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Association of serum and fecal microRNA profiles in cats with gastrointestinal cancer and chronic inflammatory enteropathy

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Abstract

Background: Differentiation of gastrointestinal cancer (GIC) from chronic inflammatory enteropathies (CIE) in cats can be challenging and often requires extensive diagnostic testing. MicroRNAs (miRNAs) have promise as non-invasive biomarkers in serum and feces for diagnosis of GIC.

Hypothesis/Objectives: Cats with GIC will have serum and fecal miRNA profiles that differ significantly from healthy cats and cats with CIE. Identify serum and fecal miRNAs with diagnostic potential for differentiation between cats with GIC and CIE as compared to healthy cats.

Animals: Ten healthy cats, 9 cats with CIE, and 10 cats with GIC; all client-owned.

Methods: Cats were recruited for an international multicenter observational prospective case-control study. Serum and feces were screened using small RNA sequencing

Abbreviations: %CV, coefficient of variance; ANOVA, analysis of variance; AUC, area under the curve; CE, chronic enteropathy; CI, confidence interval; CIE, chronic inflammatory enteropathy; CPM, counts per million; DLH, Domestic longhair; DSH, Domestic shorthair; FCEAI, feline chronic enteropathy activity index; FNCB, fine needle cytological biopsy; fPLI, feline pancreatic lipase immunoreactivity; GI, gastrointestinal; GIC, gastrointestinal cancer; IBD, inflammatory bowel disease; LCL, large-cell lymphoma; LR, likelihood ratio; miRNA, microRNA; noPAP, not containing poly(A) polymerase; SCL, small-cell lymphoma; small RNAseq, small RNA sequencing; TLI, trypsin-like immunoreactivity; tT4, Total T4; qPCR, quantitative real-time PCR; ROC, receiver operating characteristic; SAA, serum amyloid A; UCPH, University of Copenhagen; WSAVA, World Small Animal Veterinary Association.

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for miRNAs that differed in abundance between cats with GIC and CIE, and healthy cats. Diagnostic biomarker potential of relevant miRNAs from small RNA sequencing and the literature was confirmed using reverse transcription quantitative real-time PCR (RT-qPCR).

Results: Serum miR-223-3p was found to distinguish between cats with GIC and CIE with an area under the curve (AUC) of 0.9 (95% confidence interval [CI], 0.760-1.0), sensitivity of 90% (95% CI, 59.6-99.5%), and specificity of 77.8% (95% CI, 45.3-96.1%). Serum miR-223-3p likewise showed promise in differentiating a subgroup of cats with small cell lymphoma (SCL) from those with CIE. No fecal miRNAs could distinguish between cats with GIC and CIE.

Conclusion and Clinical Importance: Serum miR-223-3p potentially may serve as a noninvasive diagnostic biomarker of GIC in cats, in addition to providing a much needed tool for the differentiation of CIE and SCL.

KEYWORDS
biomarker, CIE, lymphoma, miRNA, qPCR, small RNA sequencing

1 | INTRODUCTION

Chronic inflammatory enteropathy (CIE) is increasingly common in cats, and is a diagnosis of exclusion based on clinical signs, diagnostics, elimination of other gastrointestinal (GI) diseases, and GI histopathology indicating inflammatory infiltration.[1,2] The clinical signs of persistent or recurring weight loss, dysrexia, vomiting, or diarrhea for ≥3 weeks duration are non-specific and do not differentiate between CIE and gastrointestinal cancer (GIC).[2]

Gastrointestinal cancer accounts for up to 8% to 13.5% of tumors in cats, and the most common location is the small intestine.[3,4] Lymphoma is the most prevalent intestinal cancer, followed by adenocarcinoma and mast cell tumors.[1,3,5-7] Abdominal ultrasound examination and fine needle cytological biopsy (FNCB) are invaluable diagnostic tools, but only 68% of FNCB from GI lesions in cats and dogs were clinically useful, and 66% of useful cytological samples were in complete agreement with histopathology.[7] Hence, histopathology still may be required for a diagnosis, and additional diagnostic assessment such as immunohistochemistry or clonality testing can be necessary to reach a final diagnosis and more accurately determine tumor immunophenotype and differentiate between CIE and GIC, because doing so can be challenging in individual cases.[2,8,9] Diagnostic evaluation is expensive and potentially invasive, emphasizing the need for reliable non-invasive biomarkers to diagnose these conditions in order to initiate specific treatments and improve patient quality of life and survival.

MicroRNAs (miRNAs) represent a promising array of non-invasive diagnostic biomarkers in cats. They are small non-coding RNAs that contribute to post-transcriptional regulation of gene expression by binding to and repressing translation of their target messenger RNA transcripts. In humans, miRNAs have shown great potential as biomarkers because of their stability and accessibility in serum and feces, and their ubiquitous involvement in cancer development and progression.[10,11] Likewise, several miRNAs are abundant in GIC and chronic enteropathies in dogs.[12,13] MiRNAs already have been successfully measured in feces from healthy cats,[14] but their potential as non-invasive diagnostic biomarkers in serum and feces for differentiation between GIC and CIE in cats remains to be investigated.

To investigate serum and fecal miRNAs in cats as non-invasive diagnostic biomarkers for the differentiation between GIC and CIE as compared to healthy cats, we first aimed to use small RNA sequencing (small RNAseq) combined with known published data from the human and veterinary medical literature to identify relevant miRNAs. Secondly, we aimed to establish these relevant miRNAs as a panel for reverse transcription quantitative real-time PCR (RT-qPCR) to differentiate cats with GIC and CIE from healthy cats. We hypothesized that serum and fecal miRNA profiles from cats with GIC would be significantly different from those of healthy cats and cats with CIE.

2 | MATERIALS AND METHODS

2.1 | Study design and ethics approval

This study was an international multicenter prospective observational case-control study conducted from 2018 to 2020 at the University of Copenhagen (UCPH). In addition to UCPH participating international academic centers included: University of Edinburgh, Norwegian University of Life Sciences, and University of Glasgow. Each institution obtained their own local ethical approval (#2017-9 + 2017-12; VERC#41.18; 14/04723-72; Ref 05a/18). The study was approved by the Animal Experiments Inspectorate under the Ministry of Food, Agriculture, and Fisheries of Denmark, Danish Veterinary and Food Administration (case #2017-15-0201-01353). Owners provided

informed consent before enrollment. Cats with GIC and CIE were recruited at UCPH, the University of Edinburgh, the Norwegian University of Life Sciences, University of Glasgow, or at Evidensia Karlslunde Animal Hospital, Denmark, or Evidensia Faxe Animal Hospital, Denmark. All healthy cats were recruited at UCPH. A study investigating miRNA in feces and serum from dogs using the same methodology has previously been published by this group.[12]

2.2 | Cat recruitment

Client-owned cats were prospectively recruited into 1 of 3 groups: GIC, CIE, and healthy cats (Figure 1). All cats included in the study were either directly seen by, or had their medical records evaluated by 1 board-certified internist (JGL) to assess their eligibility for final enrollment into the study.

For inclusion, cats in all 3 groups had to be ≥1 year of age and > 2 kg in body weight. Cats in all 3 groups underwent routine diagnostic evaluation including history, physical examination, fecal score based on the Purina 7-point fecal scoring system,[15] CBC, serum biochemistry, serum amyloid A (SAA) concentration, FIV/FeLV testing, urinalysis, fecal flotation (Fecalyzer, Vetoquinol, Lure, France), and *Giardia*/*Cryptosporidium* screening (Immunocard STAT! Crypto/Giardia, Meridian Bioscience, San Diego, CA). These diagnostic tests could be performed by the study investigators, other veterinarians at the same referral hospital, or by a referring veterinarian. Serum cobalamin and folate concentrations were measured at the clinician's discretion.

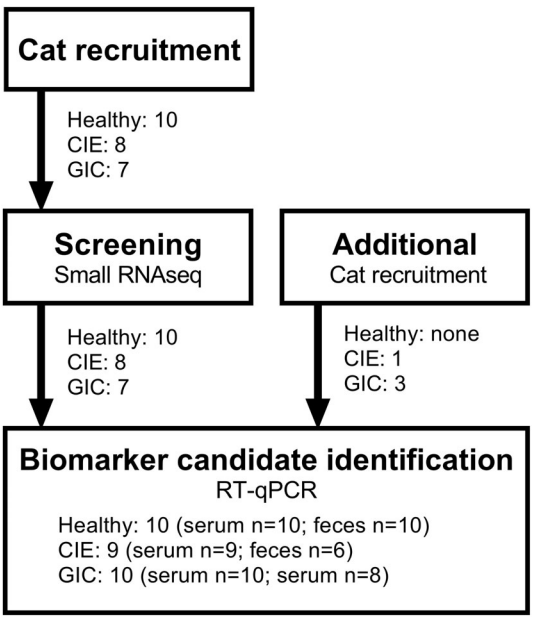

FIGURE 1 Cat recruitment and workflow. 25 cats (10 healthy, 8 CIE cats, 7 GIC cats) were recruited prior to the initial small RNAseq screening was performed. Serum was included in small RNAseq from all 25 cats, whereas feces was included in small RNAseq from 23 cats (feces not obtained from 1 CIE and 1 GIC cat). Following this, 1 additional CIE cat and 3 additional GIC cats were recruited for the study for a total number of 29 cats (10 healthy, 9 CIE cats, and 10 GIC cats). These additional cats were only included in the RT-qPCR analysis, not small RNAseq. Serum was obtained from all 29 cats. Feces was obtained from 26 cats (1 CIE cat and 2 GIC cats did not supply feces); additionally, cDNA synthesis from fecal RNA from 2 of the CIE cats was unsuccessful, leaving 6 CIE cats in the qPCR analysis of fecal miRNAs. CIE, chronic inflammatory enteropathy; GIC, gastrointestinal cancer; Small RNAseq, small RNA sequencing.

Cats in the GIC and CIE groups had feline chronic enteropathy activity index (FCEAI) score determined,[16] and if endoscopy was performed, the final FCEAI was reported. Duration of disease was defined as the number of days from first clinical signs to the first visit for study participation, and was based on the history from the owner and medical records. In addition, cats in the GIC and CIE groups had abdominal ultrasonography and endoscopy or laparotomy with biopsies for histologic assessment performed. A reference laboratory performed the histopathological assessment, and inflammatory alterations were graded using the World Small Animal Veterinary Association (WSAVA) standardization grading system.[17,18] Clonality testing, immunohistochemistry, or both were performed at the clinician's discretion.

In addition, cats in the GIC group required a histopathological diagnosis of GIC. For cats with a GIC diagnosis, full staging, including thoracic imaging and FNCB of regional lymph nodes, was encouraged. No specific exclusion criteria existed for the GIC group, and cats in this group were allowed to have co-morbidities and receive medications and supplements.

Cats in the CIE group were included if they had chronic GI signs, defined as persistent or recurrent vomiting, diarrhea, dysrexia, or abdominal pain for ≥2-3 weeks, if other causes of GI signs had been systematically ruled out, and if they had a histopathological diagnosis of mucosal inflammation.[2] For CIE cats only, to rule out other causes of enteropathy, additional diagnostic tests were performed at the clinician's discretion, based on current recommendations.[2] These included ≥1 diet trials a minimum of 2 weeks in duration using a hydrolyzed or novel protein diet, serum total T4 (tT4) concentration, serum trypsin-like immunoreactivity (TLI), and serum feline pancreatic lipase immunoreactivity (fPLI). Cats with CIE were excluded if they had food-responsive enteropathy, antibiotic-responsive enteropathy, protein-losing enteropathy, or if they had received antimicrobials, corticosteroids, or other immunomodulatory drugs within the previous 6 weeks, anthelmintics within the previous 4 weeks, or nonsteroidal anti-inflammatory drugs within the previous 2 weeks. Cats in the CIE group were allowed to have co-morbidities.

Healthy cats were included, if they were deemed healthy, without any clinically relevant abnormalities on the above-mentioned diagnostic tests in addition to serum tT4 concentration. Cats were excluded from the healthy group if there was any known disease or suspicion of disease. This determination was made based on history, physical examination, the diagnostic tests mentioned above, or episodes of GI signs within 2 months of enrollment. In addition, healthy cats were excluded if they had received antimicrobials, corticosteroids, or other immunomodulatory drugs within the previous 6 weeks, anthelmintics

within the previous 4 weeks, or nonsteroidal anti-inflammatory drugs within the previous 2 weeks.

2.3 | Sample collection

Blood was collected at the first visit by the investigators of the study and processed within 30 min of sampling. Serum samples were aliquoted into 0.5 mL cryotubes and initially frozen at −20°C to mimic a standard household freezer available in most veterinary clinics.

Within 1 hour of defecation, fecal samples were collected and aliquoted (approximately 1 g per aliquot) into cryotubes, which then were frozen at −20°C. Clients were provided detailed written and verbal instruction and the necessary supplies for at-home fecal sample collection if feces were not collected while the cats were in the hospital.[17,18] All frozen serum and fecal samples were transferred from −20°C to −80°C and kept at this temperature until batched RNA isolation.

2.4 | RNA isolation

The miRNeasy Mini Kit (Qiagen, Hilden, Germany) was used for isolation of total RNA from 100 mg feces as described previously.[14] Total RNA was isolated from 200 µL serum using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) without including the accompanying *C. elegans* miR-39 spike-in extraction control. Concentration yield and purity of each RNA sample were assessed by measuring the 260 nm/280 nm and 260 nm/230 nm absorbance ratios on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Hvidovre, Denmark). The RNA was stored at −80°C until batch analysis could be performed.

2.5 | Small RNAseq screening

The NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs, Ipswich, MA) was used for small RNAseq library preparation of fecal and serum samples using 6 µL total RNA from each sample. All samples obtained from cats that had been enrolled in the study up to this point were included in the small RNAseq screening. Library preparation and sequencing was carried out by a sequencing service provider (Genomics Unit, Center for Genomic Regulation, Barcelona Biomedical Research Park, Barcelona, Spain) using the Illumina HiSeq 2500 system to produce 50 bp single-end reads. Small RNAseq raw data was supplied to the authors as fastq files.

2.6 | RT-qPCR identification of miRNA biomarker candidates

Samples from all cats included in the small RNAseq screening in addition to 1 CIE cat and 3 additional GIC cats that were subsequently enrolled in the study (10 healthy, 9 CIE cats, and 10 GIC cats) were used in RT-qPCR biomarker candidate identification (see Figure 1). The qPCR was carried out using the high-throughput platform Biomark HD (Fluidigm, San Francisco, California) and the 96.96 Dynamic Array integrated fluidic circuit chip format (Fluidigm, San Francisco, California). All details on RT-qPCR analysis can be found in File S1 and in Table S1.[12-14,19-47]

2.7 | Data analyses

2.7.1 | Clinical data

The data was analyzed using GraphPad Prism (version 9.3.1). Normality was assessed using the Shapiro–Wilk test. Numerical data was expressed as median and range, or as number of animals and percentage. For continuous variables in general (eg, age, body weight) all 3 groups were compared using the Kruskal–Wallis test, and, if significantly different, a 2-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli was applied to correct for multiple comparisons. When assessing disease-related continuous variables (eg, duration of disease, serum cobalamin, serum folate, serum SAA) a pair-wise comparison between CIE and GIC using the Mann-Whitney test was used. Fisher's exact or Chi-squared tests were used to compare categorical data. A P value <.05 was considered significant.

2.7.2 | Small RNAseq data

Open source bioinformatics tools were used for small RNAseq data analysis. All details can be found in File S1.[48-54]

2.7.3 | qPCR data

Quality control and processing of qPCR data was carried out using the Fluidigm Real-Time PCR Analysis software (v.4.7.1, Fluidigm) and GenEx Pro software (v.7.1.1.118, MultiD Analyses AB), as described previously.[12] One-way analysis of variance (ANOVA) was used to identify significant differences in serum and fecal miRNA amounts among GIC, CIE, and healthy cats, and among SCL, CIE, and healthy cats ($P < .05$) using GraphPad Prism (v.9.2.0, GraphPad). For miRNAs with $P < .05$, the Tukey–Kramer post-hoc test was used for subsequent pairwise comparisons among the 3 groups. MiRNA fold changes were determined as the ratio of the average miRNA amounts in the 2 groups being compared. The diagnostic performance of individual miRNAs in relation to discriminating between GIC and CIE cats was analyzed if a miRNA was present at significantly different amounts in GIC relative to CIE cats. The ability of miRNAs to discriminate between GIC and CIE cats or GIC and healthy cats was evaluated using receiver operating characteristic (ROC) curve analysis in GraphPad Prism. Using the maximum value of Youden's index as cutoff, diagnostic performance of miRNAs was evaluated by computing sensitivity, specificity, and positive and negative likelihood ratios (LR+, LR−).

TABLE 1 Cat characteristics.

Group characteristic	Healthy	CIE	GIC	*P* value[α]
Total number, n	10	9	10	
Cat characteristics				
Age in months, median (range)	44 (19-133)	103 (44-149)	111 (34-202)	**<.05**
Gender, *n* female/male	6/4	4/5	3/7	.40
Body weight in kg, median (range)[a]	4.7 (2.9-6.4)	5.5 (2.9-6.7)	4.46 (2.9-5.2)	.16
Breeds, *n*	DSH, 5	DSH, 5	DSH, 7	
	Birman, 3	NFC, 2	NFC, 1	
	DLH, 1	DLH, 1	Maine coon, 1	
	Rag doll, 1	Toyger cat, 1	Bengal cross, 1	
Clinical parameters				
Disease duration in days, median (range)	NA	180 (5–1080)	30 (0–540)	.14
Clinical signs, *n* (%)	NA			
-Vomiting	NA	8/9 (89%)	4/10 (40%)	.06
-Diarrhea	NA	4/9 (44%)	4/10 (40%)	>.99
-Weight loss	NA	3/9 (33%)	7/10 (70%)	.18
-Dysrexia	NA	5/9 (56%)	7/10 (70%)	.65
-GI bleeding	NA	5/9 (56%)	5/10 (50%)	>.99
Clinicopathological parameters				
Serum cobalamin concentration in pmol/L, median (range)[a]	879 (603-2283)	876 (111–1323)	267 (152–1226)	.22
Serum folate concentration in nmol/L, median (range)[a]	NA	39.3 (13.2–98.3)	28.3 (19.6-49.8)	**.03**
SAA concentration mg/L, median (range)[a]	0 (0-0.2)	0.3 (0-126.7)	0.4 (0-104.9)	.88

Note: "Cat characteristics" (ie, age, gender, and body weight) are compared between GIC, CIE, and healthy cats. Descriptive statistics are based on multiple comparisons for the "clinical parameters" (ie, duration of disease and clinical signs) and "clinicopathological parameters" (ie, serum cobalamin, serum folate, serum amyloid A), as the healthy cats are not included in the statistical comparisons even if median and range are listed. α, Significant difference between groups ($P < .05$) is indicated with boldface values.

Abbreviations: CIE, chronic inflammatory enteropathy; DLH, Domestic longhair; DSH, Domestic shorthair; GI, gastrointestinal; GIC, gastrointestinal cancer; NFC, Norwegian forest cat; SAA, serum amyloid A.

[a]Data missing on 1 cat.

3 | RESULTS

3.1 | Cat characteristics

Twenty-nine cats were enrolled from 2018 to 2020. Cat characteristics are presented in Table 1.

Ten cats were included in the GIC group. Four of these cats had received medical treatment when sampled for the study. The treatments included meloxicam (n = 1), cannabidiol drops (n = 1), chlorambucil and prednisolone (n = 1), and metronidazole and robenacoxib (n = 1). Two cats had odontoclastic resorptive lesions, 1 cat had a history of giardiasis, and 1 cat had a history of focal seizures- and facial twitching. Weight loss and dysrexia were the most prevalent clinical signs, seen in 70% of cats. Median duration of disease was 30 days (range 0-540 days).

All but 1 GIC cat (9/10) had serum cobalamin and folate concentrations measured. The median serum cobalamin concentration was 267 pmol/L (range, 152-1226 pmol/L). Four of 9 GIC cats had serum cobalamin concentrations <250 pmol/L and 5/9 GIC cats had serum cobalamin concentrations <400 pmol/L. Hypofolatemia was seen in 4/9 cats defined as serum folate concentration < 25.2 nmol/L and 1 cat had hyperfolatemia defined as serum folate concentration > 49.0 nmol/L. All cats but 1 (9/10) had known FIV/FeLV status and were all negative.

Abdominal ultrasound examination was performed in 9/10 GIC cats. Four cats had a focal mass (2 jejunal, 1 colon, and 1 localized to a non-specified small intestinal segment). Two cats had diffuse gastric wall thickening with loss of normal wall layering, and 2 cats had diffuse small intestinal wall thickening of the mucosa and muscularis layers. One cat had hyperechoic mucosal stippling in the jejunum, but no other findings were noted in the GI tract.

One cat had thoracic radiographs performed with no evidence of metastases. Six GIC cats had endoscopic biopsies performed, and the remaining 4 cats had full thickness biopsies performed at laparotomy. In the 6 cats that had endoscopic biopsies, the median FCEAI score was 8.5 (range, 0-14). Eight cats were diagnosed with lymphoma,

including SCL (n = 4), high grade small-intermediate cell lymphoma (n = 1), and large-cell lymphoma (LCL; n = 3). Two LCL were B-cell, transmural, and located in the stomach or jejunum, respectively. The third LCL was transmural, located in the small intestine, and was CD20, PAX5, CD3, and c-KIT negative on immunohistochemistry, but was consistent with T-cell lymphoma on clonality testing. The small to intermediate T-cell lymphoma was a transmural solitary lesion originating from the jejunum. Of the cats with mucosal SCL, 3 were of T-cell, and 1 of B-cell origin. Lastly, 2 cats had a colonic papillary adenocarcinoma and rectal carcinoma with sebaceous differentiation, respectively.

Disease involvement elsewhere or metastases were not cytologically or histologically confirmed in any cat. However, 1 cat with colonic adenocarcinoma had local lymphadenopathy visualized on ultrasound examination and during laparotomy, and the tumor was transmural and expanded into the abdomen.

Nine cats with CIE were enrolled. Two of these cats had a concurrent cardiac murmur, 3 had a dermatological problem with pruritus, alopecia or both and 1 cat had a history of spontaneous pneumothorax. All 9 cats had clinical signs of enteritis alone or in combination with gastritis or colitis. Median duration of disease was 180 days (range, 5-1080 days). The most prevalent clinical signs were vomiting (89%) and dysrexia (56%). Serum tT4 concentrations were normal or low in all CIE cats, and all cats were FIV/FeLV negative. Serum TLI was assessed in 8/9 cats and was normal in 4 and above normal in 4. Serum feline pancreatic lipase concentration was assessed in 8/9, being normal in 4, between 3.6-5.3 μg/L in 2, and > 5.4 μg/L in 1 cat. Serum folate and cobalamin concentrations were assessed in all 9 CIE cats. The median serum cobalamin concentration was 876 pmol/L (range, 111-1323 pmol/L), and 3/9 CIE cats had serum cobalamin concentrations <250 pmol/L or <400 pmol/L. The median serum folate concentration was 39.3 nmol/L (range, 13.2-98.3 nmol/L), and hypofolatemia, defined as serum folate concentration < 25.2 nmol/L, was seen in 1/9 cats. Hyperfolatemia, defined as a serum folate concentration > 49.0 nmol/L, was seen in 2/9 cats.

All 9 CIE cats had endoscopic biopsies performed, but 2 did not have ileal biopsy specimens obtained because of anesthetic complications (n = 1) and difficulty passing the endoscope beyond the ileocolic valve (n = 1). The median FCEAI score was 8 (range, 4-13). Histopathologic diagnoses in this group were mild-moderate or moderate lymphoplasmacytic enteritis (n = 5), mild-moderate or moderate lymphocytic and eosinophilic enterocolitis (n = 2), and mild or mild-moderate lymphocytic enterocolitis (n = 2).

Ten healthy cats were enrolled. No clinically relevant abnormalities were noted on any of the diagnostic tests, including urinalysis, fecal testing, FIV/FeLV testing, tT4, and serum cobalamin concentration. Serum folate concentrations were measured in 3 cats, but were within the normal reference range in these cats.

Significant differences in age among all 3 groups were found and remained significant between healthy cats and cats with GIC on post-hoc analysis with GIC cats being older ($P = .02$). Cats with GIC had significantly lower serum folate concentrations compared to those with CIE ($P = .03$), but no significant differences in sex, body weight, duration of disease, clinical signs, serum cobalamin concentration, and serum SAA concentration were found among groups (Table 1).

3.2 | RNA isolation

Total RNA was isolated from cat feces and serum for use in small RNAseq and RT-qPCR. For serum, RNA was obtained from 29 samples (healthy, n = 10; CIE, n = 9; GIC, n = 10). For feces, RNA was obtained from 26 samples (healthy, n = 10; CIE, n = 8; GIC, n = 8). See also Figure 1. Results on RNA concentration and purity are summarized in Table 2. All samples were considered to be of acceptable quality for inclusion in further analysis.

3.3 | Small RNAseq screening of cat serum and feces

3.3.1 | Serum

Small RNAseq was performed on 25 serum samples (healthy, n = 10; CIE, n = 8; GIC, n = 7; Figure 1). Small RNAseq of serum yielded an average of 9.7 M reads per sample. For serum samples, 99.2% of reads passed quality control and adapter trimming and, of these, 63.8% of reads mapped to the *F. catus* genome. Of the mapped reads in serum, 2.2% mapped to known or novel cat miRNA sequences. Serum miRNA abundance is summarized in Figure 2A. Comparison of average serum miRNA amounts showed that miR-1224, miR-320a-3p, and miR-296-3p were present in significantly lower amounts in GIC cats compared to CIE cats, miR-27a-5p was present in significantly higher amounts in GIC cats compared to healthy cats, and miR-320a-3p was present in significantly lower amounts in CIE cats compared to healthy cats after correcting for multiple testing (Table S2 and Figure S1). Details on small RNAseq results can be found in Tables S3 and S4. MiRDeep2 identified several putative novel cat miRNAs in the small RNAseq data. These putative novel miRNAs were included in DESeq2 analysis (Table S2) and are further summarized in Table S5.

3.3.2 | Feces

Small RNAseq was performed on 23 fecal samples (healthy, n = 10; CIE, n = 7; GIC, n = 6; Figure 1). Small RNAseq of feces yielded an average of 10.9 M reads per sample. For fecal samples, 99.6% of reads passed quality control and adapter trimming and, of these, 18.7% of reads mapped to the *F. catus* genome. Of the mapped reads in feces, 0.07% mapped to known or novel cat miRNA sequences. Fecal miRNA abundance is summarized in Figure 2B. When comparing average fecal miRNA amounts in the 3 groups of cats, statistical analyses showed that no miRNAs were present in statistically significant amounts in any comparisons (ie, GIC vs. CIE cats, GIC vs. healthy cats, or CIE vs. healthy cats) when correcting for multiple testing (Table S2

TABLE 2 RNA isolation: Median and range.

Group	RNA concentration (ng/μl)	A260/280 ratio	A260/230 ratio
Serum			
Healthy cats	11.9 (7.1-37.6)	1.3 (1.1-1.4)	0.37 (.10–.62)
CIE cats	13.1 (8.2-35.7)	1.3 (.95-1.5)	0.22 (.08–.42)
GIC cats	11.2 (6.2-22.4)	1.3 (1.1–1-5)	0.23 (.10–.39)
All cats	12.0 (6.2-37.6)	1.3 (.95–1.5)	0.29 (.08–.62)
Feces			
Healthy cats	249.6 (66.0-398.8)	2.0 (1.8-2.1)	1.6 (.45-1.9)
CIE cats	152.0 (56.3-791.0)	1.9 (1.4-2.0)	0.78 (.30-2.0)
GIC cats	344.5 (162.6-1069.4)	2.0 (1.8–2.1)	1.5 (.53-2.1)
All cats	258.2 (56.3-1069.4)	2.0 (1.2-2-1)	1.5 (.30-2.1)

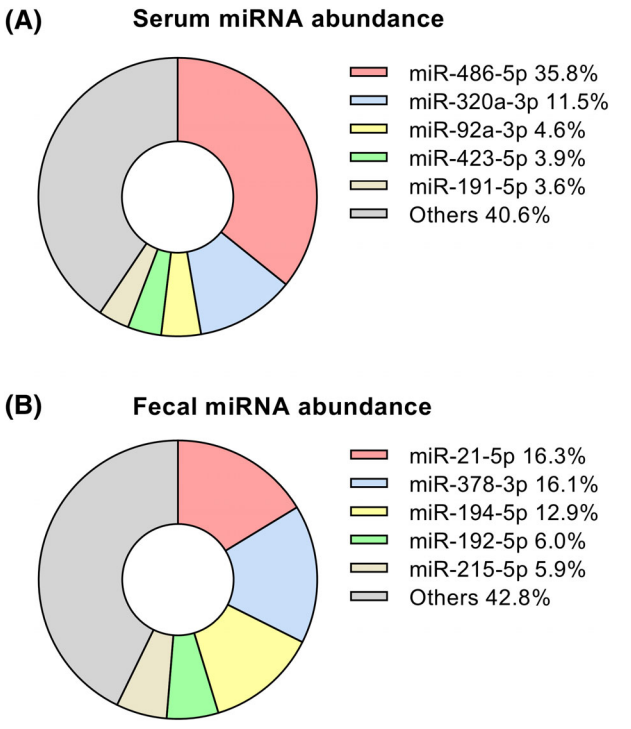

FIGURE 2 (A) The most abundant of the detected miRNAs in cat serum were miR-486-5p, miR-320a-3p, miR-92a-3p, miR-423-5p, and miR-191–5p, accounting for a total of 59.4% of all miRNA reads. (B) The most abundant of the detected miRNAs in cat feces were miR-21-5p, miR-378-3p, miR-194-5p, miR-192-5p, and miR-215-5p, accounting for a total of 57.2% of all miRNA reads.

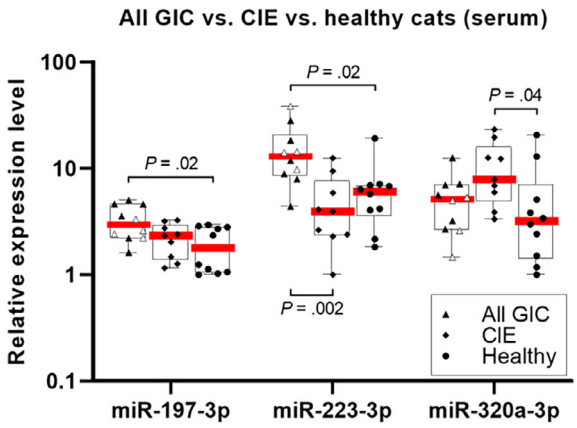

FIGURE 3 Relative expression levels of miR-197-3p, miR-223-3p, and miR-320a-3p in healthy cats, cats with CIE, and cats with GIC. *P* values from Tukey's post-hoc test. Boxes extend from 25th to 75th percentile; whiskers extend to minimum and maximum values; horizontal red lines indicate median value. For the GIC groups, white triangles highlight the 4 cats diagnosed with SCL.

and Figure S1). Details on fecal small RNAseq results and putative novel cat miRNAs can be found in Tables S3–S5.

3.4 | qPCR identification of cat miRNA biomarkers that differentiate GIC from CIE

3.4.1 | Serum

Forty-seven of the 91 assayed miRNAs (representing 52 of the 96 included primer pairs) were quantifiable by qPCR in cat serum (Table S6). Of these, let-7f-5p, let-7i-5p, and miR-21-5p were found suitable for data normalization based on evaluation using NormFinder[55] and geNorm.[56] A significant difference was found for 3 miRNAs in 1-way ANOVA (miR-223-3p, miR-197-3p, and miR-320a-3p) among the 3 groups of cats (Figure 3 and Table 3). Of these, only miR-223-3p was present in significantly different quantities in GIC and CIE cats by Tukey-Kramer's post hoc test (Figure 3 and Table 3), and miR-223-3p likewise was present in significantly higher quantities in GIC compared to healthy cats. No difference however was found for miR-223-3p between CIE and healthy cats. MiR-197-3p was significantly different between GIC and healthy cats, and miR-320a-3p was significantly different between CIE and healthy cats, but neither were significantly different between GIC and CIE cats (Figure 3 and Table 3).

3.4.2 | Feces

Twenty-three of the 91 assayed miRNAs (representing 26 of the 96 included primer pairs) were quantifiable by qPCR in cat feces

TABLE 3 qPCR identification of serum miRNA biomarker candidates for differentiation between cats with GIC and CIE, and cats with SCL and CIE.

		Comparisons						
		All GIC vs. CIE		All GIC vs. healthy		CIE vs. healthy		RNAseq-qPCR correlation (Pearson's *r*)
miRNA	*P*[a]	FC (±95%CI)	*P*[b]	FC (±95%CI)	*P*[b]	FC (±95%CI)	*P*[b]	
Serum								
miR-223-3p	**.002**	**3.2 (±1.3)**	**.002**	**2.4 (±1.0)**	**.02**	.76 (±.39)	.57	NA
miR-197-3p	**.02**	1.5 (±.34)	.15	**1.7 (±.24)**	**.02**	1.2 (±1.6)	.62	$r = .42$, $P = .05$
miR-320a-3p	**.04**	.50 (±.19)	.17	.95 (±.36)	.74	**1.9 (±.83)**	**.04**	$r = .44$, $P = .03$
Feces								
None								
		SCL vs. CIE		SCL vs. healthy		CIE vs. healthy		
Serum								
miR-223-3p	**.008**	**3.9 (±1.6)**	**.006**	**3.0 (±1.3)**	**.03**	.76 (±.39)	.58	NA
miR-320a-3p	**.04**	.34 (±.11)	.13	.65 (±.21)	.98	1.9 (±.83)	.06	$r = .45$, $P = .04$
Feces								
miR-148b-3p	**.03**	**.36 (±.34)**	**.02**	**.45 (±.43)**	**.05**	1.23 (±.31)	.72	$r = .39$, $P = .14$

Note: CI, confidence interval, NA, not available—miR-223-3p not quantified with small RNAseq.
[a]One-way ANOVA.
[b]Tukey-Kramer's post hoc test. Statistically significant changes are highlighted with bold font.

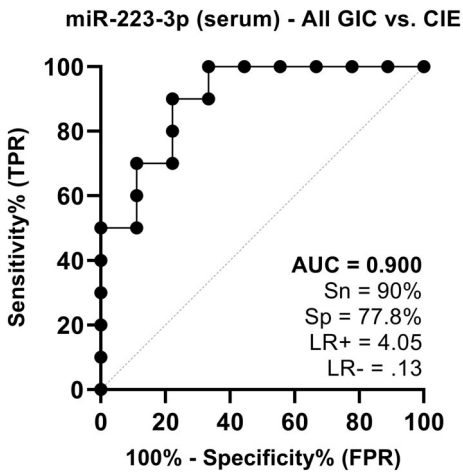

FIGURE 4 ROC curve for miR-223-3p for distinguishing all GIC from CIE cats. AUC, area under the curve; FPR, false positive rate; LR, likelihood ratio; Sn, sensitivity; Sp, specificity; TPR, true positive rate. 95% Cis, AUC 0.760-1.0; Sn, 59.6%-99.5%; Sp, 45.3%-96.1%; LR+, 1.2-14.0; LR−, 0.019-0.85.

(Table S6). Of these, let-7a-5p and miR-192-5p were found suitable for data normalization based on evaluation using NormFinder[55] and geNorm.[56] The qPCR results showed that cDNA synthesis from 2 fecal RNA samples (both CIE) had been unsuccessful, and these had to be taken out of the analysis, leaving 6 CIE cats for the remaining analysis. Statistical analysis showed no significant differences in fecal miRNA amounts among the 3 groups of cats for any of the quantified miRNAs (1-way ANOVA), and therefore no further investigation of fecal miRNA biomarker potential was conducted.

3.5 | Diagnostic performance of serum miR-223-3p for differentiation of GIC from CIE or healthy cats

The ROC curve analysis was carried out for miR-223-3p to evaluate its diagnostic potential in serum. For the differentiation between GIC and CIE, this analysis yielded an AUC of 0.90 (95% CI, 0.76-1.0; Figure 4). Using the maximum value of Youden's index, sensitivity and specificity were determined to be 90.0% (95% CI, 59.6-99.5%) and 77.8% (95% CI, 45.3-96.1%), respectively. Positive likelihood ratio was found to be 4.1 (95% CI, 1.2-14.0) and LR- was found to be 0.13 (95% CI, 0.019-0.85). For differentiation between GIC and healthy cats, this analysis yielded an AUC of 0.87 (95% CI, 0.693-1.0), sensitivity of 90.0% (95% CI, 59.6-99.5%), specificity of 90.0% (95% CI, 59.6-99.5%), LR+ of 9.0 (95% CI, 1.4-58.4), and LR- of 0.11 (95% CI, 0.017-0.72; Figure S2).

3.6 | qPCR identification of cat miRNA biomarkers that differentiate SCL from CIE

3.6.1 | Serum

All quantified miRNAs were evaluated for their ability to distinguish among SCL, CIE, and healthy cats. In 1-way ANOVA, significant difference among these 3 groups was seen for miR-223-3p and miR-320a-3p (Figure 5 and Table 3), but significant differences in Tukey-Kramer's post-hoc test were only seen for miR-223-3p between SCL and CIE cats, as well as between SCL and healthy cats. No investigation of diagnostic performance of miR-223-3p in relation to

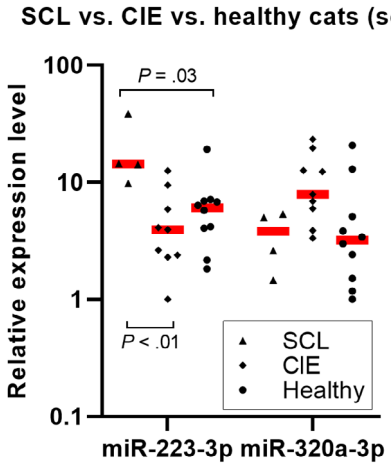

FIGURE 5 Serum quantities of miR-223-3p and miR-320a-3p in cats with SCL and CIE and healthy cats. Horizontal red lines represent median values. *P* value from Tukey–Kramer post hoc test after one-way ANOVA.

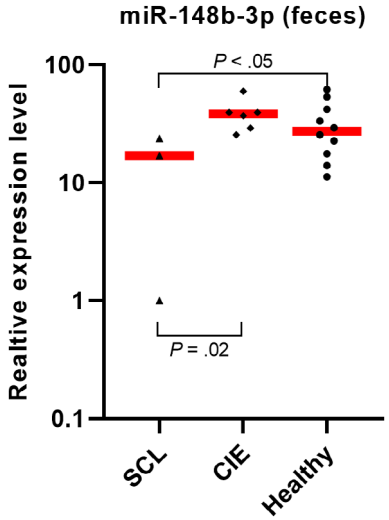

FIGURE 6 Fecal quantities of miR-148b-3p in cats with SCL and CIE and healthy cats. Horizontal red lines represent median values. *P* value from Tukey–Kramer post hoc test after one-way ANOVA.

distinguishing between SCL and CIE or healthy cats was made because of the low sample size for serum from SCL cats (n = 4).

3.6.2 | Feces

In contrast to the results of the comparison of all GIC cats to CIE and healthy cats (Section 3.4), 1 miRNA was significantly different in 1-way ANOVA when comparing only SCL, CIE, and healthy cats, namely miR-148b-3p (Figure 6 and Table 3). In Tukey-Kramer's post hoc test, miR-148b-3p was significantly different between SCL and CIE cats, as well as between SCL and healthy cats. No investigation of diagnostic performance of miR-148b-3p in relation to distinguishing between SCL and CIE or healthy cats was made because of the low sample size for feces from SCL cats (n = 3).

4 | DISCUSSION

Our study is the first to examine serum and fecal miRNAs as potential biomarker candidates for the differentiation of GIC, CIE, and healthy cats. Although fecal miRNAs could not differentiate these conditions in our study groups, serum miR-223-3p was present in significantly different quantities and was able to distinguish GIC from CIE as well as healthy cats with high sensitivity and specificity. These findings lay the potential foundation for the development of a novel assay in cats for detection and differentiation of GIC from both CIE and healthy cats.

In cats, serum amounts of miR-223-3p were significantly higher in GIC relative to CIE cats. In this context, it is relevant to note that when comparing only SCL and CIE cats, serum amounts of miR-223-3p still were high in SCL cats, suggesting that miR-223-3p might have the potential to differentiate these conditions. In our study, serum miR-223-3p could distinguish between cats with GIC and CIE with an AUC of 0.90 (95% CI, 0.760-1.0). A previous study reported similar performance with an AUC of 0.92 for this miRNA in feces for differentiating between GIC and CIE dogs.[12] MiR-223-3p was included in our study because it was previously found to be a potential fecal biomarker candidate for differentiation between GIC and CIE in dogs.[12] Another study determined miR-223-3p to be present in lower amounts in serum and colonic mucosa of dogs with CIE compared to healthy dogs,[13] supporting the notion of it as a marker of only GIC in dogs and cats. In our study, miR-223-3p serum amounts were slightly lower in cats with CIE compared with healthy cats, but this difference was not significant. Diagnostic performance of miR-223-3p in GI disease in humans has been evaluated in the context of cancer or inflammatory bowel disease (IBD) in relation to differentiation from healthy individuals, but to the best of our knowledge never in relation to differentiation between the 2 diseases. However, quantities of serum and fecal miR-223-3p have been shown to be increased in patients with GIC or IBD compared to healthy individuals. Likewise, good discriminatory ability of miR-223-3p in plasma or feces has been reported both between healthy individuals and GIC patients[42,57] and between healthy individuals and IBD patients.[29,44] Consequently, miR-223-3p might not be able to discriminate between these conditions in humans, as it appears able to in cats and dogs.

Analysis of feces yielded no miRNAs that were significantly different among the GIC, CIE, and healthy cats, neither using small RNAseq nor RT-qPCR. However, when comparing only SCL cats to CIE and healthy cats, miR-148b-3p was significantly lower in SCL than in both CIE and healthy cats. This result however should be interpreted with caution because of the small sample size for fecal samples from SCL cats (n = 3). Additionally, the relevance for miR-148b-3p as a biomarker for discrimination of SCL and CIE requires further investigation. Our reasoning for including miR-148b-3p in our analysis was its possible potential as an endogenous qPCR normalizer based on our small RNAseq

screening (Tables S1 and S2) as well as literature findings showing it to be significantly different in serum of dogs with GIC and CIE[14] and in serum of human colorectal cancer patients and healthy controls.[43]

Despite the consistency of finding miR-223-3p to be a biomarker candidate for the differentiation of GIC and CIE in cats as it also previously has been in dogs,[12] there is a discrepancy regarding sample matrix. In a previous study, miR-223-3p proved a good fecal biomarker candidate, whereas GIC and CIE cats had no differences in fecal miR-223-3p amounts.[12] In contrast, serum miR-223-3p amounts in dogs with GIC and CIE were not significantly different, contrary to our findings in cats. Evidently, care should be taken to not extrapolate findings on biomarker suitability between these species. There are a number of reasons why assessing fecal miRNAs might be subject to more variation, thus limiting their diagnostic use. Feces is generally easy to obtain from indoor cats, but it proved challenging to ensure collection of newly evacuated feces (<1 hour), because many of the enrolled cats defecated at night or while alone, especially cats that normally were allowed outdoor access. However, evaluating a more diverse group of cats with different subtypes of CIE and GIC is warranted. Dysrexic cats produced only small amounts of fecal material, and it likewise proved challenging to collect feces if the fecal score was 6-7. A prolonged time from defecation to collection as well as presence of hair or undigested plant material within the fecal content could have influenced the quality of the RNA isolated from fecal material and therefore the present fecal miRNA results. Furthermore, outdoor cats with potential access to birds or small rodents were allowed in our study. In humans, diet-derived xenomiRs have been detected in blood and feces, but this occurrence has not been assessed in cats, and hence it is unknown if specific meat-rich diets or ingestion of prey can influence the fecal or serum miRNA profiles in cats.[58,59] The difficulties relating to fecal miRNA quantification have been described previously.[12] Only 27% of the miRNA primer pairs (26/96) included in qPCR analysis in our study were successful in feces, compared with 54% (52/96) in serum. Proper storage is crucial to achieving high quality RNA from any sample, and the less controlled method of owner-conducted at-home feces collection applied in our study might have contributed to suboptimal conditions for preventing RNA degradation. We found that serum provided a more reliable sample type for the identification of GIC biomarkers in cats, and is a commonly applied minimally-invasive sample type for biomarker measurement, including miRNA biomarkers.[12,34,38,60]

A limitation of our study is the relatively small sample size. Because ours was the first study to detect miRNAs in serum and feces from healthy cats and cats with CIE and GIC, future investigation assessing the role of miR-223-3p in a larger study group would be indicated. All CIE cats and the majority of GIC cats had endoscopic biopsies performed, and 2/9 CIE cats did not have ileal biopsy specimens obtained. Previously, it was believed that ileal biopsy specimens were critical to obtaining the correct diagnosis, but such may not be the case.[61–63] Histopathologic scoring and interobserver variation remain challengeing[62,63] and the final diagnoses on some CIE or SCL cats could have been misinterpreted, but immunohistochemistry and clonality testing were performed when relevant and available.

All types of malignant neoplasia were eligible for inclusion, but only cats with lymphomas and carcinomas were enrolled and hence only these cancer types were investigated. These findings are consistent with the current literature because lymphoma and carcinomas are the most prevalent GI tumors.[3] Therefore, our findings do not necessarily apply to cats with other tumor types such as mast cell tumors or mesenchymal tumors.[3]

Because only 1 cat with GIC had thoracic radiography performed, pulmonary neoplasia or other pathology cannot be ruled out in the remaining cats, even though none of the cats with GIC had a history of clinical signs relating to the respiratory system at the time of inclusion.

A subset of cats had comorbidities, received medications or both. The effect of these comorbidities and medications are unknown both for the CIE and GIC groups, but could cause alterations of the microbiota that could affect the fecal miRNA.[64] A number of diseases, including stable hypertrophic cardiomyopathy, have shown distinct deregulated miRNAs, but none of the investigated miRNAs were significant in our study.[65]

In conclusion, we identified miR-223-3p as a potential serum biomarker candidate for the differentiation of cats with GIC and CIE from healthy cats. Additionally, serum miR-223-3p showed potential in differentiating cats with SCL from those with CIE and healthy cats.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All small RNA sequencing data will be available from the Sequencing Read Archive (https://www.ncbi.nlm.nih.gov/sra) upon final publication of the manuscript (BioProject ID: PRJNA844553).

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 local ethics committees (University of Copenhagen: #2017-9 + 2017-12; Norwegian University of Life Sciences: 14/04723-72, University of Glasgow: Ref 05a/18, University of Edinburgh: VERC#41.18) at all involved institutions as well as by the

Animal Experiments Inspectorate under the Ministry of Food, Agriculture, and Fisheries of Denmark, Danish Veterinary and Food Administration (case #2017-15-0201-01353).

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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