

# Association between Nitrogen Stable Isotope Ratios in Human Hair and Serum Levels of Leptin

Song Vogue Ahn,<sup>1,2</sup> Sang-Baek Koh,<sup>1,2</sup> Kwang-Sik Lee,<sup>3</sup> Yeon-Sik Bong<sup>3</sup> and Jong-Ku Park<sup>1,2</sup>

<sup>1</sup>Department of Preventive Medicine, Yonsei University Wonju College of Medicine, Wonju, Korea

<sup>2</sup>Institute of Genomic Cohort, Yonsei University, Wonju, Korea

<sup>3</sup>Korea Basic Science Institute, Cheongju, Korea

Stable isotope ratios have been reported to be potential biomarkers of dietary intake and nutritional status. High serum levels of leptin, a hormone which regulates energy metabolism and food intake, are associated with insulin resistance and metabolic syndrome. However, little is known about the association between stable isotope ratios and the metabolic risk in humans. We investigated whether the carbon and nitrogen stable isotope ratios in hair are associated with serum leptin levels. Hair samples were collected from 399 healthy adults (233 men and 166 women) aged 40 to 70 years of a community-based cohort in Korea and the bulk stable isotope ratios of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) were measured for all hair samples. Serum leptin levels were analyzed by radioimmunoassay.  $\delta^{15}\text{N}$  showed positive correlations with serum leptin levels. In multivariate models, increasing  $\delta^{15}\text{N}$  were associated with elevated serum leptin levels (defined as  $\geq$  the median values), whereas  $\delta^{13}\text{C}$  were not significantly associated with serum leptin levels. The odds ratio (95% confidence interval) per 1‰ increase in  $\delta^{15}\text{N}$  for an elevated serum leptin level was 1.58 (1.11-2.26). In participants with high body mass index,  $\delta^{15}\text{N}$  showed positive associations with serum leptin levels, whereas these associations were not seen in participants with low body mass index. The nitrogen stable isotopic ratio in hair is positively associated with serum leptin levels. The hair  $\delta^{15}\text{N}$  could be used as a clinical marker to estimate metabolic risk.

**Keywords:** carbon; leptin; metabolism; nitrogen; stable isotope ratio

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## Introduction

The natural abundance ratio of stable isotopes has been used to provide quantitative information on the cycle of materials and food uptake in the bodies of animals and humans in ecological and archeological studies (O'Connell and Hedges 1999). These assessments are based on the fact that body proteins of animals and humans reflect their dietary history, which can be determined by analyzing stable isotopes. Recently, stable isotope ratios of carbon ( $^{13}\text{C}/^{12}\text{C}$ ,  $\delta^{13}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ,  $\delta^{15}\text{N}$ ) were proposed as potential biomarkers for dietary intake and nutritional status (Macko et al. 1999; Petzke et al. 2010). Individuals with high intake levels of animal protein are known to have higher  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  levels (Petzke et al. 2005; Huelsemann et al. 2009; Yeung et al. 2010). Diet is an important risk factor for diabetes and metabolic syndrome (Baxter et al. 2006; Lutsey et al. 2008; Oh et al. 2010), but little is known about the association of stable isotope ratios in relation to metabolic risk in humans. Only one previous study has

attempted to investigate the association between stable isotope ratios and metabolic syndrome (Park et al. 2015).

Leptin, known as a satiety hormone, is an adipokine synthesized and secreted by adipocytes and is a key regulator of food intake and body fat deposition (Friedman and Halaas 1998). It acts on the hypothalamus in the brain to control body weight and induces proinflammatory cytokines (Yamagishi et al. 2001; Patel et al. 2008). High serum levels of leptin are strongly associated with obesity, insulin resistance, and metabolic syndrome (Segal et al. 1996; Patel et al. 2008), and it has been proposed as a good predictor of cardiovascular disease (Wallace et al. 2001). However, it remains largely unknown whether stable isotope ratios of carbon and nitrogen are related to serum leptin levels. We thus explored the association of carbon and nitrogen stable isotope ratios in human hair with serum leptin levels.

## Methods

### *Study participants*

We analyzed the data from an ancillary study with a cross-sec-

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Correspondence: Song Vogue Ahn, Department of Preventive Medicine, Yonsei University Wonju College of Medicine, 20 Ilsan-ro, Wonju, Gangwon-do 26426, South Korea.  
e-mail: dodge@yonsei.ac.kr

tional design within the Korean Genome and Epidemiology Study on Atherosclerosis Risk of Rural Areas in the Korean General Population, a community-based cohort study that was designed to estimate the prevalence of, incidence of, and risk factors for cardiovascular and metabolic diseases such as hypertension, diabetes, dyslipidemia, and metabolic syndrome (Kim et al. 2013). Hair samples were collected from study participants for the stable isotopic analysis in a 2011 survey for the second follow-up (2011-2013). From May 24, 2011 to October 6, 2011, a total of 547 participants visited the survey center, and underwent comprehensive health examinations. However, 32 participants were excluded because their dietary habits during the past 1 year had changed relative to previous years. We further excluded 63 participants without hair samples and 53 participants who had not provided informed consent for the present study. We included 399 participants (233 men and 166 women) in the final analyses. Institutional Review Board of Yonsei University Wonju College of Medicine approved the study design and protocol.

#### Data collection

Body weights and heights were measured in light indoor clothing without shoes. A venous blood sample was drawn from study participants after fasting for  $\geq 12$  hours. Serum leptin levels were analyzed by radioimmunoassay (LINCO Research, Inc., St Charles, MO, United States). The intra-assay and inter-assay coefficients of variation for serum leptin levels ranged from 3.0% to 6.2%. The fasting serum glucose and insulin levels were determined with a glucose oxidase-based assay and double-antibody radioimmunoassay. The intra-assay and inter-assay coefficients of variation for serum insulin ranged from 2.1% to 8.3%. Serum levels of triglycerides and high-density lipoprotein (HDL) cholesterol were determined by enzymatic methods (ADVIA 1800, Siemens Healthcare Diagnostics, Tarrytown, NY, United States). Insulin resistance was calculated using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) method with the following formula: fasting insulin ( $\mu\text{IU/mL}$ )  $\times$  fasting blood glucose ( $\text{mg/dL}$ )/405 (Matthews et al. 1985).

#### Stable isotopic analysis

We preprocessed hair samples taken from the participants by a standard procedure (O'Connell and Hedges 1999; Fuller et al. 2005; Park et al. 2015). We measured bulk carbon and nitrogen isotopes by an isotope-ratio mass spectrometer (GV IsoPrime, Manchester, United Kingdom) which is interfaced with an elemental analyzer (EuroVector EuroEA3000 series, Milano, Italy) at the Korea Basic Science Institute. We expressed isotopic ratios in delta per mil notation ( $\delta$ , ‰), in parts per thousand corresponding to international standards: atmospheric air for nitrogen isotopes and Vienna Pee Dee Belemnite (PDB) for carbon isotopes. Each ratio is calculated as follows:

$$\delta (\text{‰}) = [(R_x/R_s) - 1] \times 1000$$

where  $R_x = (^{13}\text{C}/^{12}\text{C})$  or  $(^{15}\text{N}/^{14}\text{N})$  of the sample,  $R_s = (^{13}\text{C}/^{12}\text{C})$  or  $(^{15}\text{N}/^{14}\text{N})$  of the standard.

#### Statistical analysis

Data are expressed as the mean with standard deviation, median with interquartile range, or frequency with percentage. Data were assessed for normality, and natural logarithms of the leptin data were used for the analyses. Regression analyses were conducted to visualize the association between  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  values and serum leptin lev-

els. Odds ratios (ORs) with 95% confidence intervals (CIs) for the elevated serum leptin levels  $\geq$  the median values were calculated using multivariate logistic regression models. We used three models of adjustment for possible confounding variables. In model 1, age and sex were adjusted. In model 2, serum levels of fasting glucose, triglycerides, HDL cholesterol, and HOMA-IR were additionally adjusted. In model 3, body mass index (BMI) were additionally adjusted. Further, we examined the independent association between  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  values and serum leptin levels stratified by BMI. The "high BMI" group was defined as participants with BMI  $\geq$  the median values, and the "low BMI" group was defined as participants with BMI  $<$  the median values. The median BMI was 23.82  $\text{kg/m}^2$  for men and 23.77  $\text{kg/m}^2$  for women. Statistical significance was considered at  $P < 0.05$  for all comparisons. All statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, United States).

## Results

### Anthropometric and metabolic characteristics

Study participants were divided by median values for serum leptin levels. The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were higher in participants with high serum leptin levels than in participants with low serum leptin levels ( $\delta^{13}\text{C} = -20.49\text{‰}$  in participants with low serum leptin and  $\delta^{13}\text{C} = -20.30\text{‰}$  in participants with high serum leptin,  $P = 0.034$ ;  $\delta^{15}\text{N} = 11.42\text{‰}$  in participants with low serum leptin and  $\delta^{15}\text{N} = 11.72\text{‰}$  in participants with high serum leptin,  $P < 0.001$ ). HDL cholesterol was lower and BMI, HOMA-IR, serum levels of triglycerides, and fasting glucose were higher in participants with high serum leptin levels than in participants with low serum leptin levels (Table 1).

### Relation of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values to serum leptin levels

We performed regression analyses to visualize the association between  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  values and serum leptin levels. Natural logarithmic transformations of leptin values were used after checking for normality. The  $\delta^{15}\text{N}$  values showed positive correlations with serum leptin levels ( $P < 0.001$ ,  $R^2 = 0.059$  in men;  $P = 0.001$ ,  $R^2 = 0.063$  in women). The  $\delta^{13}\text{C}$  values showed no significant correlations with serum leptin levels (Fig. 1).

### ORs for elevated serum leptin levels according to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values

ORs were calculated for the elevated serum leptin levels, which were defined as greater than or equal to the median values of serum leptin levels. The median serum leptin level was 3.59  $\text{ng/mL}$  for men and 9.67  $\text{ng/mL}$  for women. Increasing quartiles of  $\delta^{15}\text{N}$  values were associated with elevated serum leptin levels, whereas  $\delta^{13}\text{C}$  values were not significantly associated with serum leptin levels. The ORs for elevated serum leptin levels when comparing participants in the highest to the lowest quartile of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were 1.54 (95% CI 0.69-3.41;  $P$  for trend = 0.293) and 2.67 (95% CI 1.09-6.51;  $P$  for trend = 0.034), respectively, in multivariable models adjusted for age, sex, fasting serum

Table 1. Characteristics of the study participants by serum leptin levels.

	Serum leptin		<i>P</i>
	Low (< median)	High (≥ median)	
N	197	202	
Age, years	62.0 ± 8.2	61.3 ± 8.2	NS
Men, n (%)	115 (58.4)	118 (58.4)	NS
Body mass index, kg/m <sup>2</sup>	22.3 ± 2.4	25.8 ± 3.0	< 0.001
Fasting glucose, mg/dL	96.5 ± 18.0	101.0 ± 13.6	0.005
HDL cholesterol, mg/dL	54.0 ± 12.0	52.9 ± 12.5	NS
Triglycerides, mg/dL	111.0 (78.0, 140.0)	129.0 (97.0, 179.0)	< 0.001*
HOMA-IR, units	1.39 (1.10, 1.78)	1.94 (1.54, 2.59)	< 0.001*
δ <sup>13</sup> C, ‰ vs. PDB	-20.49 ± 0.89	-20.30 ± 0.87	0.034
δ <sup>15</sup> N, ‰ vs. air	11.42 ± 0.83	11.72 ± 0.84	< 0.001
Leptin, ng/mL	3.13 (2.41, 5.16)	6.99 (4.34, 13.78)	< 0.001 <sup>a</sup>

Values are expressed as the mean ± standard deviation or median (25<sup>th</sup> quartile, 75<sup>th</sup> percentile). The median serum leptin level was 3.59 ng/mL for men and 9.67 ng/mL for women.

\**P* value from Mann-Whitney *U* tests.

HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; PDB, Pee Dee Belemnite; NS, not significant.

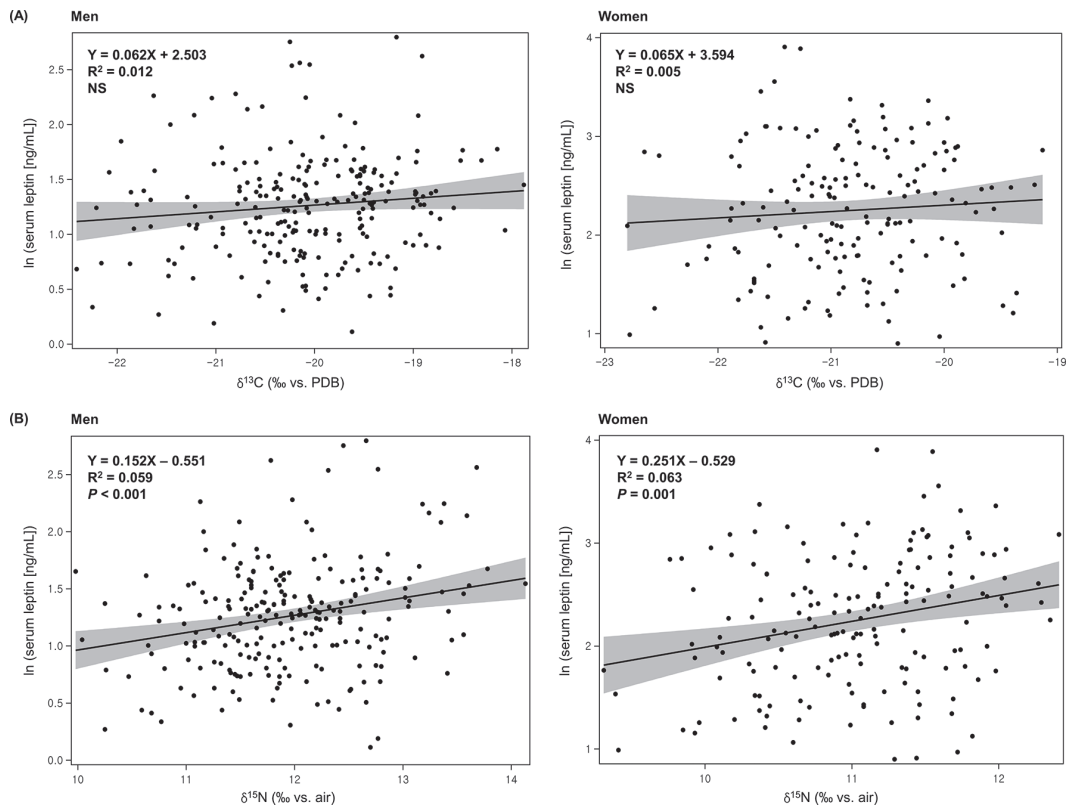


Fig. 1. Relation of δ<sup>13</sup>C and δ<sup>15</sup>N to serum leptin.

(A) δ<sup>13</sup>C. (B) δ<sup>15</sup>N. Scatter plot with regression line (black solid line) and 95% CI (grey area).

glucose, triglycerides, HDL cholesterol, HOMA-IR, and BMI (Table 2).

The OR (95% CI) per 1‰ increase in  $\delta^{15}\text{N}$  for elevated

serum leptin levels was 1.58 (1.11-2.26) after adjusting for age, sex, fasting serum glucose, triglycerides, HDL cholesterol, HOMA-IR, and BMI. When we divided participants

Table 2. Association between  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and serum leptin levels.

	OR* (95% CI)				P for trend
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	
$\delta^{13}\text{C}$ , ‰ vs. PDB	< -21.00	-21.00 to -20.38	-20.37 to -19.77	$\geq -19.76$	
Model 1	1.00	1.13 (0.64-2.00)	1.49 (0.82-2.70)	1.69 (0.89-3.21)	NS
Model 2	1.00	1.19 (0.63-2.25)	1.24 (0.63-2.43)	1.82 (0.88-3.76)	NS
Model 3	1.00	1.13 (0.55-2.29)	1.22 (0.58-2.60)	1.54 (0.69-3.41)	NS
$\delta^{15}\text{N}$ , ‰ vs. air	< 11.48	11.48 to 11.89	11.90 to 12.44	$\geq 12.45$	
Model 1	1.00	1.90 (1.05-3.44)	2.83 (1.51-5.31)	3.62 (1.78-7.38)	< 0.001
Model 2	1.00	2.52 (1.28-4.95)	3.37 (1.64-6.92)	3.96 (1.73-9.03)	0.001
Model 3	1.00	2.32 (1.11-4.82)	2.66 (1.22-5.84)	2.67 (1.09-6.51)	0.034

\*ORs were calculated for the elevated serum leptin levels  $\geq$  median values. The median serum leptin level was 3.59 ng/mL for men and 9.67 ng/mL for women.

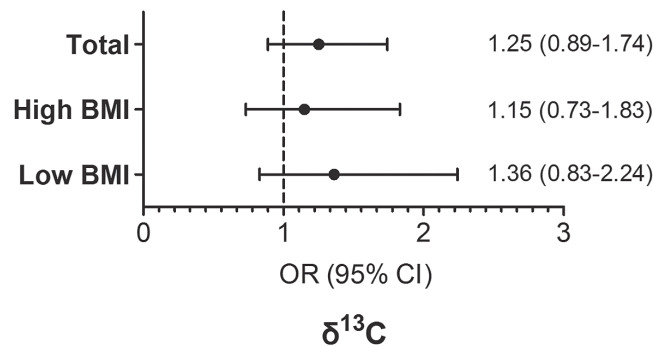
Model 1: adjusted for age, sex.

Model 2: Model 1 + additional adjustment for fasting serum glucose, triglycerides, HDL cholesterol, HOMA-IR.

Model 3: Model 2 + additional adjustment for BMI.

OR, odds ratio; CI, confidence interval; NS, not significant; PDB, Pee Dee Belemnite; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; BMI, body mass index.

(A)



(B)

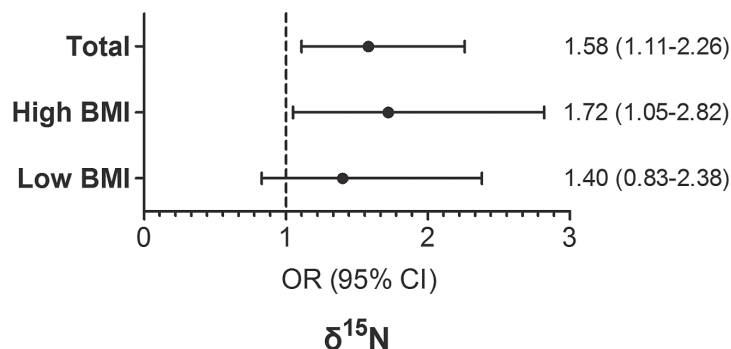


Fig. 2. Association between  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and serum leptin levels by body mass index.

(A)  $\delta^{13}\text{C}$ . (B)  $\delta^{15}\text{N}$ . ORs were calculated for the elevated serum leptin levels  $\geq$  median values after adjusting for age, sex, fasting serum glucose, triglycerides, HDL cholesterol, HOMA-IR, and BMI. The median serum leptin level was 3.59 ng/mL for men and 9.67 ng/mL for women. The high BMI group was defined as participants with BMI  $\geq$  median values; the low BMI group was defined as participants with BMI of < median values. The median BMI was 23.82 kg/m<sup>2</sup> for men and 23.77 kg/m<sup>2</sup> for women.

by BMI, the positive association between a 1‰ increase in  $\delta^{15}\text{N}$  and an elevated serum leptin level remained intact in participants with high BMI (OR 1.72, 95% CI 1.05–2.82), whereas the association between a 1‰ increase in  $\delta^{15}\text{N}$  and an elevated serum leptin level was not significant in participants with low BMI. A 1‰ increase in  $\delta^{13}\text{C}$  was not associated with an elevated serum leptin level (Fig. 2).

### Discussion

This is the first study evaluating the association of stable isotope ratios of carbon and nitrogen with serum leptin levels. In the present study, hair  $\delta^{15}\text{N}$  values showed a positive association with serum leptin levels after adjusting for age, sex, fasting serum glucose, triglycerides, HDL cholesterol, HOMA-IR, and BMI. This association was not apparent in participants with low BMI. Leptin is an adipokine that has an important role in the regulation of energy homeostasis. Leptin, serving as a cofactor for TGF- $\beta$  activation, promotes endothelial cell proliferation (Wolf et al. 1999, 2002; Wolf and Ziyadeh 2006). Leptin also activates the sympathetic nervous system and causes chronic elevations in blood pressure (Carlyle et al. 2002). Epidemiological evidence suggests that serum leptin levels are positively associated with obesity, insulin resistance, and metabolic syndrome and that they are a good predictor of cardiovascular disease (Segal et al. 1996; Wallace et al. 2001; Patel et al. 2008). These results indicate that  $\delta^{15}\text{N}$  could be a surrogate marker to estimate metabolic and cardiovascular risk.

A possible explanation for the association between  $\delta^{15}\text{N}$  values and serum leptin levels is that nitrogen stable isotope ratios are nutritional biomarkers for dietary meat intake (O’Connell and Hedges 1999; Petzke et al. 2005; Patel et al. 2014). Previous studies have reported that high levels of meat consumption are associated with metabolic risk (Baxter et al. 2006; Lutsey et al. 2008; Oh et al. 2010), which might have been an important factor in the association between  $\delta^{15}\text{N}$  and serum leptin levels. Elevated  $\delta^{15}\text{N}$  is in relation to the kinetic distribution of nitrogen stable isotope in amino acid transamination, which causes higher excretion of lighter nitrogen stable isotope and abundance of  $^{15}\text{N}$  in the body (Hulsemann et al. 2009). In a United Kingdom study, individuals with high levels of animal protein intake had higher  $\delta^{15}\text{N}$  values than vegans, which suggests that nitrogen stable isotope ratios could be a nutritional biomarker for meat intake (O’Connell and Hedges 1999).

It is also possible that  $\delta^{15}\text{N}$  values might be an indicator of metabolic risk factors, such as persistent organic pollutants and heavy metals, which are known to be related to metabolic risk (Lee et al. 2007; Ruzzin et al. 2012; Lee and Kim 2013). Nitrogen stable isotopes, persistent organic pollutants, and heavy metals show similar bioaccumulation patterns in the marine food chain (Lee et al. 2006; Dempson et al. 2010; Berntssen et al. 2011). A previous study reported an abundance of nitrogen stable isotope ratios in

Inuit populations with high levels of marine food consumption (Buchardt et al. 2007). Given that  $\delta^{15}\text{N}$  were associated with fish intake, the  $\delta^{15}\text{N}$  related to fish intake could be associated with persistent organic pollutants or heavy metals, which may result in the positive correlation of  $\delta^{15}\text{N}$  with metabolic risk (Williams and O’Connell 2002; Kuhnle et al. 2013). Nitrogen stable isotope ratio has been correlated to mercury concentration (Yoshinaga et al. 1992). It has also been reported that leptin is related to exposure to heavy metals, such as lead (Yang et al. 2014). Further studies are needed to understand the association between  $\delta^{15}\text{N}$  values and serum leptin levels in relation with metabolic risk.

In the present study,  $\delta^{15}\text{N}$  values were associated with serum leptin levels in participants with high BMI. Serum leptin levels are closely associated with the amount of body fat. Excess adiposity is associated with the up-regulation of leptin production, contributing to insulin resistance and metabolic disorders, such as type 2 diabetes and metabolic syndrome (Patel et al. 2008). Leptin levels are reported to be proportional to body adiposity and to be increased in obese individuals (Beltowski 2006). These findings suggest that adiposity may play an important role in the association between nitrogen stable isotope ratios and serum leptin levels.

This study indicates that  $\delta^{13}\text{C}$  values of hair are not associated with serum leptin levels. The carbon stable isotope ratio in the body is contributed by the proportion of C4 plants (e.g., cane and maize) and C3 plants (e.g., wheat, rice, and vegetables) in the diet of an individual (Jahren et al. 2006; Hulsemann et al. 2009). We have reported no significant association between carbohydrate intake and  $\delta^{13}\text{C}$  values, which may be attributed to the main source of carbohydrate intake, which is rice, a C3 plant in Korean diets (Park et al. 2015). Previous studies have reported a positive correlation between  $\delta^{13}\text{C}$  and animal protein consumption (Petzke et al. 2005; Petzke and Lemke 2009). On the contrary,  $\delta^{13}\text{C}$  values are negatively associated with diabetes (Patel et al. 2009, 2014). Our result showing no association of  $\delta^{13}\text{C}$  with serum leptin levels may be contributed by the conflicting associations between  $\delta^{13}\text{C}$  and dietary meat consumption and diabetes.

Several limitations of this study should be considered. First, our analyses were based on a single measurement of serum leptin, which may be a source of random error. Second, the generalization of the results might be limited because the study participants were restricted to middle-aged and elderly Koreans. Third, because this was a cross-sectional study, the direction of causal relationship between  $\delta^{15}\text{N}$  and serum leptin levels may not be confirmed. A prospective study may be needed to confirm the direction of causality.

In conclusion, the nitrogen stable isotope ratio of hair has an independent association with serum leptin levels. This association was not seen in the low BMI group. The  $\delta^{15}\text{N}$  of hair could be used as a clinical marker to estimate



metabolic risk. Further studies are necessary to interpret the association of stable isotope ratios with clinical disease.

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### Conflict of Interest

The authors declare no conflict of interest.

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