REVIEW

Journal of Veterinary Internal Medicine
ACVIM
Open Access
American College of Veterinary Internal Medicine

Clinical utility of currently available biomarkers in inflammatory enteropathies of dogs

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Chronic inflammatory enteropathies (CIE) in dogs are a group of disorders that are characterized by chronic persistent or recurrent signs of gastrointestinal disease and histologic evidence of mucosal inflammation. These CIEs are classified as either food-responsive, antibiotic-responsive, or immunosuppressant-responsive enteropathy. Patients not clinically responding to immunomodulatory treatment are grouped as nonresponsive enteropathy and dogs with intestinal protein loss as protein-losing enteropathy. Disease-independent clinical scoring systems were established in dogs for assessment of clinical disease severity and patient monitoring during treatment. Histopathologic and routine clinicopathologic findings are usually not able to distinguish the subgroups of CIE. Treatment trials are often lengthy and further diagnostic tests are usually at least minimally invasive. Biomarkers that can aid in defining the presence of disease, site of origin, severity of the disease process, response to treatment, or a combination of these would be clinically useful in dogs with CIE. This article summarizes the following biomarkers that have been evaluated in dogs with CIE during the last decade, and critically evaluates their potential clinical utility in dogs with CIE: functional biomarkers (cobalamin, methylmalonic acid, folate, α_1-proteinase inhibitor, immunoglobulin A), biochemical biomarkers (C-reactive protein, perinuclear anti-neutrophilic cytoplasmic antibodies, 3-bromotyrosine, N-methylhistamine, calprotectin, S100A12, soluble receptor of advanced glycation end products, cytokines and chemokines, alkaline phosphatase), microbiomic biomarkers (microbiome changes, dysbiosis index), metabolomic biomarkers (serum metabolome), genetic biomarkers (genomic markers, gene expression changes), and cellular biomarkers (regulatory T cells). In addition, important performance criteria of diagnostic tests are briefly reviewed.

KEYWORDS

antibiotic-responsive enteropathy, dog, food-responsive enteropathy, inflammation, inflammatory bowel disease

Abbreviations: α_1PI, alpha$_1$-proteinase inhibitor; AP, alkaline phosphatase; ARE, antibiotic-responsive enteropathy; 3-BrY, serum 3-bromotyrosine; CCL, CC chemokine ligand; CIE, chronic inflammatory enteropathy; cPLI, canine specific pancreatic lipase; CR, complete remission; CRP, C-reactive protein; CXCL, CXC chemokine ligand; DAMP, damage-associated molecular pattern; FRE, food-responsive enteropathy; IgA, immunoglobulin A; IL, interleukin; IRE, immunosuppressant-responsive enteropathy; MMA, methylmalonic acid; NMH, N-methylhistamine; NPV, negative predictive value; NR, no response; NRE, nonresponsive (immunosuppressant-refractory) enteropathy; pANCA, perinuclear anti-neutrophilic cytoplasmic antibodies; PLE, protein-losing enteropathy; PPV, positive predictive value; PR, partial response; RAGE, receptor for advanced glycation end products; sRAGE, soluble RAGE; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor; UPC, urine protein-to-creatinine ratio.

1 | INTRODUCTION

Chronic inflammatory enteropathies (CIE) in dogs comprise a group of disorders that are characterized by chronic persistent or recurrent gastrointestinal signs, histologic evidence of mucosal inflammation, and the exclusion of other underlying gastrointestinal or extra-gastrointestinal diseases.[1–4] Clinicopathologic variables, fecal parasite examination, and diagnostic imaging are important means to rule out other etiologies with a similar clinical presentation. The CIEs are

retrospectively classified based on the response to empirical treatment as food-responsive (FRE), antibiotic-responsive (ARE), or immunosuppressant-responsive enteropathy (IRE). Patients that do not respond to immunomodulatory treatment are categorized as having nonresponsive enteropathy (NRE).[3–6] The term protein-losing enteropathy (PLE) is used for a subgroup of affected CIE dogs with intestinal protein loss, overall carrying a worse prognosis.[3,6,7] Disease-independent clinical scoring systems have been established in dogs[8,9] and can be used to semi-objectively assess the severity of clinical signs at the time of diagnosis and to monitor patient improvement during treatment. Histopathologic and routine clinicopathologic findings usually are not able to distinguish different subgroups of CIE,[5,10] but younger dogs with less severe clinical signs are more likely to be diagnosed with FRE.[5,9] Treatment trials often are lengthy and further diagnostic tests, after appropriately designed dietary and antibiotic trials have failed, are usually invasive.[3–6] Biomarkers that can aid in diagnostic evaluation or patient monitoring or that can assess the response to various forms of treatment thus would be clinically useful in dogs with CIE. Several functional, biochemical, microbiomic, metabolomic, genetic, and cellular biomarkers have been evaluated in dogs with CIE during the last decade. This article will summarize selected biomarkers that have been evaluated in dogs with CIE and will critically evaluate their potential clinical utility. In addition, important performance criteria of diagnostic tests are discussed.

2 | BIOMARKER CHARACTERISTICS

Understanding the clinical utility andas the limitations of biomarkers is very important when using biomarker data in clinical cases and to prevent pitfalls in the interpretation of biomarker results in clinical practice.[11] Thus, the goals of using biomarkers and the parameters of importance for the interpretation of biomarker assays in the clinical setting are briefly reviewed.

2.1 | Clinical utility of biomarkers

For a biomarker to be clinically useful, it must aid in evaluating organ function, assess the risk for disease development, diagnose a specific disease process, assess disease activity or severity, predict the patient's response to treatment or predict disease outcome, monitor disease progress, or some combination of these.[11,14] Because a single biomarker is very unlikely to satisfy all of these goals, it is very important to consider what clinical information is expected to be gained from using a specific biomarker, and in what clinical situation it will serve as a good surrogate marker for existing algorithms (eg, definition of baseline disease activity, response to treatment, or maintenance of clinical remission) to improve diagnostic decision making, treatment recommendations, patient monitoring, or even prophylactic measures (Table 1).[11–13]

2.2 | Classification of biomarkers

Disease biomarkers are either static or dynamic, and based on the desired characteristics described above, they typically are subclassified

TABLE 1 Biomarker goals

- Assessment of the risk for disease development
- Diagnosis of a disease process
- Evaluation of organ function
- Determination of organ origin
- Assessment of disease severity
- Assessment of response to treatment
- Prediction of individual outcome
- Monitoring of a disease process
- Detection of disease flares

as functional, biochemical, cellular, genomic, proteomic, metabolomic, microbiomic, behavioral, or outcome biomarkers.[14–16] Of these, functional, biochemical, and genomic biomarkers currently are the most practical to use and most widely available and are currently of particular clinical interest for CIE management in dogs (Figure 1).

An important consideration for the interpretation of biomarker data is that based on the characteristics of the biomarker assay, either a qualitative or quantitative test result is obtained. Qualitative assays yield a dichotomized (eg, positive or negative) test result, whereas quantitative assays offer a numerical test result (ie, continuous or ordinal data). Interpretation of quantitative assays usually requires the use of either population- or patient-based cut-off values,[18–20] and the results of these assays might also fall within a gray range (ie, ambiguous test results).

2.3 | Biomarker performance criteria

Understanding the performance characteristics of diagnostic tests is important for correct interpretation. Any diagnostic test result must be interpreted in light of patient history, clinical signs, physical examination findings, and also in combination with the results of other diagnostic tests (eg, clinicopathologic variables, diagnostic imaging findings) performed.[11,14,17]

Although often assumed (and usually beyond the clinician's responsibility), it is important to recognize that the diagnostic performance of an assay relies heavily on its analytical performance.[21] This includes preanalytic effects associated with the collection and handling (including storage) of samples and analytical variability arising from the methodology, instrumentation, and also technical skills.[18,21,22] This aspect becomes especially important for the clinician when utilizing patient-side (ie, in-house) diagnostic tests.[23] Important variables that are determined for the preanalytical validation of a biomarker assay include the lower detection limit of the assay, assay sensitivity, dilutional parallelism and linearity, assay accuracy, precision, and reproducibility.[14,21] In addition, quality assurance of a biomarker test requires that a quality control program be in place.[22,23]

2.3.1 | Biological variability

Biological variability, an inherent characteristic of all biochemical analytes, is an important aspect in the interpretation of the diagnostic performance of a biomarker.[18] Normal biological variability comprises within-subject (intraindividual) and among-subject (interindividual)
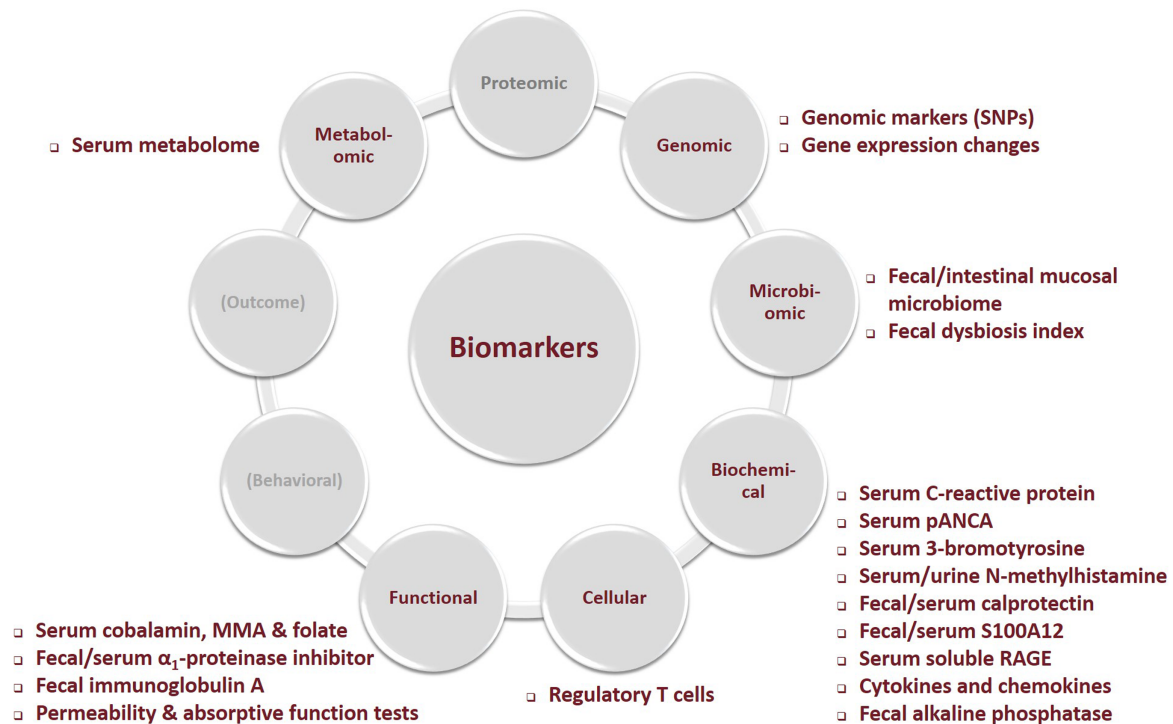
FIGURE 1 Groups of biomarkers in dogs with chronic inflammatory enteropathies (CIE). The figure shows the functional, biochemical, metabolomic, microbiomic, genomic, and cellular biomarkers that have been evaluated in dogs with CIE. MMA, methylmalonic acid; pANCA, perinuclear anti-neutrophilic cytoplasmic antibodies; RAGE, receptor for advanced glycation end products; SNP, single-nucleotide polymorphism

variation, both of which might be similar or different between health and various disease states.[18,24]

Sources of biological variation include rhythmic expression or secretion, variation in gastrointestinal passage or patchy distribution of cells expressing and releasing a biomarker measured in feces, and the metabolism and half-life of a biomarker (determined by its size, charge, hydrophobicity, and route of excretion).[18,24] Such sources of biological variation must be recognized and strategies developed to counterbalance such variation (eg, by collecting specimens on multiple days or at a certain time of the day).

Components of biological variability (ie, intra- and interindividual variability) usually are estimated from a relatively small set of specimens (depending upon the biological variability) obtained from a small group of healthy individuals during a relatively short time period.[18] For most biomarkers, this approach allows for determining the critical change value (based on the indices of individuality and heterogeneity) and thus assessing the utility of a conventional population-based reference interval.[18]

The critical change value is the percentage (or concentration) that a biomarker must change between sequential measurements to be considered a clinically relevant alteration and not merely a reflection of biological or analytical variability.[18,24] This variable also is an important determinant of the ability of a biomarker to distinguish diseased individuals from healthy individuals and from disease controls.

Biomarkers for which the use of a conventional population-based reference interval is appropriate generally are useful for diagnosing a disease (ie, as an "event marker").[18] In contrast, biomarkers for which using a traditional population-based reference interval is not feasible generally are better for monitoring a condition (ie, as a "chronic disease marker").[18]

Establishing the lower and upper limits of the reference interval for a quantitative biomarker requires a large reference sample group (ideally $n > 120$),[19,20] and can be done by several different methods, depending on the number of individuals included in the reference sample group and the distribution of the data.[19,20] Because some biomarkers can vary with age, sex, neuter status, body condition, muscle condition, or other environmental factors (eg, diet, medications), stratification of the reference population based on such variables may be necessary.

2.3.2 | Diagnostic accuracy

Diagnostic accuracy of a test typically is described by means of its estimated sensitivity and specificity.[14,25,26]

Sensitivity of a diagnostic test is the likelihood of a positive test result in a patient affected with the disease (true positive rate) that was diagnosed based on an independent gold standard diagnostic method or technique. Sensitivity is calculated as the number of all true positive individuals divided by the number of all diseased individuals. The higher the sensitivity, the lower the false negative rate (ie, great confidence exists that a negative test result is true). Consequently, diagnostic tests with a high sensitivity are useful to rule out a disease (ie, as screening tests).[11,14,25]

Specificity of a diagnostic test is the likelihood of a negative test result in a patient not having the disease (true negative rate) as determined by an independent gold standard diagnostic test. Specificity is calculated as the number of all true negative individuals divided by

the number of all nondiseased individuals. The higher the specificity, the lower the false positive rate (ie, great confidence exists that a positive test result is true). Thus, diagnostic tests with a high specificity are useful for confirming a diagnosis (confirmatory tests).[11,14,25]

2.3.3 | Diagnostic performance

Predictive values of a diagnostic test can be determined if (in addition to the diagnostic accuracy of the test) the prevalence of the disease in the population tested is known.[14,25] Predictive values are important variables in clinical practice when using a biomarker to determine the patient's disease status, because they can help select the patient population in which a specific test is likely to yield meaningful results.[14,20,26]

The positive predictive value (PPV) is the chance that a patient with a positive test result has the disease.[26] It is calculated as the number of all true positive test results divided by the number of all individuals testing positive. Diagnostic tests with a low specificity have a low PPV when the disease prevalence is low. These tests are good confirmatory tests in patients with a strong suspicion for the disease and absent any factors (eg, medication) that could produce false-positive results, but they should not be used to screen for an uncommon disease. The negative predictive value (NPV) is the chance that a patient with a negative test result does not have the disease.[26] It is calculated as the number of all true negative test results divided by the number of all individuals testing negative. Diagnostic tests with a low sensitivity have a low NPV when the disease prevalence is high. These tests are good screening tests for large patient populations, but should not be used to rule out the diagnosis when there is a strong suspicion for the disease.[11,14]

Because PPV and NPV depend on the disease prevalence, studies reporting PPV and NPV should be critically evaluated and interpreted with caution if predictive values are estimated based on the study prevalence as it might not reflect the prevalence of the disease in the population for which the test will be used. The study prevalence might be different from the disease prevalence in a specific clinical practice setting and, unfortunately, the true prevalence of few diseases is known in veterinary medicine.

3 | BIOMARKERS IN CHRONIC INFLAMMATORY ENTEROPATHIES OF DOGS

3.1 | Routine clinicopathologic variables in CIE

Routine clinicopathologic variables usually are not specific for a diagnosis of CIE but are important to rule out other diseases causing similar clinical signs and to assess the overall status of the patient.[1,3,6]

Serum albumin concentration can be decreased in some dogs with CIE because of gastrointestinal protein loss (PLE) and is of prognostic value.[7,9,27,28] Serum calcium and cholesterol concentrations also may be decreased in dogs with PLE.[6,7,29,30] Serum bile acid concentrations can help further evaluate the patient for hepatopathy, and urinalysis with a urine protein-to-creatinine (UPC) ratio can help exclude renal loss of protein if the UPC is <0.5.

A baseline serum cortisol concentration or an ACTH stimulation test can exclude or confirm a diagnosis of (atypical)

hypoadrenocorticism. A fecal parasite examination also should be performed. Subnormal serum canine trypsin-like immunoreactivity concentrations can identify dogs with exocrine pancreatic insufficiency.[31] Serum canine specific pancreatic lipase (cPLI) concentration also should be measured to evaluate the patient for concurrent pancreatitis. Increased serum cPLI concentrations appeared to be a negative prognostic factor in dogs with CIE in 1 study.[32] Serum gastrin concentrations may indicate a gastrinoma if increased >10 times above the normal reference interval.[33]

Despite the importance of the minimum database comprised of routine clinicopathologic tests, these diagnostic tests usually are not specific for CIE in dogs, and additional biomarkers that are more disease-specific, organ-specific, or both appear to be a very useful additional tool for the management of dogs with CIE.

3.2 | Clinical utility of biomarkers for CIE

Biomarkers that could be clinically useful in dogs with CIE are markers that reflect the individual risk to develop CIE, evaluate gastrointestinal function (ie, digestion, absorption, secretion, or a combination of these), aid in diagnosing the inflammatory disease process, indicate disease activity or severity, predict individual response to specific forms of treatment or disease outcome, monitor the severity of gastrointestinal inflammation, or even a combination of these.[12-14] The utility of a marker in routine clinical practice also requires that it be easy to evaluate, inexpensive, and minimally invasive, in addition to being stable in routine biological specimens under clinical conditions.[11]

3.3 | Classes of biomarkers for CIE

Biomarkers with potential clinical utility that have been evaluated in dogs with CIE are functional, biochemical, and genomic markers (Figure 1). Selected biomarkers are discussed below.

3.4 | Functional biomarkers

The functional biomarkers include fecal and serum alpha$_1$-proteinase inhibitor (α_1PI) concentrations, serum folate (vitamin B$_9$), and cobalamin (vitamin B$_{12}$) concentrations, markers to evaluate gastrointestinal permeability and absorptive function (eg, ^{51}Cr-EDTA or iohexol absorption, serum or urine lactulose/rhamnose ratio, or the xylose/methylglucose ratio), fecal immunoglobulin A (IgA) concentrations, serum metabolite profiles, and the fecal dysbiosis index. Because markers to assess gastrointestinal permeability and absorptive function are fairly invasive and impractical, these tests are not useful clinically and will not be discussed in detail.

3.4.1 | Fecal and serum alpha$_1$-proteinase inhibitor (α_1PI)

Alpha$_1$-proteinase inhibitor (α_1PI, also known as alpha$_1$-antitrypsin in human medicine) is a major proteinase inhibitor that is primarily synthesized in the liver.[34] Despite being protective against the effects of trypsin, chymotrypsin, and neutrophil proteases, the role of canine α_1PI as an acute-phase reactant still is controversial.[35-38] Canine α_1PI has a molecular weight similar to that of albumin,[39] and with diseases

TABLE 2 Performance characteristics of selected biomarkers evaluated in dogs with chronic inflammatory enteropathies

Biomarker	Group comparison	Cut-off	Sensitivity	Specificity	Critical change value	Reference
Fecal α_1PI (3-day mean)	PLE versus non-PLE-CIE[a]	≥19.0 µg/g	44%	85%	nd	[43]
Serum α_1PI		nd ≤1,087 mg/L	nd 64%	nd 93%	510 mg/L nd	[48] [43]
Serum-to-fecal α_1PI ratio		≤53.6 g/mL	49%	89%	nd	[43]
Serum CRP	IRE/NRE versus FRE/ARE	≥9.1 mg/L nd	72% nd	100% nd	nd 270%	[50] [92]
Serum pANCA	IRE/NRE versus other causes of diarrhea or healthy	pANCA positivity	51%	83%	nd	[98]
	FRD versus IRE/NRE		62%	77%	nd	[100]
	IRE/NRE versus lymphoma		37%	83%	nd	[101]
	FRD versus IRE/NRE/healthy		61%	100%	nd	[99]
Fecal calprotectin	IRE/NRE: PR/NR versus CR	≥15.2 µg/g	80%	75%	nd	[50]
Serum calprotectin	IRE/NRE versus healthy	≥296 µg/L nd	82% nd	68% nd	nd 6.4 mg/L	[94] [173]
Fecal S100A12	Endoscopic score: ≤1 versus ≥2	≥273 ng/g	71%	89%	nd	[133]
	IRE/NRE versus FRE/ARE	≥490 ng/g	64%	77%	nd	[45]
	IRE/NRE: NR versus CR/PR	≥2,700 ng/g	100%	73%	nd	[45]
Serum S100A12	nd nd	nd nd	nd nd	nd nd	85% 115%	[132] [131]
Serum sRAGE	IRE/NRE versus. healthy	≤340 ng/L	90%	73%	nd	[27]
Fecal dysbiosis index	CIE versus healthy	≥0	74%	95%	nd	[68]

Abbreviations: α_1PI, alpha$_1$-proteinase inhibitor; ARE, antibiotic-responsive enteropathy; CIE, chronic inflammatory enteropathy; CRP, C-reactive protein; CR, complete remission; FRE, food-responsive enteropathy; IRE, immunosuppressive-responsive enteropathy; nd, not determined; NR, no response; pANCA, perinuclear anti-neutrophilic cytoplasmic antibodies; PLE, protein-losing enteropathy; PR, partial response; sRAGE, soluble receptor for advanced glycation end products.
[a] All dogs with CIE independent of the disease classification.

causing gastrointestinal protein loss both should be lost at approximately the same rate. Unlike albumin, α_1PI is resistant against proteolysis, allowing its extraction and quantification in fecal samples.[40]

Increased fecal canine α_1PI concentrations are clinically useful as a marker for gastrointestinal protein loss and histologic lesions seen with PLE in dogs (ie, lacteal dilatation, crypt abscesses, or both).[41–43] Relatively large day-to-day variation in fecal α_1PI concentrations necessitates that fecal samples be collected on 3 consecutive days,[40,44] and a 3-day mean fecal α_1PI concentration ≥13.9 µg/g or a 3-day maximum fecal α_1PI concentration ≥21.0 µg/g is interpreted as abnormal.[40] A 3-day mean fecal α_1PI concentration ≥19.0 µg/g[43] is a good confirmatory test (PPV > 80%) for histologic lesions typically seen with PLE in dogs with CIE that have failed elimination diet and antibiotic trials (where the prevalence of hypoalbuminemia is 50–56%[27,45]; Table 2).

Fecal canine α_1PI appears to be particularly useful for the early detection of gastrointestinal protein loss because it can be increased before the onset of clinical signs, hypoalbuminemia (or panhypoproteinemia), or both.[41] The α_1PI test also might be useful to differentiate gastrointestinal protein loss from hepatic causes of hypoalbuminemia. Using a 3-day mean fecal α_1PI concentration of ≥4.0 µg/g appears to be a good test to screen (NPV > 90%) for PLE in dogs with CIE (the prevalence of histologic lesions typically seen with PLE is 20% of dogs with hypoalbuminemia and 20%-30% of all dogs with CIE[5,9,43,45]; Table 2).[43] However, although a value of ≥4.0 µg/g has high sensitivity for PLE, it is not very specific and many dogs with values ≥4.0 µg/g but <19.0 µg/g do not have PLE. Also, increased

fecal canine α_1PI concentrations in dogs <6–12 months of age should be interpreted with caution and should be verified at ≥1 year of age.[40]

Chronic gastrointestinal loss of α_1PI caused by PLE will deplete systemic α_1PI concentrations, resulting in a decreased serum α_1PI concentration[43,46,47] and presumably an altered plasma proteinase-proteinase inhibitor balance. Consequently, the serum-to-fecal α_1PI ratio appears to further improve the diagnostic accuracy for PLE in hypoalbuminemic dogs,[43] but caution must be exercised with the interpretation of serum α_1PI concentrations in corticosteroid-treated dogs.[48] Also, the serum concentration of α_1PI must decrease by at least 510 mg/L (minimum critical difference) for that decrease to be considered clinically relevant.[48]

3.4.2 | Serum cobalamin, methylmalonic acid (MMA), and folate concentrations

Cobalamin (vitamin B$_{12}$) and folate (vitamin B$_9$) are water-soluble vitamins that can be measured in serum and might serve as indicators of CIE.

Cobalamin is exclusively absorbed in the distal small intestine (ileum) where specific receptors internalize the intrinsic factor–cobalamin complex. Hypocobalaminemia frequently is detected in dogs with chronic enteropathies (19%-54%)[5,9,43,45,49,50] and is presumed to reflect distal small intestinal malabsorption, secondary small intestinal dysbiosis (with increased utilization of cobalamin by the intestinal microbiota), or both. Hypocobalaminemia is a negative prognostic factor in dogs with CIE and is associated with hypoalbuminemia.[5,9] However, hypocobalaminemia is not specific for CIE and also can be

observed in dogs with exocrine pancreatic insufficiency.[31] Also, a normal serum cobalamin concentration does not rule out a diagnosis of CIE. Subnormal or low normal serum cobalamin concentrations (<400 ng/L) indicate a need for parenteral (50 μg/kg SQ once weekly for 6 weeks, then every 2-4 weeks) or PO supplementation (50 μg/kg PO q24h for at least 12 weeks) with cyanocobalamin.[51,52]

Methylmalonic acid (MMA) is a metabolite that accumulates when the activity of methylmalonyl-CoA mutase is decreased because of a lack of intracellular cobalamin.[53] Thus, increased MMA production is a useful marker for cobalamin deficiency at the cellular level as a result of cobalamin malabsorption, decreased cobalamin transport, or both.[53,54] Concentrations of MMA can be determined in serum and urine samples,[49,53,54] and measurement of both serum cobalamin and serum or urine MMA appears to be superior to serum cobalamin concentration alone for proper assessment of cobalamin status in dogs. However, measurement of MMA currently is not routinely performed in companion animals[51] because of the cost and technical difficulty of the assay, but it might be a reasonable future strategy. Serum MMA concentrations can be increased in patients with renal insufficiency[49] and must be interpreted in light of the serum creatinine or symmetric dimethylarginine (SDMA) concentration.

Folate is primarily absorbed in the proximal small intestine (ie, duodenum and proximal jejunum) as folate monoglutamate via folate carriers. Hypofolatemia can result from chronic malabsorption in the proximal small intestine and was detected in 14% of dogs with CIE in 1 study,[50] but a decreased serum folate concentration is not specific for CIE and normofolatemia does not exclude a diagnosis of CIE. Serum folate concentration can be falsely normal or increased because of secondary small intestinal dysbiosis (with increased folate production by some members of the intestinal microbiota) or hypocobalaminemia and should be monitored after cobalamin supplementation.[55] Oral folic acid supplementation (10 μg/kg or 200-400 μg/dog PO q24h for 30 days) is recommended in patients with moderate or marked hypofolatemia.

3.4.3 | Fecal immunoglobulin A

Concentrations of IgA can be measured in canine feces presumably reflecting IgA synthesis and secretion by the intestinal mucosa.[56–58] Impaired function of IgA-producing plasma cells and decreased fecal IgA concentrations (relative or absolute IgA deficiency) have been detected in German Shepherd dogs with chronic gastrointestinal disease, which could be a cause or consequence of the disease process.[56,59–62] Although decreased fecal IgA concentrations might be detected in dogs with CIE, currently published data do not lend enough support for the clinical utility of measuring fecal IgA concentrations in dogs suspected to have CIE.

3.5 | Microbiomic biomarkers

3.5.1 | Fecal and mucosal intestinal microbiome

Alterations in the fecal bacterial microbiome have been found in dogs with CIE.[63–66] The most significant decreases were detected in *Faecalibacterium* spp. and Fusobacteria, which are short-chain fatty acid producers of importance for intestinal health.[65] Some differences in the abundance of individual bacterial taxa (ie, an unclassified genus of Neisseriaceae and *Bilophila* in the duodenum, *Burkholderia* in the

colon) also were identified between the intestinal mucosal microbiome in IRE dogs and dogs with FRE.[66]

3.5.2 | Fecal dysbiosis index

The fecal *dysbiosis index* indicates changes in the gut bacterial microbiome (ie, presence of normobiosis versus dysbiosis) by evaluating 8 bacterial groups that commonly are altered in dogs with CIE, particularly dogs diagnosed with IRE (ie, *Blautia*, *Clostridium hiranonis*, *Escherichia coli*, *Faecalibacterium*, *Fusobacterium*, *Streptococcus*, *Turicibacter*, and total bacteria) and distinguishes IRE from health with 74% sensitivity and 95% specificity.[67,68]

3.6 | Metabolomic biomarkers

3.6.1 | Serum metabolite profile

Serum metabolome changes in dogs with CIE were shown to have a distinct signature (ie, a higher abundance of 3-hydroxybutyrate, hexuronic acid, ribose, and gluconic acid lactone) indicating CIE in dogs to be associated with oxidative stress and also functional changes in the gut microbiome.[63]

Serum metabolite profiles together with the fecal dysbiosis index might be clinically useful tools and offer an approach to individualized treatment and monitoring, but high expense and lack of wide availability currently limit their application in clinical practice.

3.7 | Inflammatory biomarkers

For an inflammatory biomarker to be clinically useful, it ideally should be quantifiable without any temporal delay in expression, secretion, or both to reflect disease severity.[11,14] Specificity for the gastrointestinal tract (ie, organ-specific expression or by type of specimen) would be another desirable characteristic for which fecal biomarkers hold great promise.[12,13,17]

Biochemical markers that have been evaluated in dogs with CIE are serum C-reactive protein (CRP), perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA), serum 3-bromotyrosine (3-BrY), urine and fecal N-methylhistamine (NMH) concentrations, serum and fecal S100A8/A9 complex (calprotectin) concentrations, serum and fecal S100A12 concentrations, serum soluble receptor of advanced glycation end products (sRAGE), several cytokines and chemokines, and intestinal alkaline phosphatase (AP) activity.

3.7.1 | Serum C-reactive protein

C-reactive protein is a positive type II acute phase protein expressed in the liver[69] in response to infection, inflammation, or cancer,[70] and the serum CRP concentration is a nonspecific marker of inflammation.[71–80] Quantification of canine CRP in serum can be done using several assay formats,[81–88] all of which have a reference interval of approximately 0–8 mg/L. A high-sensitivity CRP (hs-CRP) assay with improved sensitivity for lower serum CRP concentrations[89,90] currently is not routinely available in veterinary medicine.[91]

High biological variability of serum CRP concentrations in dogs[92] limits its utility as a diagnostic biomarker in dogs with CIE. Serum CRP appears to be clinically more useful as a surrogate marker to assess disease progression and response to treatment in dogs with

CIE,[8,50,93–95] although some studies did not find a relationship with the severity of clinical signs or histologic lesions.[9,96,97] The serum concentration of CRP must increase or decrease at least 2.7-fold for this change to be considered clinically relevant.[92]

A recent study found that a serum CRP concentration ≥9.1 mg/L distinguished dogs with CIE requiring anti-inflammatory or immunosuppressive treatment from those dogs responding to an elimination diet or antibiotic trial with a sensitivity of 72% and a specificity of 100%.[50] With a pretest probability of ~30%, serum CRP has high PPV (~100%) for the diagnosis of IRE or NRE.

3.7.2 | Perinuclear anti-neutrophilic cytoplasmic antibodies

Perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA) are serum autoantibodies against neutrophil granule components (eg, nuclear histone, proteinase 3, myeloperoxidase) and suspected cross-reactivity with a gastrointestinal bacterial antigen[98] that can be detected by indirect immunofluorescence assays.[98,99]

Seropositivity for pANCA (and also pANCA titers) is higher in dogs with FRE (61%-62%) than in those with IRE or NRE (0%-37%).[95,99–101] However, pANCA seropositivity is not specific for CIE and also can occur with other immune-mediated, infectious, or neoplastic diseases.[101,102] Positivity for pANCA is associated with PLE, protein-losing nephropathy, or both in Soft Coated Wheaten Terriers, with pANCA positivity being detected >2 years before the onset of hypoalbuminemia.[103] Although pANCA is a difficult assay to acquire, it might be useful for diagnosing dogs with FRE and for early detection of protein-losing disease in Soft Coated Wheaten Terriers (where fecal α_1PI also might predict gastrointestinal protein loss before the development of hypoalbuminemia).

3.7.3 | Serum 3-bromotyrosine

3-Bromotyrosine (3-BrY) is the stable metabolite of eosinophil peroxidase,[104] which is released from eosinophils after their activation and degranulation.[105] Thus, serum 3-BrY is a biomarker of eosinophilic inflammation.[104]

Histopathologic findings in dogs with CIE can vary and, although the lamina propria cellular infiltrate often is primarily lymphoplasmacytic, an eosinophilic component or mixed inflammation also can be present.[2] Dogs with CIE have increased serum 3-BrY concentrations,[106] with dogs requiring anti-inflammatory or immunosuppressive treatment having higher serum 3-BrY concentrations compared to dogs responding to an elimination diet.[107] However, sensitivity and specificity of the serum 3-BrY concentration to differentiate dogs with IRE or NRE from those with FRE or ARE have not been determined. Therefore, further research is needed before serum 3-BrY can be recommended as a biomarker in routine clinical practice.

3.7.4 | N-methylhistamine

N-methylhistamine (NMH), a stable product of histamine metabolism, is a proinflammatory biomarker of mast cell activation and degranulation.[108] It can be measured in serum, urine, and fecal specimens.[109,110]

Mast cell-mediated inflammation appears to be a component in some dogs with CIE.[110–112] Mast cell degranulation and fecal NMH

concentrations were increased in Norwegian Lundehunds and Soft Coated Wheaten Terriers with CIE.[111,112] Some dogs of other breeds with CIE (36–43%) also have increased urine NMH concentrations.[109,110] The NMH concentrations correlated with the severity of histologic lesions in 1 study,[110] but not with the number of mast cells in the duodenal mucosa or the severity of clinical signs.[109,110] N-methylhistamine might be a clinically useful biomarker, but sensitivity and specificity remain to be determined.

3.7.5 | Fecal and serum S100A8/A9 protein complex

Calprotectin, the S100A8/A9 protein complex, belongs to the S100/calgranulin family of damage-associated molecular pattern (DAMP) molecules that accumulate at sites of inflammation.[113] Calprotectin is expressed and released by activated macrophages and neutrophils, but expression also can be induced in epithelial cells. Calprotectin is a ligand for Toll-like receptor (TLR) 4, which plays a role in acute and chronic inflammation.[114] Mucosal S100-mRNA concentrations also are upregulated in dogs with CIE.[115]

Serum calprotectin concentrations are increased in dogs with CIE,[47,94,116] but serum calprotectin is not specific for the gastrointestinal tract and must be interpreted with caution in corticosteroid-treated dogs.[94,95,117,118]

Calprotectin is a stable protein complex that can be extracted and measured in fecal samples.[50,119,120] Fecal calprotectin concentrations have been evaluated in dogs with chronic gastrointestinal inflammation[50,95,121] and appear to serve as a useful biomarker of intestinal inflammation in dogs. Fecal calprotectin concentrations are a good surrogate marker of disease severity in dogs with CIE,[50,95,121] with a cut-off value of ~50 μg/g feces to identify dogs with severe clinical disease.[121] Fecal calprotectin concentration also appears to have utility in predicting the response to treatment in dogs with CIE,[50] and concentrations ≥15.2 μg/g distinguish partial responders or nonresponders (PR/NR) from dogs with IRE that achieve complete clinical remission (CR) with a sensitivity of 80% and a specificity of 75%. Given the 45% prevalence of PR/NR in that study, PPV (77%) and NPV (82%) for fecal calprotectin to identify PR/NR are fairly high.

Because fecal calprotectin has been shown to be an indicator of overall gastrointestinal health[58] and also can be increased in acute gastrointestinal inflammatory conditions,[122] the patient population for fecal calprotectin testing must be carefully selected to gain relevant information from this marker. The possibility of an effect of gastrointestinal neoplasia on fecal calprotectin concentrations remains to be determined.

3.7.6 | Fecal and serum S100A12

Calgranulin C (S100A12) is another DAMP molecule that also belongs to the S100/calgranulin protein family[123] and has a cellular distribution similar to that of calprotectin.[124] The S100A12 protein has a number of different target proteins, such as the pattern recognition receptor RAGE (receptor for advanced glycation end products)[125,126] and plays a central role in the innate and acquired immune response.[113,127]

It is a very sensitive and specific marker of localized inflammatory processes, such as gastrointestinal inflammation,[128] and can be increased

in serum with various inflammatory disorders.[47,116,118,129,130] In contrast to calprotectin, corticosteroid treatment does not affect serum S100A12 concentrations.[131]

The S100A12 protein also is stable in fecal samples from which it can be extracted and measured using a species-specific assay.[131,132] Fecal S100A12 concentration also appears to be a useful biomarker of gastrointestinal inflammation in dogs (Table 2).[14,27,45,50,133] Fecal canine S100A12 concentrations are correlated with the severity of clinical signs and endoscopic lesions, but not with the severity of histopathologic changes.[50,133] A fecal S100A12 concentration ≥490 ng/g can distinguish dogs requiring anti-inflammatory or immunosuppressive treatment (IRE/NRE) from those with FRE or ARE with a sensitivity of 64% and a specificity of 77%.[45] Thus, there is a high chance for a dog to respond to elimination diet and antibiotic trial (NPV > 80%) if the fecal S100A12 concentration is <490 ng/g (20%-40% prevalence of IRE/NRE in CIE dogs[5,45]). Fecal S100A12 concentrations ≥2,700 ng/g distinguished dogs with IRE/NRE that were refractory to treatment (NR) from those with at least a PR with a sensitivity of 100% and a specificity of 76%.[45] Thus, dogs with IRE are very likely to have at least PR (NPV ~100%) if fecal S100A12 concentration is <2,700 ng/g (11%-16% pretest probability[45,50]). A significant association between increased fecal S100A12 concentrations and disease outcome also was reported in dogs with CIE.[27]

Fecal S100A12 concentrations also can be increased with acute gastrointestinal inflammatory conditions,[122] thus patient selection for use of this marker is important. The possibility of an effect of gastrointestinal neoplasia on fecal S100A12 concentrations remains to be determined.

3.7.7 | Soluble receptor for advanced glycation end products

Soluble RAGE (sRAGE) is a truncated variant of transmembrane RAGE, a pattern-recognition receptor involved in chronic inflammatory disease processes.[134] Soluble RAGE functions as an anti-inflammatory decoy receptor sequestering ligands of RAGE (eg, DAMP molecules such as S100A12) and thus abrogating cellular proinflammatory RAGE signaling,[134] rendering sRAGE also a potential therapeutic target in patients with chronic inflammatory diseases.[135] The lack of a correlation between serum sRAGE and S100A12 concentrations suggests that sRAGE is a nonspecific decoy receptor in dogs.[27]

Similar to inflammatory bowel disease in humans,[136] serum sRAGE concentrations are significantly decreased in dogs with CIE but do not correlate with the severity of clinical signs, overall histologic lesions, or outcome.[27] However, a weak correlation exists between serum sRAGE concentrations and histologic duodenal lesions (Cabrera Garcia, personal communication 2018). Serum sRAGE concentration also may be a good surrogate marker to assess response to treatment in dogs with CIE because serum sRAGE concentrations increased (normalized) only in dogs achieving complete clinical remission.[27] However, further studies are needed to evaluate the potential clinical utility of serum sRAGE as a biomarker in dogs with CIE.

3.7.8 | Cytokines and chemokines

Several reports describe various components of the cytokine signature (interleukin [IL]-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-17, IL-18, IL-23, IL-25, IL-33, IFN-γ, TNF-α, and TGF-β1) in dogs with CIE,[137–143] all of which do not support a Th1 nor Th2 polarization as described for inflammatory bowel diseases in humans.[144,145] There also is no evidence of a Th17 signature in dogs with CIE.[146,147] One problem with using cytokines as biomarkers in dogs is that there are many cytokine assays available. However, most of them either have not been analytically validated or have failed basic analytical validation. Recently, a multiplex assay that concurrently measures concentrations of IL-2, IL-6, IL-8, and TNF-α has been analytically validated, and initial studies have shown that dogs with CIE commonly have increases of serum concentrations of IL-2, IL-6, and TNF-α, but not IL-8 (Buono, personal communication 2018).

The chemokine signature identified in dogs with CIE (ie, CC chemokine ligand [CCL]2, CCL20, CCL25, CCL28, and CXC chemokine ligand [CXCL]8) is characterized by an increase in chemotaxins for T and B lymphocytes.[148]

Other signaling molecules of the innate immune response that are dysregulated in dogs with CIE include intracellular signaling (eg, nuclear factor-κB) and intercellular adhesion molecules (eg, vascular cell adhesion molecule [VCAM]-1, mucosal vascular addressin cell adhesion molecule [MAdCAM]-1, and intercellular adhesion molecule [ICAM]-1),[141,149] as well as other gene products involved in innate immunity, inflammation, cell replication, cellular detoxification, iron and calcium transport, intestinal barrier function, and extracellular matrix degradation.[115,150] Evidence of alterations in the serotonergic neuroendocrine system also have been identified in dogs with CIE.[151]

3.7.9 | Intestinal alkaline phosphatase (AP)

Mucosal expression and activity as well as fecal concentrations of intestinal AP were shown to be decreased in dogs with CIE, especially those with moderate or severe disease.[152] More research is needed to determine the potential role of fecal AP concentration as a biomarker in dogs with CIE.

3.8 | Genomic biomarkers

Genomic biomarkers include single nucleotide polymorphisms (SNPs) in *TLR4*, *TLR5*, *nucleotide oligomerization domain (NOD)2*, and the *neutrophil cytosolic factor (NCF2)* gene,[153–161] a reduction of genetic diversity (eg, T cell receptor repertoire),[162] and also alterations in the expression of several other genes (eg, genes encoding TLR2, TLR4, TLR5, TLR9, caspase-3, and B-cell lymphoma [Bcl]-2).[115,163–166] Overexpression of some marker genes also was associated with clinical disease severity.[164–166] To date, these genomic markers have been utilized as research tools to investigate the pathogenesis of CIE in dogs, but none of these markers currently have any relevance in routine clinical practice.

3.8.1 | Genetic polymorphisms

Polymorphisms in the genes encoding TLR4 (risk-associated SNPs: A1571T, G1807A), TLR5 (risk-associated SNP: G22A; protective SNPs: C100T, T1844C; leucine rich repeat domain-SNP: T443C), and NOD2 (a cytosolic pattern recognition receptor that recognizes peptidoglycan

motifs of Gram⁻ and Gram⁺ bacteria) have been linked to CIE in dogs.[153–158] In addition to breed-unspecific SNPs, individual breeds may harbor unique SNPs. The functional implication of the *TLR5* SNPs associated with CIE is a hyper-responsiveness of TLR5 to stimulation with flagellin.[159,160] Genetic polymorphisms in the *NCF2* gene (which encodes for a subunit of neutrophil nicotinamide adenine dinucleotide phosphate hydrogen [NADPH] oxidase involved in intracellular autophagy) were detected in Boxer dogs with granulomatous colitis.[161]

3.8.2 | Alterations in gene expression

Mucosal expression of TLR9 (intracellular) as well as TLR2 and TLR4 (apical epithelial surface) is increased in dogs with CIE, but correlates only weakly or not at all with disease severity and response to treatment.[163–165] Decreased TLR5 expression can be observed in dogs with CIE, which however also is not correlated with the severity of the disease.[164] However, these results remain to be confirmed at the protein level.[167]

3.9 | Cellular biomarkers

3.9.1 | Regulatory T cells

Regulatory T cells (T_{reg}) play an important role in cell-mediated mucosal immunotolerance and were decreased in duodenal biopsy specimens from dogs with CIE.[168,169] Numbers of circulating T_{reg} were also decreased in peripheral blood samples from dogs with CIE (IRE and PLE), and this cellular biomarker might be useful to monitor disease progression (ie, disease activity) and assess response to treatment.[170] However, further studies are needed to determine the clinical utility of assessing the disturbance in T_{reg} homeostasis as a cellular biomarker in dogs with CIE.

4 | SUMMARY AND OUTLOOK

Several biomarkers have been evaluated and are potentially clinically useful in dogs with CIE. Although the lack of wide availability, expense, and low stability in biological specimens limits the routine analysis of some of these clinically useful small molecules (eg, cytokines and chemokines), a number of biomarkers can easily be quantified in serum, urine, and even feces, or a combination of these biological samples.

Some functional biomarkers, such as serum cobalamin and folate concentrations as well as fecal α_1PI are already widely used in dogs with suspected CIE in the clinical setting. Genomic biomarkers currently are not readily available, but may be able to provide the clinician more information about the individual risk of a dog to develop CIE or even a specific form of CIE in the future. Genomic biomarkers also may represent an avenue to individualized treatment and monitoring. Inflammatory biomarkers beyond serum CRP, such as serum 3-BrY, fecal calprotectin, or fecal S100A12, currently are not widely available, but a human fecal calprotectin assay holds promise for fecal calprotectin measurement in dogs.[120] Inflammatory markers appear to be useful surrogate markers for guiding the management of dogs with CIE, especially to predict response to treatment because therapeutic trials often are tedious, invasive diagnostic tests are impractical for evaluating response to treatment, and routine clinicopathologic and histopathologic findings usually are not able to distinguish different forms of CIE in dogs.[171,172] Combining the results of several inflammatory biomarkers (eg, serum CRP and fecal calprotectin[50]) also may increase the diagnostic accuracy of each individual biomarker.

Incorporating the information gained from several inflammatory biomarkers (ie, cellular components and severity of the inflammatory disease process) and functional biomarkers (ie, digestive, absorptive, and secretory capacity of the gastrointestinal tract as well as the degree of dysbiosis) into an algorithm or several algorithms may provide an improved strategy to manage dogs with CIE in the future. Such a biomarker panel also may be a useful tool to further characterize CIE in dogs, and further research is warranted to identify such algorithms for different subgroups of dogs with CIE.

5 | CONCLUSION

Several functional, biochemical, and genomic biomarkers have been evaluated in dogs with CIE over the last 2 decades and great strides have been made increasing the diagnostic and monitoring armamentarium in canine gastroenterology. However, further prospective and longitudinal studies are now needed to critically evaluate the utility of currently established biomarker assays in dogs with CIE in the routine clinical setting.

CONFLICT OF INTEREST DECLARATION

Dr Jörg Steiner is affiliated with the Gastrointestinal Laboratory at the Texas A&M University College of Veterinary Medicine and Biomedical Sciences where some of the biomarker assays reviewed in this article are offered on a fee-for-service basis.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

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