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Relationship of Circulating Spexin with Markers of Cardiovascular Disease: A Pilot study in Adolescents with Obesity

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Abstract

Purpose—Spexin, a novel peptide, has potential implications in obesity, satiety and energy homeostasis. The current study examined the relationship of spexin with various biomarkers of cardiovascular disease (CVD) and endothelial function in adolescents with obesity.

Methods—Nineteen adolescents with obesity [age, 15.8 ±1.7 years] were studied. Spexin, leptin and various CVD biomarkers were measured. Endothelial function was assessed by high-resolution Doppler ultrasonography of the right brachial artery.

Results—Spexin concentration [median (IQR) 0.38 ng/mL (0.29-0.59 ng/mL)] was inversely correlated (r=-0.50, *P*=0.03) with leptin. When participants were clustered into two groups ("high spexin and low leptin" vs. "low spexin and high leptin"), the odds of having "low spexin and high leptin" in participants with higher hs-CRP (≥ 3mg/L) was 12.25 times (95% CI- 1 to139, *P*=0.026) higher than that of participants with lower hs-CRP (<3mg/L). Spexin levels, however, were not associated with measures of endothelial function.

Conclusions—The inverse association between spexin and leptin and the presence of higher concentrations of hs-CRP in adolescents with obesity in the setting of "low spexin and high leptin" suggest a potential role for spexin in the regulation of satiety and certain cardiovascular risk factors in children with obesity.

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SK, JH, AJ, IK and BB have nothing to disclose.

SK and AJ carried out experiments, SK, AJ and BB conceived experiments and JH and AJ analyzed data. SK and BB interpreted the data and all authors were involved in writing the paper and had final approval of the submitted and published versions.

Keywords

Spexin; Leptin; Cardiovascular Risk; Obesity; Endothelial Function

Background

Spexin is a novel peptide recently identified using Markov modeling [1]. The gene encoding spexin (Cg12orf39) is the most down regulated gene in obese omental and subcutaneous human fat [2]. The physiological significance of spexin remains mostly unclear. Previous studies in rat and goldfish have demonstrated expression of spexin mRNA and protein in several tissues including the hypothalamus, hippocampus, cerebral cortex, stomach, kidney, adrenal gland and ovary [3, 4]. Notably, circulating spexin concentrations are lower in children and adults with obesity than in their lean counterparts [5, 6]. Previous animal studies have also described spexin-induced weight loss in rodents with diet induced obesity (DOI) [5, 7]. Studies in humans on spexin are sparse and Walewski et al reported an inverse association between spexin and leptin[5]. Although in a previous study in children we reported spexin's ability to discriminate between lean and obese children[6], its potential physiological role in children and adolescents with obesity and/or its relationships with satiety hormones, biomarkers of cardiovascular disease (CVD) and endothelial dysfunction are less clear. The objective of the current study was to examine the relationship between spexin and leptin along with specific biomarkers of cardiovascular disease (CVD) and endothelial function in adolescents with obesity.

Materials and Methods

After obtaining approval from the Institutional Review Board of Mayo Clinic, Rochester, MN and consents from participants and parents, a total of 19 adolescents with BMI at or above the 95th percentile [age, 15.8 ±1.7 years] were studied. Sample size calculation was not performed *a priori* due to scant data on spexin, especially in children. Study design and other details can be found in detail in our previous study[8]. Height, weight, and blood pressure (BP) were obtained after overnight fast. Study participants were normotensive and non-diabetic.

Biochemical Measurements

Blood samples were obtained after overnight fast of 12 hours. The measurements included spexin, leptin, glucose, insulin, lipid profile, high sensitivity C-reactive protein (hs-CRP), total adiponectin and high molecular weight adiponectin. Spexin was measured using a specific enzyme linked immunoassay (ELISA) from Phoenix Pharmaceuticals, Inc, Burlingame, CA. The intra-assay and inter-assay coefficients of variation for spexin were ≤0% and ≤15% respectively. The assay sensitivity was 0.11 ng/mL with a linear range of 0.11 - 1.07 ng/mL.

Glucose was measured by oxidase method (Analox Glucose Analyzer; Analox Instruments), insulin using commercial electrochemiluminescence immunoassay kits (Roche E Modular, Roche Diagnostics, Indianapolis, IN), total- and high molecular weight (HMW) adiponectin

and leptin by ELISA (R&D Systems Inc., Minneapolis, MN) and total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides by enzymatic colorimetric assay (Roche Diagnostics, Indianapolis, IN) and low-density lipoprotein (LDL) cholesterol was calculated. Hs-CRP was measured using particle-enhanced immunonephelometry (Siemens Healthcare Diagnostics, Deerfield, IL). A concentration of CRP >3 mg/L was considered high risk [10-13] for data analysis.

The homeostatic model assessment (HOMA-IR) index was calculated as: HOMA-IR = fasting serum glucose(mmol/L) × fasting insulin(mcIU/mL)/22.5.

Assessment of Endothelial Function

Endothelial function was assessed by high-resolution Doppler ultrasonography examination of the right brachial artery as previously reported [8].

Statistical Analysis

Demographics, clinical characteristics and biochemical measurements were summarized by mean [standard deviation (SD)] or median [Interquartile Range (IQR)]). Pearson correlation or Spearman rank correlation was performed, as appropriate, to examine the association of spexin with leptin as well as with various biochemical measurements and brachial artery FMD.

A principal component analysis (PCA) was performed to combine the concurring characteristics of spexin and leptin levels. Principal components (PC) are independent linear combinations based on the patterns of the correlations of the original variables. PCs are structured by the magnitude of the total variability in the data set that they represent, such that the first PC (PC1) accounts for highest proportion of the total variance, followed by PC2 and so on. In case of two correlated variables, PC1 represents the concurring characteristics of the two variables. In the current study, both spexin and leptin met all assumptions for PC and a scatterplot of these two variables showed an estimated linear relationship between them. There were no outliers and the variables were not significantly deviated from the normality ($p > 0.05$ for Shapiro-Wilk test). A value of 0.7 for Kaiser-Meyer-Olkin (KMO) test indicated the sampling adequacy of the data. A significant Bartlett test of sphericity ($p = 0.03$) justified the application of principal component analysis of these two correlated variables. PC1 represents more than 75% of the variation in the data of these two variables. Spearman rank correlation or Pearson correlation coefficient, as appropriate, was used to examine the association between PC1 and cardiometabolic biomarkers. A two-sample test was used to compare the mean score of PC1 between the two groups based on hs-CRP concentration: one group with hs-CRP > 3 mg/L, which represented greater risk for CVD and the other group with hs-CRP < 3 mg/L with relatively lower risk for CVD. A cluster analysis of PC1 was performed to classify patients with distinct patterns of correlation between spexin and leptin levels. Variables with substantial correlation with PC1, including spexin and leptin, were characterized for the patients in distinct cluster groups. Median and interquartile ranges of these variables are presented by groups. Mann-Whitney U test was used to compare the median of these variables between groups. These variables were grouped in ordinal categories of median splits for further investigating the association with

cluster groups. Chi-square or Fisher exact test was used to compare the distribution. In addition, logistic regression analysis was performed to determine the association of the newly created ordinal categorical variables with cluster groups. The same analysis was performed to detect the association of the hs-CRP with cluster groups. Odds ratio (OR) and confidence intervals were calculated.

Results

Demographic, anthropometric and laboratory characteristics of the participants are provided in Table 1. A total of 19 children with obesity (age 15.8 ± 1.7 years, BMI 36.1 ± 6.03 kg/m^2, BMI z score 2.3 ± 0.5) were studied. Of the 19 participants, 11 had severe obesity as defined by BMI ≥ 120% of the 95th percentile values or a BMI ≥ 35 kg/m^2(whichever is lower) and 12 (63%) of the participants were male. The majority of the participants were non-Hispanic white (89.5%). All participants were ≥ Tanner stage 4. For clarity of discussion of the new data on spexin, adipokines and related data on certain factors that were reported previously[8] were also included in Table 1.

Median (IQR) for spexin for the patients in this study was 0.38 ng/mL (0.29-0.59 ng/mL). Spexin concentration in the study subjects was inversely correlated with leptin (Pearson correlation coefficient, r=-0.50, P=0.03) even after adjusting for age, sex, BMI and BMI z score (Figure 1).The first principal component (PC1) of spexin and leptin levels, that combined these two variables, accounted for 75% of the variance of these two variables. This composite variable was associated positively with spexin (r=0.87) and inversely with leptin (r=-0.87). The composite factor was also associated with insulin and hs-CRP. For insulin the Spearman rank correlation parameters were, ρ= -0.53, P=0.02. The means (SE) of PC1 were 0.43(.26) and -0.59(0.22) in the two hs-CRP groups of <3 mg/L and CRP>3 mg/L, P=0.02. Based on previous data [10-13] we divided the participants into high risk (hs-CRP ≥3 mg/L) and lower risk (hs-CRP <3 mg/L) groups. Cluster analysis of the PC1 resulted in two distinct groups. Reflecting the inverse concurrence of spexin and leptin levels, one group (group 1) represented the obese patients with high spexin (median 0.62 ng/mL, IQR 0.43,0.95) and low leptin (median 19.7 ng/mL, IQR 18.2,32.7) levels and the other group (group 2) represented those with low spexin (median 0.31 ng/mL, IQR 0.27, 0.40) and high leptin concentrations(median 58.2 ng/mL, IQR 48.9, 80.7). There were 8 patients in the high spexin and low leptin group and 11 patients in the low spexin and high leptin group. As expected, the spexin and leptin levels in the two groups were significantly different (P= 0.004 and P <0.001 respectively). Potential confounders such as sex, BMI, BMI z score, fasting glucose and lipids were not significantly different in the two groups. Insulin levels were lower in group with high spexin and low leptin (median 138.9 pmol/L, IQR: 99.3 pmol/L, 254.9 pmol/L) than in the group with low spexin and high leptin (median 247.2 pmol/L, IQR: 169.5pmol/L, 293.1 pmol/L) but these did not reach statistical significance (P=0.091). Table 2 presents the distribution of spexin, leptin, insulin and hs-CRP for patients by cluster groups. The number of patients for lower and upper than medians are presented. Odds ratio (OR) in the Table 2 compared the likelihood (odds) of having low spexin and high leptin (group 2) in patients with upper median split to the patients with lower median split for the corresponding variable. The odds (likelihood) of having the characteristics of the group with low spexin and high leptin in patients with high

hs-CRP (> 3 mg/L) was 12.3 times as high as it was in patients with low hs-CRP (<3 mg/L). Additionally, the odds (likelihood) of having the characteristics of the group with low spexin and high leptin in patients with high insulin (upper median split) was 2.9 times as high as it was in patients with low insulin (lower median split). However, spexin levels did not correlate with fasting glucose, HOMA-IR, lipids, total adiponectin and high molecular weight adiponectin, systolic and diastolic blood pressure, brachial FMD and RHI (P >0.05 for all).

Discussion

In the current study of post-pubertal adolescents with obesity, we demonstrate an inverse correlation between diminished circulating spexin concentration and hyperleptinemia. Another novel finding of the study is that the adolescents with “low spexin and high leptin” were significantly more likely to have higher levels of hs-CRP and insulin. To the best of our knowledge these findings are novel and are being reported for the first time in children and/or adolescents.

The inverse association between circulating spexin and leptin levels is novel in adolescents and/or children with obesity, but similar to the sparsely available data in adults[5]. Leptin is a satiety factor produced predominantly in adipocytes and plays an active role in appetite regulation and metabolism at the level of the hypothalamus via melanocortin receptors [14]. Hyperleptinemia in adults and children/adolescents with obesity has been consistently reported [15-18]. It reflects obesity-related leptin resistance or its lack of sensitivity for satiety related functions, potentially leading to its blunted effects on food intake [19-21]. In this context, the inverse association between spexin and leptin in the current study is important and it is tempting to speculate that this may suggest a potential role for spexin in the regulation of body weight and energy homeostasis. In fact, in animals (rats and mice) with diet induced obesity, spexin administration has been shown to decrease caloric intake and increase locomotion [5, 7]. Similarly, spexin decreased appetite in goldfish [3]. Quite intriguingly, our previous study that included normal weight children as well as children with obesity did not show a relationship between spexin and leptin [6]. Although we do not know the exact reasons for this discrepancy, it could be related to the differences in the characteristics of study participants including age and severity of obesity. Compared to our previous study, the participants in the current study were older, more obese and with higher degrees of insulin resistance. Leptin levels were also higher among the participants in the current study than that in our previous study, likely reflecting increased leptin resistance. Indeed, it remains to be determined if the relationship between spexin and leptin is influenced by age, severity of obesity and degrees of insulin and leptin resistance.

We further analyzed the contrasting characteristics of spexin and leptin concentrations in the participants in the current study. When combined to form a composite factor shared by spexin and leptin, it explained >75% of the variance in the dataset. Obviously, the composite variable showed strong positive and negative associations with the concentrations of spexin and leptin, respectively. More importantly, this composite variable involving spexin and leptin showed fascinating associations with hs-CRP and insulin. The likelihood of participants with high hs-CRP (>3 mg/L) was significantly higher compared to those with

lower hs-CRP (<3 mg/L) to be in a subgroup of subjects typified by low spexin and high leptin vs. high spexin and low leptin. Previous studies have shown that hs-CRP concentration of ≥3mg/L manifests significantly greater risk for CVD [10-13, 22]. Thus the subgroup with "low spexin and high leptin", which emerged from our analysis, represents a greater risk profile compared to those with "high spexin and low leptin". This observation is novel and important in adolescents with obesity considering the well-established fact that hs-CRP is a nonspecific, downstream marker of subclinical inflammation and its elevated concentrations independently predict CVD events in diverse populations [13, 22-25]. Various biomarkers and risk factors of CVD including elevated hs-CRP, are evident in children and adolescents[10, 13, 16, 17, 23-27]. Additionally, puberty in children and adolescents is associated with worsening of CVD risk factors including insulin resistance and carotid intima media thickness [13, 25-27]. If the composite factor shared by spexin and leptin can serve as a potential marker to stratify CVD risk in adolescents with obesity needs to be investigated in future studies with larger sample size. Further, we also observed an intriguing relationship between spexin and insulin levels as well in the current study. There was a higher chance of having the characteristics of the subgroup with "low spexin and high leptin" in participants with high insulin concentrations compared to those with lower insulin concentrations. While we consider this as an interesting observation, this should be viewed cautiously because the lack of direct and more sophisticated measures of insulinemia and/or insulin resistance in the current study. On the other hand, Gu and colleagues reported lower spexin levels in adults with type 2 diabetes relative to healthy counterparts [28]. They also noted negative association between spexin and fasting blood glucose, HbA1C and triglycerides[28]. Such associations were not evident in the participants of our study, however, and these data are similar to our previous study [6]. The risk for CVD risk factors such as fasting blood glucose and fasting insulin is dependent upon the duration and severity of obesity[13, 25]. Therefore, the lack of relationships observed in the current study, in contrast to the study in adults[28] may likely be related to the factors such as the age, duration, severity and complications of obesity in the participants in our study. The participants in the current study were overall healthier compared to the two previous studies in adults [5, 28]. Physical activity patterns also, most likely, differed in the participants in our study and those in the previous studies[5, 28]. Taken together, the relationship of spexin with leptin and presence of higher hs-CRP and insulin in those with low spexin/high leptin found in the current study, warrant further investigations on the role of obesity-related 'hypospexinemia' on long term health implications in children and adolescents.

The pathophysiological mechanisms that link spexin with obesity, insulin resistance and CVD remain unclear. Walewski et al reported that the gene encoding spexin (Cg12orf39) was the most down regulated gene in fat tissue in humans with obesity[2]. Circulating spexin concentrations, as well, were lower in children and adults with obesity than in their lean counterparts [5, 6]. Inverse correlations between spexin and leptin[5], spexin and fasting glucose, glycated hemoglobin and triglycerides[28] have been reported in adults. Spexin also appears to influence energy homeostasis by altering energy consumption as well as energy expenditure. In rodents with diet induced obesity (DOI), spexin administration reduced caloric intake and resulted in weight loss[5]. Spexin also reduced adipocyte uptake of long chain fatty acids in DIO mice and improved glucose tolerance and insulin resistance in DOI mice with type 2

diabetes mellitus [5, 7]. Reductions in the concentration of liver enzymes in mouse models of hepatic steatosis have also been reported with spexin administration[7]. In this context, although preliminary, the inverse association between spexin and leptin found in the current study along with the propensity for higher levels of hs-CRP and insulin in the "low spexin-high leptin" group suggests its potential role in obesity and risk for CVD. The current study is not designed to look at the underlying mechanisms. Interestingly, recent studies suggest that spexin mediated multiple biological processes are likely through the activation of the galanin receptor 2 (GALR2) in competition with galanin[7, 29].

Important strengths of our study include the ethnic homogeneity of the participants and the absence of obesity related comorbidities that are potential confounders. Limitations of our study include the small sample size, cross sectional nature of the study, lack of serial measurements and the absence of a non-obese control group for comparisons. Despite the strong evidence for inverse correlation between spexin and leptin, the small number of patients in the two subgroups ("high spexin/low leptin" and "low spexin/high leptin") resulted in limited statistical power to detect significant differences between the groups (if any) in other outcome variables. In the current study we were able to use only the brachial artery FMD for measurement of endothelial function. Further studies should examine arterial function using other methods including carotid intima thickness and pulse wave velocity. Another limitation, as noted above as well, was lack of a more sophisticated measurement of insulin resistance. The findings of the inverse correlation between spexin and leptin and differences in hs-CRP and insulin between the two subgroups based on spexin and leptin levels may not necessarily prove a causative role for spexin in obesity or obesity related comorbidities such as diabetes and CVD.

Conclusion

The inverse association between spexin and leptin in adolescents with obesity and the likelihood of having higher hs-CRP and insulin concentrations in the setting of low spexin and high leptin levels found in the current study are novel. Overall the data from the current study are intriguing and warrant further validation in larger diverse cohorts not only to further validate the clinical significance of the findings, but to delineate the underlying mechanisms involved as well.

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Abbreviations

DIO	diet induced obesity
CVD	cardiovascular disease
BMI	body mass index
SBP	systolic blood pressure
DBP	diastolic blood pressure
BP	blood pressure
hs-CRP	high sensitivity C- reactive protein
HDL cholesterol	high density lipoprotein cholesterol
LDL cholesterol	low density lipoprotein cholesterol
HMW adiponectin	high molecular weight adiponectin
FMD	flow-mediated dilation
RHI	reactive hyperemia index

What is already known about this subject?

- Spexin is a novel peptide, which is implicated in obesity and energy homeostasis.
- Circulating spexin levels are decreased in children and adults with obesity

What this study adds

- Spexin levels were inversely correlated with leptin levels in adolescents with obesity
- The odds of having “low spexin and high leptin” in participants with higher CRP (≥ 3mg/L) was significantly higher than that of participants with lower CRP
- The findings suggest a potential role for spexin in the regulation of satiety and certain cardiovascular risk factors in children with obesity.

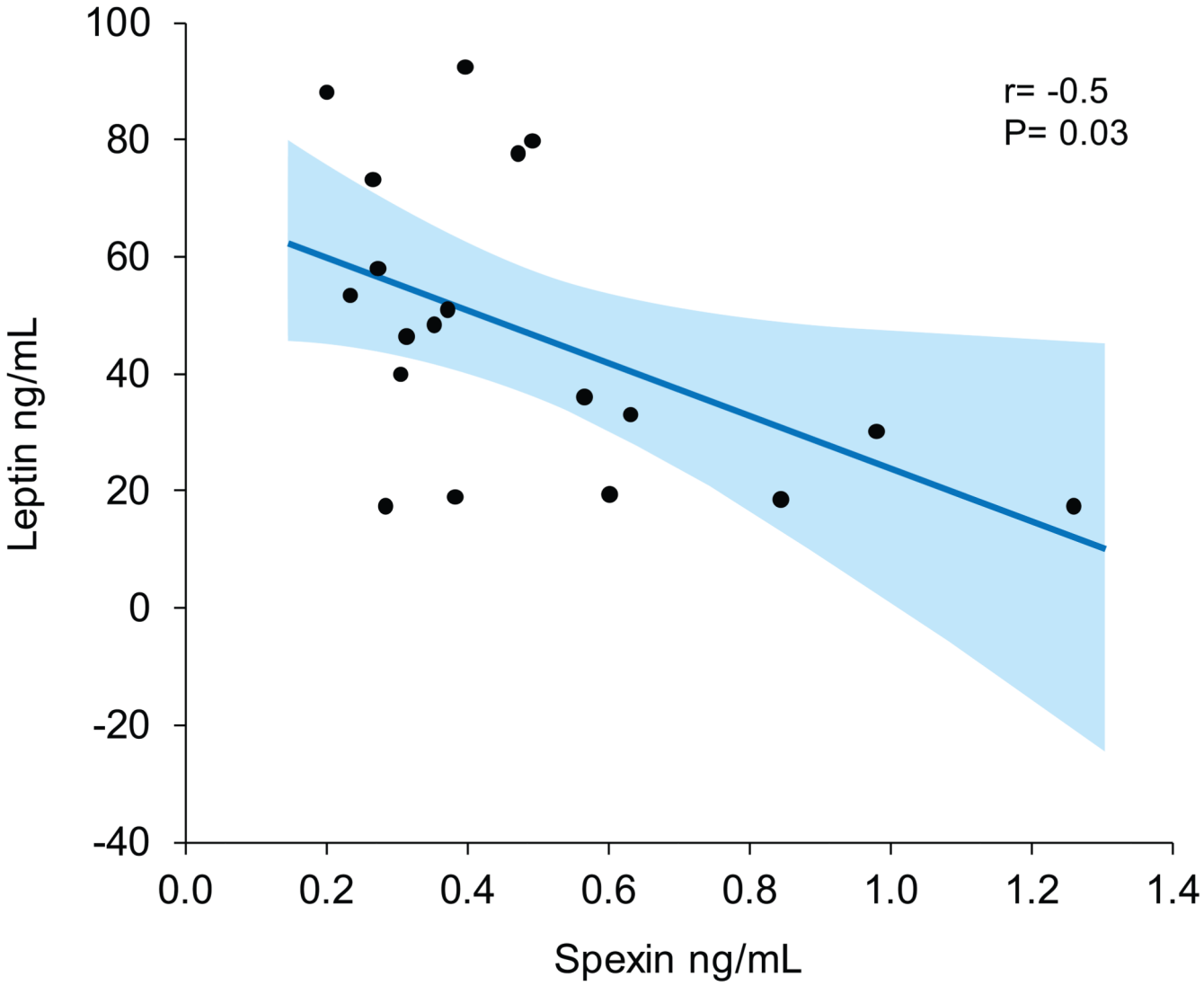

Figure 1. Correlation between spexin and leptin concentrations

Relationship between spexin (ng/mL) and leptin (ng/mL) in adolescents with obesity (n=19). Pearson correlation coefficient (*r*) and p-value (*P*) are included

Table 1
Anthropometric and laboratory characteristics of study participants

Characteristic or Measurement	Mean(SD) or median(IQR)*
Age,years	15.8(1.7)
Weight,kg	109.2(26.33)
Height,cm	168.7(16.6)
BMI, kg/m2	36.1(6.03)
BMI z score	2.35(0.56)
SBP, mm Hg	116.8 (8.11)
DBP, mm Hg	69.2 (8.75)
Total adiponectin μg/mL	5.44(1.26)
HMW adiponectin μg/mL	1.99(1.04)*
Leptin, ng/mL	47.86(25.27)
Fasting glucose, mmol/L	4.9 (0.28)
Fasting insulin, pmol/L	225.71(127.93)
FMD (%)	9.5 (3.52)
RHI (%)	449.3 (243.5)
HOMA-IR	7.11(4.18)
Spexin, ng/mL	0.38(0.31)*

*Values are presented as mean (standard deviation) unless otherwise indicated.

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure

HMW adiponectin, high molecular weight adiponectin; FMD, flow-mediated dilation; RHI, reactive hyperemia index; HOMA-IR, homeostatic model assessment insulin resistance

Table 2

Association of cluster groups with outcomes

Variable	Median (IQR)		Median Split	Number (Percentage) of Patients		*P*-value	OR (95% CI)
	Group 1*	Group 2 ɨ		Group 1*	Group 2 ɨ		
	N=8	N=11					
Spexin (ng/mL)	0.62 (0.43, 0.95)	0.31 (0.27, 0.40)	LEM	1 (11.1%)	8 (88.9%)	0.009	Ref
			GEM	7 (70.0%)	3 (30.0%)		0.05 (.004, 0.64)
Leptin (ng/mL)	19.7 (18.2, 32.7)	58.2 (48.9,80.7)	LEM	8 (88.9%)	1 (11.1%)	<0.001	n/a
			GEM	0 (0.0%)	10 (100.0%)		n/a
Insulin mcIU/mL	20 (14.3, 36.7)	35.6 (24.4, 42.2)	LEM	5 (55.6%)	4 (44.4%)	0.260	Ref
			GEM	3 (30.0%)	7 (70.0%)		2.9 (0.4, 19.2)
Hs-CRP mg/L	<3 mg/L	≥ 3 mg/L	LEM	7 (63.6%)	4 (36.4%)	0.026	Ref
			GEM	1 (12.5%)	7 (87.5%)		12.3 (1, 139)

Note: LEM(Lower median split) contains subjects with a value less than median; GEM(Upper median split) contains the subjects with a value greater than or equal to median; OR = odds ratio; CI= confidence interval; Ref= referent; OR is not available (n/a) as frequency of a cell is 0.

*(high level of spexin and low level of leptin)

ɨ(low level of spexin and high level of leptin)

Group 1 (min, max): Spexin (0.28, 1.26); Leptin (17.74, 36.47); Insulin (12.9, 94.9)

Group 2 (min, max): Spexin (0.20, 0.49); Leptin (40.51, 93.51); Insulin (20.1, 45.1)