Int J Clin Exp Med 2016;9(3):6580-6588
www.ijcem.com /ISSN:1940-5901/IJCEM0020951

Original Article

Serum betatrophin level increased in subjects with nonalcoholic fatty liver disease

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Received December 1, 2015; Accepted February 29, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Background: Betatrophin is a novel adipokine/hepatokine that promotes pancreatic β-cell proliferation and regulates lipid metabolism. Aims: This study is to evaluate the role of serum betatrophin in nonalcoholic fatty liver disease (NAFLD). Methods: A hospital-based age-/gender-/Body Mass Index-matched case-control study of 194 subjects was conducted in Hangzhou, China. Serum level of Betatrophin was measured and validated by enzyme-linked immunosorbent assay and western blotting. The serum levels of 12 adipokines were evaluated by commercial Adipokine Magnetic Bead Panel kits. Statistical analyses included receiver operating characteristic (ROC) curve, bivariate correlation, and multivariate stepwise linear regression. Results: Serum Betatrophin witnessed a higher level in NAFLD patients (1094.98 ± 541.31 pg/mL, P < 0.001), compared with controls (730.03 ± 431.10 pg/mL). Compared with the lowest tertile of serum Betatrophin level, the highest tertile indicated an association with higher odds of NAFLD (adjusted Odds Ratio = 2.012, 95% Confidence interval [CI] [1.285-3.148], P = 0.002). ROC curve of Betatrophin was developed to predict the presence of NAFLD (Area under ROC = 0.702 [95% CI 0.628-0.777t, P < 0.001). Furthermore, Betatrophin correlated with several parameters, including age, Waist-to-hip ratio, Fasting plasma glucose, Haemoglobin A1C, Homeostasis model assessment of insulin resistance, and Fasting C Peptide, and various adipokines, including Resistin, Interleukin-8, and tumor necrosis factor-α. Conclusions: Serum Betatrophin level increased in subjects with NAFLD and was associate with parameters of β-cell function and inflammation.

Keywords: Betatrophin, angiopoeitin-like 8 (ANGPTL8), adipokine, nonalcoholic fatty liver disease (NAFLD), case-control

Introduction

The global prevalence of obesity-related metabolic disorders is mounting to epidemic proportions [1-3]. Obesity, insulin resistance (IR), hyperglycemia, dyslipidemia and hypertension are often coincident in one individual, a disorder known as Metabolic Syndrome (MetS) [4]. As one of the most prevalent chronic liver diseases in the world now, nonalcoholic fatty liver disease (NAFLD), regarded as the hepatic manifestation of IR and MetS, is best characterized by aberrant lipid deposition in the hepatocytes [5].

In 2013, Yi et al. [6] identified Betatrophin, encoded by Gm6484 in mice/C19 or f80 in human and also known as angiopoeitin-like 8 (ANGPTL8)/Lipasin/refeeding-induced fat and liver protein (RIFL), is a 22-kDa novel hormone provokes a significant pancreatic beta cells proliferation and expand beta cells mass in a IR mice model. Meanwhile, more evidences support a close association between Betatrophin and lipid metabolism. Quagliarini et al. [7] revealed that Betatrophin (named ANGPTL8) regulates postprandial triglyceride (TG) and fatty acid metabolism, associated with a higher activity of lipoprotein lipase (LPL) and a reduced secretion of Very Low-density lipoprotein (VLDL) [8]. Furthermore, Zhang et al. showed that the expression of Betatrophin in brown adipose dramatically increased when exposed to the cold environment or high-fat diet, however downregulated in fasting [9].

Inspired by the lab experiments, a series of observational studies were conducted to inves-

tigate the role of Betatrophin in obesity-related metabolic disorders, e.g. diabetes and overweight/obesity [10-14]. This hospital-based case-control study was conducted, for the first time, to evaluate the role of serum Betatrophin in NAFLD.

Subjects and methods

Subjects

A total of 92 NAFLD patients were consecutively recruited from the subjects who attended to the outpatient department of the First Affiliated Hospital, Zhejiang University from January to May 2015. Based on a principle of age-, gender- and Body Mass Index (BMI)-match, 92 controls were selected from the annual health examination during the same period. Participants were excluded if they had malignant tumor, severe cardiopulmonary disorders, renal/thyroid dysfunction, severe inflammatory diseases, viral/drug-induced/autoimmune liver diseases, pregnancy, or excessively alcoholic consumption. All subjects gave written informed consent before participation. This study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University, in accordance with the Helsinki Declaration in 1975.

Anthropometric and biochemical examinations and ultrasonography

Anthropometric and biochemical examinations as well as upper abdominal ultrasonography, were performed as described before [15].

Measurement of serum betatrophin

Serum Betatrophin was evaluated using a commercial enzyme-linked immunosorbent assay (ELISA) (Catalogue No. E11644h; N-terminus; Wuhan Eiaab Science, Wuhan, China; Intra-assay coefficient of variation (CV) < 4.8%; Interassay CV < 7.2%) [12]. Serum levels were validated by another ELISA kit (Catalogue No. SEW803Hu; USCN Life science Inc., Wuhan, China; Intra-assay CV < 10%; Interassay CV < 12%) [14] and western blotting (Anti-C19 or f80, N-terminus, Catalogue No. ab180915; Abcam Ltd., Cambridge, UK and β-Actin Rabbit mAb, Catalogue No. 8457; Cell Signaling Technology, Inc., Danvers, MA) [16].

MILLIPLEX® human adipokine magnetic bead panel kits

The MILLIPLEX® Human Adipokine Magnetic Bead Panels were performed to evaluate twelve serum adipokines, according to the manufacturer's protocol (Cat. # HADK1MAG-61K and Cat. # HADK2MAG-61K).

Definition of NAFLD

NAFLD was diagnosed base on the guidelines for diagnosis and treatment of NAFLD issued by Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association [17, 18].

Statistical methods

Normally distributed variables were presented as mean ± standard deviation (SD); variables with a skewed distribution underwent a lg (x) transformation to achieve a normal distribution and were presented as median value (interquartile range). Normality of distribution was tested with the Kolmogorov-Smirnov test. The Student's t test or Mann-Whitney U test for continuous variables, and χ^2 test or Kruskal-Wallis test for categorical variables were used to compare the parameters between two groups. Comparisons of serum Betatrophin levels among multiple NAFLD groups used One-way ANOVA, following with *post hoc* comparisons of Bonferroni. To assess the relationship between Betatrophin and obesity-related metabolic disorders, we calculated the adjusted Odds Ratio (OR) and 95% Confidence interval (CI) with a multivariable binary logistic regression. Bivariate correlation analyses were performed using Pearson's correlation analysis or Spearman rho correlation analysis. Receiver operating characteristic (ROC) curve of Betatrophin was developed to predict the presence of NAFLD. Multivariate stepwise linear regression analysis was conducted for Betatrophin (dependent variable), including the variables that significantly correlated with Betatrophin as independent variables. All statistical analyses and plotting were performed using Stata (version MP 11.2, StataCorp LP, College Station, Texas, USA) and GraphPad Prism (version 6.0, GraphPad Software, Inc., San Diego, CA, USA). Power of sample size was calculated by G*Power (version 3.1, Heinrich-Heine-

Table 1. Characteristics of subjects according to NAFLD

Parameters	Control	NAFLD	*P* value
No. of subjects	92	92	
Age (years)	53.10 ± 10.06	55.07 ± 12.02	0.230
Male, n (%)	60, 65.2%	60, 65.2%	1.000
Overweight & Obesity, n, n (%, %)	35, 2 (38.0%, 2.2%)	27, 9 (29.3%, 9.8%)	0.64
Type 1 Diabetes, n, (%)	0, 0%	71, 77.2%	< 0.001#
BMI (kg/m^2)	24.40 ± 3.00	24.70 ± 3.88	0.552
WHR	0.892 ± 0.087	0.925 ± 0.062	0.004#
Betatrophin (pg/ml)	730.03 ± 431.10	1094.98 ± 541.31	< 0.001#
FPG (mmol/L)	5.02 (4.64-5.54)	7.09 (5.67-9.56)	< 0.001#
HbA1c (%)	5.75 (5.40-6.20)	8.55 (6.83-10.50)	< 0.001#
FINS (μU/mL)	12.25 (9.54-16.40)	13.10 (8.88-19.90)	0.383
HOMA-IR	2.67 (2.08-3.91)	4.12 (2.59-6.72)	< 0.001#
Fasting C Peptide (ng/ml)	0.940 (0.580-1.355)	1.005 (0.670-1.648)	0.256
AFP (μg/L)	2.50 (1.80-3.50)	2.40 (1.80-3.10)	0.434

Data are mean ± SD or median (interquartile range) for continuous variables; AFP, Alpha-fetoprotein; FINS, Fasting insulin; #*P* value is less than 0.01.

Universität Düsseldorf, Germany) [19]. A two-sided $P < 0.05$ was considered statistically significant.

Results

Validation of ELISA

The validation of Betatrophin levels presented a fine consistency ($r = 0.8155$, $P < 0.001$) between two ELISA kits (Supplementary Figure 1). Representative western blot detection of Betatrophin in four serum samples revealed a consistent trend with the ELISA result (Supplementary Figure 2).

Power of sample size

Given the serum Betatrophin concentrations and the numbers of the case and the control, the power of the sample size was 0.999 (Effect size $d = 0.746$, Critical $t = 1.973$, Supplementary Figure 3).

Characteristics of all subjects

The characteristics of 184 participants (92 cases and 92 age-/gender-/BMI-matched controls) are presented in **Table 1**.

Serum Betatrophin witnessed a higher level in NAFLD patients (1094.98 ± 541.31 pg/mL, $P < 0.001$), compared with controls (730.03 ± 431.10 pg/mL, **Table 1**; **Figure 1A**). Furthermore, there was no difference of serum Betatrophin level between female (840.24 ± 433.18 pg/mL) and male (951.05 ± 556.23 pg/mL, $P = 0.170$, **Figure 1B**).

Betatrophin tertiles

All 184 subjects were divided into 3 groups, according to the tertiles of serum Betatrophin concentrations. The frequency of NAFLD showed an upward trend (T1: 31.1%, T2: 50.8%, and T3: 67.7%, $P < 0.001$), as Betatrophin concentration increased among its tertiles (**Figure 1C**).

Odds ratios of NAFLD

Table 2 reveals that compared with the 1st tertile, the 2nd of Betatrophin indicated no association with the presence of NAFLD (adjusted OR = 2.218, 95% CI [0.933-5.271], $P = 0.071$), after controlling age, gender, BMI, Waist-to-hip ratio (WHR), and parameters of liver enzymes and lipid metabolism. However, the 3rd tertile revealed its association with higher odds of NAFLD (adjusted OR = 2.012, 95% CI [1.285-3.148], $P = 0.002$), in comparison with the 1st tertile.

ROC curve of Betatrophin

ROC curve of Betatrophin was developed to predict the presence of NAFLD (**Figure 1D**). Area under ROC was 0.702 ([95% CI 0.628-

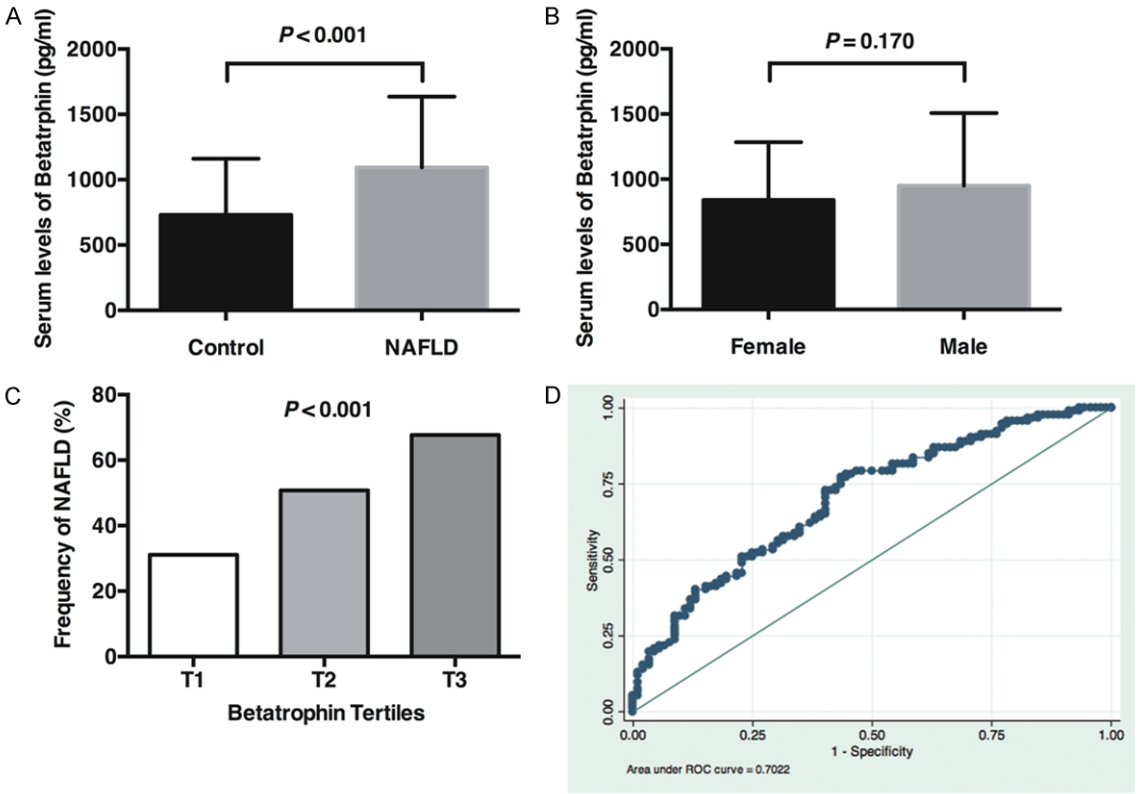

Figure 1. A: Comparisons of serum betatrophin levels (pg/ml, mean ± SD) between Control (730.03 ± 431.10) and NAFLD (1094.98 ± 541.31), $P < 0.001$; B: Comparisons of serum betatrophin levels (pg/ml, mean ± SD) between female (840.24 ± 433.18) and male (951.05 ± 556.23), $P = 0.170$; C: Frequency (%) of NAFLD according to betatrophin tertiles (T1: 31.1%, T2: 50.8%, and T3: 67.7%, $P < 0.001$). D: ROC curve of betatrophin to predict the presence of NAFLD (AUROC 0.702 [95% CI 0.628-0.777], $P < 0.001$).

Table 2. ORs and 95% CIs of the presence of NAFLD by betatrophin tertiles

		Tertile 1	Tertile 2	P for trend	Tertile 3	P for trend
NAFLD	Model 1	Reference	2.284 (1.091-4.781)	0.028*	2.155 (1.474-3.150)	< 0.001#
	Model 2	Reference	2.304 (1.089-4.875)	0.029*	2.090 (1.408-3.102)	< 0.001#
	Model 3	Reference	1.976 (0.906-4.308)	0.087	1.955 (1.303-2.934)	0.001#
	Model 4	Reference	2.223 (0.980-5.043)	0.056	1.963 (1.291-2.985)	0.002#
	Model 5	Reference	2.218 (0.933-5.271)	0.071	2.012 (1.285-3.148)	0.002#

Model 1: unadjusted; Model 2: adjusted for age and gender; Model 3: adjusted for age, gender, BMI and WHR; Model 4: adjusted for age, gender, BMI, WHR, ALT, AST, and GGT; Model 5: adjusted for age, gender, BMI, WHR, ALT, AST, GGT, TG, HDL-C, LDL-C, and VLDL-C; *P value is less than 0.05; #P value is less than 0.01.

0.777], $P < 0.001$), with the sensitivity of 78.26%, the specificity of 55.43% and the accuracy of 66.85%, when cut-off value of Betatrophin was 690.33 pg/mL.

Correlations with anthropometric/biochemical parameters and adipokines

In **Table 3**, all subjects witnessed positive associations of Betatrophin with age ($r = 0.239$, $P = 0.001$) and WHR ($r = 0.179$, $P = 0.015$).

In terms of lipid and cholesterol metabolism, Betatrophin presented inverse associations with Total cholesterol (TC) ($r = -0.189$, $P = 0.010$) and LDL cholesterol (LDL-C) ($r = -0.152$, $P = 0.040$), whereas Betatrophin correlated positively with Fasting plasma glucose (FPG) ($r = 0.229$, $P = 0.002$), Haemoglobin A1C (HbA1c) ($r = 0.233$, $P = 0.001$), Homeostasis model assessment of insulin resistance ($r = 0.218$, $P = 0.003$), and Fasting C Peptide ($r = 0.156$, $P =$

Table 3. Correlations of serum betatrophin with various anthropometric/biochemical parameters and adipokines

Categories	Parameters	*r* value	*P* value	Categories	Parameters	*r* value	*P* value	Functions	Adipokines	*r* value	*P* value
Anthropometry	Age	0.239	0.001#	Lipid and Cholesterol Metabolism	TG†	-0.071	0.336	Appetite and Energy Homeostasis	Leptin‡	0.035	0.767
	Height	-0.098	0.184		TC	-0.189	0.010*	Insulin Sensitivity and Vascular Function	Resistin	0.570	< 0.001#
	Weight†	0.032	0.667		HDL-C	-0.134	0.071		Adiponectin‡	-0.023	0.847
	BMI	0.108	0.146		LDL-C	-0.152	0.040*	Inflammation	Interleukin-6‡	0.134	0.254
	Hip circumstance	-0.020	0.789		VLDL-C†	-0.029	0.692		Interleukin-8‡	0.256	0.028*
	Waist circumstance	0.106	0.153	Glucose Metabolism and Insulin Function	FPG‡	0.229	0.002#		MCP-1	0.015	0.898
	WHR	0.179	0.015*		HbA1c‡	0.233	0.001#		Lipocalin-2‡	0.127	0.279
Liver Enzymes	ALT†	-0.054	0.466		FINS‡	0.108	0.145		TNF-α	0.295	0.011*
	AST†	-0.021	0.777		HOMA-IR†	0.218	0.003#		NGF‡	0.004	0.972
									HGF	0.079	0.502
	GGT†	0.047	0.527		Fasting C Peptide†	0.156	0.034*		Adipsin	0.038	0.745
	AFP†	0.088	0.233					Fibrinolysis	PAI-1	0.130	0.268

†lg(x) transformation was performed because of a skewed distribution; ‡Spearman correlation analysis; AFP, Alpha-fetoprotein; ALT, Alanine transaminase; AST, Aspartate transaminase; FINS, Fasting insulin; GGT, γ-Glutamyltransferase; **P* value is less than 0.05; #*P* value is less than 0.01.

Table 4. Multiple linear regression analyses with betatrophin as dependent variable

	Variable	r^2	β	*P* value
Model 1		0.315		< 0.001#
	Resistin		0.570	< 0.001#
Model 2		0.386		< 0.001#
	Resistin		0.501	< 0.001#
	NAFLD		0.288	0.003#
Model 3		0.456		< 0.001#
	Resistin		0.493	< 0.001#
	NAFLD		0.285	0.002#
	Fasting C Peptide		0.276	0.002#
Model 4		0.483		< 0.001#
	Resistin		0.459	< 0.001#
	NAFLD		0.282	0.002#
	Fasting C Peptide		0.245	0.005#
	Age		0.188	0.034*

Values are corrected r^2 (r^2), standardized coefficients (β) and associated *P* values; **P* value is less than 0.05; #*P* value is less than 0.01.

0.034), which are parameters of glucose metabolism and insulin sensitivity.

Table 3 also reveals the associations between Betatrophin and adipokines, including Resistin (r = 0.570, P < 0.001), Interleukin-8 (r = 0.256, P = 0.028), and Tumor necrosis factor-α (r = 0.295, P = 0.011).

Multivariate linear regression

In the multiple linear regression analysis (**Table 4**), the model (corrected r^2 = 0.483, P < 0.001) that best predicted Betatrophin levels included Resistin, age, fasting C peptide and NAFLD as predictive variables.

Discussion

This case-control study found that there was a higher serum level of Betatrophin in subjects with NAFLD, compared with controls. Furthermore, serum Betatrophin levels presented a positive association with the presence of NAFLD. Additionally, Betatrophin correlated a series of parameters concerned glucose and lipid metabolism. It also reveals the correlations between Betatrophin and three adipokines, i.e.Resistin, Interleukin-8, and TNF-α. Lastly, it found serum level of Betatrophin was dependent on age, Fasting C Peptide, Resistin and NAFLD.

NAFLD is regarded as the hepatic manifestation of IR and dyslipidemia [5], while Betatrophin is identified as an adipokine/hepatokine related to the function of pancreatic islets and the regulation of lipid metabolism [6, 7, 20, 21]. Thus, we conducted a case-control study to evaluate the hypothesis that serum Betatrophin related to NAFLD.

First of all, we found that serum Betatrophin presented a higher level in subjects with NAFLD, in comparison with controls. Meanwhile, a higher level of Betatrophin was found to be associated with the presence of NAFLD.

Interestingly, our results revealed a positive association between age and serum Betatrophin level. Even though evidence supports a declined rate of beta cells replication with age in both rodent and human [22], an autopsy study of 167 non-diabetic subjects with a large age spectrum by Saisho et al. [23] revealed that the mean nuclear diameter of beta cells increased with age and the amount of apoptotic beta cells was constant. The correlation between age and circulating Betatrophin level, thus, may represent a compensatory mechanism in response to aging. Besides, serum level of Betatrophin witnessed a correlation with WHR that is a widely accepted parameter of central obesity and an indicator of NAFLD risk [24, 25].

In terms of glucose metabolism and insulin sensitivity, the correlations were observed between Betatrophin and multipleparameters, i.e. FPG, HbA1c, HOMA-IR and Fasting C Peptide, besides Resistin, a well-studied adipokines related toIR [26]. Higher serum levels of Betatrophin in subjects with NAFLD may be attributable to a defensive regulation, which might lead to an adaption to hepatic IR or elevated plasma glucose, via promoting beta cells proliferation and insulin secretion [13].

More evidence supports that Betatrophin involved in lipid metabolism, possibly via regulating VLDL secretion and LPL activity [7, 21, 27-29]. Our study also suggests close associations of Betatrophin with serum TC and LDL-C, however, not TG or High-density lipoprotein cholesterol.

As higher nutrient concentrations in whole body, inflammation can be observed in all tis-

sues of energy homeostasis, including muscle, liver, fat, and islets [30-34]. It has been proven that hepatic tissue was associated with a remarkable accumulation of immune cells during obesity [34]. In this study, the serum adipokines of inflammation, i.e. TNF-α andInterleukin-8, witnessed significant correlations with serum Betatrophin [26, 35]. Lastly, multiple linear regression analysis indicated a predicting model, including age, Resistin, NAFLD and Fasting C Peptide, explained 48.3% of the total variability of serum Betatrophin levels.

In terms of the strengths, to begin with, it is the first clinical study to assess the role of serum Betatrophin in NAFLD. Moreover, to validate the reliability, we measured serum Betatrophin level with ELISA kits from two providers and performed western blotting detection. Lastly, the serum concentrations of 12 adipokines related to inflammation and insulin sensitivity were measured, using Human Adipokine Magnetic Bead Panel kits.

Some limitations of the study merit comment. Firstly, the case-control design makes it difficult to determine the role of serum Betatrophin in the development of NAFLD. In the future, a long-term cohort study with a larger population is required. Secondly, the golden standard of NAFLD is liver pathological examination, rather than ultrasonography that evaluatedthe degrees of NAFLD (mild, moderate and severe) in this study [36]. Thirdly, the parameters of inflammation, e.g. C-reactive protein, erythrocyte sedimentation rate failed to be included in our study. Lastly, due to lack of information concerned physical activity, diet, economic status and etc., the impact of these factors was not taken into consideration.

In summary, our study found a higher serum level of Betatrophin in subjects with NAFLD, compared with controls. Serum Betatrophin presented an association with higher odds of NAFLD. Furthermore, circulating Betatrophin correlated with anthropometric parameters, i.e. age, WHR, and biochemical parameters, i.e. TC, LDL-C, HbA1c, HOMA-IR, FPG, and Fasting C Peptide. Lastly, Betatrophin presented correlations with several adipokines of inflammation and insulin sensitivity, including TNF-α, Resistin, and Interleukin-8. It suggests that serum Betatrophin is a potential biomarker and therapeutic target for NAFLD, possibly related to the regulation of inflammation and insulin sensitivity.

Acknowledgements

This work was supported by National Key Basic Research Development Program (No. 2012CB524905), National Science and Technology Support Plan Project (No. 2012BAI 06B04), National Natural Science Foundation of China (No. 81170378 and No. 81230012).

Disclosure of conflict of interest

None.

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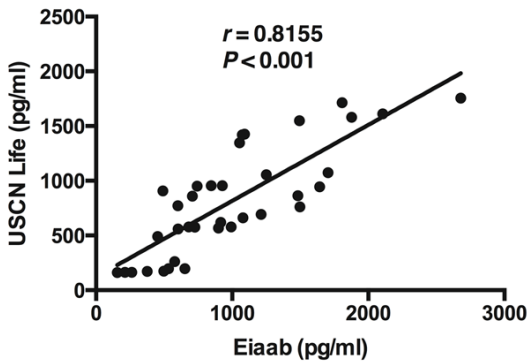
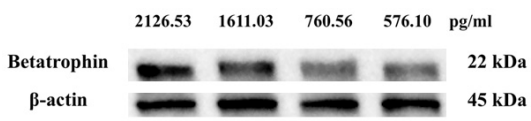
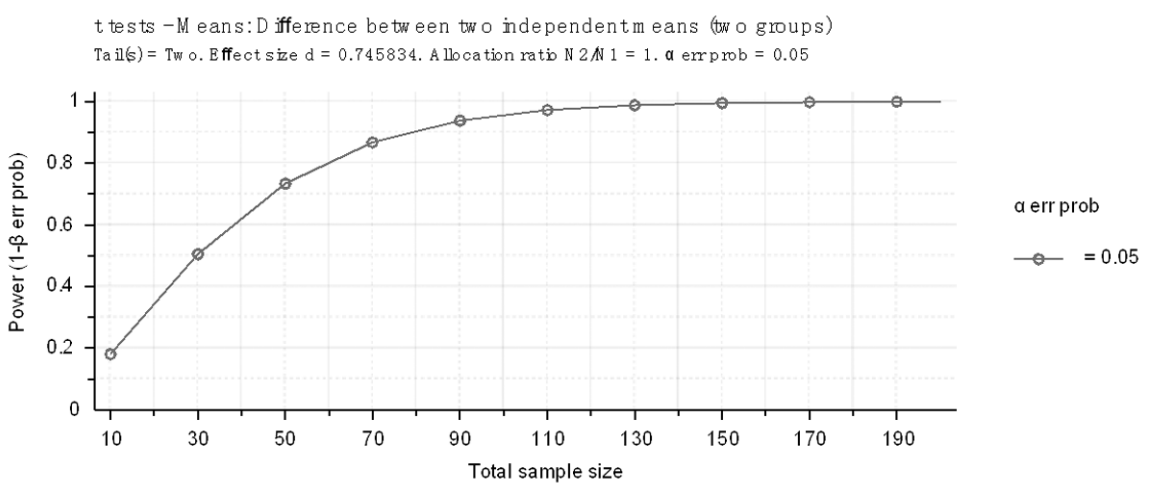


Supplementary Figure 1. Correlation of serum betatrophin levels between USCN Life ELISA kit and Eiaab ELISA kit (n = 36, *r* = 0.8155, *P* < 0.0001).



Supplementary Figure 2. Representative western blot detection of betatrophin in four serum samples.



Supplementary Figure 3. Power of sample size. Given the serum betatrophin concentrations and the numbers of the case (n = 92) and the control (n = 92), the power of the sample size was 0.999 (Effect size *d* = 0.746, Critical *t* = 1.973).