Canine C-reactive Protein

Validation of Two Automated Canine-specific C-reactive Protein Assays and Studies on Clinical and Research Applications

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Cover: Schematic figure of latex particle covered with antibodies, used in immunoturbidimetric assays (Nenne Jonsson).
Canine C-reactive protein. Validation of two automated canine-specific C-reactive protein assays and studies on clinical and research applications

Abstract
C-reactive protein (CRP) is a sensitive and specific marker of systemic inflammation in dogs, valuable for diagnosing and monitoring inflammatory diseases. The use of CRP in canine medicine has however been hampered by the lack of automated assays optimized for measuring CRP in this species. The need for improved CRP assays was the reason for initiating the current project, with the goal to generate automated, canine-specific CRP tests that could reliably measure serum CRP over the whole concentration range expected to occur in dogs. Two different assays were developed for this purpose. One was designed for routine diagnostic testing, and one was a high-sensitivity CRP test intended for research. Method validation studies were performed, demonstrating that both tests met the predefined quality criteria. Using the two novel CRP tests, it was possible to reliably measure serum CRP concentrations in the range of 0.5-1200 mg/l. After successful termination of the validation studies, the CRP assays were used in clinical research studies. C-reactive protein concentrations were measured in dogs with pyometra undergoing ovariohysterectomy, to evaluate how surgical treatment affected degree of systemic inflammation in these patients. Two other studies were performed to evaluate the usefulness of CRP as a diagnostic test. C-reactive protein concentration was found to discriminate well between dogs with suppurative arthritis and dogs with osteoarthritis, whereas measurement of CRP was not efficient for diagnosing late post-operative bacterial infections after orthopaedic surgery because these infections often did not elicit a systemic inflammatory response.

In conclusion, two novel automated canine-specific CRP assays were developed and validated with satisfactory results. The tests showed high practicability for measuring CRP in samples from clinical research studies. Availability of these assays will facilitate the use of CRP as a routine diagnostic test in veterinary medicine, and can improve quality in research on canine inflammatory diseases.

Keywords: Acute phase protein, arthritis, C-reactive protein, method validation, inflammation, immuno-turbidimetric assay, osteoarthritis, post-operative infection, pyometra, snake envenomation

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List of Publications

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


Paper I, II and III are reproduced with the permission of the publishers.
The contribution of Anna Hillström to the papers included in this thesis was as follows:

I Took major part in planning of the study, performed the laboratory work, performed data analysis, interpreted results together with supervisors, and had the main responsibility for writing of the manuscript.

II Took major part in planning of the study, performed most of the laboratory work and data analysis, interpreted results together with supervisors and co-authors, and had the main responsibility for writing of the manuscript.

III Took minor part in planning of the study, performed CRP analyses, took major part in analysis and interpretation of CRP data, and contributed to writing of the manuscript.

IV Took major part in planning of the study, sampled some of the dogs, performed all the laboratory work except for cytokine analyses, performed part of the data analysis, interpreted results together with co-authors and supervisors, and had the main responsibility for writing of the manuscript.
Additional publications related to the thesis


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
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<tr>
<td>ACVIM</td>
<td>American College of Veterinary Internal Medicine</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>ADH</td>
<td>Anti-diuretic hormone</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
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<td>cCRP</td>
<td>Gentian canine-specific C-reactive protein</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<td>CV&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Analytical coefficient of variation</td>
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<tr>
<td>CV&lt;sub&gt;G&lt;/sub&gt;</td>
<td>Between animal coefficient of variation</td>
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<td>CV&lt;sub&gt;I&lt;/sub&gt;</td>
<td>Within animal coefficient of variation</td>
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<td>hsCRP</td>
<td>Gentian canine-specific high-sensitivity C-reactive protein</td>
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<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
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<td>IQR</td>
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<td>LoB</td>
<td>Limit of blank</td>
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<td>LoD</td>
<td>Limit of detection</td>
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<td>LoQ</td>
<td>Limit of quantification</td>
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<tr>
<td>MMVD</td>
<td>Myxomatous mitral valve disease</td>
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<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<td>OA</td>
<td>Osteoarthritis</td>
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<td>OR</td>
<td>Odds Ratio</td>
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<td>QQ-plot</td>
<td>Quantile-quantile plot</td>
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<td>ROC</td>
<td>Receiver operating characteristics</td>
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<td>SAA</td>
<td>Serum Amyloid A</td>
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<tr>
<td>SLU</td>
<td>Swedish University of Agricultural Sciences</td>
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<tr>
<td>STARD</td>
<td>Standards for reporting of diagnostic accuracy</td>
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<tr>
<td>TE</td>
<td>Total error</td>
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<tr>
<td>TE&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Total error allowable</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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<td>TSH</td>
<td>Thyroid stimulating hormone</td>
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1 Introduction

Canine C-reactive protein (CRP) is a well-established inflammatory marker in dogs. Healthy dogs have low circulating concentrations of CRP, whereas there is a rapid and marked increase in CRP concentration during systemic inflammatory disease. The magnitude of increase in CRP concentration reflects the degree of systemic inflammation, and successful treatment leads to a rapid decline in CRP concentration. These properties make CRP a valuable marker both for diagnosing and monitoring systemic inflammatory diseases in dogs (Eckersall & Bell, 2010; Cerón et al., 2005; Kjelgaard-Hansen, 2004).

The use of CRP as a diagnostic test in canine medicine has however been hampered by the lack of automated, canine-specific CRP assays. Instead, measurements of canine CRP in routine practice have commonly been done with immunological assays developed for measuring CRP in human beings. Because human and canine CRP are not identical, the human-based assays did not perform optimally when measuring canine CRP (Kjelgaard-Hansen, 2010; Caspi et al., 1984). Moreover in the research setting, there was a demand for better CRP tests. For example, automated canine-specific assays that could measure very low CRP concentrations with high precision were needed.

The need for improved assays for measuring canine CRP was the main reason for initiating the current project. Availability of such assays should enable a more widespread use of CRP as a marker of systemic inflammation in dogs, both in clinical practice and in research.
2 Inflammation

Inflammation is a non-specific response to noxious stimuli, which can remove harmful agents and initiate repair of damaged tissue (Hansson, 1996). Causes of inflammation include infection, aseptic tissue injury, neoplastic growth, and immunological disorders (Heinrich et al., 1990). In early descriptions of inflammation, focus was on the local inflammatory signs redness (*rubor*), swelling (*tumour*), heat (*calor*), pain (*dolor*), and loss of function (*functio laesa*) (Scott et al., 2004; Benaroyo, 1994; Rocha e Silva, 1978). With the availability of more advanced technologies to study biological processes, the concept of inflammation broadened. Using microscopy it was possible to identify infiltration of leukocytes in damaged tissue, and today direct measurements of pro-inflammatory mediators such as cytokines can be performed (Scott et al., 2004). If inflammation is defined as a condition where the concentrations of pro-inflammatory mediators are increased, i.e. using a biochemical definition of inflammation, low-grade systemic inflammation can be found also in subjects without clinical signs of inflammation (Pedersen & Febbraio, 2008; Scott et al., 2004).

Cytokines play a major role in the induction and regulation of inflammation. They constitute a heterogeneous group of proteins and polypeptides, regulating intercellular communication. After an inflammatory stimulus, cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)α are produced by various cell types including macrophages, lymphocytes, endothelial cells and fibroblasts (Thomson & Lotze, 2003). The cytokines operate in a complex signalling network, where combinations of cytokines can have additive, inhibitory and synergistic effects (Mackiewicz et al., 1991). Locally, the cytokines are involved in chemotaxis, angiogenesis and apoptosis (Epstein & Luster, 1998; Mangan & Wahl, 1991). Cytokines also induce systemic effects, because they act on organs distant from the local inflammatory site activating receptors on different target cells (Gruys et al., 2005). This leads to a large number of cellular, metabolic and hormonal changes (Bode et al., 2012; Epstein et al., 1999; Hansson, 1996; Heinrich et al., 1990) (Figure 1).
In the clinical setting it is often appropriate to make a distinction between local and systemic inflammation (Figure 2). The reason for needing to separate local from systemic inflammation is that diagnostic procedures and patient management differ in the two conditions. In local inflammation, diagnosis is based on direct examination of the affected area for example by palpating a swollen, painful structure, or by evaluating cell numbers in samples from the tissue (Bauer et al., 2012). This is in contrast to systemic inflammation, which can be diagnosed based on haematological and biochemistry changes in peripheral blood samples (Figure 1). Whereas local inflammation may cause discomfort and pain, the general condition of the animal is typically not affected. Animals with systemic inflammation, on the other hand, often show clinical signs such as depression and anorexia. Systemic inflammation may develop into a life-threatening condition (Gebhardt et al., 2009), and these patients thus need to be more closely monitored compared with dogs with local inflammation only. There is no sharp line between local and systemic inflammation, but rather a continuum. Mild local inflammation does not cause significant systemic inflammation (Bauer et al., 2012), but in cases of more severe local inflammation a systemic inflammatory response is expected (Yamashita et al., 1994).
To summarize, inflammation is a broad concept which can be defined by clinical, cellular and biochemical changes (Scott et al., 2004). In a clinical setting it is helpful to distinguish between local and systemic inflammation, as this may affect the choice of diagnostic tests and treatments.

2.1 The acute phase proteins

In systemic inflammation, the protein synthesis in the liver changes as a consequence of increased concentrations of pro-inflammatory cytokines (Figure 1). Proteins whose circulating concentrations change by more than 25% during inflammation are called acute phase proteins (Eckersall & Bell, 2010). They are classified as positive or negative acute phase proteins, depending on whether their concentration increases or decreases during inflammation. Positive acute phase proteins are further classified by the magnitude of their increase, with moderate acute phase proteins showing a 2- to 10-fold increase, and major acute phase proteins a 10- to 100-fold increase, during systemic inflammation (Table 1) (Cerón et al., 2005). In healthy animals, the concentrations of major acute phase proteins are generally lower than the concentrations of moderate acute phase proteins (Cerón et al., 2005; Murata et al., 2004). Thus, the difference in
protein concentration between healthy and diseased dogs is more pronounced for the major acute phase proteins compared with the moderate ones.

Table 1. *Examples of canine acute phase proteins and their functions. Data are derived from review articles, where references to original studies are available (Cerón et al., 2005; Murata et al., 2004). APP: acute phase protein*

<table>
<thead>
<tr>
<th>Name</th>
<th>Classification</th>
<th>Function(s)</th>
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<tbody>
<tr>
<td>Fibrinogen</td>
<td>Moderate, positive APP</td>
<td>Fibrin formation, tissue repair</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Moderate, positive APP</td>
<td>Involved in host defence, binding of free haemoglobin.</td>
</tr>
<tr>
<td>Alpha 1-acid glycoprotein</td>
<td>Moderate, positive APP</td>
<td>Anti-inflammatory and immunomodulatory effects</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Moderate, positive APP</td>
<td>Protection against oxidant compounds, transport of copper</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>Major, positive APP</td>
<td>Opsonisation, induction of cytokine production</td>
</tr>
<tr>
<td>Serum Amyloid A</td>
<td>Major, positive APP</td>
<td>Lipid metabolism and transport, regulation of inflammation</td>
</tr>
<tr>
<td>Albumin</td>
<td>Negative APP</td>
<td>Maintain colloidal osmotic pressure, transport protein</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Negative APP</td>
<td>Iron transport</td>
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</table>

For diagnostic purposes, major positive acute phase proteins are the most relevant to measure because they efficiently discriminate between dogs with and without systemic inflammation (Christensen et al., 2014). Their concentrations start to increase already hours after initiation of inflammation and decline rapidly when the inflammatory stimulus is removed, in contrast to the moderate acute phase proteins whose concentrations change more slowly (Cerón et al., 2005; Murata et al., 2004). When comparing the two major acute phase proteins Serum Amyloid A (SAA) and CRP as diagnostic tests in dogs, SAA seems to be the more sensitive inflammatory marker of the two (Christensen et al., 2014). However, SAA is a protein that is difficult to purify (Christensen et al., 2013) and automated canine-specific SAA assays have not been commercially available. Thus, CRP has emerged as the most widely used major acute phase protein in canine medicine.
3  Canine C-reactive protein

3.1  General characteristics of C-reactive protein

Canine CRP has a molecular weight of approximately 100 kDa and consists of five non-covalently associated subunits arranged symmetrically around a central pore (Jasensky et al., 2014; Caspi et al., 1984) (Figure 3). Two of the subunits of canine CRP are glycosylated (Caspi et al., 1984), in contrast to human CRP that is not a glycoprotein (Das et al., 2003). Protein sequencing of native canine CRP has been performed, showing 60% homology with human CRP (Jasensky et al., 2014).

![Figure 3. Molecular structure of human CRP. Reprinted with permission (http://www.ebi.ac.uk).](image)

The liver is the main site of CRP production (Kushner & Feldmann, 1978; Hurlimann et al., 1966). The transcription of the CRP gene in the hepatocytes is upregulated by IL-6, and is enhanced by the presence of IL-1 (Kushner et al., 1995; Castell et al., 1989; Ganapathi et al., 1988). There is no storage pool of CRP (Kushner & Feldmann, 1978), and consequently increased CRP concentration during inflammation can be explained by de novo synthesis. Extrahepatic CRP production in low concentrations has been reported, but is not considered to significantly affect circulating CRP concentrations (Casals et al., 1998; Kuta & Baum, 1986).
In humans, the half-life of radiolabelled CRP was reported to be 19 hours both in healthy subjects and in patients with systemic inflammatory disease (Vigushin et al., 1993). The half-life of canine CRP is not known.

Various functions of CRP have been described. The protein binds to pathogens and ligands on damaged cells and function as an opsonin, and thus plays part in the early defence of the innate immune system (Hart et al., 2004; Du Clos, 2000; Volanakis & Wirtz, 1979). Furthermore, CRP activates the complement system (Mold et al., 1999), induces cytokine production (Ballou & Lozanski, 1992) and play a role as a regulatory protein (Du Clos, 2003; Gershov et al., 2000). The fact that CRP has been well-preserved through evolution and is present in many different organisms, also in primitive ones (Robey & Liu, 1981), indicates that it is a protein of importance for the immune system.

3.2 Physiological factors affecting C-reactive protein concentrations

In order to successfully apply CRP as a diagnostic test, knowledge about physiological factors affecting CRP concentrations is required. Baseline CRP concentrations are generally low, < 10 mg/l (Kjelgaard-Hansen et al., 2003b; Kuribayashi et al., 2003; Yamamoto et al., 1994b; Caspi et al., 1987), and were reported not to be affected by diurnal variation, sex or age (Kuribayashi et al., 2003; Otabe et al., 1998). The effect of breed on CRP concentration has been scarcely studied. There was no difference in CRP concentrations when comparing Greyhounds with other breeds (Lucas et al., 2015; Couto et al., 2009) whereas other studies indicate that minor breed differences in CRP concentration may exist (Fergestad et al., 2016; Wong et al., 2011). In human beings, there is a CRP gene polymorphism which can explain differences in CRP concentration in healthy people of different ethnicities (Zhu et al., 2013). It remains to be investigated whether this is the case also in dogs.

C-reactive protein concentrations in female dogs were not affected by oestrous cycle stage (Ulutas et al., 2009). In contrast, pregnancy did affect CRP concentrations with maximal levels observed day 30-45 after ovulation (Kuribayashi et al., 2003; Eckersall et al., 1993). The mean CRP concentration in pregnant beagles during this period was 77.5 mg/l, compared to < 10 mg/l in non-pregnant healthy dogs (Kuribayashi et al., 2003). It has been proposed that the increase in CRP concentration during pregnancy is associated with implantation of the foetuses (Eckersall et al., 1993), or that CRP is influenced by altered levels of endocrine hormones (Kuribayashi et al., 2003). However, no clear cause for the increased CRP concentrations found during pregnancy has
been established. There are other studies where no increase in CRP concentration was observed in pregnant dogs (Ulutas et al., 2009; Concannon et al., 1996), and the reason for this discrepancy is not known. The possibility that analytical problems occurred in some of the studies cannot be excluded because it is difficult to find a biological explanation for this marked variation.

Knowledge about effect from exercise on CRP concentration is important for clinicians using CRP as a diagnostic test, because physical activity is highly variable in dogs admitted to veterinary care. In sled dogs participating in long-distance endurance exercise, an increased CRP concentration was observed after racing compared to before the race. Mean CRP concentrations after racing were between 39 mg/l and 263 mg/l in different studies, which corresponded to a clinically significant increase (Fergestad et al., 2016; Spoo et al., 2015; Yazwinski et al., 2013; Kenyon et al., 2011; Wakshlag et al., 2010b). The increased CRP concentrations could be explained by strenuous exercise eliciting an inflammatory response in various organs or by release of IL-6 from skeletal muscles during exercise, as has been described in humans (Trayhurn et al., 2011; Pedersen & Febbraio, 2008). After exercise of short duration, only mild and clinically insignificant increases of CRP concentrations were observed (Lucas et al., 2015; Krogh et al., 2014; Wakshlag et al., 2010a). It can be concluded that the effect from exercise on CRP concentration depends on the duration and magnitude of the physical effort. Therefore, it is only for dogs that have been subjected to long-term strenuous exercise that the clinician needs to take physical activity into consideration when interpreting CRP results.

Physiological stress induced by road transportation did not lead to a clinically significant increase in CRP concentration (Fazio et al., 2015). The lack of effect from the stress hormone cortisol on baseline CRP concentrations was also illustrated by the finding that dogs with hyperadrenocorticism did not have altered CRP concentrations compared to healthy dogs (Caldin et al., 2009).

In conclusion, little variation is expected to occur in CRP concentrations due to physiological variations, except during pregnancy and after strenuous exercise. Therefore, an increased CRP concentration in a dog is highly specific for systemic inflammation.

3.3 C-reactive protein as a marker of systemic inflammation

Measurement of CRP is valuable in a clinical setting to diagnose systemic inflammation in dogs. Increased CRP concentrations have been reported in a large number of conditions, including bacterial, viral and parasitic infection (Méndez et al., 2015; Fransson et al., 2004; Yamamoto et al., 1993), immune-
mediated disease (Griebsch et al., 2009; Ohno et al., 2006), neoplasia (Tecles et al., 2005), sterile inflammation (Yuki et al., 2015; Bayramli & Ulutas, 2008; Otabe et al., 2000; Yamashita et al., 1994), and surgical trauma (Christensen et al., 2015). In studies investigating large numbers of dogs with and without systemic inflammatory diseases, it was shown that CRP concentration efficiently discriminated between the two groups (Christensen et al., 2014; Nakamura et al., 2008).

The kinetics of CRP have been studied both in natural and experimentally induced inflammation. Generally, an increase in blood CRP concentration occurs within 4-6 hours after an inflammatory stimulus, reaching the maximum concentration after about 24-48 hours (Kuribayashi et al., 2015; Kjelgaard-Hansen et al., 2013; Kum et al., 2013; Ishida et al., 2011; Nevill et al., 2010; Yamashita et al., 1994; Yamamoto et al., 1992; Ganrot, 1973). The magnitude of increase in CRP concentration reflects the degree of tissue trauma (Kjelgaard-Hansen et al., 2013; Otabe et al., 2000; Yamamoto et al., 1993), and CRP concentrations decline with successful treatment of inflammatory diseases (Méndez et al., 2015; Yuki et al., 2015; Seo Kw Fau - Lee et al., 2012; Merlo et al., 2007; Nielsen et al., 2007; Ohno et al., 2006; Ndung'u et al., 1991). It can thus be concluded that CRP concentration not only provides information on whether systemic inflammation is present or not, but it can also be used to quantify the degree of inflammation.

It has been hypothesised that measuring CRP concentration would be of prognostic value, which is plausible bearing in mind that CRP is a quantitative inflammatory marker. The idea is that severe systemic inflammation should be associated both with a high CRP concentration, and with a poor prognosis. However, a single CRP measurement was not effective for judging prognosis in dogs with immune-mediated haemolytic anaemia (Griebsch et al., 2009; Mitchell et al., 2009), sepsis (Torrente et al., 2015; Gebhardt et al., 2009), pancreatitis (Yuki et al., 2015; Mansfield et al., 2008), babesiosis (Koster et al., 2009), acute abdomen syndrome (Galezowski et al., 2010), or in critically ill dogs (Chan et al., 2009). One explanation for this could be that CRP concentration only provides information about a limited part of the complicated inflammatory cascade. The cytokines involved in inflammation have both pro- and anti-inflammatory properties (Blackwell & Christman, 1996), and CRP concentration reflects the activity of the pro-inflammatory ones. It is possible that knowledge about the balance between pro- and anti-inflammatory mediators would enable more effective prognostication, but this information is currently not available in routine clinical practice. Another reason for why a single CRP value is not prognostic in inflammatory disease could be that the location of the inflammation is of importance. For example, inflammation in the central nervous
system may be devastating, even if it does not elicit a marked systemic inflammatory response (Vestergaard et al., 2013). On the other hand, a disease such as pyometra which causes severe systemic inflammation often have favourable prognosis, because effective treatment is available. Finally, the time point of sampling is important, considering that there is a delay of about 24 hours from an inflammatory stimulus until the maximal CRP concentration is observed. Therefore, if a dog is sampled early in the disease process, the CRP concentration does not yet correctly reflect the degree of inflammation.

Although a single measurement of CRP is not of prognostic value, it has been shown that decreasing CRP concentration over time in a patient is a favourable sign (Gebhardt et al., 2009; Ohno et al., 2006). Marked changes in CRP concentration can occur over a short period of time, with a decrease in CRP concentration of ~50% in 24 hours (unpublished data) to 48 hours (Gebhardt et al., 2009; Dabrowski et al., 2007; Ohno et al., 2006). This justifies the use of iterated measurements of CRP in dogs with systemic inflammatory diseases for monitoring. Considering how rapidly CRP concentrations can change over time, sampling with no more than 24 hours interval is probably relevant in selected cases.

A frequently asked question in veterinary practice is whether systemic inflammation in a dog is caused by bacterial infection or not, which is important because the answer often affects the choice of treatment. Because CRP is an unspecific marker of systemic inflammation, it cannot be used as a standalone test for diagnosing bacterial infection (Gebhardt et al., 2009; Bathen-Noethen et al., 2008). However, in people it has been shown that CRP measurements can be useful for indicating presence of bacterial infection when used in a specific, well-defined context (Cals et al., 2010; Muller et al., 2008). In dogs too, there are situations where this may be applicable, for example in respiratory disease (Viitanen et al., 2014). It must be emphasized though that it is only by interpreting CRP results together with anamnestic history and clinical findings, that the test has the potential to provide such information.

Because CRP is produced by hepatocytes, (Hurlimann et al., 1966), liver dysfunction could theoretically affect CRP concentrations. This has been investigated in human beings, where it was shown that CRP production was maintained during systemic inflammation despite advanced liver dysfunction (Pieri et al., 2014; Park et al., 2005). There are no similar studies in dogs, but increased CRP concentrations have been reported in dogs with chronic hepatitis and congenital portosystemic shunts indicating that dogs affected by these diseases are able to produce CRP (Gow et al., 2012; Nakamura et al., 2008). In people it was described that the more severe the underlying liver dysfunction, the lower the increase in CRP concentration was in the case of concurrent
bacterial infection (Pieri et al., 2014). This suggests that the CRP response may be blunted in humans with hepatic dysfunction, and it remains to be investigated whether this is the case also in dogs. A blunted CRP response after an inflammatory stimulus has previously been reported in canine patients with hyperadrenocorticism (Caldin et al., 2009), and in dogs of age ≤ 3 months (Hayashi et al., 2001). Thus, clinicians should be aware of the risk of underestimating the magnitude of systemic inflammation when interpreting CRP results in the above-mentioned patient groups.

Besides being a marker of systemic inflammation in a clinical setting, CRP concentrations have also been measured in research investigating conditions suspected to be associated with low-grade inflammation, such as obesity (Eirmann et al., 2009; Veiga et al., 2008; Yamka et al., 2006). The purpose of performing this type of studies is to learn more about the pathophysiology behind these conditions, rather than to find new clinical applications for CRP. Currently, there are no indications that exact determinations of CRP at low concentrations are useful in a clinical setting, with the possible exception of monitoring treatment response in dogs with inflammatory bowel disease (Jergens et al., 2010; Jergens et al., 2003). Potentially, situations will be identified in the future where measurement of CRP in the low range is beneficial, for example for detecting relapse in dogs under treatment for conditions associated with mild systemic inflammation.

In conclusion, measurement of CRP is valuable for diagnosing and monitoring systemic inflammation in dogs. C-reactive protein is an unspecific inflammatory marker, and cannot by itself be used for determining the cause of inflammation.

3.3.1 Effect of pharmaceuticals on C-reactive protein concentration

If treatment with pharmaceuticals directly affects the concentration of an inflammatory marker, it may diminish the marker’s diagnostic value. This is apparent when using the white blood cell count for diagnosing systemic inflammation, because a stress leukogram caused by glucocorticoid treatment may be identical to the leukogram present in chronic inflammatory disease (Jasper & Jain, 1965). This is in contrast to CRP, whose concentrations were not affected by glucocorticoid treatment neither in healthy dogs, nor in dogs with systemic inflammation (Martinez-Subiela et al., 2004; Yamamoto et al., 1994a). The lack of direct effect from glucocorticoids on CRP concentration makes CRP a suitable marker for monitoring inflammation in dogs under prednisolone treatment for immune-mediated disease (Lowrie et al., 2009; Kjelgaard-Hansen et al., 2006; Ohno et al., 2006). In such a patient, a change in CRP concentration
directly mirrors the inflammatory activity, without being biased by the drug itself.

No effect from NSAID treatment on CRP concentration was reported in dogs undergoing surgery (Kum et al., 2013) or in dogs with experimentally induced synovitis (Borer et al., 2003). Treatment with low-dose ketamine was described to decrease postoperative CRP concentrations in dogs undergoing ovariohysterectomy due to pyometra, and it was hypothesized that ketamine had an anti-inflammatory effect in these patients (Liao et al., 2014). Considering that ketamine is not a common treatment like glucocorticoids and NSAIDs, and that it is not for long-term use, this finding is probably of limited importance for veterinarians using CRP in a clinical setting.

To summarize, CRP concentration is not affected by treatment with glucocorticoids or NSAIDs, which is highly advantageous for a clinical marker of systemic inflammation.

3.4 Measurement of C-reactive protein

Canine CRP was first measured in 1972, using a capillary immune-reaction (Riley & Zontine, 1972). Since, several canine-specific CRP immunoassays have been developed including but not limited to ELISAs (Kjelgaard-Hansen et al., 2003b; Yamamoto et al., 1992; Eckersall et al., 1989), electroimmunoassays (Caspi et al., 1984), time resolved immunofluorometric assays (Parra et al., 2006), laser nephelometric immunoassays (Onishi et al., 2000), and immunoturbidimetric assays (Eckersall et al., 1991).

In 2003, a canine-specific ELISA test was validated and later became widely used in research on canine inflammatory diseases for quantification of CRP concentrations both in the high and the low range (Ljungvall et al., 2010; Fransson et al., 2004; Kjelgaard-Hansen et al., 2003b). However, the ELISA method is time consuming, lacks random-accessibility during analytical runs, and has relatively high imprecision. Automated CRP tests were thus requested by veterinary laboratories, and a few such assays were developed including canine-specific immunoturbidimetric and nephelometric methods (Onishi et al., 2000; Eckersall et al., 1991). However, these tests never reached a broad commercial market. Instead, an assay developed for measuring human CRP was validated for use in dogs (Klenner et al., 2010; Kjelgaard-Hansen et al., 2003a), and became a routine test at several veterinary diagnostic laboratories (Kjelgaard-Hansen, 2010). The heterologous determination of canine CRP with this immunoturbidimetric assay was possible because of sufficient cross-reactivity towards canine CRP by the polyclonal anti-human CRP antibodies of
the test (Kjelgaard-Hansen et al., 2003a). However, poor cross-reactivity between human and canine CRP had previously been reported (Yamamoto et al., 1992; Caspi et al., 1984), and stable performance of the human-based immunoturbidimetric assay could not be guaranteed across reagent batches. Therefore, batch level validation studies were required, and it was recommended to use purified canine CRP for calibration (Kjelgaard-Hansen, 2010; Ekersall, 1995). Because of this, the human-based immunoturbidimetric test worked satisfactory only in laboratories where the above-mentioned challenges could be properly addressed.

In conclusion, although canine CRP had been measured by several different methods throughout the years, there was a need for assays that were both automated and canine-specific, enabling precise CRP measurements and stable performance over time.
4 Validation of analytical performance

4.1 Method validation experiments

Method validation studies are performed to confirm that the requirements of a specified intended use for a test are fulfilled (Taverniers et al., 2004a). For an assay that will be used in a clinical setting, this corresponds to reassuring that analytical error will not negatively impact patient management. Guidelines on recommended protocols for method validation studies are available from regulatory agencies such as the Clinical and Laboratory Standards Institute (CLSI), and validation study protocols can be found in the literature (Magnusson, 2014; Westgard, 2008; Taverniers et al., 2004b).

Various performance criteria of a method are evaluated to judge whether or not the assay has acceptable performance. One such criterion is precision, which is defined as the “closeness of agreement between independent measurements obtained under stipulated conditions” (CLSI, 2004). Precision is a measure of random error. If precision is low, there is large variation in the results from repeated analysis of the same, single sample (Figure 4). Precision is usually determined in two forms. Analysing the same sample repeatedly within one run determines the intra-assay variation, and analysing the sample repeatedly over an extended period of time determines the inter-assay variation. The circumstances under which precision studies take place affects the result, with the highest precision being expected when experiments are carried out under controlled conditions over a short period of time. For a laboratory analysing routine patient samples, it is important to determine long-term precision covering multiple calibrator and reagent batches, operators and environmental conditions (White & Farrance, 2004).

Laboratory test reports released to practitioners generally do not contain information about analytical precision. It has been suggested that exact values reported from the laboratory should be accompanied by a range in which the true value is likely to lie with a stated probability (White, 2008). For example, a reported value of 50 units could have 95% probability to be somewhere between 40 and 60 units if using an assay with low precision, or between 49 and 51 units if using a highly precise assay. This information is important when the test result is interpreted. However, for practical reasons and by habit, information about precision is not included in laboratory test reports. It is the responsibility of the laboratory performing the test not to release results that are afflicted by random error of a magnitude that may negatively impact patient management.
Bias is a measure of systematic error, defined as the difference between the measured value and the true value of a measurand (Taverniers et al., 2004a) (Figure 4). Bias is of importance if results from different analytical methods or laboratories are to be compared to each other or if reference intervals and clinical decision limits are transferred between different settings. If, for example, a new assay systematically measures analyte concentrations that are twice as high as a previously used method, misinterpretations due to bias will occur if the same clinical decision limits are used for both methods. However, problems caused by bias can be overcome by establishing reference intervals and clinical decision limits for each specific test, and by avoiding direct comparison of test results obtained with different assays. This is commonly performed in veterinary laboratories where bias is often unknown because reference methods, or reference materials with known analyte concentrations, are not available. In some situations, such as when canine CRP is measured directly with assays developed for measuring human CRP, a significant systematic error is expected to occur (Kjelgaard-Hansen, 2010; Klenner et al., 2010). However, if necessary actions are undertaken to handle the problem, such methods may still be useful in veterinary laboratories.

In the validation of a CRP assay, it is also crucial to evaluate whether a prozone effect is present. The prozone effect, also called the high-dose hook effect, is a phenomenon that occurs in immunological assays when there is antigen excess in a sample, preventing successful formation of appropriate antigen-antibody complexes (Jacobs et al., 2015). There is an increased risk of prozone effect if the measured analyte is present in a wide concentration range (Jacobs et al., 2015), which is true for major positive acute phase proteins such as canine CRP. A possible consequence of prozone effect is that false low results are reported for samples that actually have extremely high concentrations of the
analyte, which may cause severe misinterpretation directly affecting patient care (Jacobs et al., 2015). Therefore, absence of relevant prozone effect must be confirmed prior to introducing a new analytical method for measuring CRP.

In conclusion, the purpose of a method validation study is to reassure that an assay has acceptable performance before it is taken into use. The number and type of experiments performed in a method validation study depends on the intended use of the assay. Experiments considered relevant to perform in the validation of canine CRP assays are described in detail in Paper I and II of this thesis.

4.2 Setting analytical quality specifications and analysis of data

As previously stated, the purpose of a method validation is to confirm that an assay has acceptable performance. The next question then is how to determine what constitutes an acceptable performance. There are different methods available for setting analytical quality specifications, and the preferred one is to directly evaluate the effect of analytical performance on clinical outcomes (Sandberg et al., 2015; Kenny et al., 1999). Because such studies are complicated and resource-demanding to perform, they are often lacking in veterinary medicine. Other approaches for determining analytical quality goals include using data on biological variation, or relying on expert recommendations (Kenny et al., 1999). What is considered to be acceptable performance varies substantially between different analytes, and may also vary for the same analyte depending on its concentration or the situation where it is applied. Thus, acceptable performance must be judged in a context where the intended use of the assay is specified.

In the current work, biological variation was used to set quality requirements. Although this is not the superior method for determining quality goals, it is still considered as a relevant measure that has the advantage of being objective, in contrast to several of the other proposed methods (Kenny et al., 1999). Biological variation is defined as the random fluctuation of an analyte around a homeostatic setting point. It is composed of CV₁, reflecting changes occurring within the same individual over time, and CV₇, representing the difference between individuals (Fraser, 2001). When data on biological variation are used to set analytical quality specification, the attempt is to minimize the ratio of “analytical noise” to the biological signal used in diagnosis (Sandberg et al., 2015). The analytical desirable performance goal, CVₐ, can be calculated according to the formula 0.5 x CV₁. This should be interpreted as follows: the analytical variability added to the already existing intra-individual variability
will be equivalent to 11.8% if $CV_A$ is $0.5 \times CV_I$. The formula for calculating desirable bias is $0.25(CV_I^2 + CV_G^2)^{0.5}$ (Fraser, 2001). Knowledge about desirable bias is useful for example when a reference interval obtained with a reference test is transferred to a new method. Normally, reference intervals include 95% of the healthy population. If the bias of the new assay is equal to $0.25(CV_I^2 + CV_G^2)^{0.5}$ it means that 5.8% instead of 5% of the normal population will fall outside the reference interval, which is considered to be acceptable.

Data on desirable bias and imprecision can be combined to create a single measurement representing the total error allowed for a test (TEa) (Harr et al., 2013; Westgard, 2008). In method validation studies, the total error (TE) can be determined by adding a test’s imprecision and bias as determined in the validation experiments (Westgard, 2008). The calculated TE is then compared to the TEa, and if TE < TEa it can be concluded that the assay has acceptable performance.

The concept of TE has met criticism (Oosterhuis & Theodorsson, 2015; White, 2008), and it can be questioned whether it is relevant to add two such different entities as imprecision and bias. One argument is that either bias is known, and then it should be corrected for, or bias is unknown and then it cannot be used for calculating TE. Instead of using TE, it has been proposed that focus should be on measurement uncertainty. This is defined as “a parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand” (JCGM, 2008). While error is a value describing the difference between an individual analytical result and the true value of the measurand, the uncertainty of a measurement is the doubt that exists about the result of any measurement (White, 2008; Taverniers et al., 2004a). The International Organization for Standardization (ISO) advocates use of measurement uncertainty (JCGM, 2008), while the TE approach is recommended by the American Society for Veterinary Clinical Pathologists (Harr et al., 2013). It has been suggested that TE and measurement uncertainty can be implemented simultaneously, but for different purposes, in the laboratory (Rozet et al., 2011).

To summarize, at this point there is no consensus regarding how TE and measurement uncertainty should be handled, and the topic is currently being discussed within the veterinary laboratory community.
5 Aims of the thesis

The overall aim of the thesis was to develop and validate new assays optimized for measuring canine CRP. Availability of such assays should improve the use of CRP as an inflammatory marker both in clinical practice and in research. An additional aim was to conduct clinical studies to evaluate CRP as a quantitative marker of systemic inflammation in dogs, and to examine the diagnostic value of measuring CRP in specific settings.

Specific aims were to:

- Validate two immunoturbidimetric automated canine-specific CRP assays for measuring serum CRP in the whole concentration range expected to occur in dogs.

- Evaluate CRP as a quantitative marker of systemic inflammation.
  - Investigate whether CRP concentration was correlated to oedema, a clinical sign of local inflammation, in dogs envenomed by *Vipera berus*. It was hypothesised that the more severe the local inflammation, the more pronounced the systemic inflammation and increase in CRP concentration would be.
  - Measure CRP concentration in dogs with pyometra undergoing ovariohysterectomy, to evaluate how surgical treatment affected degree of systemic inflammation in these patients.

- Evaluate the diagnostic value of measuring CRP concentration.
  - Investigate the usefulness of CRP for discriminating between dogs with suppurative arthritis and osteoarthritis.
  - Investigate the usefulness of CRP for diagnosing post-operative infections after orthopaedic surgery.

- Investigate whether inflammation, defined as an increased CRP concentration, was associated with severity of canine myxomatous mitral valve disease in order to increase the knowledge about the pathophysiology of this disease.
6 Materials and methods

This section summarizes the methods used in Paper I-IV, and describes additional projects not included in these papers.

In the clinical studies, client-owned dogs were enrolled prospectively with written consent given by the owner prior to participation. The studies were approved by the Local Ethical Committee on Animal Experiments, Uppsala. Blood samples were collected from the jugular or cephalic vein, and sera for CRP analysis were stored at -20°C for a maximum of 5 months, or at -80°C for a maximum of 6.5 years, prior to analysis.

6.1 Method validation studies (Paper I and II)

A reagent containing polyclonal chicken anti-canine CRP antibodies, conjugated to latex, was used in an immunoturbidimetric, automated canine-specific CRP assay (Gentian AS, Moss, Norway). Analyses were performed on a fully automated, open-system clinical chemistry/immunoassay analyser (Abbott Architect c4000, Abbott Park, IL, USA). Sample and reagent were mixed in the instrument, and when antibodies reacted with canine CRP agglutination occurred, leading to an increased turbidity that was measured spectrophotometrically (Figure 5).

![Figure 5. Principle of the latex-enhanced immunoturbidimetric assay. Anti-canine C-reactive protein (CRP) antibodies conjugated to latex particles in the reagent react with CRP in the sample (blue structures), leading to agglutination and an increased turbidity of the solution.](image-url)
Two different instrument applications were used on the same chemistry analyser. The first application, referred to as the cCRP test, was adapted for measuring the high CRP concentrations expected in dogs with systemic inflammatory disease. The second application, referred to as the hsCRP assay, was adapted for measuring low CRP concentrations. The hsCRP assay was created by increasing the sample volume of the cCRP test ten-fold, from 2 µl to 20 µl, while keeping the reagent volume unchanged. Canine-specific calibrator material provided from the manufacturer was used to create a six-point calibrator curve. For the hsCRP assay, calibrators were manually diluted 1:10 with saline prior to calibration. The reason for developing two different applications was that it was not technically possible to create one single assay that could measure CRP concentration in the whole concentration range expected to occur in dogs.

An overview of the method validation experiments performed are displayed in Table 2. Analytical quality goals were set based on data of biological variation in healthy dogs (Kjelgaard-Hansen et al., 2003c). Data analysis was performed following CLSI guidelines or other recommendations (Jensen & Kjelgaard-Hansen, 2006; Bland & Altman, 1986).

Table 2. Experiments performed in the validation studies of the canine-specific C-reactive protein assay (cCRP) and the high-sensitivity canine-specific C-reactive protein assay (hsCRP).

<table>
<thead>
<tr>
<th>Evaluated parameter</th>
<th>cCRP</th>
<th>hsCRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Consistency upon dilution</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Limit of Blank</td>
<td>ND</td>
<td>x</td>
</tr>
<tr>
<td>Limit of Detection</td>
<td>ND</td>
<td>x</td>
</tr>
<tr>
<td>Limit of Quantification</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Prozone effect</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Recovery after spiking</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Interference from haemoglobin/triglycerides</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Storage stability (room temperature and 4°C)</td>
<td>x</td>
<td>ND</td>
</tr>
<tr>
<td>Difference between methods (method comparison study)</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

x = experiment performed  
ND = not determined
In the spike and recovery studies, as well as in the study of prozone effect, commercially available purified canine CRP was used (LifeDiagnostics, West Chester, PA, USA). A fat emulsion (Intralipid 200 g/l, Fresenius Kabi AB, Uppsala, Sweden) was used to test effect of lipemia, whereas a haemolytic solution was prepared from canine samples to test interference from haemolysis (CLSI, 2005).

Two method comparison studies were performed. The cCRP assay was compared to a fully automated immunoturbidimetric CRP assay developed for measuring CRP in humans, previously validated for use in dogs (Kjelgaard-Hansen et al., 2003a). The method was calibrated with canine CRP. The hsCRP assay was compared to a previously validated canine-specific ELISA (Kjelgaard-Hansen et al., 2003b). The reason for choosing these methods as comparative assays was that they were used at the Clinical Pathology Laboratory, University Animal Hospital, SLU, for measuring CRP in the high and low concentration range, respectively, when starting these studies. Data from the method comparison studies were evaluated using Bland-Altman plots (Bland & Altman, 1986) and Passing-Bablok regression analysis (Passing & Bablok, 1983).

6.2 C-reactive protein as a quantitative marker of systemic inflammation

6.2.1 C-reactive protein in dogs envenomed by Vipera berus (Paper III)

This study was a randomized, placebo-controlled double-blind clinical study where the original aim was to evaluate the effect of prednisolone treatment on dogs envenomed by Vipera berus (V. berus) (Paper III). In this thesis, the data set from the study was used to investigate the correlation between oedema, which is a clinical sign of local inflammation, and CRP concentration. The hypothesis was that the more severe the local inflammation, the more pronounced the systemic inflammatory response would be, i.e. there would be a positive correlation between degree of oedema and CRP concentration.

Inclusion criteria were a reported snake bite within the previous 24 hours prior to admission, and presence of clinical signs typical of envenomation by V. berus. Pregnant dogs and dogs with underlying disease including hyperadrenocortism, renal disease, diabetes mellitus and inflammatory disease were excluded. Although dogs treated with glucocorticoids, NSAID or antivenom were excluded in the original publication (Paper III), they were included in this evaluation of correlation between oedema and CRP.
concentration. Dogs were examined by the attending veterinary surgeon on presentation (Day 1), 24±6 h after presentation (Day 2), and at re-examination after 10-28 days. Blood samples were collected on the same three occasions. Oedema on Day 1 and 2 was assessed by measuring the circumference of the affected body part, i.e. the head or limb region. This measure was compared to the circumference measured at re-examination, when no oedema persisted, and the increase in circumference was expressed in percent. C-reactive protein concentration was measured with the cCRP assay in duplicate in one run.

The correlation between degree of oedema and CRP concentration on Day 2 was assessed with Spearman’s rank correlation coefficient (rₛ). Significance level was set at p < 0.05.

6.2.2 C-reactive protein in dogs with pyometra undergoing ovariohysterectomy

In this study, CRP concentration was used as a quantitative marker of systemic inflammation in dogs with pyometra undergoing ovariohysterectomy. Dogs with pyometra typically have a systemic inflammatory response with neutrophilia, left shift and increased concentrations of acute phase proteins (Fransson et al., 2004). The preferred treatment for pyometra is ovariohysterectomy, a procedure that in itself causes systemic inflammation due to the tissue trauma caused by surgery (Kjelgaard-Hansen et al., 2013; Kum et al., 2013; Dabrowski et al., 2007). It could be speculated that the degree of systemic inflammation would temporally increase when ovariohysterectomy is performed in a dog with pyometra, because of the additional inflammation caused by surgery. On the other hand, ovariohysterectomy is an effective treatment for pyometra, and the inflammation is expected to gradually dissolve when the inflammatory stimulus is removed. The aim of the current study was to investigate the overall effect on the degree of systemic inflammation in dogs with pyometra undergoing ovariohysterectomy. To do so, an objective, quantitative marker of systemic inflammation was required, and measurement of CRP was chosen as the preferred test.

Dogs admitted to the University Animal Hospital, SLU, Uppsala, and AniCura Bagarmossen Small Animal Hospital, Stockholm, were included in the study. Inclusion criteria were diagnosis of pyometra, based on the finding of an enlarged, fluid filled uterus and pre-operative serum CRP concentration ≥ 20 mg/l in a dog with history and clinical findings suggestive of pyometra. Dogs with known concurrent disease expected to cause systemic inflammation were excluded. An open ventral midline approach ovariohysterectomy was performed while dogs were under general inhalation anaesthesia. Analgesic treatment included NSAIDs and opioids, and some of the dogs received antibiotics as
decided by the veterinary surgeon in charge. Information about post-operative complications were retrieved from the medical records. Blood samples were collected immediately prior to and 24±3 hours after initiation of surgery, defined as start of the first skin incision. C-reactive protein concentration was measured with the cCRP assay in duplicate in one run. A Wilcoxon sign-ranked test was performed to compare pre- and post-operative CRP concentrations.

6.3 C-reactive protein as a diagnostic test

6.3.1 Measurement of C-reactive protein concentration for discriminating between suppurative arthritis and osteoarthritis in dogs (Paper IV)

The study was performed in order to evaluate if CRP concentration could be used as a test for discriminating between dogs with suppurative arthritis and dogs with osteoarthritis (OA). Dogs admitted to University Animal Hospital, SLU, Uppsala or Evidensia Södra Djursjukhuset, Stockholm, were included if they had at least one painful joint as determined by a veterinarian at physical examination. It was further required that the dog should have arthrocentesis, arthroscopy or arthrotomy performed for diagnostic or therapeutic purposes. Exclusion criteria were pregnancy and glucocorticoid treatment within four weeks prior to sampling. Dogs were classified to have suppurative arthritis or OA based on findings from synovial fluid analysis, arthroscopy and radiographic examination. Measurement of CRP was first performed with the cCRP test, and samples with CRP concentrations < 6.8 mg/l were immediately reanalysed with the hsCRP assay.

Statistical analyses included multiple logistic regression analysis with disease (suppurative arthritis or OA) as outcome, and CRP, sex, age, body weight, NSAID treatment, and presence of concurrent disease as explanatory variables. CRP data were transformed to the base 2-logarithmic scale (log$_2$CRP) to facilitate interpretation of results: for a doubling of CRP concentration, the odds of having inflammatory arthritis increased by a factor corresponding to the odds ratio. Backward step-wise elimination of explanatory variables based on p-values was used to reduce the model until all included variables had a p-value < 0.05. Explanatory variables whose inclusion changed the beta coefficient of log$_2$CRP by > 20% were considered possible confounders, and were retained in the model. The goodness of fit was tested with the Hosmer and Lemeshow test.

The diagnostic performance of CRP for discriminating between suppurative arthritis and OA was further assessed by receiver operating characteristic (ROC) curve analysis. C-reactive protein concentrations were also compared against a locally established clinical decision limit for CRP, 20 mg/l. The decision limit
was determined by performing ROC curve analysis of CRP data from dogs with and without known systemic inflammatory disease (data not shown), similarly to what has been previously described (Christensen et al., 2014). Correct classification was defined as CRP concentration $\geq 20$ mg/l in a dog with suppurative arthritis, and CRP concentration $< 20$ mg/l in a dog with OA.

6.3.2 C-reactive protein concentration as a test for diagnosing post-operative infections after orthopaedic surgery

The objective of the study was to investigate whether measurement of CRP concentration was useful for diagnosing late post-operative bacterial infections in dogs after orthopaedic surgery. It was hypothesised that dogs with post-operative infections would have more pronounced systemic inflammations, and therefore higher CRP concentrations, compared to dogs with non-infectious complications such as implant loosening.

Inclusion criteria were that the dog had clinical signs compatible with post-operative infection including swelling, pain, and/or drainage at the surgical site, and/or joint effusion in a previously operated joint. Furthermore, the dog should have had orthopaedic surgery performed at least 20 days earlier, but no longer than 365 days earlier. Exclusion criteria were other known inflammatory disease, pregnancy and treatment with antibiotics. Blood samples were collected prior to performing any other diagnostic or therapeutic interventions.

Diagnostic procedures included bacterial culture of samples collected from the suspected infected area, cytology, and radiographic examination. Bacterial culturing was performed at the Section of Bacteriology, National Veterinary Institute, Uppsala, Sweden. All dogs had at least two of the above-mentioned diagnostic procedures performed. Dogs were classified as infected if there was significant growth of bacteria at bacterial culture, and/or if neutrophils and intracellular bacteria were found at cytological examination. Dogs that had no signs of infection with any of the diagnostic tests performed were classified as not infected. Dogs with inconclusive test results were excluded.

A group of control dogs that showed no signs of complications after orthopaedic surgery were included. Blood samples were collected from the control dogs during the same time period after surgery as for the cases, after performing a physical examination where no clinical signs compatible with post-operative infection were detected.

Information about the dogs, history, clinical findings, surgical procedures, and treatments was obtained from medical records. C-reactive protein concentration was measured with the cCRP assay in duplicate in one run, and concentrations in the different groups were compared using the Mann-Whitney
U test. Significance level was set at $p < 0.05$. C-reactive protein concentrations were further compared against the locally established clinical decision limit for CRP, 20 mg/l. Correct classification was defined as CRP concentration $\geq 20$ mg/l in a dog with infection and $< 20$ mg/l in a dog without infection.

6.4 Application of the high-sensitivity C-reactive protein assay on samples from dogs with myxomatous mitral valve disease

The hsCRP assay was applied on samples from dogs with myxomatous mitral valve disease (MMVD), with the aim to evaluate whether CRP concentrations reflected well the severity of MMVD. This was considered interesting to study, as a link between systemic inflammation and cardiovascular disease has been suggested to exist in dogs (Cunningham et al., 2012). Based on this, it was hypothesised that CRP concentrations would be positively correlated to severity of MMVD.

The included dogs had been enrolled in previous investigations in Sweden and Denmark (Reimann et al., 2014a; Reimann et al., 2014b; Ljungvall et al., 2011a; Ljungvall et al., 2011b; Ljungvall et al., 2010). The dogs underwent physical examinations including blood pressure measurements and echocardiography, and a full clinical history was obtained from each owner. Dogs with history or signs of cardiovascular disease other than MMVD, or other significant disease, were excluded. MMVD severity was determined on the bases of the American College of Veterinary Internal Medicine (ACVIM) consensus statement (Atkins et al., 2009). Group A consisted of MMVD predisposed dogs with no auscultatory heart murmur and normal echocardiogram, group B1 of dogs with mild signs of MMVD, group B2 of dogs with moderate signs of MMVD, and group C of dogs with MMVD and congestive heart failure (Reimann et al., 2016).

C-reactive protein concentrations were measured with the hsCRP assay in duplicate in one run. Prior to using the hsCRP test, samples were confirmed to have CRP concentration $< 30$ mg/l to avoid error from prozone effect (Reimann et al., 2016).

Statistical tests included the Kruskal-Wallis test to assess differences in CRP concentration, age and weight between dogs in the different ACVIM groups. If significant ($p < 0.05$), pair-wise comparisons between groups were performed using the Mann-Whitney U test. Significance level was set to $p < 0.008$, using Bonferroni adjustment for multiple comparisons.
Multiple regression analysis was performed to evaluate associations between CRP concentration (response variable) and the explanatory variables ACVIM group, age, sex, body weight, systolic blood pressure, serum storage time, sub-study, and breed (Cavalier King Charles Spaniel/other breed) (Reimann et al., 2016). Backward step-wise elimination of explanatory variables based on p values was performed, until only variables with p < 0.05 remained in the model.
7 Results and discussion

7.1 Method validation studies (Paper I and II)

Results from the validation studies of the cCRP and hsCRP assays are summarized in Table 3. Slightly different validation protocols were used for the two methods. For example, the LoB and LoD were not determined for the cCRP test, because this was judged to be irrelevant for an assay intended for measuring markedly increased CRP concentrations in dogs with systemic inflammation. While both inter- and intra-assay variation were thoroughly evaluated for the cCRP assay, determination of intra-assay variation was prioritized for the hsCRP assay considering that research samples are preferably analysed in one run.

Table 3. Results from method validation studies of the canine-specific C-reactive protein assay (cCRP) and the canine-specific high-sensitivity C-reactive protein assay (hsCRP).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>cCRP</th>
<th>hsCRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement range</td>
<td>6.8-1200 mg/l</td>
<td>0.5-30 mg/l</td>
</tr>
<tr>
<td>Intra-assay CV</td>
<td>≤ 1.7%</td>
<td>≤ 2.7%</td>
</tr>
<tr>
<td>Inter-assay CV</td>
<td>≤ 1.9%</td>
<td>≤ 3.0%</td>
</tr>
<tr>
<td>Limit of Blank</td>
<td>ND</td>
<td>0.10 mg/l</td>
</tr>
<tr>
<td>Limit of Detection</td>
<td>ND</td>
<td>0.22 mg/l</td>
</tr>
<tr>
<td>Limit of Quantification</td>
<td>6.8 mg/l</td>
<td>0.50 mg/l</td>
</tr>
<tr>
<td>Presence of relevant prozone effect</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Recovery after spiking</td>
<td>116-123%</td>
<td>114-117%</td>
</tr>
<tr>
<td>Interference from haemoglobin/triglycerides</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Storage stability at room temperature and 4°C</td>
<td>≥ 14 days</td>
<td>ND</td>
</tr>
<tr>
<td>Acceptable agreement in method comparison study</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

1 Dilutions required for samples with CRP concentrations > 300 mg/l.  
CV = coefficient of variation  
ND = not determined

The imprecisions of both the cCRP and hsCRP assay were lower than the maximal allowable imprecision based on biological variation (12%), (Kjelgaard-Hansen et al., 2003c), and similar or lower compared to imprecisions reported for other CRP assays (Klenner et al., 2010; Parra et al., 2006; Kjelgaard-Hansen et al., 2003a; Kjelgaard-Hansen et al., 2003b). The LoQ for the cCRP assay, 6.8 mg/l, was below the locally established clinical decision limit of 20 mg/l which was considered sufficient for clinical use. The LoQ of the hsCRP assay was 0.5
mg/l, which was below the mean CRP concentrations measured in healthy dogs with this assay (Reimann et al., 2016, Paper II and III).

The presence of possible prozone effect was investigated both for the cCRP and hsCRP assay. With the cCRP test, a spiked sample with CRP concentration of approximately 1200 mg/l was correctly reported to have CRP concentration > 300 mg/l by the assay, and it was concluded that no relevant prozone effect was present. The tested concentration of 1200 mg/l was well above the highest CRP concentration measured in a routine sample at the Clinical Pathology Laboratory, University Animal Hospital, SLU, about 800 mg/l (data not shown). Therefore, an acceptable safety zone was considered to be present.

The hsCRP assay, on the other hand, did report erroneous CRP results due to prozone effect. For example, a sample with CRP concentration of 228 mg/l was reported to have CRP concentration 20 mg/l by the hsCRP test, instead of being correctly reported to have CRP concentration > 30 mg/l. This prozone effect occurred because the amount of CRP in this sample was too high in relation to the amount of anti-canine CRP antibodies in the reagent, preventing successful formation of antigen-antibody complexes (Figure 6). Because of the prozone effect, it is strongly recommended not to use the hsCRP as a single test. Instead, CRP concentration should first be measured with an assay not affected by prozone effect, followed by re-analysis with the hsCRP test only for samples with low CRP concentrations.

![Figure 6. Schematic illustration of prozone effect. The amount of CRP (blue structures) in the sample is inappropriately high in relation to the amount of anti-canine CRP antibodies, which prevents successful formation of antigen-antibody complexes.](image-url)
No interfering effect from haemolysis or lipemia was detected with the cCRP test, which is important considering that these changes are commonly present in samples submitted for routine analysis. Interference from lipemia was tested by adding a fat emulsion (Intralipid®) to canine serum samples, which is a widely used and feasible approach. However, transferrence of results from the experimental setting to naturally lipemic samples can be questioned, because the particle sizes of the lipids are different in the commercial fat emulsion compared to what is encountered in vivo (Nikolac, 2014). When samples from dogs in the clinical studies were analysed with the hsCRP assay, there were occasional lipemic samples in which CRP concentrations were not possible to measure. Instead, the instrument reported that the absorbance of the sample exceeded the upper optical limit. The explanation for this error message was that the turbidity of the lipemic sample was too high to allow for enough light to be transmitted through the solution. The triglyceride concentrations in the naturally lipemic samples were lower than the triglyceride concentration in the lipemic samples used in the interference studies (data not shown). It was concluded that, despite the fact that experimentally induced lipemia had no or only mild interfering effect, this could not be granted in naturally lipemic samples. The same conclusion was drawn in another study comparing the effect of Intralipid® and natural lipemia (Bornhorst et al., 2004). Because of this, high-speed or ultracentrifugation of lipemic samples is recommended prior to performing CRP measurements. This is important particularly for the hsCRP assay, which is more susceptible to interfering substances because of the larger sample volume used with this test.

CRP was stable at room temperature for at least two weeks, which is relevant information for veterinarians sending samples to referral laboratories for CRP analysis. For long-term storage of CRP samples, freezing at -80°C can be recommended; at this temperature, human CRP was reported to be stable for 11 years (Doumatey et al., 2014).

Bias could not be assessed in the validation studies, due to the lack of reference material or a gold standard method for accurate determination of canine CRP. Therefore, the method comparison studies did not aim to determine true bias, but rather to evaluate whether different assays could be used interchangeably. The results showed that this was not possible, and it is thus necessary to establish reference intervals and clinical decision limits for each individual method.

In the method validation studies performed in this thesis, analytical quality requirements were set based on data on biological variation in healthy dogs.
However, the relevance of using such data for setting quality requirements for CRP tests can be questioned, considering that healthy animals have much lower CRP concentrations compared with dogs with systemic inflammation (Antonsen, 1994). An increase in absolute CRP concentration of 1 mg/l would represent a 100% increase if the original CRP concentration was 1 mg/l, but only a 1% increase if the original CRP concentration was 100 mg/l. Studies for determining analytical quality goals based on criteria other than biological variation needs to be performed for canine CRP, to obtain more relevant data for this purpose.

After successful termination of the method validation studies in this project, the cCRP and hsCRP assays were applied on samples from clinical research studies. The performance of the assays could thus be assessed over time when analysing samples from a large number of dogs, using different reagent batches and with analyses performed under various conditions. This provided an opportunity to identify problems that went unnoticed in the validation studies, such as the differences in interfering effect between naturally and experimentally induced lipemia. Furthermore, precision and dilution experiments confirmed stable performance across batches (data not shown). It is required that an assay is used over an extended period of time, during different times of the year, is operated by different persons and with different reagent batches, for the laboratory to conclude that they have a stable working assay. This can now be considered to be accomplished for both the cCRP and hsCRP test at the Clinical Pathology Laboratory, University Animal Hospital, SLU.

In conclusion, the method validation studies showed that the cCRP and hsCRP assays met most of the predefined quality criteria. Using these two novel assays, canine CRP could be reliably measured in the range of 0.5-1200 mg/l, which corresponds to the CRP concentrations expected to occur in dogs.

7.2 C-reactive protein as a quantitative marker of systemic inflammation

7.2.1 C-reactive protein in dogs envenomed by Vipera berus (Paper III)

Viper venom has proteolytic, fibrinolytic, anticoagulant and phospholipase A₂ effects, causing tissue damage and inflammation in the envenomed animal (Goddard et al., 2011). Increased CRP concentrations have previously been reported in dogs envenomed by V. berus (Langhorn et al., 2013b), and were found also in the current study where the median (IQR) CRP concentration on Day 2 was 68 (36-101) mg/l in 57 envenomed dogs. There was a moderate positive correlation between degree of oedema and CRP concentration (rₛ=0.45,
It has previously been reported that CRP concentrations reflected well the extent of local tissue damage in experimental settings (Kjelgaard-Hansen et al., 2013; Otabe et al., 2000). In these studies, degree of tissue injury could be determined with high accuracy. Such exact quantification was not possible in the current study, where local inflammation was assessed by various veterinarians measuring the circumference of the bitten body parts. Despite this, a positive correlation between degree of oedema and CRP concentration was found as hypothesized.

The fact that CRP is a quantitative marker of systemic inflammation is advantageous both in the clinical setting and in research; for example, measurement of CRP concentration has been used to grade and compare the inflammatory response after different types of surgery (Liu et al., 2013; Michelsen et al., 2012; Freeman et al., 2010). The next section, describing CRP concentrations before and after ovariohysterectomy in dogs with pyometra, constitutes an example where measurement of CRP was applied to quantify a systemic inflammatory response.

7.2.2 C-reactive protein in dogs with pyometra undergoing ovariohysterectomy

Twenty-nine dogs were included in the study investigating degree of systemic inflammation in dogs with pyometra undergoing ovariohysterectomy. The dogs were of 12 different breeds, with a median (IQR) age of 7.5 (5-12) years and median (IQR) weight of 29 (21-39) kg. No dog was excluded because of concurrent disease.

The median (IQR) pre-operative CRP concentration was 254 (177-364) mg/l. The day after surgery, median (IQR) CRP concentration had decreased significantly to 199 (165-271) mg/l (p < 0.001). Of the 29 dogs, 22 (76%) had decreased CRP concentration the day after surgery compared to the pre-operative CRP concentration. The conclusion was that the removal of the inflamed uterus had a larger impact on degree of systemic inflammation, compared to additional tissue trauma caused by surgery. It would not have been possible to draw this conclusion based on other routinely available measures of systemic inflammation, such as the leukocyte count or body temperature. The leukocyte numbers were generally increased the day after ovariohysterectomy, explained by the fact that neutrophils were no longer consumed in the uterus (data not shown). Measurement of body temperature was of limited value for monitoring systemic inflammation, because most of the dogs were treated with NSAID which has anti-pyretic effect. These findings illustrate the benefits of using CRP concentration as a marker for quantifying systemic inflammation.
Post-operative complications were reported in two of the dogs, and included one case of post-operative infection and one case of acute gastroenteritis. Repeated measurements of CRP concentration have previously been shown to be useful for detecting post-operative complications in dogs undergoing ovariohysterectomy (Dabrowski et al., 2009). In the current study, data about complications were not collected in a standardized way and it was therefore not possible to evaluate the efficacy of CRP as a diagnostic test for this purpose. However, it was notable that the most pronounced increases in CRP concentrations the day after surgery compared to pre-operative levels were found in the two dogs with reported complications.

Previous studies of the effects of ovariohysterectomy on CRP concentration in dogs with pyometra have shown diverging results. Two studies reported on an increase in mean CRP concentration the day after ovariohysterectomy (Dabrowski et al., 2009; Dabrowski et al., 2007), whereas another study found the opposite result (Yuki et al., 2010). There are several possible explanations for the diverging results. The use of different surgical techniques could not be excluded with certainty, although a similar open approach ovariohysterectomy was presumably performed in all of the experiments (Yuki et al., 2010; Dabrowski et al., 2009; Dabrowski et al., 2007). The severity of pyometra may have differed between studies, as well as medical treatment strategies. It has also been suggested that surgeon experience and use of anaesthetics may affect degree of post-operative inflammation (Liao et al., 2014; Michelsen et al., 2012). This is something that needs to be further studied, so that optimal treatment strategies can be established for dogs with pyometra undergoing ovariohysterectomy.

7.3 C-reactive protein as a diagnostic test

7.3.1 Measurement of C-reactive protein concentration for discriminating between suppurative arthritis and osteoarthritis in dogs (Paper IV)

Thirty-one dogs with suppurative arthritis and 34 dogs with OA were included in the study. Median (IQR) CRP concentrations in the two groups were 107 (43-145) mg/l and 0.73 (< 0.5-2.45) mg/l, respectively. In the logistic regression analysis, the final model included log2 CRP (p=0.03, OR 7.37) and sex (p=0.12, OR 0.005) as explanatory variables. The interpretation of OR for log2 CRP was that for a doubling of serum CRP concentration, the odds of having suppurative arthritis increased with 7.37. Although sex remained as an explanatory variable in the final model, it was not considered to be of clinical importance.
In the ROC curve analysis, the AUC (95% CI) was 0.99 (0.97-1.00). When comparing CRP results to the previously established clinical decision limit of 20 mg/l, there were two false negative and one false positive result among the 65 dogs. Based on these findings, it was concluded that CRP concentration discriminated well between dogs with suppurative arthritis and dogs with OA.

The study on dogs with joint disease was designed according to the STARD criteria, as recommended (Christopher, 2007; Bossuyt et al., 2004). Although such reports are important to enhance evidence-based use of diagnostic tests, they are not sufficient. It remains to be investigated whether measurement of CRP actually makes a difference for outcome, or other relevant factors, in a clinical setting. In dogs with joint disease, it would be interesting to determine whether knowledge about CRP concentration could improve decision making regarding diagnostic interventions. Especially in a dog with known OA, a sudden increase in pain and lameness could be attributed to a worsening of the underlying disease. However, dogs with OA may be predisposed for septic arthritis, which constitutes an important differential diagnosis (Clements et al., 2005). While arthrocentesis is not required in a dog with OA, it is crucial for diagnosis of septic arthritis, and knowledge on CRP concentration might be valuable for determining which patients that should undergo arthrocentesis. It could be argued that a physical examination is usually sufficient to adequately distinguish between a dog with suppurative arthritis and OA (Foster et al., 2014), making measurement of CRP concentration superfluous. However, clinical signs may be unspecific (Rondeau et al., 2005; Jacques et al., 2002) and physical examination is subjective, in contrast to CRP which is an objective marker and thus not biased by level of clinical expertise.

7.3.2 C-reactive protein concentration as a test for diagnosing post-operative infections after orthopaedic surgery

In the study of dogs undergoing orthopaedic surgery, in total 15 dogs with suspected post-operative infections were included (Figure 7). One dog was excluded because of inconclusive test result. The remaining dogs were of 10 different breeds, with median (IQR) age and weight of 4 (3-6) years and 34 (23-42) kg, respectively. Surgical procedures included tibial plateau leveling osteotomy (n=4), carpal arthrodesis (n=4), fracture surgery (n=2), lateral suture stabilisation after cruciate ligament rupture (n=2), ulnar osteotomy (n=1), and arthroscopy (n=1). Dogs were sampled on day 20-360 after surgery. Seven dogs were classified as infected based on positive bacterial culture result (n=2), positive bacterial culture result and cytological signs of infection (n=3), positive culture result and radiographic findings suggestive of osteomyelitis (n=1), and
cytological signs of infection (n=1). The remaining dogs were classified as not infected (n=7).

Four of the infected dogs had CRP concentrations below the clinical decision limit for CRP, 20 mg/l, and two dogs with no infection had CRP concentration > 20 mg/l (Figure 7 and 8). There was no significant difference in CRP concentration between dogs with infections and dogs with non-infectious complications (p=0.44).

Figure 7. Flow diagram of dogs with suspected post-operative infections. Reference tests for diagnosing infection included bacterial culture, cytological examination, and/or radiographic examination. CRP=C-reactive protein
Figure 8. Individual value plot of serum C-reactive protein (CRP) concentrations in dogs with suspected post-operative infections after orthopaedic surgery. Dogs were classified as infected (n=7) or not infected (n=7). The horizontal line represents the locally established clinical decision limit of CRP for diagnosing systemic inflammation, 20 mg/l.

Seven control dogs were sampled between day 20 and 65 days after surgery. The control dogs were of 7 different breeds, with median (IQR) age and weight of 4 (1-9) years and 29 (10-47) kg, respectively. Surgical procedures in the control dogs included tibial plateau leveling osteotomy (n=4), lateral suture stabilisation after cruciate ligament rupture (n=2), and fracture surgery (n=1). All control dogs had CRP concentration < 6.8 mg/l, which was significantly lower compared to the other group of dogs with suspected infections (p=0.003). However, this finding was considered to be of limited interest in a clinical setting, where the problem is to distinguish between dogs with infectious and non-infectious complications rather than between dogs with complications and dogs with uneventful recoveries.

The plausible explanation for the poor performance of CRP for detecting post-operative infections was that these infections did not always elicit a systemic inflammatory response. This is similar to conditions such as mild sterile subcutaneous inflammation (Bauer et al., 2012) and inflammation in the central nervous system (Vestergaard et al., 2013), where signs of local inflammation were present without a simultaneous increase in CRP concentration. C-reactive protein is a marker of systemic inflammation only, and
presence of local inflammation cannot be excluded based on a low CRP concentration. The reason for the increased CRP concentrations found in two dogs that were not infected was not determined, but traumatic injury was a possibility.

No specific diagnostic tests were performed in the control dogs to exclude presence of infection, which is a limitation of the study. The reason for not collecting samples for bacterial culture and cytology in the control dogs was that this would have required invasive procedures in apparently healed areas, with a minor but still relevant risk of inducing complications. In the review of the dogs’ medical records, none of the control dogs was reported to suffer from a post-operative complication during the first month after sampling. Therefore, erroneous inclusion of infected dogs in the control group was considered less likely.

In conclusion, CRP concentration was not efficient for discriminating between dogs with late bacterial infections after orthopaedic surgery and dogs with post-operative complications not caused by infection. The likely explanation to this finding was that post-operative infections often did not elicit a systemic inflammatory response enough to induce an increased CRP concentration.

7.4 Application of the high-sensitivity C-reactive protein assay on samples from dogs with myxomatous mitral valve disease

A total of 188 dogs were included in the study investigating whether CRP concentrations were associated with severity of MMVD (Reimann et al., 2016). Dogs with congestive heart failure (group C) had higher CRP concentrations compared with dogs in all other groups (Table 4). However, there was no difference in CRP concentration between dogs without MMVD and dogs with mild to moderate MMVD without congestive heart failure. There were statistically significant differences between groups for age and body weight (Table 4), but these variables were not significant in the multiple regression analysis. The final multiple regression model had an adjusted $r^2$ of 0.13, and ACVIM group was the only significant explanatory variable ($p < 0.001$) (Reimann et al., 2016).
Table 4. Dog characteristics and serum C-reactive protein (CRP) concentration in 188 dogs without and with different severities of myxomatous mitral valve disease (MMVD) according to American College of Veterinary Internal Medicine (ACVIM) criteria. Median and interquartiles are reported. Superscript letters a, b1, b2 and c represents ACVIM group from which there was a statistically significant difference. Modified from Reimann et al (2016).

<table>
<thead>
<tr>
<th>ACVIM group</th>
<th>A</th>
<th>B1</th>
<th>B2</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of dogs</td>
<td>58</td>
<td>56</td>
<td>38</td>
<td>36</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>36/22</td>
<td>36/20</td>
<td>17/21</td>
<td>9/27</td>
</tr>
<tr>
<td>Age (years)</td>
<td>5.5 (4.2;6.8) b1,b2,c</td>
<td>6.9 (6.2;10.0) a,c</td>
<td>8.4 (7.1;9.6) a,c</td>
<td>10.8 (9.0;11.7) a,b1,b2</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>8.6 (7.6;9.7) b1,b2,c</td>
<td>9.8 (8.0; 10.7)a</td>
<td>10.0 (8.5;11.0)a</td>
<td>10.4 (8.2;12.3)a</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.97</td>
<td>0.78</td>
<td>0.60</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.5; 1.97)c</td>
<td>(&lt;0.5; 1.73)c</td>
<td>(&lt;0.5; 1.23)c</td>
<td>(1.09; 5.09)a,b1,b2</td>
</tr>
</tbody>
</table>

Although there was a statistically significant difference in CRP concentration between dogs with congestive heart failure and dogs with milder forms of MMVD, CRP concentrations were low and overlapping. This was expected based on results from previous investigations (Cunningham et al., 2012; Ljungvall et al., 2010; Rush et al., 2006), and the current study did not aim to evaluate CRP as a diagnostic test in these patients. Instead, the research was performed to map the pathophysiological changes occurring in dogs with MMVD. Traditionally, MMVD has been considered to be a non-inflammatory disease. However, up-regulation of genes involved in inflammation, and increased concentrations of pro-inflammatory cytokines, have been reported in late stages of canine MMVD (Zois et al., 2012; Oyama & Chittur, 2006). In human beings, a positive correlation between cardiovascular disease and CRP concentration is well-described (Hingorani et al., 2009; Ridker et al., 2009; Ridker et al., 2004). In dogs, on the other hand, studies on CRP concentrations in heart disease have shown diverging results. Myxomatous mitral valve disease was reported to be associated with increased CRP concentrations in one study (Rush et al., 2006), whereas another study did not support this finding (Ljungvall et al., 2010). Similarly, information on CRP concentrations in dogs with congestive heart failure has been inconclusive (Cunningham et al., 2012; Rush et al., 2006). Several possible explanations exist for the inconsistency in reported results.

First, not all studies adjusted for baseline variables such as age, sex and breed when analysing CRP data. Although these factors are probably irrelevant when studying the marked increased CRP concentrations present in dogs with systemic inflammatory disease, it is possible that they influence results when investigating minor changes at low CRP concentrations.
Second, presence of underlying diseases that might affect CRP concentrations was not excluded with certainty in the different studies. Low-grade inflammatory conditions such as osteoarthritis and periodontitis have been associated with mildly increased CRP concentrations (Yu et al., 2010; Hurter et al., 2005), and these diseases frequently occur in dogs reported to be healthy by their owners (Hillström et al., 2015). Such conditions may be difficult to detect on physical examination, and will not be identified by performing routine haematology and biochemistry testing. It cannot be excluded that presence of underlying diseases, not necessarily affecting different populations to the same extent, may have biased interpretation of CRP results.

Finally, the discrepancy between studies could be explained by the use of analytical assays with low precision. Measurements of CRP concentrations in research have most commonly been performed with ELISA tests, where a limited number of samples can be analysed on a single plate. When several ELISA plates are used for CRP measurements, an inter-assay variation due to plate differences will inevitably be introduced. The reported inter-assay variation for samples with low CRP concentrations was reported to be up to 26% for a commercial ELISA commonly used in research (Kjelgaard-Hansen et al., 2003b). This high imprecision may mask minor changes in CRP concentrations that are of interest to study. Moreover, if study samples from different groups of dogs are not placed randomly over the plates, erroneous conclusions could be drawn because of plate differences. One of the greatest advantages with using an automated high-sensitivity CRP assay is that inter-assay variation can be limited. In the current study of CRP concentration in dogs with MMVD, all 188 samples were analysed in duplicate in one single run. The reported intra-assay variation for the hsCRP assay was ≤ 2.7% (Paper II), which was confirmed by calculating intra-assay variation of the duplicate measurements of the study samples (data not shown). The high analytical precision makes it possible to detect minor changes in CRP concentrations that might otherwise have gone unnoticed had an assay with lower precision been used.
8 Conclusions

- Two fully automated, canine-specific CRP assays, where of one was a high-sensitivity CRP test, were validated. The performance of the assays met the pre-defined quality requirements. Using these assays, canine CRP could be reliably measured in the concentration range of 0.5-1200 mg/l.

- C-reactive protein concentration in dogs envenomed by *Vipera berus* was positively correlated to degree of oedema. This finding supports the suitability of using CRP as a quantitative marker of systemic inflammation in dogs.

- C-reactive protein concentration was useful for quantifying the degree of systemic inflammation in dogs with pyometra undergoing ovariohysterectomy. The median CRP concentration decreased the day after surgery compared with pre-operative levels, indicating that the removal of the inflamed uterus had a greater impact on systemic inflammation compared with the additional tissue trauma caused by surgery in most of the dogs.

- CRP concentration discriminated well between dogs with suppurative arthritis and osteoarthritis, suggesting that CRP has the potential to be a valuable diagnostic marker in dogs with joint disease.

- CRP concentration was not efficient for diagnosing late post-operative infections in dogs after orthopaedic surgery. These infections often caused inflammations that were purely local, and therefore could not be detected by measuring CRP which is a marker of systemic inflammation.

- Congestive heart failure in dogs with myxomatous mitral valve disease was associated with significant, but very mild increased CRP concentrations, indicating that inflammation might be involved in the pathogenesis of this condition. The availability of an automated hsCRP test enabled measurement of the 188 study samples in one single run, avoiding imprecision caused by inter-assay variation.
9 Concluding remarks and future perspectives

It has been proposed that diagnostic tests should be subjected to clinical trials similar to what therapeutic agents undergo, to ensure evidence-based use of them (Kjelgaard-Hansen & Jacobsen, 2011; Ransohoff, 2007; Zweig & Robertson, 1982). A series of step-wise investigations has been recommended (Table 5) (Kjelgaard-Hansen & Jacobsen, 2011). The first step (Phase I) includes method validation, as availability of practicable assays with acceptable performance is a prerequisite for successful introduction of a new diagnostic test. In the next step (Phase II), the overlap performance is investigated where analyte concentrations in healthy subjects and in subjects affected by the condition of interest are compared. The idea is that if the test does not distinguish well between these two groups, it will not be valuable as a diagnostic marker. Failure at this step will prevent further, costly, investigations being initiated (Kjelgaard-Hansen & Jacobsen, 2011).

The capability of a test to discriminate between healthy and diseased animals does not automatically imply that it is a diagnostically useful test. Premature introduction of new diagnostic methods, as well as overestimation of test performance, will occur if relying on overlap performance data for judging the efficacy of a diagnostic marker (Ransohoff, 2007). Once it has been concluded that a test can distinguish between diseased and healthy individuals, it is therefore necessary to continue with studies investigating test performance in a clinical setting (Phase III). Recommended guidelines on how to conduct and report such studies should be followed, in order to ensure transparency of results (Bossuyt et al., 2004).

The last, most resource-demanding step is to evaluate whether the use of a test has an impact on patient outcome or other relevant parameters (Phase IV). The question is whether patients that undergo the test fare better than similar patient not taking it, i.e. if using the test improves survival, shortens treatment time, saves expenses, or provides other advantages compared to existing diagnostic methods.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Investigation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Analytical performance</td>
<td>Method validation study and investigation of applicability</td>
</tr>
<tr>
<td>II</td>
<td>Overlap performance</td>
<td>Measuring analyte concentration in healthy animals and animals affected by the condition of interest</td>
</tr>
<tr>
<td>III</td>
<td>Clinical performance</td>
<td>Evaluating performance of the test in a clinical setting</td>
</tr>
<tr>
<td>IV</td>
<td>Outcome and usefulness</td>
<td>Evaluating if performing the test has beneficial effects on outcome, or other relevant parameters</td>
</tr>
</tbody>
</table>

So, where should canine CRP be placed in this process of marker validation? As shown in the current thesis (Paper I and II), functional assays for measuring canine CRP are available, which means that Phase I requirements are fulfilled (Table 5). Phase II studies have shown that CRP efficiently discriminates between dogs with and without systemic inflammation (Christensen et al., 2014; Nakamura et al., 2008; Caspi et al., 1987). C-reactive protein has become a well-established inflammatory marker in dogs, even used in research to define presence of, or quantify, systemic inflammation (Liao et al., 2014; Langhorn et al., 2013a; Langhorn et al., 2013b; Liu et al., 2013; Michelsen et al., 2012; Freeman et al., 2010).

Studies in a clinical setting (Phase III studies) have indicated that CRP is also a valuable diagnostic test. Repeated CRP measurements can be used to follow response to treatment and for judging prognosis, as previously described. In dogs with similar clinical findings, CRP concentration can discriminate between plausible differential diagnoses such as pyometra and mucometra (Fransson et al., 2004), bacterial pneumonia and other respiratory diseases (Viitanen et al., 2014), and suppurative arthritis and OA (Paper IV). Probably there are other clinical situations where knowledge on CRP concentration is helpful for establishing an aetiological diagnosis, as long as the CRP result is interpreted in a specific, well-defined context.

The final step, to investigate whether measurement of CRP affects outcome (Phase IV), still awaits to be realized. Although such studies are resource-demanding, they are crucial to conduct to improve the use of CRP as a diagnostic test. There are numerous possible study designs that could be used. One example would be to let veterinarians judge clinical cases first without, and then with, knowledge of CRP concentration, to see whether the additional information allowed for a more correct interpretation of the case. Another relevant study would be to randomly measure CRP in only part of the dogs admitted to veterinary care, and evaluate whether outcome was improved in this group. In
hospitalised patients, it would be interesting to investigate if repeated CRP measurements enabled more effective decision making regarding whether a dog should be discharged or not. Conducting this type of Phase IV studies will be of great importance for evidence-based use of CRP as an inflammatory marker, and the fact that automated canine-specific CRP assays are now available should facilitate the research.

I ett första steg i detta forskningsprojekt utvecklades en metod som kunde mäta de kraftigt förhöjda nivåer av CRP som förekommer hos hundar med inflammatoriska sjukdomar. Syftet var att detta test skulle kunna användas rutinmässigt av kliniskt verksamma veterinärer för att upptäcka och följa inflammation hos sjuka hundar. I ett andra steg utvecklades en så kallad hög-känslig CRP-metod för att möjliggöra exakta mätningar av CRP vid mycket låga koncentrationer. Sådana mätningar är av intresse för att undersöka tillstånd som tros ha samband med låggradig, långvarig inflammation, exempelvis fetma. Hos människa är det visat att denna typ av låggradig inflammation är skadlig, och det behövs forskning för att undersöka om detsamma gäller hundar.

Omfattande tester av de båda nya CRP-metoderna genomfördes i syfte att säkerställa att de fungerade tillfredsställande. Resultaten redovisas i två publikationer som är inkluderade i avhandlingen. I artiklarna finns detaljerat information om hur de nya metoderna fungerar och hur de kan användas. Bland annat beskrivs det hur CRP analyseras direkt i ett stort analysinstrument, vilket innebär att mätningen är helt automatiserad. Detta möjliggör tillförlitliga och snabba analyssvar, vilket är en fördel både i klinisk verksamhet och inom forskning.

Ett ytterligare mål med doktorandprojektet var att utföra studier som syftade till att öka kunskapen om CRP som ett diagnostiskt test hos hund. I avhandlingen beskrivs bland annat hur analys av CRP var effektivt för att särskilja hundar som hade inflammatorisk ledsjukdom från hundar med artros, som är en sjukdom där
ledbrosket gradvis bryts ned. Vid misstanke om inflammatorisk ledsjukdom behöver ett prov tas från ledvätskan och ofta krävs behandling med antibiotika eller kortison. Vid artros är detta inte nödvändigt, utan hunden kan ofta skickas hem på smärtstillande medicin som en första åtgärd. Genom att analysera CRP kan veterinären få hjälp i sin bedömning av huruvida en hund med ledsmärta mest sannolikt har en inflammation i leden eller artros, vilket påverkar hur patienten utreds och behandlas.


Ytterligare två studier som finns beskrivna i avhandlingen handlar om CRP hos ormbitna hundar och hos hundar med livmoderinflammation. Resultaten visade att CRP var en användbar markör för att upptäcka och följa inflammation vid dessa tillstånd.

Sammanfattningsvis finns nu automatiserade metoder speciellt utvecklade för att mäta CRP hos hund tillgängliga. Detta är en stor fördel både inom djursjukvården och inom forskning och kommer att möjliggöra ett mer effektivt användande av CRP som diagnostiskt test i framtiden.
11 References


Gow, A.G., Marques, A.I., Yool, D.A., Crawford, K., Warman, S.M., Eckersall, P.D., Jalan, R. & Mellanby, R.J. (2012). Dogs with congenital porto-systemic shunting (cPSS) and hepatic encephalopathy have higher serum
concentrations of C-reactive protein than asymptomatic dogs with cPSS. *Metab Brain Dis*, 27(2), pp. 227-9.


(cCRP) and the corresponding liver mRNA in dogs. *Biochem Biophys Res Commun*, 452(3), pp. 462-7.


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