Received: 3 January 2020 | Accepted: 14 December 2020

DOI: 10.1111/jvim.16019

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Serum spexin concentration, body condition score and markers of obesity in dogs

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Funding information
Ministerstwo Nauki i Szkolnictwa Wyższego, Grant/Award Number: 005/RID/2018/19; Narodowe Centrum Nauki, Grant/Award Number: 2015/19/N/NZ4/00572; Uniwersytet Przyrodniczy w Poznaniu, Grant/Award Number: Young Researcher Program (Pawel A Kolodziejski)

Abstract

Background: Spexin (SPX) is a peptide hormone that regulates body weight, adipose tissue metabolism, and food intake.

Hypothesis: Serum SPX concentration correlates with body condition score (BCS) and markers of obesity in dogs.

Animals: Fifty-seven dogs of varying body condition assessed using a 5-point BCS.

Methods: Prospective, nonblinded, observational cohort study. Serum SPX concentration was measured using commercially available radioimmunoassay (RIA) in dogs with varying BCS. Spexin mRNA and protein expression were detected using real-time quantitative polymerase chain reaction and immunofluorescence staining.

Results: Serum SPX concentration was lower in dogs with BCS4 (8.56 +/- 2.86) and BCS5 (6.7 +/- 2.12) compared to BCS2 (11.96 +/- 2.23) and BCS3 (10.51 +/- 2.19; BCS2 vs BCS5, $P < .001$ and BCS2 vs BCS4, $P = .005$; BCS3 vs BCS5, $P = .002$). Spexin mRNA was detected in adipose tissue, liver and pancreas. Spexin protein was expressed in adipose tissue and liver but not in pancreas. There were negative correlations between SPX and serum concentration of insulin ($P < .05$); leptin ($P < .01$), triglycerides ($P < .01$), total cholesterol ($P < .01$), nonesterified fatty acids ($P < .01$), and fructosamine ($P < .01$). There was a positive correlation between SPX and serum concentration of adiponectin ($P < .01$).

Conclusions and Clinical Importance: Spexin could be involved in pathogenesis of obesity in dogs, and might be considered as a potential marker for obesity.

KEYWORDS
BCS, hormones, insulin, metabolism

Abbreviations: BCS, body condition score; GAPDH, glyceraldehyde 3-phosphate dehydrogenase gene; NEFAs, nonesterified fatty acids; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction/real-time PCR; RIA, radioimmunoassay; SPX, spexin; TChol, total cholesterol; TGs, triglycerides.

1 | INTRODUCTION

The effect of lifestyle changes of modern societies is the increase in prevalence of overweight and obesity in humans.[1] There is evidence that obesity in human beings corresponds to that in companion animals.[2,3] It is estimated that overweight and obesity affect approximately 25% to 35% of adult cats and 35% to 40% of adult dogs worldwide.[4,5] Although obesity in human beings is defined based on variable such as body mass index, there is not 1 indicator in dogs and cats. Therefore, in the case of pet animals, several variables are used in the diagnosis of obesity. Overweight and obesity in pets are diagnosed when body weight exceeds the ideal body weight by 10% to 20% and 20% to 30%, respectively.[6] Another variable that helps in detecting overweight and obesity in pets is body condition scoring (BCS).[7,8] The 2 most commonly used BCS scales are a 5-point system and a 9-point system. However, BCS, as well as other noninvasive methods, including percent body mass calculated from morphometric measurements, are relative because of breed differences. Body condition score and percent body fat are highly correlated with dual-energy X-ray absorptiometry (DEXA) method. There is a need for additional markers of obesity that would be universal for many breeds.[8]

There are numerous peptides involved in the regulation of energy homeostasis on the level of both the CNS and peripheral tissues. One of them is spexin (SPX), discovered in 2007, which contains 14 amino acids the order of which is very conservative in various animal species, including mammals.[9] The similarity of SPX sequences between different species is over 90%, and expression of this peptide is widely distributed in various animal species.[10-12] Spexin inhibits food intake, decreases body weight,[13] and regulates adipose tissue metabolism and insulin secretion in rats and mice.[14-16] We investigated the potential association between obesity and serum SPX concentration in dogs. In addition, we investigated whether there is a correlation between the concentration of this peptide in blood and other indicators of obesity and metabolic dysfunction in dogs.

2 | MATERIALS AND METHODS

2.1 | Blood collection

Blood samples from dogs were collected in plastic tubes containing gel to separate serum from cellular components using routine veterinary procedures in accordance with applicable law and after permission from dog owners. All procedures were performed according to Polish Law. Tissue samples for quantitative polymerase chain reaction (qPCR) and immunofluorescence analysis were obtained from dogs that were euthanized because of accidental injuries (breeds, age, weight, and BCS are placed in Table 1). A fragment of the liver was taken from the left lateral lobe, visceral adipose tissue from the vicinity of the kidney fat, a fragment of the pancreas from a part of the tail of this organ. Immediately after resection, the tissue was either placed in Bouin's solution (part for immunofluorescence) or in liquid nitrogen (for qPCR). After 30 minutes of incubation at room temperature, the blood was centrifuged at 3500*g* for 15 minutes at 4°C. The serum was divided into the aliquots and stored in a veterinary clinic at −20°C for a maximum of 24 hours. Then, the samples were transported to a laboratory (on dry ice) and placed in ultrafreezer (−80°C) for a longer storage. All samples were stored for a maximum of 3 months until further analyses. Dogs were fasted at least 12 to 14 hours before blood sampling.

TABLE 1 Characteristic of dogs used for tissue analysis

Breed	Weight (kg)	Age (years)	Sex	BCS
Labrador Retriever	30	2	♀	3
Labrador Retriever	29	5	♂	3
Mix	12.5	5	n	3
German Shepherd	30	8	♂	3
Mix	16.1	8	♀	3

Abbreviations: ♂, male; ♀, female; n, neuter.

2.2 | Assignment to groups

Dogs were assigned to research groups based on Hill's weight guide.[7] The dogs were not eligible for the study if they were less than 1 year old, pregnant, already participated in a weight reduction program or had diseases that affect body weight, such as hyperthyroidism, chronic renal disease, diabetes, cancer, or hepatic disease.

Breed distribution, weight, sex, age, and number of animals in investigated groups are shown in Table 2.

2.3 | Quantitative PCR and immunofluorescence detection of SPX

Quantitative PCR and immunofluorescence of SPX was detected as previously.[16] In brief, RNA from tissues was isolated using TriPure Isolation Reagent (Roche, Germany). cDNA was synthetized using 1 μg of total RNA and the High Capacity cDNA Reverse Transcription Kit reagent (Applied Biosystems, Foster City, California). Quantitative PCR was performed on QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) using 5× HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Estonia) and specific primers. Primers were designed using Primer-Blast software based on *Canis lupus familiaris* sequence. The sequences of primers used for PCR were as follows: SPX forward 5′ TACCTCCAACACACACCCCA 3′ and reverse 5′ ACCAATGAGGCCAAAGCTGA 3′ and glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) forward 5′ GGTCACCAGGGCTGCTTT 3′ and reverse 5′ ATTTGATGTTGGCGGGAT.[17] The specificity of reaction products was tested by determining the melting points (0.1 C/s transition rate). Relative gene expression was evaluated by Delta Delta CT (ΔΔCT) with GAPDH as a reference.

Immunofluorescence: Dissected tissue was rinsed in 0.9% NaCl solution and immersed in Bouin's solution. After a 24-hour fixation

TABLE 2 Breed distribution in investigated groups. Number of animals in parentheses

BCS			
2 (n-10)	**3 (n-16)**	**4 (n-16)**	**5 (n-15)**
Pug dog (1) (5.8, 8, ♀)	Golden retriever (3) (30, 4, n), (31, 6, ♀), (31.5, 7, ♂)	Golden Retriever (3) (30, 4, n), (31, 6, ♀), (31.5, 7, ♂)	Beagle (3) (18 kg, 21 kg, (22, 7, ♀)
Akita/Mix (1) (28, 10, ♂)	Labrador Retriever (4) (31, 3, n), (33, 3, ♀), (30.5, 4, ♂), (35, 7, n)	German Shepherd (2) (46, 8, ♂), (45, 10, ♀)	Labrador Retriever (4) (45, 11, ♀), (47, 8, ♂), (41, 7, n), (43.4, 7, n)
Mix (3) (5.7, 4, ♀), (6, 9, 5, ♂) (10.4, 3, n)	Beagle (3) (13, 2, n), (12, 3, ♀), (12, 5, 8, ♂)	Australian Shepherd (2) (30, 8, ♂), (32, 5, n)	Mix (1) (14, 5, n)
Miniature Schnauzer (1) (4.9, 3, ♂)	Mix (3) (18, 2, n), (18, 7, ♀), (6, 10, ♂)	Mix (3) (43, 6, n), (25, 5, ♀), (36, 9, ♂)	German Shepherd (1) (50, 8, n)
German Shepherd (2) (30, 9, ♂), (31, 2, ♀)	Border Collie (1) (13.6, 2, ♂)	Pug dog (1) (9, 5, n)	Dalmatian (1) (35, 12, ♀)
Labrador Retriever (1) (30, 8, n)	German Shepherd (1) (31, 10, ♂)	Dachshund (1) (11, 12, ♀)	Cocker Spaniel (2) (18, 7, ♂), (20, 11, ♀)
Fox Terrier (1) (5.8, 7, ♀)	Dalmatian (1) (24, 11, ♂)	Yorkshire Terrier (1) (3.6, 8, ♂)	Golden Retriever (3) (34, 11, ♀), (39, 7, n), (41, 12, ♂), (37, 8, n),
		Labrador Retriever (3) (40, 8, ♂), (30, 2, ♀), (37, 9, n)	
		Akita (1), (40, 10, ♀)	

Note: The description contains: breed (number of individuals) in the first parentheses, the next in parentheses contains (weight, age, sex) of individual dogs. Abbreviations: ♂, male; ♀, female; n, neuter.

TABLE 3 Serum concentration of basic biochemical variables in dogs with different BCS

	BCS			
Variable	**2**	**3**	**4**	**5**
Glucose (mg/dL)	88.87 ± 7.86	91.60 ± 13.89	92.77 ± 17.23	109.0 ± 19.54 (2 vs 5; *P* = .04) (3 vs 5; *P* = .04) (4 vs 5; *P* = .03)
Triglicerides (mg/dL)	27.88 ± 8.21	43.60 ± 16.11	72.67 ± 27.88 (2 vs 4; *P* = .005) (3 vs 4; *P* = .04)	105.8 ± 46.68 (2 vs 5; *P* < .001) (3 vs 5; *P* < .001) (4 vs 5; *P* = .01)
Cholesterol (mg/dL)	196.5 ± 75.61	233.0 ± 58.09	274.5 ± 59.04 (2 vs 4; *P* = .01)	335.4 ± 59.18 (2 vs 5; *P* < .001) (3 vs 5; *P* = .002)
NEFA (mmol/L)	0.349 ± 0.12	0.488 ± 0.17	0.540 ± 0.13 (2 vs 4; *P* = .01)	0.876 ± 0.14 2 vs 5; *P* < .001) (3 vs 5; *P* < .001) (4 vs 5; *P* < .001)
Albumin (g/dL)	3.42 ± 0.19	3.48 ± 0.37	3.50 ± 0.41	3.92 ± 0.66
Total protein (g/dL)	5.02 ± 0.82	4.89 ± 0.73	5.53 ± 0.92	5.85 ± 1.14
Fructosamine (mmol/L)	0.295 ± 0.057	0.318 ± 0.05	0.356 ± 0.086 (4 vs 5; *P* = .01)	0.463 ± 0.12 (2 vs 5; *P* < .001) (3 vs 5; *P* < .001) (4 vs 5; *P* = .01)

Abbreviations: BCS, body condition score; NEFA, nonesterified fatty acids.

period, tissue samples were dehydrated using increasing concentrations of ethanol (70%-96%), and subsequently embedded in Paraplast (Sigma-Aldrich). Paraplast blocks were cut into thin sections of 4.5 to 5 μm. Next, slides with sections were heated (58°C) in a laboratory oven for 45 minutes, deparaffinized in xylene for 30 minutes and rehydrated using gradient ethanol in water. For antigen retrieval, sections were heated in a citrate buffer (pH 6.0) for 10 minutes at 95°C in a microwave oven. After cooling to room temperature, nonspecific antibody binding to proteins in sections was blocked using a blocking solution containing 0.2% gelatin and 15 mM glycine. Control

incubation of tissue slices with anti-SPX primary antibody (Phoenix Pharmaceuticals, Burlingame, California) and blocking peptide-SPX protein (Phoenix Pharmaceuticals) for 24 hours was performed.

2.4 | Biochemical parameters

Biochemical variables in serum were determined using commercially available colorimetric and enzymatic assays (Table 3). The concentrations of triglycerides (TGs), total cholesterol (TChol), glucose, fructosamine, albumin, and total protein were measured using Pointe Scientific (Canton, Michigan). The concentration of nonesterified fatty acids (NEFAs) was determined using an enzymatic test from Wako (Oxoid, Dardilly, France). Optical density of samples was measured using a microplate reader Synergy 2 (Biotek, Winooski, Vermont).

2.5 | Hormonal profile

Spexin concentration was determined using Spexin/Neuropeptide Q radioimmunoassay (RIA) kit (Phoenix Pharmaceuticals). Given the fact that the assay was not species-specific, a series of dilutions were made to determine specificity of the binding. Validation of SPX RIA kit was performed based on the literature.[18,19] The first step in the validation was to determine the similarity between human, rat, and canine SPX. Based on the databases similarity and query, cover was determined at 92.86% and 100%, respectively (Figure S1A). Next, 2 canine samples were also run 10 times on to get an intra-assay coefficient of variation that was 7.26% (Figure S1B). Moreover, in each determination serum samples from 4 dogs were diluted 2, 4, 6, and 8 times, and concentration obtained from these dilutions were 2, 4, 6, and 8 times lower compared with undiluted samples to determine linearity (Figure S1C).

Glucagon concentration was determined using a RIA kit (Merck Millipore, Burlington, Massachusetts),[20] whereas the concentration of insulin and adiponectin was measured using enzyme-linked immunosorbent assays: Canine Leptin (MerckMillipore), Insulin DuoSet ELISA (R&D Systems, Inc, Minneapolis, Minnesota) and Adiponectin ELISA (BioVendor, Brno, Czech Republic).[21]

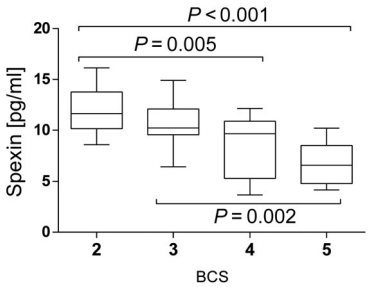

FIGURE 1 Effect of body condition score (BCS) on serum blood spexin (SPX) concentration in dogs. The boxes represent the 25th and 75th quartiles with the horizontal line representing the median. The whiskers represent the range of the data. Statistically significant differences between the experimental groups and *P*-values are presented above the graphs

2.6 | Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software. The significance of differences was determined using 1-way analysis of variance (ANOVA) followed by Tukey's range test for multiple comparisons (for data with normal distribution and equality of variance) or nonparametric Kruskal-Wallis test (when variables had other than normal distribution and had no equality of variance). Statistical significance was defined as $P < .05$. Relationships among SPX concentration, hormones, and biochemical variables of blood were analyzed using Pearson's correlation model and linear regression. Correlation coefficient values below 0.3 were considered as weak, between 0.3 and 0.5 as mild, 0.5-0.7 as moderate, and 0.7 or more as a high correlation.

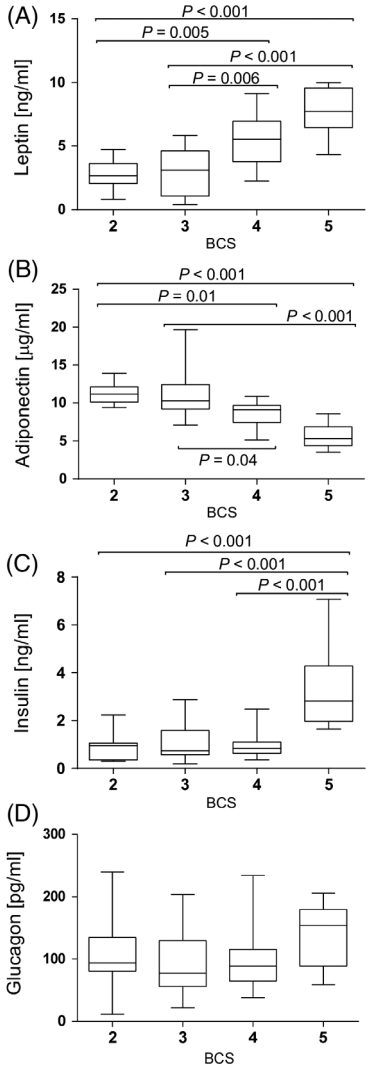

FIGURE 2 Effect of body condition score (BCS) on serum blood leptin (A), adiponectin (B), insulin (C), and glucagon (D) concentrations in dogs. The boxes represent the 25th and 75th quartiles with the horizontal line representing the median. The whiskers represent the range of the data. Statistically significant differences between the experimental groups and *P*-values are presented above the graphs

3 | RESULTS

3.1 | Hormonal profile

The serum concentration of SPX was lower in the BCS4 and BCS5 dog groups compared with that in BCS2 and BCS3 groups (BCS2 vs BCS5, $P < .001$ and BCS2 vs BCS4, $P = .005$; BCS3 vs BCS5, $P = .002$). We did not detect differences in the serum SPX concentration between BCS2 and BCS3 and between BCS4 and BCS5 (Figure 1). There was a higher leptin concentration in BCS4 and BCS5 groups compared with BCS2 and BCS3 groups (BCS2 vs BCS5; BCS3 vs BCS5, $P < .001$ and BCS2 vs BCS4, $P = .005$; BCS3 vs BCS4, $P = .006$; Figure 2A), whereas adiponectin concentration was lower in the same groups (BCS2, BCS3 vs. BCS5, $P < .001$ and BCS3 vs BCS4, $P = .04$ and BCS2 vs BCS4, $P = .01$; Figure 2B). Insulin concentration was higher in BCS5 group compared with BCS2, BCS3, and BCS4 groups (Figure 2C; $P < .001$). There was not statistically significant differences in concentration of glucagon among groups (Figure 2D).

3.2 | Metabolic profile

There was a higher concentration of glucose in BCS5 compared with other groups (BCS2, BCS3 vs BCS5, $P = .04$ and BCS4 vs BCS5 $P = .04$) and TGs in BCS4 and BCS5 groups compared with BCS2 and BCS3 (BCS3 vs BSC4, $P = .005$; BCS3 vs BCS5, $P < .01$; BCS2, BCS3 vs BCS5, $P < .001$; BCS4 vs BCS5, $P = .01$). Concentration of cholesterol in BCS4 and BCS5 was higher than in BCS2 (BCS2 vs BCS5, $P < .001$; BCS3 vs BCS5, $P = .002$) and BCS3 vs BCS4 ($P < .01$). Concentration of NEFA was higher in BCS5 (BCS2, BCS3, BCS4 vs BCS5, $P < .001$), and BCS2 vs BCS4 ($P = .01$). Fructosamine concentration was higher in BSC5 compared with the rest of the groups (BCS2, BCS3, vs BCS5, $P < .001$; BCS4 vs BCS5, $P = .01$) and BCS2 vs BCS4 ($P = .01$).

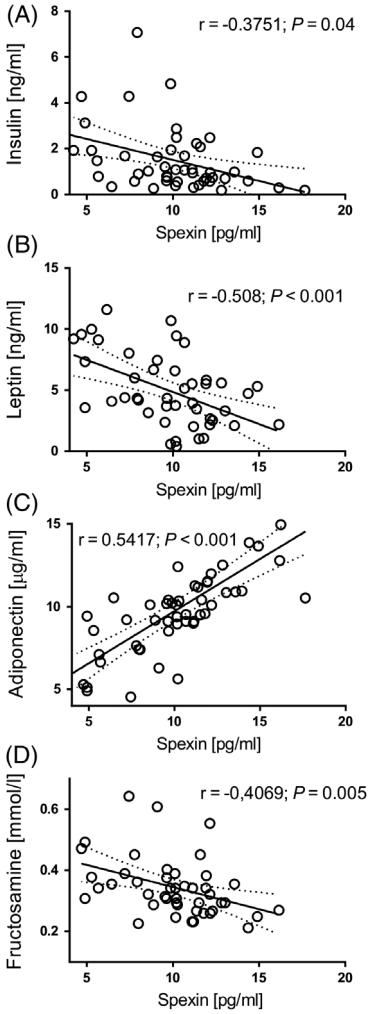

FIGURE 3 Correlations between circulating spexin (SPX) concentration and insulin (A), leptin (B), adiponectin (C), and fructosamine (D). Values for *r* and *P* are indicated in each graph. Solid and dashed lines show the mean and 95% confidence intervals, respectively, after linear regression analysis; symbols show *r*-Pearson and *P*-value. *r*-Pearson shows the correlation and *P*-value shows significance of the correlation

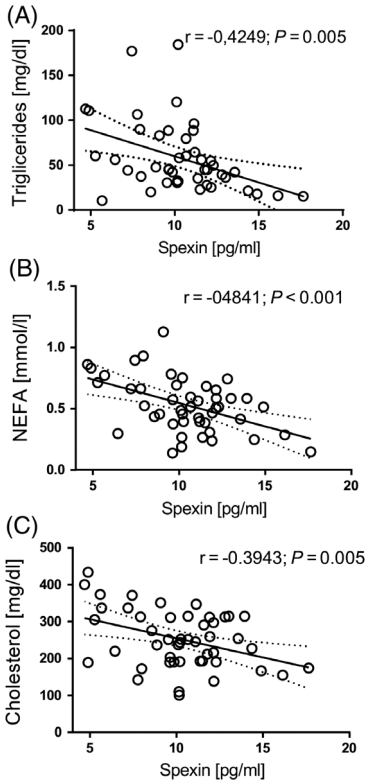

FIGURE 4 Correlations between circulating spexin (SPX) concentration and triglycerides (TGs) (A), nonesterified fatty acid (NEFA) (B), and cholesterol (C). Values for *r* and *P* are indicated in each graph. Solid and dashed lines show the mean and 95% confidence intervals, respectively, after linear regression analysis; symbols show *r*-Pearson and *P*-value. *r*-Pearson shows the correlation; *P*-value shows significance of the correlation

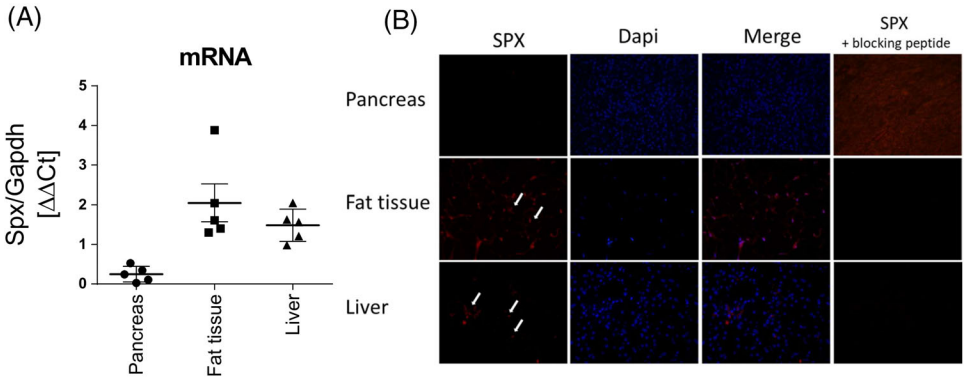

FIGURE 5 A, mRNA and B, protein expression of spexin (SPX) in dog pancreas, adipose tissue, and liver. Positive signals for SPX in adipose tissue and liver are marked with white arrows

3.3 | Correlations

There was a negative correlation between SPX and insulin (r = –0.3751, P = .04; Figure 3A) as well as between SPX and leptin (r = –0.508, P < .001; Figure 3B), and a positive correlation between SPX and adiponectin (r = 0.5417, P < .001; Figure 3C). There was no correlation between SPX and glucagon concentrations (data not shown). There was a negative correlation between SPX and TGs (r = –0.4249, P < .005; Figure 4A), NEFA (r = –0.4941, P < .001; Figure 4B), TChol (r = –0.3943, P < .005; Figure 4C), and fructosamine (r = –0.4069, P < .005; Figure 3D). There was no correlation between SPX and glucose.

3.4 | mRNA and protein expression of SPX in pancreas, liver, and adipose tissue

The mRNA of SPX was expressed in pancreas, liver, and fat (Figure 5A), whereas expression of this peptide was demonstrated in only fat and liver (Figure 5B).

4 | DISCUSSION

In the present study, we show lower SPX concentration in dogs with BCS4 and BCS5 compared to BCS2 and BCS3. We also report that SPX concentration correlates with other indicators of obesity, such as blood hormone concentrations (leptin, adiponectin, and insulin) and TGs, TChol, NEFA, and fructosamine.

There is lower SPX concentration in obese laboratory animals[13] and obese human.[22] However, there are no literature data describing the role of this peptide in pet animals such as dogs. Therefore, we investigated putative changes in SPX concentration in blood serum in dogs with varying BCS. Our results are consistent with those of studies on laboratory animals and human beings in which there was a lower concentration of this peptide in obese individuals. However, SPX concentration was lower only in obese children and adults, and this correlation was not present in adolescents.[23]

Leptin and adiponectin are hormones secreted by adipocytes, and their function is associated with the regulation of the energy homeostasis. In metabolic disorders, they are associated with the development of obesity in humans and animals (including dogs).[24,25] In addition, in laboratory animals and humans there is correlation between these adipokines and SPX.[13,22] Our current data supported such interaction also in dogs, in that we found a negative correlation between SPX and leptin, and a positive correlation between SPX and adiponectin.

Insulin and glucagon are pancreatic hormones whose abnormal levels of synthesis and secretion are associated with the development of obesity and metabolic syndrome.[26] There was a negative correlation between SPX and insulin. Spexin is able to regulate expression and secretion of insulin from isolated pancreatic islets, INS-1E cells, and in vivo in rats.[14] However, the SPX effect in vivo was observed only in obese rats. Spexin expression is regulated by glucose concentration, which might also indicate the role of this peptide in the regulation of the insulin expression and secretion.[15] However, this relationship needs to be confirmed directly using in vitro or in vivo studies such as glucose tolerance test. Finally, we investigated the expression of SPX on mRNA and protein levels in tissues involved in glucose homeostasis, such as pancreas, adipose, and liver. There was no expression of SPX in the pancreas at the protein level. Spexin is widely expressed in different tissues in rats.[13,27] However, SPX expression in the pancreas varies from species to species. Spexin is expressed in pancreatic islets in pigs and human beings but not in rats.[11,14,15] Lack of SPX protein expression in the rat and canine pancreas is surprising, as there was a correlation between insulin and SPX concentration in serum. These results could suggest that if there is an interaction between SPX and insulin, secretion adjustment is done via SPX, which goes to the pancreas via blood from other tissues. The second important hormone in the context of the development of disorders related to glucose metabolism is glucagon. The lack of observed changes in glucagon concentration might stem from different reasons, such as short half-life in the blood and its rapid degradation in the liver,[28,29] the effect of feed with holding from dogs before blood sampling, which is repeatedly highlighted in the literature,[30] or species. On the other hand, the lack of changes in glucagon concentrations confirms the absence of obesity comorbidities in these dogs.[20] However, it is hard to clearly indicate reasons for lack of changes in glucagon concentrations in our study, as the metabolism of this hormone in obese dogs is still unclear and data available on this subject remain equivocal.

Although we have obtained novel data on SPX concentration in obese dogs, we are aware of the limitations of our study. Among

these are the small number of animals studied and the fact that the research was performed based on serum samples collected from different dog breeds. This, in turn, did not allow us to measure changes in SPX concentrations during development of obesity. Another limitation of our research is the lack of in vitro studies showing the direct effect of SPX on canine tissues to confirm our findings. In addition, we also realize that the test for SPX determination in blood serum was species-non-specific, which, despite validation, allows some errors.

One should also keep in mind that mechanisms of obesity development are complex and depend on both genetic and epigenetic (eg, environmental) factors. Hence, although it is inadvisable to draw direct conclusions from correlations we present in our study, in our opinion they might be the starting point for further studies on interplay between SPX and other adipokines.

ACKNOWLEDGMENTS

The research was partially supported by the 2019 grant of the Young Researcher Program (Pawel A. Kolodziejski) of the Faculty of Veterinary Medicine and Animal Science Poznan University of Life Sciences, financed by the Polish Ministry of Science and Higher Education and partially by the National Science Centre, Poland 2015/19/N/NZ4/00572 PRELUDIUM grant. The research was also cofinanced within the framework of Ministry of Science and Higher Education program as "Regional Initiative Excellence" 2019-2022, Project No. 005/RID/2018/19 (Pawel A. Kolodziejski).

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

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

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

How to cite this article: Kolodziejski PA, Pruszynska-Oszmalek E, Nowak T, et al. Serum spexin concentration, body condition score and markers of obesity in dogs. *J Vet Intern Med*. 2021;35:397–404. https://doi.org/10.1111/jvim.16019