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Correlation between metabolomic profile constituents and feline pancreatic lipase immunoreactivity

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Abstract

Background: Feline pancreatic lipase immunoreactivity (fPLI) is commonly used to diagnose pancreatitis in cats (FP). Untargeted metabolomics has been extensively applied in human and veterinary medicine, but no metabolomic studies regarding FP have been conducted.

Objectives: To identify metabolites significantly associated with increased fPLI.

Animals: Forty-nine client-owned cats: 11 clinically healthy and 38 with various clinical conditions.

Methods: Analytical cross-sectional study with convenience sampling. A panel of 630 metabolites belonging to 26 biochemical classes was quantified in plasma using a commercial metabolomic assay. The correlation between plasma metabolite concentrations and serum fPLI was evaluated using Spearman's rank correlation coefficient (R_s) with Bonferroni correction. Multivariable analysis then was performed to control for glomerular filtration rate, liver damage, and blood glucose concentration. The accuracy of selected metabolites in discriminating between cats with normal (≤3.5 μg/L) and increased (>5.3 μg/L) fPLI was estimated using the area under the receiver operating characteristic curve (AUROC).

Results: Four hundred and seven of 630 metabolites (64.6%) were quantified in all cats. When controlled for potential confounders only 3 sphingolipids were significantly positively correlated with fPLI: 2 cerebrosides: HexCer(d18:1/24:0); ($R_s = .56$), and HexCer(d18:1/24:1); ($R_s = .58$) and 1 sphingomyelin: SM C18:0 ($R_s = .55$). Their AUROCs in identifying cats with increased fPLI were 82% (95% confidence interval [CI 95%], 70%-94%), 84% (CI 95%, 72%-96%), and 78% (CI 95%, 65%-92%), respectively.

Abbreviations: AKI, acute kidney injury; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under receiver operating characteristic curve; CE, cholesterol ester; Cer, ceramide; CI 95%, 95% confidence interval; CK, creatine kinase; CoS, coefficient of skewness; DG, diglyceride (diacylglycerol); DGGR, 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin); DM, diabetes mellitus; EDTA, potassium ethylenediaminetetraacetic acid; FIA-MS/MS, flow injection analysis-tandem mass spectrometry; FP, feline pancreatitis; fPLI, feline pancreatic lipase immunoreactivity; GalCer, galactocerebrosides; GD, disialoganglioside; GGT, gamma-glutamyltransferase; GlcCer, glucocerebrosides; GM, monosialoganglioside; Hb, hemoglobin concentration; HexCer, hexosylceramide (cerebroside); HRS, high-rise syndrome; Ht, hematocrit; IMHA, immune-mediated hemolytic anemia; IQR, interquartile range; J, Youden's index; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LDH, lactate dehydrogenase; LOD, limit of detection; NANA, N-acetylneuraminic acid; PC, phosphatidylcholine; PITC, phenyl isothiocyanate; PLT, platelet count; RBC, red blood cell count; RI, reference interval; R_s, Spearman's rank correlation coefficient; SDMA, symmetric dimethylarginine; Se, diagnostic sensitivity; SM, sphingomyelin; Sp, diagnostic specificity; TB, total bilirubin; TG, triglyceride (triacylglycerol); TP, total protein; WBC, white blood cell count.

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Conclusions and Clinical Importance: Selected sphingolipids are moderately positively correlated with fPLI and appear to have fair to moderate diagnostic accuracy in discriminating between cats with normal and increased fPLI.

KEYWORDS
cerebrosides, feline pancreatitis, fPLI, sphingolipids, sphingomyelins, untargeted metabolomics

1 | INTRODUCTION

Pancreatitis in cats (FP) appears to be a common disease,[1] but its diagnosis is hampered by nonspecific clinical manifestation and only moderate accuracy of currently used diagnostic tests.[2,3] The most common signs of FP include lethargy and anorexia, less often accompanied by vomiting, abdominal pain, diarrhea or some combination of these signs.[3-8] Although 3 histopathological forms of FP have been described (acute necrotizing pancreatitis, acute suppurative pancreatitis, chronic pancreatitis[9,10]) their clinical presentations markedly overlap, which precludes reliable antemortem differentiation.[11] Currently, no strict clinical criteria distinguish between acute and chronic or chronic active pancreatitis.[2-4] Moreover, FP is commonly accompanied by other diseases such as cholangiohepatitis, inflammatory bowel disease, kidney disease or diabetes mellitus (DM),[12-16] which makes it difficult to determine the role of pancreatic inflammation in the overall clinical picture of the patient. Currently, FP is diagnosed on the basis of the history and clinical signs, along with the combination of an increased pancreatic lipase concentration quantified using ELISA (feline pancreatic lipase immunoreactivity, fPLI) or a colorimetric 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) assay and abdominal ultrasonography.[3,17] Consistent results of laboratory and imaging diagnostic tests allow highly accurate diagnosis, but agreement between them is low.[18] Diagnostic sensitivity (Se) and specificity (Sp) of fPLI for FP have been estimated at 50% to 70% and 60% to 100%, respectively.[19-22] High variability of these estimates results from the reference standard used (histopathology vs clinical diagnosis), definition, form and severity of FP, as well as the cut-off used for fPLI interpretation. The currently used fPLI reference interval (RI) for fPLI is 0.7 to 3.5 μg/L, and the critical value indicating FP is considered >5.3 μg/L. Because these values are based on a single study reported in the form of an abstract over a decade ago,[23] they should be used cautiously.

Over the last decade, quantifying large sets of metabolites in various biological fluids, referred to as metabolomics, has gained in popularity as a potentially useful method of diagnosing and monitoring many conditions in human medicine,[24] including pancreatic diseases.[25] Several studies have investigated application of metabolomics to the diagnosis and differentiation among major types of pancreatitis in humans.[26] Fewer metabolomic studies have been carried out in veterinary medicine[27,28] and only a few of them in cats. These studies focused on feline metabolome in DM,[29,30] metabolic syndrome,[31] inflammatory bowel disease and alimentary small cell lymphoma,[32] mammary carcinoma,[33] and chronic kidney disease.[34]

In light of promising results of human studies and the need for improvement in diagnostic tests for FP we performed an untargeted metabolomic study aiming to identify metabolites significantly correlated with fPLI and attempted to determine how well they distinguish between cats with normal and increased fPLI. Because it was a hypothesis-generating initial study, it did not involve a priori hypotheses about any individual metabolites.

2 | MATERIALS AND METHODS

2.1 | Study population

This analytical cross-sectional study was carried out in 2 private veterinary clinics located in central Poland in 2018 and 2019. The study was based on convenience (opportunity) sampling. Cats were enrolled as they were presented at the clinics based on the information provided in the history (presence or lack of clinical signs potentially indicative of FP), organizational issues (opportunity to centrifuge blood immediately after collection and place the harvested plasma at −80°C within 1 hour of blood collection), and owners' consent to participate in the study. The enrollment lasted until the study population consisted of 52 cats, which ensured 95% power of correlation analysis assuming a correlation coefficient of .5. The cats were followed until a diagnosis was made or they recovered from the condition for which they had been admitted.

All cats underwent routine clinical and blood laboratory examination, which included CBC and serum biochemistry and was performed in a commercial veterinary laboratory. Also, fPLI was quantified in all cats. Other laboratory and diagnostic imaging tests as well as necropsy examination were performed if needed to make a diagnosis.

2.2 | Laboratory measurements

The CBC included total white blood cell (WBC) and differential count, red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), and platelet count (PLT). Serum biochemistry included the activity of alanine (ALT) and aspartate (AST) aminotransferase, alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), creatine kinase (CK), lactate dehydrogenase (LDH), and the concentrations of total protein (TP), albumin, total bilirubin (TB), urea, creatinine, total

cholesterol, triglycerides, glucose, fructosamine, and electrolytes (sodium, potassium, chloride, total calcium, phosphorus, magnesium). All results are reported in Table S1. The CBC and serum biochemistry were performed using a Mythic 18 Vet Hematology analyzer (PZ Cormay S.A., Poland) and automatic photometric clinical chemistry analyzer ACCENT-200 (PZ Cormay S.A., Poland), respectively, and interpreted according to the reference intervals developed by the veterinary laboratory.[35] In all cats, serum fPLI was determined using ELISA based on monoclonal antibodies (Spec fPL) at IDEXX Laboratories GmbH (Ludwigsburg, Germany). The fPLI was considered normal when ≤3.5 μg/L and increased when >5.3 μg/L. During routine blood collection an additional tube of blood containing potassium ethylenediaminetetraacetic acid (EDTA) was collected for the metabolomic analysis. The blood was centrifuged immediately after collection, plasma was harvested and within 1 hour of collection it was stored at −80°C until testing. Informed consent for collecting an additional blood sample was obtained from each cat's owner, but no ethics commission approval was required according to Polish legal regulations (the Act on the Protection of Animals Used for Scientific or Educational Purposes of 15 January 2015) because only routine diagnostic procedures essential given the clinical status of the cat were performed.

2.3 | Metabolomic analysis

The concentrations of metabolites, including symmetric dimethylarginine (SDMA), were determined by mass spectrometry using a commercial MxP Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria). The MxP Quant 500 kit can quantify 630 endogenous metabolites belonging to 26 biochemical classes (detailed list available from the manufacturer's website: https://biocrates.com/wp-content/uploads/2021/01/biocrates-Quant500-list-of-metabolites-v4-2021.pdf). Small molecules were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and lipids and hexoses by flow injection analysis-tandem mass spectrometry (FIA-MS/MS). Both were performed using a 5500 QTRAP instrument (AB Sciex, Darmstadt, Germany) with an electrospray ionization source. The experimental metabolomics measurement technique is described in detail by patents EP1897014B1 and EP1875401B1 (accessible online at https://patents.google.com/patent/EP1897014B1 and https://patents.google.com/patent/EP1875401B1). Briefly, a 96-well based sample preparation device was used to quantitatively analyze the metabolite profile in the samples. This device consisted of inserts that had been impregnated with internal standards, and a predefined sample amount was added to the inserts. Next, a phenyl isothiocyanate (PITC) solution was added to derive some of the analytes, and after the derivatization had been completed, the target analytes were extracted using an organic solvent, followed by a dilution step. The obtained extracts then were analyzed by LC-MS/MS or FIA-MS/MS method, using multiple reaction monitoring to detect the analytes. Data were quantified using appropriate mass spectrometry software (Sciex Analyst) and imported into Biocrates MetIDQ software (Biocrates Life Sciences AG, Innsbruck, Austria) for further analysis.

The analysis was performed in the laboratory of the Biocrates Life Sciences AG company in Innsbruck, Austria (Test report 5540 Sub2/2019) in December 2019. The micromolar concentrations of all metabolites were calculated (μmol/L) and normalized with respect to the internal quality control samples. For each metabolite, an individual limit of detection (LOD) was calculated and only concentrations above LOD were reported by the laboratory. Only metabolites that were above LOD in all 49 cats were included in the statistical analysis.

2.4 | Classification of metabolites

Metabolites belonged to 4 basic categories:

1. Amino acids and related compounds: proteinogenic amino acids, nonproteinogenic amino acids, biogenic amines, amino acid derivatives, indoles and derivatives (metabolites of tryptophan), p-cresol sulfate (metabolite of tyrosine and phenylalanine);
2. Lipids: simple lipids (diglycerides and triglycerides), acylcarnitines, sphingolipids (ceramides and dihydroceramides, glycosphingolipids [cerebrosides and globosides], and sphingomyelins), phosphatidylcholines (lyso-phosphatidylcholines, diacyl-phosphatidylcholines, and acyl-alkyl-phosphatidylcholines), free fatty acids, and cholesterol derivatives (cholesteryl esters, bile acids, and glucocorticosteroids);
3. Hexoses (monocarbohydrates);
4. Others: choline, trimethylamine N-oxide (metabolite of choline), xanthine and hypoxanthine (metabolites of purine nucleotide bases), trigonelline (alkaloid), and carboxylic acids.

The lipid side chain composition was abbreviated as "Cx:y," where "x" signified the total carbon number of 1 or both side chains and "y" the total number of double bonds. In diacylglyceroles (diglycerides) the number of carbon atoms (x and u) and double bonds (y and v) present in the fatty acid residues at *sn-1* and *sn-2* position, respectively, were denoted as "DG(x:y_u:v)." In triacylglycerols (triglycerides) the number of carbon atoms (x) and double bonds (y) present in the fatty acid residues at *sn-1* position and the total number of carbon atoms (n) and total number of double bonds (m) of the 2 fatty acid residues at sn-2 and sn-3 position were denoted as TG(x:y_n:m). The "_" sign indicated that the positions (*sn-1*/*sn-2*/*sn-3*) of the fatty acid residues were unknown. In acylcarnitines, the number of carbon atoms (x) and, if present, double bonds (y) in the fatty acid residue were denoted as "C x:y." The presence of a hydroxyl group or a (methyl-) dicarboxyl group was indicated by "OH" or "DC(-M)," respectively. In sphingolipids, the number of carbon atoms and double bonds present in the sphingosine or sphinganine (dihydrosphingosine) backbone (dx:y) and in the fatty acid residue (u:v) were denoted as "Cer(dx:y/u:v)," "HexCer(dx:y/u:v)," "Hex2Cer(dx:y/u:v)," or "Hex3Cer(dx:y/u:v)," with x and u indicating the number of carbon atoms and y and v the number of double bonds. Presence of a hydroxyl group was indicated by "OH." In phosphatidylcholines, the letter "a" indicated the

presence of an ester bond whereas the letter "e" indicated the presence of an ether bond in the glycerol moiety. Two letters "aa" (= diacyl) and "ae" (= acyl-alkyl) indicated the presence of 2 fatty acid residues at the *sn-1* and *sn-2* positions in the glycerol backbone, whereas a single letter "a" (=acyl) indicated the presence of a single fatty acid residue at the *sn-1* position in the glycerol backbone. On this basis phosphatidylcholines (PCs) were classified as diacyl-PCs (PC aa x:y; choline lecithins), acyl-alkyl-PCs (PC ae x:y; choline plasmalogens), and lyso-PCs (lyso-PC a x:y; choline lyso-lecithins). In cholesterol esters, the number of double bonds (y) and carbon atoms (x) present in the fatty acid residue were denoted as "CE(x:y)."

2.5 | Statistical analysis

Categorical variables were presented as counts in groups and percentages from the study population and compared among groups using the likelihood ratio test (*G* test). Numerical variables were expressed as the median, interquartile range (IQR) and range, and compared among groups using the Mann-Whitney *U* test. Their correlations were tested using Spearman's rank correlation coefficient (R_s). The 95% confidence interval (CI 95%) for R_s was calculated using the method of Bonnett and Wright.[36]

The number of cats to enroll and the power of correlation analysis were determined using the Fisher *z* transformation for the expected $R_s = .5$.[37]

The correlation between fPLI and metabolite concentration was analyzed in 2 steps. First, individual R_s was calculated for each metabolite and the Bonferroni correction for multiple comparisons was applied (corrected *P* value for R_s = *P* value for $R_s \times$ number of comparisons). Strength of correlation was classified as follows[38]: $R_s = .00$ to .19, very weak; .2 to .49, weak; .5 to .69, moderate; .7 to .89, strong; and .9 to 1, very strong.

If R_s was significant, linear regression was carried out with each metabolite and potential confounders as independent variables and fPLI as a dependent variable to clarify which of the metabolites was truly linked to fPLI. Before performing linear regression, normality of distribution of fPLI, metabolite concentration and potential confounders were evaluated using the Shapiro-Wilk W test, coefficient of skewness (CoS) with CI 95% and coefficient of shape (kurtosis) with CI 95%. The distribution was considered symmetrical when CI 95% for the CoS and kurtosis covered 0. Metabolites that violated at least 1 criterion of normality were transformed using the Box-Cox transformation[39] according to the formula:

$$y_T = \begin{cases} \frac{y^\lambda - 1}{\lambda} & \text{if } \lambda \neq 0 \\ \ln(y) & \text{if } \lambda = 0 \end{cases}$$

where *y* was the metabolite concentration, λ was an exponent parameter estimated using the profile likelihood function and took values from -5 to 5, ln was the natural logarithm, and y_T was the transformed metabolite concentration. Then, the normality of distribution of Box-Cox transformed variables was reevaluated using the same methods. Results of the normality analysis are presented in Table S3.

Metabolites significantly and independently correlated with fPLI then were evaluated in terms of their potential to distinguish between cats with normal (≤3.5 μg/L) and increased (>3.5 μg/L) fPLI by estimating the AUROC. The AUROCs were interpreted as follows: >90%, a highly accurate test; >80% to 90%, a moderately accurate test; >70% to 80%, a fairly accurate test; and ≤70%, a poorly accurate test.[40,41] The highest value of the Youden's index (J) was used to indicate the optimal cut-off at which diagnostic sensitivity (Se) and diagnostic specificity (Sp) were estimated.[42] All statistical tests were 2-tailed and the significance level (α) was set at .05. Statistical analysis was performed using TIBCO Statistica 13.3 (TIBCO Software Inc, Palo Alto, California).

3 | RESULTS

3.1 | Study population

Three of 52 plasma samples collected were disqualified because of improper storage (several freeze-thaw cycles). Therefore, the study population consisted of 49 neutered cats, 28 males (57%) and 21 females (43%), aged 1 to 18 years with a median (IQR) of 11 (9-13) years. Breeds included domestic shorthair (37 cats), Sphinx (4 cats), Siberian and Devon rex (2 each), and Maine coon, British shorthair, Russian, and Ragdoll (1 each).

Eleven cats (23%) were presented for routine prophylactic clinical and laboratory examination and had no clinical or laboratory signs of disease. Nine cats (18%) had been diagnosed with DM at least 1 month earlier and were presented for a routine evaluation during glargine insulin treatment (Lantus SoloStar, Sanofi-Aventis). These cats did not show any abnormal clinical signs and their fructosamine concentrations were <450 μmol/L and thus their DM was considered well-controlled. The following diagnoses were made in the remaining 29 cats (59%): FP (10 cats; 6 of them had concurrent DM diagnosed >1 month before), acute kidney injury (AKI; 5 cats), neoplasia (4 cats; liver neoplasia in 2 cats, pancreatic and adrenal gland neoplasia in 1 cat each), acute cholangiohepatitis (2 cats), undiagnosed acute general infection (2 cats), immune-mediated hemolytic anemia (IMHA), hyperthyroidism, aortic thromboembolism, high-rise syndrome (HRS), panleukopenia, and feline infectious peritonitis (1 each; Table S2).

3.2 | Pancreatic lipase measurement in serum

Serum fPLI ranged from 0.6 to 50 μg/L with a median (IQR) of 2.6 (1.3-6.8) μg/L. Serum fPLI was normal (≤3.5 μg/L) in 28 cats (57%) and > 5.3 μg/L in 18 cats (37%). Ten of the latter were suspected to have FP based on the presence of suggestive clinical signs and lack of other diagnoses explaining their clinical condition. Another 8 had the following concurrent acute diseases: AKI (4 cats), adrenal gland tumor and AKI (1 cat), IMHA and AKI (1 cat), HRS (1 cat), and panleukopenia (1 cat; Table S2).

TABLE 1 The accuracy of three sphingolipid metabolites significantly correlated with fPLI in distinguishing between cats with normal fPLI (≤3.5 μg/L) and increased fPLI (>5.3 μg/L)

Sphingolipid metabolite	Concentration (μmol/L)[a] in cats with normal fPLI (≤3.5 μg/L) (n = 28)	Concentration (μmol/L)[a] in cats with increased fPLI (>5.3 μg/L) (n = 18)	AUROC (CI 95%) (%)	*P*-value	Optimal cut-off (μmol/L)	Se (CI 95%) (%)	Sp (CI 95%) (%)	J (CI 95%) (%)
HexCer(d18:1/24:0)	0.36, 0.27-0.45 (0.19-0.86)	0.6, 0.45-0.76 (0.3-1.15)	82 (70-94)	<.001	0.45	78 (55-91)	75 (57-87)	53 (40-66)
HexCer(d18:1/24:1)	1.04, 0.87-1.29 (0.59-2.45)	1.77, 1.43-2.66 (0.89-4.47)	84 (72-96)	<.001	1.4	78 (55-91)	82 (64-92)	60 (48-72)
SM C18:0	28.7, 24.9-40.5 (15.1-70.8)	52.5, 32.7-66.8 (22.3-92.8)	78 (65-92)	<.001	51	56 (34-75)	93 (77-98)	48 (36-61)

Abbreviations: AUROC, area under receiver operating characteristic curve; CI 95%, 95% confidence interval; fPLI, feline pancreatic lipase immunoreactivity; HexCer, cerebroside; J, Youden's index; Se, diagnostic sensitivity; SM, sphingomyelins; Sp, diagnostic specificity.
[a]Presented as the median, interquartile range, and range.

Serum fPLI was not correlated with age (R_s = .14; CI 95%, −0.148 to 0.406; P = .34). No significant difference was found in fPLI between males (median, 3.6 μg/L; IQR, 1.4-7 μg/L; range, 0.8-50 μg/L) and females (median, 1.9 μg/L; IQR, 1.1-6.7 μg/L; range, 0.6-50 μg/L; P = .27) nor between diabetic (median, 3.6 μg/L; IQR, 1.4-7.8 μg/L; range, 1-50 μg/L) and nondiabetic (median, 2.3 μg/L; IQR, 1-6.8 μg/L; range, 0.6-23.8 μg/L; P = .2) cats.

Serum fPLI was significantly positively correlated with serum creatinine concentration (R_s = .295; CI 95%, 0.009-0.537; P = .04), plasma SDMA concentration (R_s = .379; CI 95%, 0.099-0.603; P = .007), serum ALT activity (R_s = .356; CI 95%, 0.074-0.585; P = .01), and serum glucose concentration (R_s = .296; CI 95%, 0.01-0.537; P = .04).

3.3 | Correlation between serum fPLI and metabolite concentration in plasma

In total, 407 of 630 metabolites (64.6%) were quantified in all cats (Table S4). Without Bonferroni correction, significant correlation with fPLI was observed for 67 of 407 metabolites (16.5%): 14 amino acids and derivatives, trimethylamine N-oxide, p-cresol sulfate, 5 acylcarnitines, 3 cholesterol esters, 9 ceramides, 12 cerebrosides, 10 globosides (5 dihexosylceramides and 5 trihexosylceramides), 4 sphingomyelins, 3 diacyl-phosphatidylcholines, 4 diglycerides, and hexoses. The lowest significant correlation was R_s = .28 for glycine betaine (N,N,N-trimethylglycine) and the highest was R_s = .58 for HexCer(d18:1/24:1). The correlation was weak (R_s from .2 to .39) for 44 metabolites and moderate (R_s from .4 to .59) for 23 metabolites (Table S4).

After application of the Bonferroni correction only 5 sphingolipids were significantly positively correlated with fPLI, namely 4 cerebrosides containing the following long-chain fatty acids: stearic acid: HexCer(d18:1/18:0) (R_s = .526; CI 95%, 0.27-0.713; P < .001), arachidic acid: HexCer(d18:1/20:0) (R_s = .536; CI 95%, 0.282-0.72; P < .001), lignoceric acid: HexCer(d18:1/24:0) (R_s = .556; CI 95%, 0.306-0.734; P < .001), nervonic acid: HexCer(d18:1/24:1) (R_s = .58; CI 95%, 0.336-0.751; P < .001), and 1 sphingomyelin: SM C18:0 (R_s = .553; CI 95%, 0.303-0.732; P < .001).

No correlation was observed among the 5 sphingolipids and serum creatinine concentration. On the other hand, all 5 sphingolipids were significantly correlated with SDMA, ALT and glucose (Table S5). Because SDMA, ALT and glucose in turn were significantly correlated with fPLI, they were considered potential confounders. When controlled for these 3 potential confounders in the multivariable linear regression, 3 of 5 sphingolipids remained significantly linked to fPLI: HexCer(d18:1/24:0); (β = .424; CI 95%, 0.096-0.752; P = .01), HexCer(d18:1/24:1); (β = .403; CI 95%, 0.067-0.739; P = .02), and SM C18:0 (β = .362; CI 95%, 0.005-0.72; P = .05; Table S6).

3.4 | Accuracy of plasma metabolites in discriminating between cats with normal and increased fPLI

Twenty-eight cats with normal fPLI and 18 cats with increased fPLI (10 with suspected FP and 8 with other acute conditions) were used in the analysis. The 2 groups did not differ significantly in terms of age (median of $10^{9\text{-}13}$ years and $11^{9\text{-}14}$ years, respectively; P = .67) and sex distribution (14 [50%] males and 11 [61%] males, respectively; P = .46). The concentrations of HexCer(d18:1/24:0), HexCer (d18:1/24:1), and SM C18:0 were significantly higher in cats with increased fPLI (Table 1). Their potential to distinguish between cats with normal and increased fPLI was fair to moderate with the lower confidence limit of the AUROC <70% for HexCer(d18:1/24:0) and SM C18:0. Only for HexCer(d18:1/24:1) did the entire CI 95% of the AUROC fall above 70% (Table 1), which indicated that the accuracy could be truly considered as moderate.

4 | DISCUSSION

We evaluated the relationship between a panel of >400 various metabolites in plasma and serum fPLI in 49 cats. We identified 3 metabolites, all belonging to a class of sphingolipids (2 cerebrosides and 1 sphingomyelin), that were significantly positively correlated with fPLI when controlled for glomerular filtration rate (assessed by plasma SDMA concentration), liver damage (assessed by serum ALT activity), and serum glucose concentration. The strength of the correlations was moderate. The 3 sphingolipids proved to have only fair to moderate potential to discriminate between cats with normal and increased fPLI.

Sphingomyelin SM C18:0 is composed of palmitate-derived ceramide (d18:1), with a stearic acid chain (saturated fatty acid built of 18 carbon atoms) and 1 polar phosphocholine head group. Two cerebrosides, HexCer(d18:1/24:0) and HexCer(d18:1/24:1), are composed of palmitate-derived ceramide (d18:1), with a 24-carbon atom fatty acid chain (saturated lignoceric acid and ω-9 monounsaturated nervonic acid, respectively), and a single sugar group linked to ceramide.[43] Cerebrosides may contain galactose or glucose. Galactocerebrosides (GalCer) are found predominantly in neuronal cell membranes whereas glucocerebrosides (GlcCer) rarely occur in cell membranes.[43] They are, however, typically intermediates in the synthesis or degradation of more complex glycosphingolipids such as sulfatides (3-sulfate esters of GalCer), globosides (dihexosylceramides and trihexosylceramides) and gangliosides (globosides containing N-acetylneuraminic acid [sialic acid; NANA]). Sphingolipids are elements of outer cell membranes participating in intercellular recognition (cell-cell interactions) and binding of different ligands.[44,45] The untargeted metabolomic analysis used in our study could not distinguish between GalCer and GlcCer, nor was it able to identify gangliosides. In our opinion, cerebrosides identified in our study were most likely GluCer and semiproducts of monosialoganglioside (GM) or disialoganglioside (GD) synthesis or degeneration because various GM and GD have been shown to be abundant in both the exocrine and endocrine pancreas[46,47] and their concentrations have been shown to be increased in people with pancreatic adenocarcinoma.[48] However, data on sphingolipid concentrations in people and animals with pancreatic disorders are scarce and inconsistent. Plasma concentrations of nervonic acid(C24:1)-based ceramide and cerebroside were shown to be markedly increased in people with both pancreatitis and pancreatic carcinoma.[49] HexCer(d18:1/24:1) concentration was highest in people with pancreatitis and it was significantly higher even than in patients with pancreatic cancer.[49] On the other hand, concentrations of sphingolipids were decreased rather than increased in 2 other studies in people[50] and rats[51] with pancreatitis. In our study, the concentrations of all species of sphingolipids containing nervonic acid (ie, Cer(d18:1/24:1), HexCer(d18:1/24:1), Hex2Cer (d18:1/24:1), and Hex3Cer(d18:1/24:1)) were significantly positively correlated with fPLI in the uncorrected univariable analysis, and were excluded from the multivariable analysis only because of the very stringent criteria for statistical significance that we applied.

We decided to use such strict criteria to minimize the risk of a family-wise error, which exceeds 99% when 407 comparisons are made at a significance level of .05.[52] Given that we carried out an untargeted metabolomic analysis without any initial criteria for metabolite selection and most metabolites were mutually correlated because they often constituted subsequent products of sequential chemical reactions, the risk of coincidental correlation was even higher. Cats with FP are likely to suffer from kidney failure, hepatic damage, and disrupted glucose metabolism.[4,5,7,8,53] In our study, fPLI was significantly positively correlated with SDMA (considered a sensitive indicator of an impaired glomerular filtration rate[54,55]), ALT (considered a sensitive indicator of liver damage[56]), and glucose concentration. Therefore, we decided to include these measurements in the multivariable analysis to narrow down the spectrum of possibly useful metabolites to only those with an independent link to fPLI that would raise no doubts.

Recent studies in people and laboratory animals have identified significant changes in various metabolites in the course of pancreatitis.[57-61] The metabolites significantly affected by the disease were mainly energy-related sugars, ketones, organic acids, amino acids, lipids, and purines. Nevertheless, large discrepancies between results occurred in these studies. These discrepancies could be related to the different panels of metabolites quantified as well as to different etiological and pathogenetic types of pancreatitis investigated in these studies. Given that, contrary to what is observed in human medicine, most FP cases are idiopathic,[3] we did not investigate the etiology of suspected FP in our study. Neither were histopathologic types of FP determined in our study because pancreatic samples for histopathology were not available. As a consequence, cats enrolled in our study were likely to represent various types, stages and degrees of severity of pancreatic inflammation, often accompanied by other conditions. To overcome this problem, we based our analysis on the correlation between metabolites and fPLI, which is considered the most accurate proxy of pancreatic damage so far available in veterinary medicine.[62] We assumed that metabolites highly correlated with fPLI would be good candidates for potential alternative biomarkers of FP.

In light of this assumption, the results of our study are disappointing. When controlled for family-wise error and confounding factors, only 3 metabolites were significantly correlated with fPLI. Even more important is the fact that the strongest correlation observed was only moderate ($R_s = .58$) whereas the Spearman's correlation between fPLI and DGGR lipase, considered as an alternative laboratory diagnostic modality in FP, has been shown to be as high as 0.8.[19] None of the metabolites included in our analysis were biochemically related to pancreatic lipase activity, neither as substrates or products of the reaction catalyzed by pancreatic lipase nor as cofactors of this enzyme. Therefore, we could not expect as strong a correlation as for the assays proposed to measure the same chemical compound (ie, pancreatic lipase). However, the only moderate strength of correlation indicates that the metabolites are unlikely to constitute an alternative for fPLI in FP diagnosis. To complement the analysis based on correlations, we evaluated the accuracy of the 3 sphingolipids in discriminating between cats with normal (≤3.5 μg/L) and increased (>5.3 μg/L) fPLI concentration. Although little has been published to support the manufacturer's fPLI interpretation ranges, these cut-offs are commonly used in veterinary practice. Our analysis shows that the discriminatory potential of the 3 sphingolipids is fair or moderate at best. Only fair to moderate association with fPLI does not necessarily correspond to low accuracy of the sphingolipids in diagnosing FP because fPLI itself is not completely accurate. However, in our opinion it is very unlikely that measuring HexCer(d18:1/24:0), HexCer(d18:1/24:1) or SM C18:0 concentrations will play any important role in FP diagnosis in the foreseeable future, for at least 2 reasons. First, any strong theoretical background for the

physiological link between the concentration of these particular sphingolipids and FP is lacking. Sphingolipids are distributed throughout the body and thus their increase is more likely to reflect damage to any tissues and organs, rather than being specific for the pancreas. Although we applied strict statistical criteria to avoid coincidental findings, our study remains an untargeted metabolomic investigation, which is more likely to generate scientific hypotheses than to provide definitive answers. Further physiological studies are needed to confirm the role of sphingolipids in FP and make our results clinically applicable. Second, highly sophisticated and expensive methods such as FIA-MS/MS are still necessary to quantify sphingolipids. Moreover, blood cells must be separated immediately after collection and plasma must be frozen at −80°C and transported or stored under such conditions until testing. All of these factors make sphingolipid quantification very inconvenient from a practical standpoint.

Two main limitations to the design of our study are low sample size and lack of definitive confirmation or exclusion of FP. The number of cats enrolled was restricted by the limited financial resources of the study and the high cost of metabolomic analyses. Although the study population size was sufficient to ensure the high power of individual correlation analyses, the power of final analysis was markedly decreased by the use of Bonferroni correction. At $\alpha = .000123$ (0.05/407 comparisons) the power of correlation analysis assuming $R_s = .5$ is only 48% compared to 95% at $\alpha = .05$. Nevertheless, we believe that avoiding falsely significant findings should have priority in untargeted metabolomic analyses. Lack of definitive diagnosis of FP was caused by the fact that plasma samples could only be collected when we were able to centrifuge plasma and place it at −80°C within 1 hour of blood collection. Because the cats were patients presented to private veterinary clinics and the −80°C freezer was located at the university several kilometers away, we could only enroll patients when all operational conditions were satisfied. Unfortunately, cats with pancreatitis with owners willing to finance all of the diagnostic tests necessary to make a definitive diagnosis of FP and also who would agree to participate in the study could not arrive precisely when needed. Therefore, we had to carry out our study on a convenience population and hence restrict our study only to correlation analysis. Regardless, our study provides useful information for further investigation of the pathogenesis and diagnosis of FP using metabolomics.

In conclusion, 2 cerebrosides (HexCer(d18:1/24:0), HexCer (d18:1/24:1)) and 1 sphingomyelin (SM C18:0) are moderately positively correlated with fPLI and appear to have fair to moderate diagnostic accuracy in discriminating between cats with normal and increased fPLI.

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CONFLICT OF INTEREST DECLARATION

Authors declare no 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

An informed consent for collecting an additional blood sample was obtained from each cat's owner, however no ethics commission approval was required according to the Polish legal regulations (the Act on the Protection of Animals Used for Scientific or Educational Purposes of 15 January 2015) as only routine diagnostic procedures essential given the clinical status of the cat were performed.

HUMAN ETHICS APPROVAL DECLARATION

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

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