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Serum metabolomic profiles in dogs with chronic enteropathy

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Abstract

Background: Metabolic profiles differ between healthy humans and those with inflammatory bowel disease. Few studies have examined metabolic profiles in dogs with chronic enteropathy (CE).

Hypothesis: Serum metabolic profiles of dogs with CE are significantly different from those of healthy dogs.

Animals: Fifty-five dogs with CE and 204 healthy controls.

Methods: A cross-sectional study. The serum concentrations of 99 metabolites measured using a canine-specific proton nuclear magnetic resonance spectroscopy platform were studied. A 2-sample unpaired t-test was used to compare the 2 study samples. The threshold for significance was set at $P < .05$ with a Bonferroni correction for each metabolite group.

Results: Nineteen metabolites and 18 indices of lipoprotein composition were significantly different between the CE and healthy dogs. Four metabolites were significantly higher in dogs with CE, including phenylalanine (mean and SD) (healthy: 0.0417 mmol/L; [SD] 0.0100; CE: 0.0480 mmol/L; SD: 0.0125; P value: $<.001$) and lactate (healthy: 1.8751 mmol/L; SD: 0.7808; CE: 2.4827 mmol/L; SD CE: 1.4166; P value: .003). Fifteen metabolites were significantly lower in dogs with CE, including total fatty acids, and glycine (healthy: 0.2273 mmol/L; SD: 0.0794; CE: 0.1828 mmol/L; SD CE: 0.0517; P value: $<.001$).

Conclusions and Clinical Importance: The metabolic profile of dogs with CE is significantly different from that of healthy dogs, this opens novel research avenues to develop better diagnostic and prognostic approaches as well as therapeutic trials.

KEYWORDS

amino acid, canine, glycoprotein acetyls, lipid, metabolomics

Abbreviations: ¹H NMR, proton nuclear magnetic resonance; ACTH, adrenocorticotropic hormone; APTT, activated partial thromboplastin time; CE, chronic enteropathy; CI, confidence interval; cPLI, canine pancreas specific lipase; GC-MS, gas chromatography coupled to mass spectrometry; GPA, glycoprotein acetyls; HDL, high density lipoproteins; HfSA, Hospital for Small Animals; IBD, inflammatory bowel disease; LC-MS, liquid chromatography coupled with single-stage or tandem mass spectrometry; PT, prothrombin time; PUFAs, polyunsaturated fatty acids; R(D)SVS, Royal (Dick) School of Veterinary Studies; TLI, trypsin-like immunoreactivity; VLDL, very low-density lipoprotein.

1 | INTRODUCTION

Chronic enteropathy (CE) is a common cause of gastrointestinal disease in dogs. It is characterized by a several week history of signs of gastrointestinal (GI) disease, and histopathological evidence of

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inflammatory infiltrates in the GI tract, in the absence of any underlying metabolic, infectious, or neoplastic causes.¹ It is subtyped based upon response to treatment as either food-responsive,² antibiotic-responsive,³ immunosuppression-responsive,⁴ or nonresponsive.⁵

The etiology of CE is hypothesized to be a series of complex interactions between the host genome, microbiome, lack of immune tolerance and intraluminal dietary components.^{1,6} The degree of inflammation shows marked interobserver variability dependant on scoring system used,⁷ does not consistently correlate with clinical signs,⁸ nor does it predict the response to treatment,⁹ and clinical remission often does not correlate with histological remission.¹⁰ Therefore, it remains challenging for the clinician to both diagnose and prognosticate based on current diagnostic approaches. In an effort to improve the diagnostic accuracy, reduce invasive testing and to better predict and monitor the response to treatment of dogs with CE, the clinical utility of measuring multiple biomarkers has been explored.¹¹ Of these, few are routinely used in clinical practice, mainly because of limitations concerning availability of testing and individual variation.^{11,12}

Similar challenges exist in the human field, where the clear need to noninvasively assess and personalize treatment for diseases such as ulcerative colitis and Crohn's disease resulted in the extensive study of metabolic profiling. Several serum metabolites consistently differ between healthy individuals and those suffering from inflammatory bowel disease (IBD), which might allow differentiation between subtypes.^{13–15} This not only provides a series of potential biomarkers, but also offers insights into the potential pathogenesis of these conditions. This gives rise to the hope that measuring serum metabolites might be able to reduce the clinical need of more invasive testing such as endoscopic biopsies in dogs.

Assessment of serum metabolites can be undertaken using a wide range of analytical platforms. In contrast to other commonly used metabolomics techniques such as gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled with mass spectrometry (LC-MS), proton nuclear magnetic resonance (¹H NMR) spectroscopy provides a high speed, high throughput, and highly reproducible method.¹⁶ Increased availability of these techniques has facilitated the recent expansion of metabolomic studies in both human and veterinary medicine.

Metabolomic profiles between dogs with CE and healthy individuals are not well characterized. Several recent studies documented differences in lipid and amino acid profiles in dogs with CE,^{17,18} however comprehensive metabolomic studies are lacking. Significant variance has been identified in the phospholipid profiles of dogs with IBD and food responsive enteropathy, and before and after treatment.¹⁷ Alterations in amino acid profiles are identified in dogs with IBD, and whilst correlating with clinical disease severity, these did not reflect the histological inflammation or C-reactive protein concentrations.¹⁸

This study measures a large panel of serum metabolites in dogs with a CE using a canine-specific ¹H NMR metabolomics platform. We hypothesize that serum metabolic profiles of dogs with CE are significantly different from healthy dogs. The aim of this study is to, therefore, compare the metabolomic profiles between healthy dogs and dogs with histopathologically confirmed CE.

2 | MATERIALS AND METHODS

This study was performed at the Royal (Dick) School of Veterinary Studies and was approved by the University of Edinburgh Veterinary Ethics Review Committee.

Dogs were classified as having a CE if they had a >3-wk history of signs of GI disease, confirmation of an inflammatory infiltrate of the lamina propria on histopathology from endoscopic intestinal biopsies, and exclusion of other causative processes. Signs of GI disease were classified as 1 or more of: vomiting, diarrhea, increased borborygmi or flatus, abdominal pain, increased or decreased appetite and weight loss or bloating. In order to exclude other causative processes, dogs were required to have no clinically relevant abnormalities on hematology, biochemistry, abdominal ultrasound, fecal parasitology including giardia antigen and a resting or post ACTH stimulated cortisol >2 µg/dL (>55 nmol/L) to exclude hypoadrenocorticism. Other investigations performed at the discretion of the clinician included canine pancreas-specific lipase, trypsin-like immunoreactivity, urine analysis +/- urine protein: creatinine ratio, abdominal and/or thoracic radiographs, T4 and TSH, and coagulation parameters (PT and APTT). A canine IBD activity index (CIBDAI) score was recorded in all dogs with a CE, where this was not available it was calculated from clinical records.¹⁹ Empiric deworming was performed at the discretion of the clinician but was not considered an exclusion criterion.

Healthy controls were defined as dogs presenting for an annual vaccination to the university teaching hospital without any abnormalities detected on history or physical examination. Samples were collected between November 2014 and July 2018.

After sampling, blood was placed into plain blood collection tubes (as per manufacturer's instructions), which were refrigerated immediately after collection. Serum was separated by centrifugation within 4 hours of collection and aliquoted. Samples were stored frozen at -80°C until analysis. Before analysis samples were inspected for any evidence of hemolysis, or lipemia and sample quality noted.

All samples were analyzed using a Bruker AVANCE III HD 500 ¹H NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). The canine-specific platform has been recently validated and reference intervals for the individual metabolites determined by Ottka et al.²⁰ Ninety-nine metabolites were analyzed, from 8 metabolite group, consisting of amino acids, cholesterol, fatty acids, glycolysis related metabolites, inflammatory markers, ketone bodies, lipoprotein subclass particles, and triglycerides, as outlined in Figure 1.

Statistical analysis was performed using the R statistical system (R version 4.0.3 [October 10, 2020]). An unpaired 2-sample t-test was used to compare the 2 study samples as group sizes were sufficient, under the central limit theorem, to assume that sample means were normally distributed. The threshold for significance was set at $P < .05$ with a Bonferroni correction for each metabolite group by setting the critical P -value for multiple comparisons to be .05 divided by the number of comparisons in each metabolite group.

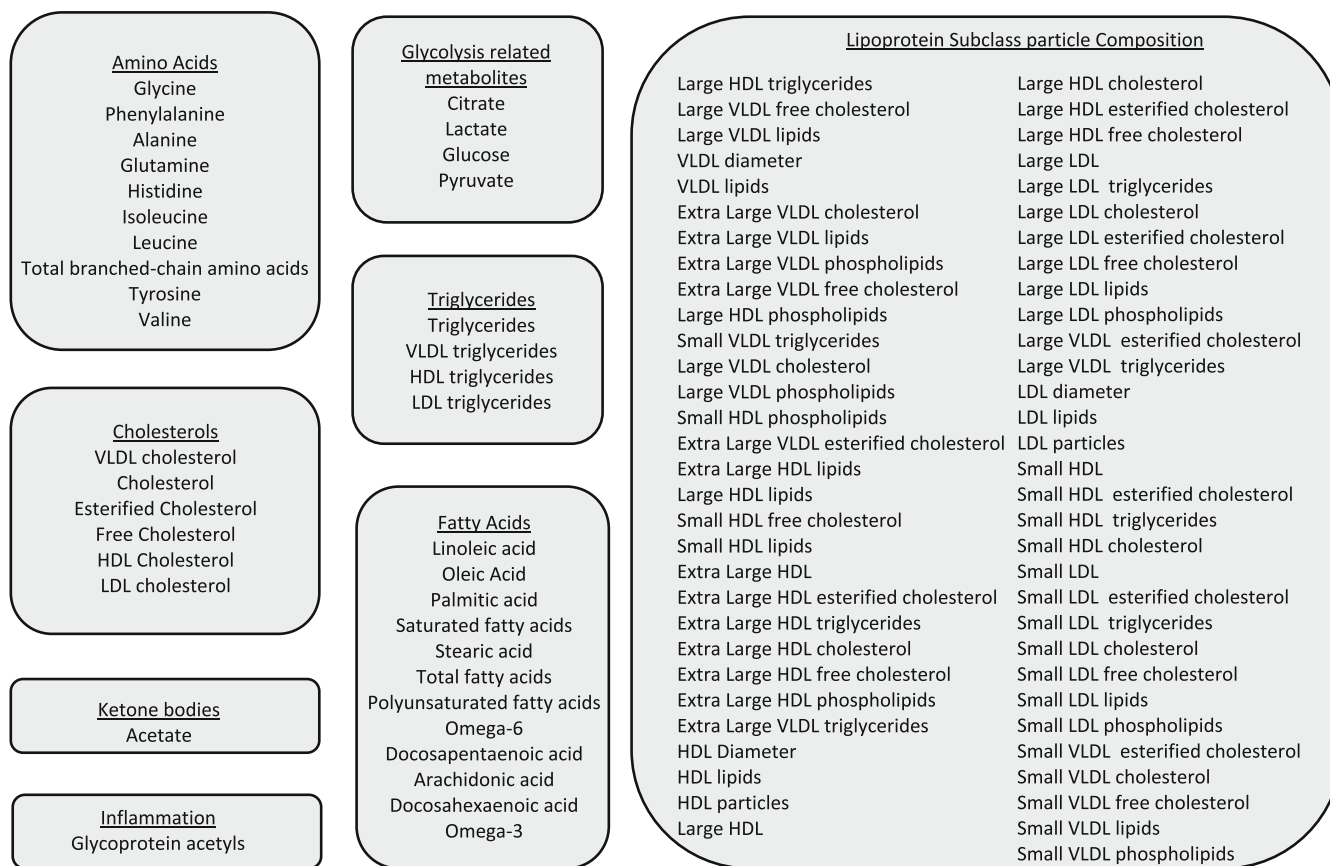


FIGURE 1 Metabolite subgroups tested in dogs with 55 dogs with chronic enteropathy compared to a healthy study sample of 204 dogs, measured using ^1H NMR Spectroscopy

TABLE 1 Ten most prevalent breeds of 55 dogs with chronic enteropathy and 204 healthy controls used in metabolomic analysis using ^1H NMR Spectroscopy

Ten most prevalent breeds of 55 dogs with chronic enteropathy	Number of Dogs	Percentage	Ten most prevalent breeds of 204 healthy controls	Number of Dogs	Percentage
Labrador	13	24	Mixed Breed	52	26
Mixed Breed	7	13	Labrador	28	14
Border terrier	3	5	English Cocker Spaniel	17	8
Border Collie	3	5	English Springer Spaniel	13	6
Irish Setter	3	5	Golder Retriever	8	4
Lurcher	2	4	Jack Russel Terrier	6	3
Golder Retriever	2	4	Greyhound	6	3
Jack Russel Terrier	2	4	Border Terrier	5	2
Rottweiler	2	4	Whippet	4	2
Rhodesian Ridgeback	2	4	Hungarian Vizsla	4	2

3 | RESULTS

3.1 | Dogs

Fifty-five dogs with CE were included in the analysis. The median age was 6 y (range, 6 mo-12.5 y). These were from 25 breeds, and 7 dogs were mixed breed. The CIBDAI scores ranged from 1 to 15 with a median of 7. Five entire females, 19 neutered females, 13 entire males and

18 neutered male dogs were included in the CE cohort. The median weight was 22.25 kg (range, 4.3-80 kg). The 10 most prevalent breeds included are outlined in Table 1. Drugs used before referral included: histamine blockers (famotidine, cimetidine, and ranitidine), antibiotics (metronidazole, enrofloxacin, trimethoprim sulfonamide, and potentiated amoxicillin), hyoscine butylbromide, probiotics, omeprazole, meloxicam, tramadol, metoclopramide, phenylpropranolamine, oclacitinib, maropitant, thyroxine, and chlorphenamine. One dog was receiving prednisolone

TABLE 2 Metabolite concentrations with significant differences in dogs with chronic enteropathy compared to a healthy study sample, measured using ¹H-NMR Spectroscopy

Metabolite	Group	Unit	Reference interval	Mean Healthy	SD Healthy	Range Healthy	Mean CE	SD CE	Range CE	P value	Critical Value
Acetate	Ketone Bodies	mmol/ L	0.021-0.037	0.0227	0.0054	0.013-0.047	0.0256	0.0077	0.011-0.052	.010	0.05
Citrate	Glycolysis related metabolites	mmol/ L	0.061-0.123	0.0871	0.0249	0.053-0.302	0.1000	0.0284	0.056-0.158	.003	0.0125
Docosapentaenoic acid	Fatty Acids	mmol/ L	0.125-0.361	0.2447	0.0724	0.107-0.579	0.2057	0.0801	0.062-0.481	.0016	0.0041
Glycine	Amino Acids	mmol/ L	0.130-0.454	0.2273	0.0794	0.074-0.631	0.1828	0.0517	0.100-0.353	<.001	0.005
Glycoprotein acetyls	Inflammation	mmol/ L	0.597-1.028	0.8230	0.2207	0.506-1.829	0.7656	0.1626	0.546-1.297	.034	0.05
HDL triglycerides	Triglycerides	mmol/ L	0.003-0.076	0.0517	0.0348	0.000-0.186	0.0327	0.0254	0.000-0.120	<.001	0.0125
Lactate	Glycolysis related metabolites	mmol/ L	1.184-3.581	1.8751	0.7808	0.553-6.846	2.4827	1.4166	0.792-9.441	.0033	0.0125
Linoleic acid	Fatty Acids	mmol/ L	2.495-5.892	4.2844	1.0007	2.350-8.203	3.4460	1.1153	2.039-7.757	<.001	0.0041
Oleic Acid	Fatty Acids	mmol/ L	1.265-2.796	2.1554	0.7010	1.056-4.662	1.5565	0.5276	0.796-3.271	<.001	0.0041
Omega-6	Fatty Acids	mmol/ L	4.148-9.810	6.8996	1.5983	3.678-12.890	5.9244	1.8162	3.261-13.036	<.001	0.0041
Palmitic acid	Fatty Acids	mmol/ L	1.837-3.616	2.9405	0.7081	1.760-5.668	2.3799	0.5846	1.592-4.413	<.001	0.0041
Phenylalanine	Amino Acids	mmol/ L	0.030-0.065	0.0417	0.0100	0.024-0.098	0.0480	0.0125	0.024-0.095	<.001	0.005
Polyunsaturated fatty acids	Fatty Acids	mmol/ L	4.723-11.141	7.8617	1.7837	4.100-15.413	6.7669	2.0155	3.783-14.517	<.001	0.0041
Saturated fatty acids	Fatty Acids	mmol/ L	3.588-7.431	5.7232	1.3384	3.241-10.381	4.6880	1.2452	2.749-9.034	<.001	0.0041
Stearic acid	Fatty Acids	mmol/ L	1.744-3.842	2.7828	0.6501	1.482-5.077	2.3082	0.6817	1.041-4.621	<.001	0.0041
Total fatty acids	Fatty Acids	mmol/ L	9.741-20.972	15.7403	3.7177	8.773-30.089	13.0114	3.7015	7.805-26.747	<.001	0.0041
Triglycerides	Triglycerides	mmol/ L	0.190-1.004	0.7942	0.5226	0.152-3.790	0.5067	0.3379	0.124-1.584	<.001	0.0125
VLDL cholesterol	Cholesterol	mmol/ L	0.027-0.311	0.1970	0.1529	0.004-0.925	0.1261	0.1356	0.013-0.905	.0012	0.0083
VLDL triglycerides	Triglycerides	mmol/ L	0.000-0.705	0.5540	0.4832	0.030-3.304	0.2822	0.3086	0.000-1.291	<.001	0.0125

TABLE 3 Lipoprotein subclass particle composition with significant differences in dogs with chronic enteropathy compared to a healthy study sample measured using ¹H-NMR Spectroscopy

Metabolite	Unit	Reference Interval	Mean Healthy	SD Healthy	Range Healthy	Mean CE	SD CE	Range CE	P value	Critical Value
Extra Large VLDL esterified cholesterol	mmol/L	0.001-0.028	0.0193	0.02	0.001-0.094	0.01	0.01	0.001-0.048	<0.0008	0.0008
Extra Large VLDL cholesterol	mmol/L	0.000-0.060	0.0452	0.0544	0.000-0.284	0.0167	0.0263	0.000-0.127	<0.0008	0.0008
Extra Large VLDL free cholesterol	mmol/L	0.001-0.045	0.0340	0.0364	0.001-0.191	0.0139	0.0180	0.001-0.087	<0.0008	0.0008
Extra Large VLDL lipids	mmol/L	0.001-0.264	0.2205	0.2594	0.001-1.394	0.0911	0.1294	0.002-0.653	<0.0008	0.0008
Extra Large VLDL phospholipids	mmol/L	0.001-0.054	0.0405	0.0485	0.001-0.275	0.0146	0.0205	0.001-0.106	<0.0008	0.0008
Large HDL lipids	mmol/L	4.966-8.260	6.1179	0.7381	3.986-8.349	5.5368	1.0208	2.915-7.441	<0.0008	0.0008
Large HDL phospholipids	mmol/L	2.481-3.936	2.9923	0.3259	2.174-3.851	2.7367	0.3796	1.853-3.447	<0.0008	0.0008
Large HDL triglycerides	mmol/L	0.003-0.028	0.0200	0.0106	0.004-0.060	0.0127	0.0081	0.001-0.039	<0.0008	0.0008
Large VLDL cholesterol	mmol/L	0.004-0.132	0.0877	0.0762	0.002-0.410	0.0468	0.0586	0.002-0.314	<0.0008	0.0008
Large VLDL free cholesterol	mmol/L	0.002-0.080	0.0587	0.0532	0.003-0.284	0.0277	0.0310	0.002-0.132	<0.0008	0.0008
Large VLDL lipids	mmol/L	0.010-0.600	0.4304	0.3815	0.004-2.414	0.2047	0.2468	0.005-1.173	<0.0008	0.0008
Large VLDL phospholipids	mmol/L	0.001-0.105	0.0676	0.0666	0.001-0.333	0.0367	0.0370	0.001-0.154	<0.0008	0.0008
Small HDL free cholesterol	mmol/L	0.093-0.193	0.1283	0.0240	0.068-0.224	0.1098	0.0341	0.022-0.191	<0.0008	0.0008
Small HDL lipids	mmol/L	1.268-2.174	1.5239	0.2230	0.941-2.288	1.3557	0.3217	0.432-1.865	<0.0008	0.0008
Small HDL phospholipids	mmol/L	0.724-1.187	0.8549	0.1123	0.572-1.205	0.7657	0.1524	0.332-1.020	<0.0008	0.0008
Small VLDL triglycerides	mmol/L	0.016-0.157	0.1350	0.0795	0.023-0.539	0.0884	0.0598	0.001-0.288	<0.0008	0.0008
VLDL diameter	mmol/L	35.261-43.770	41.6472	3.0774	35.358-57.942	39.0502	3.5107	35.005-57.046	<0.0008	0.0008
VLDL lipids	mmol/L	0.087-1.154	0.8901	0.7528	0.107-4.750	0.4684	0.4844	0.075-2.503	<0.0008	0.0008

orally at physiological dose (0.2 mg/kg q24 hours). Empiric deworming with fenbendazole at 50 mg/kg orally for 5 consecutive days was performed in 38 dogs.

Two hundred four healthy controls were used. These were from 47 breeds, and 52 dogs were mixed breed. Of the healthy controls, 96 were neutered females, 10 were entire females, 72 were neutered males, 26 were entire males. They had a median age of 3 y (range, 6 mo-16 y). The median weight of control dogs was 20.95 kg (range, 3.1-70 kg). The 10 most prevalent breeds from the healthy control group are outlined in Table 1. An unpaired t-test did not identify a significant difference between the ages of the 2 groups of dogs ($P = .15$) or the weights of the 2 groups ($P = .26$).

3.2 | Metabolites

A significant difference was identified in 19 metabolites and 18 indices of lipoprotein composition between the healthy and CE groups. Four metabolites were significantly higher in dogs with CE than healthy controls: the amino acid phenylalanine, the ketone body acetate, and the glycolysis related metabolites citrate and lactate. Fifteen metabolites were significantly lower in dogs with CE than healthy dogs, outlined in Table 2, including several fatty acids; the amino acid glycine, total triglycerides, very low-density lipoprotein triglycerides and high-density lipoprotein triglycerides, glycoprotein acetyls and very low-density lipoprotein cholesterol. The 18 indices of lipoprotein subclass particle composition that showed statistical significance are outlined in Table 3. No samples were recorded to be grossly lipemic or hemolytic.

4 | DISCUSSION

This study highlights a significant difference in the metabolomic profile between dogs with CE and healthy controls, using a recently validated ^1H NMR metabolomics platform. Alterations were identified in the serum concentrations of amino acids, fatty acids, glycolysis related metabolites, and lipid subclass composition.

Amino acid profiling has been widely investigated in human inflammatory enteropathies, and more recently has been studied in dogs with CE. Investigation of alterations in the amino acid signature of dogs with CE has highlighted tryptophan and serine as potential biomarkers in IBD.^{18,21,26} In this study, glycine was significantly lower in dogs with CE than healthy dogs. This is in contrast to previous studies, in which no significant difference was identified in glycine levels between dogs with IBD or protein losing enteropathy and healthy controls.^{18,21} Glycine is a simple amino acid, which functions as an anti-inflammatory, immunomodulatory and cytoprotective molecule.²² It suppresses TLR4 and NOD2 signaling in piglets challenged with lipopolysaccharide.²³ mRNA expression of TLR4 and NOD2 is dysregulated in CE.^{24,25} Supplementation with glycine also decreases the clinical severity and inflammatory infiltrates in animal models of inflammatory enteropathies.²² A possible explanation for the discordance between our findings and those of previous studies might lie in the smaller number of dogs used in one of these previous cohorts, or the differences in

study samples analyzed, as previous studies included only dogs with immunosuppressive - responsive enteropathy²⁶ and protein-losing enteropathy.²¹

In our study, in contrast to a previous pilot study,²⁶ phenylalanine concentration was found to be significantly higher in dogs with CE. This is also identified in humans, as people with ulcerative colitis have higher blood levels of phenylalanine than healthy controls.²⁷ The metabolism of phenylalanine relates to the enzyme phenylalanine hydroxylase, which is functionally reduced by inflammation, therefore this increased serum level might be an indirect result of inflammatory cytokines.²⁸

Aberrations in glycolysis related metabolites are documented in many disease states, driven by alterations in energy requirements. In this study, lactate was significantly higher in dogs with CE compared to the healthy study sample. Lactate measured by this ^1H NMR spectrometer encompasses both L-lactate and D-lactate. Within human IBD, serum D-Lactate has been identified to be significantly higher in people with Crohn's disease than healthy controls, because of increased intestinal production, and absorption facilitated by abnormal intestinal barrier function.²⁹ Within veterinary medicine, significant increases in fecal lactate concentrations are observed in dogs with CE.³⁰ Down-regulation of genes responsible for maintaining intestinal barrier function is identified in dogs with CE, therefore the pathogenesis of increased lactate might mirror that seen in human medicine.³¹ Alternatively, the increase in serum lactate might be a result of L-lactate production, because of tissue hypoperfusion.

Total fatty acids were significantly lower in dogs with CE than healthy controls. Essential fatty acids are important mediators of inflammation, acting as both pro- and anti-inflammatory intermediaries. Pro-inflammatory mediators are metabolized via arachidonic acid and the cyclooxygenase pathway to form prostaglandins, thromboxanes, and leukotrienes.³² In contrast, specialized pro-resolving mediators are synthesized from omega-3 essential fatty acids (eicosapentanoic acid and docosahexaenoic acid) to resolvins, protectins, and maresins and from omega-6 fatty acids to lipoxins.³³ Dysregulation in these mediators in the face of chronic inflammation, is a feature of CE in people but has not been extensively investigated in dogs with CE. Interestingly, in comparison to that of amino acids, evidence of therapeutic response to essential fatty acids in people is limited,³⁴ However there is some evidence that supplementation of eicosapentanoic acid can improve inflammation and affect colonic differentiation markers and microbiota in people with ulcerative colitis.³⁵

Lipoprotein particle composition analysis is an emerging tool in human medicine. Whilst primarily used to look for dyslipidemias surrounding cardiovascular disease, there is some emerging data that dyslipidemias in human IBD might be an important potential focus for profiling and treating these patients.^{36,37} Dyslipidemias are widely reported in dogs with CE,¹⁷ and the role of proinflammatory cytokines on mediators of lipolysis such as lipoprotein lipase are suggested to have a role in these pathways in human medicine.³⁸

Glycoprotein acetyls (GPA) were significantly lower in dogs with CE than healthy controls. Glycoprotein acetyls are a novel NMR biomarker, which reflect the concentrations of certain acute-phase glycoproteins, and in human medicine correlate with chronic inflammation.

A recent NMR study in people identified GPA to be significantly higher in people with active IBD compared with healthy controls.³⁹ In addition, they accurately reflected the mucosal recovery to a healthy state in people with both Crohn's disease and ulcerative colitis.³⁹ The lower level of GPA in dogs with CE, compared with healthy controls is unexpected, although the literature surrounding the use of GPA as biomarkers in dogs is limited. Glycoprotein acetyl profiles in dogs differ from human profiles, and are affected by various medications.^{40,41} As the medications before referral were not standardized the impact of these cannot be excluded. Equally, the lower level of GPA in dogs with CE might represent GI protein loss in our cohort.

The limitations of this study include a lack of standardized diets, lack of subtyping of CE, and the smaller number of dogs with CE than healthy controls. Six dogs had a serum albumin level below the lower reference limit which could have impacted the metabolome. The dogs were also exposed to different medications before referral including antibiotics and probiotics. Two dogs did not have a trypsin like immunoreactivity performed to exclude exocrine pancreatic insufficiency, and not all cases had empirical deworming. The body condition score of the healthy controls was not available to compare with the CE group, which could have impacts on the metabolome.

A number of animal and sample factors can influence the metabolome, and clinicians should endeavor to standardize these in follow up experiments.⁴² Animal factors which could have affected the metabolome include a difference in the fasting status between the CE and control dogs, differences in medication exposure, and time of day of sampling. The dogs were not subdivided into age categories to enhance the statistical power, however again this represents a potential for variance. Sample factors which could have impacted the metabolome include the potential for different brand of serum tubes to be used over the 4 y collection period, differences in clotting time between dogs, and a difference in time to centrifugation. Whilst most amino acids are stable for 4 hours, there is some evidence that others might be affected.⁴³ There was also a difference in storage times between the samples before analysis. Samples were stored at -80°C for several years before analysis. In a previous study validating the NMR platform, all sample metabolites were stable at -80°C for 12 mo,²⁰ however evidence into the impact of longer storage on canine samples is lacking. Studies looking at human metabolites, however, have shown negligible effects after storage at -80°C for 2.5 y when using an NMR platform,⁴⁴ despite this, the effect of storage on the current study's samples cannot be excluded. No freeze thaw cycles were performed.

In conclusion, dogs with CE have significant alterations in lipid, amino acid, fatty acid and glycolysis related metabolites compared to healthy dogs. This study might open novel research avenues which could explore the diagnostic and prognostic utility of metabolite changes and guide the development of future therapeutic trials.

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CONFLICT OF INTEREST

Claudia Ottka was an employee and Hannes Lohi is a shareholder and the chairman of the board of PetBIOMICS Ltd, who developed and provides the metabolomics test. No other authors have a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approved by the University of Edinburgh Animal Welfare and Ethics Review Board.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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