EVALUATION OF CANINE S100A12 AND sRAGE AS NOVEL DISEASE MARKERS IN DOGS WITH INFLAMMATORY BOWEL DISEASE

A Dissertation

by

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ABSTRACT

Inflammatory bowel disease (IBD) is a common condition in dogs that is challenging to diagnose. A dysregulated innate immunity plays a major role in its pathogenesis, and surrogate inflammatory markers that reflect disease severity would be clinically useful. S100A12, a damage-associated molecular pattern molecule, is involved in phagocyte activation. S100A12 binds to the receptor of advanced glycation end products (RAGE), a pattern-recognition receptor, and results in human studies suggest a role of S100A12 and RAGE in chronic inflammation. Soluble RAGE (sRAGE), a decoy receptor, functions as an anti-inflammatory molecule. S100A12 and RAGE/sRAGE have not been studied in canine IBD. Canine S100A12 has not been purified, and while an immunoassay for measurement of S100A12 in humans is available, human antibodies do not cross-react with canine S100A12 (cS100A12). Canine RAGE has been cloned and characterized.

The aims of this project were to purify and partially characterize cS100A12, to develop and analytically validate an immunoassay for cS100A12, and to determine the relationship between cS100A12 and systemic sRAGE concentrations and clinical, endoscopic, and histologic disease severity in dogs with IBD. Markers of gastrointestinal inflammation were also evaluated in dogs with acute hemorrhagic diarrhea syndrome (AHDS).

Canine S100A12 was successfully purified from canine whole blood, and a competitive liquid-phase radioimmunoassay was developed and analytically validated.

Fecal cS100A12 concentrations were shown to be increased in dogs with IBD and were associated with clinical disease activity, the severity of endoscopic lesions, and the severity of colonic inflammation. Serum sRAGE concentrations were decreased in dogs with IBD, but were not correlated with disease severity, cS100A12 concentrations, or outcome. Dogs that were euthanized had higher fecal cS100A12 concentrations than dogs that were alive at the end of the study, and serum sRAGE concentrations increased only in IBD dogs with complete clinical remission. A significant but transient increase in fecal cS100A12 was also seen in dogs with AHDS.

Fecal cS100A12 may be clinically useful as a biomarker of inflammation in dogs with IBD, and the sRAGE/RAGE axis appears to be altered in canine IBD. Lack of correlation between sRAGE and cS100A12 is consistent with sRAGE being a non-specific decoy receptor.

DEDICATION

To Nerl, Desy, Axel, Marie, Mickey, Maja, and Moses

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NOMENCLATURE

AA	Amino Acid
Ab	Antibody
α_1PI	Alpha$_1$-Proteinase Inhibitor
ACN	Acetonitrile
ACTH	Adrenocorticotropic Hormone
AGE	Advanced Glycation End Product
AHDS	Acute Hemorrhagic Diarrhea Syndrome
ANOVA	Analysis of Variance
ARD	Antibiotic-Responsive Diarrhea
AUC	Area Under the Curve
AUP	Animal Use Protocol
B_0	Zero Standard
BSA	Bovine Serum Albumin
CAI	Colitis Activity Index
cα_1PI	Canine Alpha$_1$-Proteinase Inhibitor
cS100A12	Canine S100A12 Protein
CCECAI	Canine Chronic Enteropathy Clinical Activity Index
CCL	CC Chemokine Ligand
cCP	Canine Calprotectin
CD	Crohn's Disease

CD4	Cluster of Differentiation-4
cDNA	Complementary DNA
CFA	Complete Freund's Adjuvant
CHCA	Alpha-Cyano-4-Hydroxy Cinnamic Acid
CI	Confidence Interval
CIBDAI	Canine IBD Activity Index
CLSI	Clinical and Laboratory Standards Institute
CP	Calprotectin
cRAGE	Canine RAGE protein
CRP	C-Reactive Protein
cTLI	Canine Trypsin-like Immunoreactivity
CV	Coefficient of Variation
CV_A	Analytical Variation
CV_I	Intra-individual Variation
CV_G	Inter-individual Variation
CV_T	Total Variation
CXCL	CXC Chemokine Ligand
DAMP	Damage-Associated Molecular Pattern
DDT	Dithiothreitol
DFP	Diisopropylfluorophosphate
DNA	Deoxyribonucleic Acid
dsRNA	Double-Stranded RNA

ED	Extracellular Domain
EDTA	Ethylenediamine Tetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EN-RAGE	Extracellular Newly Identified RAGE-Binding Protein
EPI	Exocrine Pancreatic Insufficiency
ESI-MS	Electrospray Ionization Mass Spectrometry
fcA12	Fecal cS100A12
Fc Region	Fragment Crystallizable Region
FPLC	Fast-Protein Liquid Chromatography
FRD	Food-Responsive Diarrhea
FU	Fluorescence Unit
GC	Granulomatous Colitis
GI	Gastrointestinal
GLP	Good Laboratory Practice
$H_{NT(14)}$	Homology of the 14 N-Terminal Amino Acids
H_{ES}	Homology of the Entire Sequence of Amino Acids
H&E	Hematoxylin and Eosin Stain
HGE	Hemorrhagic Gastroenteritis
HIC	Hydrophobic Interaction Chromatography
HMGB-1	High-Mobility Group Box-1
HRP	Horseradish Peroxidase

hS100A12	Human S100A12 Protein
hs-CRP	High-Sensitivity CRP
IBD	Inflammatory Bowel Disease
ICAM	Intercellular Adhesion Molecule
IEF	Isoelectric Focusing
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IH	Index of Heterogeneity
II	Index of Individuality
IL	Interleukin
IQR	Interquartile Range
IKK	I-kappa B Kinase
IRAK	IL-1 Receptor-Associated Kinase
IRF	Interferon Regulatory Factor
ISU	Iowa State University
JNK	C-Jun N-Terminal Kinase
KRAS	V-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog
LM	Lower Marker
LPC	Lymphoplasmacytic
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeat

MA	Mucosal Atrophy
MAdCAM	Mucosal Addressin Cell Adhesion Molecule
MAPK	Mitogen-Activated Protein Kinase
$MCD_{0.05}$	Minimum Critical Difference (at $p \leq 0.05$)
MDP	Muramyldipeptide
M_M	Molecular Mass
MMP	Matrix Metalloproteinase
M_r	Relative Molecular Mass
mRNA	Messenger RNA
MRP	Migration Inhibitory Factor-Related Protein
MS	Mass Spectrometry
M_W	Molecular Weight
MyD88	Myeloid Differentiation Factor 88
m/z	Mass-to-Charge Ratio
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form)
NA-HRP	NeutrAvidin Horseradish Peroxidase
NSB	Non-Specific Binding
NCF	Neutrophil Cytosolic Factor
NF-κB	Nuclear Factor-kappa B
NMH	N-Methylhistamine
NOD	Nucleotide Oligomerization Domain
NPV	Negative Predictive Value

NT	N-Terminus
O/E	Observed-to-Expected Ratio
OR	Odds Ratio
pAb	Polyclonal Antibody
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen-Associated Molecular Pattern
pANCA	Perinuclear Anti-Neutrophilic Cytoplasmic Antibodies
pASCA	Perinuclear Anti-*Saccharomyces Cerevisiae* Antibodies
PCR	Polymerase Chain Reaction
p*I*	Isoelectric Point
5-PL	5-Parameter Logistic Regression
PLE	Protein-Losing Enteropathy
PLN	Protein-Losing Nephropathy
PMF	Peptide Mass Fingerprint
PMN	Polymorphonuclear Neutrophil
POCT	Point-Of-Care
PPV	Positive Predictive Value
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Fluoride
QC	Quality Control
RAGE	Receptor for Advanced Glycation End Products
RBC	Red Blood Cell

rc	Recombinant Canine
RDI	Radial Double Immunodiffusion
rh	Recombinant Human
RI	Reference Interval
RIA	Radioimmunoassay
RIAB	RIA Buffer
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
RT-PCR	Reverse Transcriptase PCR
RVC	Royal Veterinary College
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SELDI-TOF	Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
SNP	Single Nucleotide Polymorphism
SP	System Peak
SPA	Scintillation Proximity Assay
sRAGE	Soluble RAGE
TAMU	Texas A&M University
TBS	Tris-Buffered Saline
TC	Total Count
TCR	T Cell Receptor
TFA	Trifluoroacetic Acid

TGF	Transforming Growth Factor
Th	Helper-T Lymphocyte
TIR	Cytoplasmic Domain Toll/IL-1R
TIRAP	TIR Adaptor Protein
t_M	Migration Time
TMB	Tetramethylbenzidine
TNF	Tumor-Necrosis Factor
TLR	Toll-like Receptor
TOF	Time-of-Flight
TRAF	TNF-Receptor Associated Factor
TRAM	TLR-4 Adaptor Protein
TRIF	TIR-Domain-Containing Adapter-Inducing IFN-β
TR-IFMA	Time-Resolved Immunofluorometric Assay
UM	Upper Marker
VCAM	Vascular Cell Adhesion Molecule
v/v	Volume/Volume
WBC	White Blood Cell
WSAVA	World Small Animal Veterinary Association
w/v	Weight/Volume
X	Unambiguously Identified Amino Acid

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1. INTRODUCTION

1.1. Canine Inflammatory Bowel Disease (IBD)

1.1.1. Definition

Idiopathic inflammatory bowel disease (IBD) in dogs is a chronic inflammation of the small and/or large intestine that may also involve the stomach, the exact cause of which is unknown (Hall and German, 2008). It is a chronic relapsing condition and its diagnosis and management can be challenging for the clinician and frustrating to the owner. The diagnosis of IBD requires a comprehensive diagnostic work-up, including invasive diagnostic procedures, to exclude other causes of chronic gastrointestinal signs such as food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), infiltrative infectious diseases (e.g., gastrointestinal histoplasmosis), or neoplasia (e.g., lymphoma) (Day et al., 2008; Washabau et al., 2010; Simpson and Jergens, 2012; Jergens and Simpson, 2012; Suchodolski et al., 2010).

1.1.2. Pathogenesis

The current consensus concerning the pathogenesis of IBD in dogs stipulates an impaired immunoregulation in a genetically predisposed host that results in an inflammatory response against dietary and/or microbial antigens (Fig. 1) (Jergens and Simpson, 2012; German et al., 2001; Burgener et al., 2008). Cells of the adaptive immune system have long been associated with the pathogenesis of idiopathic IBD in dogs, but evidence is mounting that impaired innate immunity also plays a critical role in

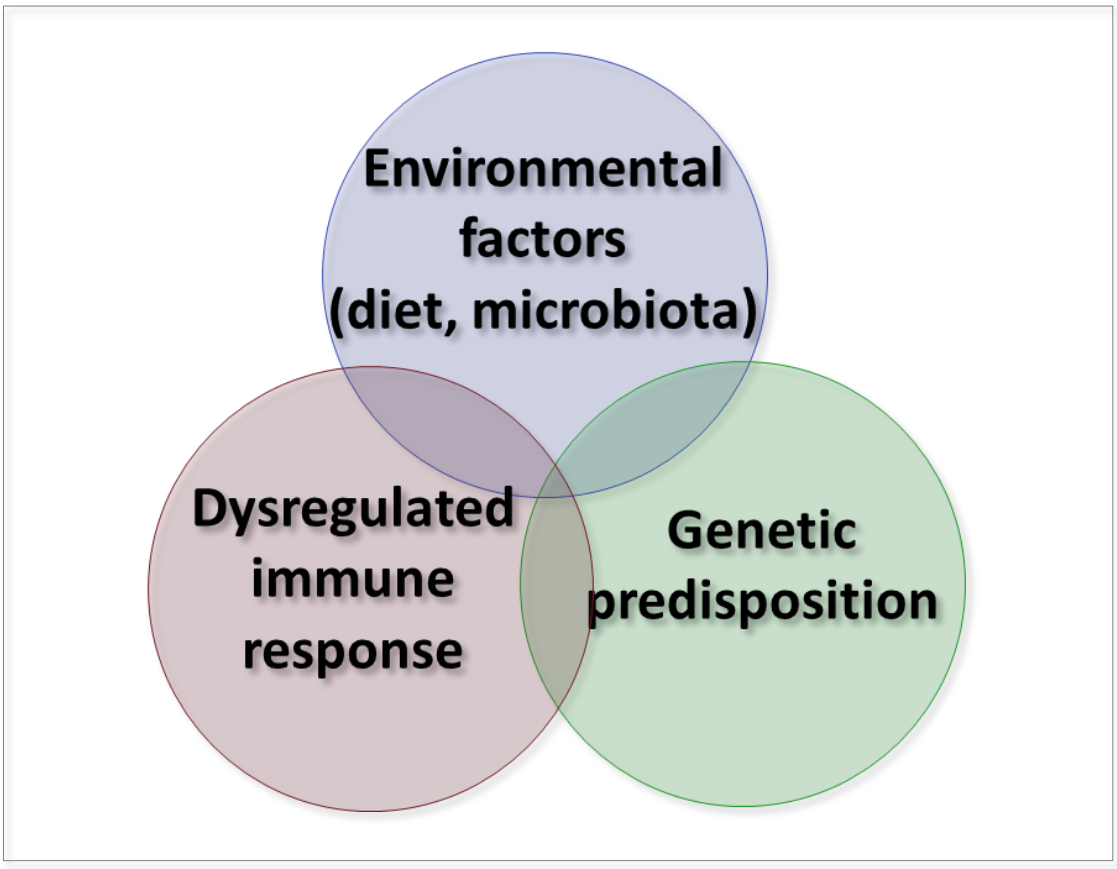

Figure 1 – Pathogenesis of canine inflammatory bowel disease (IBD). The exact cause of canine IBD is unknown. The pathogenesis appears to be complex and the current consensus is that it is multifactorial in nature and that innate immunity appears to play a central role.

the development of idiopathic IBD (German et al., 2001; Burgener et al., 2008; Luckschander et al., 2010; Allenspach, 2011a; Wilke et al., 2012). Thus, biomarkers of phagocyte activation may potentially serve as clinically useful biomarkers of inflammation in dogs with idiopathic IBD.

1.1.3. Biomarkers for canine IBD

Biomarkers are used as an aid to evaluate organ function, assess the risk for disease development, diagnose a specific disease process, assess disease severity, predict the patient's response to treatment or predict the disease outcome, and/or to monitor the disease process. Disease biomarkers are generally said to be either static or dynamic, and they are classified as functional, biochemical, cellular, genomic, or proteomic biomarkers.

The few minimally invasive biomarkers that have been evaluated in dogs with idiopathic inflammatory bowel disease (IBD) belong to the groups of biochemical, functional, and genomic biomarkers. These include (1) *functional biomarkers*: (i) serum and fecal alpha$_1$-proteinase inhibitor (α_1PI) concentrations, (ii) serum folate (vitamin B_9) and cobalamin (vitamin B_{12}) concentrations, and (iii) tests evaluating gastrointestinal permeability and absorptive function, such as ^{51}Cr-EDTA absorption, iohexol absorption, serum or urine lactulose/rhamnose ratio or the xylose/methylglucose ratio (the last group of markers is impractical and not used clinically); (2) *biochemical biomarkers*: (i) serum C-reactive protein (CRP), (ii) perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA), (iii) perinuclear anti-*Saccharomyces cerevisiae* yeast

antibodies (pASCA), (iv) urine and fecal N-methylhistamine (NMH) concentrations, (v) serum and fecal calprotectin concentrations, (vi) serum and fecal immunoglobulin A concentrations, and (vii) various cytokines and chemokines; and (3) *genomic biomarkers*: (i) single nucleotide polymorphisms (SNPs; e.g., in *TLR4*, *TLR5*, *NOD2*, *NCF2*), (ii) reduction of genetic diversity (e.g., Ig and TCR repertoire), and (iii) alterations in gene expression (e.g., TLR2, TLR4, TLR5, TLR9, CCL2, CCL20, CCL25, CCL28, CXCL8, Casp3, Bcl-2, and other genes involved in cell replication, iron/calcium transport, innate immunity/inflammation, cellular detoxification, intestinal barrier function, and extracellular matrix degradation). Selected biomarkers are discussed below.

1.1.3.1. Fecal alpha$_1$-proteinase inhibitor (α_1PI)

Alpha$_1$-proteinase inhibitor (α_1PI; also referred to as alpha$_1$-antitrypsin) is a major serum proteinase inhibitor that is mainly produced in the liver and protects the body from the detrimental effects of neutrophil proteases, trypsin, and chymotrypsin (Travis et al., 1988). Compared to humans, there is no definitive evidence to suggest that canine α_1PI is an acute-phase reactant (Conner et al., 1988; Ganrot et al., 1973). Canine α_1PI is a proteolysis-resistant protein that has a molecular weight similar to that of albumin (Melgarejo et al., 1996). It is theoretically only present at high concentrations in the gastrointestinal lumen when there is loss of proteins as a result of gastrointestinal disease, and the rate of α_1PI loss should be approximately that of albumin. However, unlike albumin, cα_1PI is expected to resist proteolysis due to its inhibitory activity

towards proteinases, and can therefore be quantified in fecal samples (Fig. 2) (Heilmann et al., 2011c).

Pathologic chronic losses of cα_1PI into the gastrointestinal lumen may (in theory) deplete systemic cα_1PI concentrations, as hepatic α_1PI synthesis and secretion is a continuous process that normally does not involve intracellular storage of α_1PI (Carlson, 1984), and change the systemic proteinase-proteinase inhibitor balance. Decreased serum cα_1PI concentrations have been measured in dogs with protein-losing enteropathy due to IBD (Equilino et al., 2015) and in cobalamin-deficient Yorkshire Terriers (Grützner et al., 2013).

The concentration of canine α_1PI in feces has been shown to be clinically useful as a marker for gastrointestinal protein loss in dogs as it increased before the onset of clinical signs and/or hypoalbuminemia or panhypoproteinemia in Soft Coated Wheaten Terrier dogs with familial protein-losing enteropathy (PLE) (Vaden et al., 2002). Thus, fecal α_1PI appears to be useful for the early detection of gastrointestinal protein loss. In a recent study, fecal α_1PI concentration distinguished dogs with moderate or severe gastrointestinal crypt abscesses and/or lacteal dilation from those without such lesions or only mild lacteal dilation with moderate sensitivity and specificity; the serum-to-fecal α_1PI ratio may improve the diagnostic accuracy in hypoalbuminemic dogs (Heilmann et al., 2014c).

Concentrations of α_1PI in fecal extracts from individual healthy dogs vary greatly from day-to-day (with %CVs up to 102% reported), and collection of a fecal sample for

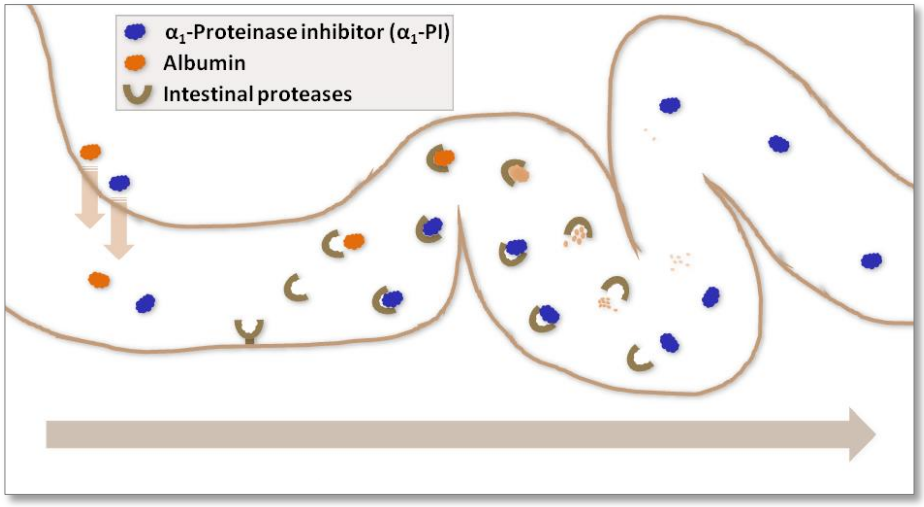

Figure 2 – Fecal canine α_1-proteinase inhibitor (α_1PI). Canine α_1-proteinase inhibitor (α_1PI) has a molecular weight similar to that of albumin and is only present at high concentrations in the gastrointestinal lumen when there is loss of proteins as a result of gastrointestinal disease. Unlike albumin, α_1PI resists proteolysis due to its inhibitory activity towards proteinases, and can therefore be quantitatively analyzed in fecal samples. (With permission from: Heilmann, R.M., Paddock, C.G., Ruhnke, I., et al., 2011. Development and analytical validation of a radioimmunoassay for the measurement of alpha$_1$-proteinase inhibitor concentrations in feces from healthy puppies and adult dogs. *Journal of Veterinary Diagnostic Investigation* 23: 476-485, © 2011 by SAGE)

each of three consecutive days from the same patient has been suggested to counterbalance this physiological variation (Steiner et al., 2003; Heilmann et al., 2011c).

A fecal α_1PI of ≥13.9 µg/g for the mean of 3 samples from consecutive days and/or ≥21.0 µg/g for the maximum of these 3 fecal extracts collected on 3 consecutive days is considered abnormal. Increased fecal canine α_1PI concentrations in dogs <1 year

of age, and especially in dogs <6 months old, should be interpreted with caution as fecal α_1PI levels have been shown to be significantly higher in healthy dogs <1 year of age (Heilmann et al., 2011c).

1.1.3.2. Serum cobalamin and folate concentrations

Cobalamin (vitamin B_{12}) and folate (vitamin B_9) are water-soluble vitamins that are often measured in dogs with chronic gastrointestinal signs. Cobalamin absorption occurs exclusively in the distal small intestine (ileum) via specific cobalamin receptors that take up intrinsic factor-cobalamin complexes. Hypocobalaminemia is frequently detected in dogs with chronic small intestinal disease (18-54%), and this can be due to distal small intestinal malabsorption, but may also reflect an increased utilization of cobalamin by the intestinal microbiota due to small intestinal dysbiosis (Allenspach et al., 2007; Berghoff et al., 2013; Heilmann et al., 2014a). Hypocobalaminemia (<200 ng/L) has been shown to be a risk factor for negative outcome and to be associated with hypoalbuminemia (<20 g/L) in dogs with IBD (Allenspach et al., 2007). Hypocobalaminemia, however, is not a specific finding for IBD, and exocrine pancreatic insufficiency is an important differential diagnosis in patients with hypocobalaminemia and chronic gastrointestinal signs (especially weight loss and voluminous stools) (Heilmann et al., 2014b). Parenteral supplementation with cyanocobalamin (once weekly for 6 weeks, then every 2-4 weeks) is indicated if serum cobalamin concentrations are <350 ng/L in a patient with chronic gastrointestinal signs. Patients with EPI may need

life-long cobalamin supplementation. Oral cobalamin supplementation may also be effective if given daily (Toresson et al., 2014), but this needs further investigation.

Folate is predominantly absorbed in the proximal small intestine (duodenum, proximal jejunum) via folate carriers and after dietary folate has been broken down from folate polyglutamate to folate monoglutamate. Serum folate concentrations can thus be decreased due to malabsorption in dogs with chronic proximal small intestinal disease, but a decreased serum folate is also not specific for IBD and a normal serum folate concentration does not rule out significant small intestinal disease (Ruaux, 2008). Serum folate can also be falsely normal or increased due to small intestinal dysbiosis (with an increased production of folate by the intestinal microbiota) or due to hypocobalaminemia (and should thus be monitored with cobalamin supplementation) (Ruaux, 2008). Oral folic acid supplementation (10 µg/kg/day or 200-400 µg/day for 30 days) is recommended if folate is severely decreased.

1.1.3.3. Serum C-reactive protein (CRP)

CRP is a positive acute-phase protein of the pentraxin family and is produced by the liver in response to IL-6 and IL-1β during states of infection, inflammation, or cancer (Rhodes and Fürnrohr, 2011). The expression of CRP in the liver is regulated by NF-κB. CRP in its annular pentameric form can opsonize bacteria and/or apoptotic/necrotic cells to facilitate complement (C3b) binding and opsonin-mediated phagocytosis. CRP monomers may bind to cell surfaces in inflamed tissue and activate neutrophils and monocytes (Rhodes and Fürnrohr, 2011).

CRP is a nonspecific biomarker of inflammation that can be useful to evaluate disease progression and response to treatment. Several assay formats are available for measurement of canine CRP in serum, these include canine-specific enzyme-linked immunosorbent assays (ELISAs) (Berghoff et al., 2006), immunoturbidimetric canine CRP-assays (Eckersall et al., 1991; Klenner et al., 2010; Hillström et al., 2014), an immunofluorometric assay (TR-IFMA) (Parra et al., 2006), and a point-of-care (POCT) lateral flow immunoassay (Plickert et al., 2011). Most canine CRP assays have a reference interval in the 0-8 mg/L range. A high-sensitivity CRP (hs-CRP) test, which has been shown to be superior to standard CRP assays for the detection of very low systemic levels of CRP (<5 mg/L) in humans (Poullis et al., 2002; Roberts et al., 2000; Shine et al., 1985), is currently not available for use in dogs.

The concentration of C-reactive protein (CRP) in serum, measured by a canine-specific enzyme-linked immunosorbent assay (ELISA), has been suggested to be clinically useful for assessment of disease progression and response to treatment in dogs with idiopathic IBD (Jergens et al., 2003; Jergens et al., 2010a; Heilmann et al. 2012a). However, in other studies, serum CRP concentration was not associated with clinical or histopathologic disease severity in dogs with idiopathic IBD (Allenspach et al., 2007; McCann et al., 2007; and Münster et al., 2010). Also, a high biological variability of serum CRP concentrations within individual dogs (Carney et al., 2011) and nonspecific increases in serum CRP concentration in response to infectious, neoplastic, or other inflammatory conditions (Dabrowski et al., 2007; Nakamura et al., 2008; Griebsch et al., 2009; Lowrie et al., 2009; Chan et al., 2009; Gebhardt et al., 2009) appear to limit the

clinical usefulness of serum CRP concentration for diagnosing and/or monitoring idiopathic IBD in dogs.

1.1.3.4. Perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA)

Perinuclear anti-neutrophilic cytoplasmic antibodies are circulating auto-antibodies directed against neutrophil granule constituents, which are detected by indirect immunofluorescence staining (Allenspach et al., 2004). It has been suggested, but remains undefined, that pANCA is directed to a nuclear histone, proteinase 3 (Pr3), and/or myeloperoxidase (MPO) and represents an autoantibody representative of cross-reactivity with a luminal bacterial antigen.

In dogs, pANCA positivity was found to have a low sensitivity (23-51%) but moderate to high specificity (83-95%) for the detection of IBD (Mancho et al., 2011; Luckschander et al., 2006). The positive rate of pANCA and pANCA titers were shown to be higher in dogs with food-responsive diarrhea (FRD; 62%) than in dogs with idiopathic IBD (23%; Luckschander et al., 2006). Detection of pANCA does not distinguish dogs with IBD from dogs with intestinal lymphoma (Mancho et al., 2011). In a Soft Coated Wheaten Terrier colony, a positive pANCA test was significantly associated with hypoalbuminemia due to protein-losing enteropathy (PLE) and/or protein-losing nephropathy (PLN), with the positive test result preceding the development of hypoalbuminemia by >2 years (Allenspach et al., 2008). Thus, pANCA may be a useful test for the detection of FRD in dogs and/or the early detection of PLE and/or PLN in Soft Coated Wheaten Terriers.

1.1.3.5. N-methylhistamine (NMH)

N-methylhistamine (NMH) is a stable metabolite of histamine and is a biomarker of mast cell degranulation. After its release from mast cells, histamine (and other inflammatory mediators) has proinflammatory effects on other immune cells and has similar effects also on nerve and smooth muscle cells (Berghoff and Steiner, 2011). NMH can be measured in urine and fecal samples. Fecal NMH and mast cell degranulation have been shown to be increased in Norwegian Lundehunds (Berghoff et al., 2008) and Soft Coated Wheaten Terriers (Vaden et al., 2000) with chronic enteropathies, respectively. Recently, urine NMH was shown to be increased in a subgroup of dogs with chronic gastrointestinal disease and to correlate with the severity of histologic lesions and serum C-reactive protein (CRP) concentrations (Berghoff et al., 2014) suggesting that NMH may be a marker of mast cell-mediated inflammation in dogs with chronic enteropathies. However, another study did not find a correlation between NMH and the number of mast cells in the duodenal mucosa or the clinical disease severity (as assessed by the CCECAI scoring system [Allenspach et al., 2007]) (Anfinsen et al., 2014).

1.1.3.6. Cytokines and chemokines

Several studies have investigated the cytokine and chemokine signature in canine IBD. The first study by German et al. (2000) found a heightened immune response in the duodenal mucosa of German Shepherd dogs with chronic enteropathy, but the expression of IL-2, IL-4, IL-5, IL-10, IL-12p40, IFN-γ, TNF-α, and TGF-β1 mRNA did

not demonstrate a polarization toward Th1 or Th2 as described in humans with Crohn's disease and ulcerative colitis, respectively (Neurath et al., 2002). Peters et al. (2005) evaluated the duodenal mucosal expression of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, IFN-γ, TNF-α, and TGF-β by RT-PCR, and did not find a predominant $CD4^+$ cell type in dogs with chronic enteropathies. A meta-analysis by Jergens et al. (2009) revealed that the inflamed mucosa in dogs with IBD fails to show either Th1- or Th2-cytokine skewing as only IL-12 mRNA expression was consistently increased in small-intestinal IBD with no differences seen in canine IBD colitis. Kołodziejska-Sawerska et al. (2013) evaluated the mucosal expression of pro-inflammatory (IL-1, IL-2, IL-5, IL-6, IL-12, IL-18, IFN-γ, and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10), and reported no distinct T lymphocyte signature. Okanishi et al. (2013) found no significant difference in duodenal mucosal mRNA levels for TNFα or IL-1β in dogs with lymphoplasmacytic enteritis compared to healthy controls. Tamura et al. (2014) investigated the mRNA expression of helper-T-lymphocyte cytokines (IFN-γ, IL-4, IL-17, and IL-10) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-8, IL-12, and IL-23) in the colonic mucosa from dogs with lymphoplasmacytic colitis, and as opposed to human IBD, did also not show a predominant cytokine profile in the inflamed colonic mucosa in dogs with lymphoplasmacytic colitis.

Few canine studies have evaluated the Th17 subset of T lymphocytes. Schmitz et al. (2012) evaluated the duodenal mucosal expression of IL-17A, IL-22, IL-10, IFN-y, and TGF-β, and did not find evidence for the involvement of Th17 signature cytokines in canine IBD. A similar study by Ohta et al. (2014) evaluated the gene and protein

expression of duodenal mucosal IL-17A, IFN-γ, and IL-10, and found also no significant difference between dogs with IBD and healthy controls, thus concluding that there is no evidence of a distinct T cell signature in canine IBD.

Maeda et al. (2011) studied the mRNA expression of chemokines of the CC and CXC family in canine IBD, and found an increase in chemokines that are chemotactic for T and B lymphocytes and were shown to be up-regulated in human IBD. However, the role of chemokines in the Th1/Th2/Th17 polarization is unclear.

The results of studies in dogs have to be interpreted with caution as several factors, including the method of mRNA quantification, the possibility of mRNA instability, the stage, severity, and location of IBD, as well as other factors (antimicrobial therapy, diet, and demographic differences) differed significantly among the different groups of dogs evaluated. Further limitations of most studies included a small sample size and the lack of a disease control group.

Analyses of the expression of other signaling molecules of the innate immune response have revealed a dysregulation in the expression of several cytokines and chemokines (e.g., CCL2, CCL20, CCL25, CCL28, and CXCL8) (Maeda et al., 2011), intracellular signaling (e.g., nuclear factor-κB) and intercellular adhesion molecules (e.g., VCAM-1, MAdCAM-1, and ICAM-1) (Luckschander et al., 2010; Okanishi et al., 2013), and other genes involved in cell replication, innate immunity/inflammation, cellular detoxification, iron/calcium transport, intestinal barrier function, and extracellular matrix degradation (Jergens et al., 2010b; Wilke et al., 2012).

1.1.3.7. Genomic biomarkers

Genetic studies have shown that polymorphisms in the genes encoding the Toll-like receptor (TLR)-4, TLR5, and nucleotide oligomerization domain (NOD)-2 are significantly associated with canine IBD in German shepherd dogs and other breeds (Allenspach, 2011a). The *TLR4* SNPs A1571T and G1807A (both recessive) and the *TLR5* SNP G22A (likely dominant) were associated with an increased risk of IBD, whereas the *TLR5* SNPs C100T and T1844C (both dominant) were protective for IBD (Kathrani et al., 2010a). Another breed-independent candidate gene approach confirmed that the *TLR5* SNPs C100T (dominant) and T1844C (recessive) are protective for IBD (Kathrani et al., 2011a). A mutational analysis in Boxer dogs with IBD revealed another SNP (T443C) in the leucine rich repeat domain of *TLR5* (Kathrani et al., 2011b), suggesting that individual breeds may harbor unique SNPs in addition to shared polymorphisms among breeds. Analysis of the significance of the 2 *TLR5* SNPs associated with IBD (C100T and T1844C) and the 3 *TLR5* SNPs in German shepherd dogs with IBD (G22A, C100T, and T1844C) on receptor function revealed a hyper-responsiveness of TLR5 to stimulation with flagellin (Kathrani et al., 2011c; Allenspach et al., 2011b). Mutational analyses in German shepherd dogs and other breeds also revealed an association between IBD and polymorphisms in the gene encoding nucleotide oligomerization domain-2 (*NOD2*), a cytosolic pattern recognition receptor that recognizes MDP of $Gram^-$ and $Gram^+$ bacteria (Kathrani et al., 2010b; Kathrani et al., 2011d; Kathrani et al. 2014). Although no longer classified as a subgroup of canine IBD, a genome-wide analysis of granulomatous colitis (GC) in boxer dogs has revealed

polymorphisms in the phagocytic cytosolic factor (*NCF*)2 gene, which codes for a subunit of neutrophil NADPH oxidase and is involved in bacterial killing and the cellular autophagy pathway (Craven et al., 2010).

Gene expression studies revealed a significantly higher mucosal expression of TLR2, TLR4, and TLR9 mRNA in dogs with IBD at the time of diagnosis, but no correlation with histology or clinical disease activity (as assessed using the CIBDAI scoring system [Jergens et al., 2003]) and also no change post-treatment despite clinical improvement (Burgener et al., 2008). Also, TLR2 and TLR4 were shown to be expressed at the apical epithelial surface whereas TLR9 was expressed intracellular. Another study showed a 20-fold increase of TLR2-mRNA in IBD dogs and a weak correlation between TLR2-mRNA expression and clinical disease severity (as assessed by the CCECAI scoring system [Allenspach et al., 2007]) but no correlation with histopathology scores (McMahon et al., 2010). Expression of *TLR4* was up-regulated and that of *TLR5* down-regulated in all segments of the intestine evaluate in German shepherd dogs with IBD, and the expression of TLR did not correlate with clinical disease activity (CCECAI), histopathology scores, or the number of bacterial clones in the duodenum (Allenspach et al., 2010). However, none of these studies confirmed their findings at the protein level.

1.1.4. Characteristics for an ideal biomarker

For a marker of inflammation to be clinically useful, it needs to be measurable without temporal delay in expression and/or secretion following changes of the

inflammatory activity. The marker should also be organ-specific in expression or the type of specimen used to determine its concentration, and be specific for the disease process. Moreover, such a marker needs to be easy, inexpensive, and minimally-invasive to measure, and be stable in the specimen under clinical conditions. Fecal biomarkers that are specific for gastrointestinal disease, correlate with disease severity, and objectively assess gastrointestinal inflammation would be very useful in clinical practice but such markers are currently lacking in veterinary medicine; and the lack of stability in fecal samples hampers the routine analysis of many small molecular inflammatory proteins (e.g., cytokines or chemokines). Thus, additional biomarkers that are more specific for IBD would be useful for the diagnosis and assessment of disease progression in dogs with IBD.

1.2. Calgranulin/S100 Proteins

S100/calgranulins, a group of three phagocyte-specific damage-associated molecular pattern (DAMP) molecules (Pietzsch and Hoppman, 2008), accumulate at sites of inflammation and appear to be candidates for fecal markers because of their stability in feces (Föll et al., 2009) and their increase at the mucosal mRNA level in dogs with IBD (Wilke et al., 2012).

The S100 proteins represent a genetically highly conserved family of intracellular Ca^{2+}-modulated signaling proteins with a tightly clustered organization of their respective genes (Schäfer and Heizmann, 1996), and were eponymously termed after its first member was shown to be soluble in 100% $(NH_4)_2SO_4$ (Moore, 1965).

Calprotectin was the first calgranulin/S100 complex discovered in the cytosol fraction of human neutrophilic granulocytes (PMN) (Fagerhol, 1980) and consists of two Ca^{2+}-binding proteins assigned to the S100 protein family: S100A8 and S100A9 (Schäfer and Heizmann, 1996). Both calprotectin proteins contain two Ca^{2+}-binding motifs of the EF-hand type (α-helix-loop-α-helix model) with disparate Ca^{2+} affinities, flanked by hydrophobic regions at either terminus that are exposed upon Ca^{2+}-binding. It has been postulated that these regions play an important role in the hydrophobic interactions with S100-specific target proteins (Hunter and Chazin, 1998; Kligman and Hilt, 1988; Odink et al., 1987; Schäfer and Heizmann, 1996; Yousefi et al., 2005).

1.2.1. S100A8/A9 protein complex

Calprotectin, the S100A8/A9 complex, is expressed and released into the extracellular space by activated macrophages and neutrophils and can be induced in epithelial cells. Serum concentrations of calprotectin are often increased in human patients with IBD (Leach et al., 2007; Lügering et al., 1995). Calprotectin may be involved in the expression of proinflammatory cytokines and chemokines downstream of Toll-like receptor-4 (Fig. 3) (Vogl et al., 2007), which is up-regulated in dogs with idiopathic IBD (Burgener et al. 2008). Canine calprotectin has been recently purified and partially characterized (Heilmann et al., 2008a), and immunoassays for the quantification of calprotectin in serum and fecal samples from dogs have been developed and analytically validated (Heilmann et al., 2008b; Grützner et al., 2014). Serum and fecal calprotectin concentrations have been evaluated in dogs with chronic gastrointestinal

inflammation (Grellet et al., 2013; Heilmann et al., 2012a; Otoni et al., 2012) and may serve as a biomarker of inflammation in dogs.

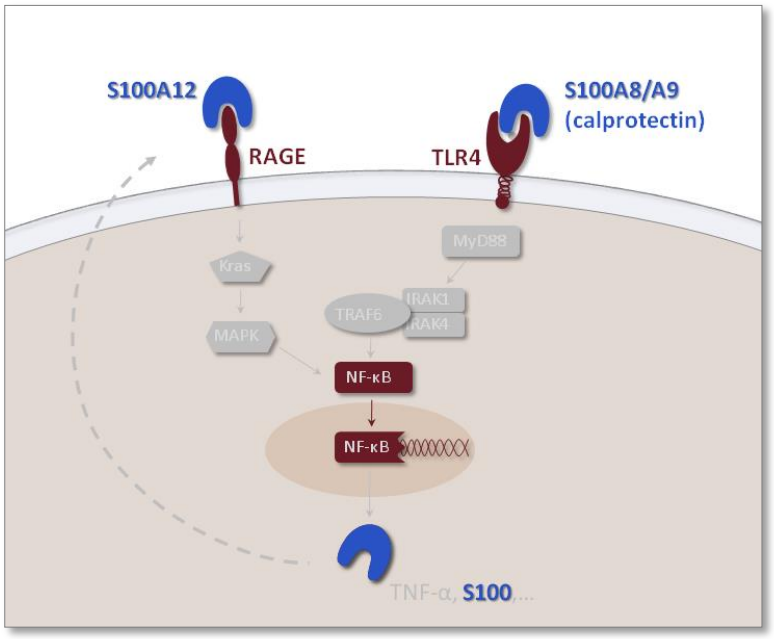

Figure 3 – S100A8/A9 and S100A12 play a role in the immune response. Both S100A8/A9 and S100A12 represent endogenous damage-associated molecular pattern (DAMP) molecules that bind to receptors of the innate immune system. Upon ligand binding, these receptors have been shown to activate NF-κB, leading to the production of pro-inflammatory cytokines and chemokines. A positive feedback on RAGE expression has been shown for S100A12.

IRAK = IL-1 receptor-associated kinase; Kras = V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; MAPK = mitogen-activated protein kinases; MyD88 = myeloid differentiation factor 88; NF-κB = nuclear factor-kappa B; RAGE = receptor for advanced glycation end products; TLR4 = Toll-like receptor 4; TNF-α = tumor-necrosis factor α; TRAF = TNF-receptor associated factor.

1.2.2. S100A12 protein

S100A12, also referred to as calgranulin C, migration inhibitory factor-related protein (MRP)-6, p6, or extracellular newly identified RAGE-binding protein (EN-RAGE) (Hofmann et al., 1999; Ilg et al., 1996; van den Bos et al., 1998), was first identified in the cytosol of human neutrophils and monocytes (Guignard et al., 1995). It belongs to the calgranulin subfamily of the highly conserved S100 proteins, a superfamily of EF-hand proteins with a high Ca^{2+}-binding capacity and binding sites for other divalent cations such as Zn^{2+} and Cu^{2+} (Heizmann, 2002). S100A12 has been reported to show a subcellular distribution pattern similar to that of S100A8/A9 (Guignard et al., 1995; Vogl et al., 1999). As has been demonstrated for several other S100 proteins, the essentiality to oligomerize in the presence of Ca^{2+} and Zn^{2+} has been confirmed for the target protein interaction and function of S100A12 (Moroz et al., 2003; Moroz et al., 2009; Xie et al., 2007). A number of different target proteins of S100A12 have been identified and led to the proposal of various regulatory cellular functions for this protein (Hofmann et al., 1999; Pietzsch and Hoppmann, 2008; Hatakeyama et al., 2004). Extracellular S100A12 was shown to exert cytokine-like effects and to function as a phagocyte-specific damage associated molecular pattern (DAMP) molecule, and therefore appears to play a central role in innate and acquired immune responses (Föll et al., 2007). The S100A12 protein has been postulated to play a role in host defense against microorganisms and parasites (Gottsch et al., 1999) and to have chemotactic properties (Hofmann et al., 1999; Miranda et al., 2001; Yang et al., 2001).

Following its release from neutrophils/monocytes due to cell damage, infection, or inflammation, S100A12 was shown to present a ligand for the receptor for advanced glycation end products (RAGE), a pattern recognition receptor (PRR), and to activate the nuclear factor kappa B (NF-kB) signal transduction pathway (Fig. 3) (Hofmann et al., 1999). As a result of the proinflammatory activation of this PRR, expressed by mononuclear phagocytes, endothelial cells, and lymphocytes, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) were shown to be released, which in turn modulate the inflammatory response (Hofmann et al., 1999; Miranda et al., 2001; Yang et al., 2001). The S100A12 protein has been suggested to be involved in the amplification and perpetuation of the traditional inflammatory response by triggering a positive feedback cycle on the expression of RAGE (Fig. 4) (Hofmann et al., 1999; Gebhardt et al., 2008), and thus to be linked to the pathogenesis of inflammatory disorders such as inflammatory bowel disease (IBD) in humans (Föll et al., 2003b). Moreover, soluble RAGE (sRAGE), a truncated variant of RAGE, has been shown to function as a decoy receptor sequestering ligands such as damage-associated pattern molecules (e.g., S100A12), thus preventing ligand interaction with cell surface RAGE and abrogating cellular RAGE signaling (Fig. 5) (Bierhaus et al., 2005). In human medicine, S100A12 has been reported to be a very sensitive and specific marker of localized inflammatory processes, such as gastrointestinal inflammation (Föll et al.,

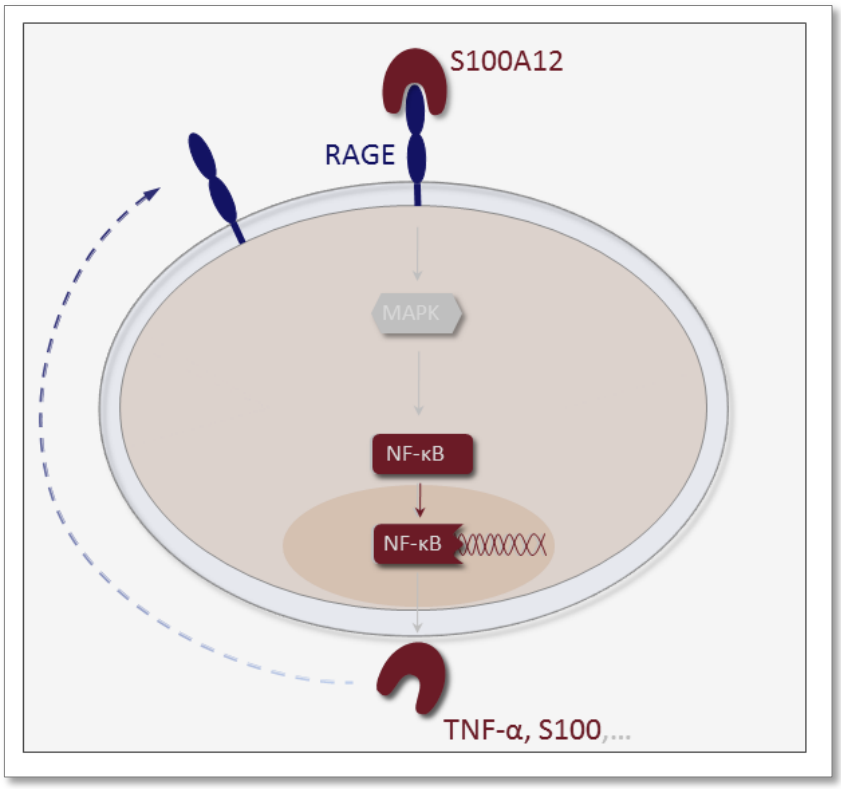

Figure 4 – S100A12-RAGE interaction. In humans, S100A12 has been shown to bind to the receptor of advanced glycation end products (RAGE), a multi-ligand pattern-recognition receptor that has been shown to play a role in chronic inflammation (Hofmann et al., 1999; Bierhaus et al., 2005). The ligand-RAGE interaction has been shown to induce sustained post-receptor signaling, including activation and nuclear translocation of nuclear factor-kappa B (NF-κB), and the up-regulation of RAGE (positive feedback loop) is believed to lead to perpetuation and amplification of the inflammatory response (Schmidt et al., 2000).

MAPK = mitogen-activated protein kinases; NF-κB = nuclear factor-kappa B; RAGE = receptor for advanced glycation end products; TNF-α = tumor-necrosis factor α.

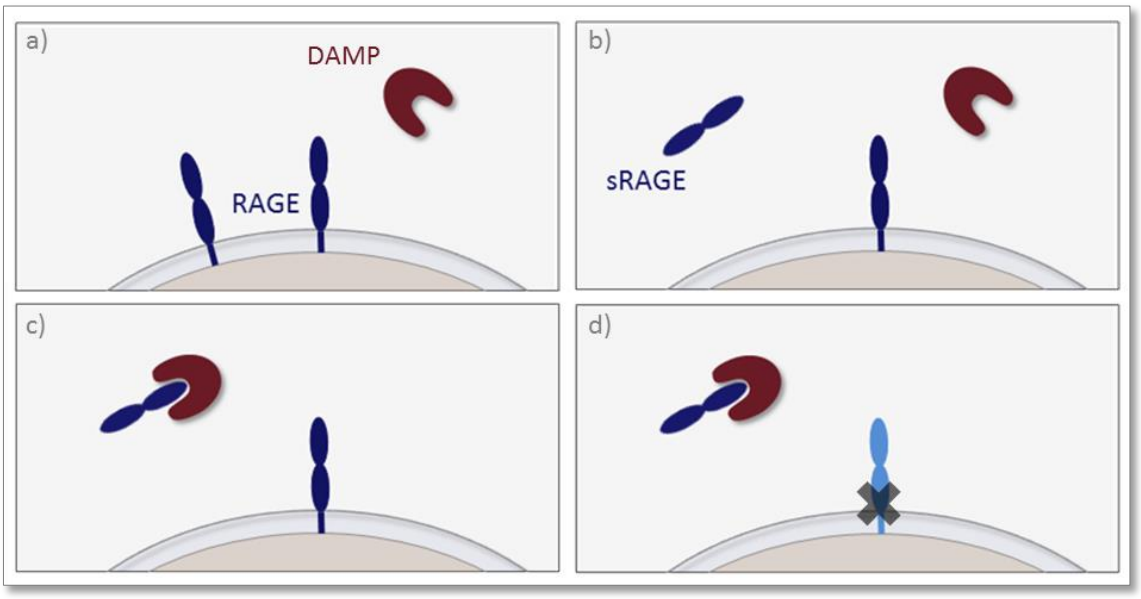

Figure 5 – Blockage of the RAGE pathway by sRAGE. As a truncated variant of RAGE (panel A), soluble RAGE (sRAGE; panel B), has been shown to function as a decoy receptor (panel C) sequestering ligands such as damage-associated pattern molecules (e.g., S100A12), thus preventing ligand interaction with cell surface RAGE (panel D) and abrogating cellular RAGE signaling (Bierhaus et al., 2005). Soluble RAGE has been shown to be decreased in inflammatory diseases in people and has been suggested as a potential therapeutic target in chronic inflammatory conditions (Hudson et al., 2003).
DAMP = damage-associated molecular pattern; RAGE = receptor for advanced glycation end products; sRAGE = soluble receptor for advanced glycation end products.

2009; Kaiser et al., 2007), and to be increased in plasma and serum in patients with various inflammatory disorders (Föll et al., 2003a; Föll et al., 2003c; Basta et al., 2006).

Only human, porcine, bovine, and rabbit S100A12 have been purified to date (Dell'Angelica et al., 1994; Hitomi et al., 1996; Ilg et al., 1996; van den Bos et al., 1998;

Yamashita et al., 1999; Yang et al., 1996), and an immunoassay for the quantification of S100A12 is available only for use in human patients. A high sequence divergence has been reported for S100A12 proteins from different species, thus substantiating the necessity of using species-specific immunologic methods for the detection of S100A12 (Moroz et al., 2003). Availability of an immunological method for the measurement of canine S100A12 would allow to further study the role of canine S100A12 in inflammatory diseases, such as IBD, in canine patients. Moreover, although the function of S100A12 may be species-specific, the fact that a homologue of this calgranulin exists in dogs while rodents lack S100A12 (Fuellen et al., 2004) (and S100A8 appears to functionally resemble S100A12 [Föll et al., 2004; Hsu et al., 2009]) also renders a canine IBD model superior to rodent models for studying S100/calgranulins and the S100A12-RAGE/sRAGE axis in human IBD. Thus, future studies into the function of S100/calgranulins and the S100A12-RAGE/sRAGE axis in chronic inflammatory conditions and their proposed use as novel selective therapeutic targets should benefit from such studies in dogs.

1.3. Pattern Recognition Receptors (PRRs)

Toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE) are pattern-recognition receptors (PRRs) that are expressed by innate immune cells and recognize pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules. Both TLRs and full-length RAGE are transmembrane proteins with a similar but distinct structure (Fig. 6). TLRs consist of (1)

an ectodomain of leucine-rich repeats (LRRs) that mediates PAMP/DAMP recognition and has a horseshoe-like structure, (2) a transmembrane domain functioning as an anchor, and (3) the cytoplasmic domain Toll/IL-1R (TIR), which initiates downstream signaling. Full-length RAGE consists of five domains: (1) a variable (V-type) domain responsible for ligand binding, (2-3) two constant (C-type) domains, (4) a transmembrane domain (anchor), and (5) a cytosolic domain that initiates signal transduction.

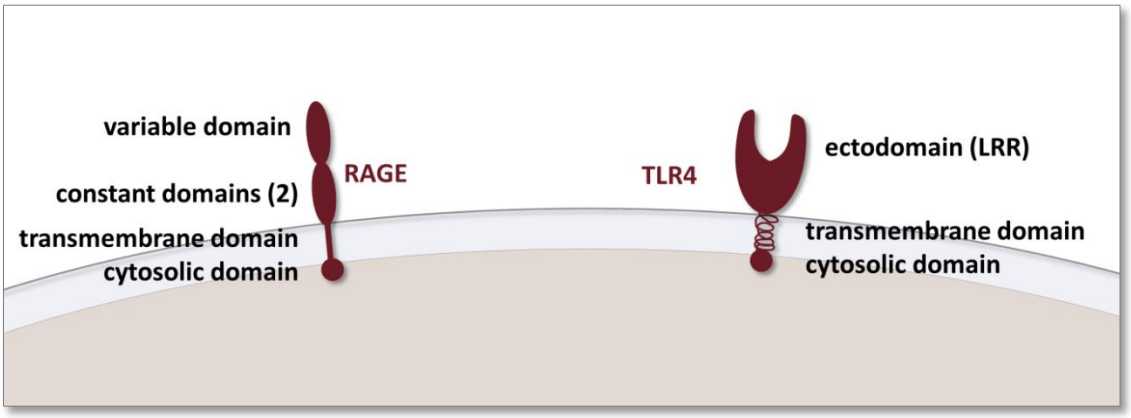

Figure 6 – Structure of the receptor for advanced glycation end products (RAGE) and Toll-like receptors (TLRs).
LPP = leucine-rich repeats; RAGE = receptor for advanced glycation end products; TLR4 = Toll-like receptor 4.

Natural alternative splicing or protease cleavage can produce isoforms that lack the cytosolic domain (e.g., dominant-negative RAGE), isoforms lacking a ligand-binding

domain (e.g., N-truncated RAGE), or decoy receptors (e.g., soluble RAGE [sRAGE]). Their presence can modulate (i.e., abrogate) cell signaling, and thus neutralize the effect of receptor ligands (Fig. 5).

1.3.1. Ligands of TLRs and RAGE

While most TLRs recognize distinct PAMPs (e.g., TLR4 recognizes bacterial lipopolysaccharide [LPS], TLR5 recognizes flagellin) there is some overlap between the different TLRs. Depending on the receptor and its ligand(s), TLRs are localized to either the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) or the intracellular space (TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13).

In contrast to TLRs, RAGE is a multi-ligand receptor that can bind several different ligands, including advanced glycation end products (AGEs, a heterogeneous group of non-enzymatically altered proteins that are enhanced at sites of inflammation), S100/calgranulin-like molecules, high-mobility group box-1 (HMGB-1, amphoterin), amyloid-β-protein (which plays a role in Alzheimer's disease), Mac-1 (CD11b/CD18, also known as integrin or complement receptor-3), and phosphatidylserine. RAGE has also been shown to bind bacterial lipopolysaccharide (LPS), and for some ligands the presence of glycans and/or redox state of the ligand appears to be essential for the mediation of the ligand-RAGE interaction.

Several TLR/RAGE ligands have also been shown to form complexes, such as HMGB-1 and LPS. These complexes are believed to elicit stronger responses than the individual partner molecules alone. Furthermore, the binding affinity and preferential

receptor activation by some ligands of TLR/RAGE (such as S100/calgranulins) appears to also depend on the cell type, concentration and 3D-conformation of the ligand, and the underlying pathophysiological condition of the tissue or the organism.

1.3.2. Signaling pathways of TLRs and RAGE

Both TLRs and RAGE can be activated by microbes (PAMPs) and/or danger signals released from damaged cells (DAMPs or alarmins). Signaling pathways of both TLRs and RAGE involve the activation and nuclear translocation of nuclear factor-κB (NF-κB) leading to the downstream activation of innate immune responses as an immediate host defense response (e.g., against microbes) as well as the modulation of adaptive immune responses (i.e., against specific antigens) and cell growth and proliferation.

1.3.2.1. Toll-like receptors (TLRs)

Depending on type and ligand(s) of TLR, PAMPs/DAMPs are recognized by either a TLR homodimer or a heterodimer with co-receptors or accessory molecules. Ligand recognition by TLRs leads to the recruitment of an adaptor protein, the major two being myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor inducing interferon-β (TRIF):

MyD88-dependent signaling pathway – MyD88 recruits the protein kinases IRAK-4 and IRAK-1 (Myddosome), which associate with the adaptor protein TRAF6.

The IRAK1-TRAF6 complex then dissociates from the receptor and downstream activates I-κB kinase (IKK) and MAP kinases (MAPK), the latter of which causes activation/nuclear translocation of nuclear factor-κB (NF-κB). In addition, MyD88 interacts with the transcription factor IRF-5 and activates it by phosphorylation (Fig. 7).

TRIF-dependent signaling pathway – TRIF associates with TRAF3 and recruits the kinases IKK-ε and TBK-1, both of which activate the transcription factor IRF-3 via phosphorylation. TRIF can also directly associate with and activate TRAF6, resulting in the activation of I-κB kinase (IKK) and MAP kinase (MAPK), but this is to a lesser extent than via the MyD88-dependent signaling pathway.

Activation of TLRs culminates in the activation/nuclear translocation of nuclear factor-κB (NF-κB) and the activation of interferon regulatory factors (IRFs) and mitogen-activated protein kinases (MAPK; such as p38 or JNK). Most TLRs, with the exception of TLR3 (which recognizes dsRNA), signal through MyD88 and induce the expression of proinflammatory cytokines (such as TNF-α, IL-1). TLR3 signals through TRIF and induces interferon-β. TLR4 is a dual receptor and signals through both pathways, but instead of associating directly with MyD88 or TRIF it uses two additional adaptor proteins (TIRAP which binds to MyD88, and TRAM which associates with TRIF).

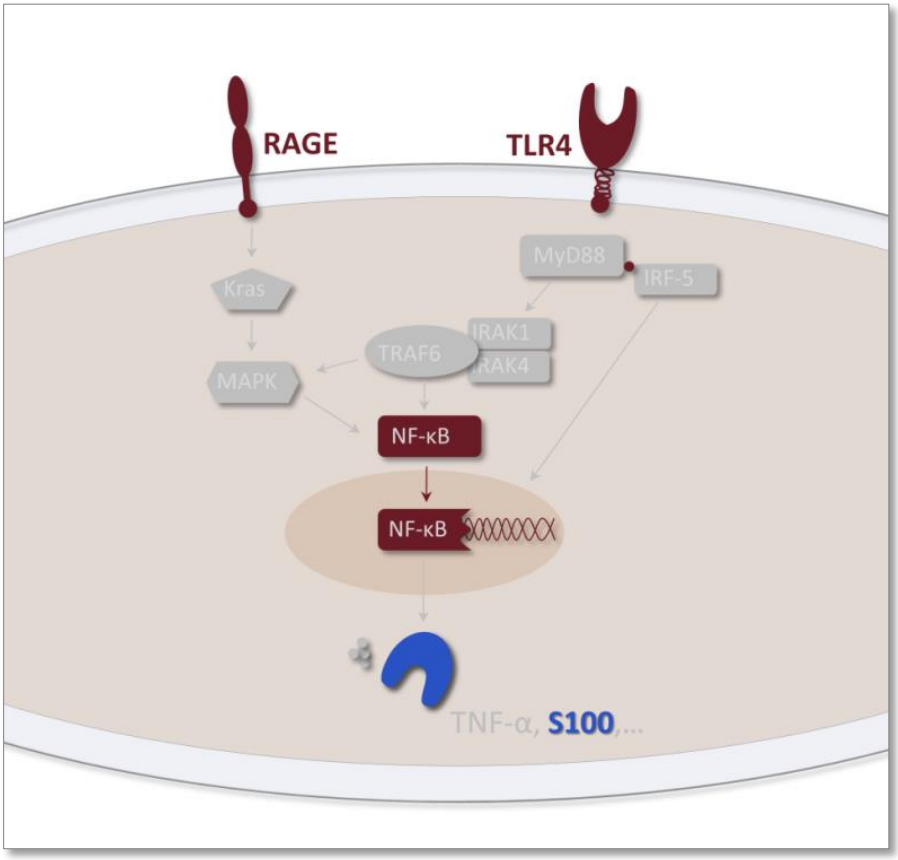

Figure 7 – RAGE and TLR/MyD88 signaling pathways play a role in the immune response. Both RAGE and TLR represent pattern recognition receptors (PRR) that recognize pathogen-associated molecular pattern (PAMP) molecules and endogenous damage-associated molecular pattern (DAMP) molecules. Upon binding of those ligands, these receptors of the innate immune system have been shown to activate NF-κB, leading to the production of cytokines (pro-inflammatory and/or interferon-β) and chemokines. A positive feedback on RAGE expression has also been shown.

IRAK = IL-1 receptor-associated kinase; IRF = interferon regulatory factor; Kras = V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; MAPK = mitogen-activated protein kinases; MyD88 = myeloid differentiation factor 88; NF-κB = nuclear factor-kappa B; RAGE = receptor for advanced glycation end products; TLR4 = Toll-like receptor 4; TNF-α = tumor-necrosis factor α; TRAF = TNF-receptor associated factor.

TLR signaling is controlled by negative regulators that target specific molecules in the TLR signaling pathways, and trafficking of TLRs after synthesis in the endoplasmic reticulum is controlled by several mechanisms. Both prevent or terminate excessive immune responses that could be detrimental to the host and lead to autoinflammation, maintaining a fine balance between positive and negative regulation of TLR signaling.

1.3.2.2. Receptor for advanced glycation end products (RAGE)

Similar to TLRs, signaling pathways of RAGE also lead to the activation of several kinases (MAPK p38/Erk, and NF-κB). Several signaling pathways, including KRAS signaling pathways (Fig. 7), have been identified to require RAGE. However, RAGE can also signal through the MyD88-dependent pathway through interaction with TIRAP and MyD88.

1.3.3. Role of TLRs and RAGE in host defense

Both types of receptors, TLRs and RAGE, can signal individually and independently from each other, but they may also interact functionally. In light of several shared ligands of both innate immune receptors (such as HMGB-1, LPS, or S100/calgranulins), this potential synergism or convergence between the TLR and RAGE signaling pathways may modulate the downstream activation of transcription factors and can potentially amplify associated cellular responses, and thus alter the pathophysiological state of the respective tissue.

TLRs and RAGE signaling pathways orchestrate a complex interplay of cellular mediators and transcription factors, and thus appear to play a central role in homeostasis and host defense. PAMP signaling is crucial for host defense responses to infection, whereas the response to cell components released in response to inflammation and organ injury (DAMPs) is an essential mechanism to stimulate cytokine production and activate cells of the innate immune system (macrophages and dendritic cells) with the goal to alert the immune system to danger. However, aberrant activation of TLR and/or RAGE signaling, mutations of the receptors and/or downstream signaling molecules (including positive and/or negative regulators), and/or DAMP/PAMP-complex-mediated TLR signaling can potentially lead to chronic autoinflammatory diseases as well as cancer. Thus, both TLR and RAGE signaling pathways appear to present an interesting new avenue for the modulation of inflammatory responses and serve as potential novel therapeutic targets.

1.3.4. Role of RAGE in inflammatory disorders

S100/calgranulins, such as S100A12, accumulate at sites of inflammation, and represent ligands for the receptor of advanced glycation end products (RAGE) (Hofmann et al., 1999). Results of studies in humans with IBD suggest a role of RAGE in chronic inflammation (Basta et al., 2002). The ligand–RAGE interaction has been shown to induce sustained post-receptor signaling, including activation and nuclear translocation of nuclear factor (NF)-kappa B (Basta et al., 2002; Bucciarelli et al., 2002), and the upregulation of RAGE itself is believed to lead to perpetuation and amplification of the

inflammatory response (Fig. 4) (Bierhaus et al., 2005; Li and Schmidt, 1997). Human RAGE is only constitutively expressed in the lung and skin, whereas in other cell lines (e.g., monocytes/macrophages, endothelial cells) postembryonic RAGE expression is induced by either accumulation of its ligands and/or activation of transcription factors (e.g., NF-kappa B) that regulate RAGE expression (Bierhaus et al., 2005; Schmidt et al. 2000). Human RAGE is believed to have the potential to convert a transient inflammatory response (e.g., cell stress, NF-kappa B activation) into a sustained cellular inflammatory response (Bierhaus et al., 2005). The expression of RAGE coincides with a proinflammatory microenvironment (e.g., in IBD), and the upregulation of RAGE has been shown to increase the recruitment of inflammatory cells through the upregulation of endothelial adhesion molecules VCAM-1 and ICAM-1 (Bierhaus et al., 2005; Schmidt et al., 2000).

Soluble RAGE (sRAGE), a truncated splice-variant of RAGE, functions as a decoy receptor sequestering ligands such as DAMPs (e.g., S100A12), thus preventing ligand interaction with cell surface RAGE (Fig. 5). Soluble RAGE has been shown to be decreased in humans with chronic inflammatory diseases (Bierhaus et al., 2005) such as inflammatory bowel disease (Meijer et al., 2014), juvenile idiopathic arthritis (Myles et al., 2011), Sjögren's syndrome (Stewart et al., 2008), asthma and chronic obstructive pulmonary disease (Sukkar et al., 2012), and acute Kawasaki disease (Wittkowski et al., 2007). Soluble RAGE has also been suggested to be a potential therapeutic target in patients with chronic inflammatory conditions (Hudson et al., 2003) based on an improved outcome in an experimental model of colitis (Hofmann et al., 1999).

Moreover, experimental studies suggest that sequestration of ligands by sRAGE prevents interaction of these ligands with RAGE, but also with other PRRs (Bierhaus et al., 2005). Canine RAGE has been characterized (Murua Escobar et al., 2006; Sterenczak et al., 2009), and the existence of its naturally occurring splicing variants (total of 24) and the expression of RAGE in different tissues has been described (Sterenczak et al., 2009; Sterenczak et al., 2011). However, the sRAGE/RAGE axis has not yet been studied in canine IBD.

1.4. Diagnostic Tests: Immunoassays

Biomarkers can be quantified by immunoassays that yield a fixed response to a given amount of analyte (Law, 1996; Crowther, 2001). The response unit depends on the detection mechanism and thus the immunoassay system used, and the quantitative nature of an immunoassay is based on the conversion of data into suitable units (e.g., concentration) requiring standardization or calibration of the immunoassay. The calibration function (i.e., standard curve) is derived from a series of standards that are samples of known and increasing concentrations of the analyte, and the standard curve is used as an equation to relate and thus estimate the immunoassay response to the concentration of the analyte in unknown samples. The relationship between immunoassay response and concentration of analyte is usually non-linear and can be affected by a large number of variables. However, for simplicity, convenience, and universal applicability, this relationship can also be standardized in a linear model (linearization).

1.4.1. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays (ELISAs) are diagnostic tests that are based on a specific antigen-antibody interaction and that use an enzyme-labeled reporter antibody to quantify or detect a specific analyte (e.g., peptides, protein, or hormones) following its immobilization to a solid surface. The activity of the enzyme label is used to quantify or detect the analyte in test samples by conversion of a substrate into a measureable product (e.g., color development) (Crowther, 2001).

ELISAs are commonly used to detect or quantify biomarkers in various types of specimens, and they can be performed using one of several different ELISA formats. Depending on the sample type, an extraction or enrichment step may be needed prior to quantification of the substance. Advantages of ELISAs are that they can be more sensitive for the measurement of protein analytes than radioimmunoassays and do not require the use of radioactive tracers (Crowther, 2001). Thus, ELISAs are often used as a first-line diagnostic assay format for the evaluation of new biomarkers.

ELISAs involve several steps (Fig. 8). First, an ELISA plate is coated with the capture antibodies by covalent attachment through the Fc region of the antibodies. Unoccupied sites are then blocked to reduce background interference and improve assay sensitivity, which is followed by incubation with test samples containing the analyte to be measured. The wells of the ELISA plate are then incubated with the detection antibodies, which can be directly labeled (primary conjugate; direct ELISA) or detected by a secondary labeled antibody (secondary conjugate; indirect ELISA). The label can be the stabilized enzyme (e.g., peroxidase) or biotin, which is then complexed with

avidin-conjugated enzyme. As a final step the substrate (chromogenic, chemifluorescent, or chemiluminescent) is added and, by the activity of the bound enzyme, is converted to a detectable product (signal), the intensity of which is proportional to the concentration of the detection antibody and thus to the respective antigen (analyte) (Crowther, 2001).

Other ELISA formats include immobilization of the antigen by direct absorption to the ELISA microplate (instead of a capture antibody) or a competitive ELISA by adding labeled antigen as competitor. An acceptable ELISA should be (1) highly sensitive to detect small amounts of the analyte, (2) robust and reproducible for the sample tested, and (3) of a wide dynamic range for the analyte.

1.4.1.1. Sandwich ELISA

The so-called "sandwich" ELISA is the most sensitive and robust type of a capture ELISA. This assay is called "sandwich" ELISA because the analyte measured or detected is bound between the capture (primary) and the detection (secondary) antibody (Fig. 8). The detection antibodies can be recognized through a secondary labeled antibody or can be directly conjugated with the enzyme or biotinylated (Crowther, 2001).

Sandwich ELISAs can also be performed as competitive assays, which is of advantage if the analyte is small or has only one antibody-binding site (epitope). A standardized amount of the labeled antigen is added to the wells together with the assay

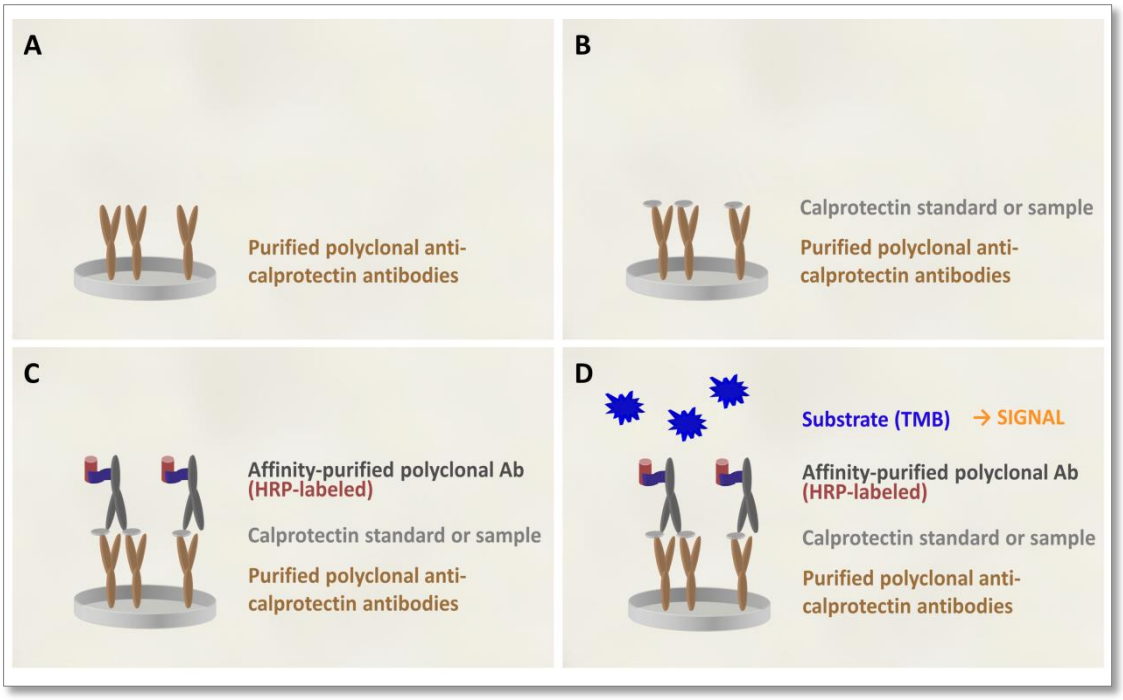

Figure 8 – Steps of an enzyme-linked immunosorbent assay (ELISA). Shown are the different steps involved in an ELISA, exemplified by the direct sandwich ELISA for canine calprotectin. (panel A) The wells of the ELISA plate are coated with the capture antibody, (panel B) test samples or assay standards are added, followed by incubation with (panel C) the peroxidase-labeled detection antibody and, finally, (panel D) the peroxidase substrate (TMB), which is converted to a soluble quantifiable signal (color). HRP = horseradish peroxidase; TMB = tetramethylbenzidine.

standards or test samples. Due to the unlabeled antigen competing for binding to the capture antibody, a decrease in signal indicates the presence of antigen in the sample.

An important consideration for „sandwich“ ELISAs is that the capture antibody and the detection antibody must recognize two non-overlapping antibody-binding sites (epitopes) of the analyte (antigen), so that when the analyte binds to the capture antibody

it does not obscure the epitope(s) recognized by the detection antibody. This concept of a “matched pair“ is particularly important when using a monoclonal antibody due to its inherent monospecificity toward a single epitope. As opposed to monoclonal antibodies, polyclonal antibodies may be used in a "self-sandwich" ELISA (where the same polyclonal antibody is used as capture and detection antibody) but this approach can limit the dynamic range as well as the sensitivity of the assay (Crowther, 2001). The most sensitive and specific design of a “sandwich” ELISA is by using a polyclonal antibody as capture antibody to allow for binding of as much analyte as possible, and to use a monoclonal antibody to allow specific detection.

1.4.2. Radioimmunoassay (RIA)

Radioimmunoassays are diagnostic tests that are also based on a specific antigen-antibody interaction but that use a radioactively labeled antigen (called tracer) as a competitor for antibody binding to quantify the amount of bound and/or free antigen. RIAs are usually liquid-phase assays (Fig. 9); the most commonly used radioactive isotope is ^{125}I, which is a gamma emitter. The performance and stability of an RIA is mainly affected by the quality of the antibody and the tracer, but also depends on the type of buffer and its molarity, protein additives, incubation volume and temperature, and the incubation time, separation system, and sample matrix.

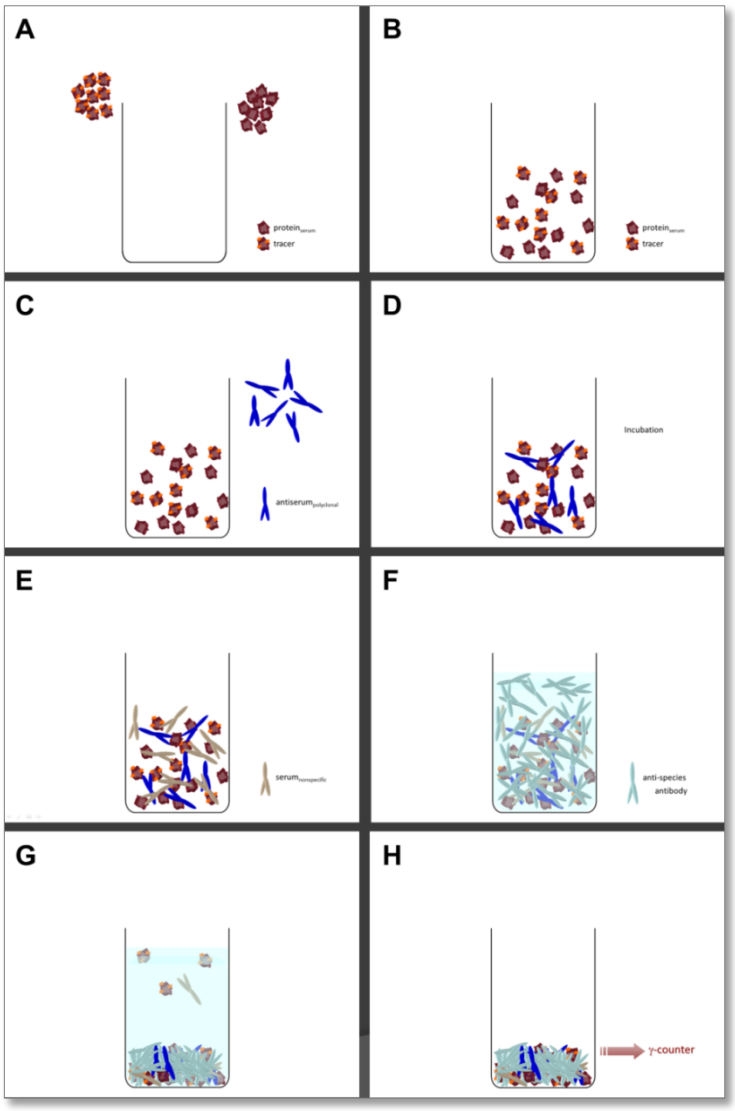

Figure 9 – Steps in a radioimmunoassay (RIA). Shown are the different steps involved in an RIA, exemplified by a liquid phase, double antibody RIA for canine calprotectin. (A) Test samples or assay standards and ^{125}I-tracer are added to the test tubes followed by (B) vortexing the tubes, (C) adding anti-calprotectin rabbit antiserum, and (D) incubation of the reaction mixture. (E) Rabbit serum is added. (F) Larger complexes are formed using anti-rabbit antibody and these larger complexes (G) are precipitated with polyethylene glycol. (H) After the supernatant has been decanted, the tubes are placed in a γ-counter to the measure the radioactivity (in counts per minute) in the test tubes.

There are three categories of separating systems that can be used for RIAs. Their selection is largely based on the expected efficiency (i.e., accuracy with which bound and free analyte are separated) and practicality (i.e., speed, simplicity, applicability, reproducibility, and cost). Adsorption methods use dextran-coated charcoal to which the tracer, but not antibodies or antibody-bound tracer, is adsorbed. This method requires that the incubation time and temperature, amount of charcoal used, and the sample type and buffer are consistent. Fractional precipitation methods use a separation agent (e.g., 18% polyethylene glycol 6000 or half-saturated $[NH_4]_2SO_4$) to "salt out" and precipitate the antibody fraction regardless if it has bound antigen or not, and the radioactivity is measured in the compacted pellet (bound fraction) after separation of the supernatant (free fraction). Second antibody methods use anti-species antibodies to precipitate the antibody fraction, either in a liquid-phase (i.e., secondary antibodies and primary antibody-bound antigen produce an insoluble macromolecular complex) or solid-phase assay (i.e., antibodies attached to the surface of the assay tubes or to small cellulose beads) format. Other RIA formats include a second antibody–fractional precipitation combination and scintillation proximity assay (SPAs), which uses second antibody-coupled scintillate microspheres.

Most RIAs are highly sensitive, robust, and reproducible, and have a large capacity with the possibility of analyzing thousands of samples per day. Disadvantages are the need for expensive equipment (i.e., a gamma counter and other equipment, such as centrifuges and vacuum sources/aspirators, designated for the use with radioactive materials) and the use of a radioactive tracer (i.e., a labeled antigen with a relatively

short shelf-life and the limitation to be used at institutions with the license and oversight required to handle radioactive materials).

1.4.3. Immunoassay development and analytical validation

Limited validation work is necessary to demonstrate the validity of the method prior to establishing it for routine use (Law, 1996; Crowther, 2001; Valentin et al., 2011). Establishment of an immunoassay requires that accuracy, precision, sensitivity, specificity, suitability of the calibration model, robustness of the immunoassay, and stability of the analyte be documented (Midgley et al., 1969; Law, 1996; Crowther, 2001; Valentin et al., 2011) to demonstrate the performance and reliability of the immunoassay and to determine the confidence that can be placed in the results generated using the immunoassay.

1.4.3.1. Immunoassay development

Stages in assay development and optimization are similar for ELISAs and RIAs, and include the selection of basic operating conditions (e.g., buffer, incubation temperature), the selection of a separating system, the assessment (i.e., specificity and sensitivity), and selection of antisera, the introduction of a matrix, and the optimization of the assay conditions to give the desired characteristics. Potential difficulties or problems that require step-wise optimization strategies are also similar for ELISAs and RIAs (Law, 2005; Crowther, 2001). These include the measurement of lipophilic analytes and the encountering of bridge-recognition or matrix effects requiring

sensitization/desensitization strategies, the use of non-equilibrium assays, and/or sample pre-treatment by extraction and/or enrichment. Thus, the final selection of a specific assay format (i.e., RIA vs. ELISA) is dependent on the required sensitivity, precision, and expected sample throughput, and available equipment, and financial constraints.

1.4.3.2. Immunoassay analytical validation

For a diagnostic assay, a complete analytical validation is essential to show that all validation parameters meet the acceptance criteria outlined by the Clinical and Laboratory Standards Institute (CLSI). Assay analytical validation also includes the establishment of a reference interval from a minimum of 120 reference individuals. Good quality control (QC) practices as well as Good Laboratory Practice (GLP) are essential to maintain an established and validated diagnostic test and guarantees that it continues to meet the CLSI criteria for a diagnostic test. Method re-evaluation is usually also included into routine laboratory operations as a continual QC measure.

Validation parameters include (i) the working range of the assay, determined as the range between the minimum and maximum detection limit of the assay, (ii) dilutional parallelism to test assay linearity (Fig. 10), (iii) spiking recovery to determine assay accuracy (Fig. 11), (iv) intra-assay variability to assess assay precision (Fig. 12), (v) inter-assay variability to determine reproducibility of the assay (Fig. 13), (vi) assay analytical specificity, (vii) analyte stability, (viii) interference testing, and (ix) biological

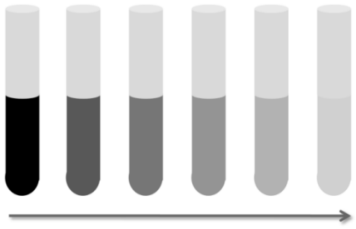

Figure 10 – Assay linearity (dilutional parallelism). Linearity of an assay is evaluated by dilutional parallelism (e.g., at serial twofold dilutions) and calculation of the observed-to-expected ratio as a percentage ([observed value/expected value]×100). Example shown: RIA.

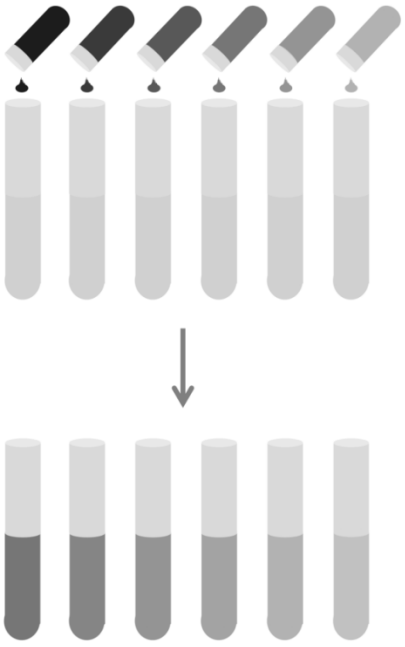

Figure 11 – Assay accuracy (spiking recovery). Accuracy of an assay is tested by spiking samples with known concentrations of the protein analyte (e.g., standard protein) followed by calculation of the percentage of standard antigen recovery ([observed value/expected value]×100). Example shown: RIA.

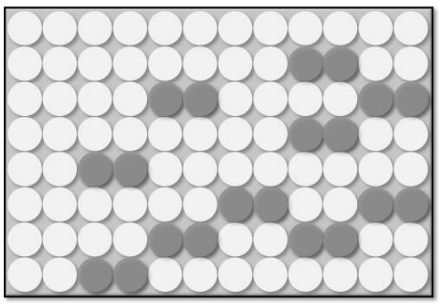

Figure 12 – Assay precision (intra-assay variability). Precision of the assay is evaluated by assaying the same sample ten times within the same assay followed by calculating the intra-assay coefficient of variation (%CV = [standard deviation/mean]×100). Example shown: ELISA.

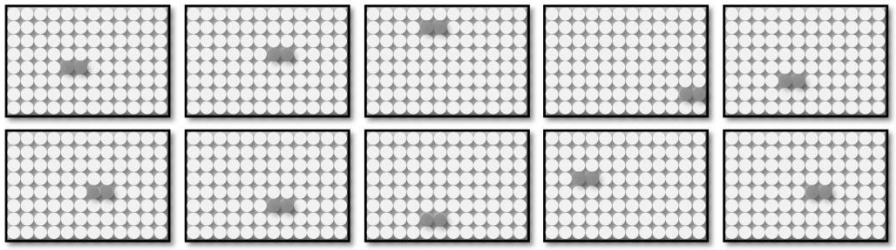

Figure 13 – Assay reproducibility (inter-assay variability). Reproducibility of the assay is determined by analyzing the same sample in ten consecutive assay runs and calculating the inter-assay coefficient of variation (%CV = [standard deviation/mean]×100). Example shown: ELISA.

variability of the analyte (Midgley et al., 1969; Law, 1996; Crowther, 2001; Valentin et al., 2011).

1.4.4. Immunoassay clinical validation

Understanding the diagnostic performance properties of diagnostic tests is an important prerequisite for its correct interpretation, and the integration of the test results with the patient's clinical signs, physical examination findings, and potentially the results of other diagnostic tests performed are important to arrive at a definitive diagnosis. While this is often not within the area of the clinician's responsibility, it is important to realize that determining the diagnostic performance of an assay assumes that its analytical performance has been established. This aspect is equally important for diagnostic tests performed patient-side (i.e., in-house).

Descriptive statistics for the performance of diagnostic tests (diagnostic accuracy) are expressed as estimates of the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). It is important to understand that sensitivity and specificity are inherent characteristics of a diagnostic test, whereas the predictive values are dependent on the prevalence of the disease and the diagnostic accuracy of the test in question. Further, when evaluating diagnostic accuracy of a test it is crucial to understand the difference between qualitative and quantitative assays. Qualitative assays produce a dichotomized result (i.e., a positive or negative result) whereas quantitative assays yield a numerical result (i.e., continuous data for which a 'cut-off' concentration will be or have to be established and that allows for results to fall within a 'gray range').

1.4.4.1. Sensitivity

Sensitivity of a test, in essence, is the chance of testing positive for a patient that truly has the disease (i.e., the true positive rate), which has been diagnosed based on an independent gold-standard diagnostic method or technique. Sensitivity is calculated as the quotient of the number of all individuals with the disease that test positive and the number of all individuals with the disease. The higher the sensitivity, the lower the false negative rate (i.e., greater confidence can be placed on negative test results being true). Thus, a test with a high diagnostic sensitivity would be useful for ruling out a disease (i.e., as a screening test). The sensitivity of a diagnostic test can be overestimated when selecting patients that are obviously diseased.

1.4.4.2. Specificity

Specificity of a test is the rate of testing negative if the patient truly does not have the disease (i.e., the true negative rate) as has been determined by an independent gold-standard diagnostic method. Specificity is calculated as the quotient of the number of all individuals without the disease that test negative and the number of all individuals without the disease. The higher the specificity, the lower the false positive rate (i.e., greater confidence can be placed on positive test results being true). Thus, a test with a high diagnostic specificity is useful to rule-in a disease (i.e., as a confirmatory diagnostic test). Specificity of a diagnostic test can be overestimated when selecting healthy individuals as a control group instead of patients with a similar signalment and/or clinical signs that overlap with the disease in question.

1.4.4.3. Positive and negative predictive values

While sensitivity and specificity are important to assess the performance of a test in a well-defined population of diseased patients, the predictive values are far more important in clinical practice when the diagnostic test is used to determine the patient's disease status. Predictive values allow inferences regarding the probability of a test result (positive vs. negative) to indicate the correct diagnosis. They are a function of the sensitivity, specificity, and the prevalence of the disease in the population, and therefore reflect the behavior of a diagnostic test given the prevalence of the disease in the population tested. Predictive values can help the clinician understand and correctly interpret a test result obtained given the presentation of the patient in whom the test was performed and/or to decide in which patient population a diagnostic test is likely going to yield a meaningful result (also called pre-test probability). Thus, evidently performing the test in a patient population with a high likelihood of having the disease (e.g., based on signalment, clinical signs, results of other diagnostics, etc.) will improve the pre-test probability. This is particularly important for diagnostic tests with a relatively low sensitivity or specificity.

Positive predictive value (PPV) is the chance for a patient to have the disease if the test result is positive. The PPV is calculated as the quotient of the number of all individuals with the disease that test positive and the number of all individuals with a positive test result. Tests with a low diagnostic specificity (i.e., a high false positive rate) have a low PPV if the disease prevalence is low (e.g., when the test is used as a screening test for an uncommon disease). Thus, such a test performs better as a

confirmatory test in a patient with a strong suspicion for the disease and in the absence of factors (e.g., medication, concurrent conditions) that can produce a false positive result.

Negative predictive value (NPV) is the chance that the patient does not have the disease if the test result is negative. The NPV is calculated as the quotient of the number of all individuals without the disease that test negative and the number of all individuals with a negative test result. Tests with a low diagnostic sensitivity (i.e., a high false negative rate) have a low NPV if the disease prevalence is high (e.g., when the test is used as a confirmatory test in a patient with a strong suspicion of the disease being present). Thus, such a test performs better when screening large populations of patients.

Because predictive values are dependent on the disease prevalence (pre-test probability), it is important for the clinician to critically evaluate studies that report PPV and NPV as these values are most likely derived using the study prevalence. However, chances are high that the pre-test probability is going to be different when using the test in clinical practice and, unfortunately, for most diseases in veterinary medicine the true prevalence is unknown. Thus, any reported sensitivity, specificity, PPV, and NPV for a given diagnostic test should be interpreted with caution and in light of the respective study design.

1.5. Acute Hemorrhagic Diarrhea Syndrome (AHDS)

The syndrome of acute hemorrhagic diarrhea (AHDS), formerly known as idiopathic hemorrhagic gastroenteritis (HGE), is characterized by peracute hemorrhagic

diarrhea, vomiting, severe hemoconcentration, depression, and anorexia, and can be associated with a high mortality if untreated (Burrows, 1977; Post and Feldman, 1978; Spielman and Garvey, 1993). The etiology of AHDS is unknown, but it is speculated that an abnormal response to bacterial endotoxins, bacteria (such as enterotoxin-producing *C. perfringens*), or dietary components may play a role (Cave et al., 2002; Schlegel et al., 2012). AHDS is characterized by an increased vascular and mucosal permeability, thought to represent a type I-hypersensitivity reaction, whereas gastrointestinal inflammation and mucosal necrosis appear to be rarely seen in canine patients with AHDS (Hall and German, 2010; Unterer et al., 2014). Markers of gastrointestinal inflammation and changes in the intestinal microbiota have not been studied extensively in dogs with AHDS (Suchodolski et al., 2012).

1.6. Hypotheses and Research Objectives

1.6.1. Hypotheses

The hypotheses of this study are that: (i) S100A12 concentrations in canine serum and fecal samples and sRAGE reflect the degree of intestinal inflammation; (ii) serum and/or fecal S100A12 and/or serum sRAGE concentrations in dogs with inflammatory bowel disease (IBD) can serve as a potential minimally- or non-invasive marker of the degree of inflammation in tissues; and (iii) dogs with acute hemorrhagic diarrhea syndrome (AHDS) have an altered fecal microbiota and show increases in fecal S100A12 concentrations.

1.6.2. Research objectives

The objectives to prove or disprove the aforementioned hypotheses are: (i) to purify canine S100A12 from canine whole blood and to partially characterize canine S100A12; (ii) to generate and purify antibodies against canine S100A12, and to develop and analytically validate an immunoassay for the measurement of canine S100A12 concentrations in canine serum and fecal samples; (iii) to evaluate canine S100A12 concentrations and determine its biological variation in serum and fecal samples from healthy dogs; (iv) to evaluate canine S100A12 concentrations in serum and fecal samples from dogs with idiopathic inflammatory bowel disease (IBD), and to correlate fecal canine S100A12 concentrations with the histologic, endoscopic, and clinical disease severity to determine the clinical usefulness of fecal S100A12 concentrations as a marker of intestinal inflammation in dogs with IBD; (v) to develop and analytically validate an immunoassay for the measurement of sRAGE in serum samples from dogs; (vi) to evaluate serum sRAGE concentrations in dogs with IBD and to correlate serum sRAGE concentrations with the severity of clinical disease, histologic lesions, outcome, and the concentration of S100A12 in serum and fecal specimens from dogs; and (vii) to evaluate fecal canine S100A12 and bacterial groups that have previously been shown to be decreased (i.e., *Faecalibacterium* spp., Ruminococcaceae, *Bifidobacterium* spp.) or increased (i.e., Proteobacteria) in fecal samples from dogs with AHDS.

2. PURIFICATION AND PARTIAL CHARACTERIZATION OF CANINE S100A12*

2.1 Introduction

S100A12, also referred to as calgranulin C, migration inhibitory factor-related protein (MRP)-6, p6, or extracellular newly identified RAGE-binding protein (EN-RAGE) (Ilg et al., 1996; Hofmann et al., 1999; van den Bos et al., 1998), was first identified in the cytosol of human neutrophils and monocytes (Guignard et al., 1995). It belongs to the calgranulin subfamily of the highly conserved S100 proteins, a superfamily of EF-hand proteins with a high Ca^{2+}-binding capacity and binding sites for other divalent cations such as Zn^{2+} and Cu^{2+} (Heizmann, 2002). S100A12 has been reported to show a subcellular distribution pattern similar to that of S100A8/A9 (Guignard et al., 1995; Vogl et al., 1999). As has been demonstrated for several other S100 proteins, the essentiality to oligomerize in the presence of Ca^{2+} and Zn^{2+} has been confirmed for the target protein interaction and function of S100A12 (Moroz et al., 2003; Moroz et al., 2009; Xie et al., 2007).

A number of different target proteins of S100A12 have been identified and led to the proposal of various regulatory cellular functions for this protein (Hofmann et al., 1999; Pietzsch et al., 2008; Hatakeyama et al., 2004). Extracellular S100A12 was shown to exert cytokine-like effects and to function as a phagocyte-specific damage associated molecular pattern (DAMP) molecule, and therefore appears to play a central role in

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innate and acquired immune response (Föll et al., 2007). The S100A12 protein has been postulated to play a role in host defense against microorganisms and parasites (Gottsch et al., 1999) and to have chemotactic properties (Hofmann et al., 1999; Yang et al., 2001; Miranda et al., 2001).

Following its release from neutrophils/monocytes due to cell damage, infection, or inflammation, S100A12 was shown to present a ligand for the receptor for advanced glycation end products (RAGE), a pattern recognition receptor (PRR), and to activate the nuclear factor kappa B (NF-kB) signal transduction pathway. As a result of the proinflammatory activation of this PRR, expressed by mononuclear phagocytes, endothelial cells, and lymphocytes, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) were shown to be released that modulate the inflammatory response (Hofmann et al., 1999; Yang et al., 2001; Roth et al., 2003). Moreover, the S100A12 protein has been suggested to be involved in the amplification and perpetuation of the traditional inflammatory response by triggering a positive feedback cycle on the expression of RAGE (Hofmann et al., 1999; Gebhardt et al., 2008), and thus to be linked to the pathogenesis of inflammatory disorders such as inflammatory bowel disease (IBD) in humans (Föll et al., 2003a).

In human medicine, S100A12 has been reported to be a very sensitive and specific marker of localized inflammatory processes, such as gastrointestinal inflammation (Kaiser et al., 2007; Föll et al., 2009), and to be increased in plasma/serum in patients with various inflammatory disorders (Föll et al., 2003a; Föll et al., 2003b; Basta et al., 2006).

To the authors' knowledge, only human, porcine, bovine, and rabbit S100A12 have been purified to date (Ilg et al., 1996; van den Bos et al., 1998; Dell'Angelica et al., 1994; Yamashita et al., 1999; Yang et al., 1996; Hitomi et al., 1996), and an immunoassay for the quantification of S100A12 is available only for use in human patients. A high sequence divergence has been reported for S100A12 proteins from different species, thus further substantiating the necessity of using species-specific immunologic methods for the detection of S100A12 (Moroz et al., 2003). In view of the current lack of both sensitive and specific inflammatory markers in veterinary medicine, the aims of this study were to establish a rapid and reproducible protocol for the purification of canine S100A12 (cS100A12) from canine whole blood, and to partially characterize this protein as a prelude to the development of an immunological method for its detection and quantification in serum and fecal specimens from canine patients. The availability of such an immunological method would allow to further study the role of cS100A12 in inflammatory diseases, such as IBD, in canine patients.

2.2. Materials and methods

2.2.1. Materials

Unless otherwise indicated, all research materials were purchased from VWR, West Chester, PA, USA. All chemical reagents were obtained from Sigma-Aldrich, St. Louis, MO, USA, and were at least of analytical grade. All solutions were prepared in aqua bidestillata (Milli-Q), filtered (0.2 μm), and degassed. Whole blood from healthy

dogs was collected in accordance with a protocol approved by the University Laboratory Animal Care Committee at Texas A&M University (AUP# 2006-145).

2.2.2. Preparation of leukocytes and extraction of the cytosol fraction

Leukocytes were isolated as previously reported (Heilmann et al., 2008). Briefly, after mixing two volumes of EDTA blood with one volume of a 6% (w/v) dextran (T-500) solution containing 0.9% (w/v) NaCl and sedimentation at room temperature (approximately 23 °C) for 180 min, the supernatant layer was collected and leukocytes were pelleted by centrifugation at 2000×g for 10 min at 4 °C. Remaining erythrocytes were lysed by incubating the sediment with RBC lysis solution (Qiagen, Valencia, CA, USA), followed by centrifugation as described above and storage of the pellets at -80 °C until further use.

Leukocyte pellets were resuspended in 20 mM Tris/HCl, 50 mM NaCl, pH 7.6 containing 15 mM diisopropylfluorophosphate (DFP) and the cytosol fraction was extracted as has been described previously (Heilmann et al., 2008). Briefly, after homogenizing the solution for 5 min at room temperature (approximately 23 °C), the cell suspension was sonicated for 12 min at 50% intensity (150 W) on ice and subjected to two successive cycles of freezing (-20 °C), thawing and sonication. The soluble cytosolic fraction was then separated from cell debris by centrifugation at 16,000×g for 12 min at 4 °C.

2.2.3. Ammonium sulfate precipitation and hydrophobic interaction chromatography (HIC)

The soluble cytosol fraction of leukocytes was precipitated by adding ammonium sulfate to a final concentration of 30% (w/v). After centrifugation at 12,000×g for 25 min at 1 °C, the supernatant was collected and adjusted to a final concentration of 2 mM $CaCl_2$. Afterwards, the solution was filtered through a 0.1 μm-pore size filter and applied to a phenylsepharose HIC column (HiTrap™ Phenyl FF, GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 column volumes of 20 mM Tris/HCl, 2 mM $CaCl_2$, 1.55 M $(NH_4)_2SO_4$, pH 7.6 at a flow rate of 0.5 ml/min at room temperature on a fast protein liquid chromatography (FPLC) system (ÄKTA basic, GE Healthcare). Proteins bound to the column were eluted in a linear gradient of 0-100% 20 mM Tris/HCl, 5 mM EGTA, pH 7.6 over 200 min, and were analyzed by automated fluorescence-based reducing gel electrophoresis in a chip format (Protein 80 assay) using a Bioanalyzer 2100 system according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, USA).

2.2.4. Ion-exchange chromatography

Canine S100A12 containing fractions eluted from the HIC column were pooled and dialyzed against 50 mM CH_3CO_2Na, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaCl, pH 5.0 (buffer A) using dialysis bags (2000 M_W cutoff; Thermo Scientific, Waltham, MA, USA), clarified by 0.1 μm-filtration and subjected to strong cation-exchange chromatography. The column (Econo-Pac® High S, Bio-Rad Laboratories,

Hercules, CA, USA) was equilibrated with 25 column volumes buffer A at a flow rate of 1 ml/min at approximately 23 °C, and fractions were eluted with a linear gradient of 0.05-0.5 M NaCl in buffer A over 120 min and were again analyzed by the Protein 80 assay.

Fractions containing cS100A12 were then pooled and dialyzed against 50 mM Tris/HCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 25 mM NaCl, pH 8.5 (buffer B) using dialysis cassettes (2000 M_W cutoff; Thermo Scientific), filtered (0.1 μm-pore size) and applied to a strong anion-exchange chromatography column (HiPrep™ Q, GE Healthcare) equilibrated with 12 column volumes buffer B at a flow rate of 1 ml/min at room temperature. Elution of proteins bound to the column was achieved by application of a linear gradient of 0.025-0.25 M NaCl in buffer B over 180 min, and the fractions eluted were again analyzed by the Protein 80 assay. Fractions containing cS100A12 were pooled, dialyzed against 50 mM NH_4HCO_3, pH 7.64 (buffer C) and lyophilized (Freeze Dry System, Labconco, Kansas City, MO, USA), followed by re-dissolution in 20 mM CH_3CO_2Na, 3 mM $CaCl_2$, pH 7.6 (buffer D) and storage at -80 °C.

2.2.5. Gel electrophoresis

Protein purity was assessed by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% Bis/Tris) in a vertical mini-gel format (Invitrogen, Carlsbad, CA, USA), and protein bands were visualized by staining with Coomassie brilliant blue R-250 (Thermo Scientific) according to the manufacturers' instructions.

Native polyacrylamide gel electrophoresis (PAGE) was performed by use of the Mini-Protean® 3 electrophoresis cell and a discontinuous buffer system (Ornstein-Davis) according to the standard procedures given by the manufacturer (Bio-Rad Laboratories). The acrylamide concentration of the separating gel was optimized at 8, 10, 12 and 15%, and electrophoresis was performed at a constant voltage of 125 V for 70 min followed by Coomassie brilliant blue staining of the gels. Using native PAGE, pure cS100A12 was evaluated in the presence of 0.3 mM Ca^{2+}, 3 mM Ca^{2+}, 10 mM Ca^{2+}, 3 mM Ca^{2+} and 5 mM or 10 mM Zn^{2+}, 10 mM Ca^{2+} and 5 mM or 10 mM Zn^{2+} and 10 mM EGTA.

Scanned images of the respective gels were analyzed using the Quantity One® v4.6.5 software (Bio-Rad Laboratories).

2.2.6. Protein concentration assay

Protein concentration was measured using the Bradford dye-binding method (Bradford et al., 1976) with bovine serum albumin (BSA) as a reference protein in accordance with the manufacturer's instruction (Bradford protein assay, Thermo Scientific).

2.2.7. Western blotting

Following reducing SDS-PAGE, electroblotting of proteins onto 0.2 μm-pore size polyvinylidene fluoride (PVDF) membranes was performed as described elsewhere (Heilmann et al., 2008a). Membranes were then blocked overnight at 4 °C by immersion in 10 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) polyoxyethylene sorbitan monolaurate,

pH 7.6 (blot buffer) supplemented with 5% (w/v) BSA (blocking buffer). All subsequent steps were carried out at approximately 23 °C on an orbital shaker at 80 rpm, unless otherwise stated. Blots were incubated for 3 h with the rabbit polyclonal anti-recombinant human (rh) S100A12 antiserum (Novus Biologicals, Littleton, CO, USA) at a dilution of 1/300 or with the rabbit polyclonal anti-rhS100A12 ab37657 (Abcam, Cambridge, MA, USA) at a dilution of 1/250 in blocking buffer, then washed 4×5 min in blot buffer, and incubated with biotin-conjugated goat anti-rabbit IgG (Thermo Scientific) as secondary antibody for 2.5 h (dilution of 1/10,000 in blocking buffer). After 4×5 min washes with blot buffer, the membranes were incubated for 2 h with horseradish peroxidase-conjugated NeutrAvidin (Thermo Scientific) at a concentration of 0.3 mg/l in blot buffer supplemented with 5% (w/v) BSA. Again, the membranes were washed in blot buffer, and the antigen-antibody complexes were detected by incubation for 20 min at 37 °C with a stabilized 3,3',5,5'-tetramethylbenzidine blotting substrate (Thermo Scientific).

2.2.8. Determination of molecular weight and relative molecular mass

Automated fluorescence-based reducing gel electrophoresis (Protein 80 assay; Agilent Bioanalyzer 2100) was used to estimate the molecular weight (M_W) of cS100A12.

The relative molecular mass (M_r) of cS100A12 was estimated by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS; ProteinChip® SELDI System, Bio-Rad Laboratories). Briefly, 220 ng of purified

cS100A12 were immobilized onto a Normal Phase (NP) 20 ProteinChip® array using 12.5 mg/ml 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SPA) in ACN:H_2O:TFA (50:49.5:0.5) to allow desorption/ionization of the protein. Ten different TOF spectra were generated by 210 laser shots with a laser intensity of 1400 nJ each (partition 9.8%), and M_r accuracy was calibrated externally by use of the All-in-One protein standard solution covering a mass-to-charge ratio (m/z) range between 6,964 and 147,300.

2.2.9. Isoelectric point

The isoelectric point (p*I*) was estimated by native isoelectric focusing (IEF) on a vertical format pre-cast polyacrylamide IEF gel covering a linear pH gradient from 3 to 10 (Invitrogen). Hereafter, proteins were applied to the second dimension (2D-PAGE) consisting of native and non-reducing SDS-PAGE (12% Tris-glycine, Invitrogen), respectively. Protein bands were visualized by Coomassie blue stain.

2.2.10. Specific absorbance

The specific absorbance of cS100A12 was determined by the quotient of the spectrophotometric absorbance of serially diluted samples containing pure cS100A12 measured at a wavelength of 280 nm (NanoDrop™ 1000, Thermo Scientific) and the corresponding protein concentrations determined by means of a Bradford assay.

2.2.11. Tryptic peptide mass fingerprint (PMF)

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) was used for the fingerprinting of peptides generated by tryptic digestion (PMF). Following SDS-PAGE, Coomassie blue staining and SDS removal, an in-gel proteolytic digestion was performed by incubation of the excised and destained protein band in 20 mg/ml bovine pancreatic trypsin in 22.5 mM ammonium bicarbonate, 1 mM HCl, pH 8.0 for 16 h at 37 °C. Then, 2 ml of the extraction mixture were applied to a ProteinChip® NP20 array using 200 mg/ml alpha-cyano-4-hydroxy cinnamic acid (CHCA) in ACN:H_2O:TFA (50:49.5:0.5) as a matrix to facilitate desorption and ionization of peptides (Devarajan and Ross, 2008). Ten different TOF spectra were acquired at 210 laser shots with a laser intensity of 900 nJ each (partition 9.8%), and M_r accuracy was calibrated externally by use of the ProteinChip® Peptide mass standard array covering a mass range between 1,084 and 5,964 (Bio-Rad Laboratories). After peptide masses were extracted from the mass spectra by use of the Peak-Erazor software (GPMAW. PeakErazor V.2.01; available at: http://www.gpmaw.com; accessed 08-12-2009), *in silico* analysis was performed by searching the data obtained against a comprehensive database (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009) using the ProFound search site (PROWL. ProFound; available at: http://prowl.rockefeller.edu; accessed 08-12-2009). The mass tolerance for matching peptide average masses was set at 100 ppm, and a maximum of 1 missed cleavage of trypsin was allowed in the search

that was restricted to the species (*Canis familiaris*) as well as the biochemical properties (p*I* and mass range) of the protein.

2.2.12. N-terminal amino acid sequence analysis

To ascertain protein identity, the N-terminal amino acid (AA) sequence of cS100A12 was analyzed by the Edman degradation method on a Model 492 automated protein sequencing system (Applied Biosystems, Foster City, CA, USA) at the Protein Chemistry Laboratory (Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA), followed by sequence comparison against a comprehensive database (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009).

2.2.13. Purification of canine S100A8

The complex of the proteins cS100A8 and cS100A9 (canine calprotectin) was isolated from canine whole blood as described previously (Heilmann et al., 2008). During strong cation-exchange chromatography a minor fraction of cS100A8 was separated from the complex protein, and the identity of cS100A8 was ascertained by N-terminal AA sequencing using the Edman degradation method.

2.2.14. Immunologic cross-reactivity

Polyclonal antibodies against cS100A12 were raised in New Zealand White rabbits by repeated inoculation with pure cS100A12 emulsified in complete and

incomplete Freund's adjuvant as described previously (Vaitukaitis et al., 1971). Immunologic cross-reactivity between canine, feline, bovine, porcine, ovine, caprine, equine, marmoset, meerkat, sea lion and penguin S100A12 was evaluated by precipitation of the respective serum (all surplus samples from other research studies) against rabbit anti-canine S100A12 antiserum using radial double immunodiffusion (Ouchterlony test). An Ouchterlony test was also performed for extracts from fecal samples collected from dogs with hemorrhagic gastroenteritis as well as from healthy dogs, the purified cS100A8 monomer and the cS100A8/A9 complex (canine calprotectin). To demonstrate specificity of the rabbit anti-cS100A12 antiserum, a modified Western blot was used to analyze the affinity of the antibody against canine serum samples (at a dilution of 1:20), canine fecal extracts (at a dilution of 1:500), the soluble cytosol fraction of leukocytes as well as a sample from each of the following purification stages, pure cS100A12 in buffer D (positive control), and BSA (negative control). Immunodetection was performed as described above except that rabbit anti-cS100A12 was used as primary antibody (primary antiserum dilution 1/2000 in blocking buffer) and the secondary antibody (biotin-conjugated goat anti-rabbit IgG) was diluted 1/25,000 in blocking buffer.

2.3. Result

2.3.1. Purification of canine S100A12

Canine S100A12 was successfully purified from the cytosol fraction of canine leukocytes, and the summary of an exemplary purification procedure of cS100A12 is

presented in Table 1 and Fig. 14. The extracted cytosol fraction of canine leukocytes containing a maximum of 200 mg total protein was subjected to ammonium sulfate

Table 1 – Purification of cS100A12 after various purification steps.

Purification stage	*Total protein content (mg)*
Extraction of leukocyte cytosol fraction	165.3
Ammonium sulfate precipitation	119.2
Hydrophobic interaction chromatography	7.7
Cation-exchange column chromatography	1.9
Anion-exchange column chromatography	1.5
Concentration (lyophilization and storage)	1.5

precipitation followed by injection onto a phenylsepharose hydrophobic interaction chromatography cartridge, where the majority of cS100A12 was eluted at approximately 53.3% buffer B (Fig. 15a). Fractions containing cS100A12 were further fractionated on an S-Sepharose™ cation-exchange and a Q-Sepharose™ anion-exchange column, where cS100A12 eluted in a symmetric peak each at approximately 0.19 M NaCl and 0.09 M NaCl, respectively (Figs. 16a and 17a). During the Protein 80 assay, cS100A12 revealed a distinct peak at approximately 7,730 (Figs. 15b and 16b, and 17b). Once polyclonal antibodies (pAb) were produced in rabbits, the progress of the purification procedure could be monitored by Western blot analysis. With the detection system used, the rabbit anti-cS100A12 antiserum strongly recognized cS100A12, and there was no cross-

reactivity observed with either the complex of canine S100A8/A9 (canine calprotectin) or cS100A8 alone. Using the pAb anti-rhS100A12 and ab37657, however, did not result

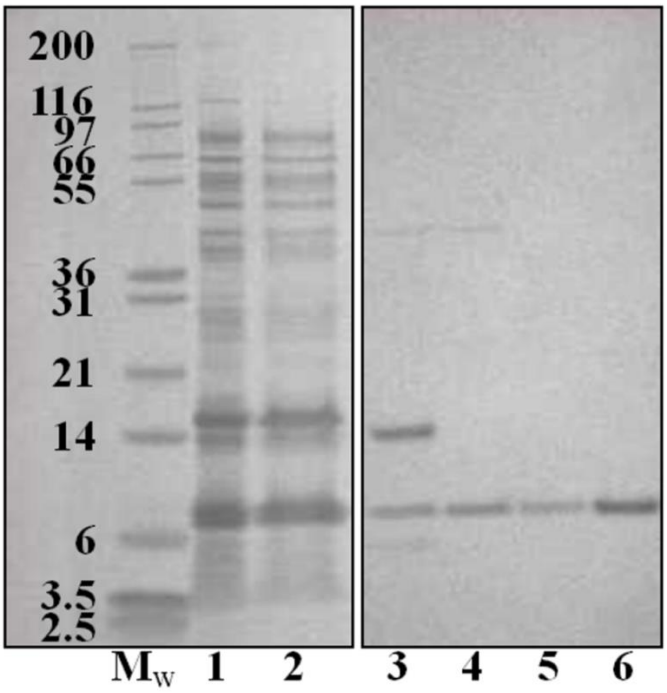

Figure 14 – Purification of cS100A12. This figure shows an SDS-PAGE of samples collected after each purification step. The following material was loaded into the lanes: lane 1, extracted cytosol fraction of canine leukocytes; lane 2, supernatant collected after ammonium sulfate precipitation; lane 3, pool of fractions containing cS100A12 after hydrophobic interaction chromatography; lane 4, pool of fractions that contained cS100A12 following strong cation-exchange chromatography; lane 5, pure cS100A12 after strong anion-exchange chromatography; lane 6, pure cS100A12 after concentration by lyophilization and reconstitution in storage buffer.

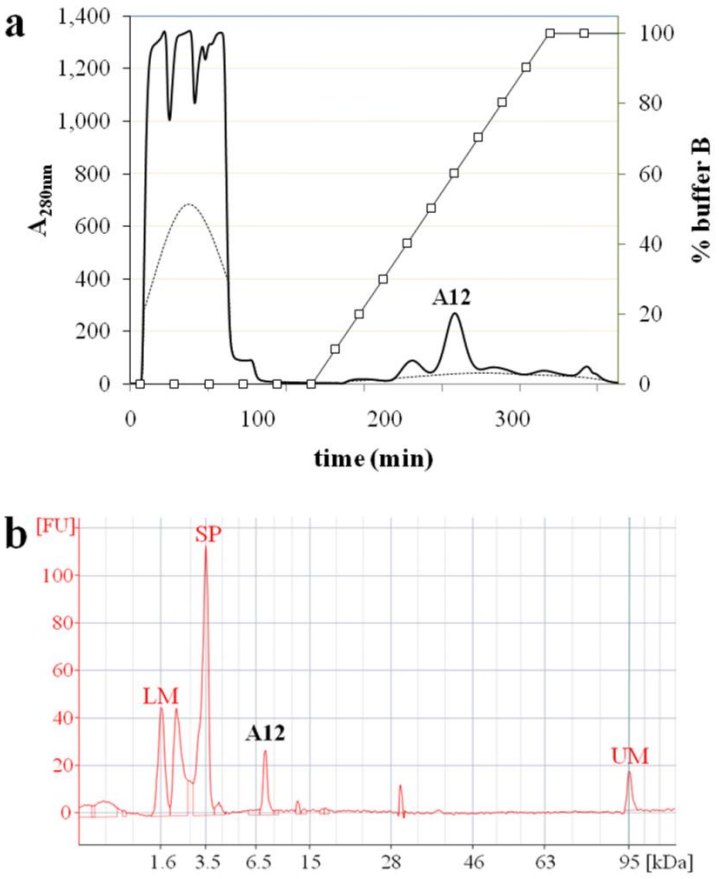

Figure 15 – (a) Hydrophobic interaction chromatography of the extracted cytosol fraction of canine leukocytes. Proteins bound to the column were eluted by a linear gradient of 1.55-0.0 M $(NH_4)_2SO_4$, 2.0-0.0 M $CaCl_2$, and 0.0-5.0 mM EGTA in buffer A (open squares). The protein elution profile was obtained by UV absorption at 280 nm (bold line). Peak labeled A12: canine S100A12. (b) Protein 80 assay (reducing conditions) of the fractions eluted from the hydrophobic interaction chromatography column. The majority of cS100A12 eluted in a single major peak (labeled A12). Migration time (t_M) proportional to the size (M_W) of the proteins is indicated on the x-axis. A standard curve of t_M versus M_W is plotted and used to calculate M_W for each fragment in the sample based on t_M measured. M_W markers: 1600 (lower marker, LM), 3500 (system peak, SP; quality control for each sample run), 6500, 15,000, 28,000, 46,000, 63,000 and 95,000 (upper marker, UM).

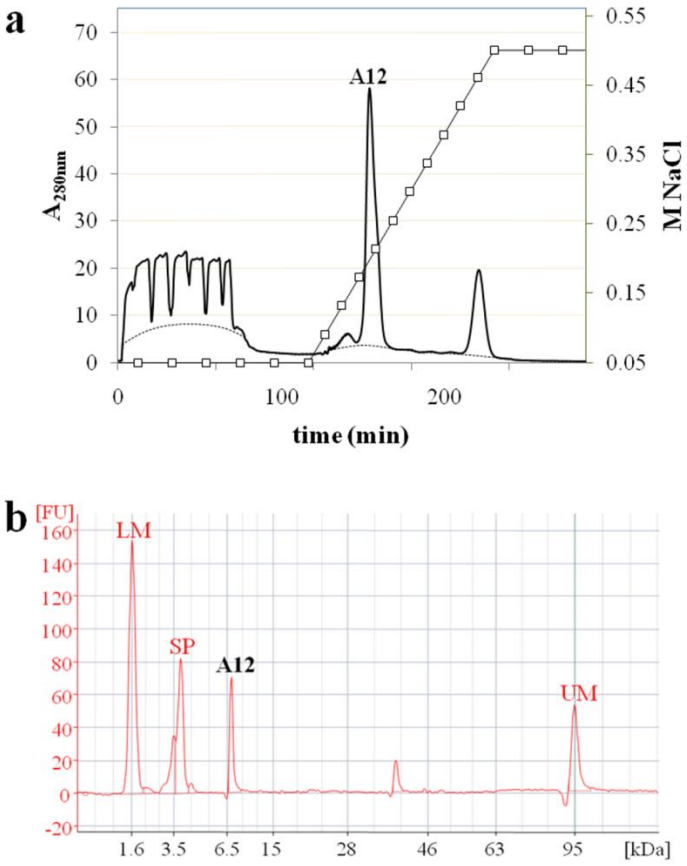

Figure. 16 – (a) Cation-exchange column chromatography of fractions containing cS100A12 from hydrophobic interaction chromatography. Bound proteins were eluted by a linear NaCl gradient of 0.05-0.50 M in buffer A (open squares). The protein elution profile was obtained by UV absorption at 280 nm (bold line). Peak labeled A12: canine S100A12. (b) Protein 80 assay (reducing conditions) of the fractions eluted from the cation-exchange column. Canine S100A12 (peak A12) eluted in a single major peak.

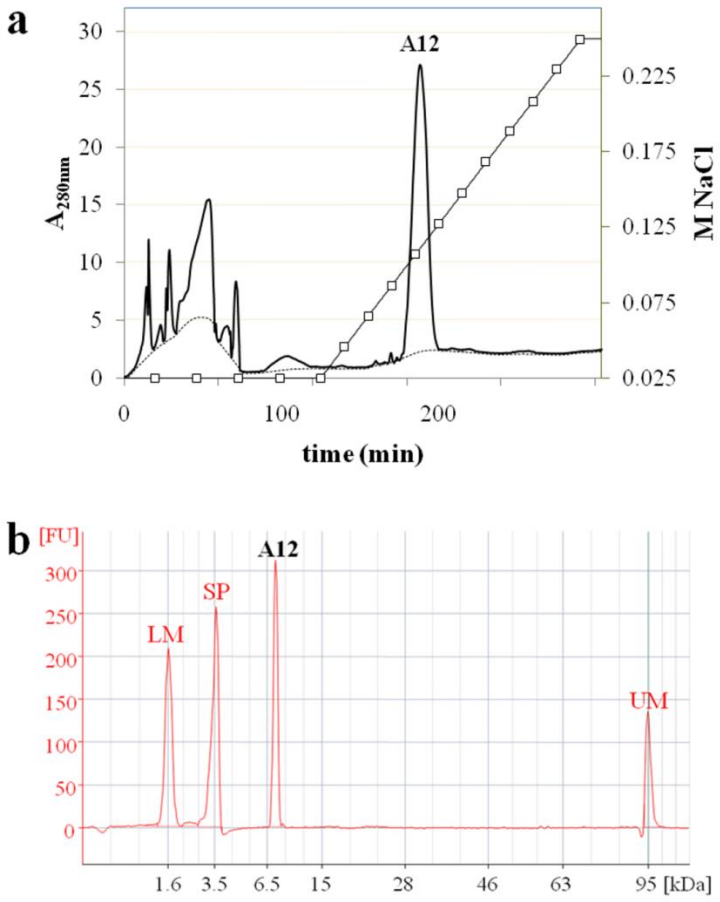

Figure 17 – (a) Anion-exchange column chromatography of cS100A12-containing fractions following cation-exchange chromatography. Proteins bound to the column were eluted with an increasing concentration of buffer B containing 0.25 M NaCl (open squares) and monitored at 280 nm absorbance (bold line). Peak labeled A12: canine S100A12. (b) Protein 80 assay (reducing conditions) of purified fractions obtained from anion-exchange chromatography. Canine S100A12 (peak A12) was localized in one peak.

in the detection of any protein bands. The overall yield of the purification protocol was 1.5 mg per 900 ml canine whole blood, and similar proportions were obtained by repeating the protocol two times. Stability of purified cS100A12 in buffer D (20 mM CH_3CO_2Na, 3 mM $CaCl_2$, pH 7.6) was demonstrated after storage at -80 °C for 9 months by means of the Bradford assay and SDS-PAGE.

Due to the restricted M_W analysis range of the Protein 80 assay (5,000-80,000), purity of cS100A12 in buffer D (storage buffer) was evaluated by SDS-PAGE (under reducing conditions) and revealed a single band in the expected M_W range for cS100A12 (7,300; Figs. 14 and 18). On SDS-PAGE under non-reducing conditions, a single protein band consistent with the respective monomeric form of cS100A12 was also observed (not shown). However, native PAGE revealed a major protein band consistent with the homodimeric form of cS100A12 and this was seen regardless of the presence of Ca^{2+} ions or EGTA in the buffer (16,800; Fig. 18). A very minor fraction of the protein migrated as a homotetramer (with a M_W of approximately 36,800) on native PAGE when the buffer contained 10 mM Ca^{2+} (Fig. 18), 10 mM Ca^{2+} and 5 mM Zn^{2+}, or 10 mM Ca^{2+} and 10 mM Zn^{2+}, respectively. In buffers containing various concentrations of only Ca^{2+}, Ca^{2+} and Zn^{2+} ions, or EGTA, no precipitation of cS100A12 was observed.

2.3.2. Purification of canine S100A8

A minor fraction of cS100A8 eluted with a distinct peak from the strong cation-exchange column at approximately 0.25 M NaCl and was successfully isolated from the cS100A8/A9 protein complex. For the 15 N-terminal AA a sequence with the single

letter code MLTELESAINSLIEV was identified and revealed complete identity with the primary sequence published for cS100A8 (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009).

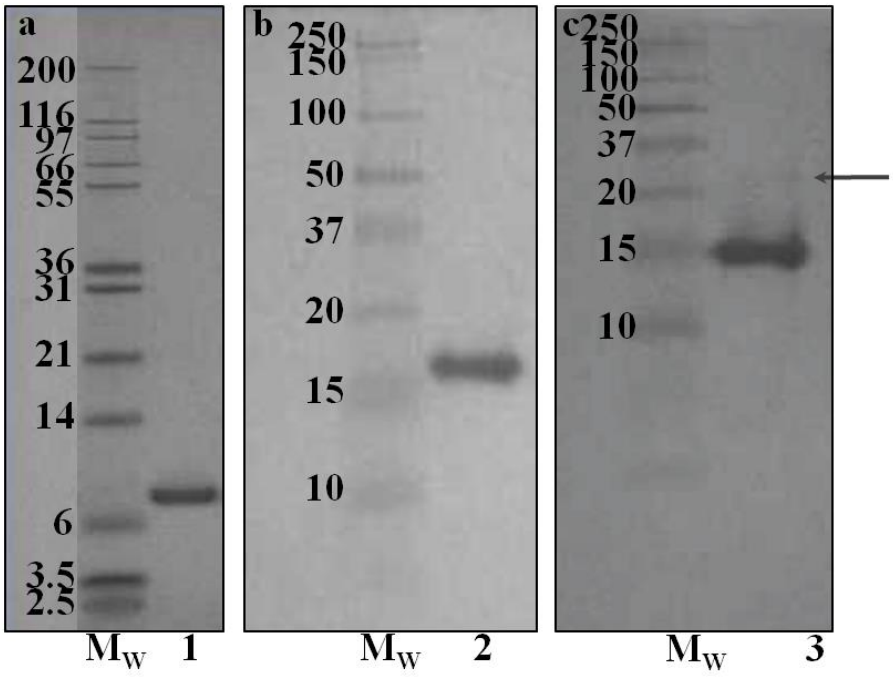

Figure 18 – Purity and dimer formation of purified cS100A12. (a) On the SDS-PAGE (10% Bis/Tris gel) under reducing conditions, cS100A12 represents a polypeptide chain with a M_W of approximately 7300 (lane 1). (b, c) Native PAGE (10% Tris-glycine gel) yielded a molecule with a M_W of approximately 16,800 (lane 2) and a very small fraction with an M_W of approximately 36,800 (arrow) when the buffer contained 10 mM Ca^{2+} (lane 3; 15% Tris-glycine gel), 10 mM Ca^{2+} and 5 mM Zn^{2+}, or 10 mM Ca^{2+} and 10 mM Zn^{2+}, respectively (not shown). M_W, molecular weight markers.

2.3.3. Partial characterization of canine S100A12

The M_r of cS100A12 was estimated at 10,379.5, and a doublet peak corresponding to the monomer of cS100A12 was the most intense signal in each of the spectra. A minor doublet peak detected was assigned to a non-specific double charged monomer of the protein (Fig. 19), and reducing SDS-PAGE excluded the possibility that this minor peak may have arisen from a contaminating substance.

Isoelectric focusing of cS100A12 revealed a single band in the presence as well as the absence of Ca^{2+} or EGTA in the solution (Fig. 20), which migrated as a homodimer in the second dimension consisting of native PAGE compared to a monomer during SDS-PAGE.

The approximate specific absorbance of cS100A12 at 280 nm was determined to be 1.78 for a 1 mg/ml solution.

Characterization of peptides generated by tryptic digestion revealed the separation of 16 different peaks with m/z values of 955, 1,399, 1,615, 1,679, 1,864, 1,909, 2,072, 2,275, 2,528, 2,874, 2,987, 3,217, 3,286, 3,629, 4,252, and 4,861. As determined by *in silico* comparison, the m/z ion values of these peaks correlated well with the expected m/z values of digest fragments for the complete AA sequence of cS100A12 (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009; PROWL. ProFound; available at: http://prowl.rockefeller.edu; accessed 08-12-2009) and covered 68% of the total length of the AA sequence of the protein.

The AA sequence of the first 15 N-terminal residues was obtained for this protein, with the single letter code TKLEDHXEGIVDVFH (X denotes the AA at position 7, which was not unambiguously identified) (Table 2). A database search (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at:

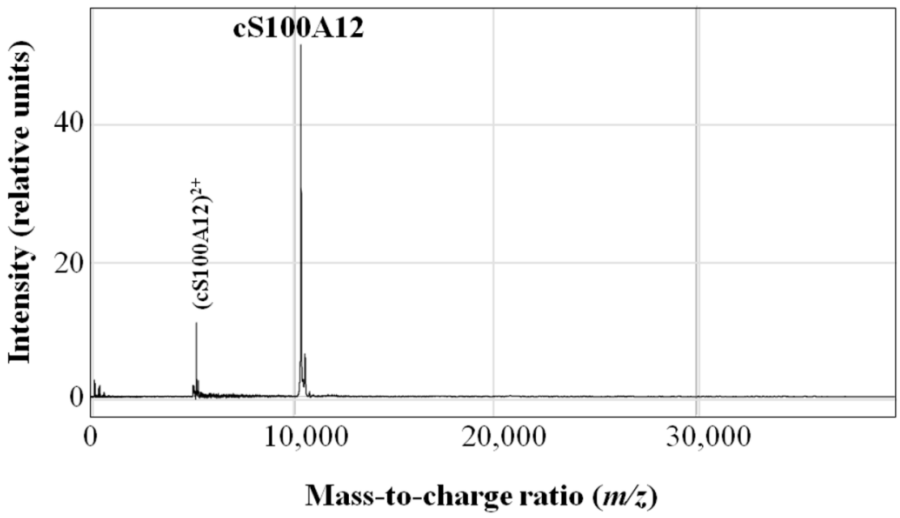

Figure 19 – SELDI-TOF-mass spectrum of the purified cS100A12. M_r (m/z) range was 5000-40,000. Average of 10 single shot spectra. To ensure accurate protein M_r assignments, generated TOF-mass spectra were externally calibrated using M_r standards (recombinant hirudin (6964), bovine cytochrome c (12,230), equine cardiac myoglobin (16,951), bovine RBC carbonic anhydrase (29,023), yeast (*S. cerevisiae*) enolase (46,671)). A doublet peak corresponding to the singly charged cS100A12 monomer (m/z: 10,379.5; peak A12) yielded the most intense signal. A non-specific double charged monomer of the protein was also detected (m/z approximately 5178.3), but no other multimeric forms were evident.

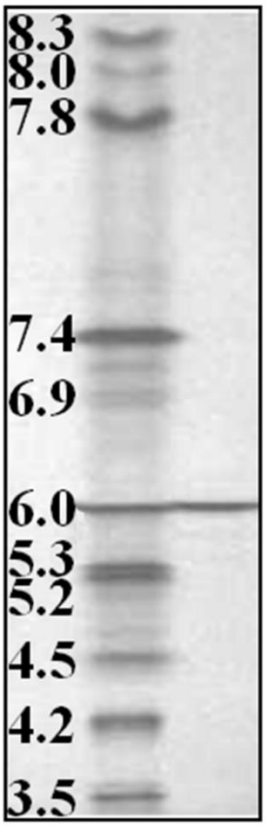

Figure 20 – IEF of purified cS100A12. In a Ca^{2+}-free solution as well as in a buffer containing 3 mM Ca^{2+}, one band was detected with an approximate pI of 6.0, which migrated to a 6.9 kDa position in the second dimension on a gel containing SDS and to a 15.8 kDa position in the second dimension on a native gel (not shown). Left lane, IEF markers.

Table 2 – N-terminal amino acid (AA) sequence of canine, feline, equine, bovine, porcine, and human S100A12.

Species	*S100A12 NT (15)*																$H_{NT(14)}$ *(%)*	H_{ES} *(%)*
Canis familiaris (dog)	(M)	T	K	L	E	D	H	X	E	G	I	V	D	V	F	H	100.0	100.0
Felis catus (cat)	(M)	T	K	L	E	**E**	H	(L)	E	G	I	**I**	**N**	V	F	H	78.6	73.6
Equus caballus (horse)	(M)	T	K	L	E	D	H	(L)	E	G	**V**	**I**	**N**	**I**	F	H	71.4	72.5
Bos taurus (cow)	(M)	T	K	L	E	D	H	(L)	E	G	I	**I**	**N**	**I**	F	H	78.6	70.3
Sus scrofa (pig)	(M)	T	K	L	E	D	H	(L)	E	G	I	**I**	**N**	**I**	F	H	78.6	68.1
Homo sapiens (human)	(M)	T	K	L	E	**E**	H	(L)	E	G	I	V	**N**	**I**	F	H	78.6	63.7

http://www.ensembl.org; accessed 08-10-2009) using the 14 AA analyzed revealed complete identity with the primary sequence predicted from the cDNA sequence for cS100A12 with the exception that the first methionine residue was cleaved off from the protein. Thus, the protein purified was identified as pure canine S100A12 (cS100A12). The highest homology was found with cS100A8 and cS100A9 (38.5 and 44.6% identity, respectively [Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009]).

Radial double immunodiffusion yielded a single strong precipitin line between rabbit anti-cS100A12 antiserum and some canine sera from dogs with an unknown disease status, extracts of fecal samples collected from dogs with acute hemorrhagic gastroenteritis, and porcine serum (Fig. 21). However, no reactivity was found against feline, bovine, ovine, caprine, equine, marmoset, meerkat, sea lion and penguin serum. No precipitation occurred between rabbit anti-cS100A12 antiserum and extracts of fecal samples from healthy dogs, purified cS100A8 and cS100A8/A9, respectively. Western blot analysis using the anti-cS100A12 antiserum clearly revealed one band at a M_M of approximately 7.8 kDa in samples from each of the purification stages, canine fecal extracts, and purified cS100A12, whereas the rabbit anti-cS100A12 pAb only weakly recognized cS100A12 in canine serum samples. No bands were detected for BSA.

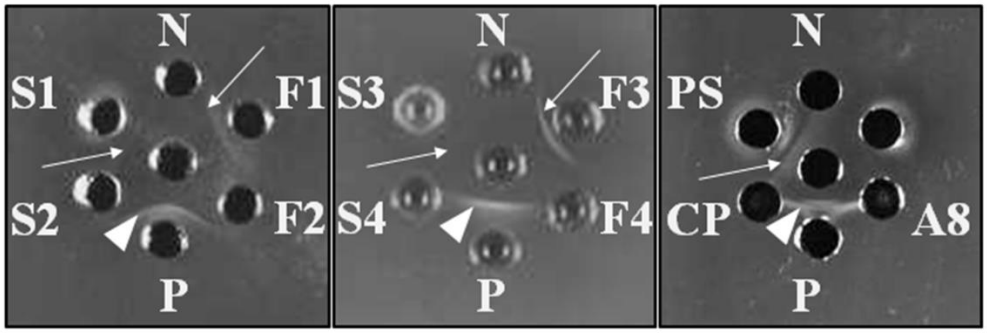

Figure 21 – Radial double immunodiffusion (Ouchterlony test). Radial double immunodiffusion yielded a single strong precipitin line between rabbit anti-cS100A12 antiserum and some canine sera (S1, S3), extracts of fecal samples collected from dogs with acute hemorrhagic gastroenteritis (F1, F3), and porcine serum (PS). However, no precipitation occurred between rabbit anti-cS100A12 antiserum and some canine sera (S2, S4), extracts of fecal samples collected from healthy dogs (F2, F4), purified cS100A8 (A8) and cS100A8/A9 (CP), respectively. P, positive control (purified canine S100A12 protein); N, negative control (BSA).

2.4. Discussion

Here we report the first purification of canine S100A12 (cS100A12) from canine whole blood using a rapid and reproducible protocol. Peptide mass fingerprinting and N-terminal AA sequence analysis together allowed to confirm the identity of cS100A12. A rabbit polyclonal antibody raised against purified cS100A12 recognized the protein as determined by Western blot analysis and radial double immunodiffusion. Despite the use of a relatively sensitive detection system used for Western blot analysis, cS100A12 was not detected by two different pAb against recombinant human S100A12, where the efficiency of blotting was controlled for by staining the marker lane of the respective

PVDF membrane as well as the gel with Coomassie blue following the transfer. This finding demonstrates that canine S100A12 (cS100A12) cannot be detected by use of these polyclonal antibodies produced against the human (recombinant) counterpart, the entire sequence of which has a 63.7% interspecies similarity with cS100A12 (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009), and therefore substantiates the need for a species-specific immunologic method for the detection and quantification of cS100A12 in serum and fecal specimens from dogs.

The cS100A12 protein showed greatest homology with canine S100A8 (MRP8) and S100A9 (MRP14), both of which are consistent with those reported for human S100A12 (40% and 46% identity with human S100A8 and human S100A9, respectively [Ilg et al., 1996]). In this study, pAb raised against cS100A12 did not detect purified cS100A8 or the cS100A8/A9 complex as determined by radial immunodiffusion. While this finding is consistent with that for the respective human counterparts in one study (Robinson et al., 2000), it differs with those of other studies where polyclonal anti-human S100A12 was found to be slightly cross-reactive to human S100A8 (Ilg et al., 1996) or the human S100A8/A9 complex (Yang et al., 2001). Based on this finding, we speculate that the rabbit anti-cS100A12 pAb produced in this study is of valuable potential for the development of an analytically specific immunologic method for the detection of cS100A12.

The exposure of hydrophobic domains of the effector protein in order to interact with the respective targets has been shown for human S100A12 and several other

proteins belonging to the S100 superfamily (Hilt and Kligman, 1991). Leading to an altered chromatographic behavior of cS100A12 in the presence of Ca^{2+}, this property was taken advantage of during the initial step of the purification procedure consisting of hydrophobic interaction chromatography, where cS100A12 was bound in the presence of 2 mM Ca^{2+} and could be eluted from the cartridge by an increasing concentration of a Ca^{2+}-free buffer containing 5 mM EGTA. To prevent any artefactual interaction with other reagents or materials due to an increased hydrophobicity in the presence of Ca^{2+}, the buffers employed throughout the remaining purification stages contained DTT, EDTA, and EGTA, whereas the amount of Ca^{2+} in the storage buffer (3 mM) was chosen at a concentration similar to that found in extracellular biological fluid (Mischke et al., 1996).

The p*I* value observed for cS100A12 is in agreement with the expected p*I* calculated based on the AA sequence (5.9) (ProteinProspector web-site. MS-Digest; available at: http://prospector.ucsf.edu; accessed 08.28.2009) and compares to that reported for human and porcine S100A12 (Ilg et al., 1996; Dell'Angelica et al., 1994). However, unlike in humans and porcines (Guignard et al., 1995; Dell'Angelica et al., 1994), only one isoform was found for cS100A12. As the two different isoforms of the human counterpart have been demonstrated to exert different affinities for the binding of Ca^{2+} (Guignard et al., 1995), the possibility that cS100A12 molecules with a lower Ca^{2+}-binding affinity may have been eliminated during the HIC step of the purification procedure in this study cannot be ruled out.

The M_W of cS100A12 was determined to be approximately 7,730, which is consistent with the M_M estimated by SDS-PAGE. As some Ca^{2+}-binding proteins have been reported to show aberrant mobilities on SDS-PAGE gels, such as human S100A12 due to its distinct nonspherical shape (Xie et al., 2007), a more precise determination of the relative molecular mass (M_r) of cS100A12 was aimed at using SELDI-TOF-MS analysis. Although purified cS100A12 appeared to be homogeneous during IEF, Protein 80 assay, native and SDS-PAGE (revealing only one single band or peak formation, respectively) and N-terminal AA sequencing, SELDI-TOF-MS spectra unveiled the presence of two components. The m/z value of 10,379.5 that was revealed for the major component of cS100A12, is in good agreement with the expected masses derived from the AA sequence in the absence of the first methionine residue (10,376) (ProteinProspector web-site. MS-Digest; available at: http://prospector.ucsf.edu; accessed 08-28-2009), and compares to the M_r of human S100A12 as determined by SELDI-TOF-MS (10,444) (de Seny et al., 2008) and electrospray ionization mass spectrometry (ESI-MS; 10,444) (Ilg et al., 1996) but also to that of porcine (10,614 and 10,654) (Dell'Angelica et al., 1994), bovine (10,554) (Hitomi et al., 1996) and rabbit S100A12 (10,680) (Yang et al., 1996). The absence of the N-terminal methionine residue has also been reported for human, porcine, bovine, and rabbit S100A12 (Ilg et al., 1996; Yang et al., 1996; Hitomi et al., 1996). The presence of a doublet peak with a difference in mass of about 208.2 and an m/z value for the second minor peak of approximately 10,587.7, could be speculated to indicate posttranslational modification of a minor fraction of cS100A12. This difference being the result of divalent cation binding

to the monomer cannot be ruled out but seems rather unlikely, especially because a buffer devoid of any such ions with the exception of Ca^{2+} (m/z ≈ 40) was used. A doublet peak has also been found for porcine S100A12 using electrospray ionization mass spectrometry (ESI-MS) (Dell'Angelica et al., 1994), where this difference in m/z was much smaller and therefore suggestive to be the result of Ca^{2+} binding to the S100A12 protein. The generated laser desorption/ionization-mass spectra also showed a smaller doublet peak with an m/z value of approximately 5178.3 and a peak area that was exceeded by that of the major peak by a factor of about 4–6. Therefore, this additional minor peak can be considered to represent a non-specific, double charged monomer of cS100A12.

In this study, the formation of a homodimer has been demonstrated for cS100A12 (Fig. 5) and was similar to the dimerization pattern shown for human S100A12 (with 2 Ca^{2+} ions per subunit) in the presence of >1 mM Ca^{2+} (Moroz et al., 2003). Homohexamers were, however, not revealed for the cS100A12 protein but were for human S100A12 (with 3 Ca^{2+} ions per subunit) and proposed to be the extracellular form (Moroz et al., 2003). A possible explanation for this discrepancy could be that, while the AA residues found to participate in coordinating the canonical Ca^{2+} ions within the EF-hand (Moroz et al., 2003) are highly conserved between human and canine S100A12 (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009), only one of the four residues shown to coordinate interdimer Ca^{2+} binding necessary for hexamer formation of human S100A12 (Moroz et al., 2003) exists in the AA sequence of cS100A12 as predicted from

cDNA analysis (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009). These residues have been shown to vary widely among S100A12 from different species (Moroz et al., 2003), and the existence of two lysines instead of Glu-55 and Gln-64, both of which appear to be necessary for hexamer formation of human S100A12, suggests a structural similarity of cS100A12 with its bovine pendant, which has also been shown to lack a homohexameric form (Moroz et al., 2003; Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009). Further investigation comprising crystallography is, however, required to test this hypothesis.

The weak nature of non-covalent macromolecular interactions renders them relatively fragile to the disruptive nature of SDS and desorption/ionization processes during SELDI-TOF-MS analysis, thus resulting in their dissociation. In the presence as well as the absence of a strong reducing agent (DTT) during SDS-PAGE, cS100A12 migrated to the same M_W position within the gel that is consistent with the monomeric form, whereas during native PAGE it was shown to migrate as a homodimer (Fig. 5), which is consistent with results obtained for the human pendant (Miranda et al., 2001) and confirms our findings for cS100A12 in a preparative PAGE experiment under native conditions (data not shown). As has been reported for human S100A12 (Hatakeyama et al., 2004), the formation of a homodimer was found to occur both in the presence as well as in the absence of Ca^{2+}, indicating that Ca^{2+} is not essential for cS100A12 dimerization. These findings suggest that the native state of cS100A12 is dimeric with a non-covalent association of cS100A12 protein monomers.

Homotetramerization of cS100A12 in a buffer with a higher Ca^{2+} molarity (with or without micromolar amounts of Zn^{2+}) was evident under native conditions in this study, and thus interpreted to be also non-covalent in nature. The formation of higher order oligomers in the presence of Ca^{2+} and Zn^{2+} has been reported in humans and porcines (Moroz et al., 2009; Dell'Angelica et al., 1994), and the N-terminal AA His-15 and Asp-25 together with the AA His-85 and His-89 in the C-terminal region of the protein that have been suggested to participate in the binding of Zn^{2+} ions (Moroz et al., 2009) are also contained in the AA sequence of cS100A12 (Ensembl Genome Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009). In view of the different composition of the intracellular and the extracellular milieu, further experiments are warranted to determine whether the homodimeric, the monomeric, or a higher order oligomeric form of cS100A12 occurs *in vivo*.

Immunologic cross-reactivity between different species serves as an indicator of high AA sequence similarity in the antigenic sites of a protein in these species. Given a high sequence divergence of a protein belonging to the highly conserved S100 superfamily, as it has been reported for S100A12 from different species (Moroz et al., 2003), a crossreactivity with porcine serum but not with feline, equine, or bovine serum was unexpected. This finding leads the authors to speculate that porcine S100A12 may present the highest sequence similarity with cS100A12 in the antigenic sites, although it is only conserved to 68.1% (as opposed to a 73.6, 72.5, and 70.3% AA sequence similarity of the feline, equine, and bovine pendant, respectively) (Ensembl Genome

Browser web-site. Canis Familiaris [CanFam2.0]; available at: http://www.ensembl.org; accessed 08-10-2009). Radial double immunodiffusion (RDI) yielded a precipitin line between rabbit anti-cS100A12 antiserum and canine sera from some dogs with an unknown disease status as well as extracts of fecal samples collected from dogs with acute hemorrhagic gastroenteritis but not from healthy dogs. While this may support the functional characterization of cS100A12, it should be noticed, however, that RDI is not a quantitative technique and an immunologic method for the quantification of cS100A12 in serum and fecal samples from dogs is currently being developed.

In summary, this study is the first report of the successful purification and a partial characterization of canine S100A12. Similarities were found between canine, human, porcine, bovine, and rabbit S100A12 with regard to structural as well as biochemical properties (such as M_r and p*I*). It remains to be determined whether cS100A12 occurs as a homodimer, a monomer, or a higher order oligomer *in vivo*.

3. DEVELOPMENT AND ANALYTIC VALIDATION OF AN IMMUNOASSAY FOR THE QUANTIFICATION OF CANINE S100A12 IN SERUM AND FECAL SAMPLES AND ITS BIOLOGICAL VARIABILITY IN SERUM FROM HEALTHY DOGS[†]

3.1 Introduction

In canine medicine, laboratory markers that aid in the detection and/or monitoring of localized (e.g., gastrointestinal, urogenital, or cerebrospinal) and/or systemic inflammation are needed but are currently scarce. Inflammatory diseases of the gastrointestinal tract, for example, often represent a diagnostic challenge for the veterinarian as it requires the integration of clinical signs, laboratory results, and frequently more invasive diagnostic modalities (i.e., upper and lower gastrointestinal endoscopy and histopathologic evaluation of tissue samples) (Washabau et al., 2010). Of markers examined to date, only the acute-phase protein C-reactive protein in serum appears to be useful for monitoring of canine patients with idiopathic gastrointestinal inflammation, although there is as yet no consensus as to this marker's utility (Jergens et al., 2003; Jergens, 2004; Allenspach et al., 2007; McCann et al., 2007). Thus, objective markers that are minimally or noninvasive and will aid in the diagnosis and monitoring of disease activity and/or severity are needed in canine gastroenterology.

[†]Reprinted with permission from Heilmann, R.M., Lanerie, D.J., Ruaux, C.G., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2011. Development and analytic validation of an immunoassay for the quantification of canine S100A12 in serum and fecal samples and its biological variability in serum from healthy dogs. *Veterinary Immunology and Immunopathology* 144, 200-209, Copyright (2011) by Elsevier.

A group of proteins that appear to represent good candidates as inflammatory markers are the calgranulins (S100A8, A9, and A12) as they reflect phagocyte activation and turnover *in vivo*. An immunoassay for the quantification of the canine S100A8/A9 (or calgranulin A/B) complex has become available (Heilmann et al., 2008) and is currently being clinically validated. S100A12 (also referred to as calgranulin C, extracellular newly identified RAGE-binding protein (EN-RAGE), migration inhibitory factor-related protein (MRP)-6, or p6) is also a member of the calgranulin subfamily of S100 proteins (Ilg et al., 1996) that has been linked to both innate and acquired immune responses and thus, to acute and chronic inflammation (Hofmann et al., 1999; Föll et al., 2004; Goyette and Geczy, 2011). S100A12 plays a role in intracellular homeostasis and is involved in extracellular processes such as chemotaxis, migration, activation, and sustained recruitment of leukocytes, oxidant scavenging, and generation of proinflammatory cytokines and chemokines (Miranda et al., 2001; Yang et al., 2001, 2007), and thus represents an endogenous damage-associated molecular pattern (DAMP) molecule, or alarmin. Although its expression and functions are species-specific (Hsu et al., 2009), S100A12 is predominantly expressed in activated phagocytes (Vogl et al., 1999), but can also be induced in keratinocytes (Mirmohammadsadegh et al., 2000) and eosinophils (Yang et al., 2007). Physiologic as well as pathologic (e.g., traumatic) stressors have been suggested to be the main modulators of calgranulin expression (Yang et al., 2001).

In humans, over-expression and/or increased concentrations of S100A12 in serum and/or stool samples have been detected in patients with inflammatory diseases,

such as rheumatoid arthritis (Föll et al., 2003a), respiratory disease (Föll et al., 2003b; Lorenz et al., 2008), and inflammatory bowel disease (Föll et al., 2003c; de Jong et al., 2006; Kaiser et al., 2007; Leach et al., 2007; Sidler et al., 2008) for all of which serum S100A12 concentrations correlated well with disease activity.

An immunoassay for fecal and serum S100A12 measurement is currently available for human patients but antibodies against the human protein were shown not to immune cross-react with the canine analogue (Heilmann et al., 2010). The availability of an immunoassay for the measurement of cS100A12 would allow to further study the role and the diagnostic potential of cS100A12 in inflammatory diseases in canine patients.

Biological variation is an inherent characteristic of analytes in clinical chemistry and comprises within- as well as among-subject biological variation. Assessment of biological variation in healthy individuals may be used to assess the utility of population-based reference intervals for clinical chemistry analytes, and to derive critical change values (Fraser and Harris, 1989). Compared to establishing a reference interval, which requires a large reference sample group (Geffré et al., 2009), only a relatively small set of samples collected from a small group of individuals during a short time period is needed to study the biological variation of an analyte (Fraser and Harris, 1989). Given the possible future use of cS100A12 as a marker for disease activity in canine patients with inflammatory disorders, the investigation of its normal biological variation is warranted.

This study therefore aimed to develop and analytically validate an RIA for the quantification of cS100A12 in canine serum and fecal extracts as a potential clinical

marker in dogs with inflammatory conditions, and to evaluate the biological variability of serum cS100A12 in healthy dogs to determine the utility of a population-based reference interval for serum cS100A12.

3.2 Materials and Methods

3.2.1 Sampling population

Three groups of dogs were included in our study (Table 1): a group of 124 healthy dogs of various breeds and ages that were used as reference individuals for serum cS100A12 concentrations (group A), 65 healthy dogs of various breeds and ages that were used as the reference sample group for fecal cS100A12 concentrations and to assess biological variability of cS100A12 concentrations in fecal samples (group B), and 12 healthy pet dogs that were repeatedly sampled to determine the biological variability of cS100A12 concentrations in serum (group C). Surplus material was used for all remaining experiments.

3.2.2. Immunoassay development

3.2.2.1. Production of the tracer

A tracer was produced by labeling pure cS100A12 with radioactive iodine (^{125}I) using the chloramine T method (Hunter and Greenwood, 1962). Briefly, 7.4 μg of previously purified cS100A12 (Heilmann et al., 2010) were dissolved in 0.25 M sodium phosphate (pH 7.5), and 7.4 μL Na^{125}I (0.74 mCi at the time of production) and 20 μg chloramine T were added. After 40 s incubating at approximately 23 °C, 40 μg sodium

metabisulfite and 1.72 mg potassium iodide were added to a final volume of 1 mL. ^{125}I-tagged cS100A12 and free ^{125}I were separated by size exclusion (PD-10 Desalting column, GE Healthcare Bio-Sciences, Piscataway, USA) and buffer-exchanged against 0.05 M sodium phosphate, 0.02% (w/v) NaN_3, 0.5% (w/v) BSA; pH 7.5 (RIAB). The ^{125}I-labeled cS100A12 containing fractions were tested with the antiserum and selected based on a low non-specific binding (NSB, <1%) at a tracer-binding ratio for the zero standard (B_0/TC) of approximately 30% (Berson and Yalow, 1968). Tracer was diluted in RIAB to approximately 30,000 cpm, and stored until further use during the RIA. Specific activity of the tracer was estimated (Chiang, 1987) and stability of ^{125}I-labeled cS100A12 at different storage conditions investigated over 42 days (Law, 1996): aliquots of tracer were stored at +4 or −20°C at approximately 0.2 or 2.3 mCi/L in polypropylene containers, or at 4°C and approximately 0.2 mCi/L in non-inert glass containers.

3.2.2.2. Polyclonal antiserum production

Polyclonal antisera against purified cS100A12 (Heilmann et al., 2010) were generated in two New Zealand White rabbits (*Oryctolagus cuniculus*). Both animals were repeatedly inoculated s.c. with pure cS100A12 emulsified in complete (CFA) and incomplete Freund's adjuvant (IFA). The initial inoculation of both rabbits consisted of 200 µg cS100A12 in CFA, and was followed by two booster injections with 150 µg cS100A12 each in IFA every three weeks and another booster four weeks later. Due to an insufficient Ab response, one rabbit continued to receive four monthly boosters of

200 μg cS100A12 in IFA s.c. into the prescapular nodal area. Ten days after each inoculation, blood samples were collected for evaluation of Ab titers using a simplified RIA and reactivity of the antisera was tested by radial double immunodiffusion as described (Heilmann et al., 2010). Anti-cS100A12 antiserum from the rabbit that received four injections, collected following the third booster injection, was selected for the RIA with a final dilution of 1:12,000.

3.2.2.3. Radioimmunoassay development

The RIA was conducted in polypropylene tubes. Each tube received 100 μL cS100A12 standard (serial 2-fold dilution from 200.0 to 0.2 μg/L), serum (1:20 diluted) or fecal extract (in a 1:8 or 1:160 dilution) in RIAB, 100 μL antiserum (diluted 1:4000) in RIAB containing 0.05% polyoxyethylene sorbitan monolaurate and 10 mM EDTA, and 100 μL tracer. For the zero standard (B_0), the cS100A12 standard was replaced by RIAB. NSB tubes received 200 μL RIAB with 0.025% polyoxyethylene sorbitan monolaurate and 5 mM EDTA, and 100 μL ^{125}I-labeled cS100A12. Total count (TC) tubes contained 100 μL of tracer each. Following 4 hours of incubation at approximately 23 °C, 100 μL rabbit carrier serum (Rabbit serum [sterile-filtered], Sigma–Aldrich, St. Louis, USA; diluted 1:100 in RIAB) and 1 mL 2° antibody solution (Precipitating solution (N6), Diagnostic Products Corporation, Los Angeles, USA) were added to all tubes except TC, free fractions were separated from bound fractions by centrifugation for 30 min at 3,360×g and 4°C, and the remaining pellets were washed with RIAB. Tubes were analyzed using an automated γ-counter (Wallac 1470 WIZARD, Perkin

Elmer Life and Analytical Sciences, Wellesley, USA) and software (MultiCalc® software, Perkin Elmer Life and Analytical Sciences, Wellesley, USA) to calculate a 5PL-curve fit ($y = f[x] = d + [(a–d)/(1 + (x/c)^b)^e]$; y is the dependent variable, x is the independent variable, and a through e describe the shape of the curve) (Gottschalk and Dunn, 2005). Standard concentrations of cS100A12 (log scale) defined the abscissa, and ordinates were calculated as $y = [(B_{std}/B_0)\times100]$ (Midgley et al., 1969) where B_{std} is the NSB-subtracted cpm for the standard and B_0 the NSB-subtracted cpm obtained for B_0. Sample concentrations of cS100A12 (in μg/L serum or ng/g feces) were determined by plotting the NSB-subtracted cpm against the standard curve. Samples with a cS100A12 concentration beyond the standard range of the assay were further diluted and re-assayed. Aliquots of diluted fecal extracts with low, moderate, and high cS100A12 concentrations were stored at −80°C, and served as quality controls.

3.2.3. Immunoassay analytical validation

3.2.3.1. Collection and processing of serum and fecal specimens

Serum was obtained from 124 healthy dogs (group A; Table 3). The protocol for collection of blood samples from healthy dogs was reviewed and approved by the Clinical Research Review Committee at Texas A&M University. Fecal samples were collected from 65 healthy dogs (group B). All dogs were vaccinated, had been de-wormed regularly, did not show clinical signs of any disease, and did not have any condition or receive any medications known to affect the gastrointestinal tract. Feces were collected, placed in pre-weighed polypropylene tubes (Fecal collection tube,

Table 3 – Number of animals, age, and sex distribution within the groups of dogs included in the study.

Study part	Group	N	Age, median [range]	Sex male [neutered] / female [spayed]
Determination of reference intervals				
Reference interval, serum	A	124	4.5 [0.8–13.5]	55 [40] / 69 [56]
Reference interval, feces‡	B‡	65‡	4.0 [0.8–11.1]	32 [31] / 43 [20]
Analyte biological variation				
Biological variability, serum	C	11	3 [2–8]	4 [4] / 7 [6]
Biological variability, feces‡	B‡	65‡	4.0 [0.8–11.1]	32 [31] / 43 [20]

‡same dogs/samples

Sarstedt AG & Co., Nümbrecht, Germany), and immediately frozen until further use. Feces were then thawed and extracted using a previously established protocol (Heilmann et al., 2008). Fecal extracts were stored at −80°C until assayed.

3.2.3.2. Radioimmunoassay analytical validation

The minimum detection limit of the RIA was determined by analyzing 20 duplicates of B_0 in the same assay run, and calculating the mean and SD. The cS100A12 concentration that corresponded to the mean cpm minus three SD plotted against the standard curve, was defined as lower limit of detection of the RIA. The upper limit of the working range was determined by evaluating ten duplicates of a solution containing cS100A12 in a concentration ten times the top standard in the same assay run (i.e., 2000 μg/L), and calculating the mean and SD. The cS100A12 concentration that corresponded to the mean cpm plus three SD was defined as maximum cS100A12 concentration detectable by the RIA (upper limit of the working range). Analytical specificity was determined by assessing cross-reactivity of the RIA with 0.2–20,000 μg/L cS100A8/A9, the closest structural analogues of cS100A12 available. To determine linearity, accuracy, precision, and reproducibility of the RIA, surplus samples with low, moderate, and high cS100A12 concentrations were tested. Assay linearity was evaluated by dilutional parallelism for six different serum samples at serial twofold dilutions from 1:12 to 1:96. Linearity of the RIA for canine fecal extracts was tested using three different extracts at dilutions of 1:100–1:1600, and three and two different extracts with a higher cS100A12 concentration diluted 1:200–1:3200 and 1:400–1:6400, respectively. Three different

fecal extracts with low cS100A12 concentrations were diluted 1:40–1:160. Assay accuracy was tested by spiking seven different sera and seven different fecal extracts with known concentrations of cS100A12 (i.e., 0, 0.2, 1, 2, 5, 10, 20, 50, and 100 µg/L for serum samples and 0, 200, 800, 1600, 4000, 8000, 16,000, 40,000, and 80,000 ng/g for fecal extracts). The percentage of standard antigen recovery was calculated as [observed value (µg/L or ng/g)/expected value (µg/L or ng/g)]×100. Precision of the assay was evaluated by assaying seven different serum samples and seven different fecal extracts from dogs ten times within the same assay followed by calculating the intra-assay coefficients of variation (%CV = [SD/mean]×100). Reproducibility of the assay was determined by analyzing seven different canine sera and seven different canine fecal extracts in ten consecutive assay runs and calculating inter-assay %CVs. Species specificity of the RIA was tested by evaluating two different porcine sera at dilutions of 1:10–1:80.

3.2.3.3. Assessment of interference with the RIA due to hyperlipidemia

Endogenous interference of increased concentrations of lipids as common sample matrix components with the assay was tested by spiking four different surplus canine serum samples (with low, moderate, and high cS100A12 concentrations) with known concentrations of a commercially available intravenous lipid formulation (Liposyn II® 20% [Intravenous fat emulsion], Hospira, Lake Forest, USA; i.e., 0, 100, 250, 500, 750, 1100, 1250, 1500, 1750, 2000, and 3000 mg/dL) followed by a Friedman test for data analysis. Also, concentrations of cS100A12 were measured in 32 grossly lipemic surplus

serum samples before and after high speed centrifugation at 16,000×g for 15 min followed by a Wilcoxon signed rank test for paired data analysis.

3.2.3.4. Reference interval for serum cS100A12

A reference interval for serum cS100A12 was established by evaluating serum samples from 124 healthy adult dogs (group A) and calculating the central 95th percentile. Serum cS100A12 concentrations were compared between healthy male and female dogs using a Mann–Whitney *U* test, and between dogs of different age groups (<2, 2–4, 4–6, 6–8, and >8 years) by a Kruskal–Wallis test.

3.2.3.5. Reference interval and intra-individual variation of fecal cS100A12

Distribution of cS100A12 in feces was assessed by determining the variation within a single defecation for 12 dogs. Three random aliquots of approximately 1.0 g were collected from different portions of feces (spot samples) and extracted as described. The remainder of the feces was diluted 1:2 in extraction buffer, homogenized (Tissue grinder Polytron® PTMR-2100, Kinematica, Littau, Switzerland), and two aliquots (homogenates) were sampled and extracted with a final dilution of 1:5. Variability of fecal cS100A12 concentrations was evaluated for each dog by calculating the %CV for cS100A12 quantified in all extracts. Short-term intra-individual variation of fecal cS100A12 over five consecutive days was evaluated for 40 of the 65 healthy dogs (group B). To determine a reliable and efficient sampling strategy, mean fecal cS100A12 concentrations and intra-individual variation (i.e., CV) were evaluated for various

numbers of sampling days (day 1; days 1 and 2; days 1–3; days 1–4; and days 1–5) by use of a Friedman test with a Dunn's post hoc test. A reference interval for fecal cS100A12 concentration was established from the central 95th percentile of the mean fecal cS100A12 concentrations of three samples (collected on days 1–3) from each of the 65 healthy dogs (group B). Mean fecal cS100A12 concentrations of three sampling days were compared between healthy dogs of different age groups (<3, 3–6, and >6 years) using a Kruskal–Wallis test.

3.2.4. Biologic variation of cS100A12 in serum

Twelve apparently healthy pet dogs of different breeds (group C) were enrolled into the study, the protocol for which had been reviewed and approved by the Institutional Animal Care and Use Committee at Oregon State University and has been detailed elsewhere (Carney et al., 2011). Briefly, biologic variation of serum cS100A12 concentrations was evaluated over a period of 2.6 months in 11 of the dogs; one dog was excluded from the study due to the development of inflammatory skin disease immediately following the sample collection period. From each dog, blood was collected by venipuncture daily for seven days, weekly for six weeks, and a final sample one month later. Immediately after collection, serum was separated from the samples followed by storage at −80°C until analysis.

Serum cS100A12 was measured simultaneously in all specimens using standard assay criteria (i.e., samples that yielded a replicate CV of ≥15% were re-assayed). Samples were analyzed over five assay runs ensuring use of the same lots of reagents,

standards, and quality controls. To eliminate between-run analytical variation, serial samples from the same individual were assayed in the same RIA. Tests for outliers were carried out at three levels (i.e., within-run analytical variance, and intra- and inter-individual variation), and a nested analysis of variance (ANOVA) model was used to calculate analytical and biological components of variation (Fraser and Harris, 1989): analytical (CV_A), intra-individual (CV_I), inter-individual (CV_G), and total variation (CV_T) (Fraser and Harris, 1989; Wu et al., 2009). Indices of biological variation were expressed as index of individuality (II; an II of ≤ 0.6 would indicate that individuals tend to be distinct from each other and thus, use of a population-based reference interval would not be appropriate whereas an II of ≥ 1.4 suggests a population-based reference interval may be useful), index of heterogeneity (IH; the ratio of CV_I to the theoretical CV), and minimum critical difference ($MCD_{0.05}$; the percentage difference in concentration that is significant at $p \leq 0.05$ and is calculated using the 90^{th} percentile of the observed distribution of within-subject variances) (Fraser and Harris, 1989; Wu et al., 2009).

Assumptions of normality and equality of variances were tested using a Shapiro–Wilk *W* test and a Bartlett's test, respectively. Microsoft Excel (Microsoft® Office Excel® 2007, Microsoft, Redmond, USA), GraphPad Prism (GraphPad Prism® [v.5.0], GraphPad Software, San Diego, USA) or JMP (JMP [v.9.0], SAS Institute, Cary, USA) software were used for all calculations and statistical analyses.

3.3. Results

3.3.1. Radioimmunoassay development and analytical validation

Immunization with pure cS100A12 yielded a moderate titer of anti-cS100A12 Ab in both rabbits. Specific activity of the tracer was 636.4 ± 80.8 Ci/mmol (mean ± SD). ^{125}I-labeled cS100A12 was least stable when stored at −20 °C and approximately 2.3 mCi/L. For optimal stability, the tracer was stored in polypropylene tubes at −20 °C and a radiochemical concentration of approximately 0.2 mCi/L, and yielded a good performance of the RIA up to 42 days without any effect on NSB.

The assay was linear from 1 to 100 μg/L and had a working range between 0.6 and 432.7 μg/L (Fig. 22). Thus, for serum and fecal samples assayed in a 1:20 and 1:800 [1:40] dilution, respectively, the minimum detection limit of the assay was 11.2 μg/L and 500 ng/g [24 ng/g], respectively, with a maximum detection limit of 8654.4 μg/L and 346,200 ng/g [17,400 ng/g], respectively. No cross-reactivity was observed with the cS100A8/A9 protein complex up to 20,000 μg/L (Fig. 22). Dose–response curves for the serial dilution of serum and fecal samples with a wide range of cS100A12 concentrations paralleled the standard curve (Table 4), and expected and observed values for spiking recovery of the assay were closely correlated (Table 5). For precision and reproducibility testing of the RIA, mean values and intra- and inter-assay CVs are presented in Table 6. Aliquots of cS100A12 standard solutions were stored at −80 °C, and no change in the standards was observed after storage of 18 months. Spiking of sera with various concentrations of lipid components did not affect the results (p = 0.104) whereas cS100A12 concentrations (median: 201.2 μg/L) were significantly decreased after high

speed centrifugation (median: 138.1 μg/L; $p < 0.0001$). By use of this RIA, S100A12 was detected in one of two porcine sera, the serial dilution of which yielded observed-to-expected ratios of 103.2 ± 16.4% (mean ± SD).

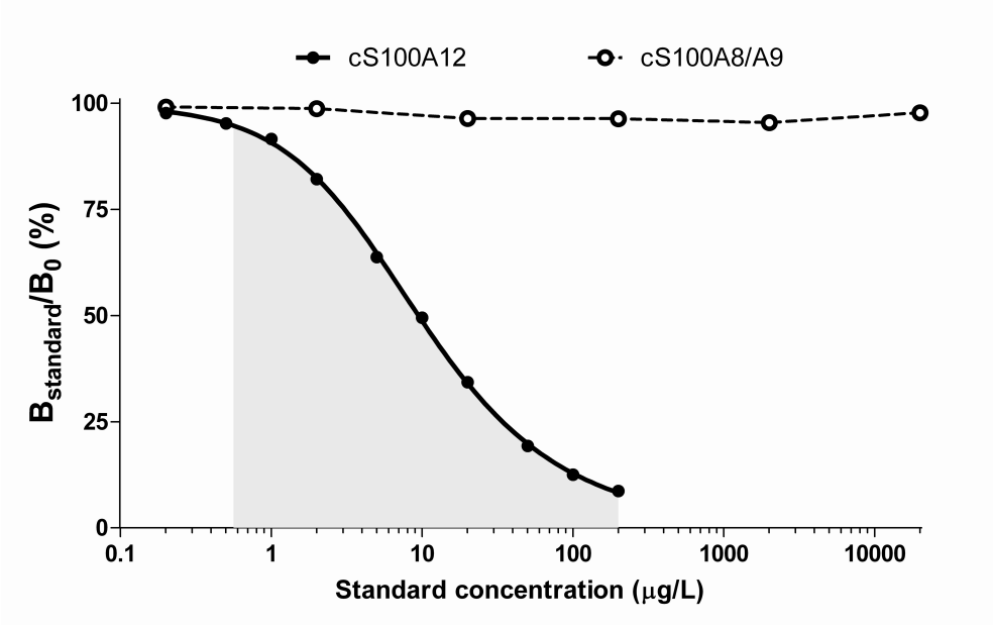

Figure 22 – Representative calibration plot for the estimation of cS100A12 (●) by a competitive, double antibody RIA. The gray shaded portion of the curve was used to quantify cS100A12 in serum and fecal extracts. No cross-reactivity was observed with the canine S100A8/A9 (canine calprotectin) protein complex (○).

Table 4 – Results for dilutional parallelism of serum samples and fecal extracts for the newly developed cS100A12 RIA. Observed-to-expected ratios for the serial dilution of six serum samples ranged from 97.2–146.8% (mean ±SD: 122.5 ±14.9%) and of 11 fecal extracts from 75.3–129.8% (104.8 ±12.5%).

Specimen	Dilutions	cS100A12 (µg/L or ng/g)	Observed/ expected ±SD (%)
Serum 1	1:2–1:16	181.7	123.1 ±16.2
Serum 2	1:2–1:16	191.6	123.8 ± 8.5
Serum 3	1:2–1:16	197.0	117.7 ±16.5
Serum 4	1:2–1:16	217.6	132.1 ±17.5
Serum 5	1:2–1:16	312.6	133.5 ± 9.5
Serum 6	1:2–1:16	335.3	104.5 ± 7.1
Fecal 1	1:40–1:160	186	104.4 ±24.3
Fecal 2	1:40–1:160	648	95.3 ±15.0
Fecal 3	1:40–1:160	1,887	112.7 ± 3.5
Fecal 4	1:100–1:1,600	612	109.6 ±14.6
Fecal 5	1:100–1:1,600	4,127	107.5 ±18.3
Fecal 6	1:100–1:1,600	14,572	100.9 ±15.0
Fecal 7	1:200–1:3,200	2,228	97.1 ±16.0
Fecal 8	1:200–1:3,200	5,043	101.9 ± 8.8
Fecal 9	1:200–1:3,200	12,159	110.0 ± 5.6
Fecal 10	1:400–1:6,400	48,434	101.8 ± 5.2
Fecal 11	1:400–1:6,400	688,947	115.8 ± 9.3

SD: standard deviation

Table 5 – Results for spiking recovery of cS100A12 as determined by the newly developed RIA. Observed-to-expected ratios obtained by spiking seven canine serum samples and seven fecal extracts with eight different concentrations of cS100A12 ranged from 87.8 to 130.4% (mean ±SD: 100.6 ±6.5%) and from 84.8 to 143.8% (103.7 ±10.6%), respectively.

Specimen	cS100A12 (µg/L or ng/g)	Observed/ expected ±SD (%)
Serum 1	83.5	97.1 ± 3.9
Serum 2	103.1	97.2 ± 5.0
Serum 3	113.2	98.0 ± 4.2
Serum 4	187.3	100.6 ± 3.2
Serum 5	402.3	104.0 ± 3.3
Serum 6	442.0	99.7 ± 6.2
Serum 7	772.2	107.7 ±10.4
Fecal 1	6,623	97.4 ± 6.2
Fecal 2	6,935	105.1 ± 4.8
Fecal 3	8,054	109.6 ± 7.7
Fecal 4	14,139	94.6 ± 6.7
Fecal 5	24,847	99.7 ± 4.1
Fecal 6	71,045	113.8 ±15.3
Fecal 7	146,089	106.1 ±12.0

SD: standard deviation

Table 6 – Precision and reproducibility of the RIA for cS100A12.

	Serum sample	Mean (µg/L)	CV (%)	Fecal sample	Mean (ng/g)	CV (%)
	1	196.5	1.5	1	5,236	3.8
	2	204.1	8.1	2	6,728	5.5
Intra-assay	3	224.0	6.3	3	7,299	3.8
variability	4	342.1	4.5	4	18,900	4.2
	5	437.5	6.6	5	79,720	6.1
	6	713.0	5.7	6	189,851	7.4
	7	2,021.3	6.2	7	215,221	7.8
	1	169.2	5.6	1	6,977	5.3
	2	246.4	5.9	2	7,684	5.9
Inter-assay	3	265.5	7.8	3	10,190	5.7
variability	4	272.9	5.8	4	12,694	4.0
	5	299.2	5.1	5	26,025	2.9
	6	394.3	3.3	6	92,566	7.5
	7	463.7	6.5	7	228,744	8.7

CV: coefficient of variation

3.3.2. Biological variation and reference interval for serum cS100A12

Serum cS100A12 concentrations in samples from 124 healthy pet dogs (group A) ranged from 30.4 to 300.2 µg/L (median: 84.4 µg/L) and the reference interval for serum cS100A12 concentration was established as 33.2–225.1 µg/L (Fig. 23). Serum cS100A12 concentrations were not significantly different (p = 0.819) between healthy male and female dogs. Although the number of sexually intact male (n = 15) and female (n = 13) dogs was small compared to neutered males (n = 40) and females (n = 56), serum cS100A12 concentrations were also not significantly different among those four groups (p = 0.068). Also, serum cS100A12 concentrations were not significantly different among healthy dogs of different age groups (p = 0.427).

For evaluation of the biological variability, a total of 14 serial specimens were collected from 9 dogs and 13 serial samples from 2 dogs (group C). Two within-subject outliers were detected (one each from dogs 2 and 10) and excluded from further analysis, yielding a total of 150 serum samples (Fig. 24) and slightly right-skewed data. No outlying observations (maximum variance/sum of the variances = 0.086; Cochrane test) or outliers among mean concentrations of subjects (extreme minus next highest concentration = 15% of the concentration range; Reed's criterion) were detected. CV_A was calculated as 5.7%, CV_I as 29.2%, and CV_G as 31.2% resulting in a CV_T of 66.0%. Index of Individuality (II) was determined to be 0.95 and IH was 56.4, yielding a one-sided $MCD_{0.05}$ of 84.9%.

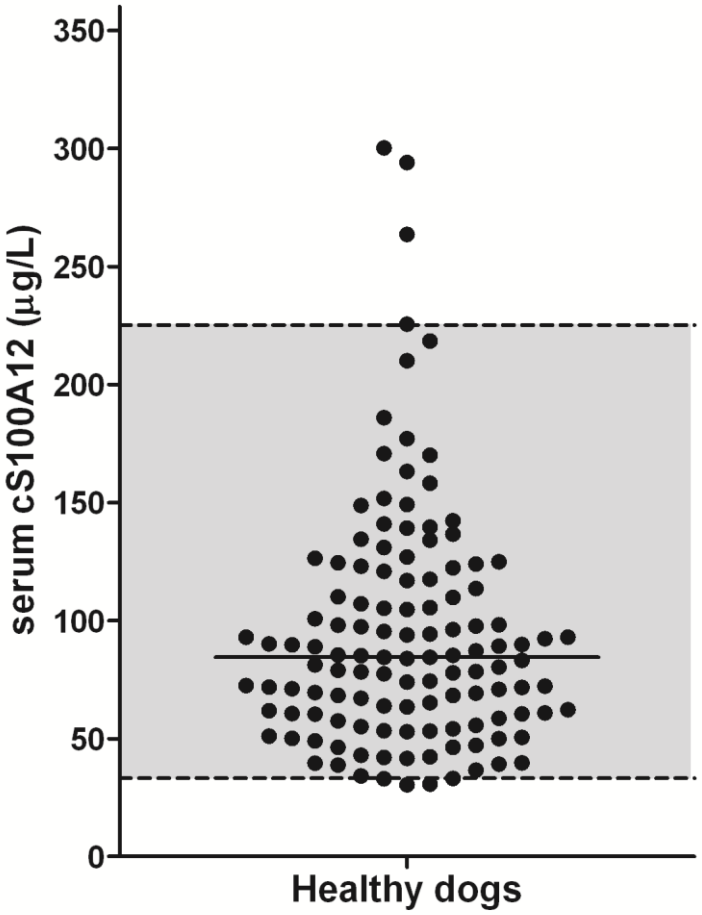

Figure 23 – Scatter plot of serum cS100A12 concentrations measured in specimens from 124 healthy dogs. Each symbol represents the concentration for a specific dog. Median cS100A12 concentration (solid horizontal line) and reference interval (gray shaded portion between dashed horizontal lines) were calculated.

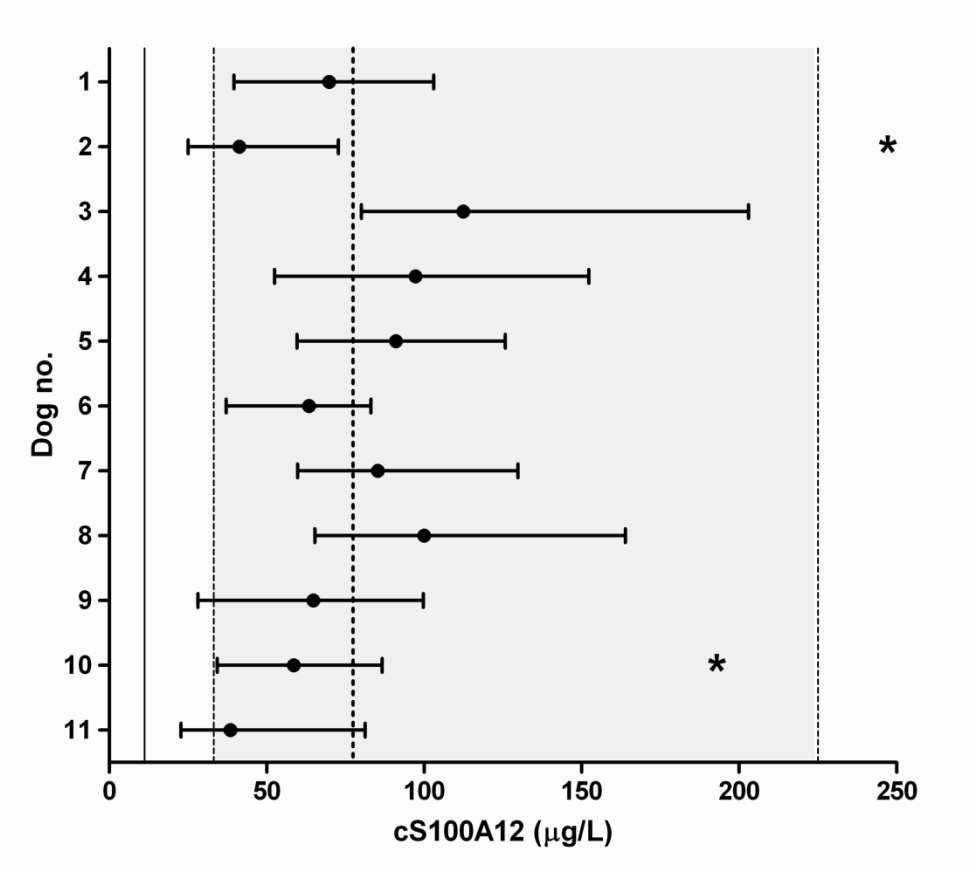

Figure 24 – Long-term biological variation of serum cS100A12 concentrations in 11 healthy dogs. For each dog, the mean (circles) and range (horizontal bars) of serum cS100A12 concentrations over 2.6 months is shown. The gray shaded area (delineated by dashed vertical lines) indicates the reference interval; the overall mean serum cS100A12 concentration (77.4 μg/L) is shown by the dotted vertical line. None of the measurements were below the lower detection limit of the RIA (11.2 μg/L; solid vertical line) and all dogs (except for one of the two outlying observations; asterisks) measured below the upper limit of the reference interval (225.1 μg/L); three dogs had cS100A12 concentrations below the lower limit of the reference interval (33.2 μg/L).

3.3.3. Intra-individual variability and reference interval for fecal cS100A12

For cS100A12 concentrations in three fecal spot samples collected from one single defecation, CVs ranged from 0.0 to 152.9% (mean ± SD: 34.7 ± 53.9%). Fecal cS100A12 in three samples from each of the 65 healthy dogs (group B) ranged from <24 to 2,305 ng/g (median: <24 ng/g), with three-day sample mean cS100A12 concentrations ranging from <24 to 926 ng/g (median: <24 ng/g) (Fig. 25). In five consecutive fecal samples from 40 dogs of group C, fecal cS100A12 ranged from <24 to 5,686 ng/g (median: <24 ng/g) and the five-day sample mean fecal cS100A12 from <24 to 1,381 ng/g (median: <24 ng/g). Mean fecal cS100A12 concentrations were not significantly different (p = 0.581) among different numbers of sampling days; significance was reached for maximum fecal cS100A12 concentrations and CVs overall (both $p < 0.001$), but not in any of the post hoc tests. The reference interval for fecal cS100A12 concentration was established as a three-day sample mean of <24–745 ng/g (Fig. 26). Three-sample mean fecal cS100A12 concentrations were not significantly different among healthy dogs of different age groups (p = 0.410) or between healthy male and female dogs (p = 0.464).

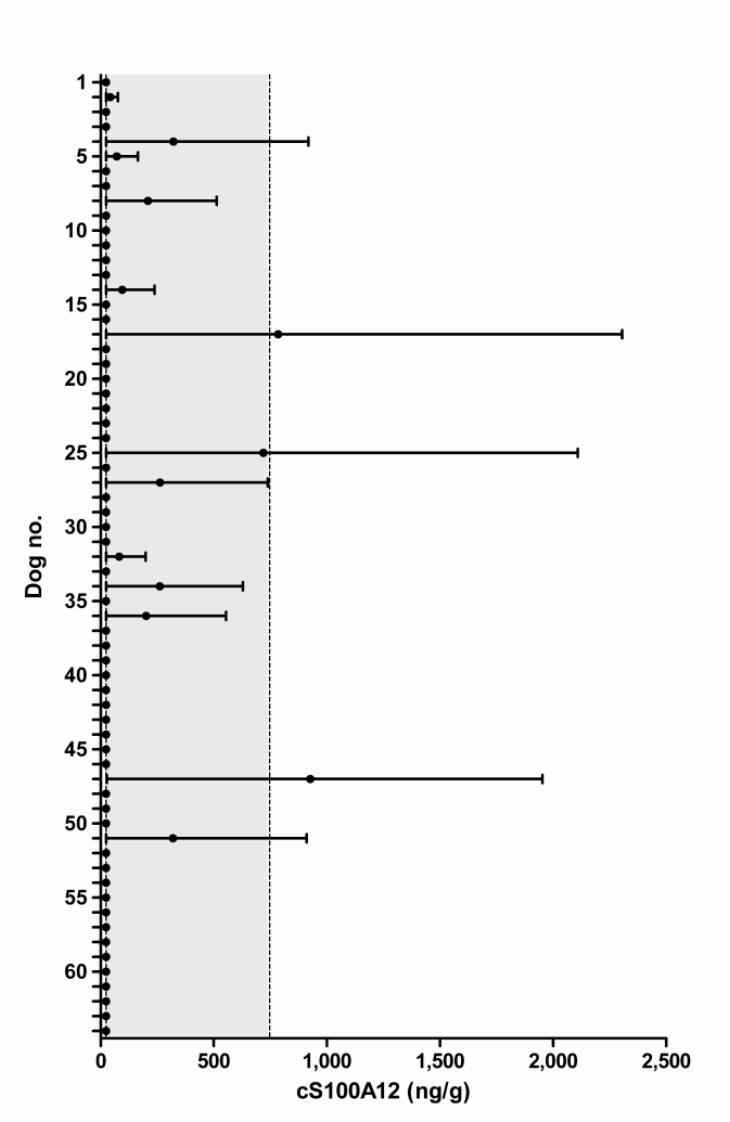

Figure 25 – Short-term biological variation of fecal cS100A12 concentrations in 65 healthy dogs. The reference interval is indicated by the gray shaded area delineated by its upper limit (745 ng cS100A12/g feces) to the right (dashed vertical line). In 13 of the 65 dogs (20%) fecal cS100A12 was detectable in at least one of the samples collected on 3 consecutive days; the majority of the dogs had a three-day sample mean fecal cS100A12 concentration of <320 ng/g.

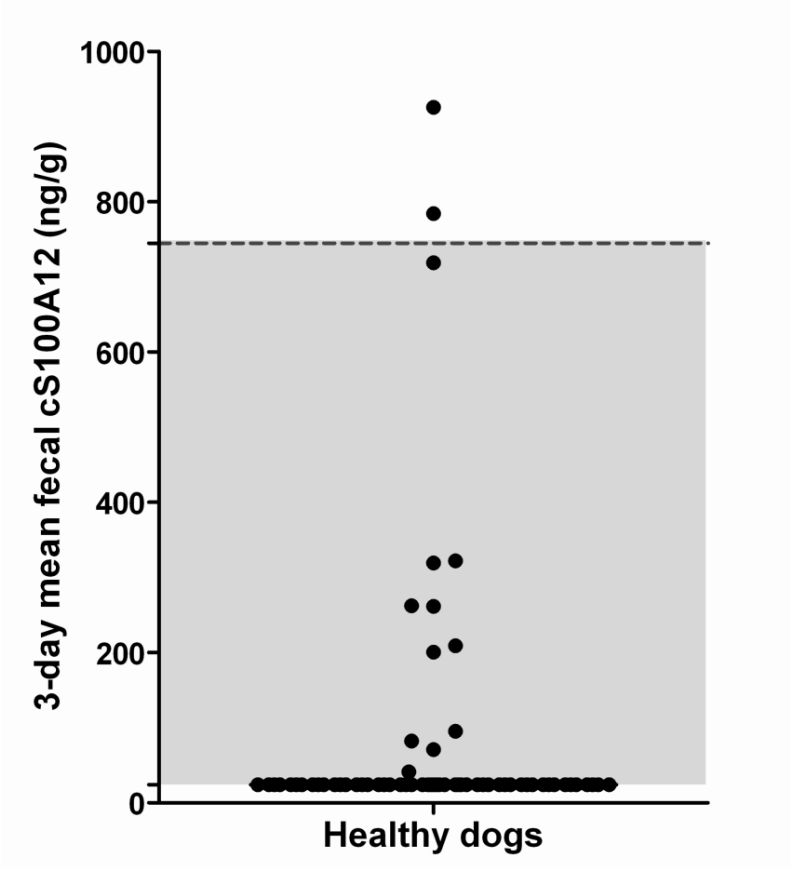

Figure 26 – Scatter plot showing the three-day fecal mean cS100A12 concentrations in specimens collected from 65 healthy dogs. Each symbol represents the three-day mean fecal cS100A12 concentration for a specific dog. Median (solid horizontal line) and reference interval (gray shaded portion between dashed horizontal lines) for 3-day mean fecal cS100A12 concentrations were calculated.

3.4. Discussion

A radioimmunoassay for the measurement of cS100A12 in serum samples and fecal extracts was successfully established. The minimum detection limit of the assay was calculated to be 11.2 μg/L for serum and 24 ng/g feces for fecal samples, respectively, which appears to be adequate considering the upper limit of the reference intervals for serum (225.1 μg/L) and fecal samples (745 ng/g), and the intent to identify dogs with increased serum and/or fecal cS100A12 concentrations. Both a very dilute antiserum and tracer were chosen to increase the sensitivity of the assay and allow measuring cS100A12 in fecal samples from healthy dogs and dogs with gastrointestinal inflammation, assumed to have increased fecal cS100A12 concentrations. This decreases the range of binding ratios (B_{std}/B_0) but appeared to not affect the overall assay performance. However, as fecal cS100A12 concentrations were below the minimum detection limit of the RIA (24 ng/g) in 51/65 healthy dogs (78.5%), further studies in canine patients with gastrointestinal inflammation will need to be conducted to show whether this assay is sufficiently sensitive for detection of cS100A12 in fecal samples from clinical patients.

Dilutional parallelism and spiking recovery of cS100A12 in canine sera and fecal extracts indicate linearity and accuracy of the RIA, and the intra- and inter-assay CVs demonstrate precision and reproducibility of the assay. Due to the availability of only the cS100A8/A9 protein complex as a cS100A12 analogue, analytical specificity of the RIA for cS100A12 could only be demonstrated by testing a wide range of cS100A8/A9 concentrations (i.e., concentrations exceeding that of the cS100A12 top standard by a

factor of 100). As more canine S100 proteins (e.g., psoriasin) and/or analogues may become available in the future, analytical specificity of this cS100A12 RIA may need to be further evaluated.

Compared to reports in humans (Larsen et al., 2007), concentrations of serum cS100A12 did not change after repeated freeze–thaw cycles in our study (data not shown), and attempts to analyze serum and fecal samples using RIAB devoid of EDTA yielded a matrix effect rendering quantification of cS100A12 impossible. Based on the signal (i.e., cpm) obtained and the fact that this was not seen in cS100A12 standards, whereas spiking cS100A12 standards into an "analyte-free" matrix consisting of feline serum samples and fecal extracts (shown to not cross-react using this assay) yielded the same effect, we speculate that ^{125}I-cS100A12 was bound to matrix components (possibly forming multimers with the native protein in specimens) and that EDTA in a concentration expected to chelate most of the Ca^{2+} in serum or extraction buffer (Ca^{2+} was added to increase the stability of S100 proteins) effectively neutralized this effect. However, an increased interaction of cS100A12 with the surface of the assay tubes (presumably due to its increased hydrophobicity upon Ca^{2+} binding) also needs to be considered.

Difficulties raising anti-cS100A12 antiserum in rabbits using a common vaccination protocol for producing pAb were unexpected and have not been reported for hS100A12. Similar difficulties were experienced when generating pAb against cS100A8/A9 in rabbits (Heilmann et al., 2008); however, S100A12 is reported to be less well conserved among species than S100A8/A9 (Moroz et al., 2003). As for

cS100A8/A9, modifying both the amount of antigen inoculated and injection site led to an increase in anti-cS100A12 titer. Thus, the insufficient pAb response against cS100A12 in one rabbit may be due to a high epitopic homology between canine and rabbit S100A12, the size of cS1000A12 being close to the minimum for immunogens (~5 kDa), or idiosyncrasy. However, sequence homology of rabbit S100A12 with the canine (63%) and human protein (67%) is similar, and cS100A12 is assumed to form homooligomeric complexes in the Ca^{2+}- containing buffer used for the inoculations (Heilmann et al., 2010).

Lipids represent matrix components that commonly interfere with immunoassay performance. While the spiking of samples with lipid components in concentrations measured in patients with severe hypertriglyceridemia/hyperlipidemia did not affect the results in our study, separating the majority of the lipid fraction from the sera by high speed centrifugation appeared to also remove a significant portion of cS100A12 from the samples. It seems plausible that a fraction of cS100A12 is found in the lipid portion of the samples (possibly due to its hydrophobicity at Ca^{2+} concentrations found in serum). Therefore, we suggest that lipemic serum samples should be assayed or an aliquot removed before separating the lipid fraction.

Concentrations of cS100A12 in feces from healthy dogs varied, which may be due to a patchy distribution of cS100A12-expressing cells within the gastrointestinal mucosa and/or variations in gastrointestinal passage leading to variation in the concentration of fecal proteins, and has also been shown for other fecal markers in dogs (Steiner et al., 2003; Heilmann et al., 2008). To counterbalance the intra-individual

variation of cS100A12 observed in this study, we recommend analyzing fecal samples collected on three consecutive days. However, results of further studies in dogs with gastrointestinal inflammation (assumed to have increased fecal cS100A12 concentrations) are needed to definitively reveal the number of consecutive fecal samples required for reliable results.

Fecal cS100A12 concentrations were not significantly different among pet dogs of various age groups, which may suggest that, in contrast to humans (de Jong et al., 2006; Kaiser et al., 2007; Sidler et al., 2008), age related changes do not occur. However, cS100A12 concentrations were not evaluated in healthy dogs <1 year, and further studies are warranted to evaluate serum and fecal cS100A12 in healthy puppies.

Detection of porcine S100A12 by this assay was expected as cross-reactivity between cS100A12 and porcine S100A12 has been shown previously (Heilmann et al., 2010). Moreover, linearity of the RIA for porcine serum samples indicates a potential application of this assay for S100A12 quantification in this species.

Biological variation is determined by pre-analytic factors associated with the collection of samples and true biological variance, whereas analytical variance arises from the methodology, instrumentation, and technical skills (Fraser and Harris, 1989). The protocol that was used for collection and analysis of samples to determine the biological variation has the advantage of eliminating long-term (i.e., inter-assay) analytical variation, thus rendering intra-assay variability (CV_A; estimated from analyzing duplicates of samples) the only component of analytical variation. The analytical goal of $CV_A \leq ½ \times CV_I$ (Fraser and Harris, 1989) has also been satisfied in this

study. Although serum cS100A12 appears to be maintained within a relatively narrow concentration window in individual dogs, it showed moderate individuality and, as indicated by the $MCD_{0.05}$, moderate changes in serum cS100A12 between sequential measurements in a dog are necessary to be considered relevant rather than reflecting biological and/or analytical variation. Thus, the use of a conventional population-based reference interval to detect increased cS100A12 concentrations may or may not be appropriate, and needs to be further evaluated in dogs with acute and chronic inflammatory diseases. Interestingly, using the $MCD_{0.05}$ with the median (84.4 μg/L) and the 75^{th} percentile determined for serum cS100A12 concentrations in the reference sample group yielded cS100A12 concentrations coinciding with the upper limit of the central 90^{th} percentile and the upper limit of the reference interval calculated (225.1 μg/L), respectively. This demonstrates that the reference interval established is within reasonable limits.

3.5. Conclusions

In summary, the RIA described here is sensitive, linear, accurate, precise, and reproducible, and has the potential to distinguish healthy dogs from dogs with inflammatory diseases, assumed to be associated with increased serum and/or fecal cS100A12 concentrations. Further research into the clinical utility of measuring serum and/or fecal cS100A12 in dogs with systemic or localized inflammatory conditions (e.g., pancreatitis or gastrointestinal inflammation) is warranted and is currently under way. Further studies in dogs with gastrointestinal inflammation will show if the RIA is

sufficiently sensitive to reliably measure cS100A12 in fecal samples. A population-based reference interval for serum cS100A12 may or may not be a reasonable tool, but this will depend on the degree of change observed in dogs with acute and chronic inflammatory diseases. Further studies to evaluate the clinical utility of serum and/or fecal cS100A12 for the diagnosis (i.e., as "event marker") and/or monitoring (i.e., as "chronic disease marker") (Fraser and Harris, 1989) of canine patients with inflammatory diseases, and to determine whether stratification of the reference population based on variables other than age and sex (e.g., body condition score) may be necessary, are currently under way.

4. ASSOCIATION BETWEEN FECAL S100A12 CONCENTRATION AND HISTOLOGIC, ENDOSCOPIC, AND CLINICAL DISEASE SEVERITY IN DOGS WITH IDIOPATHIC INFLAMMATORY BOWEL DISEASE[‡]

4.1 Introduction

Idiopathic inflammatory bowel disease (IBD) in dogs is a chronic relapsing condition. Its diagnosis and management can be challenging, requiring invasive diagnostic procedures after a tedious work-up to exclude other causes of chronic gastrointestinal (GI) signs (Washabau et al., 2010; Simpson and Jergens, 2012). Fecal markers that are specific for GI disease, correlate with disease severity, and objectively assess GI inflammation would be very useful in clinical practice but such markers are currently lacking in veterinary medicine.

S100/calgranulins, a group of 3 phagocyte-specific damage-associated molecular pattern (DAMP) molecules (Pietzsch and Hoppmann, 2009), accumulate at sites of inflammation and appear to be candidates for fecal markers because of their stability in feces (Föll et al., 2009) and their increase at the mucosal mRNA level in dogs with IBD (Wilke et al., 2012). S100A12 (also known as calgranulin C, myeloid-related protein-6 [MRP6], or extracellular newly identified receptor for advanced glycation end products [EN-RAGE]) represents a ligand for receptors of the innate immunity, such as the

[‡]Reprinted with permission from Heilmann, R.M., Grellet, A., Allenspach, K., Lecoindre, P., Day, M.J., Priestnall, S.L., Toresson, L., Procoli, F., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2014. Association between fecal S100A12 concentration and histologic, endoscopic, and clinical disease severity in dogs with idiopathic inflammatory bowel disease. *Veterinary Immunology and Immunopathology* 158, 156-166, Copyright (2014) by Elsevier.

receptor for advanced glycation end products (RAGE) (Hofmann et al., 1999). The ligand–RAGE interaction has been shown to activate nuclear factor-kappa B, leading to the production of proinflammatory cytokines and chemokines. Also, a positive feedback on the expression of RAGE has been proposed to induce sustained post-receptor signaling, thus perpetuating and amplifying the inflammatory response (Bierhaus et al., 2005).

Biomarkers of inflammation, such as the concentration of fecal S100A12, are considered useful tools for diagnosing and monitoring IBD in people (Föll et al., 2003, 2009; Kaiser et al., 2007). For a marker of inflammation to be clinically useful, it would ideally be measurable without temporal delay in expression and/or secretion following changes of the inflammatory activity. The marker should also be organ-specific in expression or the type of specimen used to determine its concentration, and be specific for the disease process. Moreover, such a marker would need to be easy, inexpensive, and non-invasive to measure, and be stable in the specimen under clinical conditions. Although the last hampers the routine analysis of many small molecular inflammatory proteins (e.g., cytokines or chemokines), measurability and stability in feces are criteria applicable to fecal S100A12 (Heilmann et al., 2011b). Also, our preliminary work has shown that serum canine S100A12 (cA12) concentrations are increased in dogs with IBD (Heilmann et al., 2011a). However, fecal concentrations of cA12 (fcA12), assumed to be more specific for the GI tract, have not yet been investigated in canine IBD.

We hypothesized that fcA12 concentrations are increased in dogs with IBD, indicating an increased infiltration and/or activation of phagocytes; and that the

expression of fcA12 correlates with clinical, endoscopic, and/or histologic disease severity. Thus, the aims of the present study were to measure fcA12 concentrations in dogs with IBD, and to assess the relationship of fcA12 concentrations to the severity of clinical signs, macroscopic (i.e., endoscopic) mucosal lesions, and histopathologic findings.

4.2 Materials and Methods

4.2.1. Animals

Dogs with chronic GI signs (vomiting, diarrhea, and/or weight loss) and a diagnosis of IBD based on clinical, clinicopathologic, endoscopic, and histopathologic criteria (Washabau et al., 2010), recruited at the Small Animal Teaching Hospital of the Royal Veterinary College (RVC) at the University of London (KA, FP) between April 2009 and May 2010 (n = 9 dogs) and at St Priest's Clinique vétérinaire des Cérisioz (PL, AG) between July 2010 and December 2010 (n = 17 dogs), were retrospectively enrolled in the study. Protocols for enrollment of dogs and use of specimens were approved by the RVC Ethics and Welfare Committee and the Royal Canin Internal Ethics Committee (St Priest's Clinique vétérinaire des Cérisioz). Informed owner consent was obtained at the time dogs were enrolled for using material.

The control group comprised 90 apparently healthy dogs that were enrolled at the Texas A&M University (TAMU) College of Veterinary Medicine and the Region-djursjukhuset Helsingborg, Sweden. An owner questionnaire served to assess the current health status of each dog and to ensure valid vaccination and other preventive

medications depending on the geographical location. No further blood testing or other invasive procedures were performed in the control group. The flow chart (Fig. 27) shows the group distribution of all 116 dogs enrolled in the study.

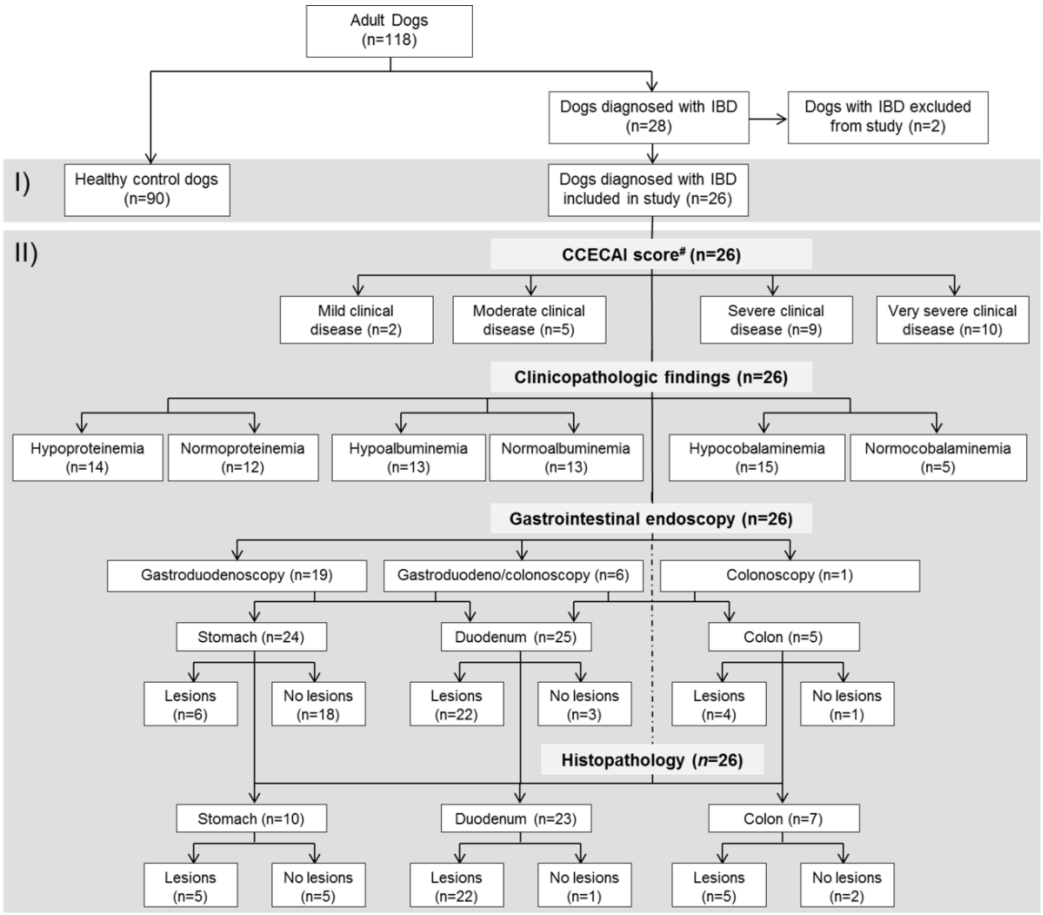

Figure 27 – Study flow chart. Shown is the group distribution of the 116 dogs enrolled in the study. The two different parts of the present study are marked by the gray shaded area: (I) evaluation of fcA12 concentrations in healthy dogs and dogs with IBD; and (II) evaluation of the relation between fcA12 with clinical, endoscopic, and histologic disease severity in dogs with IBD. CCECAI: canine chronic enteropathy clinical activity index.

4.2.2. Clinical disease activity

Dogs were diagnosed with IBD using established clinical, clinicopathologic, endoscopic, and histopathologic criteria (Washabau et al., 2010). The severity of their disease was then scored using an established clinical disease activity index (canine chronic enteropathy clinical activity index, CCECAI) (Allenspach et al., 2007), which considers general attitude and activity, appetite, frequency of vomiting, stool consistency and frequency of defecation, weight loss, serum albumin concentration, peripheral edema and ascites, and pruritus. The composite CCECAI score can range from 0 to 27, and according to the score, clinical disease is categorized as: clinically insignificant (score 0–3), mild (score of 4–5), moderate (score of 6–8), severe (score of 9–11), or very severe (score of ≥12) (Allenspach et al., 2007). A CCECAI score of at least 4 was required for inclusion into the study.

4.2.3. Clinical pathology and diagnostic imaging in dogs with IBD

A complete blood cell count, serum biochemistry profile, and the concentration of canine trypsin-like immunoreactivity (cTLI) in serum were used to rule out other GI or systemic diseases, and concentrations of serum cobalamin and folate were also measured at each institution. Diagnostic imaging consisted of an abdominal ultrasound examination in 25 of the 26 dogs with IBD to rule out non-intestinal disease.

4.2.4. Endoscopic disease activity

Each of the 26 dogs in the IBD group underwent endoscopic evaluation conforming to existing standard protocols at each center, during which the mucosa was visually inspected and the severity of lesions were documented and scored. Gastroduodenoscopy was performed in 19 dogs, gastroduodenoscopy and colonoscopy in 6 dogs, and only colonoscopy in 1 dog. At the Clinique vétérinaire des Cérisioz (n = 17 dogs), endoscopic disease severity was recorded at the time of endoscopy using a 4-point grading system with 0 = normal mucosa, 1 = mild lesions (slightly friable and erythematous mucosa), 2 = moderate changes (friable and white-speckled mucosa), and 3 = severe endoscopic disease (very friable, easily bleeding, ulcerated or cobblestone-appearing mucosa; difficulties on endoscopic insufflation) (Allenspach et al., 2007). In order to be able to compare scores between the two centers, endoscopic disease activity in dogs enrolled at the RVC (n = 9 dogs) was retrospectively graded based on the endoscopy reports in the patient medical records. The endoscopic reports had been generated by different clinicians and by using guidelines established by the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization group (Washabau et al., 2010). Endoscopic lesions were retrospectively assigned a score by the investigator (RMH) who was blinded to the clinical disease activity, severity of histologic lesions, clinicopathologic findings, and any other parameters of the dogs at the time of scoring. Visual inspection of the colon was obscured in 2 (7.7%) of the dogs where biopsies were collected but the severity of macroscopic (endoscopic) lesions could not be assessed.

4.2.5. Histologic disease activity

Endoscopically collected, formalin-fixed, and paraffin-embedded tissue biopsies were cut and H&E-stained in preparation for histological evaluation conforming to standard protocols at the respective institution. Tissues were evaluated and scored by a single, experienced pathologist at each center, using guidelines established by the WSAVA Gastrointestinal Standardization group (Washabau et al., 2010; Day et al., 2008) and with the pathologist being blinded to the results of fcA12, the severity of endoscopic lesions, and clinicopathologic data from these dogs. The severity of histopathologic lesions in each section of the GI tract was recorded using a 4-point grading system with 0 = normal, 1 = mild lesions, 2 = moderate changes, and 3 = severe histopathologic changes. For correlation analysis, the individual inflammatory and morphologic criteria, composite scores for morphologic and inflammatory criteria, and the sum of both in each of the different GI sections and over all sections examined were considered.

4.2.6. Fecal S100A12 analysis

A single spot (1 g) fecal sample was collected from each dog, all samples were stored frozen as soon as possible after collection and were shipped on ice to the Gastrointestinal Laboratory at TAMU. All samples were then stored at −80°C until further analysis (up to 15 months). To allow quantification and stabilize fcA12, all samples were extracted as previously described (Heilmann et al., 2010), and fcA12 was quantified by ELISA. Briefly, 96-well immunoassay plates (Nunc-Immuno™ plates

MaxiSorp, Sigma-Aldrich, St. Louis, MO, USA) were coated with affinity-purified polyclonal anti-cA12 Ab (Heilmann et al., 2010) and were blocked with Tris-buffered saline (TBS)-Tween-10% BSA. Plates were then incubated with duplicates of standard cA12 (Heilmann et al., 2010) solutions, assay controls, or fecal extracts (Heilmann et al., 2008) diluted in TBS-Tween-3% BSA, after which they were incubated with horseradish peroxidase-labeled (Horseradish peroxidase (HRP); Thermo Scientific, Rockford, IL, USA) anti-cA12 polyclonal Ab (Heilmann et al., 2010, 2011b). A 3,3',5,5'-tetramethylbenzidine-substrate (Tetramethylbenzidine [TMB]-substrate; 1-Step™ Ultra TMB ELISA, Thermo Scientific, Rockford, IL, USA) was used for color development; the absorbance at 450 nm was measured (Synergy 2 Alpha Microplate reader, BioTek®, Winooski, VT, USA), and a five-parameter logistic curve fit was used for the standard curve (Gen5™ Data Analysis Software [v1.11], BioTek®, Winooski, VT, USA). Lower detection limit of the assay was 1 ng fcA12/g fecal sample. Fecal cA12was measured using the same lot of reagents for all samples and was reported as μg fcA12 per g fecal wet weight. The investigator was blinded to each dog's parameters at the time fcA12 was measured.

4.2.7. Data and statistical analysis

Standards for reporting of diagnostic accuracy studies (Bossuyt et al., 2003a, 2003b) were applied. All statistical analyses were performed using a commercial software package (JMP® v10.0, SAS Institute, Cary, NC, USA; GraphPad Prism® v5.0, GraphPad Software, San Diego, CA, USA). Normality and equality of variances were

tested using a Shapiro–Wilk W and a Brown–Forsythe test, respectively. Summary statistics were reported as medians and interquartile ranges (IQR), and nonparametric tests (Wilcoxon rank sum or Kruskal–Wallis test) were used for comparison of two or more groups, respectively. A Spearman ρ was calculated for correlation analysis. Cut-offs for the concentrations of serum albumin and cobalamin shown to be associated with negative outcomes (20 g/L and 200 ng/L, respectively [Allenspach et al., 2007]) were used to compare fcA12 concentrations in patients dichotomized into 2 subgroups with concentrations below or above the respective cut-off values. A receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity of the test in distinguishing dogs with IBD from healthy dogs, and the Youden index and the likelihood ratio were used to determine the optimal cut-off concentration for the diagnosis of IBD. Statistical significance was defined as $P \leq 0.05$, and Holm's sequential Bonferroni correction (Holm, 1979), which controls the family-wise error rate, was applied for multiple comparisons with the numbers of categories or subcategories considered (i.e., adjusted P = unadjusted $P \times [n - k + 1]$; with n being the number of hypotheses tested and k being the ordered rank of the uncorrected P-values).

4.3. Results

4.3.1. Patient demographics

Twenty-eight dogs were considered for inclusion into the study. However, 1 dog was excluded because full-thickness biopsies had been obtained before sample collection, and another dog was excluded because of con-current exocrine pancreatic

insufficiency. There were no differences in age (dogs with IBD, median: 4.0 years, IQR: 2.9–7.0 years; healthy dogs, median 3.9 years, IQR: 2.3–6.6 years; *P* = 0.487, Wilcoxon rank sum test) and sex distribution (dogs with IBD, male [neutered]/female [spayed]: 16 [4]/10 [5]; healthy dogs, male [neutered]/female[spayed]: 45 [38]/45 [27]; *P* = 0.299, Pearson Chi-square test) between the 26 dogs with chronic GI inflammation (IBD) and the 90 healthy controls. Dogs in the healthy control group were of various breeds; and breeds that were represented by more than one dog included mixed breed (n = 26), German Shepherd dog (n = 8), Dachshund (n = 5), Australian Cattle dog (n = 4), Labrador Retriever (n = 4), Golden Retriever (n = 3), Australian Shepherd dog (n = 2), Border Collie (n = 2), Boston Terrier (n = 2), Boxer (n = 2), Great Pyrenees (n = 2), Rat Terrier (n = 2), Rottweiler (n = 2), Siberian Husky (n = 2), Weimaraner (n = 2), West Highland White Terrier (n = 2), and White Shepherd dog (n = 2). In the group of dogs with IBD, breeds included, German Shepherd dog (n = 4), Labrador Retriever (n = 3), Yorkshire Terrier (n = 3), Rottweiler (n = 2), mixed breed (n = 2), and 1 each of Akita Inu, Basset Hound, Belgian Malinois, Bernese Mountain dog, Boxer, Cane Corso, Cavalier King Charles Spaniel, French Bulldog, Great Dane, Shih Tzu, Siberian Husky, and Staffordshire Bull Terrier.

4.3.2. Clinical presentation and clinicopathologic findings in dogs with IBD

In the study group the most frequent clinical sign at presentation was diarrhea (25/26 dogs; 96%), followed by weight loss (20/26; 77%), anorexia/dysorexia (17/26; 65%), vomiting/regurgitation (14/26; 54%), polydipsia (6/26; 23%), hematochezia/

melena (5/26; 19%), tenesmus (5/26; 19%), and abdominal distension and pruritus (each 1/26; 4%). Clinicopathologic abnormalities included hypoproteinemia in 14/26 dogs (54%) and hypoalbuminemia in13/26 dogs (50%; median serum albumin concentration: 7 g/L, IQR: 6–13.9 g/L; reference interval: 23–40 g/L [St Priest's Clinique vétérinaire des Cérisioz] and 28–39 g/L [RVC]); 15 of the 26 dogs (54%) were hypocobalaminemic (median serum cobalamin concentration: 187 ng/L, IQR: 167–234 ng/L; reference interval: 300–800 ng/L [St Priest's Clinique vétérinaire des Cérisioz] and >200 ng/L [RVC]) and 21 dogs (81%) had a serum cobalamin concentration below 400 ng/L. Nineteen (73%) of the 26 dogs with IBD had a CCECAI score of ≥9 indicating severe or very severe clinical disease (Table 7); and endoscopic lesions in the duodenum were moderate or severe in 16/26 dogs (62%) and were at least of moderate severity in the colon in 2/5 dogs (40%). Clinical disease activity scores and the severity of endoscopic lesions and histopathologic findings are summarized in Tables 7 and 8. Eight of the dogs with IBD had been treated with steroids prior to evaluation and sample collection, and eight dogs had received an antibiotic.

4.3.3. Fecal S100A12 concentrations

Fecal cA12 concentrations in single spot samples were significantly higher in dogs with IBD than in healthy control dogs ($P < 0.0001$; Fig. 28). Using a cut-off concentration of 59 ng/g (Youden index and likelihood ratio), the sensitivity and specificity of fcA12 to distinguish dogs with IBD from healthy control dogs were 65%

Table 7 – Patient characteristics. Specific characteristics of dogs with inflammatory bowel disease (IBD; n=26) included in the study.

Duration of clinical signs, months (median [range])	3.0 [0.75–24.0]
Treated with glucocorticosteroids at the time of study (n [%])	8 [31%]
Treated with antimicrobials at the time of study (n [%])	8 [31%]
Clinical disease activity, CCECAI score (median [range])	10 [5–18]
- mild (n [%])	2 [8%]
- moderate (n [%])	5 [19%]
- severe (n [%])	9 [35%]
- very severe (n [%])	10 [38%]
Endoscopic disease severity, composite score[#] (median [range])	2 [0–6]
- gastric (median [range])	0 [0–3]
- duodenal (median [range])	2 [0–3]
- colonic (median [range])	1 [0–3]
Histologic disease severity, composite score[#] (median [range])	1 [1–3]
- gastric (median [range])	1 [0–2]
- duodenal (median [range])	1 [0–3]
- colonic (median [range])	1 [0–3]

CCECAI: canine chronic enteropathy clinical disease activity index (range of possible scores: 0–27; insignificant disease: 0–3, mild disease: 4–5, moderate disease: 6–8, severe disease: 9–11, very severe disease: ≥12) (Allenspach et al., 2007); [#]sum of all segments evaluated (range of possible scores: 0–3).

Table 8 – Histopathologic disease severity in dogs with IBD. Severity of morphologic and inflammatory histopathologic lesions found within each segment of the gastrointestinal tract evaluated in dogs with inflammatory bowel disease (IBD; n=26).

Histologic findings	N	Severity of lesions[†]			
		Normal	Mild	Moderate	Severe
Gastric fundus (composite score[$])	10	5	4	1	0
Morphologic criteria[#]					
- Surface epithelial injury	10	10	0	0	0
- Gastric pit epithelial injury	10	9	1	0	0
- Fibrosis/glandular nesting/MA	10	6	4	0	0
Inflammatory criteria[#]					
- Intraepithelial lymphocytes	10	9	1	0	0
- Lamina propria LPC	10	8	1	1	0
- Lamina propria eosinophils	10	10	0	0	0
- Lamina propria neutrophils	10	9	1	0	0
- Lamina propria macrophages	10	10	0	0	0
- Lymphofollicular hyperplasia	10	8	1	1	0
Gastric antrum (composite score[$])	9	6	2	1	0
Morphologic criteria[#]					
- Surface epithelial injury	9	8	1	0	0
- Gastric pit epithelial injury	9	7	1	1	0
- Fibrosis/glandular nesting/MA	9	8	1	0	0
Inflammatory criteria[#]					
- Intraepithelial lymphocytes	9	8	1	0	0

Table 8 – continued

Histologic findings	N	Severity of lesions[†]			
		Normal	Mild	Moderate	Severe
- Lamina propria LPC	9	7	1	1	0
- Lamina propria eosinophils	9	9	0	0	0
- Lamina propria neutrophils	9	7	1	1	0
- Lamina propria macrophages	9	9	0	0	0
- Lymphofollicular hyperplasia	9	8	1	0	0
Duodenum (composite score[$])	23	1	19	2	1
Morphologic criteria[#]					
- Villus stunting	23	3	19	0	1
- Epithelial injury	23	20	2	1	0
- Crypt distension	23	12	9	2	0
- Lacteal dilation	23	18	3	2	0
- Mucosal fibrosis	23	22	1	0	0
Inflammatory criteria[#]					
- Intraepithelial lymphocytes	23	19	3	1	0
- Lamina propria LPC	23	4	18	1	0
- Lamina propria eosinophils	23	21	2	0	0
- Lamina propria neutrophils	23	19	3	0	1
- Lamina propria macrophages	23	21	2	0	0
Colon (composite score[$])	7	2	3	1	1
Morphologic criteria[#]					
- Epithelial injury	7	5	1	0	1

Table 8 – continued

Histologic findings	N	Severity of lesions[†]			
		Normal	Mild	Moderate	Severe
- Crypt hyperplasia	7	4	3	0	0
- Crypt dilation and distortion	7	3	3	1	0
- Mucosal fibrosis and atrophy	7	6	1	0	0
Inflammatory criteria[#]					
- Lamina propria LPC	7	6	1	0	0
- Lamina propria eosinophils	7	7	0	0	0
- Lamina propria neutrophils	7	5	0	1	1
- Lamina propria macrophages	7	7	0	0	0

N: sample size; LPC: lymphocytes/plasma cells; MA: mucosal atrophy; [†]using the WSAVA criteria for histologic evaluation of gastrointestinal inflammation; [#]individual criteria were graded as normal (grade 0), mild (grade 1), moderate (grade 2), or severe lesions (grade 3); [$]the composite score reflects the highest grade for any individual morphologic and/or inflammatory criteria within the respective gastrointestinal segment.

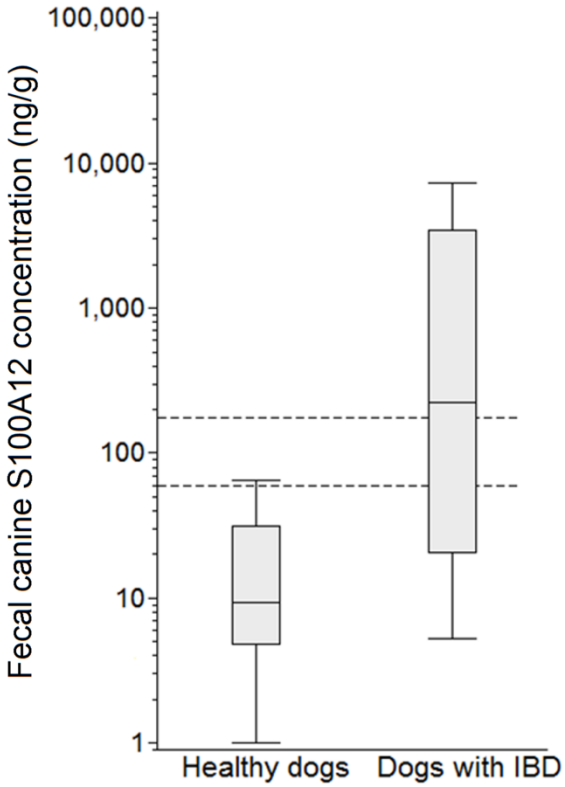

Figure 28 – Fecal canine S100A12 concentrations in healthy dogs and dogs with IBD. FcA12 concentrations in single spot samples were significantly higher in the 26 dogs with IBD (median [IQR]: 223 [21–3477] ng/g) than in the 90 healthy controls (median [IQR]: 9 [5–31] ng/g; $P < 0.0001$). Box plots indicate the median and interquartile range of fcA12 concentrations, the whiskers represent the inner Tukey fences, and the dashed lines represent the optimal criterion concentrations (59 and 174 ng/g) for distinguishing dogs with IBD from healthy controls.

(95% confidence interval [CI]: 44–83%) and 84% (95%CI: 75–91%), respectively, with an area under the ROC curve (AUC) of 81% (95%CI: 71–91%) and a misclassification of 20%. A cut-off concentration of 174 ng/g yielded a specificity of >90% (93%; 95%CI: 86–98%) but a lower sensitivity (54%, 95%CI: 33–73%) and a misclassification rate of 16%.

4.3.3.1. FcA12 and CCECAI, endoscopic lesions, and histologic disease severity in dogs with IBD

The fcA12 concentration and CCECAI score were significantly associated (Table 9). Moreover, fcA12 concentrations correlated with the severity of endoscopic lesions in the duodenum and colon (Table 9, Fig. 29), however, they failed to correlate with the sum score for histopathologic changes (Table 9). A total score comprising the composite endoscopic lesion score and CCECAI (weighted and unweighted) was found to have a slightly stronger association with fcA12 (Table 9). Only the endoscopic disease score in the duodenum and the composite endoscopic lesion score were significantly correlated with the CCECAI score (Spearman $\rho = 0.6398$, unadjusted $P = 0.0006$, Holm–Bonferroni adjusted $P = 0.0018$ and Spearman $\rho = 0.4413$, unadjusted $P = 0.0240$, Holm–Bonferroni adjusted $P = 0.0480$, respectively) whereas the severity of histologic lesions was not associated with clinical disease activity. The severity of macroscopic (endoscopic) and microscopic (histologic) disease was not correlated. Of the histopathologic criteria, only the composite inflammation score in the colon was significantly associated with the concentration of fcA12 (Table 10). Fecal cA12

Table 9 – Correlation of fcA12 with disease activity scores. Correlation between fecal S100A12 (fcA12) concentrations and the clinical disease activity, and the severity of endoscopic lesions and histopathologic changes in dogs with inflammatory bowel disease (IBD; n=26).

Parameter correlated with		**fecal canine S100A12 (ng/g)**		
	N	**Spearman ρ**	**unadjusted $P^{\dagger}$**	**adjusted $P^{\#}$**
CCECAI score	26	0.4778	**0.0136**	**0.0408**
Endoscopic disease score (sum)	26	0.4378	**0.0253**	0.0506
- Stomach	24	0.0703	0.7443	0.7443
- Duodenum	25	0.4703	**0.0177**	**0.0354**
- Colon	5	0.9747	**0.0048**	**0.0144**
Histologic disease score (sum)	26	-0.1400	0.4952	0.4952
- Stomach	10	0.1776	0.6234	0.6234
- Duodenum	23	-0.1975	0.3664	0.7328
- Colon	7	0.5426	0.2082	0.6246
CCECAI + composite endoscopic score[$]	26	0.5335 (0.4951)	**0.0050 (0.0101)**	**0.0100 (0.0303)**
CCECAI + hypocobalaminemia + composite endoscopic score[$]	26	0.5520 (0.4018)	**0.0035 (0.0419)**	**0.0105 (0.0419)**
CCECAI + hypocobalaminemia[$]	26	0.5109 (0.4298)	**0.0076 (0.0284)**	**0.0076** (0.0568)

N: sample size; CCECAI: canine chronic enteropathy clinical activity index; [†]without correction ($P < .05$); [#]after Holm-Bonferroni correction (n=3); [$]unweighed (weighed).

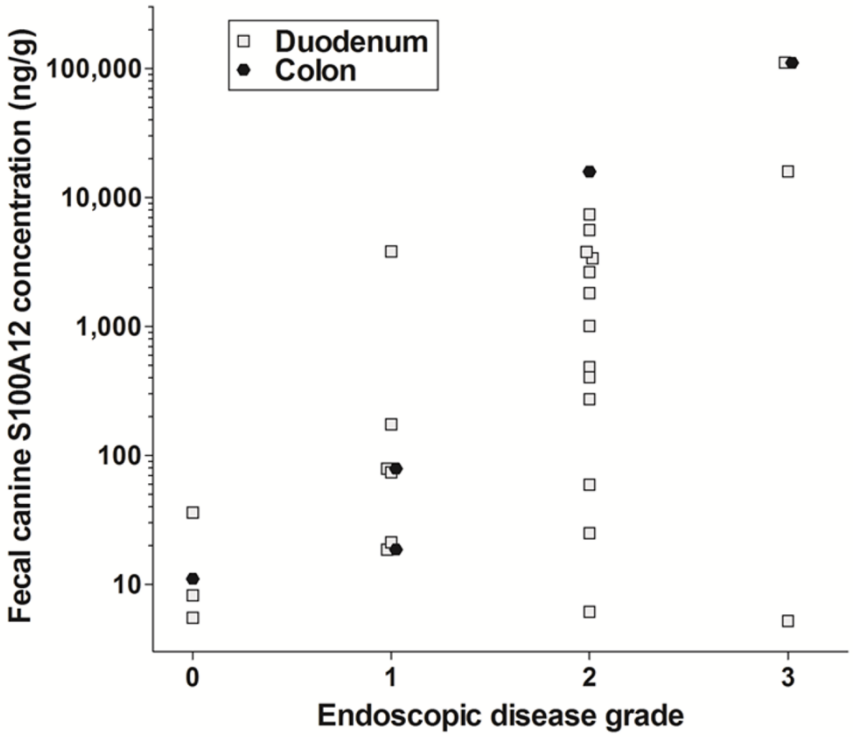

Figure 29 – Correlation of fecal canine S100A12 concentrations and macroscopic (endoscopic) disease severity. FcA12 concentrations in single spot samples correlated with the severity of endoscopic lesions in the colon ($\rho = 0.9747$, adjusted $P = 0.0144$; closed symbols) and the duodenum ($\rho = 0.4703$, adjusted $P = 0.0354$; open symbols).

Table 10 - Correlation of fcA12 with variables of the histologic score for canine IBD. Relationship between fecal S100A12 (fcA12) concentrations and the severity of morphologic and inflammatory histologic lesions found within the gastrointestinal tract in dogs with inflammatory bowel disease (IBD; n=26).

Histologic findings correlated with	**N**	**fecal canine S100A12 (ng/g)**		
		Spearman ρ	**unadjusted $P^{\dagger}$**	**adjusted $P^{\#}$**
Duodenum (composite score)	23	-0.3264	0.1285	0.2570
Morphologic criteria (sum)	23	-0.4268	**0.0422**	0.0844
- Villus stunting	23	-0.3297	0.1245	ns
- Epithelial injury	23	-0.4365	**0.0373**	0.1865
- Crypt distension	23	-0.2963	0.1698	ns
- Lacteal dilation	23	-0.2147	0.3252	ns
- Mucosal fibrosis	23	-0.3536	0.0979	ns
Inflammatory criteria (sum)	23	-0.1052	0.6328	ns
- Intraepithelial lymphocytes	23	0.0974	0.6584	ns
- Lamina propria LPC	23	0.3376	0.1151	ns
- Lamina propria eosinophils	23	-0.1861	0.3952	ns
- Lamina propria neutrophils	23	-0.5110	**0.0127**	0.0635
- Lamina propria macrophages	23	-0.4187	**0.0467**	ns
Colon (composite score)	7	0.5426	0.2082	ns
Morphologic criteria (sum)	7	0.2058	0.6579	ns
- Epithelial injury	7	0.7572	**0.0487**	0.1948
- Crypt hyperplasia	7	-0.4330	0.3318	ns
- Crypt dilation and distortion	7	0.6172	0.1398	ns
- Mucosal fibrosis and atrophy	7	-0.6124	0.1438	ns

Table 10 – continued

Histologic findings correlated with	**N**	**fecal canine S100A12 (ng/g)**		
		Spearman ρ	**unadjusted $P^{\dagger}$**	**adjusted $P^{\#}$**
Inflammatory criteria (sum)	7	0.8669	**0.0115**	**0.0230**
- Lamina propria LPC	7	0.2041	0.6606	ns
- Lamina propria eosinophils	7	-	-	-
- Lamina propria neutrophils	7	0.7572	**0.0487**	0.0974
- Lamina propria macrophages	7	-	-	-

N: sample size; LPC: lymphocytes/plasma cells; ns: non-significant; †without correction ($P < .05$); $^{\#}$after Holm-Bonferroni correction (n=2, 4, or 5).

concentrations were higher if the inflammatory infiltrate had a phagocytic component (i.e., neutrophils and macrophages) (median: 15,830 ng/g, IQR: 19–110,400 ng/g; n = 3 vs. median: 174 ng/g, IQR: 21–2640 ng/g; n = 23), but this difference did not reach significance ($P = 0.1991$).

Having a concentration of 273 ng fcA12/g or greater distinguished dogs with at least moderate endoscopic disease (a score ≥2) in any GI section from dogs with at most mild endoscopic disease (a score ≤1) with a sensitivity of 71% (95%CI: 44–90%) and a specificity of 89% (95%CI: 52–100%; AUC: 75%, 95%CI: 55–95%). Dogs with a duodenal lesion score of ≥2 were separated from dogs with a duodenal lesion score of ≤1 at the same fcA12 concentration (273 ng/g) and with a sensitivity and specificity of 75% (95%CI: 48–93%) and 89% (95%CI: 52–100%), respectively (AUC: 76%, 95%CI: 57–96%). Patients with very severe clinical disease (i.e., a CCECAI score of ≥12) were separated from those patients with a CCECAI score of <12 at the same cut-off concentration (273 ng/g) with 90% sensitivity (95%CI: 56–100%) and 75% specificity (95%CI: 48–93%; AUC: 82%, 95%CI: 66–98%) (Fig. 30).

4.3.3.2. FcA12 and clinicopathologic abnormalities and clinical presentation in dogs with IBD

In dogs with IBD, fcA12 concentrations were numerically higher in those with serum albumin concentrations <20 g/L (median 741 ng/g, IQR: 113–3,700 ng/g; n = 12) than in those with higher serum albumin concentrations (median: 55 ng/g, IQR: 10–2,297 ng/g; n = 14), but this difference was not significant ($P = 0.1358$). Also, no

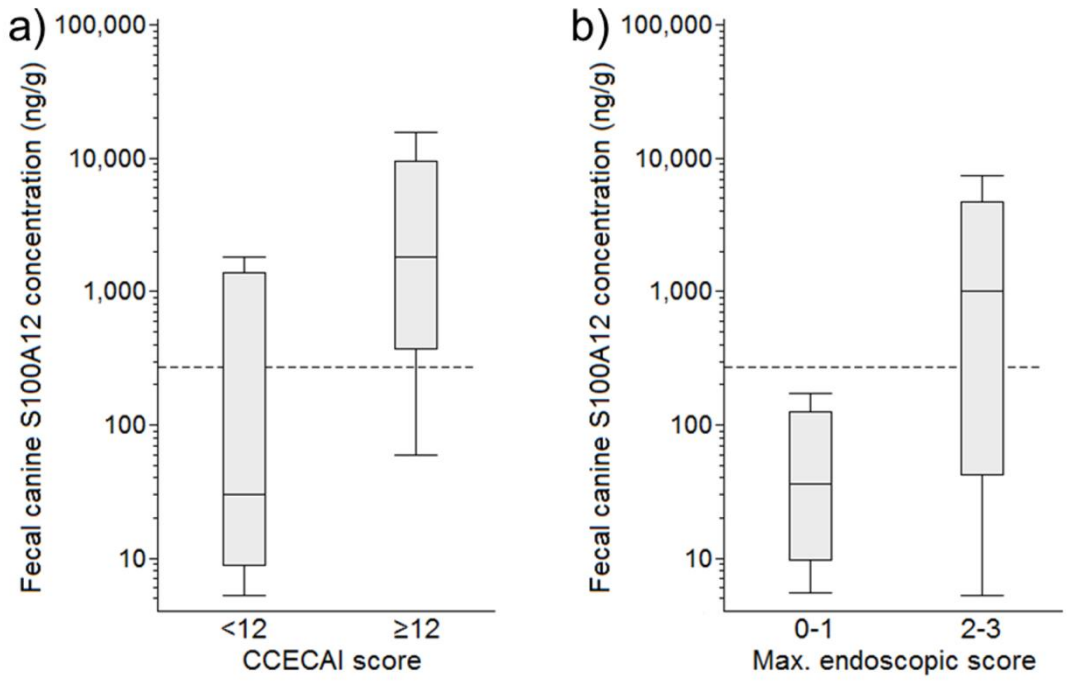

Figure 30 – Fecal canine S100A12 concentrations in IBD dogs dichotomized by the CCECAI score and the score reflecting endoscopic disease severity. (a) FcA12 concentrations were significantly higher in IBD dogs with very severe clinical disease (i.e., a CCECAI score of ≥12) (median [IQR]: 1,821 [371–9,485] ng/g) than in dogs a CCECAI score of less than 12 (median [IQR]: 30 [9–1395] ng/g; $P = 0.0078$). (b) Dogs with IBD and at least moderate severity of endoscopic lesions (i.e., a score of at least 2) in any of the GI segments had significantly higher fcA12 concentrations (median [IQR]: 1,001 [42–4,675] ng/g) than dogs with at most mild endoscopic disease severity (i.e., a score of ≤1) (median [IQR]: 36 [10–126] ng/g; $P = 0.0461$). Box plots show the median and IQR for fcA12concentration, the whiskers represent the inner Tukey fences, and the dashed lines represent the optimal fcA12 concentration (273 ng/g) for distinguishing the groups of dogs.

difference in fcA12 concentrations was seen between dogs with serum cobalamin concentrations <200 ng/L (median: 1,001 ng/g, IQR: 174–3,808 ng/g; n = 11) and those with serum cobalamin concentrations ≥200 ng/L (median: 59 ng/g, IQR: 8–2,640 ng/g; n = 15; P = 0.0821). Hypoalbuminemia and hypocobalaminemia were significantly associated (Pearson's χ^2= 7.72, P = 0.0055). The concentration of serum albumin was negatively related to the severity of clinical disease (Spearman ρ = −0.5642, unadjusted P = 0.0096, Holm–Bonferroni adjusted P = 0.0288) but was not associated with the severity of endoscopic lesions (Spearman ρ = −0.0684, P = 0.7745) or histologic changes (Spearman ρ = 0.4526, unadjusted P = 0.0451, adjusted P = 0.0902).

Fecal cA12 concentrations in IBD dogs that had received steroids at the time of evaluation (median: 155 ng/g, IQR: 22–3,700 ng/g; n = 8) did not differ from those in dogs that had not (median: 288 ng/g, IQR: 17–2,925 ng/g; n = 18; P = 0.9778). Fecal cA12 concentrations were also not different in dogs with IBD that had received antibiotics prior to evaluation (median: 49 ng/g, IQR: 7–2,782 ng/g; n = 8) compared to dogs that had not been treated with antimicrobials (median: 442 ng/g, IQR: 47–3,801 ng/g; n = 16; P = 0.3123). FcA12 concentrations did not correlate with the chronicity of GI signs observed (ρ = −0.0741, P = 0.7369). Although the number of dogs with large intestinal involvement was small (n = 5), concentrations of fcA12 did not differ significantly between this patient group (median: 174 ng/g, IQR: 10–63,115 ng/g) and dogs with only confirmed small intestinal disease (median: 273 ng/g, IQR: 23–3,008 ng/g; n = 21; P = 0.7450).

4.4. Discussion

Endoscopy, histopathologic confirmation of inflammation, and ruling out known causes of intestinal inflammation are considered the gold standard for diagnosing canine IBD (Washabau et al., 2010). However, endoscopy is time-consuming and invasive and, in veterinary medicine, it requires general anesthesia and, before colonoscopy, thorough preparation of the colon. Surrogate biomarkers that objectively reflect mucosal disease severity and could help the clinician monitor GI inflammation might be useful in clinical practice.

To our knowledge, this paper is the first to report fcA12 concentrations in dogs with IBD. Concentrations of fcA12 were significantly higher in dogs with IBD, which agrees with a more than 10-fold observed up-regulation of S100-mRNA at the mucosal level in dogs with IBD in one study (Wilke et al., 2012). However, there was also an overlap of fcA12 concentrations between dogs with IBD and healthy dogs. Concentrations of fcA12 in healthy dogs were comparable to those found in healthy people (Kaiser et al., 2007). However, the fcA12 concentrations in dogs with IBD were lower than those in people with active Crohn's disease (CD) or ulcerative colitis (Kaiser et al., 2007; van de Logt and Day, 2013). This difference could be explained by the smaller number of granulocytes found in the intestinal mucosa of dogs with IBD. Alternatively, it could be due to differences in S100A12 expression and release from canine granulocytes and other cell lines, or species-specific differences in the pathways involved in canine granulocyte activation. Likewise, the cut-off for distinguishing dogs with active IBD from healthy dogs was about 10-fold lower than that in people (Kaiser

et al., 2007). It should also be noted that immunoassays are not truly analytical because they are based on antigen–antibody binding which can be affected by several factors, such as antibody avidity. Thus, the difference between fcA12 in people and in dogs may not represent a true difference in fecal concentrations between these two species, but rather a difference in the efficiency of the two assay systems to measure S100A12. Regardless, the significant difference in fcA12 concentrations between dogs with IBD and healthy control dogs suggests that the number of infiltrating phagocytes, which tend to appear later in the course of IBD, and/or their activation status in dogs is sufficient to cause increases in fcA12. These results also support that phagocyte activation plays a role in the pathophysiology of canine IBD. An increased expression and/or secretion of the proinflammatory (Yang et al., 2001; Hofmann et al., 1999), phagocyte-specific DAMP molecule S100A12 support the important role of the innate immunity in canine IBD (Burgener et al., 2008; Luckschander et al., 2010; Kathrani et al., 2012) and could be an important factor for the recruitment of cells of the acquired immunity.

The diagnosis of IBD requires the collection of biopsies, which may not be practical to routinely re-assess disease activity at follow-up examinations. FcA12 concentrations and the CCECAI scores were equally correlated with the severity of endoscopic disease in the duodenum. FcA12 correlated slightly more closely with endoscopic disease severity in the colon, although the sample size was very small. This is consistent with findings in people with CD (van de Logt and Day, 2013) and suggests that fcA12 could be a marker to assess the severity of endoscopic lesions. A possible explanation for a closer correlation offcA12 with the endoscopic disease severity in the

colon could be an increased granulocytic expression of cA12 in canine IBD localized to the colon and/or lack of stability of extracellular cA12 as it travels aborad through the GI tract. However, further studies are needed to confirm these findings and to determine whether fecal fcA12 concentrations decrease with endoscopic mucosal healing and could thus be useful to monitor endoscopic remission. Although endoscopic disease activity has received little attention in canine IBD, severe mucosal lesions in the duodenum were associated with a negative outcome in dogs with chronic enteropathies (Allenspach et al., 2007). Also, endoscopic lesions improved with successful treatment of dogs with chronic enteropathies (García-Sancho et al., 2007). Thus, further research to determine the practical value of fcA12 as an inflammatory marker for dogs with IBD is warranted.

Like others (García-Sancho et al., 2007; McMahon et al., 2010; Mandigers et al., 2010), we found no correlation between clinical signs and the severity of histologic lesions in dogs with IBD. Also, the lack of a relationship between endoscopic and histologic disease severity (assessed by an unweighted scoring system that considers both morphologic and inflammatory changes), as seen in this study, has been reported previously (Allenspach et al., 2007; García-Sancho et al., 2007). This may reflect the localized nature of the disease process. The lack of a correlation between clinical disease severity and the degree of histologic changes could also be explained by the functional pathophysiology of canine IBD. Inflammation and inflammatory mediators (even if localized to only a segment of the GI tract) can significantly affect intestinal epithelial cell function and structure. On the other hand, a change in GI physiology and epithelial

barrier function can cause immune-mediated mucosal damage and inflammatory mediator expression.

FcA12 concentrations were not associated with the site of inflammatory lesions within the GI tract which agrees with one study in people with IBD (Kaiser et al., 2007). However, it contrasts with the strong dependence on disease location demonstrated by others, who found a significantly higher release of S100A12 from the colonic mucosa than from the ileal mucosa in patients with active CD (Föll et al., 2008). However, not all dogs in our study had both upper and a lower GI endoscopy performed. Also, the potential contribution of colonic and/or ileal lesions to fcA12 concentrations cannot be ruled out in these cases. Likewise, the possibility of lesions in sections of the GI tract that are not within reach of the endoscope (e.g., the jejunum) cannot be excluded. Another assumption made for analytes measured in fecal samples is their ability to withstand the GI passage. Although we have demonstrated the short-term stability of cA12 in fecal samples stored at −80°, −20°, +4°, and approximately +23°C for 7 days (Heilmann et al., unpublished data), its stability within the GI tract has not been investigated and further studies are needed to clarify this issue.

FcA12 and CCECAI were correlated in this study which is consistent with the relationship between fecal S100A12 and the colitis activity index (CAI; a scoring system based on clinical disease severity scores similar to the CCECAI) in human patients with ulcerative colitis but not the lack of correlation with the CD activity index (a clinical disease activity index used in people with CD) (Kaiser et al., 2007). Interestingly, 2 dogs in our study group that had ulcerative lesions in the colon and a mixed inflammatory

infiltrate were assessed as having very severe (CCECAI score of 13 and 17) clinical disease. However, these 2 cases were not found to be the main contributors to the significant relationship between fcA12 and CCECAI. Lack of a correlation between fcA12 and the severity of histologic changes is in contrast with findings in people with CD or ulcerative colitis where fecal A12 showed a moderate correlation with an inflammation score reflecting histologic disease severity (Kaiser et al., 2007). This may again reflect the localized nature of the disease process, the smaller number of granulocytes found in the intestinal mucosa of dogs with IBD, and/or species-specific differences in the functional pathophysiology of canine IBD.

Although fcA12 concentrations ranged between 1 and 1,810 ng/g in the healthy control group of dogs and were significantly different from concentrations of fcA12 in dogs with IBD (range: 5–110,400 ng/g), an overlap between both groups was noted. However, in most of the healthy dogs (93%), fcA12 concentrations were <170 ng/g, with the remaining dogs clustering between 635 and 1,810 ng/g. The higher fcA12 concentrations in 6 of the control dogs might be explained by biological variation. However, because ethical considerations prevented us from performing more invasive studies in the control dogs, the presence of subclinical intestinal inflammation in individual dogs, which may have led to falsely classifying dogs with subclinical inflammation of the GI mucosa as healthy, cannot be definitively ruled out. There was no reason to remove these 6 dogs as outliers from the data set, and establishment of fcA12 concentrations in healthy dogs and further stratification of the reference sample

group (e.g., by body condition score, recent vaccination) will be needed to clarify whether these are true outliers.

Interestingly, serum cobalamin concentration was decreased in more than 50% of dogs with IBD in this study, and more than 80% of the dogs had a serum cobalamin concentration <400 ng/L. These numbers are higher than previously reported in dogs with GI diseases (Allenspach et al., 2007; Berghoff et al., 2013), but are consistent with the frequency of hypoalbuminemia in dogs in this study. This finding indicates that, beyond the need of cobalamin supplementation in the majority of dogs with IBD, ileal lesions would have remained undiagnosed. However, cobalamin-dependent metabolites, such as the concentration of serum methylmalonic acid and/or serum homocysteine, were not evaluated in this study.

A major limitation of the study is the fact that the patients were from 2 different institutions, introducing inter-operator variation. Also, endoscopic disease severity in dogs (n = 9) enrolled at the second center (RVC) was graded retrospectively based on endoscopy reports that were generated using currently accepted guidelines (Washabau et al., 2010; Day et al., 2008). The inclusion of only dogs with a histopathologic diagnosis of IBD and no concurrent diseases that could affect the GI tract as well as the tertiary care setting are sources of potential bias. Such a bias may have artificially increased the diagnostic accuracy (Bossuyt et al., 2003a) of fcA12 found in this study. Also, fcA12 was measured in samples that had been stored for up to 15 months. Although we have demonstrated that fcA12 is stable at various temperatures for 7 days (Heilmann et al., unpublished data), the long-term stability of fcA12 warrants further study. A further

limitation of this study is that ileal lesions, which often do not correlate with lesions found in the duodenum or colon (Washabau et al., 2010; Casamian-Sorrosal et al., 2010), were not evaluated. Also, chronic enteropathies in dogs are classified according to the response to therapeutic intervention as food-responsive diarrhea, antibiotic-responsive diarrhea, or steroid-responsive idiopathic IBD (Allenspach et al., 2007). As it was the aim of this study to evaluate the association of fcA12 concentrations with clinical, endoscopic and histologic disease severity, the potential of fcA12 to distinguish dogs with IBD that may respond to various therapies was not investigated. Thus, further prospective studies are needed to evaluate this possibility by further stratifying the patient population according to the response to diet, antibiotics, or steroids and/or other immunosuppressant medications as well as according to disease outcome. Further work is also needed to determinefcA12 concentrations in infectious gastroenteritis.

The results of this study are a first step toward the clinical evaluation of fcA12 as a non-invasive marker for diagnosing and monitoring GI inflammation in dogs. Further, evaluation of the role of cA12 in spontaneous inflammatory diseases in dogs (such as IBD) can potentially be translated to human medicine, where rodent models cannot be used to study A12 as it has been lost during rodent evolution (Fuellen et al., 2004).

4.5. Conclusions

In summary, fecal canine S100A12 (fcA12) concentrations were increased in dogs with IBD compared to healthy control dogs, but were only moderately accurate in distinguishing dogs with IBD from healthy controls. In dogs with IBD, fcA12 correlated

with the clinical disease activity, the severity of intestinal endoscopic (macroscopic) lesions, and with the histologic inflammatory changes in the colon but not with the severity of histologic lesions overall. These results suggest that fcA12 may be a useful adjunct for assessing macroscopic (endoscopic) disease severity in canine IBD. Further research is needed to assess the utility of measuring fcA12 in clinical practice and to evaluate the potential of fcA12 in canine IBD to reflect clinical remission and/or mucosal healing.

5. SYSTEMIC LEVELS OF THE ANTI-INFLAMMATORY DECOY RECEPTOR SOLUBLE RAGE (RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS) ARE DECREASED IN DOGS WITH INFLAMMATORY BOWEL DISEASE[§]

5.1 Introduction

Inflammatory bowel disease (IBD) is a common disease in dogs and a dysregulated innate immunity appears to play a major role in its pathogenesis (Jergens and Simpson, 2012). S100A12 (also known as extracellular newly identified RAGE binding protein [EN-RAGE]) is an endogenous damage-associated molecular pattern (DAMP) molecule that is involved in phagocyte activation and has been shown to be increased in serum and fecal specimens from dogs with IBD (Heilmann et al., 2012b). Fecal canine S100A12 concentrations were also associated with endoscopic disease severity in dogs with IBD (Heilmann et al., 2014a). S100/calgranulins, such as S100A12, a family of small calcium-binding proteins that accumulate at sites of inflammation, represent ligands for the receptor of advanced glycation end products (RAGE) (Hofmann et al., 1999), a multi-ligand pattern-recognition receptor and member of the immunoglobulin superfamily with potential similarities to the family of Toll-like receptors. Results of studies in humans with IBD suggest a role of RAGE in chronic inflammation (Hofmann et al., 1999). The ligand–RAGE interaction has been shown to

[§]Reprinted with permission from Heilmann, R.M., Otoni, C.C., Jergens, A.E., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2014. Systemic levels of the anti-inflammatory decoy receptor soluble RAGE (receptor for advanced glycation end products) are decreased in dogs with inflammatory bowel disease. *Veterinary Immunology and Immunopathology* 161, 184-192, Copyright (2014) by Elsevier.

induce sustained post-receptor signaling, including activation and nuclear translocation of nuclear factor (NF)-kappa B (Basta et al., 2002; Bucciarelli et al., 2002), and the upregulation of RAGE itself is believed to lead to perpetuation and amplification of the inflammatory response (Bierhaus et al., 2005; Li and Schmidt, 1997).

Human RAGE is only constitutively expressed in the lung and skin, whereas in other cell lines (e.g., monocytes/macrophages, endothelial cells) postembryonic RAGE expression is induced by either accumulation of its ligands and/or activation of transcription factors (e.g., NF-kappa B) that regulate RAGE expression (Schmidt et al., 2000; Bierhaus et al., 2005). Human RAGE is believed to have the potential to convert a transient inflammatory response (e.g., cell stress, NF-kappa B activation) into a sustained cellular inflammatory response (Bierhaus et al., 2005). The expression of RAGE coincides with a proinflammatory microenvironment (e.g., in IBD), and the upregulation of RAGE has been shown to increase the recruitment of inflammatory cells through the upregulation of endothelial adhesion molecules VCAM-1 and ICAM-1 (Schmidt et al., 2000; Bierhaus et al., 2005).

Soluble RAGE (sRAGE), a truncated splice-variant of RAGE, functions as a decoy receptor sequestering ligands such as DAMPs (e.g., S100A12) thus preventing ligand interaction with cell surface RAGE. Soluble RAGE has been shown to be decreased in humans with chronic inflammatory diseases (Bierhaus et al., 2005) such as inflammatory bowel disease (Meijer et al., 2014), juvenile idiopathic arthritis (Myles et al., 2011), Sjögren's syndrome (Stewart et al., 2008), asthma and chronic obstructive pulmonary disease (Sukkar et al., 2012) and acute Kawasaki disease (Wittkowski et al.,

2007). Soluble RAGE has also been suggested to be a potential therapeutic target in patients with chronic inflammatory conditions (Hudson et al., 2003) based on an improved outcome in an experimental model of colitis (Hofmann et al., 1999). Moreover, experimental studies suggest that sequestration of ligands by sRAGE prevents interaction of these ligands with RAGE, but also with other pattern-recognition receptors (Bierhaus et al., 2005).

Canine RAGE has been characterized (Murua Escobar et al., 2006; Sterenczak et al., 2009), and the existence of its naturally occurring splicing variants and the expression of RAGE in different tissues has been described (Sterenczak et al., 2009, 2011). However, the sRAGE/RAGE axis has not been studied in canine IBD. Thus, this study aimed to validate an ELISA for the measurement of sRAGE in dogs, and to evaluate serum sRAGE and serum/fecal S100A12 concentrations in the diagnosis and treatment of canine IBD. We hypothesized that (1) serum sRAGE concentrations are decreased in dogs with IBD compared to healthy controls, and (2) the serum concentration of sRAGE in dogs with IBD correlates with clinical disease activity, severity of histologic lesions, outcome, and/or the concentration of S100A12 in serum and/or fecal specimens.

5.2 Materials and Methods

5.2.1. Study population

Serum and fecal samples were collected from 35 dogs between 2011 and 2013. The study was approved by the Iowa State University (ISU) Institutional Animal Care

and Use Committee and the Institutional Clinical Research Review Committee at Texas A&M University, and owner consent was obtained before enrollment of dogs. Information on some of the dogs used in this study has been previously reported (Otoni et al., 2012).

5.2.1.1. Patient group

Twenty of the dogs were presented to the ISU Veterinary Teaching Hospital for further evaluation of chronic gastrointestinal signs (i.e., vomiting and/or diarrhea of at least 3 weeks duration). Dietary and antibiotic trials had been performed in 8 and in 9 of the dogs, respectively. Prior to presentation, 3 dogs had received prednisone and 3 dogs had received supplemental cyanocobalamin injections weekly. A complete work-up was performed in all dogs, which included an upper gastrointestinal endoscopy (esophagogastroduodenoscopy) and collection of endoscopic tissue biopsies. The histopathologic criteria established by the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization group (Day et al., 2008; Washabau et al., 2010) were used to assess histologic disease. Clinical disease activity was assessed using the canine IBD activity index (CIBDAI) scoring system (Jergens et al., 2003). A serum sample and 3 fecal samples collected on 3 consecutive days were obtained upon presentation. Following the diagnostic workup, dogs received prednisone, prednisone and cyclosporine, or budesonide and/or metronidazole, and were given a commercial elimination (i.e., novel protein or hydrolyzed protein) diet. Six dogs received parenteral supplemental cobalamin, and a fiber supplement was added in 2 patients. An additional

serum and 3 fecal samples were collected when patients were re-evaluated approximately 3 weeks after the initial evaluation at the ISU Veterinary Teaching Hospital after initiation of medical treatment. At that time the response to treatment was assessed using the CIBDAI scoring system (complete clinical remission was defined as a ≥75% decrease in CIBDAI; partial response was defined as a 25–75% decrease in CIBDAI; and no response was defined as a ≤25% decrease in CIBDAI).

5.2.1.2. Healthy controls

Serum was collected from 15 healthy control dogs (based on a non-invasive assessment of health) at the College of Veterinary Medicine at Texas A&M University (n = 2) and at the ISU Veterinary Teaching Hospital (n = 13). Fecal samples from 3 consecutive days were also collected from the 13 healthy dogs enrolled at ISU.

5.2.2. Canine RAGE Western blot

5.2.2.1. Extraction of tissues

For anti-canine RAGE analysis, a leukocyte extract was prepared as follows: white blood cells were harvested after dextran sedimentation and hypoosmolar erythrolysis. Cells were then suspended in lysis buffer (0.05 M Tris/HCl, 0.15 M NaCl, 1% [v/v] Igepal-630 [NP-40], pH 7.4; with one proteinase inhibitor tablet [Complete EDTA-free proteinase inhibitor cocktail tablets, Roche Diagnostics, Mannheim, Germany] added per 10 mL of buffer) followed by repeated sonication and freeze-thaw

cycles, and finally, harvesting the supernatant fraction after centrifugation at 15,000×g for 12 min at +4°C and clearance by filtration through a 0.45 μm-pore size filter.

Based on the tissue expression of RAGE in humans (Schmidt et al., 2000), canine lung and cerebral cortex were included as positive control tissues. Canine lung and brain tissue extracts for anti-canine RAGE analysis were prepared by adding 30 mL of lysis buffer to 5 g of surplus tissue, followed by repeated sonication and freeze–thaw cycles, and finally, harvesting the supernatant fraction after high-speed centrifugation and filtration.

5.2.2.2. Western blotting

All samples were subjected to SDS–PAGE (10% Bis/Tris gel, Invitrogen, Carlsbad, CA, USA) followed by electrotransfer onto a PVDF membrane for 7 min at 20 V (iBlot®, Invitrogen, Carlsbad, CA, USA). Prior to immunoblot analysis, membranes were blocked overnight at 4°C with 0.01 M Tris/HCl, 0.15 M NaCl, 0.05%Tween, 10% (w/v) BSA; pH 7.6 (block buffer) followed by a 1 h-incubation at room temperature (approximately 23°C) with the primary Ab (i.e., goat polyclonal anti-recombinant human RAGE [AHP594, AbD serotec, Bio-Rad Laboratories, Hercules, CA, USA] diluted 1/500-1/5000 or sheep polyclonal anti-recombinant canine RAGE [Sheep anti-recombinant canine RAGE, R&D Systems, Minneapolis, MN, USA] at 50 μg/L). Subsequently, membranes were washed 4 times 5 min in 0.01 M Tris/HCl, 0.15 M NaCl, 0.05% Tween, pH 7.6 (wash buffer) and then incubated for 1 h at approximately 23°C with secondary biotin-conjugated Ab (Biotin-conjugated rabbit anti-goat IgG, Thermo

Scientific, Rockford, IL, USA; Biotin-conjugated rabbit anti-sheep IgG; Thermo Scientific, Rockford, IL, USA; 60 μg/L). Following another 4 times 5 min washes in wash buffer, membranes were incubated for 45 min at approximately 23°C with NeutrAvidin-Horseradish peroxidase (NeutrAvidin-horseradish peroxidase [NA-HRP], Thermo Scientific, Rockford, IL, USA; 200 μg/L). Finally, blots were washed 4 times 5 min in wash buffer and protein bands were detected by use of a stabilized substrate (1-Step™ TMB-Blotting solution, Thermo Scientific, Rockford, IL, USA).

5.2.3. Canine serum soluble RAGE (sRAGE) ELISA

5.2.3.1. ELISA procedure

Enhanced-binding ELISA plates (MaxiSorp™ Nunc-Immuno™ Plates, Thermo Scientific, Rockford, IL,USA) were coated with affinity-purified polyclonal anti-canine RAGE Ab (Sheep anti-recombinant canine RAGE, R&D Systems, Minneapolis, MN, USA; 150 ng/well) in 0.2 M carbonate-bicarbonate; pH 9.4, incubated for 1 h at 37°C, and washed twice with 0.1 M PBS, pH 7.2 and once with PBS containing 0.05% (v/v) polyoxyethylene-20 sorbitan monolaurate. Nonspecific binding sites were blocked with PBS supplemented with 30% (v/v) newborn calf serum, 1% (v/v) Triton X-100; pH 8.0 (assay buffer), plates were incubated for 1 h at 37°C and again washed three times as described. Calibrator solutions with different canine RAGE concentrations (Recombinant canine RAGE, R&D Systems, Minneapolis, MN, USA; 5, 2, 1, 0.5, 0.2, 0.1, and 0.02 ng/mL in assay buffer) and test samples (assayed in a 1:2 dilution in assay buffer) were applied to each plate in duplicates of 100 μL solution. For the blanks, the

canine RAGE standard was replaced by assay buffer. Plates were incubated again for 1 h at 37°C and washed as described. To detect captured Ag, plates were incubated with biotinylated anti-canine RAGE (Biotinylated anti-recombinant canine RAGE, R&D Systems, Minneapolis, MN, USA; 60 ng/well) for another hour at 37°C and washed three times. Then, the plates were incubated with NA-HRP diluted in PBS, 1% (w/v) BSA (20 ng/well) for another hour at 37°C, washed three times, and developed with a stabilized 3,3',5,5'-tetramethylbenzidine substrate (1-Step™ Ultra TMB-ELISA, Thermo Scientific, Rockford, IL, USA). After 10 min incubation, the reaction was stopped by adding 4 M acetic acid, 0.5 M sulfuric acid, and the absorbance was measured in each well at 450 nm using an automated plate reader (Synergy 2 Alpha Microplate Reader, BioTek®, Winooski, VT, USA). A commercial software (Gen5™ Data Analysis Software [v1.05], BioTek®, Winooski, VT, USA) was used to calculate a 5-parameter logistic curve fit ($y = f[x] = d + [(a–d)/(1 + (x/c)^b)^e]$, where y is the dependent variable, x is the independent variable, and a through e describe the shape of the curve) and to determine canine serum sRAGE concentrations in test samples.

5.2.3.2. Partial analytical validation of the ELISA

The RAGE ELISA was analytically validated by determination of the lower detection limit, dilutional parallelism, spiking recovery, and intra- and inter-assay variability. Lower detection limit of the assay was determined by calculating the mean response plus three SD for 5 replicates (10 wells) of the blank solution and transposing this value onto the standard curve. Dilutional parallelism was determined by evaluating

one serum sample at a serial 2-fold dilution from 1:2 to 1:8 and three samples with higher serum canines RAGE concentrations at serial twofold dilutions from 1:2 to 1:16. The remaining validation parameters were determined using four different serum samples in a dilution of 1:2 with assay buffer. Spiking recovery was determined by adding 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, and 2.5 ng/mL recombinant canine RAGE (R&D Systems) to each of four test samples and calculating the percentage of standard Ag recovery ([observed value (ng/mL)/expected value (ng/mL)] × 100). Intra-assay variability of the ELISA was evaluated by assaying four serum samples 8 times each within the same assay and calculating the intra-assay coefficients of variation for each sample (%CV = [SD/mean] × 100). Inter-assay variability of the assay was determined by analyzing four serum samples in 8 consecutive assay runs and calculating inter-assay %CVs.

5.2.4. Serum sRAGE and serum and fecal S100A12 analysis

Serum sRAGE concentrations were measured in serum samples (diluted 1:2) using the canine sRAGE ELISA described above. Serum and fecal canine S100A12 (cS100A12) concentrations were measured by an in-house ELISA previously described (Heilmann et al., 2014).

5.2.5. Data and statistical analysis

Statistical analyses were performed using commercial software packages (JMP® v10.0, SAS Institute, Cary, NC, USA; GraphPad Prism® v5.0, GraphPad Software, San

Diego, CA, USA). A Shapiro–Wilk W and a Brown–Forsythe test were used to assess normality and equal variances of the data. A Wilcoxon rank sum test was used to compare values in dogs with IBD vs. healthy controls, and in dogs with a positive outcome (i.e., clinical remission) vs. those that were euthanized. A Spearman rank sum correlation coefficient was used to assess the potential relationship of serum sRAGE concentrations with clinical disease activity (using the CIBDAI scoring system), serum and fecal cS100A12 concentrations, and histologic disease severity (using a 4-point semi-quantitative grading system). Associations were tested using a Fisher's exact test or the likelihood ratio test, as appropriate, and the odds ratio (OR) and the 95% confidence interval (CI) were calculated. A receiver-operating characteristic curve was generated and the likelihood ratio was used to calculate sensitivity and specificity at the optimum sRAGE concentration to distinguish dogs with IBD from healthy controls.

5.3. Results

Canine RAGE-pAb was demonstrated to be specific for canine RAGE as indicated by Western blot analysis (Fig. 31) whereas the polyclonal anti-recombinant human RAGE did not detect the canine RAGE protein. An ELISA for measuring canine sRAGE concentrations in serum samples was established and analytically validated. The lower detection limit of the canine sRAGE assay was 26 ng/L, which translates into a serum canine sRAGE concentration of 52 ng/L considering a 1:2 dilution used for serum samples. Observed-to-expected (O/E) ratios for dilutional parallelism of four serum samples ranged from 73.2 to 122.0% (mean ± SD: 103.3 ± 18.3%), and O/E ratios for

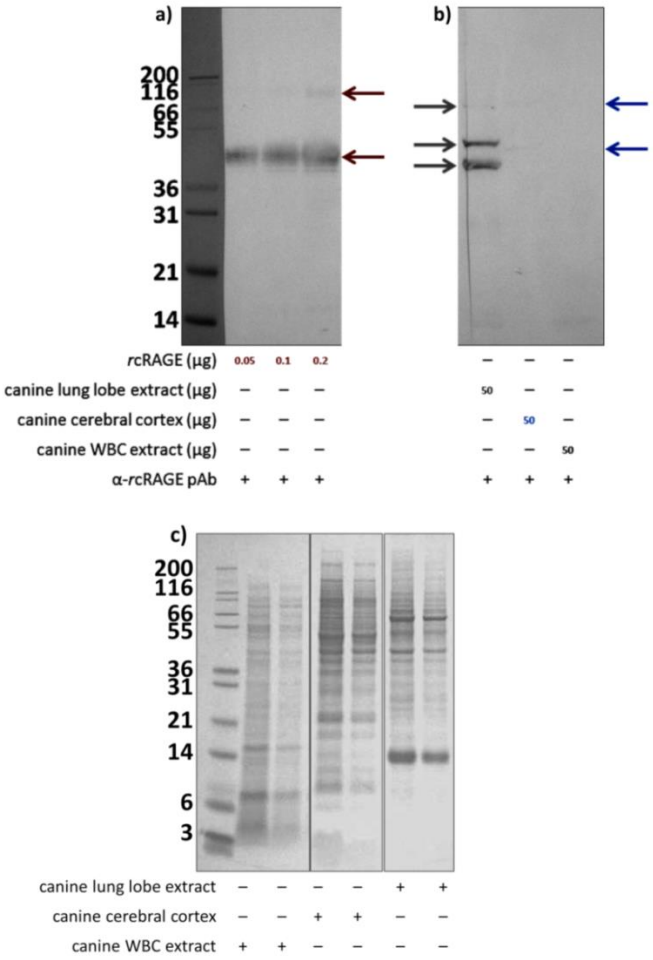

Figure 31 – Antibody specificity evaluated by Western blotting. Western blot analysis using the polyclonal anti-recombinant canine RAGE (α-rcRAGE) revealed (a) monomeric and dimeric forms of rcRAGE (red arrows) and (b) canine RAGE in tissue extracts from dogs, with two different isoforms of monomeric canine RAGE as well as an assembly of dimers found in the lung (gray arrows) where RAGE is constitutively expressed. Only faint bands of monomeric as well as dimeric canine RAGE were detected in the extract of cerebral cortex (blue arrows). No bands were detected in canine white blood cells (WBC) where postembryonic RAGE expression is induced. (c) Loading controls (SDS-PAGE) for the tissue extracts are also shown.

spiking four different serum samples with seven different canine RAGE concentrations ranged from 76.3 to 103.9% (mean ± SD:91.3 ± 7.3%). Coefficients of variation for intra- and inter-assay variability were 10.7, 8.1, 4.1, and 3.7% and 19.3, 11.5, 21.7, and 6.5%, respectively. Serum canine sRAGE concentrations in 15 healthy dogs ranged from below the detection limit of the assay (52 ng/L) – 802 ng/L (median: 448 ng/L).

Serum sRAGE concentrations were significantly lower in dogs with IBD (median [interquartile range, IQR]: <52 [<52–309] ng/L) than in healthy controls (448 [326–536] ng/L; $p = 0.0003$; Fig. 32). Although the sample size was small, using 340 ng/L as a cut-off, the sensitivity and specificity of serum sRAGE to distinguish dogs with IBD from healthy controls was 90% and 73% (area under the curve: 83%). Patient characteristics and serum and fecal variables are summarized in Tables 11 and 12, respectively. No differences were seen in age ($p = 0.174$) and sex distribution ($p = 1.000$) between both study groups. Serum sRAGE concentrations were not associated with the severity of histologic changes ($\rho = 0.2009$; $p = 0.4241$), the CIBDAI score before ($\rho = 0.3922$; $p = 0.0967$) or after treatment ($\rho = -0.3929$; $p = 0.1067$), the serum cS100A12 concentration before ($\rho = 0.0173$; $p = 0.9214$) or after treatment ($\rho = -0.1937$; $p = 0.4411$), the fecal cS100A12 concentration before ($\rho = -0.3160$; $p = 0.0732$) or after treatment ($\rho = -0.1153$; $p = 0.6596$), or with the individual outcome ($p = 0.4066$). Clinical remission ($p = 0.5727$) and the change in serum ($\rho = 0.1241$, $p = 0.6126$) and fecal cS100A12 ($\rho = -0.0500$, $p = 0.8439$) were not significantly associated with a change in serum sRAGE concentration after initiation of treatment.

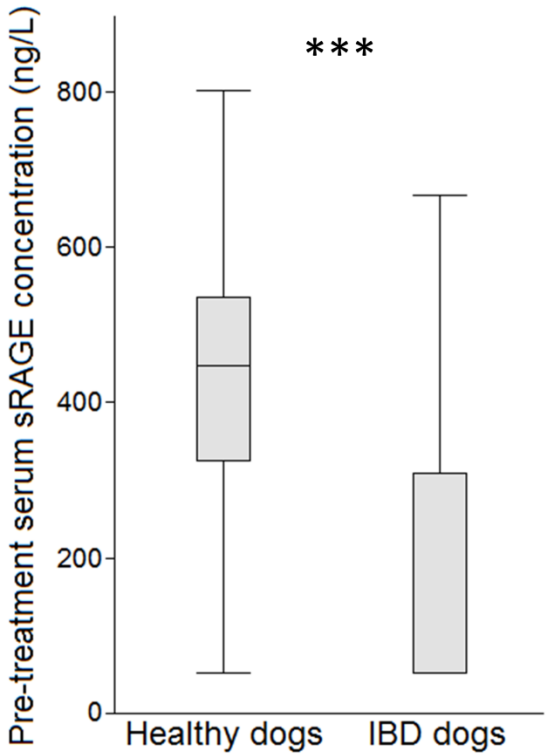

Figure 32 – Serum sRAGE concentrations in dogs with IBD compared to healthy control dogs. Pre-treatment serum sRAGE concentrations were significantly lower in dogs with IBD (median [IQR]: <52 [<52–309] ng/L) than in healthy controls (448 [326–536] ng/L; $p = 0.0003$).

Table 11 – Clinical characteristics of dogs with IBD.

Age (median [range]), years	7.5 [2-12]
Sex (male/female)	8/12
Treated with glucocorticosteroids at the time of study (n [%])	3 [15%]
Treated with antimicrobials at the time of study (n [%])	9 [45%]
Clinical disease activity, CIBDAI score (median [range])	6 [1–13]
- mild (n [%])	7 [35%]
- moderate (n [%])	6 [30%]
- severe (n [%])	7 [35%]
Histologic disease severity, grade (median [range])[#,$]	1 [1–3]
- mild, grade 1 (n [%])	4 [21%]
- moderate, grade 2 (n [%])	14 [74%]
- severe, grade 3 (n [%])	1 [5%]
IBD diagnosis	
- ARD/FRD IBD (n [%])	4 [20%]
- Idiopathic IBD (n [%])	16 [80%]
- Hypoalbuminemic (n [%])	9 [56%]
- Normoalbuminemic (n [%])	7 [44%]
Response to treatment	
- complete remission (n [%])	16 [80%]
- partial response (n [%])	3 [15%]
- no response (n [%])	1 [5%]
Outcome[‡]	
- alive at the end of the study (n [%])	13 [68%]
- euthanized due to IBD (n [%])	6 [32%]

CIBDAI: canine IBD activity index (range of possible scores: 0–18; insignificant disease: 0–3, mild disease: 4–5, moderate disease: 6–8, severe disease: ≥9) (Jergens et al., 2003); [#]sum of all segments evaluated (range of possible scores: 0–3). [$]not reported for one patient. [‡]one patient was censored due to euthanasia for a condition unrelated to IBD. ARD/FRD: antibiotic-/food-responsive disease.

Table 12 – Serum and fecal variables in dogs with IBD.

Parameter	Pre-treatment, median [IQR]	Post-treatment, median [IQR]	*P*-value
Serum soluble RAGE	<52 [<52-309] ng/L	<52 [<52-182] ng/L	0.9097
Serum cS100A12	138 [83-294] μg/L	251 [134-313] μg/L	0.0671
Fecal cS100A12	591 [28-1359] ng/g	26 [4-283] ng/g	0.0014[$]

RAGE: receptor for advanced glycation end products; IQR: interquartile range. [$]significant difference between pre- and post-treatment concentrations ($p<0.05$).

However, serum sRAGE concentrations increased only in IBD dogs with complete clinical remission (n = 15) whereas sRAGE remained the same or decreased in dogs with partial or no response to treatment (n = 4). In healthy dogs, serum sRAGE concentrations were inversely correlated with age ($\rho = -0.5658$; $p = 0.0279$), but a similar relationship was not identified in dogs with IBD ($p = 0.5852$).

Sixteen of the IBD dogs were diagnosed with idiopathic IBD based on chronic gastrointestinal signs, an inadequate response to dietary and antibiotic trials, histopathologic changes on endoscopic tissue biopsies consistent with a diagnosis of IBD, and the need for anti-inflammatory/immunosuppressive treatment. The remaining 4 dogs were classified as having antibiotic- or food-responsive disease (ARD/FRD). Dogs with idiopathic IBD had lower serum sRAGE (median [IQR]: <52 [<52–228] ng/L) and higher fecal cS100A12 concentrations (median [IQR]: 626 [39–2,147] ng/g) than dogs

with ARD/FRD (333 [121–340] ng/L and 31 [15–913] ng/g, respectively) but both differences did not reach significance (p = 0.1335 and 0.2772, respectively). No differences were seen between both groups of dogs in serum cS100A12 (p = 0.1126). Age was not significantly different between both groups of dogs (p = 0.1216).

Patients with IBD and hypoalbuminemia and thus being suspected of having protein-losing enteropathy (PLE), were older (median [IQR]: 9 [5.5–10.5] years) than those with a normal serum albumin concentration (median [IQR]: 4 [2–10] years), but this difference did not reach significance (p = 0.1970). However, outcome was significantly associated with hypoalbuminemia (p = 0.0031). Patients with hypoalbuminemia were more likely to be euthanized than patients with a normal serum albumin (OR = 39.000 [95%CI]: 1.720–884.300]). Also, dogs that were euthanized due to their chronic gastrointestinal disease had significantly higher fecal cS100A12 concentrations at the time of diagnosis (1,760 [758–3,162] ng/g; n = 6) than dogs that were alive at the end of the study (55 [12–771] ng/g; p = 0.0124; n = 13) (Fig. 33). Clinical disease severity (p = 0.3129), serum sRAGE (p = 0.9337), and serum (p = 0.5183) and fecal cS100A12 (p = 0.1106) concentrations were not significantly different between IBD dogs with or without hypoalbuminemia.

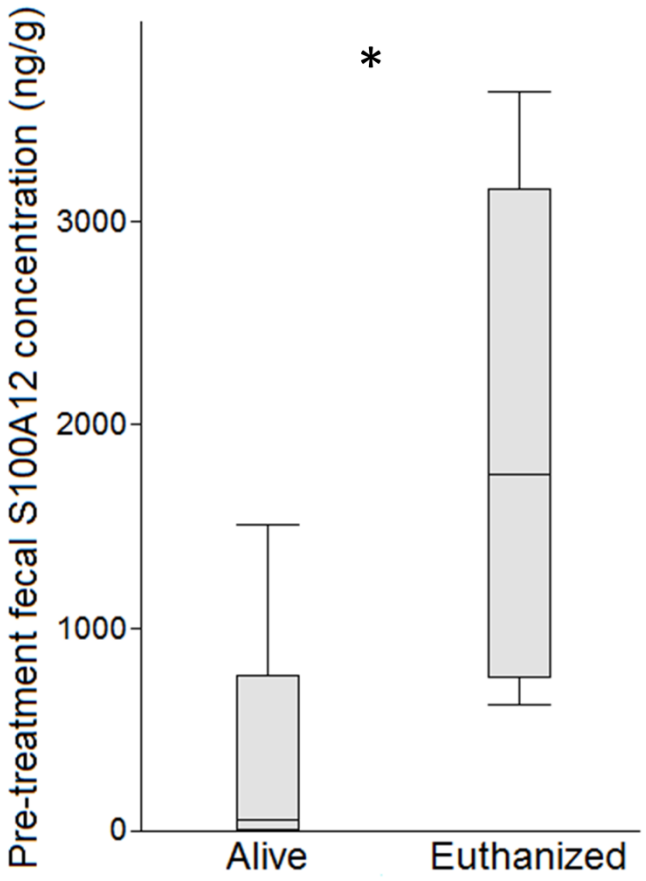

Figure 33 – Fecal S100A12 concentrations and short-term outcome in dogs with IBD. Dogs with IBD that were alive at the end of the study had significantly lower pretreatment fecal S100A12 concentrations (55 [12–771] ng/L) than dogs that had been euthanized (1760 [758–3162] ng/g; $p = 0.0124$).

5.4. Discussion

The present study is the first to describe serum sRAGE concentrations in dogs with IBD. Canine sRAGE-pAb were shown to be specific for canine RAGE and the ELISA for measuring canine sRAGE in serum was shown to be sufficiently analytically sensitive, linear, accurate, precise, and reproducible.

Similar to the situation in humans, we found two different canine RAGE isoforms in canine lung tissue extracts, presumed to be full-length RAGE (approximately 50 kDa) and its truncated variant sRAGE (approximately 42 kDa), as well as an assembly of dimers (approximately 80 kDa). The protein bands detected in canine brain extract by Western blot analysis (approximately 47 kDa and 70–80 kDa) suggest the presence of a monomeric and dimeric form of RAGE that is structurally different from that in the canine lung, a finding that is consistent with previous studies in dogs (Sterenczak et al., 2009). These molecular weights are in agreement with those expected based on the primary sequence published for cRAGE (Ensembl Genome Browser web-site: *Canis familiaris* (CanFam3.1); available at: http://useast.ensembl.org [accessed 7-6-2014]).

Further, this study showed that serum sRAGE concentrations are decreased in dogs diagnosed with IBD compared to healthy dogs, suggesting that similar to chronic inflammatory diseases in humans, such as IBD (Meijer et al., 2014), sRAGE/RAGE may be involved in the pathogenesis of chronic gastrointestinal inflammation in dogs. Lower circulating concentrations of sRAGE in dogs with IBD, acting as a decoy receptor, may allow ligand interaction with cell surface RAGE and lead to sustained activation of the

RAGE pathway, and thus a perpetuation of the inflammatory response (Bierhaus et al., 2005). Although our study did not evaluate canine RAGE at the tissue level and post-RAGE signaling has also not been studied in dogs, blockade of canine RAGE by sRAGE to neutralize RAGE activity, as demonstrated in humans (Bierhaus et al., 2005), may also be a protective mechanism in inflammatory diseases in dogs, such as canine IBD. Furthermore, as has been reported in humans (Hudson et al., 2003), blockade of canine RAGE activation by sRAGE may be an interesting therapeutic strategy for chronic inflammatory diseases in dogs.

It appears plausible that a decrease in sRAGE may result in reduced ligand trapping and may shift the sRAGE/RAGE balance toward RAGE signaling and thus a proinflammatory response. However, a cause-and-effect relationship, i.e., whether the decrease in sRAGE is due to sRAGE consumption or decreased production, cannot be extrapolated from our data. Also, RAGE ligands or even anti-sRAGE-autoantibodies could potentially cause epitope masking and interfere with binding in the assay, but this has been shown to have no significant effect in humans (Lorenzi et al., 2014).

Lack of a correlation between sRAGE and cS100A12 concentrations would be consistent with sRAGE functioning as a non-specific circulating (pattern recognition) receptor. However, this study did not evaluate cS100A12-RAGE binding or measure RAGE ligands other than cS100A12 (such as high-mobility group box-1 [HMGB-1], calgranulin A/B, or other DAMPs). Also, while sRAGE has been shown to act as a decoy receptor with the potential to bind free advanced glycation end products (AGE) and other RAGE ligands, thus competing with membrane-bound RAGE for ligand

binding, it is unknown whether sRAGE/RAGE show the same affinity for cS100A12 in dogs as demonstrated in humans (Hofmann et al., 1999). Further studies are thus needed to evaluate the formation of cS100A12-RAGE complexes.

The trends of lower serum sRAGE concentrations and higher fecal cS100A12 concentrations in dogs with idiopathic IBD compared to ARD/FRD dogs are interesting, and the lack of statistical significance could be a result of the small sample size of the subgroups. Lack of a relationship between systemic sRAGE concentrations and clinical (CIBDAI) or histologic disease scores in dogs with IBD is in line with findings in human patients with Crohn's disease (Malickova et al., 2010; Meijer at el., 2014; Leach et al., 2007), but contrasts to findings in patients with ulcerative colitis (Meijer et al., 2014). However, performance of only esophagogastroduodenoscopy in dogs in this study presents a limitation as lesions in the ileum or colon could have been missed.

Interestingly, increases in sRAGE concentrations were only seen in patients with a complete response to treatment. This effect was independent of the disease group (idiopathic IBD vs. ARD/FRD, and hypoalbuminemia vs. normal albumin), disease severity, and the serum sRAGE concentration at presentation. It would further support the hypothesis that sRAGE is an anti-inflammatory receptor and that sRAGE-ligand binding has the potential to down-regulate the proinflammatory response mediated by RAGE. Further research is warranted to evaluate the gastrointestinal mucosal expression of membrane-bound RAGE in healthy and diseased dogs, and to further evaluate the potential imbalance between sRAGE and its proinflammatory protein ligands.

An inverse correlation of sRAGE with age in healthy dogs is consistent with findings in people (Koyama et al., 2005; Geroldi et al., 2006). It is possible that the relationship between sRAGE and age reflects changes associated with aging, such as the accumulation of AGEs. This has been described in people where high sRAGE concentrations were shown to be a marker of longevity (Geroldi et al., 2006).

Commercial polyclonal Ab generated against the recombinant canine RAGE extracellular domain (ED; 315 amino acids [AA]) was used in this study. Because full-length canine RAGE consists of the ED (318 AA), the transmembrane segment (19 AA), and the cytoplasmic domain (43 AA), the ED sequence is not unique to the canine sRAGE isoform (Murua Escobar et al., 2006; Sterenczak et al., 2011). Thus, it is reasonable to assume that the Ab used in this study detect both canine sRAGE and canine full-length RAGE. Western blot analysis appears to be a suitable method to answer this question, but the sensitivity of our Western blot did not allow detection of sRAGE in canine serum samples. Thus, future studies will need to clarify this issue.

Interestingly, in this study pre-treatment fecalcS100A12 concentrations were higher in dogs with a negative outcome (euthanasia) compared to dogs that were alive at the conclusion of the study. Outcome data may be inherently biased due to euthanasia being an owner's decision. However, our group has previously shown that fecal cS100A12 concentrations are associated with the severity of endoscopic lesions in the duodenum and colon in dogs with IBD. Heilmann et al. (2014) and Allenspach et al. (2007) showed that severe endoscopic lesions in the duodenum are a negative prognostic factor in dogs with IBD (Allenspach et al., 2007). Thus, fecal cS100A12 may be a good

marker to assess outcome in dogs with IBD. However, further studies are needed to confirm this finding and to determine the impact of pre-treatment fecal cS100A12 concentrations on outcome.

5.5. Conclusion

In conclusion, the current study has demonstrated that sRAGE concentrations are decreased in dogs with IBD. These results suggest that sRAGE may serve a biomarker for inflammatory diseases such as IBD in dogs. Further studies are warranted to determine the role of sRAGE/RAGE in canine IBD and to evaluate the expression of RAGE at the tissue level.

6. FECAL INFLAMMATORY MARKERS AND MICROBIAL CHANGES IN DOGS WITH THE ACUTE HEMORRHAGIC DIARRHEA SYNDROME (AHDS)[**]

6.1 Introduction

Idiopathic acute hemorrhagic diarrhea syndrome (AHDS), formerly known as hemorrhagic gastroenteritis (HGE), is characterized by a peracute onset of hemorrhagic diarrhea, vomiting, depression, and anorexia, and can be associated with a high mortality if untreated (Hall and German, 2010). The etiology of AHDS is unknown, but it has been speculated that an abnormal response to bacterial endotoxins, bacteria (e.g., toxin-producing *C. perfringens*), or dietary components play a role (Cave et al., 2002; Schlegel et al., 2012; Busch et al., 2014).

AHDS is characterized by an increased vascular and mucosal permeability (Hall and German, 2010). Intestinal mucosal necrosis of different severity in individual patients is the principal histological lesion found in endoscopic biopsies from dogs with AHDS, with a minor influx of inflammatory cells into the intestinal mucosa (Unterer et al., 2014). However, less invasive markers that reflect gastrointestinal inflammation and intestinal microbial changes have not been studied in dogs with AHDS during the acute phase of the disease.

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6.2 Objective

This study aimed to repeatedly evaluate fecal canine calprotectin (cCP) and S100A12 (cA12, both markers of inflammation), α_1-proteinase inhibitor (cα_1PI, a marker of gastrointestinal protein loss), and bacterial groups that have previously been shown to be decreased (i.e., *Faecalibacterium* spp., Ruminococcaceae, *Bifidobacterium* spp.) or increased (i.e., Proteobacteria) in fecal samples from dogs with AHDS.

6.3 Materials and Methods

Dogs (n=7) with AHDS were enrolled into a treatment trial at the Veterinary Teaching Hospital at the Ludwig-Maximilians University Munich, Germany (Unterer et al., 2011). The study was conducted in accordance with the German animal welfare law. Each dog had a complete blood count, chemistry profile, coagulation profile, and serum total bile acids measured at the time of enrollment. A blood culture was also performed for each dog and fecal examination included a direct examination, flotation, culture (IDEXX Vet Med Labor GmbH, Ludwigsburg, Germany), and specific diagnostic testing for *Giardia* sp. (ProSpecT *Giardia* Microplate Assay, Remel, Lenexa, KS, USA), canine parvovirus (Snap Parvo Test, IDEXX Laboratories, Westbrook, ME, USA), and *C. perfringens* enterotoxin (*Clostridium perfringens* enterotoxin ELISA, Tech Lab Inc, Blacksburg, VA, USA). Diagnostic imaging and other diagnostic tests (e.g., ACTH stimulation test) were performed at the discretion of the attending clinician. Clinical disease severity was evaluated daily using the *canine HGE activity index* (Unterer et al., 2011), which considers the clinical parameters: general attitude, appetite, vomiting, stool

consistency, stool frequency, and dehydration.

Dogs were hospitalized for at least 3 days and treated using a standard protocol consisting of intravenous crystalloid fluid therapy, antiemetic (maropitant [Cerenia®, Pfizer Pharma GmbH, Karlsruhe, Germany] 1 mg/kg SC 24-hourly for 2 days), antacid (ranitidine [Sostril®, GlaxoSmithKline, Verona, Italy] 2 mg/kg IV 8-hourly for 2 days), and low-molecular-weight heparin for prophylaxis of consumption coagulopathy (dalteparin [Fragmin D®, Pfizer Pharma GmbH, Karlsruhe, Germany] SC 12-hourly for 3 days: 75 IU/kg [day 1], 38 IU/kg [day 2], and 19 IU/kg [day 3]). As part of the randomized treatment trial, one dog received amoxicillin/clavulanic acid (Synulox®, Pfizer Pharma GmbH, Karlsruhe, Germany; 12.5 mg/kg PO 12-hourly for 7 days), whereas the remaining 6 dogs were not administered antibiotics (Unterer et al., 2011).

Fecal samples were collected non-invasively as part of the routine diagnostic work-up of all dogs included in the study (Fig. 34), with no extra samples taken for study purposes. An aliquot of fecal material was frozen after collection, stored frozen at -80 °C, and shipped to the Gastrointestinal Laboratory at Texas A&M University on dry ice. Fecal samples were then thawed, extracted as described (Heilmann et al., 2011a; Heilmann et al., 2011b), and fecal cCP, cA12, and cα_1PI concentrations measured by in-house immunoassays (Heilmann et al., 2008; Heilmann et al., 2011a; Heilmann et al., 2011b). Bacterial DNA was extracted from each fecal sample and was analyzed for *Faecalibacterium* spp., Proteobacteria, Ruminococcaceae, and *Bifidobacterium* spp. using quantitative PCR assays as previously described (Suchodolski et al., 2012). Concentrations of fecal cCP, cA12, and cα_1PI and the abundance of bacterial DNA were

Figure 34 – Flowchart summarizing the fecal sample collection in dogs with acute hemorrhagic diarrhea syndrome (AHDS).

compared using a Friedman test with Dunn's *post-hoc* tests.

6.4 Results

Dogs enrolled in the study were 4.5 years (median; range: 2–8 years) of age. Breeds represented by n>1 dog included Jack Russell Terrier and mixed breed (both n=2). At the time of presentation, fecal cCP, cA12, and cα_1PI were above the suggested reference intervals in 6 (86%), 6 (86%), and 5 (71%) of the 7 dogs, respectively. Until day 3, this number decreased to 2 (29%), 1 (14%), and 4 (57%), respectively. The HGE score was not correlated with any fecal marker and a significant correlation among those markers was only seen between cCP and cA12 on day 1 (ρ=0.79) and day 3 (ρ=0.76).

Concentrations of fecal cCP, cA12, and cα_1PI and the abundance of Ruminococcaceae significantly decreased over the study period, with no differences observed in the abundances of *Faecalibacterium* spp., *Bifidobacterium* spp., or Proteobacteria (Table 13).

6.5 Discussion and Conclusions

Fecal markers of inflammation (cCP, cA12) and gastrointestinal protein loss (cα_1PI) were increased in dogs with AHDS. Although the number of patients was small, following the initiation of treatment, all fecal markers decreased significantly. This suggests a loss of protein into the intestinal lumen at the onset of AHDS, which subsides within 48 hours after initiating treatment. It also suggests the loss of inflammatory cells or intestinal inflammation in the same time period. Compared to cα_1PI, both cCP and cA12 decreased between days 2–3, suggesting that intestinal inflammation is a more likely explanation of the increased fecal cCP and cA12 concentrations than loss of inflammatory cells, though further studies need to definitively differentiate these possibilities. This differentiation is of special interest as a previous study did not identify histopathological evidence of significant intestinal inflammation in canine AHDS (Unterer et al., 2014). Lack of significant increases of *Faecalibacterium* spp., *Bifidobacterium* spp., and Ruminococcaceae, and decreases in Proteobacteria may

Table 13 – Clinical disease severity, fecal markers of inflammation or protein loss, and abundances of selected bacterial groups in dogs with AHDS. For each parameter, medians not sharing a common superscript (marked in bold) are significantly different at $P<0.05$.

Parameter	Day 1	Day 2	Day 3	*P*-value
Canine HGE activity index[#,$]	**14^{A}** [8–15]	4 [2–9]	**3^{B}** [1–7]	**0.017**
Fecal canine calprotectin (cCP), in µg/g[$]	87.7^{A,B} [31.7–127.3]	**64.8^{A}** [55.8–136.1]	**6.8^{B}** [2.9–66.9]	**0.016**
Fecal canine S100A12 (cA12), in µg/g[$]	8.9^{A,B} [3.0–163.4]	**10.1^{A}** [2.7–93.0]	**0.5^{B}** [0.5–1.2]	**0.016**
Fecal canine α_1-proteinase inhibitor ($c\alpha_1PI$), in µg/g[$]	**114.0^{A}** [15.3–191.0]	19.7^{A,B} [13.7–183.0]	**22.0^{B}** [3.7–81.4]	**0.012**
Faecalibacterium spp.[$]	7.8×10^3 [0.4×10^3–0.1×10^6]	44.0×10^3 [0.7×10^3–1.8×10^6]	0.5×10^3 [1–6.6×10^3]	0.085
Bifidobacterium spp.[$]	1 [1–6.9]	1 [1–1]	1 [1–1]	0.192
Proteobacteria[$]	1 [1–2.5×10^6]	0.6×10^6 [1–2.8×10^6]	0.2×10^6 [0.1×10^6–4.8×10^6]	0.305
Ruminococcaceae[$]	$137\times10^{3\ A,B}$ [1–833×10^3]	**$237\times10^{3\ A}$** [133×10^3–593×10^3]	**$21.7\times10^{3\ B}$** [0.1×10^3–213×10^3]	**0.008**

[#]Unterer et al., 2011; [$]shown are the medians and interquartile ranges (IQR)

suggest an ongoing intestinal dysbiosis. The abundances of most bacterial groups were unchanged over the 3-day study period, suggesting that the intestinal dysbiosis outlasts gastrointestinal protein loss. Further longitudinal studies including larger numbers of dogs with AHDS are warranted to evaluate intestinal dysbiosis in canine AHDS.

7. SUMMARY AND CONCLUSIONS

Idiopathic inflammatory bowel disease (IBD) in dogs is a chronic inflammation of the small and/or large intestine that may also involve the stomach of an unknown etiology. Canine IBD is a chronic relapsing condition, and its diagnosis and management can be challenging for the veterinarian and frustrating for the owner. The diagnosis of canine IBD requires comprehensive diagnostic testing, including invasive diagnostic procedures, to exclude other causes of chronic gastrointestinal signs such as FRD, ARD, infiltrative infectious diseases, or neoplasia. Traditionally, clinical improvement is being used to determine the success of medical management in canine patients with IBD.

The exact cause of IBD is unknown. Current consensus is that the pathogenesis of IBD in dogs involves an impaired immunoregulation in a genetically predisposed host that results in an inflammatory response against dietary and/or microbial antigens. Cells of the adaptive immune system have been associated with the pathogenesis of idiopathic IBD in dogs, but recent evidence suggests a critical role of the innate immune system in the development of canine IBD. Thus, biomarkers of phagocyte activation, such as the S100/ calgranulin Ca^{2+}-binding EF-hand protein S100A12 have potential clinical utility as markers of inflammation in dogs with IBD. Soluble RAGE (sRAGE), a truncated variant of RAGE, has been shown to function as a decoy receptor sequestering ligands such as damage-associated molecular pattern molecules (e.g., S100A12), thus preventing their interaction with cell surface RAGE and abrogating cellular RAGE signaling.

Both S100A12 and the sRAGE/RAGE axis had not previously been studied in dogs with IBD. Thus, this project sought to determine if S100A12 has clinical utility as a biomarker of gastrointestinal inflammation in dogs and if the sRAGE/RAGE axis plays a role in canine IBD. Canine IBD is also currently considered the best spontaneous animal model of human IBD (i.e., Crohn's disease, ulcerative colitis, and indeterminate colitis) owing to the similarities of the clinical features, etiopathogenesis, and diagnostic and management strategies, as well as the shared environment of dogs and humans. Thus, results of studies in dogs with IBD can potentially translate directly to human IBD, and studying S100A12 and sRAGE in dogs with IBD contributes to comparative gastroenterology involving S100A12/sRAGE for diagnosing inflammation, assessing disease progression, and as potential novel therapeutic strategies in patients with chronic autoinflammatory conditions.

7.1 Summary

Canine S100A12 was purified for the first time. Purification involved a multistep protocol comprising leukocyte enrichment from canine blood and extraction of the soluble cytosol fraction, ammonium sulfate precipitation, and hydrophobic interaction and ion-exchange chromatography. Partial characterization of the canine S100A12 protein by determination of its molecular weight and relative molecular mass, isoelectric point, specific absorbance, tryptic peptide mass fingerprint, N-terminal amino acid sequence analysis, and immunologic cross-reactivity revealed similarities between

canine, human, porcine, bovine, and rabbit S100A12 with regard to structural as well as biochemical properties.

Anti-canine S100A12 polyclonal antibodies were generated, purified by affinity chromatography, and demonstrated to be specific for canine S100A12. Canine S100A12 was successfully labelled with radioactive iodine (^{125}I), and a competitive, double antibody radioimmunoassay for the measurement of serum and fecal canine S100A12 concentrations was successfully developed. Analytical validation of the canine S100A12 radioimmunoassay included determination of the assay working range (lower and upper detection limits of the assay), analytical specificity, assay linearity (dilutional parallelism), assay accuracy (spiking recovery), precision and reproducibility (intra- and inter-assay variability), endogenous interference of hyperlipidemia, non-parametric reference intervals, biological variation of canine S100A12 in serum and fecal samples, and its distribution in feces. The radioimmunoassay was analytically sensitive and specific, linear, accurate, precise, and reproducible. Using a population-based reference interval for serum canine S100A12 may or may not be reasonable, and moderate changes in serum canine S100A12 are needed to be considered as clinically relevant.

Fecal canine S100A12 concentrations were evaluated in dogs with chronic gastrointestinal signs and a diagnosis of IBD based on clinical, clinicopathologic, endoscopic, and histopathologic criteria. Routine blood work and diagnostic imaging was performed in these dogs, and the clinical disease severity was assessed using the CCECAI scoring system. Endoscopic evaluation served to visually inspect the gastrointestinal mucosa in stomach, duodenum, ileum, and/or colon, score the severity of

lesions using a 4-point grading system, and collect tissue biopsies. Formalin-fixed and paraffin-embedded tissue biopsies were used for histological evaluation using guidelines established by the WSAVA Gastrointestinal Standardization group. The control group was comprised of healthy dogs. Canine S100A12 was quantified in fecal samples from each dog. Fecal S100A12 concentrations were compared between dogs with IBD and healthy controls, with the sensitivity and specificity calculated, followed by assessment of the relationship between fecal S100A12 concentrations and histologic, endoscopic, and clinical disease severity, and the negative prognostic factors hypoalbuminemia and hypocobalaminemia. The investigation showed that fecal canine S100A12 concentrations are increased in dogs with IBD compared to healthy control dogs, and are moderately accurate in distinguishing dogs with IBD from healthy controls. In dogs with IBD, fecal canine S100A12 concentrations correlated with the clinical disease activity, the severity of intestinal macroscopic (endoscopic) lesions in the duodenum and colon, and with the histologic inflammatory changes in the colon but not with the severity of histologic lesions overall. A fecal canine S100A12 concentration of ≥273 ng/g distinguished dogs with moderate to severe endoscopic disease in any section of the gastrointestinal tract from dogs with at most mild endoscopic disease (sensitivity: 71%, specificity: 89%). The same cut-off concentrations separated dogs with very severe clinical disease (i.e., a CCECAI score of ≥12) from dogs with a CCECAI score of <12 (sensitivity: 90%, specificity: 75%).

Specificity of the polyclonal anti-recombinant canine RAGE antibody was tested, and a sandwich enzyme-linked immunosorbent assay was successfully developed and

analytically validated for the measurement of canine sRAGE concentrations in serum. The canine sRAGE enzyme-linked immunosorbent assay was demonstrated to be analytically sensitive, linear, accurate, precise, and reproducible. Canine sRAGE concentrations were evaluated in serum samples from dogs with chronic gastrointestinal signs (i.e., vomiting and/or diarrhea of ≥3-weeks duration) that had failed dietary and antibiotic trials and were diagnosed with IBD based on further diagnostic work-up, including an upper gastrointestinal endoscopy and collection of tissue biopsies. The histopathologic criteria by the WSAVA Gastrointestinal Standardization group were used to grade histologic disease, and clinical disease activity was assessed using the CIBDAI scoring system. Following the diagnostic work-up, dogs received prednisone, prednisone and cyclosporine, or budesonide and/or metronidazole, and were given a commercial elimination diet. An additional set of samples were collected upon patient re-evaluation approximately 3 weeks later, when response to treatment was assessed using the CIBDAI score (complete clinical remission: ≥75% decrease in CIBDAI; partial response: 25–75% decrease in CIBDAI; no response: ≤25% decrease in CIBDAI). Serum was also collected from a group of healthy control dogs. Serum sRAGE was measured in all serum samples, and canine S100A12 was also quantified in serum and feces. Serum sRAGE and serum/fecal S100A12 were compared in dogs with IBD vs. healthy controls, with calculation of sensitivity and specificity, and in dogs with a positive outcome (clinical remission) vs. those that were euthanized. This was followed by testing the relationship between serum sRAGE concentrations and the CIBDAI score, histologic disease severity, and serum and fecal canine S100A12 concentrations. The

study demonstrated that serum sRAGE concentrations are significantly decreased in dogs with IBD, but are not correlated with the severity of histologic lesions, the CIBDAI score or serum canine S100A12 concentration before or after treatment, or outcome. Clinical remission and the change in serum sRAGE concentration after treatment were not associated, but an increase in serum sRAGE concentrations was only seen in IBD dogs with complete clinical remission. Dogs that were euthanized had significantly higher fecal S100A12 concentrations than those that were alive at the end of the study.

Markers of gastrointestinal inflammation (fecal canine calprotectin and canine S100A12), protein loss (α_1-proteinase inhibitor), and changes in the intestinal microbiota (*Faecalibacterium* spp., Proteobacteria, Ruminococcaceae, and *Bifidobacterium* spp.) were also studied in a group of dogs with AHDS. Fecal samples were collected from 3 consecutive days starting on the day of hospital admission. The study showed that all fecal markers evaluated were significantly increased at the time of presentation and decreased significantly within 48 hours after initiating treatment. No differences in the abundances of bacterial groups were seen over time, except for a decrease in Ruminococcaceae.

7.2 Conclusions

The results of this investigation suggest that measurement of canine S100A12 in fecal samples may be a useful adjunct for assessing macroscopic (endoscopic) disease severity in dogs with IBD, that sRAGE/RAGE may be involved in the pathogenesis of canine IBD, and that sRAGE may also serve a biomarker for inflammatory diseases such

as IBD in dogs. Lack of a correlation between sRAGE and S100A12 concentrations is consistent with sRAGE functioning as a non-specific decoy receptor.

Canine AHDS is a clinical entity that is different from canine IBD. However, fecal canine S100A12 concentrations are also increased in dogs with AHDS at the time of presentation, and canine AHDS is associated with a significant but transient intestinal loss of protein, intestinal inflammation, and an intestinal dysbiosis that appears to outlast the gastrointestinal loss of proteins.

This investigation is an important first step to evaluate canine S100A12 and sRAGE as novel disease markers in dogs with IBD. However, further research is needed to evaluate the potential of fecal canine S100A12 to reflect clinical remission and/or mucosal healing in dogs with IBD, to evaluate the gastrointestinal mucosal expression of RAGE in healthy and diseased dogs, and to demonstrate the formation of canine S100A12-canine sRAGE/RAGE complexes. Results of these studies will help to further determine the clinical utility of canine S100A12 and sRAGE as surrogate tests to aid in the diagnosis and monitoring of dogs with chronic gastrointestinal inflammation. The availability of immunological methods to quantify canine S100A12 and sRAGE will also allow for studying their role in other autoinflammatory conditions in dogs.

7.3 New Hypotheses

Based on the current knowledge, the following hypotheses are proposed regarding the mechanism of the canine S100A12-RAGE/sRAGE interaction in canine IBD: *Canine S100A12* accumulates at sites of inflammation or cell damage, forming

oligomers in the presence of Ca^{2+} and Zn^{2+} or complexes with PAMPs (e.g., LPS) or other DAMPs (e.g., AGEs, HMGB-1). In the presence of glycans and an oxidized state, this higher order canine S100A12-complex is recognized by the RAGE receptor, which is expressed by activated mononuclear phagocytes and endothelial cells. The ligand-RAGE interaction activates the NF-κB signal transduction pathway (Fig. 35), leading to an increased production of pro-inflammatory cytokines and chemokines (e.g., TNF-α, S100/calgranulins, MMPs) and an up-regulation of RAGE. This mechanism increases the availability of S100/calgranulins for host defense against microorganisms by sequestering essential trace elements (e.g., Zn^{2+}) and, via chemotaxis, attracts monocytes and mast cells to the site of inflammation or tissue damage. MMPs initially facilitate leukocyte chemotaxis by breaking down tissue matrix, but are later on counteracted by accumulating S100/calgranulins (e.g., S100A12) due to the sequestration of essential Zn^{2+}. *Soluble RAGE*, and possibly also dominant-negative and N-truncated RAGE, act as a mechanism of receptor regulation by competitively sequestering ligands such as DAMPs (e.g., S100/calgranulins) and thus preventing their interaction with full-length cell surface RAGE.

Under normal conditions, the immune response using the pathway described should be self-limiting with all steps involved being tightly controlled by positive and negative regulators. However, a dysregulation in this pathway (e.g., due to dysregulation or mutation of the full-length receptor[s], decoy receptor[s], adaptor protein[s], and/or negative or positive regulator[s]; increased accumulation of DAMPs and/or PAMPs as ligands; and/or a microenvironment that favors their higher-order complex formation)

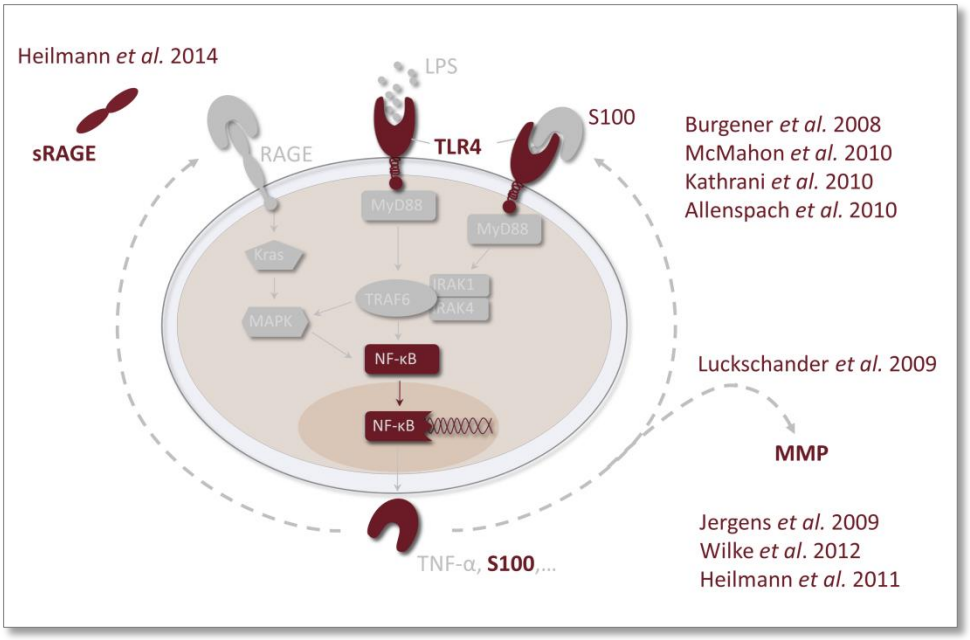

Figure 35 – Proposed mechanism of canine S100/calgranulin-RAGE/sRAGE and/or TLR interaction in view of current knowledge. Several aspects of this new hypothesis have been evaluated in canine IBD. In addition to a significant increase in S100A12 and a decrease in sRAGE, increased NF-κB activation was shown in the duodenal lamina propria in dogs with chronic enteropathies. A large gene expression study identified several genes that were up-regulated in the intestinal mucosa in dogs with IBD, which included genes encoding TNF, S100/calcium-binding proteins, and MMPs. Several studies have also analyzed and found TLR4 dysregulation and a genomic instability in the *TLR4* gene in canine IBD.

IRAK = IL-1 receptor-associated kinase; Kras = V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LPS = lipopolysaccharide; MAPK = mitogen-activated protein kinases; MyD88 = myeloid differentiation factor 88; NF-κB = nuclear factor-kappa B; RAGE = receptor for advanced glycation end products; S100 = S100 protein; sRAGE = soluble receptor for advanced glycation end products; TLR4 = Toll-like receptor 4; TNF-α = tumor-necrosis factor α; TRAF = TNF-receptor associated factor.

could modulate the immune response leading to sustained post-receptor signaling and thus, its amplification and perpetuation beyond the traditional inflammatory response. A dysregulation in the S100/calgranulin-RAGE/sRAGE pathway could also be involved in the pathogenesis of chronic autoinflammation such as IBD. However, further research is needed to confirm or disprove this hypothesis and to further evaluate the S100/calgranulin-RAGE/sRAGE axis in canine IBD.

REFERENCES

Allenspach, K., Luckschander, N., Styner, M., Seibold, F., Doherr, M., Aeschbach, D., Gaschen, F., 2004. Evaluation of assays for perinuclear antineutrophilic cytoplasmic antibodies and antibodies to *Saccharomyces cerevisiae* in dogs with inflammatory bowel disease. *American Journal of Veterinary Research* 65: 1279–1283.

Allenspach, K., Wieland, B., Gröne, A., Gaschen, F., 2007. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. *Journal of Veterinary Internal Medicine* 21: 700–708.

Allenspach, K., Lomas, B., Wieland, B., Harris, T., Pressler, B., Mancho, C., Lees, G.E., Vaden, S.L., 2008. Evaluation of perinuclear anti-neutrophilic cytoplasmic autoantibodies as an early marker of protein-losing enteropathy and protein-losing nephropathy in Soft Coated Wheaten Terriers. *American Journal of Veterinary Research* 69: 1301–1304.

Allenspach, K., House, A., Smith, K., McNeill, F.M., Hendricks, A., Elson-Riggins, J., Riddle, A., Steiner, J.M., Werling, D., Garden, O.A., Catchpole, B., Suchodolski, J.S., 2010. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German Shepherd Dogs with chronic enteropathies. *Veterinary Microbiology* 146: 326–335.

Allenspach, K., 2011a. Clinical immunology and immunopathology of the canine and feline intestine. *The Veterinary Clinics of North America (Small Animal Practice)* 41: 345–360.

Allenspach, K., Kathrani, A., House, A., Holder, A., Catchpole, B., Werling, D., 2011b. Toll-like receptor 5 mutations in German Shepherd Dogs are hyper-responsive to stimulation with flagellin. *Journal of Veterinary Internal Medicine* 25: 688 (abstract).

Anfinsen, K.P., Berghoff, N., Priestnall, S.L., Suchodolski, J.S., Steiner, J.M., Allenspach, K., 2014. Urinary and faecal N-methylhistamine concentrations do not serve as markers for mast cell activation or clinical disease activity in dogs with chronic enteropathies. *Acta Veterinaria Scandinavica* 56: 952.

Basta, G., Lazzerini, G., Massaro, M., Simoncini, T., Tanganelli, P., Fu, C., Kislinger, T., Stern, D.M., Schmidt, A.M., De Caterina, R., 2002. Advanced glycation end products activate endothelium through signaling signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. *Circulation* 105: 816–822.

Basta, G., Sironi, A.M., Lazzerini, G., Del Turco, S., Buzzigoli, E., Casolaro, A., Natali, A., Ferrannini, E., Gastaldelli, A., 2006. Circulating soluble receptor for advanced glycation end products is inversely associated with glycemic control

and S100A12 protein. *The Journal of Clinical Endocrinology and Metabolism* 91: 4628–4634.

Berghoff, N., Suchodolski, J.S., Steiner, J.M., 2006. Assessment of stability and determination of a reference range for canine C-reactive protein in serum. *Journal of Veterinary Internal Medicine* 20: 791 (abstract).

Berghoff, N., Suchodolski, J.S., Steiner, J.M., 2008. Fecal N-methylhistamine concentrations in Norwegian Lundehunds with gastrointestinal disease. *Journal of Veterinary Internal Medicine* 22: 748 (abstract).

Berghoff, N., Steiner, J.M., 2011. Laboratory tests for the diagnosis and management of chronic canine and feline enteropathies. *The Veterinary Clinics of North America (Small Animal Practice)* 41: 311–328.

Berghoff, N., Parnell, N.K., Hill, S.L., Suchodolski, J.S., Steiner, J.M., 2013. Serum cobalamin and methylmalonic acid concentrations in dogs with chronic gastrointestinal disease. *American Journal of Veterinary Research* 74: 84–89.

Berghoff, N., Hill, S., Parnell, N.K., Mansell, J., Suchodolski, J.S., Steiner, J.M., 2014. Fecal and urinary N-methylhistamine concentrations in dogs with chronic gastrointestinal disease. *The Veterinary Journal* 201: 289–294.

Berson, S.A., Yalow, R.S., 1968. General principles of radioimmunoassay. *Clinica Chimica Acta* 22: 51–69.

Bierhaus, A., Humpert, P.M., Morcos, M., Wendt, T., Chavakis, T., Arnold, B., Stern, D.M., Nawroth, P.P., 2005. Understanding RAGE, the receptor for advanced glycation end products. *Journal of Molecular Medicine (Berlin)* 83: 876–886.

Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, C.A., Glasziou, P.P., Irwig, L.M., Moher, D., Rennie, D., de Vet, H.C., Lijmer, J.G., 2003a. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Clinical Chemistry* 49: 7–18.

Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, C.A., Glasziou, P.P., Irwig, L.M., Lijmer, J.G., Moher, D., Rennie, D., de Vet, H.C., 2003b. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD Initiative. *Radiology* 226: 24–28.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* 72: 248–254.

Bucciarelli, L.G., Wendt, T., Rong, L., Lalla, E., Hofmann, M.A., Goova, M.T., Taguchi, A., Yan, S.F., Yan, S.D., Stern, D.M., Schmidt, A.M., 2002. RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. *Cellular and Molecular Life Sciences* 59: 1117–1128.

Burgener, I.A., König, A., Allenspach, K., Sauter, S.N., Boisclair, J., Doherr, M.G., Jungi, T.W., 2008. Upregulation of toll-like receptors in chronic enteropathies in dogs. *Journal of Veterinary Internal Medicine* 22: 553–560.

Burrows, C.F., 1977. Canine hemorrhagic gastroenteritis. *Journal of the American Animal Hospital Association* 13: 451–458.

Busch K., Suchodolski, J.S., Kühner, K.A., Minamoto, Y., Steiner, J.M., Mueller, R.S., Hartmann, K., Unterer, S., 2014. *Clostridium perfringens* enterotoxin and *Clostridium difficile* toxin A/B do not play a role in acute haemorrhagic diarrhoea syndrome in dogs. *Veterinary Record* doi: 10.1136/vr.102738.

Carlson, J., 1984. $Alpha_1$-antitrypsin and the liver. Clinical and biosynthetic studies. Thesis, University of Lund, Malmö, Sweden.

Carney, P.C., Ruaux, C.G., Suchodolski, J.S., Steiner, J.M., 2011. Biological variability of C-reactive protein and specific pancreatic lipase immunoreactivity (Spec cPL) in apparently healthy dogs. *Journal of Veterinary Internal Medicine* 25: 825–830.

Casamian-Sorrosal, D., Willard, M.D., Murray, J.K., Hall, E.J., Taylor, S.S., Day, M.J., 2010. Comparison of histopathologic findings in biopsies from the duodenum and ileum of dogs with enteropathy. *Journal of Veterinary Internal Medicine* 24: 80–83.

Cave, N.J., Marks, S.L., Kass, P.H., Melli, A.C., Brophy, M.A., 2002. Evaluation of a routine diagnostic fecal panel for dogs with diarrhea. *Journal of the American Veterinary Medical Association* 221: 52–59.

Chan, D.L., Rozanski, E.A., Freeman, L.M., 2009. Relationship among plasma amino acids, C-reactive protein, illness severity, and outcome in critically ill dogs. *Journal of Veterinary Internal Medicine* 23: 559–563.

Chiang, C.S., 1987. A linear method for determining specific activity of tracers in radioimmunoassays. *Clinical Chemistry* 33: 1245–1247.

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

Craven, M., Acland, G.M., Mezey, J., Boyko, A., Wang, W., Meurs, K., McDonough S., Simpson, K.W., 2010. Genome-wide association scan reveals polymorphisms in the $P67_{phox}$ subunit (*Ncf2*) of the NADPH oxidase complex in Boxer dogs with adherent and invasive E.coli-associated granulomatous colitis: a potential model of chronic granulomatous disease. *Gastroenterology* 138: S683 (abstract).

Crowther, J.R., 2001. *The ELISA Guidebook* (1^{st} ed.), Humana Press, Totowa, NJ.

Dabrowski, R., Wawron, W., Kostro, K., 2007. Changes in CRP, SAA and haptoglobin produced in response to ovariohysterectomy in healthy bitches and those with pyometra. *Theriogenology* 67: 321–327.

Day, M.J., Bilzer, T., Mansell, J., Wilcock, B., Hall, E.J., Jergens, A.E., Minami, T., Willard, M.D., Washabau, R., World Small Animal Veterinary Association Gastrointestinal Standardization Group, 2008. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. *Journal of Comparative Pathology* 38: S1–S43.

de Jong, N.S., Leach, S.T., Day, A.S., 2006. Fecal S100A12: a novel noninvasive marker in children with Crohn's disease. *Inflammatory Bowel Diseases* 12: 566–572.

Dell'Angelica, E.C., Schleicher, C.H., Santomé, J.A., 1994. Primary structure and binding properties of calgranulin C, a novel S100-like calcium-binding protein from pig granulocytes. *The Journal of Biological Chemistry* 269: 28929–28936.

de Seny, D., Fillet, M., Ribbens, C., Marée, R., Meuwis, M.A., Lutteri, L., Chapelle, J.P., Wehenkel, L., Louis, E., Merville, M.P., Malaise, M., 2008. Monomeric calgranulins measured by SELDI-TOF mass spectrometry and calprotectin measured by ELISA as biomarkers in arthritis. *Clinical Chemistry* 54: 1066–1075.

Devarajan, P., Ross, G.F., 2008. SELDI technology for identification of protein biomarkers. In: Wang, F. (Ed.), *Biomarker Methods in Drug Discovery and Development*. Humana Press, Totowa, pp. 251–271.

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

Equilino M., Théodoloz, V., Gorgas, D., Doherr, M.G., Heilmann, R.M., Suchodolski, J.S., Steiner, J.M., Burgener, I.A., 2015. Evaluation of serum biochemical marker concentrations and survival time in dogs with protein-losing enteropathy. *Journal of the American Veterinary Medical Association* 246: 91–99.

Fagerhol, M.K., Dale, I., Anderson, T., 1980. Release and quantitation of a leucocyte derived protein (L1). *Scandinavian Journal of Haematology* 24: 393–398.

Föll, D., Kane, D., Bresnihan, B., Vogl, T., Nacken, W., Sorg, C., Fitzgerald, O., Roth, J., 2003a. Expression of the pro-inflammatory protein S100A12 (EN-RAGE) in rheumatoid and psoriatic arthritis. *Rheumatology (Oxford)* 42: 1383–1389.

Föll, D., Kucharzik, T., Kraft, M., Vogl, T., Sorg, C., Domschke, W., Roth, J., 2003b. Neutrophil derived human S100A12 (EN-RAGE) is strongly expressed during chronic active inflammatory bowel disease. *Gut* 52: 847–853.

Föll, D., Seeliger, S., Vogl, T., Koch, H.G., Maschek, H., Harms, E., Sorg, C., Roth, J., 2003c. Expression of S100A12 (EN-RAGE) in cystic fibrosis. *Thorax* 58: 613–617.

Föll, D., Frosch, M., Sorg, C., Roth, J., 2004. Phagocyte-specific calcium-binding S100 proteins as clinical laboratory markers of inflammation. *Clinica Chimica Acta* 344: 37–51.

Föll, D., Wittkowski, H., Vogl, T., Roth, J., 2007. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *Journal of Leukocyte Biology* 81: 28–37.

Föll, D., Wittkowski, H., Ren, Z., Turton, J., Pang, G., Däbritz, J., Ehrchen, J., Heidemann, J., Borody, T., Roth, J., Clancy, R., 2008. Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. *The Journal of Pathology* 216: 183–192.

Föll, D., Wittkowski, H., Roth, J., 2009. Monitoring disease activity by stool analyses: from occult blood to molecular markers of intestinal inflammation and damage. *Gut* 58: 859–868.

Fraser, C.G., Harris, E.K., 1989. Generation and application of data on biological variation in clinical chemistry. *Critical Reviews in Clinical Laboratory Sciences* 27: 409–437.

Fuellen, G., Nacken, W., Sorg, C., Kerkhoff, C., 2004. Computational searches for missing orthologs: the case of S100A12 in mice. *OMICS: A Journal of Integrative Biology* 8: 334–340.

Ganrot, K., 1973. Plasma protein response in experimental inflammation in the dog. *Research in Experimental Medicine (Berlin)* 161: 251–261.

García-Sancho, M., Rodriguez-Franco, F., Sainz, A., Mancho, C., Rodriquez, A., 2007. Evaluation of clinical, macroscopic, and histopathologic response to treatment in nonhypoproteinemic dogs with lymphocytic-plasmacytic enteritis. *Journal of Veterinary Internal Medicine* 21: 11–17.

Gebhardt, C., Riehl, A., Durchdewald, M., Németh, J., Fürstenberger, G., Müller-Decker, K., Enk, A., Arnold, B., Bierhaus, A., Nawroth, P.P., Hess, J., Angel, P., 2008. RAGE signaling sustains inflammation and promotes tumor development. *The Journal of Experimental Medicine* 205: 275–285.

Gebhardt, C., Hirschberger, J., Rau, S., Arndt, G., Krainer, K., Schweigert, F.J., Brunnberg, L., Kaspers, B., Kohn, B., 2009. Use of C-reactive protein to predict outcome in dogs with systemic inflammatory response syndrome or sepsis. *Journal of Veterinary Emergency and Critical Care (San Antonio)* 19: 450–458.

Geffré, A., Friedrichs, K., Harr, K., Concordet, D., Trumel, C., Braun, J.P., 2009. Reference values: a review. *Veterinary Clinical Pathology* 38: 288–298.

German, A.J., Helps, C.R., Hall, E.J., Day, M.J., 2000. Cytokine mRNA expression in mucosal biopsies from German shepherd dogs with small intestinal enteropathies. *Digestive Diseases and Sciences* 45: 7–17.

German, A.J., Hall, E.J., Day, M.J., 2001. Immune cell populations within the duodenal mucosa of dogs with enteropathies. *Journal of Veterinary Internal Medicine* 15: 14–25.

Geroldi, D., Falcone, C., Minoretti, P., Emanuele, E., Arra, M., D'Angelo, A., 2006. High levels of soluble receptor for advanced glycation end products may be a marker of extreme longevity in humans. *Journal of the American Geriatrics Society* 54: 1149–1150.

Gottsch, J.D., Eisinger, S.W., Liu, S.H., Scott, A.L., 1999. Calgranulin C has filariacidal and filariastatic activity. *Infection and Immunity* 67: 6631–6636.

Gottschalk, P.G., Dunn, J.R., 2005. The five-parameter logistic: a characterization and comparison with the four-parameter logistic. *Analytical Biochemistry* 343: 54–65.

Goyette, J., Geczy, C.L., 2011. Inflammation-associated S100 proteins: new mechanisms that regulate function. *Amino Acids* 41: 821–842.

Grellet, A., Heilmann, R.M., Lecoindre, P., Feugier, A., Day, M.J., Peeters, D., Freiche, V., Hernandez, J., Grandjean, D., Suchodolski, J.S., Steiner, J.M., 2013. Fecal calprotectin in adult dogs with chronic diarrhea. *American Journal of Veterinary Research* 74: 706–711.

Griebsch, C., Arndt, G., Raila, J., Schweigert, F.J., Kohn, B., 2009. C-reactive protein concentration in dogs with primary immune-mediated hemolytic anemia. *Veterinary Clinical Pathology* 38: 421–425.

Grützner, N., Heilmann, R.M., Bridges, C.S., Suchodolski, J.S., Steiner, J.M., 2013. Serum concentrations of canine alpha$_1$-proteinase inhibitor in cobalamin-deficient Yorkshire Terrier dogs. *Journal of Veterinary Diagnostic Investigation* 25: 376–385.

Grützner, N., Heilmann, R.M., Suchodolski, J.S., Steiner, J.M., Holzenburg, A.K., 2014. Cold-microwave enhanced enzyme-linked immunosorbent assays – a path to high-throughput clinical diagnostics. *Analytical Biochemistry* 457: 65–73.

Guignard, F., Mauël, J., Markert, M., 1995. Identification and characterization of a novel human neutrophil protein related to the S100 family. *The Biochemical Journal* 309: 395–401.

Hall, E.J., German, A.J., 2008. Inflammatory bowel disease. In: Steiner, J.M. (Ed.) *Small Animal Gastroenterology*. Hannover, Germany: Schlütersche, pp. 312–329.

Hall, E.J., German, A.J., 2010. Diseases of the small intestine – Acute small-intestinal disease: Hemorrhagic gastroenteritis. In: Ettinger, S.J., Feldman, E.C. (Eds.), *Textbook of Veterinary Internal Medicine*. Saunders Elsevier, St. Louis, MO, USA, pp. 1526–1572.

Hatakeyama, T., Okada, M., Shimamoto, S., Kubota, Y., Kobayashi, R., 2004. Identification of intracellular target proteins of the calcium-signaling protein S100A12. *European Journal of Biochemistry* 271: 3765–3775.

Heilmann, R.M., Suchodolski, J.S., Steiner, J.M., 2008a. Purification and partial characterization of canine calprotectin. *Biochimie* 90: 1306–1315.

Heilmann, R.M., Suchodolski, J.S., Steiner, J.M., 2008b. Development and analytic validation of a radioimmunoassay for the quantification of canine calprotectin in serum and feces from dogs. *American Journal of Veterinary Research* 69: 845–853.

Heilmann, R.M., Suchodolski, J.S., Steiner, J.M., 2010. Purification and partial characterization of canine S100A12. *Biochimie* 92: 1914–1922.

Heilmann, R.M., Allenspach, K., Procoli, F., Weber, K., Suchodolski, J.S., Steiner, J.M., 2011a. Serum calgranulin concentrations in dogs with inflammatory bowel disease. *Journal of Veterinary Internal Medicine* 25: 1486 (abstract).

Heilmann, R.M., Lanerie, D.J., Ruaux, C.G., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2011b. Development and analytic validation of an immunoassay for the quantification of canine S100A12 in serum and fecal samples and its biological variability in serum from healthy dogs. *Veterinary Immunology and Immunopathology* 144: 200–209.

Heilmann, R.M., Paddock, C.G., Ruhnke, I., Berghoff, N., Suchodolski, J.S., Steiner, J.M., 2011c. Development and analytical validation of a radioimmunoassay for the measurement of alpha$_1$-proteinase inhibitor concentrations in feces from healthy puppies and adult dogs. *Journal of Veterinary Diagnostic Investigation* 23: 476–485.

Heilmann, R.M., Jergens, A.E., Ackermann, M.R., Barr, J.W., Suchodolski, J.S., Steiner, J.M., 2012a. Serum calprotectin concentrations in dogs with idiopathic inflammatory bowel disease. *American Journal of Veterinary Research* 73: 1900–1907.

Heilmann, R.M., Grellet, A., Allenspach, K., Lecoindre, P., Day, M.J., Procoli, F., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2012b. Fecal S100A12 concentrations are increased in dogs with inflammatory bowel disease. *Journal of Veterinary Internal Medicine* 26: 767 (abstract).

Heilmann, R.M., Grellet, A., Allenspach, K., Lecoindre, P., Day, M.J., Priestnall, S.L., Toresson, L., Procoli, F., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2014a. Association between fecal S100A12 concentration and histologic, endoscopic, and clinical disease severity in dogs with idiopathic inflammatory bowel disease. *Veterinary Immunology and Immunopathology* 158: 156–166.

Heilmann, R.M., Steiner, J.M., 2014b. Laboratory testing for the exocrine pancreas. In: Bonagura, J.D., Twedt, D.C. (Eds.) *Kirk's Current Veterinary Therapy XV*. St. Louis, Missouri: Saunders Elsevier, pp. 554–557.

Heilmann, R.M., Parnell, N.K., Mansell, J., Berghoff, N., Minamoto, T., Sattasathuchana, P., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2014c. Serum and fecal canine alpha$_1$-proteinase inhibitor concentrations are associated with the severity of crypt abscesses and/or lacteal dilation in dogs with inflammatory bowel disease. *Journal of Veterinary Internal Medicine* 28, 1044-1045 (abstract).

Heizmann, C.W., 2002. The multifunctional S100 protein family. In: Vogel, H.J. (Ed.), *Methods in Molecular Biology. Calcium-Binding Protein Protocols, Reviews and Case Studies*, vol. 1. Humana Press, Totowa, pp. 69–80.

Hillström, A., Hagman, R., Tvedten, H., Kjelgaard-Hansen, M., 2014. Validation of a commercially available automated canine-specific immunoturbidimetric method for measuring canine C-reactive protein. *Veterinary Clinical Pathology* 43: 235–243.

Hilt, D.C., Kligman, D., 1991. The S100-protein family: a biochemical and functional overview. In: Heizmann, C.W. (Ed.), *Novel Calcium-binding Protein Proteins: Fundamentals and Clinical Implications*. Springer-Verlag, Berlin, pp. 65–103.

Hitomi, J., Yamaguchi, K., Kikuchi, Y., Kimura, T., Maruyama, K., Nagasaki, K., 1996. A novel calcium-binding protein in amniotic fluid, CAAF1: its molecular cloning and tissue distribution. *Journal of Cell Science* 109: 805–815.

Hofmann, M.A., Drury, S., Fu, C., Qu, W., Taguchi, A., Lu, Y., Avila, C., Kambham, N., Bierhaus, A., Nawroth, P., Neurath, M.F., Slattery, T., Beach, D., McClary, J., Nagashima, M., Morser, J., Stern, D., Schmidt, A.M., 1999. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 97: 889–901.

Holm, S., 1979. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6: 64–70.

Hsu, K., Champaiboon, C., Günther, B.D., Sorenson, B.S., Khammanivong, A., Ross, K.F., Geczy, C.L., Herzberg, M.C., 2009. Anti-infective protective properties of S100 calgranulins. *Anti-inflammatory & Anti-allergy Agents in Medical Chemistry* 8: 290–305.

Hudson, B.I., Bucciarelli, L.G., Wendt, T., Sakaguchi, T., Lalla, E., Qu, W., Lu, Y., Lee, L., Stern, D.M., Naka, Y., Ramasamy, R., Yan, S.D., Yan, S.F., D'Agati, V., Schmidt, A.M., 2003. Blockade of the receptor for advanced glycation end products: a new target for therapeutic intervention in diabetic complications and inflammatory disorders. *Archives of Biochemistry and Biophysics* 419: 80–88.

Hunter, W.M., Greenwood, F.C., 1962. Preparation of ^{131}iodine-labeled human growth hormone of high specific activity. *Nature* 194, 495–496.

Hunter, M.J., Chazin, W.J., 1998. High level expression and dimer characterization of the S100 EF-hand proteins, migration inhibitory factor-related proteins 8 and 14. *The Journal of Biological Chemistry* 273: 12427–12435.

Ilg, E.C., Troxler, H., Bürgisser, D., Kuster, T., Markert, M., Guignard, F., Hunziker, P., Birchler, N., Heizmann, C.W., 1996. Amino acid sequence determination of human S100A12 (p6, calgranulin C, CGRP, CAAF1) by tandem mass spectrometry. *Biochemical and Biophysical Research Communications* 225: 146–150.

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

Jergens, A.E., 2004. Clinical assessment of disease activity for canine inflammatory bowel disease. *Journal of the American Animal Hospital Association* 40: 437–445.

Jergens, A.E., Sonea, I.M., O'Connor, A.M., Kauffman, L.K., Grozdanic, S.D., Ackermann, M.R., Evans, R.B., 2009. Intestinal cytokine mRNA expression in

canine inflammatory bowel disease: a meta-analysis with critical appraisal. *Comparative Medicine* 59: 153–162.

Jergens, A.E., Crandell, J., Morrison, J.A., Deitz, K., Pressel, M., Ackermann, M., Suchodolski, J.S., Steiner, J.M., Evans, R., 2010a. Comparison of oral prednisone and prednisone combined with metronidazole for induction therapy of canine inflammatory bowel disease: a randomized-controlled trial. *Journal of Veterinary Internal Medicine* 24: 269–277.

Jergens, A.E., Nettleton, D, Suchodolski, J.S., Dowd, S., Steiner, J.M., 2010b. Interplay of commensal bacteria, host gene expression, and clinical disease activity in the pathogenesis of canine inflammatory bowel disease. *Journal of Veterinary Internal Medicine* 24: 1570 (abstract).

Jergens, A.E., Simpson, K.W., 2012. Inflammatory bowel disease in veterinary medicine. *Frontiers in Bioscience* 4: 1404–1419.

Kaiser, T., Langhorst, J., Wittkowski, H., Becker, K., Friedrich, A.W., Rueffer, A., Dobos, G.J., Roth, J., Föll, D., 2007. Fecal S100A12 as a non-invasive marker distinguishing inflammatory bowel disease from irritable bowel syndrome. *Gut* 56: 1706–1713.

Kathrani, A., House, A., Catchpole, B., Murphy, A., German, A., Werling, D., Allenspach, K., 2010a. Polymorphisms in the TLR4 and TLR5 gene are

significantly associated with inflammatory bowel disease in German shepherd dogs. *PLoS One* 5: e15740.

Kathrani, A., House, A., Werling, D., Catchpole, B., Murphy, A., German, A.J., Lee, H., Allenspach, K., 2010b. Overdominant single nucleotide polymorphisms in the nucleotide oligomerization domain two (*NOD2*) gene are significantly associated with canine inflammatory bowel disease. *Journal of Veterinary Internal Medicine* 24: 725 (abstract).

Kathrani, A., House, A., Catchpole, B., Murphy, A., Werling, D., Allenspach, K., 2011a. Breed-independent toll-like receptor 5 polymorphisms show association with canine inflammatory bowel disease. *Tissue Antigens* 78: 94–101.

Kathrani, A., Werling, D., Allenspach, K., 2011b. Mutational analysis of the TLR5 gene in Boxer dogs with inflammatory reveals novel polymorphisms in the leucine rich repeat domain. *Journal of Veterinary Internal Medicine* 25: 696 (abstract).

Kathrani, A., Werling, D., Holder, A., Catchpole, B., Allenspach, K., 2011c. Polymorphisms in Toll-like receptor 5 results in hyper-responsiveness to flagellin. *Journal of Veterinary Internal Medicine* 25: 687–688 (abstract).

Kathrani, A., White, C., Werling, D., Allenspach, K., 2011d. Mutational analysis of the NOD2 gene in German shepherd dogs with inflammatory bowel disease reveals polymorphisms in the leucine rich repeat domain. *Proceedings 21th ECVIM-CA Congress* 236–237 (abstract).

Kathrani, A., Holder, A., Catchpole, B., Alvarez, L., Simpson, K., Werling, D., Allenspach, K., 2012. TLR5 risk-associated haplotype for canine inflammatory bowel disease confers hyper-responsiveness to flagellin. *PLoS One* 7: e30117.

Kathrani, A., Lee, H., White, C., Catchpole, B., Murphy, A., German, A.J., Werling, D., Allenspach, K., 2014. Association between nucleotide oligomerization domain two (nod2) gene polymorphisms and canine inflammatory bowel disease. *Veterinary Immunology and Immunopathology* 161: 32–41.

Klenner, S., Bauer, N., Moritz, A., 2010. Evaluation of three automated human immunoturbidimetric assays for the detection of C-reactive protein in dogs. *Journal of Veterinary Diagnostic Investigation* 22: 544–552.

Kligman, D., Hilt, D.C., 1988. The S100 protein family. *Trends in Biochemical Sciences* 13: 437–443.

Kołodziejska-Sawerska, A., Rychlik, A., Depta, A., Wdowiak, M., Nowicki, M., Kander, M, 2013. Cytokines in canine inflammatory bowel disease. *Polish Journal of Veterinary Sciences* 16: 165–171.

Koyama, H., Shoji, T., Yokoyama, H., Motoyama, K., Mori, K., Fukumoto, S., Emoto, M., Shoji, T., Tamei, H., Matsuki, H., Sakurai, S., Yamamoto, Y., Yonekura, H., Watanabe, T., Yamamoto, H., Nishizawa, Y., 2005. Plasma level of endogenous secretory RAGE is associated with components of the metabolic syndrome and

atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 25: 2587–2593.

Larsen, A., Bronstein, I.B., Dahl, O., Wentzel-Larsen, T., Kristofferson, E.K., Fagerhol, M.K., 2007. Quantification of S100A12 (EN-RAGE) in blood varies with sampling method, calcium and heparin. *Scandinavian Journal of Immunology* 65: 192–201.

Law, B., 1996. Radiolabelling procedures for radioimmunoassay. In: Law, B. (Ed.), *Immunoassay – A Practical Guide*. Taylor & Francis, Bristol, pp. 62–95.

Law, B., 2005. *Immunoassay – A Practical Guide*, Taylor & Francis, Bristol, PA.

Leach, S.T., Yang, Z., Messina, I., Song, C., Geczy, C.L., Cunningham, A.M., Day, A.S., 2007. Serum and mucosal S100 proteins, calprotectin (S100A8/S100A9) and S100A12, are elevated at diagnosis in children with inflammatory bowel disease. *Scandinavian Journal of Gastroenterology* 42: 1321–1331.

Li, J.H., Schmidt, A.M., 1997. Characterization and functional analysis of the promotor of RAGE, the receptor for advanced glycation end products. *The Journal of Biological Chemistry* 272: 16498–16506.

Lorenz, E., Muhlebach, M.S., Tessier, P.A., Alexis, N.E., Duncan Hite, R., Seeds, M.C., Peden, D.B., Meredith, W., 2008. Different expression ratio of S100A8/A9 and S100A12 in acute and chronic lung disease. *Respiratory Medicine* 102: 567–573.

Lorenzi, R., Grossin, N., Lambert, M., Daroux, M., Adjoutah, Z., Flahaut, C., Jacolot, P., Tessier, F.J., Lefranc, D., Desremaux, P., Dubucquoi, S., Boulanger, E., 2014. Soluble form of receptor for advanced glycation end-products (sRAGE): do sRAGE ligands or anti-sRAGE autoantibodies interfere with sRAGE quantification? *Annals of Clinical Biochemistry* 51: 248–257.

Lowrie, M., Penderis, J., Eckersall, P.D., McLaughlin, M., Mellor, D., Anderson, T.J., 2009. The role of acute phase proteins in diagnosis and management of steroid-responsive meningitis arteritis in dogs. *The Veterinary Journal* 182: 125–130.

Luckschander, N., Allenspach, K., Hall, J., Seibold, F., Gröne, A., Doherr, M.G., Gaschen, F., 2006. Perinuclear antineutrophilic cytoplasmic antibody and response to treatment in diarrheic dogs with food responsive disease or inflammatory bowel disease. *Journal of Veterinary Internal Medicine* 20: 221–227.

Luckschander, N., Hall, J.A., Gaschen, F., Forster, U., Wenzlow, N., Hermann, P., Allenspach, K., Dobbelaere, D., Burgener, I.A., Welle, M., 2010. Activation of nuclear factor-kappa B in dogs with chronic enteropathies. *Veterinary Immunology and Immunopathology* 133: 228–236.

Lügering, N., Stoll, R., Kucharzik, T., Schmid, K.W., Rohlmann, G., Burmeister, G., Sorg, C., Domschke, W., 1995. Immunohistochemical distribution and serum

levels of the $Ca^{(2+)}$-binding proteins MRP8, MRP14 and their heterodimeric form MRP8/14 in Crohn's disease. *Digestion* 56: 406–414.

Maeda, S., Ohno, K., Nakamura, K., Uchida, K., Nakashima, K., Fukishima, K., Tsukamoto, A., Goto-Koshino, Y., Fujino, Y., Tsujimoto, H., 2011. Quantification of chemokine and chemokine receptor gene expression in duodenal mucosa of dogs with inflammatory bowel disease. *Veterinary Immunology and Immunopathology* 144: 290–298.

Malícková, K., Kalousová, M., Fucíková, T., Bortlík, M., Duricová, D., Komárek, V., Zima, T., Janatková, I., Lukás, M., 2010. Anti-inflammatory effect of biological treatment in patients with inflammatory bowel diseases: calprotectin and IL-6 changes do not correspond to sRAGE changes. *Scandinavian Journal of Clinical and Laboratory Investigation* 70: 294–299.

Mancho, C., Sainz, Á., García-Sancho, M., Villaescusa, A., Rodríguez-Franco, F., 2011. Evaluation of perinuclear antineutrophilic cytoplasmic antibodies in sera from dogs with inflammatory bowel disease or intestinal lymphoma. *American Journal of Veterinary Research* 72: 1333–1337.

Mandigers, P.J., Biourge, V., van den Ingh, T.S., Ankringa, N., German, A.J., 2010. A randomized, open-label, positively-controlled field trial of a hydrolyzed protein diet in dogs with chronic small bowel enteropathy. *Journal of Veterinary Internal Medicine* 24: 1350–1357.

McCann, T.M., Ridyard, A.E., Else, R.W., Simpson, J.W., 2007. Evaluation of disease activity markers in dogs with idiopathic inflammatory bowel disease. *The Journal of Small Animal Practice* 48: 620–625.

McMahon, L.A., House, A.K., Catchpole, B., Elson-Riggins, J., Riddle, A., Smith, K., Werling, D., Burgener, I.A., Allenspach, K., 2010. Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease. *Veterinary Immunology and Immunopathology* 135: 158–163.

Meijer, B., Hoskin, T., Ashcroft, A., Burgess, L., Keenan, J.I., Falvey, J., Gearry, R.B., Day, A.S., 2014. Total soluble and endogenous secretory receptor for advanced glycation end products (RAGE) in IBD. *Journal of Crohn's and Colitis* 8: 513–520.

Melgarejo, T., Williams, D.A., Griffith, G., 1996. Isolation and characterization of α_1-protease inhibitor from canine plasma. *American Journal of Veterinary Research* 57: 258–263.

Midgley, A.R., Niswender, G.D., Rebar, R.W., 1969. Principles for the assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). *Acta Endocrinologica. Supplementum (Copenhagen)* 142: 163–184.

Miranda, L.P., Tao, T., Jones, A., Chernushevich, I., Standing, K.G., Geczy, C.L., Alewood, P.F., 2001. Total chemical synthesis and chemotactic activity of human S100A12 (EN-RAGE), *FEBS Letters* 488: 85–90.

Mirmohammadsadegh, A., Tschakarjan, E., Ljoljic, A., Bohner, K., Michel, G., Ruzicka, T., Goos, M., Hengge, U.R., 2000. Calgranulin C is overexpressed in lesional psoriasis. *The Journal of Investigative Dermatology* 114: 1207–1208.

Mischke, R., Hänies, R., Lange, K., Rivera Ramirez, P.A., 1996. The effect of the albumin concentration on the relation between the concentration of ionized calcium and total calcium in the blood of dogs. *Deutsche Tierärztliche Wochenschrift* 103: 199–204.

Moore, B.W., 1965. A soluble protein characteristic of the nervous system. *Biochemical and Biophysical Research Communications* 19: 739–744.

Moroz, O.V., Dodson, G.G., Wilson, K.S., Lukanidin, E., Bronstein, I.B., 2003. Multiple structural states of S100A12: a key to its functional diversity. *Microscopy Research and Technique* 60: 581–592.

Moroz, O.V., Burkitt, W., Wittkowski, H., He, W., Ianoul, A., Novitskaya, V., Xie, J., Polyakova, O., Lednev, I.K., Shekhtman, A., Derrick, P.J., Bjoerk, P., Föll, D., Bronstein, I.B., 2009. Both Ca^{2+} and Zn^{2+} are essential for S100A12 protein oligomerization and function. *BMC Biochemistry* 10: 11.

Münster, M., Suchodolski, J.S., Bilzer, T., Bilzer, C., Hörauf, A., Steiner, J.M., 2010. Influence of physiological disturbances on treatment success of dietary therapy in dogs with chronic enteropathies. *Berliner und Münchener Tierärztliche Wochenschrift* 123: 74–82.

Murua Escobar, H., Soller, J.T., Sterenczak, K.A., Sperveslage, J.D., Schlueter, C., Burchardt, B., Eberle, N., Fork, M., Nimzyk, R., Winkler, S., Nolte, I., Bullerdiek, J., 2006. Cloning and characterization of the canine receptor for advanced glycation end products. *Gene* 369: 45–52.

Myles, A., Viswanath, V., Singh, Y.P., Aggarwal, A., 2011. Soluble receptor for advanced glycation end products is decreased in patients with juvenile idiopathic arthritis (ERA category) and inversely correlates with disease activity and S100A12 levels. *The Journal of Rheumatology* 38: 1994–1999.

Nakamura, M., Takahashi, M., Ohno, K., Koshino, A., Nakashima, K., Setoguchi, A., Fujino, Y., Tsujimoto, H., 2008. C-reactive protein concentration in dogs with various diseases. *The Journal of Veterinary Medical Science* 70: 127–131.

Neurath, M.F., Finotto, S., Glimcher, L.H., 2002. The role of Th1/Th2 polarization in mucosal immunity. *Nature Medicine* 8: 567–573.

Odink, K., Cerletti, N., Brüggen, J., Clerc, R.G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R., Sorg, C., 1987. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* 330: 80–82.

Ohta, H., Takada, K., Sunden, Y., Tamura, Y., Osuga, T., Lim, S.Y., Murakami, M., Sasaki, N., Wickramasekara Rajapakshage, B.K., Nakamura, K., Yamasaki, M., Takiguchi, M., 2014. $CD4^+$ T cell cytokine gene and protein expression in duodenal mucosa of dogs with inflammatory bowel disease. *The Journal of Veterinary Medical Science* 76: 409–414.

Okanishi, H., Kabeya, H., Maruyama, S., Kagawa, Y., Watari, T., 2013. Activation of nuclear factor-kappa B and cell adhesion molecule mRNA expression in duodenal mucosa of dogs with lymphocytic-plasmacytic enteritis. *Veterinary Immunology and Immunopathology* 154: 145–152.

Otoni, C.C., Heilmann, R.M., García-Sancho, M., Suchodolski, J.S., Steiner, J.M., Jergens, A.E., 2012. Serologic and fecal markers in prediction of acute disease course in canine chronic enteropathies. *Journal of Veterinary Internal Medicine* 26: 768 (abstract).

Parra, M.D., Tuomola, M., Cabezas-Herrera, J., Cerón, J.J., 2006. Analytical and clinical validation of a time-resolved immunofluorometric assay (TR-IFMA) for canine C-reactive protein in serum. *Veterinary Research Communication* 30: 113–126.

Peters, I.R., Helps, C.R., Calvert, E.L., Hall, E.J., Day, M.J., 2005. Cytokine mRNA quantification in duodenal mucosa from dogs with chronic enteropathies by real-time reverse transcriptase polymerase chain reaction. *Journal of Veterinary Internal Medicine* 19: 644–653.

Pietzsch, J., Hoppmann, S., 2008. Human S100A12: a novel key player in inflammation? *Amino Acids* 36: 381–389.

Plickert, H.D., Einspanier, R., Arndt, G., Brunnberg, L., Kohn, B., 2011. Evaluation of a point-of-care test for canine C-reactive protein. *Veterinary Clinical Pathology* 40: 384–388.

Post, K., Feldman, E.C., 1978. Hemorrhagic gastroenteritis in a Toy Poodle. *Modern Veterinary Practice* 59: 422-426.

Poullis, A.P., Zar, S., Sundaram, K.K., Moodie, S.J., Risley, P., Theodossi, A., Mendall, M.A., 2002. A new, highly sensitive assay for C-reactive protein can aid the differentiation of inflammatory bowel disorders from constipation- and diarrhea-predominant functional bowel disorders. *European Journal of Gastroenterology and Hepatology* 14: 409–412.

Rhodes, B., Fürnrohr, B.G., Vyse, T.J., 2011. C-reactive protein in rheumatology: biology and genetics. *Nature Reviews. Rheumatology* 7: 292–298.

Roberts, W.L., Sedrick, R., Moulton, L., Spencer, A., Rifai, N., 2000. Evaluation of four automated high-sensitivity C-reactive protein methods: Implications for clinical and epidemiological applications. *Clinical Chemistry* 46: 461–468.

Robinson, M.J., Hogg, N., 2000. A comparison of human S100A12 with MRP-14 (S100A9). *Biochemical and Biophysical Research Communications* 275: 865–870.

Roth, J., Vogl, T., Sorg, C., Sunderkötter, C., 2003. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends in Immunology* 24: 155–158.

Ruaux, C.G., 2008. Laboratory testing for the diagnosis of intestinal disorders. In: Steiner, J.M. (Ed.) *Small Animal Gastroenterology*. Hannover, Germany: Schlütersche, pp. 50–55.

Schäfer, B.W., Heizmann, C.W., 1996. The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends in Biochemical Sciences* 21: 134–140.

Schlegel, B.J., Van Dreumel, T., Slavić, D., Prescott, J.F., 2012. *Clostridium perfringens* type A fatal acute hemorrhagic gastroenteritis in a dog. *The Canadian Veterinary Journal* 53: 555–557.

Schmidt, A.M., Yan, S.D., Yan, S.F., Stern, D.M., 2000. The biology of the receptor for advanced glycation end products and its ligands. *Biochimie et Biophysica Acta* 1498: 99–111.

Schmitz, S., Garden, O.A., Werling, D., Allenspach, K., 2012. Gene expression of selected signature cytokines of T cell subsets in duodenal tissues of dogs with and without inflammatory bowel disease. *Veterinary Immunology and Immunopathology* 146: 87–91.

Shine, B., Berghouse, L., Jones, J.E., Landon, J., 1985. C-reactive protein as an aid in the differentiation of functional and inflammatory bowel disorders. *Clinica Chimica Acta* 148: 105–109.

Sidler, M.A., Leach, S.T., Day, A.S., 2008. Fecal S100A12 and fecal calprotectin as noninvasive markers for inflammatory bowel disease in children. *Inflammatory Bowel Diseases* 14: 359–366.

Simpson, K.W., Jergens, A.E., 2012. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. *The Veterinary Clinics of North America (Small Animal Practice)* 41: 381–398.

Spielman, B.L., Garvey, M.S., 1993. Hemorrhagic gastroenteritis in 15 dogs. *Journal of the American Animal Hospital Association* 29: 341-344.

Steiner, J.M., Ruaux, C.G., Miller, M.D., Wright, Z.M., Teague, S.R., Vaden, S., Williams, D.A., 2003. Intra-individual variability of fecal α_1-proteinase inhibitor concentration in clinically healthy dogs. *Journal of Veterinary Internal Medicine* 17: 445 (abstract).

Sterenczak, K.A., Willenbrock, S., Barann, M., Klemke, M., Soller, J.T., Eberle, N., Nolte, I., Bullerdiek, J., Murua Escobar, H., 2009. Cloning, characterisation, and comparative quantitative expression analyses of receptor for advanced glycation end products (RAGE) transcript forms. *Gene* 434: 35–42.

Sterenczak, K.A., Kleinschmidt, S., Wefstaedt, P., Eberle, N., Hewicker-Trautwein, M., Bullerdiek, J., Nolte, I., Murua Escobar, H., 2011. Quantitative PCR and immunohistochemical analyses of HMGB1 and RAGE expression in canine

disseminated histiocytic sarcoma (malignant histiocytosis). *Anticancer Research* 31: 1541–1548.

Stewart, C., Cha, S., Caudle, R.M., Berg, K., Katz, J., 2008. Decreased levels of soluble receptor for advanced glycation end products in patients with primary Sjögren's syndrome. *Rheumatology International* 28: 771–776.

Suchodolski, J.S., Xenoulis, P.G., Paddock, C.G., Steiner, J.M., Jergens, A.E., 2010. Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. *Veterinary Microbiology* 142: 394–400.

Suchodolski, J.S., Markel, M.E., Garcia-Mazcorro, J.F., Unterer, S., Heilmann, R.M., Dowd, S.E., Kachroo, P., Ivanov, I., Minamoto, Y., Dillman, E.M., Steiner, J.M., Cook, A.K., Toresson, L., 2012. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One* 7: e51907.

Sukkar, M.B., Wood, L.G., Tooze, M., Simpson, J.L., McDonald, V.M., Gibson, P.G., Wark, P.A., 2012. Soluble RAGE is deficient in neutrophilic asthma and COPD. *The European Respiratory Journal* 39: 721–729.

Tamura, Y., Ohta, H., Yokoyama, N., Lim, S.Y., Osuga, T., Morishita, K., Nakamura, K., Yamasaki, M., Takiguchi, M., 2014. Evaluation of selected cytokine gene expression in colonic mucosa from dogs with idiopathic lymphocytic-plasmacytic colitis. *The Journal of Veterinary Medical Science* 76: 1407–1410.

Toresson, L., Steiner, J.M., Suchodolski, J.S., Spillmann, T., 2014. Oral cobalamin supplementation in dogs with chronic enteropathies and hypocobalaminemia. *Journal of Veterinary Internal Medicine* 28: 1044 (abstract).

Travis, J., 1988. Structure, function, and control of neutrophil proteinases. *The American Journal of Medicine* 84: 37–42.

Unterer, S., Strohmeyer, K., Kruse, B.D., Sauter-Louis, C., Hartmann, K., 2011. Treatment of aseptic dogs with hemorrhagic gastroenteritis with amoxicillin/clavulanic acid: a prospective blinded study. *Journal of Veterinary Internal Medicine* 25: 973–979.

Unterer, S., Busch, K., Leipig, M., Hermanns, W., Wolf, G., Straubinger, R.K., Mueller, R.S., Hartmann, K., 2014. Endoscopically visualized lesions, histologic findings, and bacterial invasion in the gastrointestinal mucosa of dogs with acute hemorrhagic diarrhea syndrome. *Journal of Veterinary Internal Medicine* 28: 52–58.

Vaden, S.L., Hammerberg, B., Orton, S.M., Stone, E.A., 2000. Mast cell degranulation responses in Soft-Coated Wheaten Terriers with protein-losing enteropathy and/or nephropathy. *Journal of Veterinary Internal Medicine* 14: 348 (abstract).

Vaden, S.L., Vidaurri, A., Levine, J.F., Harris, T., Steiner, J.M., 2002. Fecal α_1-proteinase inhibitor activity in Soft Coated Wheaten Terriers. *Journal of Veterinary Internal Medicine* 16: 382 (abstract).

Vaitukaitis, J.L., Robbins, J.B., Nieschlag, E., Ross, G.T., 1971. A method for producing specific antisera with small doses of immunogen. *The Journal of Clinical Endocrinology and Metabolism* 33: 988–991.

Valentin, M.A., Ma, S., Zhao, A., Legay, F., Avrameas, A., 2011. Validation of immunoassay for protein biomarkers: Bioanalytical study plan implementation to support pre-clinical and clinical studies. *Journal of Pharmaceutical and Biomedical Analysis* 55: 869–877.

van de Logt, F., Day, A.S., 2013. S100A12: a noninvasive marker of inflammation in inflammatory bowel disease. *Journal of Digestive Diseases* 14: 62–67.

van den Bos, C., Rammes, A., Vogl, T., Boynton, R., Zaia, J., Sorg, C., Roth, J., 1998. Copurification of P6, MRP8, and MRP14 from human granulocytes and separation of individual proteins. *Protein Expression and Purification* 13: 313–318.

Vogl, T., Pröpper, C., Hartmann, M., Strey, A., Strupat, K., van den Bos, C., Sorg, C., Roth, J., 1999. S100A12 is expressed exclusively by granulocytes and acts independently from MRP8 and MRP14. *The Journal of Biological Chemistry* 274: 25291–25296.

Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A., Nacken, W., Föll, D., van der Poll, T., Sorg, C., Roth, J., 2007. Mrp8 and Mrp14

are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nature Medicine* 13: 1042–1049.

Washabau, R.J., Day, M.J., Willard, M.D., Hall, E.J., Jergens, A.E., Mansell, J., Minami, T., Bilzer, T.W., WSAVA International Gastrointestinal Standardization Group, 2010. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. *Journal of Veterinary Internal Medicine* 24: 10–26.

Wilke, V.L., Nettleton, D., Wymore, M.J., Gallup, J.M., Demirkale, C.Y., Ackermann, M.R., Tuggle, C.K., Ramer-Tait, A.E., Wannemuehler, M.J., Jergens, A.E., 2012. Gene expression in intestinal mucosal biopsy specimens obtained from dogs with chronic enteropathy. *American Journal of Veterinary Research* 73: 1219–1229.

Wittkowski, H., Hirono, K., Ichida, F., Vogl, T., Ye, F., Yanlin, X., Saito, K., Uese, K., Miyawaki, T., Viemann, D., Roth, J., Föll, D., 2007. Acute Kawasaki disease is associated with reverse regulation of soluble receptor for advance glycation end products and its proinflammatory ligand S100A12. *Arthritis and Rheumatism* 56: 4174–4181.

Wu, A.H., Lu, Q.A., Todd, J., Moecks, J., Wians, F., 2009. Short- and long-term biological variation in cardiac troponin I measured with a high-sensitivity assay: implications for clinical practice. *Clinical Chemistry* 55: 52–58.

Xie, J., Burz, D.S., He, W., Bronstein, I.B., Lednev, I., Shekhtman, A., 2007. Hexameric calgranulin C (S100A12) binds to the receptor for advanced glycation end products (RAGE) using symmetric hydrophobic target-binding patches. *The Journal of Biological Chemistry* 282: 4218–4231.

Yamashita, K., Oyama, Y., Shishibori, T., Matsushita, O., Okabe, A., Kobayashi, R., 1999. Purification of bovine S100A12 from recombinant *Escherichia coli*. *Protein Expression and Purification* 16: 47–52.

Yang, Z., de Veer, M.J., Gardiner, E.E., Devenish, R.J., Handley, C.J., Underwood, J.R., Robinson, H.C., 1996. Rabbit polymorphonuclear neutrophils form ^{35}S-labeled S-sulfo-calgranulin C when incubated with inorganic [^{35}S] sulfate. *The Journal of Biological Chemistry* 271: 19802–19809.

Yang, Z., Tao, T., Raftery, M.J., Youssef, P., Di Girolamo, N., Geczy, C.L., 2001. Proinflammatory properties of the human S100 protein S100A12. *Journal of Leukocyte Biology* 69: 986–994.

Yang, Z., Yan, W.X., Cai, H., Tedla, N., Armishaw, C., Di Girolamo, N., Wang, H.W., Hampartzoumian, T., Simpson, J.L., Gibson, P.G., Hunt, J., Hart, P., Hughes, J.M., Perry, M.A., Alewood, P.F., Geczy, C.L., 2007. S100A12 provokes mast cell activation: a potential amplification pathway in asthma and innate immunity. *The Journal of Allergy and Clinical Immunology* 119: 106–114.

Yousefi, R., Ardestani, S.K., Saboury, A.A., Kariminia, A., Zeinali, M., Amani, M., 2005. Investigation on the surface hydrophobicity and aggregation kinetics of human calprotectin in the presence of calcium. *Journal of Biochemistry and Molecular Biology* 38: 407–413.