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Level of proinsulin in association with cardiovascular risk factors and sleep snoring

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Abstract

AIM: To explore the relationship between the level of proinsulin with cardiovascular risk factors and sleep snoring.

METHODS: Based on the random stratified sampling principle, 1 193 Chinese residents in Pizhou City, Jiangsu Province (530 males and 663 females, aged 35-59 years with an average age of 46.69 years) were recruited. Their sleep snoring habits were investigated. Biotin-avidin based double mAbs ELISA was used to detect specific insulin and proinsulin, and a risk factor score was established to evaluate the individuals according to the number of their risk factors.

RESULTS: The results of Spearman correlation analysis and covariate ANOVA analysis after age and sex were controlled, indicated that not only the level of proinsulin ($r = 0.156$, $P = 0.000$, $F = 5.980$ $P = 0.000$), but also cardiovascular risk factors score ($r = 0.194$, $P = 0.000$, $F = 11.135$, $P = 0.000$) significantly associated with the frequency of sleep snoring, and the significant relationship between true insulin and frequency of sleep snoring was only shown in the covariate ANOVA analysis ($F = 2.868$, $P = 0.022$). The result of multivariate stepwise logistic regression after age, sex, body mass index, waist circumference and true insulin were controlled showed that proinsulin (division by interval of quartile) was an independent risk factor for sleep snoring (OR = 1.220, 95%CI: 1.085-1.373, $P = 0.001$).

CONCLUSION: The interaction of cardiovascular risk factors clustering, high proinsulin level and sleep breathing disorder may be a syndrome, which has not been recognized in human beings so far.

INTRODUCTION

Dyslipidemia, hypertension, hyperinsulinemia and obesity (special central obesity) have been recognized as potent risk factors for coronary heart disease in adults, the clustering of the above cardiovascular risk factors often occurs in adults, and the condition has been termed as syndrome X[1], and insulin resistance emerges as a common pathogenetic denominator underlying the above risk factor clustering[2]. Obstructive sleep apnea syndrome is associated with the increased cardiovascular and cerebrovascular morbidity. It is also recognized that many people with obstructive sleep apnea syndrome have features of the insulin resistance syndrome, and it is suggested that insulin resistance syndrome may actually include sleep apnea syndrome and could be better considered as syndrome Z[3]. The level of circulating proinsulin (PI) has been recognized as a sensitive marker for the dysfunction of islet beta cells[4], and several groups reported that proinsulin was more strongly associated with cardiovascular disease than true insulin[5]. So far, few epidemiological studies have been focused on the relationship between true insulin *vs* proinsulin and cardiovascular risk factors clustering, and sleep breathing disorder. Although polysomnography is the definitive method for diagnosing sleep apnea syndrome, the costs of this method in combination with the high demand for accurate screening limits its utility in clinical practice and epidemiological studies. It has been proposed that snoring is a precursor to the development of obstructive sleep apnea syndrome. This theory is supported by retrospective studies in which patients reported snoring more loudly over the years, before they developed nocturnal respiratory pauses, which later became increasingly frequent[6]. To explore the relationship between PI *vs* true insulin and

cardiovascular risk factors and sleep snoring, a population-based epidemiological investigation was conducted in Pizhou city, located in the mideastern part of China.

MATERIALS AND METHODS

Study subjects

From April 2001 to May 2001, a large cross-sectional, community-based epidemiological study was conducted. The subjects for the survey were adults aged between 35 and 59 years. A two-stage cluster-sampling scheme based on existing census divisions was used to randomly select four areas, each with a population from 300 to 350 subjects, and sample was stratified by sex and age group (5 years) to ensure representation of each part of the population. Among the 1 351 individuals investigated, the response rate was 88.5%, and the random sample and random-sample responder populations closely reflected the actual distribution of age group and sex in Pizhou area. Among 1 193 rural residents, there were 530 males (44.57%) and 663 females (55.43%), and their average age was 46.69 years. Signed informed consent was obtained from all participants and the study was approved by the Nanjing Medical University Ethics Review Committee.

Questionnaires and investigators

A standard questionnaire was adopted, and the investigators were students from Nanjing Medical University, who had received special training. The questionnaire included questions about occupation, height, weight, sleep habits, sleep quality, and frequency of disruptive snoring. The question concerning snoring was "How often do you snore loudly and disturbingly?" with the following response alternatives: 1 = never, 2 = seldom, 3 = sometimes, 4 = often, 5 = very often, and those who answered 2-5 were regarded as snorers.

Anthropometric measurements

Anthropometric measurements were performed after the participants had removed their shoes and upper garments and donned an examining gown. Each measurement was performed twice and the average was used in the analysis. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Weight was measured to the nearest 0.1 kg using a hospital balance beam scale. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m²). The waist circumference was measured to the nearest 0.5 cm at the point of narrowing between the umbilicus and xiphoid process (as viewed from behind) and the waist circumference was used as a judgment of upper-body adiposity. Blood pressure was measured in the right arm with the participant seated and the arm bared. Three readings were recorded for each individual, and the average of the three readings was defined as the subject's blood pressure.

Laboratory measurement

The 12-h fasting blood samples were drawn in the morning and the sera were stored at -70 ℃ immediately after centrifugation until being assayed. All laboratory measurements were conducted at the Central Clinical Laboratory in the First Affiliated Hospital of Nanjing Medical University. Fasting blood glucose (FBG), fasting total cholesterol (TCH), fasting triglyceride (TG) and fasting high-density lipoprotein cholesterol (HDL-c) were determined by enzymatic procedures on an automated autoanalyzer (AU 2700 Olympus, 1st Chemical Ltd., Japan), and the source of reagents for the autoanalyzer was from 1st Chemical Ltd., Japan. The laboratory was monitored for precision and accuracy of glucose and lipid measurements by the agency's surveillance program. Measurements of agency-assigned quality control samples showed no consistent bias over time within or between surveys. Low-density lipoprotein cholesterol (LDL-c) was assessed by the Friedwald method[7], and the concentration of LDL-c was calculated for each subject according to the following formula: $C_{LDL-c} = C_{PLASMA} - C_{HDL} - TG/5$.

Measurement of intact insulin and proinsulin

The intact insulin level was measured using a highly sensitive two-site sandwich ELISA[8]. This assay method for insulin is based on the enzyme immunoassay principle and it is constructed as a sandwich immunoassay. Two monoclonal murine antibodies specific for insulin were employed. One of the antibodies (HUI-018), which binds to an epitope on one side of the insulin-molecule, was used for coating on the ELISA plate wells. The other antibody (OXI-005), which binds to another epitope on the other side of the insulin-molecule, was covalently bound to biotin. The biotin-antibody reagent and sample or calibrator were added to the precoated-wells followed by incubation. During incubation, a complex between plastic-surface, coated-antibody, insulin and the biotinylated-antibody was formed. This complex would not be removed by the washing procedure, which followed the incubation. After the wash, avidin-peroxidase was added. It bound to the biotin of the biotinylated-antibody and extended the complex with the enzyme peroxidase. The amount of enzyme was visualized by addition of the substrate peroxide and 3',3',5',5'-tetramethylbenzidine (TMB), which was converted to a soluble colored product. The developed color was proportional to the amount of insulin in the sample. The color-development progressed with time and it was interrupted after a fixed time by the addition of phosphoric acid. The color was stable and absorbance was measured in an ELISA-plate photometer. A calibrator curve was constructed based on the absorbance values and the insulin concentration in the serum samples could be found. The detection limit was 5.0 pmol/L. The specificity of the assay excluded intact, split (32-33) and des (31, 32) PI. There was some cross-reactivity with the less abundant split (65-66) PI (30%) and des (64, 65) PI (63%).

The PI level was measured in a similar manner using another sensitive two-site sandwich ELISA[9]. The analysis for human PI is an enzyme immunoassay constructed as a sandwich immunoassay. Two monoclonal mouse antibodies were used: one with specificity for human C-peptide (PEP-001) was used for coating, the other antibody (HUI-001) with specificity for insulin was biotin labeled and acted as detecting antibody. The plasma samples were diluted 1:2 during analysis to eliminate the plasma effect. In the first reaction, after the samples were applied to the coated wells, PI in the samples would bind to the coating antibody. In the

second reaction, the bound PI was detected by the use of another mAb directed against human insulin and biotin labeled. In a third reaction, the amount of bound detecting antibody in the coating antibody-PI-detection antibody complex was visualized by the use of streptavidin-peroxidase that bound to the biotin label. Between each step, the plate was washed four times in a washing buffer in order to measure only the bound material. Finally, a substrate for the peroxidase (tetramethylbenzidine and H_2O_2) was applied to the wells resulting in color development proportional to the amount of peroxidase, i.e., PI present. The enzymatic reaction resulted in development of a blue color, which changed into yellow, when the reaction was stopped with H_3PO_4. The plate was read in an ELISA reader at 450 nm and the absorbance at 620 nm was subtracted. The detection limit in human serum was 0.25 pmol/L. There was no cross-reactivity with human insulin and human C-peptide. However, the four major PI conversion intermediates reacted at various proportions of 65-99%.

The between- and within-assay coefficients of variation were 6.8, 7.8% for true insulin respectively and 6.7, 7.8% for PI respectively. All measurements were performed in duplicate. The four mAbs including OXI-005, HUI-018, PEP-001 and HUI-001 were kind gifts from Novo Nordisk, Bagsvaerd, Denmark, and the standard samples of true insulin and PI were supplied by the Mercodia Company, Sweden. The ELISA-plate photometer (Bio-tek EL900, USA) and ELISA plate (NUNC company, Denmark) were employed in the assay.

Definition of risk factors
To investigate the relationship between the level of PI and cardiovascular risk factors clustering, we created a risk factor score to rank individuals according to the number of the risk factors at the time of the survey. The following risk factors and cut-off points were used to build up the risk factor scores: (1) hypertension was defined when systolic blood pressure (SBP) was $\geqslant$140 mmHg and/or diastolic blood pressure (DBP) $\geqslant$90 mmHg or taking anti-hypertensive drugs because of previous hypertension according to the 1999 WHO/ISH criteria[10]; (2) hyperglycemia was diagnosed based on the FBG serum glucose $\geqslant$6.1 mmol/L according to the American Diabetes Association (ADA) criteria[11] or having a history of diabetes mellitus; (3) hypercholesterolemia was defined as fasting TCH$\geqslant$5.20 mmol/L; (4) high LDL-c was defined as LDL-c$\geqslant$3.38 mmol/L; (5) low HDL-c was defined as HDL-c$\leqslant$1.04 mmol/L; (6) hypertriglyceridemia was defined as fasting TG$\geqslant$1.70 mmol/L[12]; (7) overall overweight was considered as BMI $\geqslant$25.0 kg/m² according

to the WHO guideline[13]; (8) visceral obesity was defined as waist circumference $\geqslant$85 cm in male and $\geqslant$80 cm in female[14]. The final risk factor score varied from 0 to 5, with 0 meaning no exposure to these risk factors; one exposure to any one risk factor; 2-4 exposure to any combination of 2-4 risk factors respectively; five exposure to any combination of five or more than five risk factors simultaneously.

Statistical analysis
All data analyses were performed using statistical package for social science (SPSS for Windows, version 10.0, 1999, SPSS Inc, Chicago, IL, USA). Data of BMI, waist circumference, age and blood pressure were normally distributed parameters and presented as mean±SD, whereas skewed data including FBG, fasting lipid, fasting true insulin, and PI were logarithmically transformed before analysis and were expressed as median (quartile range).

RESULTS
Sex and age distribution of sleep snoring subjects
Tables 1 and 2 show sex and age distribution of sleep snoring in the population-based epidemiological study. The results indicated that the frequency of sleep snoring in males was significantly higher than in females ($\chi^2 = 68.227$, $P<0.001$), and the frequency of sleep snoring significantly increased with the elevation of age ($\chi^2 = 44.465$, $P<0.001$).

Table 1 Sex distribution of sleep snoring subjects

Snoring	Male	Female	Total
Never	265	481	746
Seldom	34	29	63
Sometimes	83	68	151
Often	59	39	98
Very often	89	46	135
Total	530	663	1 193
Statistical parameter	$\chi^2 = 68.227$	$P<0.001$	

Spearman correlation analysis of sleep snoring with tested parameters
The results of Spearman correlation analysis (Table 3) indicated that the frequency of sleep snoring was significantly correlated with such parameters as BMI, waist circumference, SBP, DBP, fasting TCH, fasting TG, fasting LDL-c, PI, and risk factors score ($P<0.01$). However, the significant correlation was not found between the frequency of sleep snoring and FBG, true insulin, and fasting HDL-c ($P>0.05$).

Table 2 Age distribution of sleep snoring subjects

Snoring	35~	40~	45~	50~	55~	Total
Never	225	144	143	113	121	746
Seldom	17	5	9	14	18	63
Sometimes	39	18	31	28	35	151
Often	18	13	23	18	26	98
Very often	22	18	25	30	40	135
Total	321	198	231	203	240	1 193
Statistical parameter			$\chi^2 = 44.465$	$P<0.001$		

Table 3 Spearman correlation analysis of sleep snoring with tested parameters

Parameters	BMI	WC	SBP	DBP	FBG	CH
Correlation coefficient	0.177[b]	0.268[b]	0.211[b]	0.191[b]	0.027	0.126[b]
Parameters	TG	HDL	LDL	TI	PI	RFS
Correlation coefficient	0.132[b]	-0.043	0.108[b]	0.050	0.156[b]	0.194[b]

[b]$P<0.01$ vs others. Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; CH, fasting total cholesterol; TG, fasting triglycerides; HDL, fasting high-density lipoprotein cholesterol; LDL, fasting low-density lipoprotein cholesterol; TI, true insulin; PI, proinsulin; RFS, risk factors score.

Analysis of covariate variance of sleep snoring with tested parameters

Table 4 summarizes the results of analysis of covariate variance of sleep snoring and its relevant parameters. In the analysis, age and sex were controlling variables; the frequency of sleep snoring was factor variable, and the parameters including BMI, waist circumference, SBP, DBP, FBG (logarithmically transformed value), TCH (logarithmically transformed value), TG (logarithmically transformed value), HDL (logarithmically transformed value), LDL (logarithmically transformed value), true insulin (logarithmically transformed value), PI (logarithmically transformed value), and risk factors score were employed as dependent variables, respectively. The results indicated that a significant difference in parameters including BMI, waist circumference, SBP, DBP, TCH, TG, true insulin, PI, and risk factors score was found among various sleep snoring groups respectively after controlling for age and sex, and significant difference was not found in FBG, HDL-c, LDL-c between different frequencies of sleep snoring groups.

Analysis of multivariate logistic regression with sleep snoring as an independent variable

The results of multivariate logistic regression analysis are shown in Table 5, in which the dependent variable was the frequency of sleep snoring (the never snoring was coded as 0, and the seldom, sometimes, often, and very often snoring was considered as sleep snoring and was coded as (1), and age stratum (age stratification was performed with 5-year age strata), sex (male was coded as 1, female as 0), BMI (group division by an interval of quartile), waist circumference (group division by an interval of quartile), true insulin (group division by an interval of quartile), and PI (group division by an interval of quartile) were employed as independent variables. In the analysis of multivariate logistic regression, the variables including sex, age, PI, and waist circumference were accepted by the final model, and BMI and true insulin were refused by the regression model. The final model suggested that male, age, PI, and waist circumference were risk factors for sleep snoring, and the OR (95%CI) for sex, age, PI, and waist circumference was 0.401 (0.311-0.518)

Table 4 Analysis of covariate variance with sleep snoring as factor variable, and age and sex as controlling variables

Para-meters	Never	Seldom	Some-times	Often	Very often	F	P
BMI	23.49±2.83	24.08±2.89	24.14±2.84	24.56±3.66	25.32±3.47	16.335	0.000
WC	75.89±7.78	78.68±8.31	78.83±7.89	81.65±9.98	83.37±10.63	22.000	0.000
SBP	121.03±19.05	128.77±21.08	126.39±20.28	126.80±20.29	133.93±23.16	7.169	0.000
DBP	77.25±10.66	79.48±10.75	80.20±11.58	80.68±13.89	85.59±13.59	9.957	0.000
FBG	4.44 (4.06-4.89)	4.40 (4.17-4.80)	4.53 (4.12-4.93)	4.47 (4.08-4.81)	4.53 (4.07-4.88)	0.124	0.974
CH	3.95 (3.39-4.54)	4.25 (3.32-5.01)	4.09 (3.67-4.60)	4.00 (3.49-4.65)	4.34 (3.70-5.04)	2.580	0.007
TG	0.75 (0.55-1.08)	0.77 (0.59-1.22)	0.91 (0.62-1.31)	0.79 (0.61-1.33)	0.89 (0.63-1.59)	5.778	0.000
HDL	1.07 (0.89-1.28)	1.15 (0.93-1.31)	1.02 (0.83-1.23)	1.03 (0.80-1.28)	1.04 (0.83-1.29)	1.693	0.149
LDL	2.44 (2.02-2.85)	2.55 (2.03-3.10)	2.60 (2.19-3.10)	2.53 (2.13-2.96)	2.74 (2.19-3.21)	1.392	0.234
TI	4.81 (2.89-6.98)	4.69 (2.54-7.09)	5.11 (3.55-7.18)	5.13 (2.88-7.50)	5.06 (3.16-7.58)	2.868	0.022
PI	3.26 (1.92-5.11)	3.29 (1.77-5.19)	4.03 (2.46-6.49)	4.19 (2.22-6.09)	4.43 (2.72-7.18)	5.980	0.000
RFS	1.24±1.15	1.47±1.33	1.57±1.27	1.70±1.28	2.06±1.53	11.135	0.000

Abbreviations as in Table 3.

Table 5 Analysis of multivariate logistic regression with sleep snoring as an independent variable

Variable	B	S.E.	WALD	P	OR (95%CI)
Sex	-0.913	0.130	49.133	0.000	0.401 (0.311-0.518)
Age (yr)	0.223	0.044	25.762	0.000	1.249 (1.146-1.361)
Proinsulin	0.199	0.060	10.964	0.001	1.220 (1.085-1.373)
Waist circumference	0.335	0.062	29.504	0.000	1.398 (1.239-1.578)
Constant	-1.150	0.305	14.192	0.000	-

B, SE, and WALD were statistical parameters.

(P = 0.000), 1.249 (1.146-1.361, P = 0.000), 1.220 (1.085-1.373) (P = 0.001), and 1.398 (1.239-1.578) (P = 0.000), respectively.

DISCUSSION

Snoring is the main symptom of obstructive sleep apnea syndrome, which is one of the most frequent sleep disorders affecting 2-4% of middle-aged white[15] and Asian populations[16]. A predominantly Chinese population-based epidemiological study was conducted in Singapore, in which, 220 people aged 30-60 years, were interviewed for their snoring habits, and 87.5% of loud habitual snorers had significant obstructive apneas on the polysomnogram and 72% of these apneics complained of excessive daytime sleepiness (EDS)[17]. The results from a population-based study investigating the long-term outcome in men with snoring over a 10-year period suggest, that snoring is the preceding form of obstructive sleep apnea syndrome[18]. PI is a multi-peptide with 86 amino acids and its molecular weight is 9 390. It is synthesized in the β-cells of the islets of Langerhans and is subsequently processed through enzymatic cleavage to form C-peptide and insulin[19]. The biological activity of PI is very low (approximately 10% of insulin), and it is the major storage form of insulin. Under physiological conditions, only small amounts of intact and split PI (less than 3% of insulin) are co-secreted with insulin from the pancreatic β-cells[4]. In the earlier studies, insulin concentration was measured with radioimmunoassay using polyclonal antibodies, which cross-reacted with largely inactive insulin precursor molecules such as PI and des-31, 32 PI, hence it was called immunoreactive insulin (IRI). These PI molecules can be distinguished from more biologically active true insulin molecules by using highly sensitive and specific, two site immunoassays based on monoclonal antibodies. Since it is rather common that sleep breathing disorder is accompanied with insulin resistance, it has been clinically considered that both of them are closely correlated and even syndrome Z was named for coexisting sleep breathing disorder and insulin resistance. So far the associated pathogenesis between sleep breathing disorder and insulin resistance has not been elucidated clearly. To explore the relationship between sleep snoring and true insulin *vs* PI, a population-based epidemiological investigation was conducted in Pizhou city located in the mideastern part of China.

The results from this study showed that the frequency of sleep snoring in males was significantly higher than those in females, and the frequency of sleep snoring significantly increased with age. Furthermore, the results of Spearman correlation analysis indicate that the frequency of sleep snoring was significantly correlated with the concentration of PI and risk factors score. Moreover, the results of analysis of covariate variance in which age and sex were controlling variables suggest that the frequency of sleep snoring was positively and significantly associated with the risk factors score, and this finding is in agreement with several previous studies[3-5].

In the present study, the level of circulating PI was positively and significantly associated with the frequency of sleep snoring in the analysis including Spearman correlation, covariate variance in which age and sex were controlling variables, and multivariate stepwise logistic regression in which age, sex, BMI, and true insulin were adjustment variables. To our knowledge, this finding is the first, from a Chinese population-based epidemiological study. Obesity is the accumulation of excessive fat in the body and plays a role in metabolic disorders and the mortality and morbidity of cardiovascular diseases. Obesity, especially the local obesity represented by increased waist circumference and neck circumference, is related to the narrowness of upper respiratory tract and can lead to sleep apnea and hypopnea, as well as hypoxia and hypercapnia[20]. This study demonstrated that in the multivariate stepwise logistic regression analysis, when the obesity represented as BMI and local obesity represented by waist circumference entered in the final regression model, the waist circumference rather than BMI was accepted by the model. Thus, it could be concluded that visceral obesity rather than general obesity was the independent risk factor for sleep snoring. To date, the mechanism underlying the consistent and significant association between PI and sleep snoring is unknown. In insulin resistance atherosclerosis study (IRAS), it demonstrated that the visceral fat accumulation could result in the elevation of circulating PI level[21], and the concentration of PI was independently correlated with fibrinogen, and plasminogen activator inhibitor type 1 (PAI-1)[22]. The elevation of the activity of fibrinogen, and PAI-1 following the increase of PI level can lead to the disorder of homeostasis and finally result in the clustering of cardiovascular risk factors. The above finding may be one of the mechanisms for the association between PI, sleep snoring and cardiovascular risk factors clustering.

Our conclusions are that the frequency of sleep snoring which is the main symptom of obstructive sleep apnea syndrome is independently associated with the level of PI, and is associated with the clustering of cardiovascular risk factors. This might be the first study demonstrating a relationship between sleep breathing disorder, circulating PI level, and clustering of cardiovascular risk factors in healthy middle-aged subjects from the general Chinese population.

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