2004b), steroid therapy did not modulate the CRP response compared to untreated controls in experimental *Bordetella bronchiseptica* infection (Yamamoto et al., 1994a) and neither did non-steroid anti-inflammatory therapy of experimental *Escherichia coli* sepsis (Hulton et al., 1985). Finally, CRP was not compromised as a marker of inflammation in experimentally induced synovitis by carprofen, etodolac, meloxicam or butorphanol, in a controlled study on pain relief in dogs (Borer et al., 2003). Thus, CRP seems to be a stable marker of inflammation during various frequently used anti-inflammatory and immunomodulating therapies that is known to modulate or induce other important inflammatory markers, e.g. rectal temperature and CBC (Hulton et al., 1985; Yamamoto et al., 1994a). Interleukin-6 applied as a possible therapy to modulate bacterial invasion in experimental pancreatitis was reported to modulate the CRP response (Liu et al., 2000), as expected, because IL-6 is the main inducer of the acute phase response (see Introduction). Modulation by downregulation of the CRP response by the therapeutic group of statins was also reported in both dogs and human models for vasculitis (Strandberg et al., 1999; Kumagai et al., 2004). However, signs of a lower inflammatory activity during the state of disease was also detected in the treated group (Kumagai et al., 2004), suggesting that the CRP level may still reflect this activity during statin treatment.

Measuring canine CRP

A key issue when assessing the applicability of a parameter for routine use is the general practicability, which especially includes assessment of the availability of reliable assays and the costs (regarding both time and economic expenses) of the different methods.

Several canine-specific methods have been developed to quantify CRP, mainly used in scientific scales only in different specific studies: Capillary immuno-reaction (Riley and Zontine, 1972), sandwich enzyme-linked immunosorbent assay (ELISA) (Eckersall et al., 1989; Yamamoto et al., 1992), competitive ELISA (Börngen, 1998), slide reversed passive latex agglutination (Yamamoto et al., 1994b), immunodiffusion (Conner et al., 1988; Yamamoto et al., 1992), electro-immunoassay (Caspi et al., 1984), quantitative capillary reversed passive latex agglutination (Tagata et al., 1996), laser nephelometric immunoassay (Onishi et al., 2000; Kumagai et al., 2004), time-resolved immunofluorometric assay (Parra et al., 2004) and immunoturbidimetric assay (Eckersall et al., 1991). A non-immuno enzyme-linked assay based on the ligand affinity of CRP to phosphorylcholine was also developed (Deegan et al., 2003). However, only a few quantitative canine CRP assays were made commercially available (a sandwich ELISA [Phase Range, Tridelta, Kildare, Ireland] and an immunoturbidimetric assay [K-assay, Kamiya Biomedical Company, Seattle, USA]), but none of them were independently evaluated for canine serum CRP determination for clinical use. Recently, although, an available semi-quantitative immuno-chromatographic canine CRP method was validated (McGrotty et al., 2004).

The possibility to perform heterologous determination of canine CRP have also been investigated, as heterologous determination has been reported to be fully valid for other canine parameters (Hoier and Jensen, 1993; Jensen et al., 2001), and could solve the problem of availability and maybe enhance cost- and time-effectiveness by the use of established human assays. Several reports on the cross-reactivity of canine CRP towards different polyclonal and monoclonal anti-human CRP antibodies exist and these observed either no cross-reactivity (Caspi et al., 1984; Maudsley and Pepys, 1987; Yamamoto et al., 1993c; Börngen, 1998; Parra et al., 2002) or an undetermined degree of cross-reactivity insufficient for quantitative assay purposes (Gotschlich, 1962; Dillman and Coles, 1966; Riley and Coleman, 1970; Yamamoto et al., 1992; Yamamoto et al., 1993a; Concannon et al., 1996). Thus, the potential of using anti-

human CRP antibodies for the determination of canine CRP remained an unclear matter.

Preanalytical factors

The preanalytic influence of sampling and handling procedures on the observed level of CRP have been addressed in a few canine studies, where the level was observed not to change significantly in a period of repetitive short-interval phlebotomies in a study of circadian fluctuations (Otabe et al., 1998) or as a result of administration of placebo or control material in studies on experimental infections (Rikihisa et al., 1994; Yamamoto et al., 1994a).

For some of the canine specific CRP assays various postsampling preanalytical factors have been investigated, suggesting that the use of citrated plasma may decrease the CRP level (Martínez-Subiela et al., 2004a). Haemolysis, according to method, may alter the measured level of CRP compared to fresh serum samples (Eckersall et al., 1991; Martínez-Subiela et al., 2004a). The use of ethyl-diamine tetra acetate or lithium heparine stabilized plasma seemed not to alter the measured level of CRP (Martínez-Subiela et al., 2004a). Recent investigations reported that the use of gel-separator in blood containers may have a preanalytical influence on the measured level of human CRP of a magnitude important only at low levels (Anderson et al., 2003; Chang et al., 2003; Anderson et al., 2004). In human medicine the observation of abnormal fluctuations of CRP at low levels may be indicating increased risk of chronic disease development (Kluft and de Maat, 2002). This correlation at low levels of CRP was not established in canine medicine and therefore, the influence of the gel-separator may be negligible for canine CRP.

Finally, canine C-reactive protein was reported to possess useful storage properties with no significant change in immunoreactivity after storage overnight at 4˚C, up to 2 months at -20˚C (Dillman and Coles, 1966) and up to 4 months at -10˚C (Riley and Zontine, 1972) and may be assumed to be stable at storage temperatures below -70˚C (unpublished observations). The immunoreactivity of canine CRP is reported to be eliminated after 30 minutes at 70˚C (Dillman and Coles, 1966).

Clinical applicability of canine CRP measurements

The knowledge of the properties of CRP, as a whole, indicates that an elevated level of serum CRP is consistent with an acute phase response, no matter the origin or nature of the stimulus. Furthermore, the relative level of CRP seems to reflect the relative activity of the inflammation during the state of disease, again without relation to the origin or nature of the stimulus. Thus, the CRP response doesn't seem to depend on whether the inflammatory stimulus is of, e.g. aseptic, bacterial or viral nature, but rather on how destructive and proinflammatoric the stimulus is. The diagnostic sensitivity of CRP is facilitated by the properties of a major acute phase protein, with very low physiological concentrations compared to the consistent marked increase during an acute phase response. Regarding diagnostic specificity, it seems that the only significant inducer of increased serum CRP levels is the proinflammatory cytokine IL-6, giving CRP the potential of being a diagnostically specific marker of proinflammatory hypercytokinaemia, i.e. an acute phase response. However, the diagnostic sensitivity as an inflammatory marker seemed to be impaired in very young dogs (< 3 months of age) (Hayashi et al., 2001) and diagnostic specificity impaired during mid-gestation of pregnant dogs (Eckersall et al., 1993; Concannon et al., 1996; Kuribayashi et al., 2003). The most frequently used anti-inflammatory drugs did not compromise CRP as an inflammatory marker and the only therapeutic entities reported to modulate the CRP response seem to do this through a direct modulation of the inflammatory activity and therefore possibly not compromising CRP as a marker thereof. Finally, the influence of common preanalytical factors regarding sampling, handling and storage seem not to adversely affect routine applicability, and regarding storage, the properties are superior to that reported for cellular inflammatory markers (Vogelaar et al., 2002).

Thus, the knowledge of CRP and the positive indications for its potential as a marker of inflammatory activity within different aspects of canine medicine is quite comprehensive. However, the review of the previous investigations of canine CRP also reveals that some areas of importance to an objective assessment of the applicability of CRP in canine medicine, lack attention. The lack of practical applicable validated quantitative assays for the determination of canine CRP seems to be the major impediment for the establishment of a routine use of CRP. In addition, if such assays can be identified, there will be a need for objectively derived clinical decision levels for different settings of clinical interest, e.g. for diagnostic, screening and monitoring

purposes. Finally, the utility of CRP compared to other well-established inflammatory markers in different clinical settings should be further investigated, as the knowledge on this field is still limited to a few studies.

Objectives, hypotheses and outline of the thesis

It was the objective of the present thesis to solve the major issues that currently impair implementation of canine CRP as a routine parameter in canine medicine and to sustain the applicability of CRP measurements in canine medicine.

The main hypothesis for the thesis was:

- Canine serum CRP measurements are applicable for routine purposes in canine medicine.

To facilitate the process of testing this hypothesis, the different aspects of the hypothesis were addressed in a stepwise manner. It has been suggested that the introduction of a diagnostic parameter for routine laboratory use should be done similar to the structured evolution of a new drug through a phase trial (Zweig and Robertson, 1982; Fraser, 2001) going through the following phases:

- Phase I Analytical investigations
    - Assessment of reliability and practicability characteristics
- Phase II Overlap investigations
    - Assessment of values in health and disease
- Phase III Clinical investigations
    - Evaluation of diagnostic sensitivity and specificity in clinical settings of interest
- Phase IV Outcome and utility investigations
    - Assessment of whether individual (or community) gain advantage

A test can then only be said to be truly applicable for routine purposes through acceptable and useful results throughout all phases of the trial, and at the bottom-line answering to the statement of Zweig and Robertson (1982): “A laboratory test is clinically useful only if it successfully answers a question of consequence to patient management”.

The structure of this suggested phase trial was adopted throughout the thesis-projects and the corresponding phases were implemented as sub-hypotheses to assist testing the main hypothesis of the thesis. The sub-hypotheses were as follows:

- Phase I
    - The analytical performance of commercially available assays (and available calibration material) can fulfil recognized objective analytical

performance standards, regarding imprecision, inaccuracy, total error and detection limit, thus determine canine CRP reliably in both the short- and long-term.

- Phase II and III
  - Canine CRP measurements can
    - Discriminate between healthy dogs and different subpopulations of diseased dogs or
    - Discriminate between different subpopulations of diseased dogs.
- Phase IV
  - Canine CRP measurements has a positive consequence on the case management of individuals or groups of individuals in canine medicine, e.g. by either
    - Improving the outcome of a clinical problem
    - Providing equivalent clinically useful information faster than established parameters
    - Providing equivalent clinically useful information more cost-effective than established parameters

In phase I (Chapter 2) a commercially available canine specific CRP sandwich ELISA (Paper I) and a human CRP turbidimetric immunoassay (CRP-TIA) (Paper II) were validated for the determination of canine serum CRP. In parallel the biological variation of canine serum CRP was investigated (Paper III) to establish objective analytical performance standards for imprecision, inaccuracy and total error for use in phase I, but also to provide valuable information for the clinical decision level for serially obtained CRP measurements and evaluation of the usefulness of population-based reference ranges. The successful validation of the human CRP-TIA for heterologous determination of canine CRP made it necessary to investigate the possibility to plan and implement a canine-specific internal quality control procedure for that assay (Paper IV).

Phase II and III (Chapter 3) were completed by the establishment of a population based reference range for healthy dogs and an investigation of the clinical performance of CRP in discriminating between different diagnostic groups of clinical interest (Paper V). Finally, in phase IV (Chapter 4) a case-report on the use of CRP measurements in

a specific clinical setting provided additional evidence on the clinical benefits of canine CRP measurements on case-management (Paper VI).

The overall results of this phase trial to test the applicability of serum CRP for routine purposes in canine medicine are discussed, concluded upon and put in perspective in Chapter 5.

Chapter 2

PHASE I – ANALYTICAL INVESTIGATIONS

Due to restrictions from the publishers of the journals in which paper III has been published, this paper is not present in this PDF. The paper can be found in:

Paper III:
Kjelgaard-Hansen, M., Mikkelsen, L. F., Kristensen, A. T., Jensen, A. L. (2003). Study on biological variability of five acute-phase reactants in dogs. Comparative Clinical Pathology, 12, 69-74.
DOI: 10.1007/s00580-003-0477-z

Paper VI has since the publication of this PhD been published in Acta Veterinaria Scandinavia. It is available in:

Paper VI:
Kjelgaard-Hansen, M., Jensen, A. L., Houser, G. A., Jessen, L. R., Kristensen, A. T. (2006). Use of serum C-reactive protein as an early marker of inflammatory activity in canine type II immune-mediated polyarthritis: case report. Acta Veterinaria Scandinavia, 49, 9.
DOI: 10.1186/1751-0147-48-9

The remaining papers has been published in journals published by Wiley/Blackwell, who has kindly allowed that the articles can be made publicly available in this PDF, in the institutional repository at the Faculty of Life Sciences at the University of Copenhagen.

Paper I – Evaluation of a commercially available enzyme-linked immunosorbent assay (ELISA) for the determination of C-reactive protein in canine serum

Published in
Journal of Veterinary Medicine Series A
(2003), 50, 164-168
(Reprinted with permission)

J. Vet. Med. A **50,** 164–168 (2003)

ISSN 0931–184X

Department of Clinical Studies, Central Laboratory, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark

Evaluation of a Commercially Available Enzyme-Linked Immunosorbent Assay (ELISA) for the Determination of C-Reactive Protein in Canine Serum

M. Kjelgaard-Hansen[1,3], A. T. Kristensen[2] and A. L. Jensen[1]

Addresses of authors: [1]Department of Clinical Studies, Central Laboratory, The Royal Veterinary and Agricultural University, 3 Gronnegaardsvej, DK-1870 Frederiksberg, Denmark; [2]Department of Clinical Studies, Small Animal Hospital, The Royal Veterinary and Agricultural University, 12 Ridebanevej, DK-1870 Frederiksberg, Denmark; [3]Corresponding author: Tel.: +45 35 28 3873; fax: +45 35 28 2947; E-mail: mjkh@kvl.dk

With 1 figure and 3 tables

Received for publication: September 16, 2002

Summary

The purpose of this study was to evaluate a commercially available enzyme-linked immunosorbent assay for determination of canine serum C-reactive protein (CRP). The concentration of CRP could be determined accurately and the intra- and inter-assay coefficients of variation were in the range of 6.9–10.1 and 7.5–29.0%, respectively. This level of imprecision between runs is usually considered unacceptable for diagnostic purposes, but the overall results indicated that the assay was useful in differentiating dogs suffering from infections, from dogs suffering from various other diseases (neoplastic diseases, endocrine/metabolic disorders), and healthy dogs. The assay was also able to detect dynamic changes of CRP during development and after cessation of spontaneous occuring inflammatory stimuli in two clinical cases.

Introduction

Several studies identify canine serum C-reactive protein (CRP) as an acute phase marker with a low physiological level and a rapid increase with the onset of acute inflammation or tissue destruction (Conner et al., 1988; Yamamoto et al., 1992; Burton et al., 1994; Yamashita et al., 1994; Hayashi et al., 2001). Additionally, CRP concentration seems to reflect the underlying disease activity (Ndung'u et al., 1991; Yamamoto et al., 1994a; Otabe et al., 2000), which together with the rapid drop after ended inflammatory stimuli (Conner et al., 1988; Burton et al., 1994), can make CRP useful for prognostic, monitoring and diagnostic purposes.

Recently, a species-specific enzyme-linked immunosorbent assay (ELISA) for canine serum CRP has become commercially available. The aim of this study was to evaluate this assay by means of imprecision, inaccuracy, detection limit (DL), ability to detect dynamic changes and comparison of CRP values in various groups of dogs.

Materials and Methods

Clinical assessment

Forty-six dogs were included in this part of the study. Thirty-eight dogs were client-owned dogs presented at the Small Animal Veterinary Teaching Hospital, Department of Clinical Studies, The Royal Veterinary and Agricultural University, Denmark, for various diagnostic, therapeutic or prophylactic measures. Eight dogs were clinically healthy beagle dogs from a research colony. All these dogs were subjected to clinical examination and standard haematological and clinical chemical profiles performed at our laboratory (Jensen et al., 2001). Additional diagnostic testing on each dog was conducted at the discretion of the individual clinician (e.g. endocrine testing, biopsies, radiography, smears or microbial testing).

Based on the above examinations and the final clinical diagnosis, the 46 dogs were retrospectively assigned to five groups, i.e. clinically healthy dogs, dogs with neoplastic diseases, dogs with infection, dogs with endocrine/metabolic disorders and dogs with miscellaneous diseases not belonging to any of the former groups. Specifically, clinically healthy dogs should have unremarkable findings on clinical and clinical pathological examination. Dogs with neoplastic diseases had tumours that were cytologically and/or histologically characterized as neoplastic. Dogs with infection had a pathogen identified that could be related to the pathological findings. Dogs with endocrine/metabolic disorders had a final diagnosis that could be related to a hormone or metabolic malfunction/disorder. Dogs with miscellaneous diseases had various diagnoses and no clinical findings to associate them with any of the other groups.

CRP dynamics

In experimental studies, CRP reportedly increases within the first 24 h following induction of an inflammatory response (Otabe et al., 2000; Hayashi et al., 2001) and gradually decline after cessation of the inflammatory stimuli (Yamamoto et al., 1993; Hayashi et al., 2001). A clinical case of acute mastitis caused by *Pseudomonas aeruginosa* in a 7-year-old intact female Great dane afforded the opportunity to illustrate the acute CRP dynamics in a spontaneous case from appearance of the first local inflammatory signs and after 24 h, when the fulminant clinical signs had developed. Further, to illustrate the expected decline in CRP level after cessation of an inflammatory stimulus, CRP concentrations were measured in a clinical case of pyometra in a 5-year-old intact American bulldog just before and 24 and 48 h after ovariohysterectomy.

Serum was prepared by centrifugation ($1500 \times g$, 5 min) of blood samples after collection of the blood in plain vials with

U.S. Copyright Clearance Center Code Statement: 0931–184X/2003/5003–0164 $15.00/0

clot activator and separation gel (Vacuette, Greiner bio-one, Austria). The serum samples were stored in plastic vials at −80°C until analysis for maximally 8 weeks.

CRP analysis

The analysis for canine CRP was performed with the Phase Range® canine CRP ELISA (Tridelta Development Ltd, Kildare, UK).

Assay characteristics

Serum samples from additionally 25 client-owned unclassified dogs were also included to investigate the characteristics of the ELISA assay. The intra-assay variation [coefficient of variation (CV)] was determined from the difference between duplicate determinations of CRP concentration in 63 canine serum samples. Twenty-nine of these samples were run in duplicate in another run to assess the inter-assay variation. Additionally, the inter-assay CV was determined from the mean value and the standard deviation of six to eight replicate determinations of two serum pools with different CRP concentrations. The pools were frozen in aliquots and only vials needed for each specific analytical run were thawed to prevent potential variation as a result of repetitive freeze–thaw cycles. Inaccuracy was evaluated indirectly by investigating linearity under dilution of a canine serum pool with a high CRP concentration with the sample diluent provided with the assay (Jensen, 2000). The DL was calculated based on data from 26 replicate determinations of the diluent buffer from the assay (Büttner et al., 1980).

Statistical methods

Intra- and inter-assay variations (intra- and inter-assay CV) and DL ($P < 0.01$) were calculated using routine descriptive statistical procedures (Box et al., 1978; Büttner et al., 1980). Lack-of-fit tests were performed to investigate whether straight-line regression models actually represented a straight line appropriately (Weisberg, 1985). Investigation of linearity under dilution was accomplished by regression analysis in linear and logarithmic transformation. The latter was introduced in order to achieve homogeneity of the variance (Weisberg, 1985). Kruskal–Wallis one-way analysis was performed to determine whether the samples in the different diagnostic groups belonged to different distributions. If the outcome was significant ($P < 0.05$), a multiple comparison test was performed to get a more detailed picture of the deviation (Siegel and Castellan, 1988).

Results

Assay characteristics

The intra- and inter-assay variations are given in Table 1. Analysis of dilutions of a canine serum pool having a high concentration of CRP resulted in a linear regression equation the intercept of which did not differ from zero, as shown in Table 2. The lack-of-fit test revealed that the dilution curve was in fact linear [P (LOF) = 0.99]. In the corresponding logarithmic regression analysis non-parallelity was indicated by a slope different than 1, although the deviation from parallelity was small (slope = 0.93). The DL was calculated as 0.41 μg/ml (mean value 0.26 μg/ml, SD 0.06 μg/ml), thus below the range for normal canine sera observed in earlier studies (0.8–22.6 μg/ml (Otabe et al., 1998), 2.4–30.0 μg/ml (Yamamoto et al., 1994b) and in this study (1.1–6.3 μg/ml).

Table 1. Intra- and inter-assay variations of determinations of C-reactive protein (CRP) in canine serum samples

	No. of samples	Mean value (μg/ml)	SD (μg/ml)	CV (%)
Intra-assay	39	1.6	0.11[a]	6.9
	24	44.4	4.49[a]	10.1
Inter-assay	15	2.3	0.60[a]	26.2
	14	54.9	15.91[a]	29.0
Inter-assay	6	1.2	0.09[b]	7.5
	8	92.3	20.45[b]	22.2

SD, standard deviation; CV, coefficient of variation.
[a]Based on duplicate determinations of different serum samples.
[b]Based on replicate determinations of the same serum-pool.

Table 2. Analytical inaccuracy of C-reactive protein (CRP) measurements assessed by dilution of a canine serum pool with high CRP content

Regression	y-Intercept	y-Intercept 95% CI	Slope	Slope 95% CI	P (LOF)	r
Linear	4.54	[−6.90; 15.97]	0.97	[0.89; 1.01]	0.99	0.99
Logarithmic	0.16	[0.05; 0.27]	0.93	[0.88; 0.98]	0.99	0.99

95% CI, 95% confidence interval; P (LOF), probability for model fitting, i.e. that the data follow a straight line; r, coefficient of correlation.

Clinical assessment and CRP dynamics

The clinical characteristics and CRP concentration of the 46 dogs are given in Table 3. The Kruskal–Wallis analysis revealed that the CRP level in the different groups deviated significantly from each other ($P < 0.0001$). The multiple comparison analysis showed that the serum CRP level in dogs with infection was significantly higher than in dogs from every single other group in the study ($P < 0.01$). No other pair of groups deviated significantly from each other ($P > 0.05$). The dynamics of the CRP level during development and after cessation of spontaneously occurring inflammatory stimuli in two dogs are illustrated in Fig. 1.

Discussion

Several studies show that serum CRP concentration is an acute phase marker in the dog (Conner et al., 1988; Yamamoto et al., 1992; Burton et al., 1994; Yamashita et al., 1994; Hayashi et al., 2001). The rise in CRP is rapid after various septic and aseptic inflammatory stimuli and seemingly correlates to the extent and severity of the inflammatory process (Yamamoto et al., 1994a; Otabe et al., 2000). CRP might therefore be a useful diagnostic, monitoring and prognostic parameter in the dog. The freezing and storage of the serum samples prior to analysis

Table 3. Clinical characteristics of 46 dogs on which serum C-reactive protein concentration was measured

Clinical condition	Breed	Sex	Age (years)	CRP (μg/ml)
Clinically healthy				
	Beagle	Male	6	6.3
	Beagle	Male	5	2.0
	Beagle	Male	2	1.5
	Beagle	Male	2	1.2
	Beagle	Female	6	2.0
	Beagle	Female	4	2.3
	Beagle	Female	3	1.1
	Beagle	Female	3	2.3
Range (median)			2–6 (3.5)	1.1–6.3 (2.0)
Neoplastic disease				
Myxosarcoma	Cocker spaniel	Female	8	1.5
Mammary adenocarcinoma	German shorthair	Female	10	5.4
Mammary adenocarcinoma	Mixed breed	Female	6	0.8
Mammary adenocarcinoma	German sheepdog	Female	11	3.2
Seminoma	Fox terrier	Male	13	1.0
Mastocytoma	English setter	Male	8	1.3
Mastocytoma	Fox terrier	Male	9	1.3
Lymphosarcoma	Golden retreiver	Male	7	14.2
Papilloma	Rottweiler	Female	9	1.5
Cutaneous lipoma	Mixed breed	Female	11	2.3
Cutaneous histiocytoma	American bulldog	Male	6	3.1
Range (median)			6–13 (9)	0.8–14.2 (1.5)
Infection				
Septic mastitis	Great dane	Female	12	51.7
Septic arthritis	Labrador	Male	1	86.8
Septic endometritis	Cairn terrier	Female	9	53.5
Pyometra	Rottweiler	Female	10	49.2
Pyometra	Icelandic sheepdog	Female	14	88.6
Septic pneumonia	Mixed breed	Female	12	75.7
Systemic leishmaniosis	French bulldog	Male	6	54.9
Angiostrongylosis	German sheepdog	Female	6	39.3
Crenosomiasis	Mixed breed	Male	2	28.5
Crenosomiasis	Tiberian terrier	Male	3	13.6
Crenosomiasis	Mixed breed	Female	5	29.3
Range (median)			1–14 (6)	13.6–88.6 (51.7)
Endocrine/metabolic disorder				
Diabetes mellitus	Rottweiler	Male	5	0.6
Hypothyroidism	Labrador	Female	5	1.8
Hypoadrenocorticism	German sheepdog	Male	3	0.5
Hyperadrenocorticism	Bull terrier	Male	10	0.8
Hyperadrenocorticism	Cairn terrier	Male	8	0.9
Hyperadrenocorticism	Welsh terrier	Female	11	5.2
Metabolic myopathy	English springer spaniel	Male	2	0.8
Ovarial dysfunction	Bichon friché	Female	7	3.1
Range (median)			2–10 (5)	0.5–5.2 (0.8)
Miscellaneous				
Disc prolapse	Dalmatian	Male	6	0.6
Urolithiasis	Cairn terrier	Male	7	1.1
Urolithiasis	Fox terrier	Male	7	2.9
Vasovagal dysfunction	Boxer	Female	4	1.1
Heart insufficiency	Mixed breed	Female	3	2.0
Inguinal hernia	Pekingese	Female	1	2.7
Chronic hyperplastic enteropathy	Labrador	Male	2	0.7
Chronic hyperplastic enteropathy	Jack Russell terrier	Male	1	1.3
Range (median)			1–7 (3.5)	0.6–2.9 (1.2)

could be argued to cause pre-analytical variation, but the effect should be negligible in the present study, as canine CRP was reported to be stable at −10°C for at least 3 months (Riley and Zontine, 1972).

The CRP ELISA used in this study measured CRP in a linear and nearly proportional manner with intra- and inter-assay CVs in the range of 6.9–10.1 and 7.5–29.0%, respectively. For comparison, an ELISA employing phosphorylcholine binding of CRP revealed an intra- and inter-assay CV of 12.5 and 22%, respectively (Eckersall et al., 1989). Imprecision at the level found between runs in the present study is usually considered unacceptable for diagnostic purposes and reducing measures should be taken (Fraser, 1981). However, the increase of CRP in dogs in relation to acute inflammation is of a magnitude (Caspi et al., 1987; Conner et al., 1988; Otabe et al., 2000; Hayashi et al., 2001)

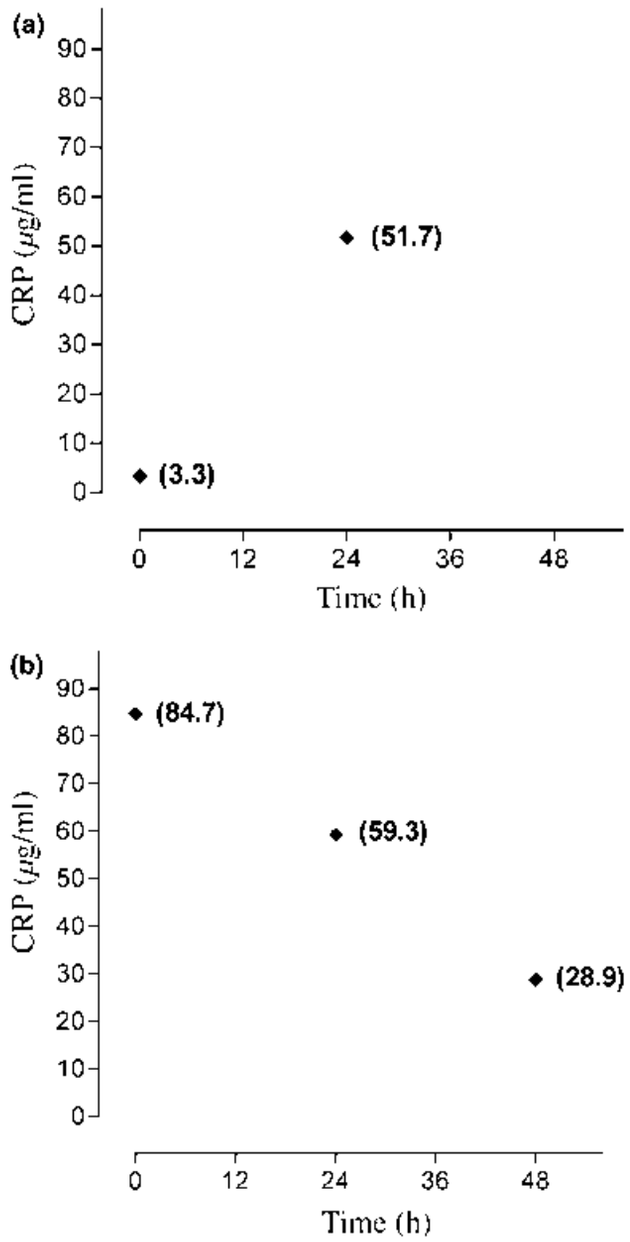

Fig. 1. Serum concentration of canine C-reactive protein (CRP) in a clinical case of acute mastitis caused by *Pseudomonas aeruginosa* in a 7-year-old intact female Great dane (a) and a clinical case of pyometra in a 5-year-old intact American bulldog subjected to ovariohysterectomy (b). The *x*-axis indicates time after first signs of local inflammation and time after ovariohysterectomy, respectively.

that would appear to diminish the consequences of a high CV. This was further sustained, as the assay was able to detect the expected dynamic changes during a spontaneous development of an inflammatory response and after the cessation of an inflammatory stimulus in two cases of acute mastitis and pyometra, respectively (Fig. 1).

The CRP level in dogs with infection was higher than in clinically healthy dogs and dogs with various other diseases (Table 3). This is in accordance to former studies on CRP and confirms that CRP could be a useful diagnostic parameter for infection.

References

Box, G. E. P., W. G. Hunter, and J. S. Hunter, 1978: Statistics for Experimenters. John Wiley and Sons, New York.

Burton, S. A., D. J. Honor, A. L. Mackenzie, P. D. Eckersall, R. J. F. Markham, and B. S. Horney, 1994: C-reactive protein concentration in dogs with inflammatory leukograms. Am. J. Vet. Res. **55,** 613–618.

Büttner, J., R. Borth, H. J. Boutwell, and P. M. G. Broughton, 1980: International federation of clinical chemistry. Approved recommendation (1978) on quality control in clinical chemistry. Part 2. Assessment of analytical methods for routine use. J. Clin. Chem. Clin. Biochem. **18,** 78–88.

Caspi, D., F. W. J. J. Snel, R. M. Batt, D. Bennett, G. R. Rutteman, E. G. Hartman, M. L. Baltz, E. Gruys, and M. B. Pepys, 1987: C-reactive protein in dogs. Am. J. Vet. Res. **48,** 919–921.

Conner, J. G., P. D. Eckersall, J. Ferguson, and T. A. Douglas, 1988: Acute phase response in the dog following surgical trauma. Res. Vet. Sci. **45,** 107–110.

Eckersall, P. D., J. G. Conner, and H. Parton, 1989: An enzyme-linked immunosorbent assay for canine C-reactive protein. Vet. Rec. **124,** 490–491.

Fraser, C. G., 1981: Analytical goals in clinical biochemistry. In: Stefani, M., and E. B. Benson (eds), Progress in Clinical Pathology, 7th edn, pp. 101–122. Grune & Stratton, New York.

Hayashi, S., T. Jinbo, K. Iguchi, M. Shimizu, T. Shimada, M. Nomura, Y. Ishida, and S. Yamamoto, 2001: A comparison of the concentrations of C-reactive protein and alpha1-acid glycoprotein in the serum of young and adult dogs with acute inflammation. Vet. Res. Commun. **25,** 117–126.

Jensen, A. L., 2000: Validation of diagnostic tests in hematology laboratories. In: Feldman, B. V., F. G. Zinkl, and N. C. Jain (eds), Schalm's Veterinary Hematology, 5th edn. pp. 20–28. Lippincott Williams and Wilkins, Philadelphia.

Jensen, A. L., M. Bomholt, and L. Moe, 2001: Preliminary evaluation of a particle enhanced turbidometric immunoassay (PETIA) for the determination of serum cystatin C-like immunoreactivity in dogs. Vet. Clin. Path. **30,** 86–90.

Ndung'u, J. M., P. D. Eckersall, and F. W. Jennings, 1991: Elevation of the concentration of acute phase proteins in dogs infected with *Trypanosoma brucei*. Acta. Trop. **49,** 77–86.

Otabe, K., T. Ito, T. Sugimoto, and S. Yamamoto, 2000: C-reactive protein (CRP) measurement in canine serum following experimentally-induced acute gastric mucosal injury. Lab. Anim. **34,** 434–438.

Otabe, K., T. Sugimoto, T. Jinbo, M. Honda, S. Kitao, S. Hayashi, M. Shimizu, and S. Yamamoto, 1998: Physiological levels of c-reactive protein in normal canine sera. Vet. Res. Commun. **22,** 77–85.

Riley, R. F., and W. Zontine, 1972: Further observations on the properties of dog C-reactive protein and the C-reactive protein response in the dog. J. Lab. Clin. Med. **80,** 698–703.

Siegel, S., and N. J. Castellan, 1988: Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

Weisberg, S., 1985: Applied Linear Regression. John Wiley and Sons, New York.

Yamamoto, S., K. Tagata, H. Nagahata, Y. Ishikawa, M. Morimatsu, and M. Naiki, 1992: Isolation of canine C-reactive protein and characterization of its properties. Vet. Immunol. Immunopathol. **30,** 329–339.

Yamamoto, S., T. Shida, S. Miyaji, H. Santsuka, H. Fujise, K. Mukawa, E. Furukawa, T. Nagae, and M. Naiki, 1993: Changes in serum C-reactive protein levels in dogs with various disorders and surgical traumas. Vet. Res. Commun. **17,** 85–93.

Yamashita, K., T. Fujinaga, T. Miyamoto, T. Hagio, Y. Izumisawa, and T. Kotani, 1994: Canine acute phase response: relationship between serum cytokine activity and acute phase protein in dogs. J. Vet. Med. Sci. **56,** 487–492.

Yamamoto, S., T. Shida, M. Honda, Y. Ashida, Y. Rikihisa, M. Odakura, S. Hayashi, M. Nomura, and Y. Isayama, 1994a: Serum C-reactive protein and immune responses in dogs inoculated with *Bordetella bronchiseptica* (phase I cells). Vet. Res. Commun. **18,** 347–357.

Yamamoto, S., T. Shida, T. Okimura, K. Otabe, M. Honda, Y. Ashinda, E. Furukawa, M. Sarikaputi, and M. Naiki, 1994b: Determination of C-reactive protein in serum and plasma from healthy dogs and dogs with pneumonia by ELISA and slide reversed passive latex agglutination test. Vet. Q. **16,** 74–77.

Paper II – Evaluation of a commercially available human C-reactive protein (CRP) turbidometric immunoassay for determination of canine serum CRP concentration

Published in
Veterinary Clinical Pathology
(2003), 32, 81-87
(Reprinted with permission)

Evaluation of a Commercially Available Human C-Reactive Protein (CRP) Turbidometric Immunoassay for Determination of Canine Serum CRP Concentration

Mads Kjelgaard-Hansen, Asger Lundorff Jensen, Annemarie T. Kristensen

Background: Serum C-reactive protein (CRP) is an acute phase marker in dogs that is useful for the diagnosis and monitoring of inflammatory disease. Rapid, reliable, and automated assays are preferable for routine evaluation of canine serum CRP concentration. **Objective**: The aim of this study was to evaluate whether canine serum CRP concentration could be measured reliably using an automated turbidometric immunoassay (TIA) designed for use with human serum. **Methods**: A commercially available TIA for human serum CRP (Bayer, Newbury, UK) was used to measure canine serum CRP concentration. Cross-reactivity of antigen was evaluated by the Ouchterlony procedure. Intra- and interassay imprecision was investigated by multiple measurements on canine serum samples and serum pools, respectively. Assay inaccuracy was investigated by linearity under dilution and comparison of methodologies (canine CRP ELISA, Tridelta Development Ltd, Kildare, UK). Then the assay was applied to serum samples from 14 clinically healthy dogs, 11 dogs with neoplasia, 13 with infections, 8 with endocrine or metabolic diseases, and 10 with miscellaneous diseases. **Results**: Cross-reactivity between canine serum CRP and the anti-human CRP antibody was found. Intra- and interassay imprecision ranged from 5.2% to 10.8% and 3.0% to 10.2%, respectively. Serum CRP concentration was measured in a linear and proportional manner. There was no significant disagreement and there was linear correlation of the results in the comparison of methodologies, except for a slight proportional discrepancy at low CRP concentrations (<10 µg/mL). Dogs with infections had a significantly higher concentration of serum CRP than did all other dogs, and dogs with neoplasia had a significantly higher concentration of serum CRP than did clinically healthy dogs. **Conclusions**: Canine serum CRP concentration can be measured reliably using the commercially available TIA designed for human CRP (*Vet Clin Pathol.* 2003;32:81-87).

Key Words: Acute phase marker, C-reactive protein, dog, immunoassay, test validation

Several researchers have identified serum C-reactive protein (CRP) as a useful acute phase marker in the dog. The normal physiologic concentration of CRP is low but increases rapidly with the onset of acute inflammation or tissue destruction, and changes in CRP during the course of disease seem to reflect the underlying disease activity.[1-11] Several methods for measuring canine serum and plasma CRP concentrations have been developed for use in individual studies, including ELISA,[6,12] slide reversed passive latex agglutination,[9] immunodiffusion,[4,6] electroimmunoassay,[11] quantitative capillary reversed passive latex agglutination,[13] and immunoturbidometric assay.[14] Recently, a commercially available ELISA specific for canine CRP was validated.[15] However, to use CRP concentration for routine diagnosis and monitoring, a rapid automated assay is needed. A turbidometric immunoassay (TIA) for measuring human CRP concentration is available (Bayer CRP TIA, Bayer, Newbury, UK). The principle of the analysis is the binding of CRP to polyclonal goat anti-human CRP antibodies to form a precipitate that is measured turbidometrically. In previous investigations of the cross-reactivity of canine CRP with different polyclonal and monoclonal anti-human CRP antibodies, either no cross-reactivity[11,16-18] or insufficient cross-reactivity for assay purposes has been recorded.[6,19,20]

The aim of this study was to evaluate the reliability of the Bayer CRP TIA for measuring canine serum CRP concentration. We investigated the cross-reactivity of canine CRP with the anti-human CRP antibody, assay characteristics (imprecision, inaccuracy, and detection limit), and the ability of the assay to differentiate between clinically healthy dogs and relevant groups of diseased dogs.

Materials and Methods

CRP analysis

The Bayer CRP TIA (lot 0198X) designed for the determination of human serum CRP concentration was used for the heterologous determination of CRP concentration in canine serum samples. According to the manufacturer's

From the Central Laboratory (Kjelgaard-Hansen, Jensen) and the Small Animal Hospital (Kristensen), Department of Clinical Studies, The Royal Veterinary and Agricultural University, DK-1870 Frederiksberg, Denmark. Corresponding author: Mads Kjelgaard-Hansen, Central Laboratory, Department of Clinical Studies, Royal Veterinary and Agricultural University, 3 Gronnegaardsvej, DK-1870 Frederiksberg, Denmark (mjkh@kvl.dk).

recommendations, the analysis was performed using an automated analyzer (ADVIA 1650 Chemistry System, Bayer); a human CRP calibrator (lot 0226X, Bayer) was used to create the calibration curve. For samples used for method comparison, the concentration of CRP was determined simultaneously using a commercially available ELISA specific for canine CRP (canine CRP ELISA, Tridelta Development Ltd, Kildare, UK) that had been validated previously.[15]

Cross-reactivity of antigen

Investigation of cross-reactivity between canine CRP and the goat anti-human CRP antibody (Bayer) was performed using an Ouchterlony procedure[21] in a 1% (w/v) agarose gel (Agarose Litex, Bie & Berntsen, Rodovre, Denmark) prepared in phosphate-buffered saline (0.01 M, pH 7.7, Bie & Berntsen). The goat anti-human CRP antibody was placed in the center well, and human CRP calibrator (Bayer) and canine serum samples were placed in evenly distributed adjacent wells. Canine sera included 2 canine serum samples with high CRP concentrations (61.0 µg/mL and 89.9 µg/mL) and a canine serum pool with a high CRP concentration (102.6 µg/mL) as determined by ELISA (Tridelta Development Ltd). The gel was scored after 24 hours of incubation in a humid chamber at room temperature (~25°C).

Assay characteristics of the TIA

The intra-assay variation between replicates was determined as the coefficient of variation (CV) from a pooled variance estimate of the difference between duplicate determinations at low (<20 µg/mL) and high (>20 µg/mL) CRP concentrations from 39 and 40 observations, respectively. The interassay CV was determined from the mean and SD of 7 replicate determinations of 2 serum pools with different CRP concentrations. The pools were frozen in aliquots, and only vials needed for each analytical run were thawed to prevent potential variation due to repetitive freeze-thaw cycles. Inaccuracy was investigated by method comparison and evaluation of linearity under dilution.[22] For the study of linearity under dilution, duplicate determinations of CRP concentration were made on dilutions of a serum pool with a high concentration of CRP diluted 0%, 10%, 20%, 40%, 50%, 60%, 90%, 95%, and 100% using physiologic saline (0.9% NaCl). The detection limit (DL) was calculated based on data from 12 replicate determinations of physiologic saline.[23]

Samples

Fifty-six dogs were included in the study. Forty-eight dogs were presented by their owners to the Small Animal Veterinary Teaching Hospital (Royal Veterinary and Agricultural University, Denmark) for various diagnostic, therapeutic, or prophylactic procedures between December 2001 and May 2002. Eight dogs were clinically healthy Beagles from a research colony. All dogs underwent clinical examination, and the results of standard hematological and clinical chemical profiles were evaluated at our laboratory.[24] Additional diagnostic tests for each dog were conducted at the discretion of the individual clinician (eg, endocrine testing, biopsies, radiography, evaluation of blood smears, or microbial testing).

Based on the results of the above examinations and the final clinical diagnosis, the 56 dogs were retrospectively assigned to 5 groups: clinically healthy dogs (n=14), dogs with neoplasia (n=11), dogs with infections (n=13), dogs with endocrine/metabolic disorders (n=8), and dogs with miscellaneous diseases not belonging to any of the former groups (n=10). Clinically healthy dogs had unremarkable findings on physical and clinical pathologic examinations. Dogs with neoplasia had tumors that were cytologically and/or histologically characterized as neoplastic (benign and malignant). Dogs with infections had a pathogen identified that could be related to the pathologic findings. Dogs with endocrine/metabolic disorders had a final diagnosis that could be related to a hormonal or metabolic malfunction or disorder. Dogs with miscellaneous diseases had various diagnoses and no clinical findings to associate them with any of the other groups.

Seventy-nine serum samples, selected from samples submitted to our laboratory between December 2001 and May 2002 from dogs presented to our hospital, were used to investigate the assay characteristics of the TIA method and for method comparison. Nineteen of these samples were from dogs noted above and the remainder from unclassified dogs. The selections were made at random from samples with low (<20 µg/mL) and high (>20 µg/mL) CRP concentrations (n=39 and n=40, respectively) as determined by ELISA (Tridelta Development Ltd). In all cases, serum was prepared by centrifugation (2000*g*, 5 minutes) of blood samples after collection in plain vials with clot activator and separation gel (Vacuette, Greiner Bio-One, Kremsmuenster, Austria). The serum samples were stored in plastic vials at −80°C until analysis, for a maximum of 6 months.

Statistical analysis

Arithmetic means, medians, intra- and interassay CVs , and DL ($P < .01$) were calculated using routine descriptive statistical procedures.[23,25] Investigation of linearity under dilution was accomplished by ordinary least-squares linear regression analysis with linear and loga-

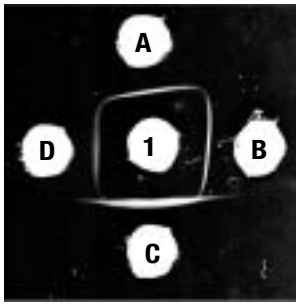

Figure 1. Ouchterlony procedure for visualization of cross-reactivity between polyclonal goat anti-human C-reactive protein (CRP) antibodies and canine sera. (**A** and **D**) Canine sera with high CRP concentrations (**A**, 89.9 µg/mL; **D**, 61.0 µg/mL). (**B**) Canine serum pool with a high CRP concentration (102.6 µg/mL). (**C**) Human CRP (TIA calibrator 1005 µg/mL). (**1**) Polyclonal goat anti-human CRP antibody (TIA reagent).

rithmic transformations. Logarithmic transformations were performed to achieve homogeneity of the variance.[26] Methods were compared with the Wilcoxon signed rank test to determine whether paired values from both methods were significantly different,[27] with an Altman-Bland plot to reveal any possible discrepancy,[22] and with Passing-Bablok regression analysis to derive regression data.[28] The Runs test was performed in all types of regression analyses to determine whether data deviated significantly from the applied model.[29] Comparison between diagnostic groups was made using the Kruskal-Wallis test; when significant results were obtained, Dunn's multiple comparison test was applied to get a more detailed picture of the deviation.[29] Significance was set at $P < .05$ unless otherwise stated.

Results

Cross-reactivity of antigens

The result of the Ouchterlony procedure indicated that the polyclonal goat anti-human CRP antibodies used in the TIA were able to precipitate with canine sera and serum pool with high CRP concentrations (Figure 1). A reaction of partial identity between the TIA human CRP calibrator and the canine sera was observed, with spurs formed, indicating that only a fraction of the polyclonal anti-human CRP antibodies formed a precipitate with canine acute phase sera, but also indicating that the precipitation with the canine sera was as specific to canine CRP as it was to human CRP (assay calibrator; no reaction of nonidentity was seen). Thus, the canine sera contained antigenic determinants identical to human CRP antigenic determinants, but no antigenic determinants different from human CRP antigenic determinants that precipitated with the polyclonal goat anti-human CRP antibodies.

Table 1. Intra- and interassay variation in determinations of C-reactive protein (CRP) concentration in canine serum samples.

Comparison	No. Samples	CRP Concentration (µg/mL)		CV* (%)
		Mean	SD	
Intra-assay†	39	4.0	0.43	10.8
	40	67.0	3.48	5.2
Interassay‡	7	3.8	0.39	10.2
	7	101.6	3.05	3.0

*CV indicates coefficient of variation.
†Based on duplicate determinations of different serum samples.
‡Based on replicate determinations of the same serum pool.

Assay characteristics

The intra- and interassay CVs ranged from 5.2% to 10.8% and 3.0% to 10.2%, respectively, with the highest CVs in serum samples with low CRP concentrations (Table 1). The investigation of inaccuracy by dilution of a canine serum pool resulted in a linear regression equation in which x = the expected level according to dilution of the serum pool and y = the measured level and in which the intercept and slope did not differ from 0 and 1, respectively (Table 2). The Runs test revealed that the data did not deviate significantly from the linear model ($P = .07$). In the corresponding logarithmic regression analysis, the slope was not different from 1, thereby indicating parallelism, and the logarithmic model also showed linearity (Runs, $P = .11$). Hence, the TIA method measured canine serum CRP concentration in a linear and proportional manner. The detection limit was 0.07 µg/mL (mean 0.02 µg/mL, SD 0.02 µg/mL), which was below the range for normal canine serum CRP concentration observed in previous studies using other methods (0.8-22.6 µg/mL,[30] 2.4-30.0 µg/mL,[9] and 1.1-6.3 µg/mL[15]) and in this study (0.5-8.4 µg/mL).

Table 2. Analytical inaccuracy of C-reactive protein (CRP) concentrations assessed by dilution of a canine serum pool with a high CRP concentration.

Value	Regression	
	Linear	Logarithmic
y-intercept (95% CI*)	6.29 (–3.35, 15.92)	0.18 (0.04, 0.32)
Slope (95% CI)	1.02 (0.90, 1.14)	0.93 (0.86, 1.02)
P (Runs)†	.07	.11
r‡	.99	1.00

*CI indicates confidence interval.
†Probability for model fitting, ie, that the data follow a straight line (Runs test).
‡Coefficient of correlation.

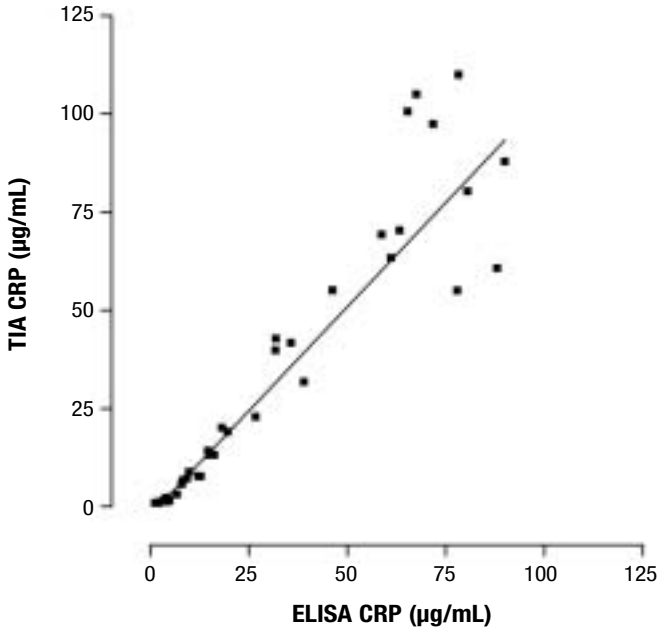

Figure 2. Passing-Bablok regression analysis for C-reactive protein (CRP) concentrations in canine serum samples using an ELISA specific for canine CRP (ELISA CRP) and a turbidometric immunoassay (TIA CRP) designed for measuring human CRP concentration (n = 38). The solid line represents the regression equation: TIA CRP (µg/mL) = 1.06×ELISA CRP (µg/mL)-2.16.

Table 3. Regression data for comparison of ELISA and turbidometric immunoassay (TIA) for evaluation of C-reactive protein concentrations.

Value	ELISA vs TIA
n	38
y-intercept (95% CI*)	–2.16 (–3.73, –1.11)
Slope (95% CI)	1.06 (0.97, 1.22)
P (Runs)†	.22
P (W) ‡	.83

*CI indicates confidence interval.
†Probability for model fitting, ie, that the data follow a straight line (Runs test).
‡Probability for Wilcoxon signed rank test, ie, whether the paired values from both methods are significantly different.

Method comparison

Results of the Wilcoxon signed rank test indicated that results from the 2 methods were not significantly different ($P = .83$), the data did not deviate significantly from the linear regression model (Runs, $P = .22$), and the slope was not different from 1 (Figure 2 and Table 3). However, the y-intercept differed from 0 (–2.16 µg/mL), and the Altman-Bland plot indicated that a proportional discrepancy at low CRP concentrations (<10 µg/mL) might be present, although the deviations were small (Figure 3).

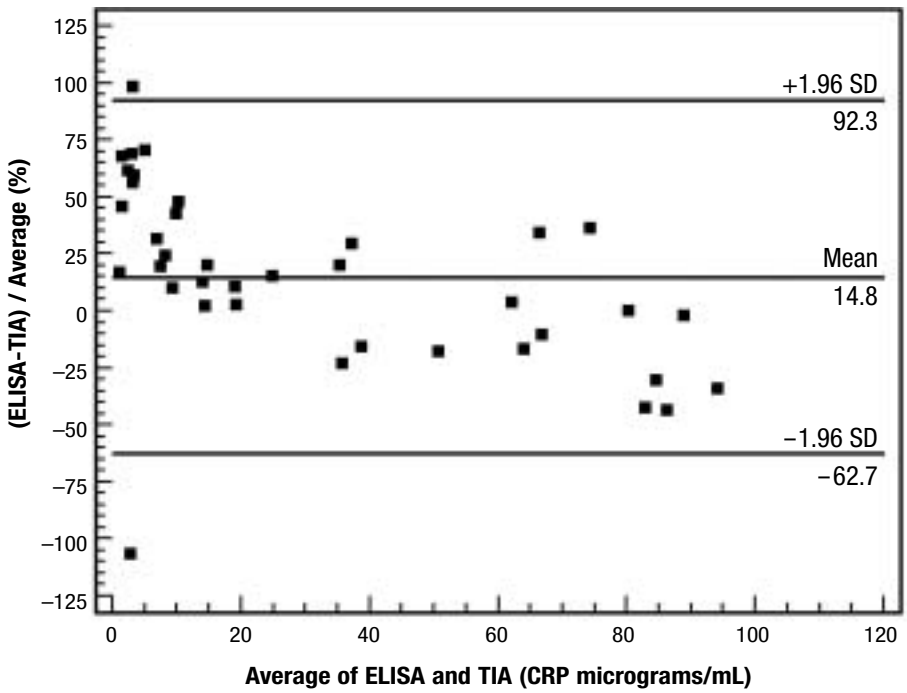

Figure 3. Altmann-Bland difference plot for C-reactive protein (CRP) concentrations in canine serum samples using an ELISA specific for canine CRP and a turbidometric immunoassay (TIA) designed for measuring human CRP concentration (n = 38). Lines indicate mean difference and ±1.96 SD of the mean difference.

Comparison of serum CRP concentrations in healthy and diseased dogs

The medians (ranges) of CRP concentrations in the 5 groups of dogs were: clinically healthy dogs, 0.9 (0.5-8.4) µg/mL; dogs with neoplasia, 5.0 (1.1-91.9) µg/mL; dogs with infections, 60.4 (33.3-88.8) µg/mL; dogs with endocrine/metabolic diseases, 2.4 (0.1-26.9) µg/mL; and dogs with miscellaneous diseases, 3.9 (0.6-13.9) (Figure 4). The results of the Kruskal-Wallis analysis indicated that the medians of the different groups were significantly different ($P < .0001$). The multiple comparison test showed that median serum CRP concentration in dogs with infections was significantly higher than that in any other group ($P < .001$). Dogs with neoplasia had a significantly higher median CRP concentration than did clinically healthy dogs ($P < .01$). Median CRP

concentration was not significantly different ($P < .05$) in any other pair of groups.

Discussion

Several researchers have shown that serum CRP concentration is a useful acute phase marker for routine diagnostic purposes in dogs.[1-11] However, none of the previous methods available for measuring canine serum CRP concentration was ideal for providing clinically useful information in a routine diagnostic setting, where a rapid, reliable, and automated method would be preferable. The reliability of the rapid automated Bayer CRP TIA evaluated in this study was found to be acceptable for determination of CRP in canine serum.

The Bayer CRP TIA measured heterologous canine serum CRP concentration with intra- and interassay CVs in the ranges of 5.2-10.8% and 3.0-10.2%, respectively. This level of intra- and interassay imprecision was comparable to that of a commercially available ELISA specific for canine CRP,[15] although with the ELISA imprecision increased with increasing CRP concentrations, whereas the opposite trend was observed for the TIA. The regression analysis of method comparison was not biased because of these differences in imprecision, because Passing-Bablok regression is insensitive to differences in imprecision.[31] The level of imprecision was within the limits of the objective analytical performance standard for imprecision (CV_{max}=12.2%) derived from data on biological variation of canine CRP concentration.[32]

Canine serum CRP concentration was measured in a linear and proportional manner by the Bayer TIA. Comparison of methodologies showed no significant differences in results and indicated a linear correlation of the results of the canine-specific ELISA and the TIA. However, a small proportional discrepancy was apparent at low CRP concentrations (<10 µg/mL). The origin of this discrepancy was not determined in the present study, and further clarification may require access to canine CRP standards. The median serum CRP concentration in dogs with infections was, as expected, higher compared to that of clinically healthy dogs and dogs with various other diseases. This finding is in agreement with those from other studies on canine serum CRP concentration. The group of dogs with neoplasia had a significantly higher median concentration of CRP than did the clinically healthy dogs, as has been shown for canine acute phase proteins[33,34] and human CRP concentrations in similar comparisons.[35,36]

The possibility that the stability of CRP was compromised during freezing is unlikely, because canine CRP has been reported to be stable at –10°C for at least 4 months.[37] The Ouchterlony procedure revealed that

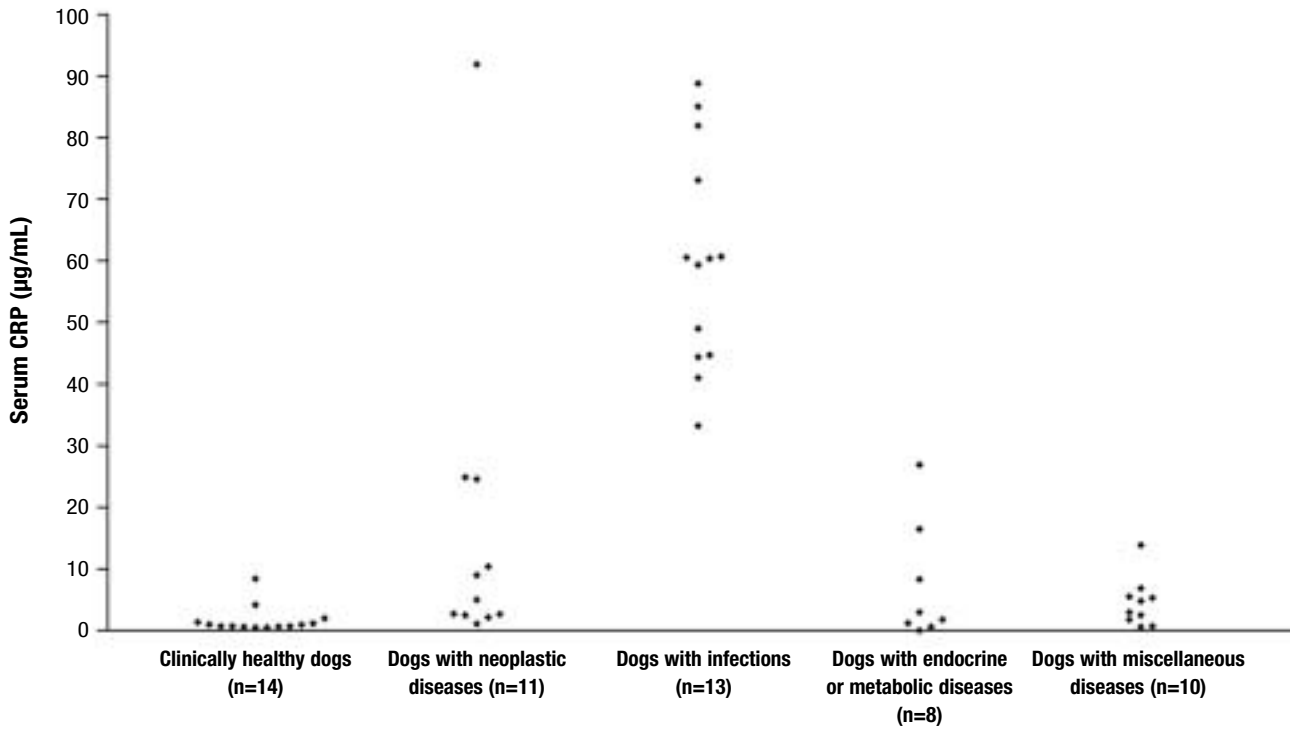

Figure 4. Serum C-reactive protein (CRP) concentrations in 5 groups of dogs, measured using the Bayer CRP TIA, a turbidometric immunoassay designed for determination of human serum CRP concentration.

the canine sera and serum pool with high CRP levels contained a heterologous cross-reacting antigen, and that none of the cross-reacting antigens differed from those in the TIA calibrator. This finding strongly indicates that the agreement in the results of the 2 techniques was not due to nonspecific reactions, but rather that the TIA most likely measured canine serum CRP concentration specifically. In several investigations of cross-reactivity between canine CRP and anti-human CRP antibodies, either no cross-reactivity has been observed[11,16-18] or cross-reactivity was insufficient for assay purposes.[6,19,20] To our knowledge, this is the first study to show enough cross-reactivity between canine CRP and anti-human CRP antibodies to be meaningful for assay purposes.

The results of the present study indicate that canine serum CRP concentration can be measured reliably using the Bayer CRP TIA, which is designed for measuring human serum CRP concentration. Because this assay method is automated and rapid, it is appropriate for routine use in diagnostic laboratories. ◊

References

1. Hayashi S, Jinbo T, Iguchi K, et al. A comparison of the concentrations of C-reactive protein and alpha1-acid glycoprotein in the serum of young and adult dogs with acute inflammation. *Vet Res Commun.* 2001;25:117-126.
2. Otabe K, Ito T, Sugimoto T, Yamamoto S. C-reactive protein (CRP) measurement in canine serum following experimentally-induced acute gastric mucosal injury. *Lab Anim.* 2000;34: 434-438.
3. Yamamoto S, Shida T, Honda M, et al. Serum C-reactive protein and immune responses in dogs inoculated with *Bordetella bronchiseptica* (phase I cells). *Vet Res Commun.* 1994;18:347-357.
4. Conner JG, Eckersall PD, Ferguson J, Douglas TA. Acute phase response in the dog following surgical trauma. *Res Vet Sci.* 1988;45:107-110.
5. Burton SA, Honor DJ, Mackenzie AL, et al. C-reactive protein concentration in dogs with inflammatory leukograms. *Am J Vet Res.* 1994;55:613-618.
6. Yamamoto S, Tagata K, Nagahata H, et al. Isolation of canine C-reactive protein and characterization of its properties. *Vet Immunol Immunopathol.* 1992;30:329-339.
7. Caspi D, Snel FWJJ, Batt RM, et al. C-reactive protein in dogs. *Am J Vet Res.* 1987;48:919-921.
8. Ndung'u JM, Eckersall PD, Jennings FW. Elevation of the concentration of acute phase proteins in dogs infected with *Trypanosoma brucei. Acta Trop.* 1991;49:77-86.
9. Yamamoto S, Shida T, Okimura T, et al. Determination of C-reactive protein in serum and plasma from healthy dogs and dogs with pneumonia by ELISA and slide reversed passive latex agglutination test. *Vet Q.* 1994;16:74-77.
10. Yamashita K, Fujinaga T, Miyamoto T, et al. Canine acute phase response: relationship between serum cytokine activity and acute phase protein in dogs. *J Vet Med Sci.* 1994;56:487-492.
11. Caspi D, Baltz ML, Snel F, et al. Isolation and characterization of C-reactive protein from the dog. *Immunology.* 1984;53:307-313.
12. Eckersall PD, Conner JG, Parton H. An enzyme-linked immunosorbent assay for canine C-reactive protein. *Vet Rec.* 1989;124:490-491.
13. Tagata K, Yokoyama S, Ginbo T, et al. Quantitative capillary reversed passive latex agglutination test for C-reactive protein (CRP) in the dog. *Vet Res Commun.* 1996;20:21-30.
14. Eckersall PD, Conner JG, Harvie J. An immunoturbidimetric assay for canine C-reactive protein. *Vet Res Commun.* 1991;15: 17-24.
15. Kjelgaard-Hansen M, Kristensen AT, Jensen AL. Evaluation of a commercially available enzyme-linked immunosorbent assay (ELISA) for the determination of C-reactive protein in canine serum. *J Vet Med A.* 2003;50:164-168.
16. Parra MD, Martinez-Subiela S, Tuomola M, Ceron JJ. Evaluation of different monoclonal antibodies against human CRP in a TR-FIA for determination of canine CRP. Available at: http://www.gla.ac.uk/faculties/vet/research/protein/03_doorn_2002/abstracts/Ceron.html. Accessed April 10, 2003.
17. Yamamoto S, Miyaji S, Abe N, et al. Canine C-reactive protein (CRP) does not share common antigenicity with human CRP. *Vet Res Commun.* 1993;17:259-266.
18. Yamamoto S, Tagata K, Otabe K, Shida T. Serum levels of C-reactive protein in dogs and simple techniques for its measurement. *Nippon Juishikai Zasshi.* 1993;46:870-873.
19. Riley RF, Coleman MK. Isolation of C-reactive proteins of man, monkey, rabbit and dog by affinity chromatography on phosphorylated cellulose. *Clin Chim Acta.* 1970;30:483-496.
20. Dillman RC, Coles EH. A canine serum fraction analogous to human C-reactive protein. *Am J Vet Res.* 1966;27:1769-1775.
21. Ouchterlony Ö. Diffusion-in-gel methods for immunological analysis. *Prog Allergy.* 1958;5:1-78.
22. Jensen AL. Validation of diagnostic tests in hematology laboratories. In: Feldman BV, Zinkl JG, Jain NC, eds. *Schalm's Veterinary Hematology.* 5th ed. Philadelphia, PA: Lippincott Williams and Wilkins; 2000:20-28.
23. Büttner J, Borth R, Boutwell HJ, Broughton PMG. International Federation of Clinical Chemistry. Approved recommendation (1978) on quality control in clinical chemistry. Part 2. Assessment of analytical methods for routine use. *J Clin Chem Clin Biol.* 1980;18:78-88.
24. Jensen AL, Bomholt M, Moe L. Preliminary evaluation of a particle enhanced turbidometric immunoassay (PETIA) for the determination of serum cystatin C-like immunoreactivity in dogs. *Vet Clin Pathol.* 2001;30:86-90.
25. Box GEP, Hunter WG, Hunter JS. *Statistics for Experimenters.* 3rd ed. New York, NY: John Wiley and Sons; 1978.
26. Weisberg S. *Applied Linear Regression.* 2nd ed. New York, NY: John Wiley and Sons; 1985.
27. Jones RG, Payne RB. *Clinical Investigation and Statistics in Laboratory Medicine.* 1st ed. London, UK: ACB Venture Publications; 1997.

28. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, part I. *J Clin Chem Clin Biol.* 1983;21:709-720.

29. Siegel S, Castellan NJ. *Nonparametric Statistics for the Behavioral Sciences.* 2nd ed. New York, NY: McGraw-Hill; 1988.

30. Otabe K, Sugimoto T, Jinbo T, et al. Physiological levels of C-reactive protein in normal canine sera. *Vet Res Commun.* 1998; 22:77-85.

31. Payne RB. Method comparison: evaluation of least squares, Deming and Passing/Bablok regression procedures using computer simulation. *Ann Clin Biochem.* 1997;34:319-320.

32. Kjelgaard-Hansen M, Mikkelsen LF, Kristensen AT, Jensen AL. Study on biological variability of five acute phase reactants in dogs. *Comp Clin Pathol.* In press.

33. Hahn KA, Freeman KP, Barnhill MA, Stephen EL. Serum alpha1-acid glycoprotein concentrations before and after relapse in dogs with lymphoma treated with doxorubicin. *J Am Vet Med Assoc.* 1999;214:1023-1025.

34. Ogilvie GK, Walters LM, Greeley SG, Henkel SE, Salman MD. Concentration of alpha1-acid glycoprotein in dogs with malignant neoplasia. *J Am Vet Med Assoc.* 1993;203:1144-1146.

35. Nozoe T, Matsumata T, Sugimachi K. Preoperative elevation of serum C-reactive protein is related to impaired immunity in patients with colorectal cancer. *Am J Clin Oncol (CCT).* 2000;23: 263-266.

36. Nozoe T, Matsumata T, Sugimachi K. Serum level of C-reactive protein may be a marker for proliferation of esophageal carcinoma. *Hepatogastroenterology.* 2000;47:1622-1623.

37. Riley RF, Zontine W. Further observations on the properties of dog C-reactive protein and the C-reactive protein response in the dog. *J Lab Clin Med.* 1972;80:698-703.

Paper III – Study on biological variability of five acute phase reactants in dogs

Published in
Comparative Clinical Pathology
(2003), 12, 69-74
(Reprinted with permission)

Paper IV – Internal quality control of a turbidimetric immunoassay for canine serum C-reactive protein based on pooled patient samples

Published in
Veterinary Clinical Pathology
(2004), 33, 139-144
(Reprinted with permission)

Internal quality control of a turbidimetric immunoassay for canine serum C-reactive protein based on pooled patient samples

Mads Kjelgaard-Hansen, Asger Lundorff Jensen, Annemarie T. Kristensen

Background: Optimized internal quality control (IQC) procedures are important to ensure that only results without medically important errors are used for medical decision-making and to ensure that unnecessary rejection of valid analytical runs is avoided. Additionally, estimates of the analytical performance can be derived from IQC data. In the absence of available species-specific standards of a compound, the use of alternative control materials based on patient samples is a possibility, although investigations on the suitability of this approach are needed. **Objectives:** The objective of the study was to plan and implement a simple IQC procedure with control material based on pooled canine serum samples for a turbidimetric immunoassay (TIA) for the determination of human C-reactive protein (CRP) that recently was validated for the determination of canine serum CRP, and to assess the clinical analytical performance of the assay. **Methods:** Proposed guidelines for the planning and implementation of IQC procedures were followed by using 2 control materials. Quality requirements of the assay were defined objectively by means of available data on biological variation, and goals for IQC performance were defined according to recommendations (probability of error detection [P_{ed}] $> .90$ and of false rejection [P_{fr}] $< .05$). Analytical performance was evaluated by means of medical decision charts. **Results:** The control rule of $1_{2.5s}$ (ie, rejection of the analytical run if at least 1 of 2 control materials deviates from the mean by more than 2.5 SD) fulfilled the criteria of predicted IQC performance ($P_{ed} = .94–1.00$, $P_{fr} = .03$). The IQC method was successfully implemented over a 14-week period. The observed coefficient of variation in the period of monitoring was 3.8% (low) and 2.9% (high), which equals excellent analytical performance. **Conclusions:** It was possible to plan and implement a simple IQC procedure for the CRP-TIA with control materials based on canine serum samples that fulfilled the criteria of high error detection and low false rejection of valid analytical runs. The assay showed excellent long-term analytical performance over a 14-week period. (*Vet Clin Pathol*. 2004;33:139–144)

Key Words: Analytical performance, control material, C-reactive protein, dog, immunoassay, quality control

Internal quality control (IQC) is an important task within the discipline of clinical chemistry. The reason to apply proper IQC is primarily to ensure that results used for medical purposes are reported only without medically important errors (ie, errors that could possibly alter the clinical decision). Another aspect of proper IQC is to minimize the frequency or risk of falsely rejecting valid analytical runs to minimize unnecessary costs and waste in the laboratory.

Several guidelines have been proposed for the implementation and optimization of statistical IQC,[1,2] which describe steps of defining quality requirements and setting up appropriate IQC procedures to optimize detection of medically important errors and reduce the risk of false rejection.

IQC usually is based on control materials traceable to a certified reference material, when available (eg, the International Federation of Clinical Chemistry and Laboratory Medicine reference material #470 for human serum components).[3] Such standards and traceable control materials are not yet available for canine acute phase proteins (eg, canine serum C-reactive protein [CRP]), although the problem is recognized and work has been initiated.[4] Thus, it could be of interest to investigate the implemention of an IQC procedure for canine CRP based on pooled patient samples as an alternative control material.

Recently, an automated commercially available human CRP turbidimetric immunoassay (CRP-TIA; Bayer, Newbury, UK) was validated for the determina-

From the Central Laboratory (Kjelgaard-Hansen, Jensen) and the Small Animal Veterinary Teaching Hospital (Kristensen), Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark. Corresponding author: Mads Kjelgaard-Hansen, Central Laboratory, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University, 3 Gronnegaardsvej, DK-1870 Frederiksberg, Denmark (mjkh@kvl.dk).

Table 1. Proposed steps for planning internal quality control (IQC).

1. Define quality requirements for the test
2. Determine method imprecision and bias
3. Identify candidate IQC procedures
4. Select goals for IQC performance
5. Select an appropriate IQC procedure

tion of canine serum CRP concentration.[5] The scope of the present study was to plan and implement an IQC procedure for the CRP-TIA according to existing guidelines[1,2] by using control material based on pooled patient samples. In addition, we estimated the long-term analytical performance of the assay under the control of the implemented IQC procedure.

Materials and methods

Assay

The Bayer CRP-TIA designed for the determination of human serum CRP was used for the heterologous determination of CRP in canine serum samples for which it was previously validated.[5] The principle of the analysis is the binding of canine CRP to polyclonal goat antihuman CRP antibodies to form a precipitate that is measured turbidimetrically. Human CRP calibrator (Bayer) was used to create a standard curve, and the analysis was performed with an automated analyzer (ADVIA 1650 chemistry system, Bayer) according to the manufacturer's description.

Control material

Serum samples originated from 15 client-owned dogs presented to the Small Animal Veterinary Teaching Hospital, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark, for various diagnostic, therapeutic or prophylactic measures. Serum was prepared by centrifugation (2000*g*, 5 minutes) within 1 hour after collection of the blood in plain vials with clot activator and separation gel (Vacuette, Greiner Bio-One, Kremsmuenster, Austria). Two serum pools with different concentrations of CRP (approximately 20 mg/L [low] and 65 mg/L [high], as determined by the CRP-TIA) were created by mixing a number (n) of individual serum samples with low and high CRP concentrations as determined by the CRP-TIA (range 15–35 mg/L [n = 6] for the low pool and 50–95 mg/L [n = 9] for the high pool). The pools were aliquoted and stored for a maximum of 4 months in plastic vials at –80°C before analysis.

Planning the IQC

The IQC procedure was planned as outlined by proposed guidelines (Table 1).[1,2]

Define quality requirements for the test. The predefined quality requirements to perform optimal IQC planning were set equal to previously derived objective analytical performance standards from a study on biological variation of canine serum CRP (maximum allowable bias = 9.5% and maximum allowable total error [TE_{max}] = 29.6%).[6]

Determine method imprecision and bias. Method imprecision was assessed in a small pilot study consisting of 5 separate analytical runs of the pools (data not shown); imprecision was approximately 4% at the 2 control levels. The true bias of the assay could not be assessed because of the lack of available external standards. Because the previous study showed no significant bias,[5] the assay was assumed to have acceptable bias (<9.5%). To assess the sensitivity of the IQC procedure to changes in bias, both extremes of acceptable observed bias (0.0% and 9.5%) were used in the following steps.

Identify candidate IQC procedures. The list of candidate IQC procedures included 4 single rules with constant limits, ie, the rule is violated if one of the controls deviates more than a given number (x) of SDs from the mean of the control material ($1_{(x)s}$, 1_{2s}, $1_{2.5s}$, 1_{3s}, and $1_{3.5s}$), and 1 multirule procedure ($1_{3s}/2_{2s}$), ie, either of the control materials deviate more than 3 SDs or both materials deviate more than 2 SDs from the mean within the same analytical run.

Select goals for IQC performance. The goals for IQC performance were set to a probability for detection of medically important systematic error (P_{ed}) of .90 or higher and a probability of false rejection of a valid analytical run (P_{fr}) of .05 or lower.

Select an appropriate IQC procedure. The predicted performance of the candidate IQC procedures was estimated by means of the commercially available software package Validator 2.0 (Westgard Quality Corporation, Ogunquit, ME, USA). The critical systematic error (ie, the systematic shift that would cause a 5% risk of reporting a medically incorrect test result ([total allowable error – observed bias]/observed imprecision – 1.65) was calculated,[2] which in the present study gave 5.75s and 3.38s for observed bias of 0.0% and 9.5%, respectively. The predicted performance was then estimated with power functions for the candidate IQC procedures, described in detail elsewhere,[1] and expressed as P_{ed} and P_{fr} for the detection of medically important systematic errors. The simplest IQC procedure to fulfill the selected goals for IQC performance was selected for implementation.

Implementation of IQC

The selected IQC procedure was implemented and performed twice weekly from January 24 to May 8, 2003, by thawing and analyzing a new vial of control material at each control point. Violation of the IQC rule was monitored by QCTool (Westgard Quality Corporation), available online.[7] If the control rule was violated, error detection was performed and new controls were run immediately after correction of the error.

Evaluation of analytical performance

A method evaluation decision chart (MEDx)[8] was used to assess the analytical performance of the CRP-TIA during stable analytical performance. The MEDx method uses the objective analytical performance standard for TE_{max} derived from data on biological variation to rank the performance as poor, marginal, good, or excellent according to defined criteria.[8] The observed imprecision of the CRP-TIA (expressed as the coefficient of variation of the non-rejected measurements of the control material) was plotted against the full range of acceptable bias (0.0–9.5%) to assess the performance.

Results

The control rule of $1_{2.5s}$ was the simplest rule to fulfill the criteria of predicted IQC performance (P_{ed} = .94–1.00, P_{fr} = .03) within the range of acceptable bias (0.0–9.5%). All candidate procedures except 1_{2s} (P_{ed} = 1.00, P_{fr} = .09) fulfilled the criteria at minimum bias (Figure 1A), but only $1_{2.5s}$ and $1_{3s}/2_{2s}$ fulfilled the criteria at the maximum allowable bias (Figure 1B). The 1_{3s} and $1_{3.5s}$ rules had a P_{ed} that was too low, and 1_{2s} had a P_{fr} value that was too high. Thirty control measurements of each control material were performed during the period of monitoring (Figure 2), and all analytical runs were in control except for 2 that exceeded the $1_{2.5s}$ criteria (run 9 [low] and run 28 [high]), thus indicating a medically important error. In both instances, troubleshooting detected a lamp error as the cause, and an acceptable control run was obtained immediately after correction of the lamp error. The observed analytical imprecisions of the assay during stable analytical performance were 3.8% (low) and 2.9% (high), not exceeding the 4% used during IQC development.

Evaluation of the CRP-TIA by MEDx chart showed that the observed imprecision at both levels of CRP corresponded to excellent analytical performance within the range of acceptable bias (Figure 3).

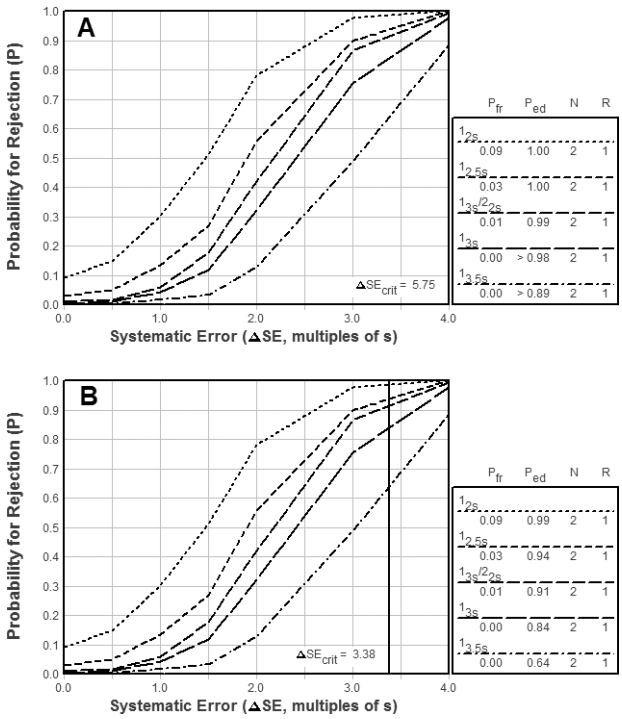

Figure 1. Power functions for systematic error for 5 different control rules of 2 control materials (N = 2) within the same analytical run (R = 1). Four single rules (1_{2s}, $1_{2.5s}$, 1_{3s}, and $1_{3.5s}$) with constant limits (ie, the rule is violated if one of the controls deviates more than a given number [x] of SDs from the mean observation of the control material [$1_{(x)s}$]) and 1 multirule procedure ($1_{3s}/2_{2s}$) (ie, either of the control materials deviate more than 3 SDs or both materials deviate more than 2 SDs from the mean observation within the same analytical run) were included. Probabilities of error detection (P_{ed}) and of false rejection (P_{fr}) for the critical systematic error (SE_{crit}) are shown in the boxes at the right. The SE_{crit} = 5.75 (**A**) and 3.38 (**B**) corresponding to an observed bias of 0.0% and 9.5%, respectively.

Discussion

Planning and implementation of IQC in clinical chemistry laboratories is important to ensure that results with medically important errors are not reported and used for diagnostic purposes and to ensure that the risk of rejecting valid analytical runs is minimized to avoid unnecessary waste of time and material. In the absence of available standards for canine serum CRP, we planned and implemented an acceptable IQC procedure for the CRP-TIA based on pooled patient serum samples as an alternative control material.

The results showed that it was possible to plan and implement an IQC procedure. The procedure ($1_{2.5s}$) fulfilled the criteria of predicted high error detection of medically important systematic errors (P_{ed} = .94–1.00) and low false rejection (P_{fr} = .03) of valid analytical runs. It was interesting to observe that the traditional criterion of Levey-Jennings charts (1_{2s}) failed to fulfil the

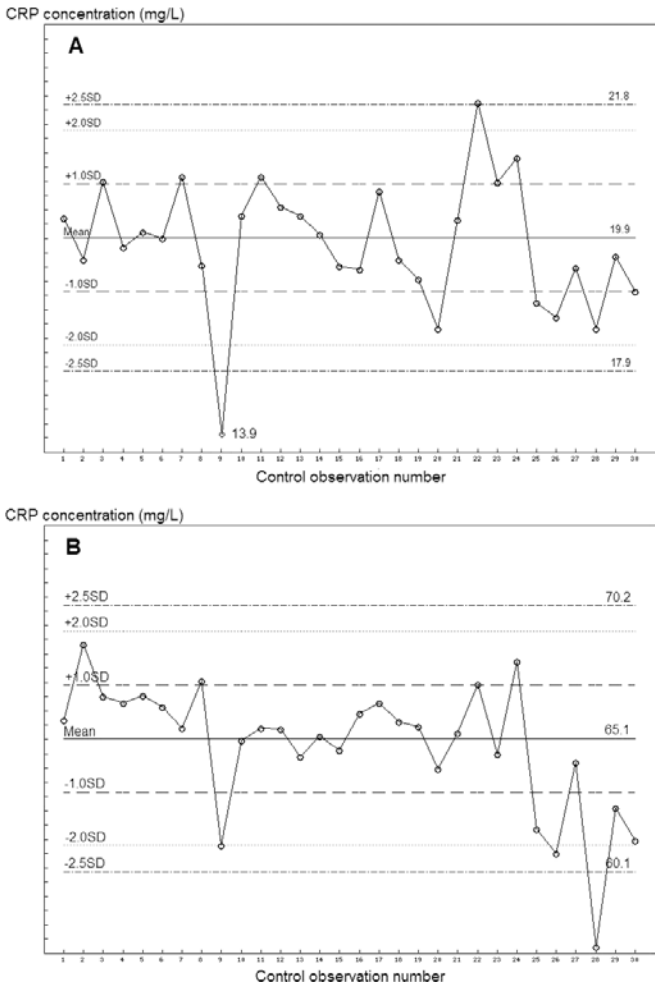

Figure 2. Control charts for low (A) and high (B) pools used as control materials for the CRP turbidimetric immunoassay (CRP-TIA) during 30 control runs over a 14-week period. Horizontal lines represent mean of control material measurements and mean ± the given number of SDs. The IQC rule is violated if the lines representing mean ± 2.5 SDs are exceeded.

criteria of predicted IQC performance (P_{ed} = .99–1.00, P_{fr} = .09) and would have resulted in 3 times as many false rejections of valid analytical runs than the chosen procedure, resulting in unnecessary waste of material and time.

Previously published guidelines for planning and implementation of IQC procedures[1,2] were followed in the planning stage. Data on biological variation were used to define quality requirements of the test; this application has been proposed as an objective way to define analytical quality requirements,[9,10] and has been accepted as the best method to define the quality requirements when a direct "evaluation of the effect of analytical performance on clinical outcome in specific clinical settings" is not available.[11] To ensure the applicability of the IQC with the number of available control materials and to ease application, the list of candidate IQC procedures was limited to include procedures using only 2 control materials within a single run. This approach may have lead to a rejection of all candidate procedures; had that occurred, more complex IQC procedures could have been added and tested. The candidate IQC procedures were chosen primarily to keep the procedure simple. The goals set for predicted IQC performance (.90 and .05 for P_{ed} and P_{fr}, respectively) have been proposed as acceptable requirements,[2] but stricter criteria were targeted. There often is a trade-off between stricter IQC performance and the wish for simple IQC procedures.

When performing IQC by including control material to monitor changes in analytical performance, the ICQ is, in a strict sense, performed only at the point concentrations of the control materials. Therefore, it is important to ensure that the levels of the compound in the control materials are close to the level(s) of clinical importance (ie, where the detection of clinically important errors is essential). Traditionally, 1 control value is selected close to or within the normal range of values for healthy individuals, because this level is important when using a cut-off limit statistically derived from data on healthy individuals. Another control value is then selected within the range of values found in pathological conditions, to control the analytical performance at abnormal levels. However, studies on changes in canine CRP concentration in systemic inflammation,[12–15] to-

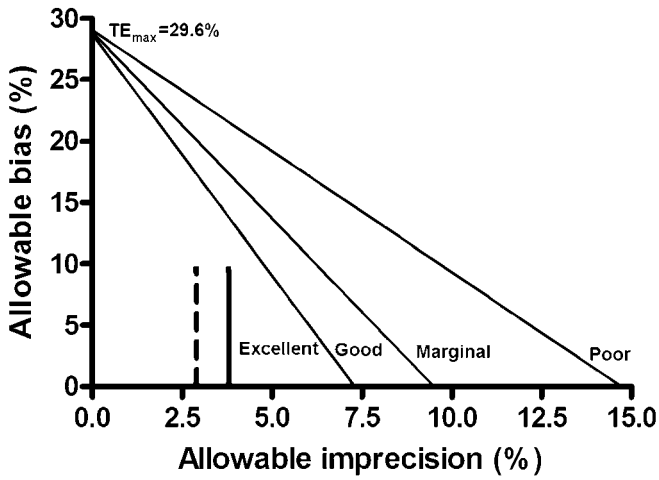

Figure 3. Medical decision chart for assessing analytical performance of the CRP turbidimetric immunoassay (CRP-TIA). Maximum allowable error (TE_{max} = 29.6%) is plotted on the *y*-axis and fractions (0.25, 0.33 and 0.5) of the TE_{max} are plotted on the *x*-axis as criteria for ranking analytical performance. Observed operating ranges of low control (solid line) and high control (dotted line) are plotted as observed imprecision (3.8% and 2.9%, respectively) versus the range of allowable bias of 0.0%–9.5%. CRP-TIA performance was excellent at both levels of control material.

gether with a study on the biological variation of canine CRP,[6] indicate that the diagnostic sensitivity of CRP for the detection of systemic inflammation most likely is provided by a large relative change in concentration rather than by the use of a cut-off level close to the normal range. The low index of individuality of canine CRP makes the use of a traditional reference interval diagnostically insensitive at the level of individuals.[6] On the contrary, the diagnostic specificity of canine CRP can be enhanced without seriously impairing diagnostic sensitivity by using a cut-off limit somewhat above the normal range. Optimally, this cut-off should be derived from CRP levels in groups of dogs with different diseases of clinical interest. Preliminary results in our laboratory suggest that a clinical decision limit for canine CRP optimized to differentiate among unhealthy dogs with or without systemic inflammation is in the range of 16–18 mg/L for this assay (unpublished results). A 3rd control within the normal range would have further optimized the IQC procedure, to also detect errors at the normal level and enhance detection of proportional errors. However, fluctuation within the normal range of CRP values has not yet been found to be clinically useful in canine medicine as it has in human medicine.[16] Thus, the control levels used in the present study were placed in the critical region of a clinical decision limit (approximately 20 mg/L) and at a pathologic level (65 mg/L) to ensure control of clinically important errors during the establishment of a diagnosis and during monitoring of inflammation after the establishment of a diagnosis.

In contrast to the use of traceable standard material that enables estimation of the absolute level of bias, the use of patient samples as control material limits the control of bias to a detection of a change in bias. Participation in multilaboratory proficiency testing programs also enables the assessment of bias relative to other laboratories, but such programs are yet to be established for canine CRP. However, because significant bias in the CRP-TIA was proven unlikely by other means,[5] the monitoring of changes in systematic error should be sufficient.

The observed imprecision at the 2 levels of CRP corresponded to excellent analytical performance of the CRP-TIA as evaluated by the MEDx chart, which is a further validation of the assay for routine use in canine medicine. Retrospective determination of assay imprecision using control materials has been argued to be biased by selection; assessment is more correctly performed by using a separate material for evaluation of imprecision only.[1] In the present study, error in the assessment of imprecision because of selection was considered minimal, since the values resembled earlier observations made using separate material[5].

The use of control material in IQC is based on an assumption of a constant concentration of the compound in the control material throughout the control period; thus, it is important to ensure that the pools of patient serum samples are stable.The period of use in the present study was limited to 14 weeks because a previous study showed canine CRP to be stable at –10°C for at least 4 months.[17] Continuous IQC based on patient samples can then be performed by preparation of pools of fresh material every 3 months. The pools can be run concurrently with established control materials for 6–8 control points to establish data for the new control material during a period of stable analytical performance under control of the established control material. This procedure can be repeated every 3 months. In the present study, a trend of decrease was present in the high pool after the 25th control point (12 weeks of storage); whether this represents a limit of stability or another source of variation is unclear. Further studies of the storage properties of canine CRP at storage temperatures used in modern laboratories are needed to elucidate this aspect and enable optimization of a continuous IQC based on patient samples. The guidelines used here for planning the IQC procedure are applicable to many types of tests. The results of the present study suggest that similar procedures could be applied to other veterinary assays and to species-specific IQC when commercial standards are not available.

Acknowledgements

The skillful and indispensable work of Ms Emma Thomsen and Ms Natascha Errebo in the laboratory is hereby acknowledged.

References

1. Petersen PH, Ricos C, Stoeckl D, et al. Proposed guidelines for the internal quality control of analytical results in the medical laboratory. *Eur J Clin Chem Clin Biochem.* 1996;34:983–999.
2. Westgard JO. Internal quality control: planning and implementation strategies. *Ann Clin Biochem.* 2003;40:593–611.
3. Bienvenu J, Later R, Pontet F. Reference material (CRM 470) for serum proteins: preparation, characteristics and conditions of use [in French]. *Ann Biol Clin (Paris).* 1995;53:499–505.
4. Skinner JG. Special report. International standardization of acute phase proteins. *Vet Clin Pathol.* 2001;30:2–7.
5. Kjelgaard-Hansen M, Jensen AL, Kristensen AT. Evaluation of a commercially available human C-reactive protein (CRP) turbidometric immunoassay for determination of canine serum CRP concentration. *Vet Clin Pathol.* 2003;32:81–87.
6. Kjelgaard-Hansen M, Mikkelsen LF, Kristensen AT, Jensen AL. Study on biological variability of five acute phase reactants in dogs. *Comp Clin Pathol.* 2003;12:69–74.
7. WesTgard® Quality Corporation. Online QC Tools, Beta version. Available at: http://www.westgard.com/qctool1.html. Accessed June 22, 2004.

8. Westgard JO. A method evaluation decision chart (MEDx chart) for judging method performance. *Clin Lab Sci.* 1995; 8:277–283.

9. Petersen PH, Fraser CG, Jørgensen L, et al. Combination of analytical quality specifications based on biological within- and between-subject variation. *Ann Clin Biochem.* 2002;39: 543–550.

10. Fraser CG, Petersen PH, Ricos C, Haeckel R. Proposed quality specifications for imprecision and inaccuracy of analytical systems for clinical chemistry. *Eur J Clin Chem Clin Biochem.* 1992;30:311–317.

11. Kenny D, Fraser CG, Petersen PH, Kallners A. Consensus agreement. *Scand J Clin Lab Invest.* 1999;59:585.

12. Hayashi S, Jinbo T, Iguchi K, et al. A comparison of the concentrations of C-reactive protein and alpha$_1$-acid glycoprotein in the serum of young and adult dogs with acute inflammation. *Vet Res Commun.* 2001;25:117–126.

13. Otabe K, Ito T, Sugimoto T, Yamamoto S. C-reactive protein (CRP) measurement in canine serum following experimentally-induced acute gastric mucosal injury. *Lab Anim.* 2000; 34:434–438.

14. Yamamoto S, Shida T, Honda M, et al. Serum C-reactive protein and immune responses in dogs inoculated with *Bordetella bronchiseptica* (phase I cells). *Vet Res Commun.* 1994;18:347–357.

15. Conner JG, Eckersall PD, Ferguson J, Douglas TA. Acute phase response in the dog following surgical trauma. *Res Vet Sci.* 1988;45:107–110.

16. Kluft C, de Maat MP. Sensitive markers of inflammation make it possible to study the chronic process: the rise of interest in low levels of C-reactive protein. *Vascul Pharmacol.* 2002;39:99–104.

17. Riley RF, Zontine W. Further observations on the properties of dog C-reactive protein and the C-reactive protein response in the dog. *J Lab Clin Med.* 1972;80:698–703.

Chapter 3

PHASE II AND III – OVERLAP AND CLINICAL INVESTIGATIONS

Paper V – Assessment of reference limits and clinical decision levels for canine serum C-reactive protein

Submitted to
Journal of Veterinary Diagnostic Investigation
(October 2004)

Title

Assessment of reference limits and clinical decision levels for canine serum C-reactive protein

Authors

Mads Kjelgaard-Hansen[1] (Corresponding author)

Asger Lundorff Jensen[1]

Michael Luntang-Jensen[2]

Inge Tarnow[3]

Henrik Duelund Pedersen[3]

Jakob Willesen[2]

Jørgen Koch[2]

Annemarie T. Kristensen[2]

[1]Central Laboratory, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University. 3 Gronnegaardsvej, DK-1870 Frederiksberg, Denmark.

[2]Small Animal Veterinary Teaching Hospital, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University. 16 Dyrlaegevej, DK-1870 Frederiksberg, Denmark.

[3]Department of Animal and Veterinary Basic Sciences, The Royal Veterinary and Agricultural University, 7 Gronnegaardsvej, DK-1870 Frederiksberg, Denmark.

Keywords: Clinical decision level, dog, immunoassay, inflammation, reference range.

1 Figure and 2 Tables

E-mail: mjkh@kvl.dk, phone: +45 35282907 and fax: +45 35282947 of corresponding author

Summary

The determination of canine serum C-reactive protein (CRP) is reported to be of clinical value for diagnostic and monitoring purposes. Recently, an automated turbidimetric immunoassay (CRP-TIA) has been validated for the determination of canine serum CRP, which facilitates routine use of CRP in canine medicine. Method-specific clinical decision levels are necessary to enable the evaluation of single measurements of CRP. The objective of this study was to estimate the reference limits of canine serum CRP determined by the CRP-TIA of healthy dogs and to assess the optimal cut-off values for CRP to differentiate dogs with conditions causing systemic inflammation from (1) healthy dogs and (2) diseased dogs without systemic inflammation. Fifty-nine healthy dogs, 35 diseased dogs without systemic inflammation and 50 dogs in three different groups with conditions causing systemic inflammation (pyometra [10 dogs], 23h post operative stress [14 dogs] and *Angiostrongylus vasorum* infection [26 dogs]) were included in the study. The lower and upper reference limits for healthy dogs were 0.4 mg/L and 15.9 mg/L, respectively. The optimal cut-off value to differentiate between dogs in setting (1) and (2) was within 11.1-14.8 mg/L and 16.0-17.8 mg/L, respectively when diagnostic specificity was favoured, seemingly without impairing the detection of a wide range of inflammatory activity. The results of the study can be used as a guideline for the evaluation of single measurements of canine CRP in different clinical settings.

Introduction

C-reactive protein (CRP) is a major acute phase protein in dogs characterized by a marked change in serum concentration consistent with systemic inflammatory activity (Conner et al., 1988; Yamamoto et al., 1994a; Hayashi et al., 2001). Several studies have reported canine serum CRP to be a fast, diagnostically sensitive and specific marker for systemic inflammatory activity very likely to be useful for routine diagnostic and monitoring purposes in canine medicine (Ndung'u et al., 1991; Otabe et al., 2000; Jergens et al., 2003; Martinez-Subiela et al., 2003a). Most studies on methodologies for canine CRP determination have been performed using locally developed non-commercial assays for the determination of serum CRP concentration, but a few commercially available assays have been validated for canine serum CRP (an automated turbidimetric immunoassay [CRP-TIA, Bayer, Newbury, UK] (Kjelgaard-Hansen et al., 2003a), a canine CRP-ELISA [Tridelta, Kildare, Ireland] (Kjelgaard-Hansen et al., 2003b) and a semi-quantitative immunochromatographic assay [CRP rapid assay pack, EVL, Woerden, The Netherlands] (McGrotty et al., 2004)), which should enable wide-spread routine laboratory use. Studies on the biological variation of canine CRP have been performed, which amongst other things facilitate the clinical evaluation of serially obtained CRP measurements from the same individual (Martinez-Subiela et al., 2003b; Kjelgaard-Hansen et al., 2003c). However, to also enable clinical decision-making based on single CRP measurements it is necessary to establish clinical decision levels such as reference limits for healthy dogs and cut-off values for specific clinical settings. The previously reported ranges of CRP in healthy dogs seem to partly depend on the method used as the range varies considerably between studies using different methods; 0.8-22.6 mg/L (Otabe et al., 1998), 2.4-30.0 mg/L (Yamamoto et al., 1994b), 0-67.4 mg/L (Börngen, 1998) and 1.2-6.4 mg/L (Kjelgaard-Hansen et al., 2003b). This problem is partly due to the lack of recognized standards for canine CRP, as a common reference standard most likely would enable a decrease of this inter-method variation. Until such standards are available it is necessary to establish method-specific clinical decision levels.

The objective of this study was to estimate the reference limits of canine serum CRP determined by the CRP-TIA of healthy dogs and to assess the optimal cut-off values for CRP to differentiate dogs with conditions causing systemic inflammation from (1) healthy dogs and (2) diseased dogs without systemic inflammation.

Materials and methods

Assay

A CRP-TIA (Bayer, Newbury, UK) designed for the determination of human serum CRP was used for the heterologous determination of CRP in canine serum samples for which it has previously been validated (Kjelgaard-Hansen et al., 2003a). The principle of the analysis is the binding of canine CRP to polyclonal goat anti-human CRP antibodies to form a precipitate that is measured turbidimetrically. Human CRP calibrator (Bayer, Newbury, UK) was used to create a standard curve and the analysis was performed using an automated analyzer (ADVIA® 1650 chemistry system, Bayer, Newbury, UK) according to the manufacturer's description. Routine canine CRP-specific internal quality control, as previously described (Kjelgaard-Hansen et al., 2004b) was also performed throughout the study period to ensure detection of possible clinically important errors.

Samples

When nothing else is stated the serum samples used were obtained from client-owned dogs presented at the Small Animal Veterinary Teaching Hospital, Department of Small Animal Clinical Studies, The Royal Veterinary and Agricultural University, Denmark for various diagnostic, therapeutic or prophylactic measures. Routine pre-anaesthetic serum samples from 59 dogs undergoing elective surgery (mainly ovariohysterectomi, orchidectomy or removal of persistent deciduous teeth) were included in the study to represent samples from healthy dogs. None of the dogs had clinical, haematological or biochemical abnormalities concordant with any disease activity. The healthy dogs were represented by 25 different breeds and 13 cross-breeds with an age-span of ½-12 years (median 2 years).

Thirty-five Cavalier King Charles Spaniels (age-span of 2½-13½ years [median 7 years]) with untreated moderate to severe mitral regurgitation (a regurgitant jet occupying 50-100% of the left atrial area on echocardiography) were included to represent diseased dogs without systemic inflammation. The dogs were enrolled in connection with a mitral valve disease screening programme and a study on assessment of changes in haemostatic markers performed at the Department of Animal and Veterinary Basic Sciences, The Royal Veterinary and Agricultural

University, Denmark. None of these dogs had clinical or haematological signs of any systemic inflammatory process.

Three different groups represented dogs with different conditions causing systemic inflammation. Ten dogs with pyometra, 26 dogs with *Angiostrongylus vasorum* infections (positive Baerman test) and 14 dogs sampled 23 hours after elective abdominal surgery (sampled to represent aseptic systemic inflammation (Conner et al., 1988; Hayashi et al., 2001)). The 3 groups represented 6 breeds and 1 cross-breed, aged 1-13 years (median 6 years); at least 13 breeds, aged 1-11 years (median 3 years) and 5 breeds, aged ½-10 years (median 4 years), respectively. No other source of systemic inflammation was detected clinically in these dogs.

For all samples serum was prepared by centrifugation (2000g, 5 minutes) within 1 hour after collection of the blood in plain vials with clot activator and separation gel (Vacuette, Greiner bio-one, Austria). The CRP determination was performed immediately after serum preparation except for dogs with mitral regurgitation and aseptic inflammation, where serum was stored in plastic vials at -80˚C for a maximum of 18 and 6 months, respectively before analysis.

Statistical methods

The reference limits (outer 90% confidence limits of the central 95% confidence interval (CI)) of healthy dogs were established as previously outlined (Solberg, 1996); a goodness-of-fit test of the reference population to a Gaussian distribution was performed by means of the Kolmogorov-Smirnov test and a logarithmic transformation was performed in case of skewed data to obtain a distribution fit for the parametric approach. The mean and standard deviation (SD) was estimated and the lower and upper reference limit calculated as the mean-1.96SD-2.81SD/$n^{½}$ and mean+1.96SD+2.81SD/$n^{½}$, respectively. Transformation back to the ordinary scale was performed if transformed data were used. Receiver operating characteristic (ROC) analysis (Zweig and Campbell, 1993) was performed to assess the optimal cut-off values to differentiate dogs with conditions causing systemic inflammation from (1) healthy dogs and (2) diseased dogs without systemic inflammation. The cut-off value for CRP was obtained at sensitivity=1 and specificity=1 and at the maximum differential positive rate (maxDPR), i.e. where sensitivity-(1-specificity) was highest.

Results

The CRP concentrations varied markedly between the different groups of dogs (Fig 1). The CRP concentrations in the group of healthy dogs were positively skewed and deviated significantly from a Gaussian distribution ($P<0.001$). No skewness or deviation from Gaussian distribution was observed after logarithmic transformation. The logarithmic reference limits were -0.38 mg/L and 1.20 mg/L (mean=0.41 mg/L, SD=0.34 mg/L and n=59), corresponding to 0.4 mg/L and 15.9 mg/L on the ordinary scale for the lower and upper limit, respectively. The optimal cut-off values for the different settings are summarized in Table 1 and 2.

Discussion

The reference limits for a diagnostic parameter is derived from data on sampled healthy individuals to assess what levels could be expected in non-diseased individuals (Solberg, 1996). In the case of canine CRP, only the upper reference limit of 15.9 mg/L is of current interest because any clinical significance of very low levels of CRP or fluctuations within normal levels has not been established in canine medicine. The outer 90% confidence limits of the traditional 95% confidence interval were used to account for the limited sample size (Solberg, 1996).

The sub-groups of dogs with conditions causing systemic inflammation were analysed separately when performing the ROC analysis, as different causes are reported to result in markedly different distributions of inflammatory responses and CRP concentrations (Yamamoto et al., 1993; Higgins et al., 2003; Kjelgaard-Hansen et al., 2004a). This was not done to resemble any clinical setting because canine CRP, as a major acute phase protein, is recognized as a diagnostic sensitive and specific marker of inflammatory activity in general, but non-specific to the cause thereof (Eckersall and Conner, 1988; Hayashi et al., 2001). The pooling of dogs belonging to very different distributions of CRP values could have influenced the assessment of cut-off values by the ROC analysis. Additionally, the separate analysis of the different groups of dogs with conditions causing systemic inflammation enabled assessing the consequence of the different distributions of CRP concentrations to the cut-off value estimates.

However, the separate use of (1) healthy dogs and (2) dogs with a non-inflammatory condition as control dogs in the assessment of clinical performance was performed to

resemble different clinical settings. Situation (1) resembled the use of CRP measurements for screening purposes, whereas (2) resembled the typical clinical situation where abilities to identify dogs with inflammatory activity from a population of diseased dogs are wanted. The estimated cut-off values optimized for high diagnostic specificity and for maxDPR, respectively were within narrow ranges regardless of the clinical setting, an indication of stable estimates. Regarding the clinical decision level optimized for high diagnostic sensitivity it should be noted that a sensitivity of 1 could only be obtained with cut-off=0 mg/L when detecting systemic inflammatory activity in *A. vasorum*-diseased dogs. A rather even distribution of CRP concentrations from low-normal to markedly increased were observed in this group (Fig 1), despite they all had active *A. vasorum* infection, which *per se* includes larval migration (Bolt et al., 1994). Some of the dogs with *A. vasorum* most likely did not have a significant acute phase response at all, as several other haematological and biochemical indicators of systemic inflammation were also within reference range (data not shown).

In general, the optimal cut-off level depends on the relative importance of diagnostic sensitivity and specificity (Linnet, 1988). Therefore, considerations should be made of whether they are of equal importance or either of them should be favoured. If, for instance, the test is used to differentiate dogs with systemic inflammation from healthy dogs, i.e. as a screening tool for systemic inflammation, a low prevalence is often expected and therefore, diagnostic specificity should be favoured to avoid a high number of false-positives (Linnet, 1988) and ensure an acceptable predictive value of a positive result. Additionally, in the case of canine CRP it appears that the diagnostic sensitivity in detecting systemic inflammatory activity lies within the large relative change in concentration, rather than in using cut-off levels close to the normal range (Conner et al., 1988; Otabe et al., 2000; Hayashi et al., 2001; Kjelgaard-Hansen et al., 2003c). The latter because of an observed low index of individuality (low ratio between intra- and inter-individual coefficients of variation) of canine CRP, which in general makes the use of a traditional reference range diagnostically insensitive in the detection of small but significant changes at the level of individuals (Harris, 1974; Kjelgaard-Hansen et al., 2003c). Furthermore, favouring diagnostic specificity that could ensure high predictive value of a positive result seemed not to adversely impair the continued detection of a wide range of inflammatory activity. Thus, the clinical

decision levels should be within 11.1-14.8 mg/L and 16.0-17.8 mg/L for screening and diagnostic purposes, respectively.

In Table 1 and 2 the cut-off levels at sensitivity=1 and at the maximum differential positive rate are also provided, should other settings require optimal diagnostic sensitivity or equal importance of diagnostic sensitivity and specificity, respectively.

The age-range and -distribution were similar in the groups used in this study and previous studies have shown no age or sex variation of the CRP level in healthy dogs (Kuribayashi et al., 2003) and only very young dogs (under 3 months) are reported to have a different CRP acute phase response compared to other age-groups (above 3 months) (Hayashi et al., 2001). Dogs under 3 months of age seemed to have a weaker CRP acute phase response than dogs in other age groups (Hayashi et al., 2001), which may impair the diagnostic sensitivity of canine serum CRP in the detection of significant systemic inflammation in this age-group. Additionally, previous studies reported that the canine pregnancy induces a significant acute phase response with elevated levels of CRP during mid-gestation (Eckersall et al., 1993; Kuribayashi et al., 2003) which will most likely alter the diagnostic specificity of the detection of systemic inflammation by means of CRP measurements in this group. Thus, the results of the present study should be applicable for all non-pregnant dogs above 3 months of age and specific studies are needed to establish separate clinical decision levels for the pregnant and very young dogs.

The result of the present study should be used as guidelines for clinical decision levels for canine serum CRP determined by the CRP-TIA.

Acknowledgements

The indispensable help of the staff at the Central Laboratory and the Small Animal Veterinary Teaching Hospital, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University, Denmark is hereby acknowledged.

References

Bolt,G., J.Monrad, J.Koch and A.L.Jensen, 1994: Canine angiostrongylosis: A review. The Veterinary Record **135,** 447-452.

Börngen,S., 1998: Nachweis von C-reaktivem protein beim Hund. Institut für Bakteriologie und Mykologie der Veterinärmedizinischen Fakultät der Universität Leipzig, Germany. pp. 1-95.

Conner,J.G., P.D.Eckersall, J.Ferguson and T.A.Douglas, 1988: Acute phase response in the dog following surgical trauma. Research in Veterinary Science **45,** 107-110.

Eckersall,P.D. and J.G.Conner, 1988: Bovine and canine acute phase proteins. Veterinary Research Communications **12,** 169-178.

Eckersall,P.D., M.J.A.Harvey, J.M.Ferguson, J.P.Renton, D.A.Nickson and J.S.Boyd, 1993: Acute phase proteins in canine pregnancy (*canis familiaris*). Journal of Reproduction and Fertility **S47,** 159-164.

Harris,E.K., 1974: Effects of intra- and interindividual variation on the appropriate use of normal ranges. Clinical Chemistry **20,** 1535-1542.

Hayashi,S., T.Jinbo, K.Iguchi, M.Shimizu, T.Shimada, M.Nomura, Y.Ishida and S.Yamamoto, 2001: A comparison of the concentrations of C-reactive protein and alpha1-acid glycoprotein in the serum of young and adult dogs with acute inflammation. Veterinary Research Communications **25,** 117-126.

Higgins,M.A., B.R.Berridge, B.J.Mills, A.E.Schultze, H.Gao, G.H.Searfoss, T.K.Baker and T.P.Ryan, 2003: Gene expression analysis of the acute phase response using a canine microarray. Toxicological sciences **74,** 470-484.

Jergens,A.E., C.A.Schreiner, D.E.Frank, Y.Niyo, F.E.Ahrens, P.D.Eckersall, T.J.Benson and R.Evans, 2003: A scoring index for disease activity in canine inflammatory bowel disease. Journal of Veterinary Internal Medicine **17,** 291-297.

Kjelgaard-Hansen,M., A.L.Jensen, J.Koch and A.T.Kristensen, 2004a: Characterization of the systemic inflammatory response in dogs naturally infected with *Angiostrongylus vasorum*. Journal of Veterinary Internal Medicine **18,** 385-386.

Kjelgaard-Hansen,M., A.L.Jensen and A.T.Kristensen, 2003a: Evaluation of a commercially available human C-reactive protein (CRP) turbidometric immunoassay for determination of canine serum CRP concentration. Veterinary Clinical Pathology **32,** 81-87.

Kjelgaard-Hansen,M., A.L.Jensen and A.T.Kristensen, 2004b: Internal quality control of a turbidimetric immunoassay for canine serum C-reactive protein based on pooled patient samples. Veterinary Clinical Pathology **33,** 139-144.

Kjelgaard-Hansen,M., A.T.Kristensen and A.L.Jensen, 2003b: Evaluation of a commercially available enzyme-linked immunosorbent assay (ELISA) for the determination of C-reactive protein in canine serum. Journal of Veterinary Medicine. A, Physiology, Pathology and Clinical Medicine **50,** 164-168.

Kjelgaard-Hansen,M., L.F.Mikkelsen, A.T.Kristensen and A.L.Jensen, 2003c: Study on biological variability of five acute phase reactants in dogs. Comparative Clinical Pathology **12,** 69-74.

Kuribayashi,T., T.Shimada, M.Matsumoto, K.Kawato, T.Honjyo, M.Fukuyama, Y.Yamamoto and S.Yamamoto, 2003: Determination of serum C-reactive protein (CRP) in healthy beagle dogs of various ages and pregnant beagle dogs. Experimental Animals **52,** 387-390.

Linnet,K., 1988: A review on the methology for assessing diagnostic tests. Clinical Chemistry **34,** 1379-1386.

Martinez-Subiela,S., L.J.Bernal and J.J.Ceron, 2003a: Serum concentrations of acute-phase proteins in dogs with leishmaniosis during short-term treatment. Am. J. Vet. Res. **64,** 1021-1026.

Martinez-Subiela,S., F.Tecles and J.J.Ceron, 2003b: Critical differences of acute phase proteins in canine serum samples. Vet J. **166,** 233-237.

McGrotty,Y.L., C.M.Knottenbelt, I.K.Ramsey, S.W.Reid and P.D.Eckersall, 2004: Evaluation of a rapid assay for canine C-reactive protein. Veterinary Record **154,** 175-176.

Ndung'u,J.M., P.D.Eckersall and F.W.Jennings, 1991: Elevation of the concentration of acute phase proteins in dogs infected with *Trypanosoma brucei*. Acta Tropica **49,** 77-86.

Otabe,K., T.Ito, T.Sugimoto and S.Yamamoto, 2000: C-reactive protein (CRP) measurement in canine serum following experimentally-induced acute gastric mucosal injury. Laboratory Animals **34,** 434-438.

Otabe,K., T.Sugimoto, T.Jinbo, M.Honda, S.Kitao, S.Hayashi, M.Shimizu and S.Yamamoto, 1998: Physiological levels of C-reactive protein in normal canine sera. Veterinary Research Communications **22,** 77-85.

Solberg,H.E., 1996: Establishment and use of reference values. In: Tietz Fundamentals in Clinical Chemistry, 4th ed. (editors: Burtis,C.A. and E.R.Ashwood), W.B.Saunders Company, Philadelphia, PA. pp. 182-191.

Yamamoto,S., T.Shida, M.Honda, Y.Ashida, Y.Rikihisa, M.Odakura, S.Hayashi, M.Nomura and Y.Isayama, 1994a: Serum C-reactive protein and immune responses in dogs inoculated with *Bordetella bronchiseptica* (phase I cells). Veterinary Research Communications **18,** 347-357.

Yamamoto,S., T.Shida, S.Miyaji, H.Santsuka, H.Fujise, K.Mukawa, E.Furukawa, T.Nagae and M.Naiki, 1993: Changes in serum C-reactive protein levels in dogs with various disorders and surgical traumas. Veterinary Research Communications **17,** 85-93.

Yamamoto,S., T.Shida, T.Okimura, K.Otabe, M.Honda, Y.Ashinda, E.Furukawa, M.Sarikaputi and M.Naiki, 1994b: Determination of C-reactive protein in serum and plasma from healthy dogs and dogs with pneumonia by ELISA and slide reversed passive latex agglutination test. Veterinary Quarterly **16,** 74-77.

Zweig,M.H. and G.Campbell, 1993: Receiver-operating characteristic (ROC) plots: A fundamental tool in clinical medicine. Clinical Chemistry **39,** 561-577.

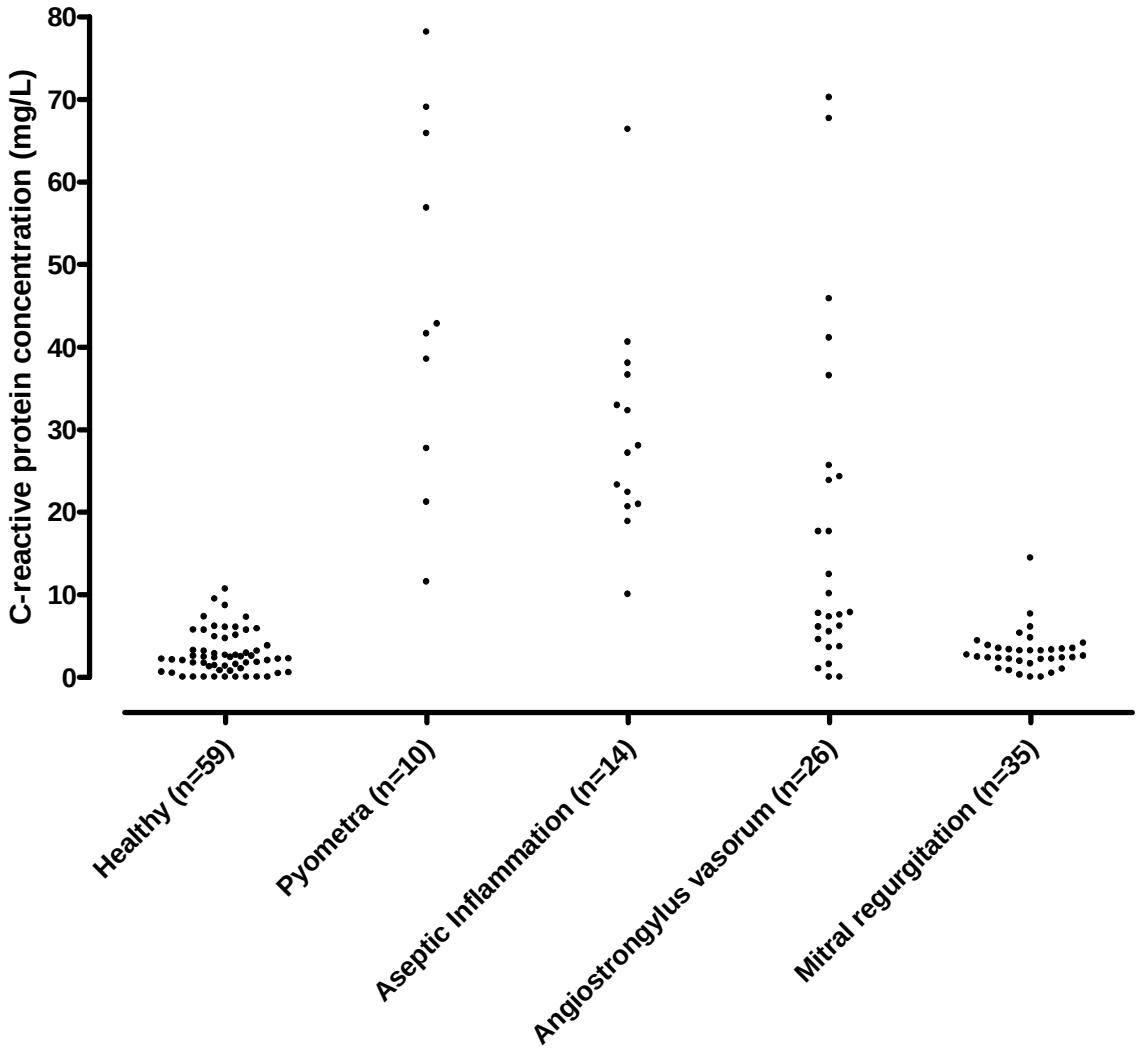

Figure 1. Serum concentration of C-reactive protein in different groups of dogs.

Table 1. Estimates of the clinical decision levels for canine C-reactive protein (mg/L) to differentiate between healthy dogs and dogs with conditions causing systemic inflammation obtained by receiver operating characteristic analysis

Groups compared: Healthy vs.	CDL based on maxDPR (Se;Sp)	CDL when Sensitivity=1 (Se;Sp)	CDL when Specificity=1 (Se;Sp)
Pyometra	11.1 (1;1)	11.1 (1;1)	11.1 (1;1)
Aseptic inflammation	9.8 (1;0.98)	9.8 (1;0.98)	14.8 (0.93;1)
Angiostrongylus vasorum	9.8 (0.46;0.98)	0 (1;0)	11.6 (0.42;1)

CDL; Clinical decision level. maxDPR; Maximum differential positive rate, i.e. where sensitivity-(1-specificity) is highest, the optimal clinical decision level if sensitivity and specificity are of equal importance. Se; Diagnostic sensitivity. Sp; Diagnsotic specificity.

Table 2. Estimates of the clinical decision levels for canine C-reactive protein (mg/L) to differentiate between diseased dogs without systemic inflammation and dogs with conditions causing systemic inflammation obtained by receiver operating characteristic analysis

Groups compared: Diseased dogs without systemic inflammation vs.	CDL based on maxDPR (Se;Sp)	CDL when Sensitivity=1 (Se;Sp)	CDL when Specificity=1 (Se;Sp)
Pyometra	9.6 (1;0.97)	9.6 (1;0.97)	17.8 (0.90;1)
Aseptic inflammation	8.8 (1;0.97)	8.8 (1;0.97)	16.7 (0.93;1)
Angiostrongylus vasorum	5.8 (0.69;0.91)	0 (1;0)	16.0 (0.38;1)

CDL; Clinical decision level. maxDPR; Maximum differential positive rate, i.e. where sensitivity-(1-specificity) is highest, the optimal clinical decision level if sensitivity and specificity are of equal importance. Se; Diagnostic sensitivity. Sp; Diagnsotic specificity.

Chapter 4

PHASE IV – OUTCOME AND UTILITY INVESTIGATIONS

Paper VI – C-reactive protein as an objective early marker of inflammatory activity during immunosuppressive therapy in a dog with type II immune-mediated polyarthritis

Submitted to
Journal of Small Animal Practice
(October 2004)

Case-report

Title

C-reactive protein as an objective early marker of inflammatory activity during immunosuppressive therapy in a dog with type II immune-mediated polyarthritis

Authors

Mads Kjelgaard-Hansen[1]

Asger Lundorff Jensen[1]

Geoffrey A. Houser[2]

Lisbeth Rem Jessen[2]

Annemarie T. Kristensen[2]

[1]Central Laboratory, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University. 3 Gronnegaardsvej, DK-1870 Frederiksberg, Denmark.

[2]Small Animal Veterinary Teaching Hospital, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University. 16 Dyrlaegevej, DK-1870 Frederiksberg, Denmark.

Abstract

Canine C-reactive protein (CRP) measurements were performed during treatment of a case of canine type II immune-mediated polyarthritis (IMPA) with several periods of inflammatory relapses to possibly provide clinically useful information not obtainable by means of complete blood cell count or clinical evaluation. Canine CRP is a diagnostically sensitive and specific marker of systemic inflammatory activity, but non-specific regarding the stimulus thereof. Additionally, it is neither induced nor impaired as such marker of systemic inflammation by corticosteroids or non-steroid therapy. These features and the observations made in the present case suggest canine CRP measurements to be of clinical value for assessment of treatment efficiency and for an early and objective detection of inflammatory relapses during treatment of canine type II IMPA. The observed early and treatment-independent detection of inflammatory relapses could possibly enable pre-emptive diagnostic measures to be taken to detect the cause of the inflammatory stimulus.

Keywords

CRP; dog; immune-mediated; inflammation; monitoring

Introduction

Monitoring the effect of steroid therapy of immune-mediated diseases may be difficult. The clinician mainly has to rely on clinical signs since objective disease markers such as white blood cell count, and absolute numbers of neutrophil granulocytes, lymphocytes, monocytes and eosinophil granulocytes may be so affected by the steroid treatment *per se* that they are maybe inadequate for reliable monitoring of the effect of treatment. Further complicating factors include the well-known side-effects such as polyuria, polydipsia and steroid hepatopathy encountered during steroid treatment. Thus, an objective disease marker that is not affected by steroid treatment *per se* could potentially be of great value to the clinician when monitoring the response to and effect of treatment.

One such disease marker could be canine C-reactive protein (CRP). Studies on canine CRP indicated that it is a clinically useful marker for monitoring fluctuations in inflammatory activity during the state of disease (Ndung'u et al., 1991; Otabe et al., 2000; Martínez-Subiela et al., 2002; Jergens et al., 2003), to assess the effect of therapy (Jergens et al., 2003; Martínez-Subiela et al., 2003) and finally, the CRP acute phase response seemed neither to be induced (Martínez-Subiela et al., 2004) nor altered (Yamamoto et al., 1994) by corticosteroid therapy as well as non-steroid therapy (Hulton et al., 1985). This should make CRP feasible for the monitoring of the inflammatory activity during corticosteroid therapy and thus make CRP a potential monitoring marker of the response to therapy and possible inflammatory relapses. Validated assays for the determination of canine CRP are commercially available (Kjelgaard-Hansen et al., 2003a; Kjelgaard-Hansen et al., 2003b; McGrotty et al., 2004) as well as guidelines for clinical decision levels for single (Kjelgaard-Hansen et al., 2004b) and serially (Kjelgaard-Hansen et al., 2003c) obtained CRP measurements in dogs. In this report, we communicate the variations in CRP levels during treatment of a type II immune-mediated polyarthritis (IMPA) in a dog.

Case report

Diagnosis

A 9-year old, female English Springer Spaniel was referred to the Small Animal Veterinary Teaching Hospital, Department of Small Animal Clinical Sciences, The Royal Veterinary and Agricultural University, Denmark with a history of weight-loss,

lethargy, intermittent lameness, generalized lymphadenopathy and recurrent febrile episodes during the preceding 8 weeks despite antibiotic and anti-inflammatory steroid treatment at dosage. Clinical investigation revealed depression, pyrexia (39.9 ˚C), lameness, reluctance to stand and joint pain in multiple joints. Diagnostic procedures included complete blood count (CBC) and biochemical profile, urinalysis and cytological evaluation of synovial fluid as well as lymph nodes. The CBC and biochemical profile revealed a regenerative anaemia characterized by spherocytosis and autoagglutination. Lymph node and joint cytology revealed reactive lymphadenopathy and neutrophilic inflammation in all joints sampled with *Ehrlichia spp.*-like inclusions in occasional neutrophils. A diagnosis of type II IMPA and immune-mediated haemolytic anaemia (IMHA) was made and antibiotic therapy (doxycycline 10 mg/kg sid [Ronaxan Vet.®, Merial, Lyon, France]) was initiated. To further confirm *Ehrlichia spp.* infection and rule out other potential suspect causes of IMHA and type II IMPA, thoracic radiographs, abdominal ultrasound, PCR tests for Canine distemper virus, *Ehrlichia spp.*, serum antibody titer-tests for *Borrelia spp., Bartonella spp.* and *Babesia spp.* and anti-nuclear antibody test were performed. All were unremarkable. A serum antibody titer for *Ehrlichia spp.* was, however, positive (IgG titer 1:640). Based on the clinical and paraclinical examinations, the dog was considered to suffer from IMHA and a type II IMPA secondary to an *Ehrlichia spp.* infection.

Immunosuppressive therapy (prednisolone 1.0 mg/kg tid [Prednisolonacetat DAK, Nycomed, Roskilde, Denmark]) and analgesic therapy (buprenorphine 0.015 mg/kg sid for 2 days [Buprenorfin 1A Farma, 1A Farma, Albertslund, Denmark]) were initiated together with protective treatment of the gastric mucosa (sucralfate [Antepsin®, Orion Corporation, Espoo, Finland] and misoprostol [Cytotec®, Pharmacia Ltd. Morpeth, England]). Antibiotic therapy (doxycycline 10 mg/kg sid) was continued.

Follow-up

In the follow-up period, the dog was monitored by means of weekly to bi-weekly clinical examinations and CBC or if the owner observed symptoms of excessive immunosuppression (secondary infection), insufficient immunosuppression (symptoms of polyarthritis) or steroid associated adverse effects.

The dosage of corticosteroid was regulated throughout the follow-up period in an attempt to titrate the dosage to an acceptable clinical outcome regarding symptoms of

the immune-mediated disease and side-effects of therapy (Figure 1). The dog had several periods with relapse of clinical symptoms of polyarthritis (Figure 1) mainly in relation to tapering of the corticosteroid therapy, suggesting insufficient immunosuppression. Azathioprine (2.0 mg/kg/day [Imurel®, Glaxo Wellcome GmbH & Co., Bad Oldesloe, Germany]) was included in the therapy regimen from day 105 in combination with prednisolone to possibly lower the necessary dose of prednisolone as the dog developed unacceptable degrees of steroid associated adverse effects at the immunosuppressive dosage needed to eliminate clinical symptoms of IMPA. For 38 days the dog did well on a combination of prednisolone (0.125-0.5 mg/kg tid) and azathioprine (2.0 mg/kg sid), with acceptable degrees of side effects at the lower doses of prednisolone. Symptoms of IMPA returned at day 143 and bone marrow suppression indicated by panleukopenia (Figure 1B) and thrombocytopenia was observed at day 170 and the azathioprine dosage was halved. The dog was euthanized at day 189 as the owner declined further therapy. The total number of white blood cells (WBC) and neutrophils (Figure 1B) were abnormally high from initiation of therapy until day 144 and day 158, respectively and decreased to leukopenic and neutropenic levels at day 170. The observed fluctuations of WBC and neutrophil count were not reflecting the clinical signs of disease activity (Figure 1B). Serum C-reactive protein (CRP) concentrations were measured (Kjelgaard-Hansen et al., 2003a; Kjelgaard-Hansen et al., 2004a) in parallel with the CBCs. The CRP values were not disclosed to the clinicians and thus, the results were not used for therapy-adjustments or changes in the monitoring regimen.

CRP variations during therapy

The CRP concentration was high at day 0-4 (65-66 mg/L) (Figure 1A) with a steep decline to a level within reference range of 13.1 mg/L (Kjelgaard-Hansen et al., 2004b) during the period of initial high-dose prednisolone (0.5-1.0 mg/kg tid [day 4-30]). From day 30 to 90, where prednisolone dosage was consistently below 0.5 mg/kg tid, CRP gradually increased. The rise in CRP preceded the relapse of clinical signs of polyarthritis (day 47-49) by approximately 14 days and was persistently high during the period of clinical signs. A second equivalent pattern of CRP was observed during the second phase of prednisolone dosage titration (day 95-160) with a rapid decline during the period of high prednisolone (0.5 mg/kg tid) and an increase shortly after halving the

prednisolone dosage, again preceding the relapse of clinical signs by approximately 14 days. The CRP declined to immeasurable values (from day 160) during the period of severe bone marrow suppression where inflammatory activity was most likely impaired by a depletion of inflammatory cells, therefore also a depletion of cellular IL-6 release necessary for CRP acute phase response induction. Thus, an increase in CRP concentration was observed on two occasions where it preceded clinical signs of increased inflammatory activity (polyarthritis) by approximately 14 days during periods of low prednisolone dosage (below 0.5 mg/kg tid). Additionally, abnormal levels of CRP were consistent with the clinical signs of polyarthritis, except just before the late period of severe bone marrow suppression.

Discussion

The observations made in the present case indicated that the variation in canine CRP during immunosuppressive therapy in dogs with type II immune-mediated polyarthritis could be useful as an objective early marker of changes in inflammatory activity. This could have a positive effect on the case management of type II IMPA by possibly enabling early assessment of treatment efficiency, unbiased by treatment itself and enabling early indications of undesirable inflammatory activity, due to either relapses of disease or the presence of secondary stimulus. These applications are sustained by previous reported features of canine CRP: C-reactive protein is a non-specific marker, regarding the cause of inflammation. Thus, a decrease in concentration during treatment indicates either an effect on the cause of inflammation or a direct medical down-regulation of the inflammatory activity (Ndung'u et al., 1991; Jergens et al., 2003; Martínez-Subiela et al., 2003; Kumagai et al., 2004). In contrast, an increase in CRP concentration during treatment indicates increased inflammatory activity, due to increased disease activity or any possible secondary inflammatory stimulus (Caspi et al., 1987; Ndung'u et al., 1991; Jergens et al., 2003). Finally, the CRP response is neither modulated nor induced directly by steroid as well as non-steroid drugs (Hulton et al., 1985; Yamamoto et al., 1994; Martínez-Subiela et al., 2004). The CBC seemed to be adversely biased by both the steroid and cytotoxic treatment regimen, as could be expected, and thus, the observed fluctuations were not reflecting the inflammatory activity of the disease, as detected by clinical signs of the IMPA in a clinically useful manner (Figure 1B).

Canine IMPA is subgrouped into 4 groups according to either the absence of defined associations (type I), associations with infection (type II), associations with gastrointestinal tract disease (type III) and association with neoplasia (type IV) (Bennet, 1987). Infections of *Ehrlichia spp.* are reported as a cause of type II IMPA in dogs (Cowell et al., 1988; Skotarczak, 2003) where it is assumed that the infectious process provides an antigenic source for immune complex formation, and either the antigen or circulating complexes are deposited within the synovium to initiate inflammation by a type III hypersensitivity reaction (Bennet and May, 1995).

In general, the therapeutic regimen of type II IMPA is directed against the infectious agent, where recovery from the polyarthritis can be seen upon clearance of the infectious agent (Bennet, 1987; Cowell et al., 1988). However, supportive immunosuppressive therapy is often part of the regimen to help resolve the joint inflammation, using either prednisolone alone (1.0-2.0 mg/kg/day) or in combination with cytotoxic drugs such as azathioprine (2.0 mg/kg/day) (Bennet, 1987). When clinical signs of polyarthritis have resolved, the dosages are gradually reduced over a couple of months and if signs return the dosage is increased (Bennet, 1987).

Canine ehrlichiosis has 3 distinct phases (Kuehn and Gaunt, 1985; Skotarczak, 2003; McQuiston et al., 2003). After an incubation period of 8-20 days, an acute phase (duration 1-3 weeks) with non-specific signs of infection is seen, including increased CRP concentrations (Rikihisa et al., 1994; Shimada et al., 2002). If untreated, affected dogs go into a subclinical phase where they remain seropositive, but clinical signs and acute phase response diminish. Finally, a third stage of chronic infection may develop with marked weight loss, lymphadenopathy, pyrexia and a possible fatal outcome. After the acute phase of canine ehrlichiosis, the infection becomes more persistent to antibiotic treatment (Wen et al., 1997) and thus may become a continuous stimulus for a type II IMPA regardless of therapy. This possible persistent stimulus for the type II IMPA could be the reason for the multiple relapses of clinical signs in the present case.

The involvement of *Ehrlichia spp.* in the present diagnosis was based on clinical signs, cytological findings and a positive antibody titer against *Ehrlichia spp.,* but a negative PCR for *Ehrlichia spp.* antigen was also obtained. However, negative PCR is induced quickly after antibiotic treatment (Lappin, 2003).

In conclusion, CRP measurements seemed valuable as an early and unbiased marker of inflammatory activity during immunosuppressive treatment of type II IMPA in this

dog, seemingly providing clinically useful information superior to that of CBC. This suggests that CRP could be beneficial to case management when used systematically in dogs with immune-mediated diseases, as an early and unbiased marker of treatment efficiency and detection of undesired inflammatory activity, due to disease relapses or secondary stimuli.

References

Bennet,D., 1987: Immune-based non-erosive inflammatory joint disease of the dog 3. Canine idiopathic polyarthritis. Journal of Small Animal Practice **28,** 909-928.

Bennet,D. and C.May, 1995: Joint diseases of dogs and cats. In: Textbook of Veterinary Internal Medicine, 4th ed. (editors: Ettinger,S.J. and E.C.Feldman), WB Saunders, Philadelphia, PA. pp. 2032-2076.

Caspi,D., F.W.J.J.Snel, R.M.Batt, D.Bennett, G.R.Rutteman, E.G.Hartman, M.L.Baltz, E.Gruys and M.B.Pepys, 1987: C-reactive protein in dogs. American Journal of Veterinary Research **48,** 919-921.

Cowell,R.L., R.D.Tyler, K.D.Clinkenbeard and J.H.Meinkoth, 1988: Ehrlichiosis and polyarthritis in three dogs. J. Am. Vet. Med. Assoc. **192,** 1093-1095.

Hulton,N.R., D.J.Johnson and D.W.Wilmore, 1985: Limited effects of prostaglandin inhibitors in Escherichia coli sepsis. Surgery **98,** 291-297.

Jergens,A.E., C.A.Schreiner, D.E.Frank, Y.Niyo, F.E.Ahrens, P.D.Eckersall, T.J.Benson and R.Evans, 2003: A scoring index for disease activity in canine inflammatory bowel disease. Journal of Veterinary Internal Medicine **17,** 291-297.

Kjelgaard-Hansen,M., A.L.Jensen and A.T.Kristensen, 2003a: Evaluation of a commercially available human C-reactive protein (CRP) turbidometric immunoassay for determination of canine serum CRP concentration. Veterinary Clinical Pathology **32,** 81-87.

Kjelgaard-Hansen,M., A.L.Jensen and A.T.Kristensen, 2004a: Internal quality control of a turbidimetric immunoassay for canine serum C-reactive protein based on pooled patient samples. Veterinary Clinical Pathology **33,** 139-144.

Kjelgaard-Hansen,M., A.L.Jensen, M.Luntang-Jensen, I.Tarnow, H.D.Pedersen, J.Willesen, J.Koch, and A.T.Kristensen, 2004b: Assessment of reference limits and clinical decision levels for canine serum C-reactive protein (submitted).

Kjelgaard-Hansen,M., A.T.Kristensen and A.L.Jensen, 2003b: Evaluation of a commercially available enzyme-linked immunosorbent assay (ELISA) for the determination of C-reactive protein in canine serum. Journal of Veterinary Medicine. A, Physiology, Pathology and Clinical Medicine **50,** 164-168.

Kjelgaard-Hansen,M., L.F.Mikkelsen, A.T.Kristensen and A.L.Jensen, 2003c: Study on biological variability of five acute phase reactants in dogs. Comparative Clinical Pathology **12,** 69-74.

Kuehn,N.F. and S.D.Gaunt, 1985: Clinical and hematologic findings in canine ehrlichiosis. J. Am. Vet. Med. Assoc. **186,** 355-358.

Kumagai,K., H.Nakashima and K.Saku, 2004: The HMG-CoA reductase inhibitor atorvastatin prevents atrial fibrillation by inhibiting inflammation in a canine sterile pericarditis model. Cardiovascular Research **62,** 105-111.

Lappin,M.R., 2003: Polysystemic rickettsial diseases. In: Small Animal Internal Medicine, 3rd ed. (editors: Nelson,R.W. and C.G.Couto), Mosby, St.Louis, MS. pp. 1265-1272.

Martínez-Subiela,S., L.J.Bernal and J.J.Ceron, 2003: Serum concentrations of acute-phase proteins in dogs with leishmaniosis during short-term treatment. American Journal of Veterinary Research **64,** 1021-1026.

Martínez-Subiela,S., P.J.Ginel and J.J.Ceron, 2004: Effects of different glucocorticoid treatments on serum acute phase proteins in dogs. Veterinary Record **154,** 814-817.

Martínez-Subiela,S., F.Tecles, P.D.Eckersall and J.J.Ceron, 2002: Serum concentrations of acute phase proteins in dogs with leishmaniasis. Veterinary Record **150,** 241-244.

McGrotty,Y.L., C.M.Knottenbelt, I.K.Ramsey, S.W.Reid and P.D.Eckersall, 2004: Evaluation of a rapid assay for canine C-reactive protein. Veterinary Record **154,** 175-176.

McQuiston,J.H., C.L.McCall and W.L.Nicholson, 2003: Ehrlichiosis and related infections. J. Am. Vet. Med. Assoc. **223,** 1750-1756.

Ndung'u,J.M., P.D.Eckersall and F.W.Jennings, 1991: Elevation of the concentration of acute phase proteins in dogs infected with *Trypanosoma brucei*. Acta Tropica **49,** 77-86.

Otabe,K., T.Ito, T.Sugimoto and S.Yamamoto, 2000: C-reactive protein (CRP) measurement in canine serum following experimentally-induced acute gastric mucosal injury. Laboratory Animals **34,** 434-438.

Rikihisa,Y., S.Yamamoto, I.Kwak, Z.Iqbal, G.Kociba, J.Mott and W.Chichanasiriwithaya, 1994: C-reactive protein and alpha1-acid glycoprotein levels in dogs infected with *Ehrlichia canis*. Journal of Clinical Microbiology **32,** 912-917.

Shimada,T., Y.Ishida, M.Shimizu, M.Nomura, K.Kawato, K.Iguchi and T.Jinbo, 2002: Monitoring C-reactive protein in beagle dogs experimentally inoculated with *Ehrlichia canis*. Veterinary Research Communications **26,** 171-177.

Skotarczak,B., 2003: Canine ehrlichiosis. Ann. Agric. Environ. Med. **10,** 137-141.

Wen,B., Y.Rikihisa, J.M.Mott, R.Greene, H.Y.Kim, N.Zhi, G.C.Couto, A.Unver and R.Bartsch, 1997: Comparison of nested PCR with immunoflourescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. Journal of Clinical Microbiology **35,** 1852-1855.

Yamamoto,S., T.Shida, M.Honda, Y.Ashida, Y.Rikihisa, M.Odakura, S.Hayashi, M.Nomura and Y.Isayama, 1994: Serum C-reactive protein and immune responses in dogs inoculated with *Bordetella bronchiseptica* (phase I cells). Veterinary Research Communications **18,** 347-357.

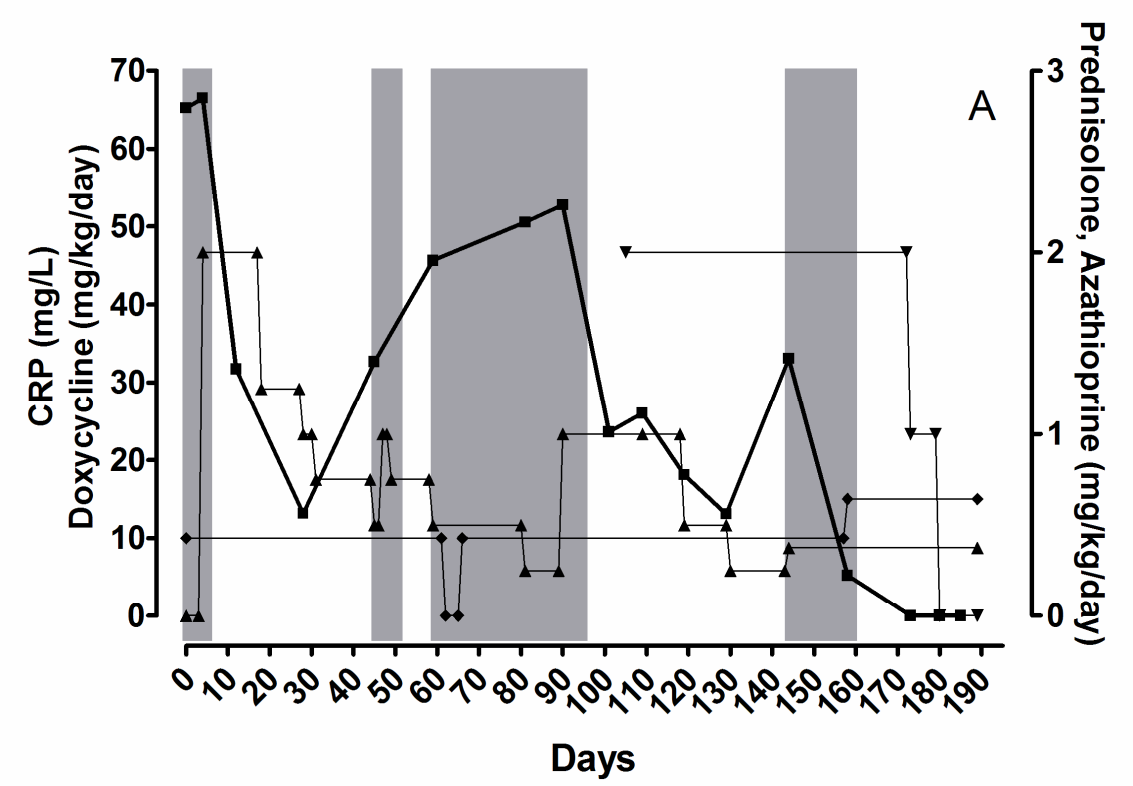

Figure 1A. Serum C-reactive protein concentrations (CRP [■]) daily dosages of antibiotic and immunosupressive therapy in a dog with type II immune-mediated polyarthritis (Doxycycline [♦], Prednisolone [▲] and Azathioprine [▼]) plotted against days after referral of patient. Shaded areas are periods with clinical symptoms of polyarthritis.

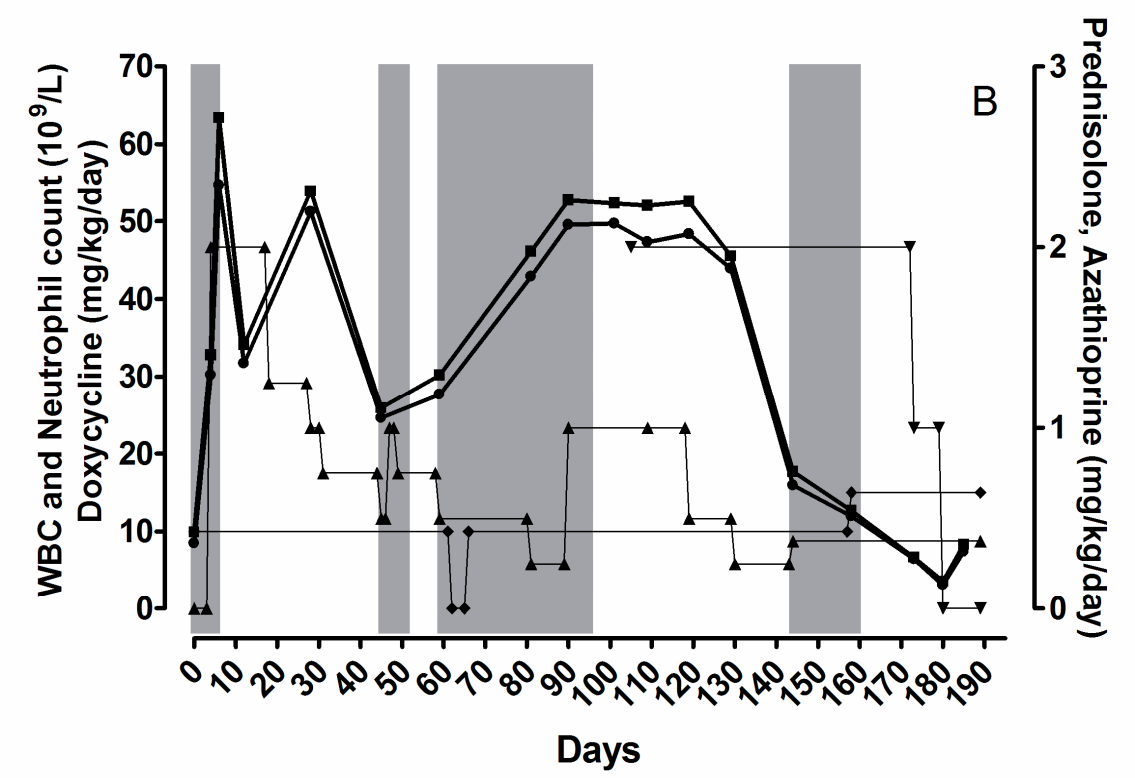

Figure 1B. White blood cell count (WBC [■]), neutrophil count [●] and daily dosages of antibiotic and immunosupressive therapy in a dog with type II immune-mediated polyarthritis (Doxycycline [♦], Prednisolone [▲] and Azathioprine [▼]) plotted against days after referral of patient. Shaded areas are periods with clinical symptoms of polyarthritis.

Chapter 5

GENERAL DISCUSSION

Objectives

The aim of the thesis was to investigate the hypothesis that “Canine serum CRP measurements are applicable for routine purposes in canine medicine”.

This was done by means of a set of sub-hypotheses covering the different phases of investigating the overall hypothesis:

- Phase I Analytical investigations
    - The analytical performance of commercially available assays (and calibration material) can fulfil recognized objective analytical performance standards, regarding imprecision, inaccuracy, total error and detection limit, thus determine canine CRP reliably in both the short- and long-term.
- Phase II and III Overlap and Clinical investigations
    - Canine CRP measurements can
        - Discriminate between healthy dogs and different subpopulations of diseased dogs or
        - Discriminate between different subpopulations of diseased dogs.
- Phase IV Outcome and Utility investigations
    - Canine CRP measurements has a positive consequence on the case management of individuals or groups of individuals in canine medicine, e.g. by either
        - Improving the outcome of a clinical problem
        - Providing equivalent clinically useful information faster than established parameters
        - Providing equivalent clinically useful information more cost-effective than established parameters

In the following the major results of the investigations are presented, discussed and concluded upon in relation to these sub-hypotheses and the main objective of the thesis.

The major findings of the present thesis were the unprecedented validation and application of an automated human CRP-TIA for the determination of canine serum CRP (Paper II) and the presentation and implementation of an optimized species-specific IQC to detect clinically important errors of the CRP-TIA (Paper IV).

Furthermore, a CRP-ELISA specific for canine CRP was validated for clinical use with a few limitations (Paper I), objective analytical performance standards (Paper III) and objective clinical decision levels for single (Paper V) and serial CRP measurements (Paper III) were reported. Finally, observations were made in a case of type II immune-mediated polyarthritis that sustained previous reports of a possible positive impact of CRP determinations on case management (Paper VI).

Phase I – Analytical Investigations

CRP-TIA

The major and most significant contribution of the thesis was the identification and validation of a fully automated commercially available human CRP turbidimetric immunoassay (CRP-TIA) for the determination of canine serum CRP (Paper II).

Analytical performance

The analytical performance standards for imprecision, inaccuracy and total error were objectively derived, as recommended (Fraser et al., 1992; Petersen et al., 2002), from observations on the biological variation of canine serum CRP (Paper III). The main purpose of the establishment of analytical performance standards were to ensure that neither imprecision, inaccuracy nor the combination (total error) would be of a magnitude that could adversely influence a clinical decision based on the reported result. A hierarchy of methods to assess such performance standards has been established as a consensus agreement of clinical pathologists (Kenny et al., 1999). The analytical performance standards derived from observations on biological variation were judged to be the best alternative to a direct ‘‘evaluation of the effect of analytical performance on clinical outcome in specific clinical settings’’ (Kenny et al., 1999). The absolute size of a possible inaccuracy of the assay could not be determined as no recognized standard or control materials are available for canine CRP. This is a general problem for veterinary acute phase proteins, and work has been initiated to solve the problem (Eckersall et al., 1999; Skinner, 2001). Instead attempts to indirectly detect a possible inaccuracy of the assay were performed by studying linearity under dilution and by comparison to another methodology of canine-specific CRP determination. The negative outcome in both methods indicated that no constant or proportional inaccuracy of significance were present at time of validation.

Species-specific Internal Quality Control

The lack of available canine CRP standards and controls also gave rise to problems within the aspect of routine internal quality control (IQC). Internal quality control is essential to monitor the everyday and long-term analytical performance to detect

possible clinically important errors (Westgard, 2001). The CRP-TIA is based on polyclonal goat anti-human CRP antibodies and thus relies on the cross-reactivity of the anti-human CRP antibodies towards canine CRP. In addition, the assay uses purified human CRP as calibration material and thus, long-term stable determination furthermore relies on a stable relative affinity of the polyclonal antibodies towards human and canine CRP, respectively. Together these aspects made species-specific IQC necessary to monitor and detect any clinically significant fluctuations in analytical performance. A method for species-specific IQC was successfully developed and implemented for the CRP-TIA (Paper IV) using pooled patient serum as surrogate control material. Recommended procedures for optimization and implementation of IQC procedures were followed (Petersen et al., 1996; Westgard, 2003) during the process. The main purpose of the IQC is to ensure that errors of possible significance for the clinical decision is detected and corrected before the result is reported. Another important aspect is to reduce the risk of falsely rejecting clinically valid runs, due to too stringent IQC procedures (Westgard, 2001). In Paper IV, it was demonstrated that the use of the traditional IQC criterion of Levey-Jennings charts would result in three times as many falsely rejected runs as the reported optimized procedure for the CRP-TIA, resulting in unnecessary waste of time and reagents. In other cases, the traditional criterion may not be stringent enough and may result in clinical decisions made on a false basis due to undetected errors of clinical importance. Thus, for every laboratory it is advisable to plan the IQC at an assay-level to optimize the IQC performance. In the absence of available recognized species-specific control material, the use of surrogates should be considered, as demonstrated in the present thesis (Paper IV). Using such surrogates as control material limits the surveillance of inaccuracy to a detection of important fluctuations instead of assessing the absolute level of inaccuracy. However, this should be sufficient if the presence of clinically important inaccuracies was proven unlikely by other measures at the initiation of the IQC procedure, as it was done in Paper II for the CRP-TIA. To the author's knowledge, no previous reports on such systematic approach of optimization of a species-specific IQC in veterinary clinical pathology have been published, even though; it is an important issue, especially when relying on a heterologous determination.

Improvement of analytical performance

In the case of the CRP-TIA, the sources of variation in analytical performance could possibly be limited by using purified canine CRP as calibration material, as this would eliminate the dependency of a stable relative cross-reactivity of the antibodies towards human and canine CRP, respectively. Preliminary results indicate an improved linearity under dilution compared to the observations made in Paper II (unpublished data) and other aspects of the performance may also improve, although this again depends on the availability of such material.

CRP-ELISA

A commercially available canine specific CRP-ELISA was also validated for clinical purposes in the present thesis (Paper I), with acceptable within-run analytical performance. However, between-run imprecision at high levels of CRP could not meet the performance standard, as also observed by others (Martínez-Subiela et al., 2003c). In practical terms this would make it necessary to perform additional measures to enable clinical use, e.g. to analyze serially obtained samples from the same individual together in the same analytical run to eliminate the inter-assay variation and thus, ensure reliable monitoring of the CRP level.

General practicability of assays

In general, TIA is considered to be superior to ELISA regarding practicability, as a TIA makes it possible to run single samples on demand compared to ELISA where batch-runs are usually necessary (Eckersall et al., 1991). This together with the poorer time-effectiveness of the CRP-ELISA compared to the automated CRP-TIA makes the CRP-ELISA an important alternative only if apparatus for running the CRP-TIA is not at hand or the CRP-TIA should fail to withhold the clinically acceptable performance in the future.

Automated assays specific for canine CRP have been developed (Eckersall et al., 1991; Onishi et al., 2000; Kumagai et al., 2004) and these homologous assays should be preferred to heterologous assays if analytical performance and practicability are equalled. However, an important additional aspect in the assessment of practicability, besides automation is general availability and economic costs, especially important

when assessing the applicability for wide-spread routine purposes. In these aspects the level of practicability of the CRP-TIA is unequalled at the present.

Conclusions of Phase I

The validation of a canine specific CRP ELISA (Paper I) and a human CRP-TIA (Paper II) for the clinical useful determination of canine serum CRP assessed by means of objective analytical performance standards (Paper III) and the successful application of a canine-specific IQC including the observation of an excellent long-term analytical performance of the CRP-TIA (Paper IV) sustain the hypothesis of phase I in the thesis.

Phase II and III – Overlap and Clinical Investigations

As expected, a marked and significant difference in the median-levels of CRP between dogs with inflammatory diseases and dogs without systemic inflammation (healthy dogs and dogs with a non-inflammatory disorder) were observed in the overlap investigations (Paper V). It is well established that CRP is a major acute phase protein in dogs and good evidence for an elevated level of canine CRP being consistent with increased systemic inflammatory activity (see Introduction).

Diagnostic properties of canine CRP

Canine CRP seemingly has a substantial potential as a clinically useful marker of systemic inflammatory activity with high diagnostic sensitivity and specificity. C-reactive protein is a non-specific marker of systemic inflammation regarding the nature of stimulus and the relative concentration seems to relate to the relative inflammatory activity of the disease process. These properties facilitate the use of canine CRP in the selection of “first-line” tests used in the phase of differential-diagnostic considerations of the diseased dog, where it can provide valuable information of possible inflammatory involvement. The properties of high diagnostic sensitivity, due to the marked increase in a wide range of systemic inflammatory activities and a high diagnostic specificity in the detection of inflammation, but being non-specific regarding the nature of stimulus, strengthen this applicability. This was in part sustained by the observations in Paper V, where a high diagnostic specificity for the CRP-TIA to discriminate between different subpopulations of diseased dogs (dogs with or without diseases causing systemic inflammation) and between healthy dogs and dogs with diseases causing systemic

inflammation could be obtained (cut-off between 16.0-17.8 mg/L). At the reported cut-off level the high diagnostic specificity was obtained without adversely impairing the capability to detect a wide range of inflammatory activity. Optimizing diagnostic specificity without impairing the detection of a wide range of inflammatory activity observed in various diseases will most likely ensure a high predictive value of a positive result under different clinical settings, as also reported for canine CRP by others (Riley and Zontine, 1972; Eckersall et al., 1988; Börngen, 1998).

If the objective is to rule out systemic inflammatory activity, e.g. in health screenings, diagnostic sensitivity should be favoured to facilitate high predictive value of a negative result. However, an observed low index of individuality for canine CRP (R=0.87), i.e. the ratio of intra-individual and inter-individual biological variation seem to impair this application (Paper III). The index of individuality can be used to assess the utility of population-based reference ranges (and also other population-based clinical decision levels) to evaluate single measurements (Harris, 1974). The observed low index of individuality characterizes canine CRP as relative tightly controlled within the individual compared to the variation observed between individuals, as also observed by others (Otabe et al., 1998). Therefore, the use of population-based decision levels are insensitive in the detection of small, but to the individual significant changes in CRP (Harris, 1974). The properties of canine CRP as an acute phase marker, thus, seem to favour its clinical applicability as a confirmative marker of systemic inflammatory activity compared to the applicability as an exclusion marker.

Monitoring properties of canine CRP

There is also substantial evidence for a correlation between the canine CRP concentrations and the activity of the inflammatory process, and that fluctuations of CRP during the state of disease is an almost “real-time” marker of the activity (see Introduction). This, together with the apparently high diagnostic specificity in detecting systemic inflammation and the lack of observed modulatory effects of a wide range of common therapeutics (see Introduction), gives canine CRP a potential as an objective marker for monitoring inflammatory activity during the state of disease and treatment thereof. The critical difference (CD) of canine CRP, i.e. the change in CRP needed between two serially obtained samples from the same individual to be significant was assessed from observations on biological variation to be 71.7% (Paper III). A similar

study on the biological variation of canine CRP also reported the different variance components of biological variation (0.82 mg/L, 2.21 $[mg/L]^2$, 1.74 $[mg/L]^2$ and 0.74 $[mg/L]^2$ for overall arithmetic mean and for intra-individual, inter-individual and analytical variance, respectively) (Martínez-Subiela et al., 2003b) corresponding to a CD as high as 583%. Unfortunately, a CD that high also indicates that outlying observations may have been included in the study causing biased estimates of the variance components and thus, a biased estimate of the critical difference (Franzini, 1998).

The CD will apply as an objective clinical decision level to both monitor clinically significant increases and decreases of CRP to detect either increased or decreased inflammatory activity between two samples (Clark and Fraser, 1993). It can also be used to evaluate differences between multiple serially obtained samples and this slightly more complicated statistical matter is described elsewhere (Kroll, 2002).

Conclusions of Phase II and III

The observations made in Paper III and V of the present thesis, together with the substantial information from other studies assessing CRP values in health and disease and investigating the clinical useful discriminative properties of canine CRP (see Introduction) sustain the presence of clinically useful discriminatory abilities of canine CRP as hypothesised. The observations made in Paper III and V provide clinical decision levels to evaluate serial and single measurements of canine CRP, respectively. The properties of canine CRP were observed to favour its clinical applicability as a confirmative marker of systemic inflammatory activity, rather than an exclusion marker.

Phase IV – Outcome and Utility Investigations

The essence of Phase IV is enclosed in the statement of Zweig and Robertson (1982): “A laboratory test is clinically useful only if it successfully answers a question of consequence to patient management”. Phase IV is only applied if the previous phases of the applicability assessment were successfully answered to, but, in the end, if the hypothesis of the final phase: “Canine CRP measurements have a positive consequence on the case management of individuals or groups of individuals in canine medicine” is rejected the results of the previous phases are of no significance.

The introduction of novel routine parameters can improve case management in several ways, e.g. by:

- Improving clinical outcome
- Providing equivalent clinically useful information faster than established parameters
- Providing equivalent clinically useful information more cost-effective than established parameters

Information on the effect on clinical outcome of established canine inflammatory markers is scarcely available. One study assessing the diagnostic sensitivity and specificity of traditional inflammatory markers (rectal temperature, CBC, band neutrophil concentration, heart rate and respiratory rate) in detecting canine sepsis, which *per se* includes an APR, identified CBC and band neutrophil number as the best performing single markers (Hauptman et al., 1997). The best detection of sepsis was, however, obtained using all parameters in a combined serial and parallel setting with observed diagnostic sensitivities and specificities of 83%-97% and 64%-77%, respectively (Hauptman et al., 1997). An observed clinical performance to which the observed diagnostic sensitivities and specificities of canine CRP seem at least comparable, especially regarding diagnostic specificity (Paper V). This can in part be explained by the stress-lability (e.g. affected by endogenous corticosteroids and catecholamines) of these traditional markers of systemic inflammation, except for band neutrophils, whereas CRP at the present state of knowledge is only induced by proinflammatory hypercytokinaemia.

It is not clear whether an increase in CRP or an altered CBC are the first appearing sign of an APR (Yamamoto et al., 1993b; Yamashita et al., 1994; Burton et al., 1994; Otabe et al., 2000; Higgins et al., 2003). Thus, it should be investigated whether the information of CRP and CBC could be complementary in the early detection of an APR and as a conglomerate improve the case management of patients during the per-acute inflammatory state of disease.

The results of Paper VI suggested an improved case management of type II immune-mediated polyarthritis (IMPA) by means of CRP measurements. Canine serum CRP seemed to be an early objective marker of the relative inflammatory activity. In the absence of secondary causes of increased inflammatory activity, CRP was a marker of treatment efficiency during steroid, antibiotic and cytotoxic treatment of the IMPA and

furthermore seemed to be capable of early objective detection of relapses of clinical symptoms. In that case, diagnostic measures should be taken to exclude secondary causes of elevated inflammatory activity. When monitoring the effect of steroid therapy of immune-mediated polyarthritis, the clinician mainly has to rely on clinical signs since objective disease markers such as white blood cell count, and absolute numbers of neutrophil granulocytes, lymphocytes, monocytes and eosinophil granulocytes may be so affected by the steroid treatment *per se* that they are maybe inadequate for reliable monitoring of the effect of treatment. The observations made in Paper VI sustained previous reported observations, where canine CRP was identified as an objective marker of treatment efficiency during treatment of leishmaniasis (Martínez-Subiela et al., 2003a), as a marker of final treatment efficiency of canine inflammatory bowel disease (Jergens et al., 2003) and as an indicator of parasitaemic relapses during insufficient treatment of experimental *Trypanosoma brucei* infections (Ndung'u et al., 1991). The early and objective detection of inflammatory relapses during treatment of IMPA, as indicated in Paper VI, has the potential to improve case management. The detection preceded the reappearance of clinical symptoms and therefore enables an earlier initiation of diagnostic measures to identify the source of the change in inflammatory activity.

Finally, serum CRP possesses superior storage properties compared to the cellular markers of inflammation (Vogelaar et al., 2002), e.g. making retrospective diagnostic measures possible by reanalysis on stored serum.

Conclusions of Phase IV

Through evaluation of previously reported clinical findings on the utility of canine CRP in graduating, detecting and monitoring the appearance and variation in inflammatory activity, canine CRP measurements must be assumed to have a positive effect on the outcome to case management under various specific clinical settings, as exemplified by the findings in Paper VI. The hypothesis of Phase IV was therefore sustained.

General Conclusions

Based on the observations made in this thesis and those previously reported on canine CRP it can be concluded that canine CRP measurements are applicable for routine purposes in canine medicine. This hypothesis was thoroughly tested throughout the thesis in a stepwise manner, where the measurement of canine serum CRP answered positively to all phases.

The major contribution of the present thesis to the assessment of the applicability of canine CRP for clinical purposes can be summarized by the following conclusions:

- Canine CRP can be measured reliably and with high practicability by means of a commercially available automated CRP-TIA designed for the determination of human CRP concentrations with excellent analytical performance during short- and long-term evaluation as assessed by objective analytical performance standards (Paper II, III, and IV). An application which is unprecedented regarding canine CRP and which by means of enhanced practicability and availability compared to other methods for canine CRP determination should facilitate routine application.
- Canine CRP can be measured reliably by means of a canine CRP specific ELISA fulfilling objective analytical performance standards except for inter-assay imprecision (Paper I). An important alternative to the CRP-TIA during circumstances where this could be non-applicable.
- A simple species-specific IQC optimized for detection of clinically important errors and minimization of false-rejection can be implemented for the CRP-TIA to ensure control of the everyday and long-term analytical performance by means of pooled patient serum as surrogates for the lacking recognized standards and control material (Paper IV). To the author's knowledge, there are no previous reports of such optimized species-specific IQC in veterinary clinical pathology and the method presented in Paper IV can with advantage be used for other veterinary assays to ensure species-specific monitoring and detection of clinically important errors.
- The properties of canine CRP were observed to favour the use of CRP measurements as a confirmative parameter in detecting systemic inflammatory

activity and clinical decision levels optimized for this purpose were established (Paper III and V).

- An objective clinical decision level for evaluation of differences between serially obtained canine CRP measurements was established (Paper III).
- Observations were made during corticosteroid treatment in a case of type II immune-mediated polyarthritis that sustained the previous observations of a positive impact of canine CRP measurements on case management in specific clinical settings by providing clinical useful objective information on inflammatory activity, otherwise not obtainable (Paper VI).

Perspectives

In this thesis a thorough systematic evaluation of the different aspects of the applicability of CRP for routine purposes in canine medicine was performed. A continuum of objectively derived information on these aspects is thus now available to sustain previously reported findings regarding canine CRP. In perspective, this will hopefully help to increase and spread the use of canine CRP as a routine parameter for diagnostic, monitoring and possibly screening purposes. A routine implementation of canine CRP will automatically enhance the knowledge of it, as measurements will no longer be performed on a material as selected as during specific investigations. This will most likely lead to new hypothesis of the applicability of canine CRP measurements, as it did in human medicine regarding a possible link to atherosclerosis and –thrombosis (Manolov et al., 2003; Jialal et al., 2004). An interesting aspect here could be the evaluation, diagnosis and monitoring of inflammation-related coagulopathies.

Regarding quality control, there is a mere need for available recognised control material to ensure comparability between results obtained throughout a geographical area and to facilitate wide-spread appropriate IQC. The approach to implement a local species-specific IQC in the lack of recognised species-specific control material for canine CRP assays, as presented in Paper IV, can be used for other veterinary assays also. All veterinary assays should optimally be subdued to species-specific IQC, but to a higher degree in assays relying on heterologous determination of species-specific molecules, e.g. proteins. The method presented in paper IV is applicable to fill the period until the work to establish species-specific control materials (Eckersall et al., 1999; Skinner, 2001) is finished.

No doubt, that additional testing of clinical hypotheses is needed to widen the spectre of possible applications of canine CRP measurements and to identify the specific clinical entities that will benefit the most of its application. To perform the latter properly, there is a mere need for appropriately designed prospective randomized controlled studies on the effect of canine CRP measurements on the outcome of specific clinical entities, which would be the next logical step to enhance the evaluation of the applicability of canine CRP measurements.

The detection and classification of naturally occurring sepsis in dogs could be an interesting field where serum CRP measurements could facilitate the investigations. In human medicine there is a focus on that imbalances in the relative level of pro-inflammatory cytokines (e.g. IL-6) and the compensatory anti-inflammatory cytokines (e.g. IL-10) during sepsis may have a casual link to the development and outcome of sepsis (Gogos et al., 2000; Loisa et al., 2003; Carrigan et al., 2004). However, as measurements of canine IL-6 requires delicate bioassays and canine CRP has been reported to follow the IL-6 response (Yamashita et al., 1994) and reflects the extent of the inflammatory activity, CRP may be useful as a IL-6 surrogate to detect and classify possible imbalances in the inflammatory response to canine sepsis.

REFERENCES

Abernethy,T.J. and O.T.Avery, 1941: The occurence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patients' sera and the effect of calcium on the flocculation reaction with C-polysaccharide of pneumococcus. Journal of Experimental Medicine **73,** 173-182.

Agrawal,A., A.K.Shrive, T.J.Greenhough and J.E.Volanakis, 2001: Topology and structure of the C1q-binding site on C-reactive protein. Journal of Immunology **166,** 3998-4004.

Anderson,H.C. and M.McCarty, 1950: Determination of C-reactive protein in the blood as a measure of the activity of the disease process in acute rheumatic fever. American Journal of Medicine **8,** 445-455.

Anderson,N.R., K.Chatha, M.R.Holland and R.Gama, 2003: Effect of sample tube type and time to separation on in vitro levels of C-reactive protein. British Journal of Biomedical Science **60,** 164-165.

Anderson,N.R., K.Chatha, M.R.Holland and R.Gama, 2004: Interference caused by the contents of serum separator tubes in the Vitros CRP assay [Letter]. Annals of Clinical Biochemistry **41,** 171-172.

Andersson,M., M.Stenstrom, M.Vatne, E.Sevelius and L.Jonsson, 1998: Disease-related variations of the glycosylation of haptoglobin in the dog. Journal of Comparative Pathology **119,** 227-238.

Baltz,M.L., F.C.de Beer, A.Feinstein, E.A.Munn, C.P.Milstein, T.C.Fletcher, J.F.March, J.Taylor, C.Bruton, J.R.Clamp, A.J.S.Davies and M.B.Pepys, 1982: Phylogenetic aspects of C-reactive protein and related proteins. Annals of the New York Academy of Science **389,** 49-75.

Baumann,H. and J.Gauldie, 1994: The acute phase response. Immunology Today **15,** 74-80.

Bharadwaj,D., M.P.Stein, M.Volzer, C.Mold and T.W.Du Clos, 1999: The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. Journal of Experimental Medicine **190,** 585-590.

Borer,L.R., J.E.Peel, W.Seewald, P.Schawalder and D.E.Spreng, 2003: Effect of carprofen, etodolac, meloxicam, or butorphanol in dogs with induced acute synovitis. American Journal of Veterinary Research **64,** 1429-1437.

Börngen,S., 1998: Nachweis von C-reaktivem protein beim Hund [Dissertation]. Institut für Bakteriologie und Mykologie der Veterinärmedizinischen Fakultät der Universität Leipzig, Germany. pp. 1-95.

Bossuyt,P.M., J.B.Reitsma, D.E.Bruns, C.A.Gatsonis, P.P.Glasziou, L.M.Irwig, J.G.Lijmer, D.Moher, D.Rennie and H.C.De Vet, 2003: Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Clinical Chemistry and Laboratory Medicine **41,** 68-73.

Burton,S.A., D.J.Honor, A.L.Mackenzie, P.D.Eckersall, R.J.F.Markham and B.S.Horney, 1994: C-reactive protein concentration in dogs with inflammatory leukograms. American Journal of Veterinary Research **55,** 613-618.

Carrigan,S.D., G.Scott and M.Tabrizian, 2004: Toward Resolving the Challenges of Sepsis Diagnosis. Clinical Chemistry

Casals,C., A.Varela, M.L.Ruano, F.Valino, J.Perez-Gil, N.Torre, E.Jorge, F.Tendillo and J.L.Castillo-Olivares, 1998: Increase of C-reactive protein and decrease of surfactant protein A in surfactant after lung transplantation. American Journal of Respiratory and Critical Care Medicine **157,** 43-49.

Caspi,D., M.L.Baltz, F.Snel, E.Gruys, D.Niv, R.M.Batt and E.A.Munn, 1984: Isolation and characterization of C-reactive protein from the dog. Immunology **53,** 307-313.

Caspi,D., F.W.J.J.Snel, R.M.Batt, D.Bennett, G.R.Rutteman, E.G.Hartman, M.L.Baltz, E.Gruys and M.B.Pepys, 1987: C-reactive protein in dogs. American Journal of Veterinary Research **48,** 919-921.

Castell,J.V., M.J.Gomez-Lechon, M.David, T.Andus, T.Geiger, R.Trullenque, R.Fabra and P.C.Heinrich, 1989: Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. FEBS Letters **242,** 237-239.

Chang,C.Y., J.Y.Lu, T.I.Chien, J.T.Kao, M.C.Lin, P.C.Shih and S.N.Yan, 2003: Interference caused by the contents of serum separator tubes in the Vitros CRP assay. Annals of Clinical Biochemistry **40,** 249-251.

Clark,G.H. and C.G.Fraser, 1993: Biological variation of acute phase proteins. Annals of Clinical Biochemistry **30,** 373-376.

Concannon,P.W., T.Gimpel, L.Newton and V.D.Castracane, 1996: Postimplantation increase in plasma fibrinogen concentration with increase in relaxin concentration in pregnant dogs. American Journal of Veterinary Research **57,** 1382-1385.

Conner,J.G., P.D.Eckersall, J.Ferguson and T.A.Douglas, 1988: Acute phase response in the dog following surgical trauma. Research in Veterinary Science **45,** 107-110.

Deegan,O., K.Walshe, K.Kavanagh and S.Doyle, 2003: Quantitative detection of C-reactive protein using phosphocholine-labelled enzyme or microspheres. Analytical Biochemistry **312,** 175-181.

Dillman,R.C. and E.H.Coles, 1966: A canine serum fraction analogous to human C-reactive protein. American Journal of Veterinary Research **27,** 1769-1775.

Dinarello,C.A., 1997: Interleukin-1. Cytokine & Growth Factor Reviews **8,** 253-265.

Dong,Q. and J.R.Wright, 1996: Expression of C-reactive protein by alveolar macrophages. Journal of Immunology **156,** 4815-4820.

Dougherty,T.J., H.Gewurz and J.N.Siegel, 1991: Preferential binding and aggregation of rabbit C-reactive protein with arginine-rich proteins. Molecular Immunology **28,** 1113-1120.

Du Clos,T.W., L.T.Zlock, P.S.Hicks and C.Mold, 1994: Decreased autoantibody levels and enhanced survival of (NZB x NZW) F1 mice treated with C-reactive protein. Clinical Immunology and Immunopathology **70,** 22-27.

Du Clos,T.W., L.T.Zlock and R.L.Rubin, 1988: Analysis of the binding of C-reactive protein to histones and chromatin. Journal of Immunology **141,** 4266-4270.

Eckersall,P.D. and J.G.Conner, 1988: Bovine and canine acute phase proteins. Veterinary Research Communications **12,** 169-178.

Eckersall,P.D., J.G.Conner and J.Harvie, 1991: An immunoturbidimetric assay for canine C-reactive protein. Veterinary Research Communications **15,** 17-24.

Eckersall,P.D., J.G.Conner and H.Parton, 1989: An enzyme-linked immunosorbent assay for canine C-reactive protein. Veterinary Record **124,** 490-491.

Eckersall,P.D., S.Duthie, M.J.M.Toussaint, E.Gruys, P.Heegaard, M.Alava, C.Lipperheide and F.Madec, 1999: Standardisation of diagnostic assays for animal acute phase proteins. Advances in Veterinary Medicine **41,** 643-655.

Eckersall,P.D., M.J.A.Harvey, J.M.Ferguson, J.P.Renton, D.A.Nickson and J.S.Boyd, 1993: Acute phase proteins in canine pregnancy (*canis familiaris*). Journal of Reproduction and Fertility **S47,** 159-164.

Eckersall,P.D., H.Parton, J.G.Conner, A.S.Nash, T.Watson, T.A.Douglas and D.J.Blackmore, 1988: Acute phase reactants in diseases of dog and cattle. In: Animal clinical biochemistry of the future, 1 ed. (editors: Blackmore,D.J. and P.D.Eckersall), Cambridge University Press, Cambridge, UK. pp. 225-230.

Egenhofer,C., K.Alsdorff, K.Fehsel and V.Kolb-Bachofen, 1993: Membrane-associated C-reactive protein on rat liver macrophages is synthesized within the macrophages, expressed as neo-C-reactive protein and bound through a C-reactive protein-specific membrane receptor. Hepatology **18,** 1216-1223.

Fiedel,B.A., R.M.Simpson and H.Gewurz, 1982: Effects of C-reactive protein (CRP) on platelet function. Annals of the New York Academy of Science **389,** 263-273.

Filep,J. and E.Foldes-Filep, 1989: Effects of C-reactive protein on human neutrophil granulocytes challenged with N-formyl-methionyl-leucyl-phenylalanine and platelet-activating factor. Life Science **44,** 517-524.

Filep,J.G., F.Herman, E.Kelemen and E.Foldes-Filep, 1991: C-reactive protein inhibits binding of platelet-activating factor to human platelets. Thrombosis Research **61,** 411-421.

Fransson,B.A., E.Karlstam, A.Bergstrom, A.S.Lagerstedt, J.S.Park, M.A.Evans and C.A.Ragle, 2004: C-reactive Protein in the Differentiation of Pyometra From Cystic Endometrial Hyperplasia/Mucometra in Dogs. Journal of the American Animal Hospital Association **40,** 391-399.

Franzini,C., 1998: Need for correct estimates of biological variation: The example of C-reactive protein [letter]. Clinical Chemistry and Laboratory Medicine **36,** 131-132.

Fraser,C.G., 2001: Biological variation: From principles to practice. AACC Press, Washington, USA. pp. 1-151.

Fraser,C.G., P.H.Petersen, C.Ricos and R.Haeckel, 1992: Proposed quality specifications for imprecision and inaccuracy of analytical systems for clinical chemistry. European Journal of Clinical Chemistry and Clinical Biochemistry **30,** 311-317.

Fujimoto,T., Y.Sato, N.Sasaki, R.Teshima, K.Hanaoka and S.Kitani, 2003: The canine mast cell activation via CRP. Biochemical and Biophysical Research Communications **301,** 212-217.

Fujise,H., 1992: Availability of acute phase protein for clinical examination in domestic animals: canine C-reactive protein. Journal of the Japan Veterinary Medical Association **45,** 731-737.

Gabay,C. and I.Kushner, 1999: Acute-phase proteins and other sytemic responses to inflammation. The New England Journal of Medicine **340,** 448-454.

Gershov,D., S.Kim, N.Brot and K.B.Elkon, 2000: C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. Journal of Experimental Medicine **192,** 1353-1364.

Gogos,C.A., E.Drosou, H.P.Bassaris and A.Skoutelis, 2000: Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. Journal of Infectious Diseases **181,** 176-180.

Gotschlich,E.C., 1962: Occurence of a substance analogous to C-reactive protein in acute phase dog sera. Federation Proceedings **21,** 14.

Gotschlich,E.C. and G.M.Edelman, 1965: C-reactive protein: a molecule composed of subunits. Proceedings of the National Academy of Science of the United States of America **54,** 558-566.

Gould,J.M. and J.N.Weiser, 2001: Expression of C-reactive protein in the human respiratory tract. Infection and Immunity **69,** 1747-1754.

Han,K.H., K.H.Hong, J.H.Park, J.Ko, D.H.Kang, K.J.Choi, M.K.Hong, S.W.Park and S.J.Park, 2004: C-reactive protein promotes monocyte chemoattractant protein-1-mediated chemotaxis through upregulating CC chemokine receptor 2 expression in human monocytes. Circulation **109,** 2566-2571.

Hansson,L.-O., 1996: C-reactive protein in clinical practice - with special regard to infectious diseases [Dissertation]. Karolinska Institute, Stockholm, Sweden. pp. 1-86.

Harris,E.K., 1974: Effects of intra- and interindividual variation on the appropriate use of normal ranges. Clinical Chemistry **20,** 1535-1542.

Hattori,Y., M.Matsumura and K.Kasai, 2003: Vascular smooth muscle cell activation by C-reactive protein. Cardiovascular Research **58,** 186-195.

Hauptman,J.G., R.Walshaw and N.B.Olivier, 1997: Evaluation of the sensitivity and specificity of diagnostic criteria for sepsis in dogs. Veterinary Surgery **26,** 393-397.

Hayashi,S., T.Jinbo, K.Iguchi, M.Shimizu, T.Shimada, M.Nomura, Y.Ishida and S.Yamamoto, 2001: A comparison of the concentrations of C-reactive protein and alpha1-acid

glycoprotein in the serum of young and adult dogs with acute inflammation. Veterinary Research Communications **25,** 117-126.

Higgins,M.A., B.R.Berridge, B.J.Mills, A.E.Schultze, H.Gao, G.H.Searfoss, T.K.Baker and T.P.Ryan, 2003: Gene expression analysis of the acute phase response using a canine microarray. Toxicological sciences **74,** 470-484.

Hoier,R. and A.L.Jensen, 1993: Evaluation of an enzyme linked immunosorbent assay (ELISA) for determination of insulin in dogs. Journal of Veterinary Medicine. A, Physiology, Pathology and Clinical Medicine **40,** 26-32.

Hokama,Y., R.Tam, W.Hirano and L.Kimura, 1974: Significance of C-reactive protein binding by lecithin: a simplified procedure for CRP isolation. Clinica Chimica Acta **50,** 53-62.

Hulton,N.R., D.J.Johnson and D.W.Wilmore, 1985: Limited effects of prostaglandin inhibitors in Escherichia coli sepsis. Surgery **98,** 291-297.

Hurlimann,J., G.J.Thorbecke and G.M.Hochwald, 1966: The liver as the site of C-reactive protein formation. Journal of Experimental Medicine **123,** 365-378.

Jabs,W.J., B.A.Logering, P.Gerke, B.Kreft, E.M.Wolber, M.H.Klinger, L.Fricke and J.Steinhoff, 2003: The kidney as a second site of human C-reactive protein formation in vivo. European Journal of Immunology **33,** 152-161.

Jensen,A.L., M.Bomholt and L.Moe, 2001: Preliminary evaluation of a particle enhanced turbidometric immunoassay (PETIA) for the determination of serum cystatin C-like immunoreactivity in dogs. Veterinary Clinical Pathology **30,** 86-90.

Jergens,A.E., C.A.Schreiner, D.E.Frank, Y.Niyo, F.E.Ahrens, P.D.Eckersall, T.J.Benson and R.Evans, 2003: A scoring index for disease activity in canine inflammatory bowel disease. Journal of Veterinary Internal Medicine **17,** 291-297.

Jialal,I., S.Devaraj and S.K.Venugopal, 2004: C-reactive protein: risk marker or mediator in atherothrombosis? Hypertension **44,** 6-11.

Kampschmidt,R.F. and H.F.Upchurch, 1974: Effect of leukocytic endogenous mediator on plasma fibrinogen and haptoglobin. Proceedings of the Society for Experimental Biology and Medicine **146,** 904-907.

Kenny,D., C.G.Fraser, P.H.Petersen and A.Kallners, 1999: Consensus agreement. Scandinavian Journal of Clinical Laboratory Investigation **59,** 585.

Kishimoto,T., 1989: The biology of interleukin-6. Blood **74,** 1-10.

Kluft,C. and M.P.de Maat, 2002: Sensitive markers of inflammation make it possible to study the chronic process: the rise of interest in low levels of C-reactive protein. Vascular pharmacology **39,** 99-104.

Koj,A., 1985: Cytokines regulating acute inflammation and synthesis of acute phase proteins. Blut **51,** 267-274.

Kolb-Bachofen,V., N.Puchta-Teudt and C.Egenhofer, 1995: Expression of membrane-associated C-reactive protein by human monocytes: indications for a selectin-like activity participating in adhesion. Glycoconjugate Journal **12,** 122-127.

Kroll,M.H., 2002: Evaluating sequential values using time-adjusted biological variation. Clinical Chemistry and Laboratory Medicine **40,** 499-504.

Kumagai,K., H.Nakashima and K.Saku, 2004: The HMG-CoA reductase inhibitor atorvastatin prevents atrial fibrillation by inhibiting inflammation in a canine sterile pericarditis model. Cardiovascular Research **62,** 105-111.

Kuribayashi,T., T.Shimada, M.Matsumoto, K.Kawato, T.Honjyo, M.Fukuyama, Y.Yamamoto and S.Yamamoto, 2003: Determination of serum C-reactive protein (CRP) in healthy beagle dogs of various ages and pregnant beagle dogs. Experimental Animals **52,** 387-390.

Kushner,I. and G.Feldmann, 1978: Control of the acute phase response. Demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit. Journal of Experimental Medicine **148,** 466-477.

Kushner,I., M.Ganapathi and D.Schultz, 1989: The acute phase response is mediated by heterogeneous mechanisms. Annals of the New York Academy of Science **557,** 19-29.

Kushner,I., W.N.Ribich and J.B.Blair, 1980: Control of the acute-phase response. C-reactive protein synthesis by isolated perfused rabbit livers. Journal of Laboratory and Clinical Medicine **96,** 1037-1045.

Kuta,A.E. and L.L.Baum, 1986: C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes. Journal of Experimental Medicine **164,** 321-326.

Liu,Q., G.Djuricin, C.Nathan, P.Gattuso, R.A.Weinstein and R.A.Prinz, 2000: The effect of interleukin-6 on bacterial translocation in acute canine pancreatitis. International Journal of Pancreatology **27,** 157-165.

Loisa,P., T.Rinne, S.Laine, M.Hurme and S.Kaukinen, 2003: Anti-inflammatory cytokine response and the development of multiple organ failure in severe sepsis. Acta Anaesthesiologica Scandinavica **47,** 319-325.

Mackiewicz,A., I.Kushner and H.Baumann, 1993: Acute phase proteins: Molecular biology, Biochemistry, and clinical applications. CRC Press, London, UK. pp. 3-665.

MacLeod,C.M. and O.T.Avery, 1941: The occurence during acute infections of a protein not normally present in the blood. III. Immunological properties of the C-reactive protein and its differentation from normal blood proteins. Journal of Experimental Medicine **73,** 191-200.

Manolov,D.E., W.Koenig, V.Hombach and J.Torzewski, 2003: C-reactive protein and atherosclerosis. Is there a causal link? Histology and Histopathology **18,** 1189-1193.

Martínez-Subiela,S., L.J.Bernal and J.J.Ceron, 2003a: Serum concentrations of acute-phase proteins in dogs with leishmaniosis during short-term treatment. American Journal of Veterinary Research **64,** 1021-1026.

Martínez-Subiela,S., P.M.Dolores and J.J.Ceron, 2004a: Influence of different preanalytical factors on an ELISA for canine CRP determination [Abstract]. Veterinary Clinical Pathology [In Press].

Martínez-Subiela,S., P.J.Ginel and J.J.Ceron, 2004b: Effects of different glucocorticoid treatments on serum acute phase proteins in dogs. Veterinary Record **154,** 814-817.

Martínez-Subiela,S., F.Tecles and J.J.Ceron, 2003b: Critical differences of acute phase proteins in canine serum samples. Veterinary Journal **166,** 233-237.

Martínez-Subiela,S., F.Tecles, P.D.Eckersall and J.J.Ceron, 2002: Serum concentrations of acute phase proteins in dogs with leishmaniasis. Veterinary Record **150,** 241-244.

Martínez-Subiela,S., F.Tecles, M.D.Parra and J.J.Ceron, 2003c: Analytical validation of different methods for determination of main acute phase proteins in canine samples [Abstract]. Available at: http://www.gla.ac.uk/faculties/vet/research/protein/04_segovia_2003/abstracts/ponencia51.pdf. Accessed 13-9-2004.

Matijatko,V. and N.Kucer, 2002: CRP concentration in dogs with uncomplicated and complicated babesiosis [Abstract]. Available at: http://www.gla.ac.uk/faculties/vet/research/protein/03_doorn_2002/abstracts/Razdorov.html. Accessed 13-10-2004.

Maudsley,S. and M.B.Pepys, 1987: Immunochemical cross-reactions between pentraxins of different species. Immunology **62,** 17-22.

McCarty,M., 1947: The occurence during acute infections of a protein not normally present in the blood. IV. Crystalization of the C-reactive protein. Journal of Experimental Medicine **85,** 491-498.

McGrotty,Y.L., C.M.Knottenbelt, I.K.Ramsey, S.W.Reid and P.D.Eckersall, 2004: Evaluation of a rapid assay for canine C-reactive protein. Veterinary Record **154,** 175-176.

Mookerjea,S., J.Francis, D.Hunt, C.Y.Yang and A.Nagpurkar, 1994: Rat C-reactive protein causes a charge modification of LDL and stimulates its degradation by macrophages. Arteriosclerosis and Thrombosis **14,** 282-287.

Moshage,H., 1997: Cytokines and the hepatic acute phase response. Journal of Pathology **181,** 257-266.

Murphy,T.M., L.L.Baum and K.D.Beaman, 1991: Extrahepatic transcription of human C-reactive protein. Journal of Experimental Medicine **173,** 495-498.

Ndung'u,J.M., P.D.Eckersall and F.W.Jennings, 1991: Elevation of the concentration of acute phase proteins in dogs infected with *Trypanosoma brucei*. Acta Tropica **49,** 77-86.

Nesbitt,G.H., L.M.Freeman and S.S.Hannah, 2003: Effect of n-3 fatty acid ratio and dose on clinical manifestations, plasma fatty acids and inflammatory mediators in dogs with pruritus. Veterinary Dermatology **14,** 67-74.

Onishi,T., H.Inokuma, K.Ohno, S.Soeda, K.Noguchi and K.Sasaki, 2000: C-reactive protein concentrations in normal and diseased dogs - measured by laser nephelometric immunoassay. Journal of the Japan Veterinary Medical Association **53,** 595-601.

Onishi,T., T.Shimizu and T.Kajikawa, 1994: Simple and efficient purification of C-reactive protein from canine serum. Journal of Veterinary Medical Science **56,** 417-419.

Osmand,A.P., B.Friedenson, H.Gewurz, R.H.Painter, T.Hofmann and E.Shelton, 1977: Characterization of C-reactive protein and the complement subcomponent C1t as

homologous proteins displaying cyclic pentameric symmetry (pentraxins). Proceedings of the National Academy of Science of the United States of America **74,** 739-743.

Otabe,K., T.Ito, T.Sugimoto and S.Yamamoto, 2000: C-reactive protein (CRP) measurement in canine serum following experimentally-induced acute gastric mucosal injury. Laboratory Animals **34,** 434-438.

Otabe,K., T.Sugimoto, T.Jinbo, M.Honda, S.Kitao, S.Hayashi, M.Shimizu and S.Yamamoto, 1998: Physiological levels of C-reactive protein in normal canine sera. Veterinary Research Communications **22,** 77-85.

Parra,M.D., S.Martínez-Subiela, M.Tuomola and J.J.Ceron, 2002: Evaluation of different monoclonal antibodies against human CRP in a TR-FIA for determination of canine CRP. Available at: http://www.gla.ac.uk/faculties/vet/research/protein/03_doorn_2002/abstracts/Ceron.html . Accessed 13-9-2004.

Parra,M.D., M.Tuomola, J.Cabezas-Herrera and J.J.Ceron, 2004: Use of a time-resolved immunofluorometric assay for determination of canine C-reactive protein concentrations in whole blood. American Journal of Veterinary Research [In Press].

Pepys,M.B. and M.L.Baltz, 1983: Acute phase proteins with special reference to C-reactive protein and related proteins (pentraxins) and serum amyloid A protein. In: Advances in immunology, 1 ed. (editors: Dixon,W.J. and H.G.Kunkel), Academic Press, New York, USA. pp. 141-212.

Pepys,M.B. and G.M.Hirschfield, 2003: C-reactive protein: a critical update. Journal of Clinical Investigation **111,** 1805-1812.

Petersen,P.H., C.G.Fraser, L.Jørgensen, I.Brandslund, M.Stahl, E.M.S.Gowans, J.-C.Libeer and C.Ricós, 2002: Combination of analytical quality specifications based on biological within- and between-subject variation. Annals of Clinical Biochemistry **39,** 543-550.

Petersen,P.H., C.Ricos, D.Stoeckl, J.C.Libeer, H.Baadenhuijsen, C.G.Fraser and L.Thienpont, 1996: Proposed guidelines for the internal quality control of analytical results in the medical laboratory. European Journal of Clinical Chemistry and Clinical Biochemistry **34,** 983-999.

Pue,C.A., R.F.Mortensen, C.B.Marsh, H.A.Pope and M.D.Wewers, 1996: Acute phase levels of C-reactive protein enhance IL-1 beta and IL-1ra production by human blood monocytes but inhibit IL-1 beta and IL-1ra production by alveolar macrophages. Journal of Immunology **156,** 1594-1600.

Rikihisa,Y., S.Yamamoto, I.Kwak, Z.Iqbal, G.Kociba, J.Mott and W.Chichanasiriwithaya, 1994: C-reactive protein and alpha1-acid glycoprotein levels in dogs infected with *Ehrlichia canis*. Journal of Clinical Microbiology **32,** 912-917.

Riley,R.F. and M.K.Coleman, 1970: Isolation of C-reactive proteins of man monkey, rabbit and dog by affinity chromatography on phosphorylated cellulose. Clinica Chimica Acta **30,** 483-496.

Riley,R.F. and W.Zontine, 1972: Further observations on the properties of dog C-reactive protein and the C-reactive protein response in the dog. Journal of Laboratory and Clinical Medicine **80,** 698-703.

Robey,F.A. and T.Y.Liu, 1981: Limulin: a C-reactive protein from *Limulus polyphemus*. The Journal of Biological Chemistry **256,** 969-975.

Salonen,E.M., T.Vartio, K.Hedman and A.Vaheri, 1984: Binding of fibronectin by the acute phase reactant C-reactive protein. The Journal of Biological Chemistry **259,** 1496-1501.

Shephard,E.G., R.Anderson, O.Rosen, M.S.Myer, M.Fridkin, A.F.Strachan and F.C.de Beer, 1990: Peptides generated from C-reactive protein by a neutrophil membrane protease. Amino acid sequence and effects of peptides on neutrophil oxidative metabolism and chemotaxis. Journal of Immunology **145,** 1469-1476.

Shimada,T., Y.Ishida, M.Shimizu, M.Nomura, K.Kawato, K.Iguchi and T.Jinbo, 2002: Monitoring C-reactive protein in beagle dogs experimentally inoculated with *Ehrlichia canis*. Veterinary Research Communications **26,** 171-177.

Shrive,A.K., G.M.Cheetham, D.Holden, D.A.Myles, W.G.Turnell, J.E.Volanakis, M.B.Pepys, A.C.Bloomer and T.J.Greenhough, 1996: Three dimensional structure of human C-reactive protein. Nature Structural Biology **3,** 346-354.

Siegel,J., A.P.Osmand, M.F.Wilson and H.Gewurz, 1975: Interactions of C-reactive protein with the complement system. II. C-reactive protein-mediated consumption of complement by poly-L-lysine polymers and other polycations. Journal of Experimental Medicine **142,** 709-721.

Skinner,J.G., 2001: Special report. International standardization of acute phase proteins. Veterinary Clinical Pathology **30,** 2-7.

Spillmann,T., J.Korrell, A.Wittker, S.Börngen and M.Krüger, 2002: Serum canine pancreatic elastase and canine C-reactive protein for the diagnosis and prognosis of acute pancreatitis in dogs [Abstract]. Journal of Veterinary Internal Medicine **16,** 635.

Strandberg,T.E., H.Vanhanen and M.J.Tikkanen, 1999: Effect of statins on C-reactive protein in patients with coronary artery disease. Lancet **353,** 118-119.

Suffredini,A.F., G.Fantuzzi, R.Badolato, J.J.Oppenheim and N.P.O'grady, 1999: New insight into the biology of the acute phase response. Journal of Clinical Immunology **19,** 203-214.

Tagata,K., S.Yokoyama, T.Ginbo, M.Honda, T.Okimura, M.Odakura, M.Nomura and S.Yamamoto, 1996: Quantitative capillary reversed passive latex agglutination test for C-reactive protein (CRP) in the dog. Veterinary Research Communications **20,** 21-30.

Taylor,A.W., N.-O.Ku and R.F.Mortensen, 1989: Both human IL-1 and IL-6 induce synthesis of C-reactive protein (CRP) by the PLC/PRF/5 hepatoma cell line. Annals of the New York Academy of Science **557,** 532-533.

Tillet,W.S. and T.Francis, 1930: Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. Journal of Experimental Medicine **52,** 561-571.

Uhlenbruck,G., J.Solter and E.Janssen, 1981: [New reaction mechanisms of C-reactive protein (CRP) and related proteins (author's translation)]. Journal of Clinical Chemistry and Clinical Biochemistry **19,** 1201-1208.

Vogelaar,S.A., D.Posthuma, D.Boomsma and C.Kluft, 2002: Blood sample stability at room temperature for counting red and white blood cells and platelets. Vascular pharmacology **39,** 123-125.

Volanakis,J.E., 2001: Human C-reactive protein: expression, structure, and function. Molecular Immunology **38,** 189-197.

Volanakis,J.E. and K.W.Wirtz, 1979: Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. Nature **281,** 155-157.

Wannemacher,R.W., Jr., R.S.Pekarek, W.L.Thompson, R.T.Curnow, F.A.Beall, T.V.Zenser, F.R.DeRubertis and W.R.Beisel, 1975: A protein from polymorphonuclear leukocytes (LEM) which affects the rate of hepatic amino acid transport and synthesis of acute-phase globulins. Endocrinology **96,** 651-661.

Westgard,J.O., 2001: Six sigma quality design and control - desirable precision and requisite QC for laboratory measurement processes. Westgard QC, Inc., Madison, WI, USA. pp. 1-296.

Westgard,J.O., 2003: Internal quality control: planning and implementation strategies. Annals of Clinical Biochemistry **40,** 593-611.

Woollard,K.J., D.C.Phillips and H.R.Griffiths, 2002: Direct modulatory effect of C-reactive protein on primary human monocyte adhesion to human endothelial cells. Clinical and Experimental Immunology **130,** 256-262.

Yamamoto,S., S.Miyaji, N.Abe, K.Otabe, E.Furukawa and M.Naiki, 1993a: Canine C-reactive protein (CRP) does not share common antigenicity with human CRP. Veterinary Research Communications **17,** 259-266.

Yamamoto,S., T.Shida, M.Honda, Y.Ashida, Y.Rikihisa, M.Odakura, S.Hayashi, M.Nomura and Y.Isayama, 1994a: Serum C-reactive protein and immune responses in dogs inoculated with *Bordetella bronchiseptica* (phase I cells). Veterinary Research Communications **18,** 347-357.

Yamamoto,S., T.Shida, S.Miyaji, H.Santsuka, H.Fujise, K.Mukawa, E.Furukawa, T.Nagae and M.Naiki, 1993b: Changes in serum C-reactive protein levels in dogs with various disorders and surgical traumas. Veterinary Research Communications **17,** 85-93.

Yamamoto,S., T.Shida, T.Okimura, K.Otabe, M.Honda, Y.Ashinda, E.Furukawa, M.Sarikaputi and M.Naiki, 1994b: Determination of C-reactive protein in serum and plasma from healthy dogs and dogs with pneumonia by ELISA and slide reversed passive latex agglutination test. Veterinary Quarterly **16,** 74-77.

Yamamoto,S., K.Tagata, H.Nagahata, Y.Ishikawa, M.Morimatsu and M.Naiki, 1992: Isolation of canine C-reactive protein and characterization of its properties. Veterinary Immunology and Immunopathology **30,** 329-339.

Yamamoto,S., K.Tagata, K.Otabe and T.Shida, 1993c: Serum levels of C-reactive protein in dogs and simple techniques for its measurement. Journal of the Japan Veterinary Medical Association **46,** 870-873.

Yamashita,K., T.Fujinaga, T.Miyamoto, T.Hagio, Y.Izumisawa and T.Kotani, 1994: Canine acute phase response: relationship between serum cytokine activity and acute phase protein in dogs. Journal of Veterinary Medical Science **56,** 487-492.

Zouki,C., M.Beauchamp, C.Baron and J.G.Filep, 1997: Prevention of In vitro neutrophil adhesion to endothelial cells through shedding of L-selectin by C-reactive protein and peptides derived from C-reactive protein. Journal of Clinical Investigation **100,** 522-529.

Zweig,M.H. and E.A.Robertson, 1982: Why we need better test evaluations. Clinical Chemistry **28,** 1272-1276.