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STANDARD ARTICLE

Association of serum calprotectin (S100A8/A9) concentrations and idiopathic hyperlipidemia in Miniature Schnauzers

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Background: Idiopathic hyperlipidemia (IH) is a common condition in Miniature Schnauzers (MS). Studies in people have linked IH to low-grade inflammation, which plays an important role in the pathogenesis of IH complications. The role of inflammation in MS with IH is unknown.

Objective: Evaluation of the inflammatory markers serum calprotectin and S100A12 in MS with IH and in response to dietary intervention for IH management.

Animals: One-hundred fifty clinically healthy MS.

Methods: Serum triglyceride, cholesterol, calprotectin, and S100A12 concentrations were measured before and after placing the dogs on an ultra-low fat diet.

Results: Hypertriglyceridemia (HTGL, $P < .001$) and hypercholesterolemia (HCHOL, $P = .01$) were independently associated with increased serum calprotectin but not S100A12 concentrations. Compared to normolipidemic MS, serum calprotectin concentrations were significantly higher in MS with HTGL ($P < .001$) or combined hyperlipidemia ($P = .02$), but not those with isolated HCHOL ($P = 1.0000$). Presence ($P = .005$) and severity ($P = .003$) of HTGL and serum cholesterol concentrations ($P = .04$) decreased in MS with IH within 14-26 weeks after being placed on the ultra-low fat diet, but neither serum calprotectin nor S100A12 concentrations changed significantly with this dietary intervention.

Conclusions and clinical importance: Subclinical (low-grade) inflammation appears to be present in some MS with IH, and an ultra-low fat diet does not decrease serum concentrations of inflammatory proteins in those dogs. Whether this presumed inflammatory phenotype in MS with IH is associated with the development of IH complications (eg, insulin resistance) requires further research.

KEYWORDS

calgranulin, dog, hypertriglyceridemia, inflammation, S100A12

1 | INTRODUCTION

Primary hyperlipidemia is a condition characterized by persistent fasting hyperlipidemia without evidence of underlying pathology.[1,2] In dogs, primary (idiopathic) hyperlipidemia often is associated with specific breeds,[1–5] and affected patients are often asymptomatic.[1,2] Although mixed fasting hypertriglyceridemia (HTGL) and hypercholesterolemia (HCHOL) can occur, a predominance of HTGL appears to exist in the Miniature Schnauzer (MS) breed[1,2] and primarily a HCHOL occurs in the Shetland Sheepdog,[3] Rough Collie,[4] and Briard[5] breeds.

Idiopathic hyperlipidemia (IH) is a common condition in MS,[2] with >75% of dogs ≥9 years old being affected and >40% of these dogs having moderate or severe fasting HTGL with or without concurrent HCHOL.[1,2] Severe HTGL in the MS has been suggested to pose an increased risk for the development of several conditions, including pancreatitis,[6,7] insulin resistance,[8] hepatobiliary disease (eg, gallbladder mucocele, vacuolar hepatopathy),[9,10] glomerular injury,[11,12] ocular

Abbreviations: BCS, body condition score; BMI, body mass index; CRP, C-reactive protein; CV, coefficient of variation; HCHOL, hypercholesterolemia; hsCRP, high-sensitivity CRP; HTGL, hypertriglyceridemia; IH, idiopathic hyperlipidemia; IL, interleukin; IQR, interquartile ranges; MS, Miniature Schnauzer; NF-κB, nuclear factor "kappa-light-chain-enhancer" of activated B cells; OR, odds ratio; sCD40L, soluble CD40 ligand; sICAM, soluble intercellular adhesion molecule; Spec cPL, specific canine pancreatic lipase concentration; TNF-α, tumor-necrosis factor alpha.

disease (eg, lipid keratopathy),[13] and neurologic conditions (eg, seizures).[14] Treatment options for preventing possible complications in dogs with IH include dietary modification with an ultra-low fat (ie, a diet with <20 g fat/1000 kcal).[1,15] Lipid-lowering medical treatment options include ω-3 fatty acid supplements (eg, Menhaden fish oil), fibrates (eg, fenofibrate or gemfibrozil), or vitamin B_3 (niacin), but very few reports of their use in dogs are available and use of these approaches is mostly empirical.[1,16,17]

Recent studies in people suggest that IH is associated with a low-grade inflammatory reaction and endothelial dysfunction, which are suspected to play a role in the pathogenesis of secondary conditions associated with IH.[18,19] The risk of such complications (eg, cardiovascular disease) also has been shown to decrease with medical control of IH in people.[20] Inflammatory mediators that have been shown to be altered in people with IH include C-reactive protein (CRP), interleukin 1 beta, tumor-necrosis factor alpha (TNF-α), and nuclear factor "kappa-light-chain-enhancer" of activated B cells (NF-κB).[21,22]

In MS with IH, the role of systemic inflammation has not been evaluated to date. Biomarkers of inflammation that are available in dogs include the positive type II acute reactant CRP,[23,24] the S100A8/A9 protein (calprotectin) complex,[25] and S100A12[26] as markers of mononuclear or mixed type inflammation, bromotyrosine as a marker of eosinophilic inflammation,[27] and N-methylhistamine as a marker of mast cell degranulation.[28] The calprotectin (S100A8/A9) complex has been linked to the risk of cardiovascular complications in people with dyslipidemia.[29,30] Furthermore, macrophage-mediated pancreatic beta-cell dysfunction because of increased fatty acid and glucose concentrations (glucolipotoxicity) has been shown to be associated with activation of the S100A8/Toll-like receptor-4 pathway and has been proposed to play an important role in systemic inflammation in people.[30] Determining the role of the S100/calgranulins (S100A8/A9 and S100A12) in the pathophysiology of spontaneous IH in dogs also will serve as a good model for human IH, because homologs of all 3 calgranulins are expressed in dogs, whereas rodents lack S100A12,[31] with S100A8 appearing to resemble S100A12 functionally.[32,33]

Given that biomarkers of inflammation have not yet been investigated in MS with IH, the aims of our prospective, observational clinical case-control study were to evaluate serum calprotectin (S100A8/A9) and S100A12 concentrations in healthy MS, MS with IH, and MS with IH after switching to an ultra-low fat diet. We hypothesized (1) that serum calprotectin or S100A12 concentrations or both are increased in MS with IH compared to healthy normolipidemic MS and (2) that serum calprotectin or S100A12 concentrations or both in MS with IH decrease with dietary intervention (ie, switching the dogs to an ultra-low fat diet). In addition, the possibilities of an effect of hyperlipidemia on serum calprotectin concentrations and an association with increased serum canine-specific pancreatic lipase concentrations (as a biochemical marker for pancreatitis) were evaluated.

2 | MATERIALS AND METHODS

2.1 | Lipidemia interference testing

Previously, we have shown that hyperlipidemia does not appear to interfere with the measurement of S100A12 concentrations in serum samples, but centrifugation to remove excess lipids from serum was shown to result in lower S100A12 concentrations.[25]

The possibility of hyperlipidemia interfering with the measurement of calprotectin in canine serum specimens was further evaluated by spiking 7 different surplus canine serum samples (with low, moderate, and high calprotectin concentrations) with known concentrations (0, 100, 250, 500, 750, 1100, 1250, 1500, 1750, 2000, and 3000 mg/dL) of a commercially available IV lipid formulation (Liposyn II 20% Intravenous lipid emulsion, Hospira, Lake Forest, Illinois) followed by measurement of calprotectin concentrations in all samples.

2.2 | Ethics approval

The study of several serum analytes in MS with or without IH was approved by the Clinical Research Review Committee at Texas A&M University (TAMU-CRRC# 2008-37). The owner of each dog enrolled in the study gave written consent before inclusion of the dog. Based on serum calprotectin (or S100A12) concentrations in a previous reference group,[25,26] a sample size of at least 50 (or 26) dogs per group (dogs with or without IH) was calculated to be required to detect an increase in serum calprotectin concentrations of at least 25% with statistical power of >80% ($\alpha = 0.05$, $\beta = 0.2$).[34]

2.3 | Sampling population

Serum samples, after withholding food for at least 12 hours, were collected by venipuncture from 162 MS that were considered for inclusion in the study (Figure 1). Dogs were included if they were clinically healthy. A study questionnaire was completed for each dog by the owner or primary care veterinarian or both to confirm health status, present or previous medical conditions, current diet, and medication history. Routine diagnostic screening (including CBC, serum biochemistry profile with baseline cortisol concentration, urinalysis, thyroid profile [total thyroxine (T4), canine thyroid-stimulating hormone (cTSH), free T4], specific canine pancreatic lipase concentration [Spec cPL], and, if indicated, serum fructosamine concentration) was performed to evaluate dogs for the possibility of being hyperlipidemic secondary to other conditions such as hypothyroidism, diabetes mellitus, or hyperadrenocorticism.[1] Twelve of the dogs were excluded based on suspicion of secondary hyperlipidemia, before corticosteroid administration, or insufficient sample material, thus leaving 150 clinically healthy MS (71 dogs with IH and 79 normolipidemic dogs) enrolled in the study.

Paired baseline serum samples were obtained from 17 of the clinically healthy MS with HTGL 3-14 weeks (median, 5 weeks) apart, and an owner questionnaire was completed for each dog at each sample collection. These 17 dogs then were placed on a commercially available ultra-low fat diet (Royal Canin Gastrointestinal Low Fat dry [18.6 g fat/1000 kcal], Royal Canin USA, St. Charles, Missouri) without any additional lipid-lowering medications (and only low-fat treats such as carrots or broccoli were allowed), followed by the collection of at least 1 follow-up serum sample 14-26 weeks (median, 18 weeks) after the diet change. None of these 17 dogs had received a fat-restricted diet at the time baseline samples were obtained.

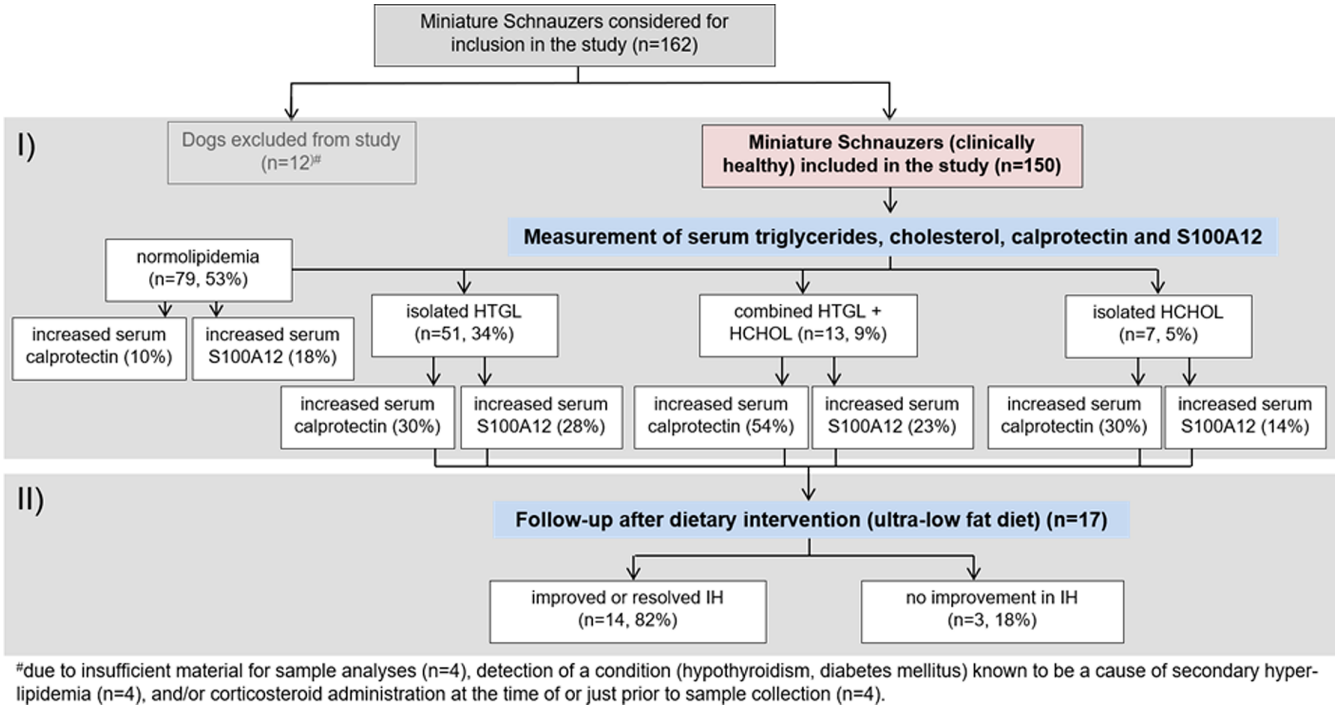

FIGURE 1 Study design. Flow chart summarizing the number of Miniature Schnauzers (MS) included in the 2 different parts of the study HCHOL, hypercholesterolemia; HTGL, hypertriglyceridemia; IH, idiopathic hyperlipidemia

2.4 | Sample analysis

Serum triglyceride and cholesterol concentrations were measured in all serum samples by means of analytically validated enzymatic assays (Roche/Hitachi Modular Analytics D2400 module, Roche Diagnostics, Indianapolis, Indiana). Reference intervals (RIs) for serum triglyceride and cholesterol concentrations were 26-108 and 124-335 mg/dL, respectively.

The same serum samples also were used to quantify serum calprotectin and S100A12 concentrations by use of established and validated species-specific in-house radioimmunoassays.[25,35] The RIs used for serum calprotectin and S100A12 concentrations were 72-234 and 33-225 μg/L, respectively.[25,26] In addition, serum-specific pancreatic lipase concentrations were measured in all serum samples (RI, 0-200 μg/L).[36]

2.5 | Data analysis

Commercially available statistical software packages (JMP v13.0, SAS Institute, Cary, North Carolina; GraphPad Prism v7.0, GraphPad Software, San Diego, California) were used for all statistical analyses. The assumptions of normality and equal variances of the data were tested using a Shapiro-Wilk W test and a Brown-Forsythe test, respectively. Summary statistics for continuous variables are reported as medians and interquartile ranges (IQR). Categorical data are presented as counts (*n*) and percentages.

Nonparametric group comparisons of continuous variables were performed using a Wilcoxon rank-sum test (2-group comparison of unpaired data), a Wilcoxon signed-rank test (2-group comparison of paired data), or a Kruskal-Wallis (multiple group comparison of unpaired data) or Friedman test (multiple group comparison of paired data) with a Dunn's post hoc test for joint ranking. A Fisher's exact or likelihood ratio test (as appropriate) with calculation of the odds ratio (OR) and 95% confidence interval (95% CI) was used to test the possibility of an association between categorical variables.

Multivariate logistic regression models with a stepwise forward model selection using Akaike's information criterion with a Wald Chi-square statistic for each effect in the model were constructed to evaluate the effect of selected (and if necessary $\log_{10}$-transformed) variables (where $P < .2$ in univariate analyses) on hyperlipidemia or increased serum calprotectin concentrations.

Statistical significance was set at $P < .05$, and Holm's sequential Bonferroni correction[37] was applied for multiple comparisons of paired data, with consideration of the numbers of categories (P_{corr} = unadjusted $P \times [n - k + 1]$, where n is the number of hypotheses tested and k is the ordered rank of the uncorrected P values).

3 | RESULTS

3.1 | Interference of lipidemia with calprotectin measurement

Compared to unspiked samples, serum calprotectin concentrations were significantly altered when samples were spiked with IV lipid emulsion at concentrations of 1250, 1500, 1750, 2000, and 3000 mg/dL (Figure 2). The total coefficient of variation (CV) for all spiked solutions from each sample ranged from 3.0 to 17.8% (mean, 10.6%).

3.2 | Study population

3.2.1 | All dogs included in the study (n = 150)

A total of 150 clinically healthy MS (median age, 7.3 years; IQR, 4.4-9.6 years; 56 males/90 females, sex not documented for 4 dogs)

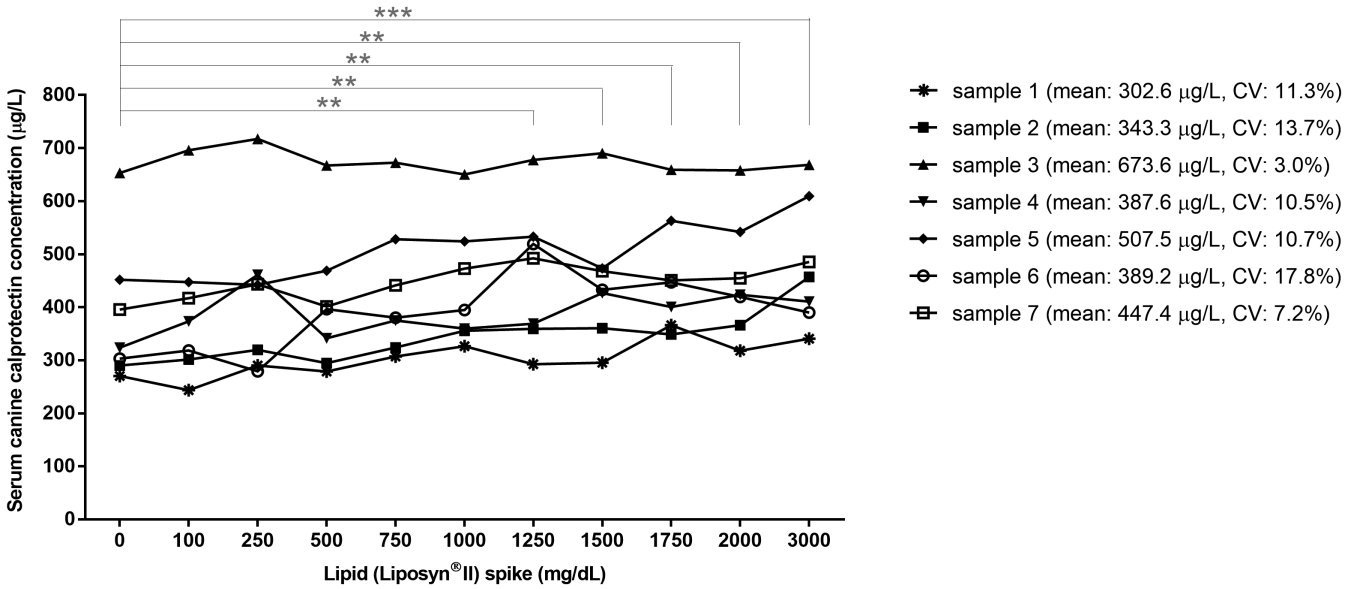

FIGURE 2 Evaluation of lipemia interference. Spiking of sera (n = 7) with increasing concentrations of lipid components significantly affected serum calprotectin measurements with spiked lipid concentrations ≥1250 mg/dL, but the overall coefficient of variation (CV) for each sample was 3.0-17.8% (mean 10.6%)

were included in the study (Figure 1; Table 1). Routine diagnostic screening together with the completed study questionnaires did not indicate any underlying conditions or administration of medications that could affect serum lipid or calgranulin concentrations in any of the dogs. Median body weight was 7.7 kg (IQR, 6.8-9.0 kg) and median body condition score (BCS) was 5/9 (IQR, 4/9-5/9). Results of thyroid profiles (serum total T4, cTSH, and free T4), serum Spec cPL, and serum cortisol concentrations are included in Tables 1 and 2. No significant differences in serum total T4, cTSH, free T4, serum Spec cPL, or serum cortisol concentrations were found between dogs with IH and normolipidemic dogs (Table 1), nor were any differences in these variables found among dogs with HTGL, HCHOL, and combined hyperlipidemia (Table 2).

Serum triglyceride concentrations ranged from 22 to 5077 mg/dL (IQR, 54-237 mg/dL) in all 150 dogs, with HTGL detected in 64 MS (43%). Hypertriglyceridemia was graded as mild (108-400 mg/dL[8]) in 40 of these 64 dogs (63%), moderate (400-900 mg/dL[8]) in 13 of the dogs (20%), and severe (>900 mg/dL[8]) in 11 dogs (17%). Serum cholesterol concentrations ranged from 120 to 783 mg/dL (IQR, 183-295 mg/dL) in all 150 dogs, with HCHOL detected in 20 dogs (13%).

Miniature Schnauzers with IH were significantly older (median, 8.8 years; IQR, 7.0-10.4 years) than normolipidemic MS (median, 5.4 years; IQR, 3.0-7.5 years; $P_{multivariate}$ < .001; Table 2).

3.2.2 | Dogs with IH included in the study (n = 71)

A total of 51 (72%), 7 (10%), and 13 MS (18%) had isolated HTGL, HCHOL, and combined hyperlipidemia, respectively (Figure 1; Table 2). Body weight and BCS did not differ among MS with isolated HTGL, HCHOL, and combined hyperlipidemia ($P_{univariate}$ = .78 and $P_{univariate}$ = .23, respectively), but dogs with HTGL were significantly older than those with isolated HCHOL ($P_{multivariate}$ = .003; Table 2).

3.3 | Serum calprotectin and S100A12 concentrations in MS with IH

Serum calprotectin concentrations were significantly higher in MS with IH ($P_{multivariate}$ < .001) compared to normolipidemic dogs, whereas the difference in S100A12 concentrations between those 2 groups of dogs was significant only during univariate analysis ($P_{univariate}$ = .04; Table 1, Figure 3). Idiopathic hyperlipidemia was significantly associated with a higher percentage of serum calprotectin concentrations above the RI (P < .001; OR, 4.5; 95% CI, 1.9-10.9), but not with increased serum S100A12 concentration (P = .25; Table 1, Figure 4).

No significant difference was found in serum calprotectin or serum S100A12 concentrations among MS with isolated HTGL, HCHOL, or combined hyperlipidemia ($P_{univariate}$ = .52 and $P_{univariate}$ = .52, respectively; Table 2, Figure 3). Also, no significant differences in serum calprotectin or S100A12 concentrations were found in dogs with severe HTGL compared to dogs with mild or moderate HTGL (calprotectin: median [IQR] = 267 [163-570] µg/L vs 204 [169-245] µg/L; P = .46; S100A12: 154 [97-244] µg/L vs 140 [115-245] µg/L; P = .99). Neither age (median [IQR] = 7.3 [4.8-10.4] years vs 7.2 [4.1-9.5] years; P = .43), body weight (median [IQR] = 8.1 [6.8-9.5] kg vs 7.7 [6.9-9.0] kg; P = .97), BCS (median [IQR] = 5 [4, 5] of 9 vs 5 [4, 5] of 9; P = .33) or BCS category (>5 of 9 = 16% vs 20%; P = .78) nor serum Spec cPL concentrations (median [IQR] = 38 [29-93] µg/L vs 33 [29-100] µg/L; P = .61) differed between MS with increased serum calprotectin concentrations and those dogs with serum calprotectin concentration within RI.

Univariate analysis identified a significant independent association of increased serum calprotectin concentrations with HTGL (P < .001; OR, 4.0; 95% CI, 1.7-9.2) and HCHOL (P = .01; OR, 3.7; 95% CI, 1.4-10.0), and both HTGL (P = .003) and HCHOL (P = .04) remained significant in multivariate analysis. However, neither of these 2 variables

TABLE 1 Characteristics of all clinically healthy Miniature Schnauzers (MS) included in the study (n = 150)

Group characteristic	Hyperlipidemia (IH)[a]	Normolipidemia	$P_{univariate}$[b]	$P_{multivariate}$[c]
Total number, *n* (%)	71 (47%)	79 (53%)	–	–
Patient characteristics				
Age in years, median (IQR)	8.8 (7.0-10.4)	5.4 (3.0-7.5)	**<.001**	**<.001**
Sex, male/female[d]	26/42	30/48	.98	–
Body weight in kg, median (IQR)	8.2 (7.1-10.5)	7.5 (6.8-8.4)	**.02**	.07
BCS, median (IQR)[e]	5 (4-5)	5 (4-5)	.96	–
BCS category, *n* (%)[e]				
≤5	50 (70%)	62 (79%)	.50	–
>5	14 (20%)	13 (17%)		
Clinicopathologic parameters				
Serum triglyceride in mg/dL, median (IQR)	245 (158-525)	57 (44-74)	**<.001**	–
Serum cholesterol in mg/dL, median (IQR)	286 (235-359)	198 (160-246)	**<.001**	–
Serum total T4 in μg/dL, median (IQR)	2.3 (1.5-2.7)	2.6 (1.9-3.2)	**.02**	–
Serum cTSH in ng/mL, median (IQR)	0.3 (0.2-0.6)	0.2 (0.1-0.3)	**<.001**	.19
Serum free T4 in ng/dL, median (IQR)	1.8 (1.2-2.3)	2.0 (1.3-2.6)	.09	–
Serum Spec cPL in μg/L, median (IQR)[f]	63 (29-188)	29 (29-47)	**<.001**	.10
Serum Spec cPL concentration[f]				
<200 μg/L, *n* (%)	51 (76%)	73 (92%)	**.01**	–
200-400 μg/L, *n* (%)	7 (11%)	4 (5%)		
>400 μg/L, *n* (%)	9 (13%)	2 (3%)		
Serum cortisol in μg/dL, median (IQR)	3.1 (2.0-4.2)	2.9 (1.6-4.9)	.65	–
Biomarkers of inflammation				
Serum calprotectin in μg/L, median (IQR)[g]	201 (168-256)	165 (120-200)	**<.001**	**<.001**
Serum calprotectin concentration[g]				
within RI[h], *n* (%)	46 (66%)	69 (90%)	**<.001**	–
above RI[i], *n* (%)	24 (34%)	8 (10%)		
Serum S100A12 in μg/L, median (IQR)	145 (106-228)	124 (75-193)	**.04**	.26
Serum S100A12 concentration				
within RI[j], *n* (%)	53 (75%)	65 (82%)	.25	–
above RI[k], *n* (%)	18 (25%)	14 (18%)		

Abbreviations: BCS, body condition score (range of possible scores: 1-9); IQR, interquartile range; RI, reference interval.
[a]Defined as hypercholesterolemia (HCHOL) and/or hypertriglyceridemia (HTGL).
[b]Significant difference between (or association with) hyperlipidemia and normolipidemia in univariate analysis.
[c]Statistical significance in a multiple stepwise logistic regression model. Bold face values indicate statistical significance at $P < .05$.
[d]Documented in n = 146 dogs.
[e]Documented in n = 139 dogs.
[f]Determined in n = 146 dogs.
[g]Determined in n = 147 dogs.
[h]Defined as 72-234 μg/L.
[i]Defined as >234 μg/L.
[j]Defined as 33-225 μg/L.
[k]Defined as >225 μg/L.

was significantly associated with increased serum S100A12 concentration (P = .18; OR, 1.7; 95% CI, 0.8-3.8; and P = .87, OR, 0.9; 95% CI, 0.3-2.9, respectively).

3.4 | Serum calprotectin and S100A12 concentrations after dietary intervention for IH

Of the 17 MS with IH included in this part of the study (Table 3), 5 dogs (29%) showed mild HTGL, 9 (53%) had moderate HTGL, and 3 dogs (18%) had marked HTGL; HCHOL was detected in 10 dogs (59%). After being placed on an ultra-low fat diet for 14-26 weeks (median, 18 weeks), 8 dogs (47%) had mild HTGL, 4 dogs (24%) had moderate HTGL, and 5 dogs (29%) were normolipidemic; 3 dogs (18%) were hypercholesterolemic (1 dog with mild HTGL and 2 dogs with moderate HTGL). The presence and severity of HTGL decreased in MS with IH within 14-26 weeks after feeding an ultra-low fat diet (P_{corr} = .03 and P = .003). Dietary intervention also yielded a significant decrease in serum cholesterol concentrations (P_{corr} = .02; Table 3).

Neither serum calprotectin nor serum S100A12 concentrations changed significantly after dogs were placed on the ultra-low fat diet, nor did the proportion of dogs with increased serum calprotectin, S100A12, or both concentrations change (all P_{corr} > .05; Table 3).

Serum Spec cPL concentrations and the proportion of dogs with increased serum Spec cPL concentration also were unchanged after feeding the ultra-low fat diet (all P_{corr} > .05; Table 3).

TABLE 2 Characteristics of all clinically healthy hyperlipidemic MS in the study (n = 71)

Group characteristic	HTGL	HGTL + HCHOL	HCHOL	$P_{univariate}$[a]	$P_{mutlivariate}$[b]
Total number, n (%)	51 (72%)	13 (18%)	7 (10%)	–	–
Patient characteristics					
Age in years, median (IQR)	9.6 (7.5-10.9)[A]	7.7 (7.0-9.9)[A,B]	4.5 (2.5-7.6)[B]	**.006**	**.003**
Sex, male/female[c]	16/33	6/6	4/3	.31	–
Body weight in kg, median (IQR)	8.0 (7.0-10.6)	8.8 (7.5-9.5)	7.3 (6.4-16.6)	.78	–
BCS, median (IQR)[d]	5 (4-5)	4 (4-5)	5 (4-6)	.23	–
BCS category, n (%)[d]					
≤5	35 (76%)	10 (83%)	5 (83%)	.81	–
>5	11 (24%)	2 (17%)	1 (17%)		
Clinicopathologic parameters					
Serum triglyceride in mg/dL, median (IQR)	277 (188-463)[A]	515 (221-1260)[A]	49 (33-79)[B]	**<.001**	–
Serum cholesterol in mg/dL, median (IQR)	270 (221-292)[A]	492 (383-562)[B]	361 (340-392)[B]	**<.001**	–
Serum total T4 in μg/dL, median (IQR)	2.3 (1.5–2.7)	2.0 (0.7-3.0)	2.5 (2.0-2.8)	.44	–
Serum cTSH in ng/mL, median (IQR)	0.3 (0.2-0.7)	0.3 (0.2-0.8)	0.1 (0.1-0.3)	.08	–
Serum free T4 in ng/dL, median (IQR)	1.6 (1.1-2.1)[A]	1.5 (0.8-2.6)[A]	2.7 (2.1-3.1)[B]	**.007**	.21
Serum Spec cPL in μg/L, median (IQR)[e]	81 (30-232)	62 (30-155)	29 (29-85)	.20	–
Serum Spec cPL concentration[e]					
<200 μg/L, n (%)	35 (73%)	10 (83%)	6 (86%)	.29	–
200-400 μg/L, n (%)	7 (15%)	0	0		
>400 μg/L, n (%)	6 (12%)	2 (17%)	1 (14%)		
Serum cortisol in μg/dL, median (IQR)	3.1 (1.9-4.5)	3.1 (2.3-3.8)	2.3 (1.0-4.0)	.50	–
Biomarkers of inflammation					
Serum calprotectin in μg/L, median (IQR)[f]	201 (168-247)	240 (172-386)	186 (166-244)	.52	–
Serum calprotectin concentration[f]					
within RI[g], n (%)	35 (70%)	6 (46%)	5 (71%)	.27	–
above RI[h], n (%)	15 (30%)	7 (54%)	2 (29%)		
Serum S100A12 in μg/L, median (IQR)	154 (115-261)	120 (89-222)	146 (78-198)	.52	–
Serum S100A12 concentration					
within RI[i], n (%)	37 (73%)	10 (77%)	6 (86%)	.72	–
above RI[j], n (%)	14 (27%)	3 (23%)	1 (14%)		

Abbreviations: BCS, body condition score (range of possible scores: 1-9); HTGL, hypertriglyceridemia; HCHOL, hypercholesterolemia; IQR, interquartile range; RI, reference interval.
[a]Significant difference between (or association with) HGTL, HTGL + HCHOL, and HCHOL in univariate analysis.
[b]Statistical significance in stepwise multiple logistic regression model. Bold face values indicate statistical significance at $P < .05$. Medians not sharing the same superscript letter are significantly different at $P < .05$.
[c]Documented in n = 68 dogs.
[d]Documented in n = 64 dogs
[e]Measured in n = 67 dogs.
[f]Determined in n = 70 dogs.
[g]Defined as 72-234 μg/L.
[h]Defined as >234 μg/L.
[i]Defined as 33-225 μg/L.
[j]Defined as >225 μg/L.

4 | DISCUSSION

We found an association between idiopathic IH in MS and increased serum calprotectin concentrations, with a weaker association detected between idiopathic IH and increased serum S100A12 concentrations. Neither serum calprotectin nor serum S100A12 concentrations changed with successful dietary intervention in a subgroup of MS with HTGL (with or without HCHOL).

Spiking experiments served to verify reliable measurement of serum calprotectin in dogs with marked hyperlipidemia. Although a statistically significant difference was found between serum calprotectin concentrations in unspiked samples and samples spiked with an intralipid concentration ≥1250 mg/dL, the overall CVs for all samples (3.0%-17.8%) were within acceptable limits for intraassay and interassay analytical variability of immunoassays (<20%).[38] Thus, serum calprotectin concentrations appear not to be affected by increased amounts of lipids.

We showed that hypercalprotectinemia consistent with subclinical systemic inflammation is more prevalent in MS with IH (approximately 34%) compared to normolipidemic MS. This finding agrees with studies in people where patients with primary combined IH or familial HCHOL had increased serum concentrations of S100A8/A9[29] and also other inflammatory markers (eg, high-sensitivity CRP [hsCRP], interleukin [IL]-6, IL-1β, TNF-α, soluble intercellular adhesion molecule [sICAM], fibrinogen, and intracellular NF-κB).[18,19,21,22,39] In people, hyperlipidemia was

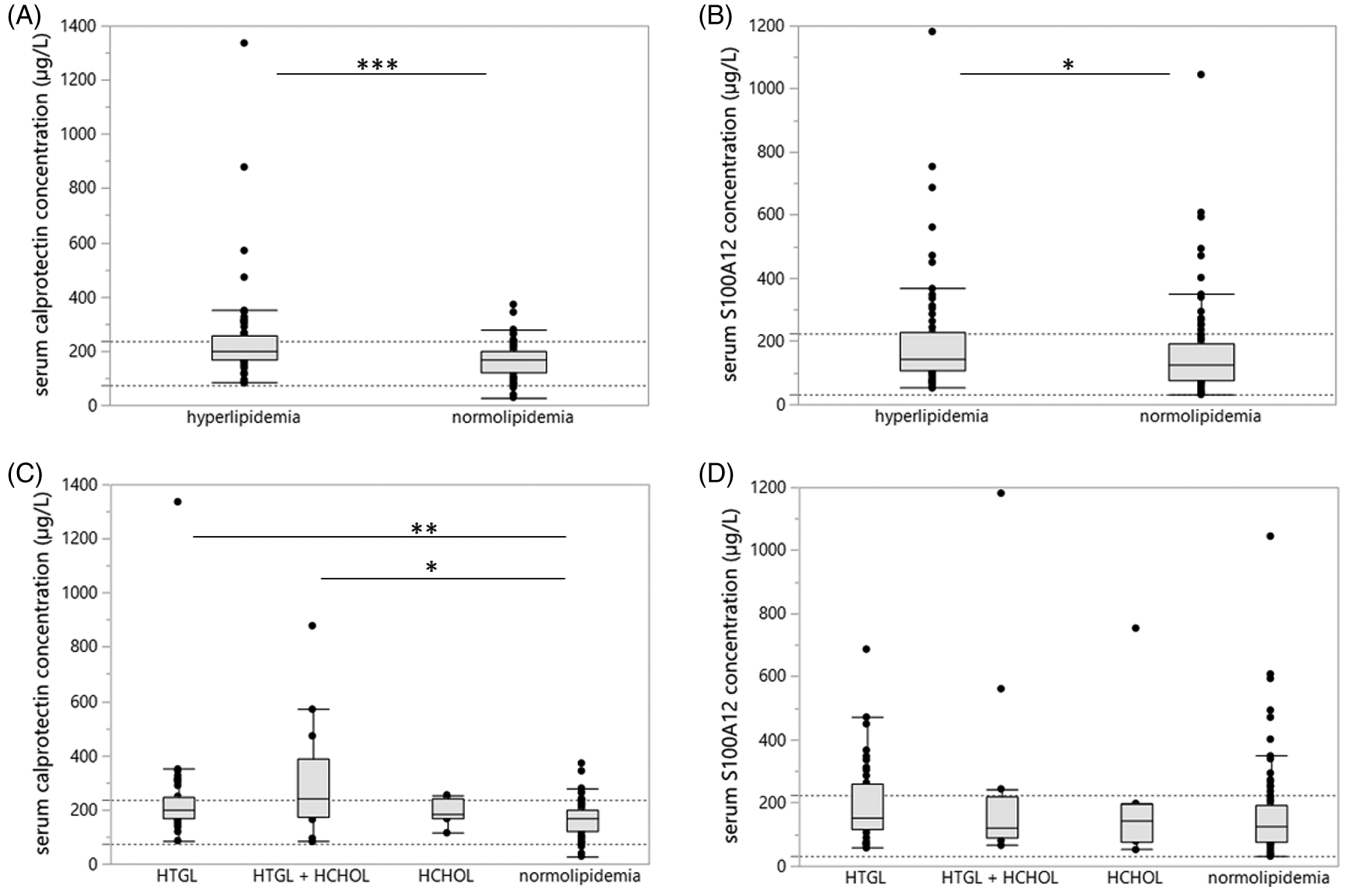

FIGURE 3 Serum calprotectin and S100A12 concentrations in relation to the fasting triglyceride and cholesterol status in clinically healthy MS (n = 150). (A) Hyperlipidemic MS had significantly higher serum calprotectin concentrations (median: 201 µg/L) compared to normolipidemic MS (median: 165 µg/L; $P_{univariate}$ < .001), (B) with a difference in S100A12 concentrations (medians: 145 and 124 µg/L, respectively) detected only in univariate analysis ($P_{univariate}$ = 0.04). (C) Compared to normolipidemic MS, serum calprotectin concentrations were significantly higher in MS with HTGL (P < .001) or combined hyperlipidemia (HTGL + HCHOL; P = .02), but not with isolated HCHOL (P = 1.0000), (D) whereas no differences in serum S100A12 concentrations were detected among all 4 groups of dogs (P = .14)
Dotted horizontal lines, upper and lower limits of the respective reference interval; HCHOL, hypercholesterolemia; HTGL, hypertriglyceridemia; HTGL + HCHOL, combined hyperlipidemia

associated with oxidative stress, low-grade systemic inflammation, and an increased risk of systemic complications.[19,22,39] Thus, further studies are warranted to evaluate if macrophage and neutrophil hyperactivity accounts for the increased serum S100/calgranulin concentrations in MS with IH. Further research also is warranted to determine whether this presumed low-grade inflammatory phenotype in MS with IH contributes to or reflects the development of possible complications of IH,[22,39,40] and to test the alternative hypothesis that the hypercalprotectinemia in MS with IH reflects an anti-inflammatory response.

In our study, a stronger association of serum calprotectin (S100A8/A9) concentration was seen with HTGL compared to HCHOL in MS. This finding is also consistent with a study in people with combined IH where a stronger association was seen between the pro-inflammatory biomarker hsCRP and serum triglyceride concentrations compared to serum cholesterol concentrations.[18]

The finding that MS with IH, particularly those with HTGL, were older than normolipidemic MS agrees with a previous study supporting that IH is a slow-onset, older-age condition in MS.[2] However, the detection of primary HCHOL without HTGL in approximately 5% of MS in our study contrasts with the results of a previous investigation in MS that did not report any cases of isolated HCHOL.[1,2] A possible explanation for this discrepancy could be a difference in the populations of dogs in both studies. However, although every effort was made to exclude dogs with a condition known to cause secondary hyperlipidemia, the possibility of missing an occult or rare cause of secondary hyperlipidemia[41,42] cannot be entirely excluded.

Lack of an association between serum calprotectin concentration and BCS in our study differs from results in nondiabetic people where body mass index (BMI) independently determined serum calprotectin concentrations.[43,44] However, calculation and interpretation of human BMI (which considers body weight, height, and also age)[45] is more objective than the 1-5 or 1-9 scale BCS currently used in veterinary medicine.[46] In addition, the majority of MS (128/139, 92%) had a BCS between 4 and 6/9 and only 7 MS were overweight or obese (BCS > 6/9). This may have prevented finding a link between BCS and serum calprotectin concentrations in our study. Furthermore, a confounder might be the fact that diabetes mellitus associated with higher BMI is typically type 2 in humans; whereas, in dogs, it is usually type 1 diabetes mellitus.

No association was found between increased serum Spec cPL concentrations and hypercalprotectinemia in our study. However, only 22 MS (15%) had a serum Spec cPL concentration above the RI (ie, 200 µg/L), with only 11 MS (8%) having a serum Spec cPL

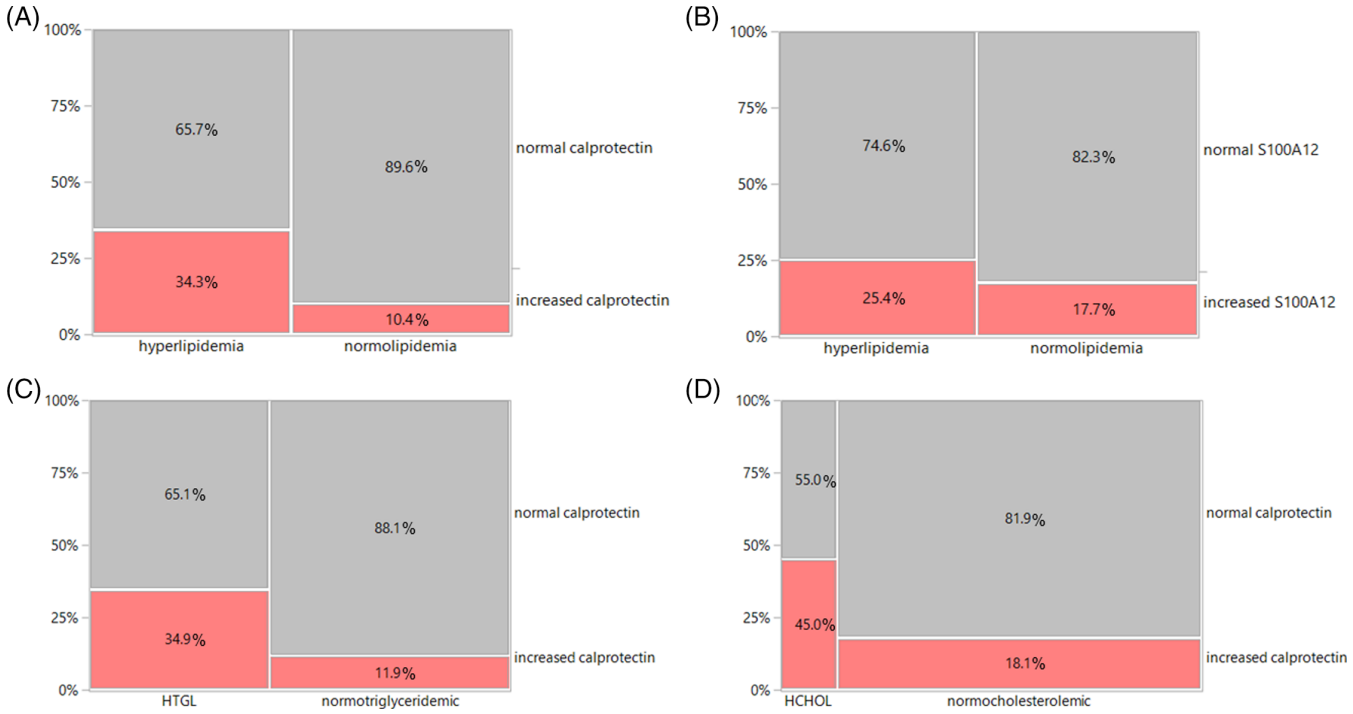

FIGURE 4 Association between idiopathic hyperlipidemia (IH) and increased serum calprotectin and S100A12 concentrations in clinically healthy MS (n = 150). (A) There was a significant association between IH and a serum calprotectin concentration increased above RI (P < .001), (B) which was not seen for serum S100A12 concentration (P = .25). (C) There was also a significant independent association of serum calprotectin concentrations increased above RI with HTGL (P < .001; OR, 4.0; 95% CI, 1.7-9.2) and (D) HCHOL (P = .01; OR, 3.7; 95% CI, 1.4-10.0). HCHOL, hypercholesterolemia; HTGL, hypertriglyceridemia

concentration > 400 μg/L (consistent with a diagnosis of pancreatitis), but abdominal imaging findings were not evaluated as part of this investigation. Thus, whether hypercalprotectinemia is associated with the presence or risk of clinical or subclinical pancreatitis cannot be evaluated.

Idiopathic HTGL in MS has been reported to be associated with proteinuria[11,40] and ultrastructural glomerular lesions,[12] but whether these findings are a consequence or cause of dyslipidemia in MS remains unknown. Presence of proteinuria (ie, urine protein/creatinine ratios) or renal biopsy specimens were not evaluated in our study. Thus, the possibility of an association between increased serum calprotectin concentrations and proteinuria, glomerular lesions, or both cannot be evaluated.

Lack of a decrease in serum calprotectin or S100A12 concentrations with successful dietary intervention (ie, feeding an ultra-low fat diet over 3-6 months) in MS with IH was an unexpected finding. These results contrast with the beneficial effect of lipid-lowering medications on serum hsCRP, IL-1β, and soluble CD40 ligand (sCD40L) concentrations in human patients with combined hyperlipidemia.[21] However, our results agree with the lack of a change in the concentrations of other inflammatory markers (CRP, sICAM, fibrinogen, IL-1β, TNF-α, sCD40L, or combinations of these) seen in people diagnosed with either combined hyperlipidemia or familial HCHOL and who received either diet-only or an intensive or long-term lipid-lowering medical treatment.[19,47–49] Our results could suggest an inflammatory

TABLE 3 Biochemical parameters in MS with IH (n = 17) before and after dietary intervention

	(1) Before dietary intervention		(2) After dietary change		Difference or association between (1) and (2)	
Variable	Median (IQR)	n (%) Values above RI	Median (IQR)	n (%) Values above RI	P	P_{corr}
Triglycerides	667 (365-840)		179 (98-369)		**<.001**	<.001
		17 (100%)		12 (71%)	**.005**	**.03**
Cholesterol	372 (268-520)		261 (238-330)		**.006**	**.02**
		10 (59%)		3 (18%)	**0.01**	**.05**
Spec cPL	118 (46-231)		134 (60-255)		.81	ns
		5 (29%)		5 (29%)	1.0000	1.0000
Calprotectin	200 (146-248)		167 (137-253)		.99	.99
		4 (24%)		6 (35%)	.45	ns
S100A12	110 (79-175)		120 (73-159)		.92	ns
		1 (6%)		2 (12%)	.54	ns

Abbreviations: IQR, interquartile range; ns, not significant (P > 1.0000); RI, reference interval; Spec cPL, specific canine pancreatic lipase; P_{corr}, after Holm-Bonferroni correction (n = 5).
P values in bold indicate significant difference or association at P < .05.

phenotype being present in some MS with IH despite a successful lipid-lowering dietary strategy. However, whether a longer treatment interval might be needed for any changes in serum S100/calgranulin concentrations or if additional lipid-lowering medications are warranted in these dogs requires further studies. Also, the relationship between the inflammatory markers evaluated in our study and evidence of insulin resistance[8,22] should be evaluated in MS with IH.

We acknowledge that our study had some limitations. Although common causes of secondary hyperlipidemia were ruled out, the possibility of occult or rare diseases (eg, lymphoma) cannot be definitively excluded in all dogs as a cause of hyperlipidemia. Also, single serum samples were evaluated, and the biological variation of serum triglyceride and cholesterol concentrations is unknown in dogs with IH. Third, the large variation in follow-up of dogs undergoing dietary intervention adds another variable and might have affected our results. Also, in contrast to the concentration of serum lipids, changes in serum inflammatory biomarkers might take longer to occur. Thus, a longer follow-up period might have shown different results. Fourth, dogs and humans differ in their lipoprotein signatures,[18,50] but we did not investigate the effect of different lipid or lipoprotein portions on serum S100/calgranulin concentrations. Furthermore, additional inflammatory markers (eg, the acute-phase protein CRP) with a spatial or temporal expression or both that differs from that of the S100/calgranulins were not evaluated. Lastly, small sample size (especially in various subgroup analyses) could result in potential for type I or type II error.

5 | CONCLUSIONS

Our results suggest that low-grade subclinical inflammation might be present in approximately every third clinically healthy MS with IH (characterized by HTGL, HCHOL, or both), and that feeding an ultra-low fat diet for 3-6 months does not appear to decrease the serum concentrations of the inflammatory S100/calgranulin proteins in hypertriglyceridemic MS with or without HCHOL. Whether this presumed inflammatory phenotype in MS with IH plays a role in the development of possible complications of IH (eg, insulin resistance, pancreatitis, or other conditions) warrants further research. Also, IH in MS appears to be a good spontaneous animal model for studying the S100/calgranulins in people with familial hyperlipidemia where rodent models cannot be utilized.

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CONFLICT OF INTEREST DECLARATION

Dr. Steiner and Dr. Suchodolski are directors of the Gastrointestinal Laboratory where calprotectin and S100A12 testing is offered on a fee-for-service basis.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

The study of several serum analytes in Miniature Schnauzers with or without idiopathic hyperlipidemia was approved by the Clinical Research Review Committee at Texas A&M University (TAMU-CRRC# 2008-37), and the owner of each dog enrolled in the study had to give written consent before the inclusion of the dog.

HUMAN ETHICS APPROVAL DECLARATION

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

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