Original Article

Association of Fetuin-B with Subclinical Atherosclerosis in Obese Chinese Adults

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Aim: We aimed to explore the independent associations of serum Fetuin-B and common genetic variants in *FETUB* locus with subclinical atherosclerosis.

Methods: A cross-sectional study of 1,140 obese adults, who underwent serum Fetuin-B testing, hepatic ultrasonography scanning, genotyping on four tagging single nucleotide polymorphisms (SNPs) in *FETUB* locus and atherosclerosis detection, was conducted in Xiamen, China.

Results: Increasing tertiles of brachial ankle pulse wave velocity (ba-PWV) were significantly associated with higher prevalence of nonalcoholic fatty liver disease (NAFLD) (48.8%, 61.5%, and 70.5% for tertiles of 1–3, respectively, $p < 0.001$) and serum Fetuin-B (3.85 ± 1.39, 4.09 ± 1.40, and 4.27 ± 1.46 µg/ml, $p = 0.047$). Multivariable linear regression analyses with adjustment for potential confounding factors, even NAFLD per se, showed that serum Fetuin-B were significantly and positively associated with ba-PWV, with standardized regression coefficients (β) ranging from 0.055 to 0.075 (all p-values < 0.05) in different models. However, the significant relationship between serum Fetuin-B and ba-PWV disappeared with further adjustment for insulin resistance. Serum Fetuin-B was not significantly associated with ankle-brachial index (ABI). All genotypes of the four tested *FETUB* tagging SNPs were not significantly associated with either ba-PWV or ABI with adjustment for potential confounding factors

Conclusion: Serum Fetuin-B was positively associated with ba-PWV and may link liver fat accumulation to subclinical atherosclerosis via insulin resistance.

Key words: Fetuin-B, Atherosclerosis, Brachial ankle pulse wave velocity, Ankle-brachial index, Single nucleotide polymorphism

Introduction

Nonalcoholic fatty liver disease (NAFLD) may be independently associated with increased cardiovascular risk[1-3]. However, factors linking NAFLD to cardiovascular disease (CVD) are not well known, although some hepatokines, such as fibroblast growth factors 21 (FGF21), Fetuin-A, and selenoprotein P are involved in liver steatosis and atherosclerosis crosstalk[4, 5]. Fetuin-B is a member of the cystatin super

family of cysteine protease inhibitors, sharing 22% homology with Fetuin-A, which has been associated with risk of CVD or atherosclerosis in a few population-based studies[6, 7]. Meex RC *et al.* reported that Fetuin-B increased in humans with liver steatosis, impaired insulin action in myotubes and hepatocytes, and caused glucose intolerance in mice[8]. We recently found that serum Fetuin-B level was positively correlated with intrahepatic triglyceride (IHTG) content and that elevated serum Fetuin-B was independently associated with increased risk of insulin resistance in Chinese adults[9].

Fetuin-B is encoded by the *FETUB* gene, which is located in the human chromosome 3q27.3 with eight exons. The *FETUB* rs4686434 SNP, an intron variant, is characterized by a A-to-G substitution. We recently found that subjects carrying the minor allele G for *FETUB* rs4686434 had lower levels of serum Fetuin-B and decreased IHTG content than their controls[10]. We further found a significant joint effect between *FETUB* rs4686434 and patatin-like phospholipase domain-containing-3 (*PNPLA3*) rs738409, another genetic variant associated with NAFLD in multi-ethnic populations in genome-wide association studies[11], on IHTG content[10]. Our findings suggest that the genetic variant on *FETUB* rs4686434 might influence hepatic triglyceride accumulation. Our and others' findings suggest that serum Fetuin-B levels and genetic variants on FETUB rs4686434 might influence hepatic triglyceride accumulation.

Available evidence has documented that both NAFLD and insulin resistance are closely associated with atherosclerosis [1, 3, 12], and we previously found that a higher serum Fetuin-B level is associated with NAFLD and increases the risk of insulin resistance. This raises the question of whether serum Fetuin-B is associated with subclinical atherosclerosis independently of traditional CVD risk factors. Also, there is no evidence currently available about the association between genetic variants in the *FETUB* locus and subclinical atherosclerosis.

In the present study, we first aimed to explore the independent association between serum Fetuin-B levels and subclinical atherosclerosis (brachial ankle pulse wave velocity (ba-PWV) and ankle-brachial index (ABI)). We also aimed to explore the independent associations of genetic variants in the *FETUB* locus with ba-PWV and ABI.

Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of the First Affiliated Hospital of Xiamen University (Xiamen, China). Written informed consent was obtained from each participant. The study complied with the Declaration of Helsinki.

Participants

Details on study participants have been described previously[9, 10]. In short, 1,523 community-living healthy adults aged 40 years or older with central obesity (waist circumference greater than 90 cm for men and 80 cm for women) living in Lianqian community, Xiamen, China, were recruited for the baseline examination of our designed cohort study in 2011. Of them, 1,140 subjects who had complete data on clinical, serum Fetuin-B, genetic variants on *FETUB* locus and subclinical atherosclerosis measurements remained for the present analysis (**Fig. 1**).

Face-to-face interviews were conducted to collect socio-demographic status, lifestyle habits, present and previous history of health and medications. Subjects underwent weight, height, and waist circumference measurements using a calibrated scale after removing shoes and heavy clothes. Arterial blood pressure was measured with a mercury sphygmomanometer after sitting for at least 15 minutes. Three readings were taken at 5-min intervals and the mean was recorded.

Anthropometric and Biochemical Measurements

Blood samples were obtained after 12-hour fasting for each subject. Plasma glucose and serum lipid profiles, including triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were determined on a HITACHI 7450 analyzer (HITACHI, Tokyo, Japan). Serum uric acid was measured by the autoanalyzer (COBAS INTEGRA 400 plus, Roche, Basel, Switzerland). Fasting plasma glucose (FPG) concentrations were measured by the hexokinase method, and serum fasting insulin concentrations were measured by electrochemiluminiscence immunoassay (Roche Elecsys Insulin Test, Roche Diagnostics, Mannheim, Germany). Homeostasis model assessment - insulin resistance (HOMA-IR) was calculated using the formula: fasting serum insulin (mU/L) *FPG (mmol/L)/22.5. Insulin resistance was defined as HOMA-IR $\geq 2.6^*10^{-6}$mol*U/L^2. Serum Fetuin-B concentration was measured using the enzyme-linked immunosorbent assay kits (Abcam, Cambridge, UK). The sensitivity of the assay was 4 ng/ml, and the linear range of the standard was 4 to 50 ng/ml. The intra-assay variation was less than 10%, and the inter-assay variation was less than 12%[9].

Hepatic ultrasonography scanning was performed by an experienced radiologist using GE LOGIQ P5 scanner (GE Healthcare, Milwaukee, USA) with a 4-MHz probe, who was blinded to the
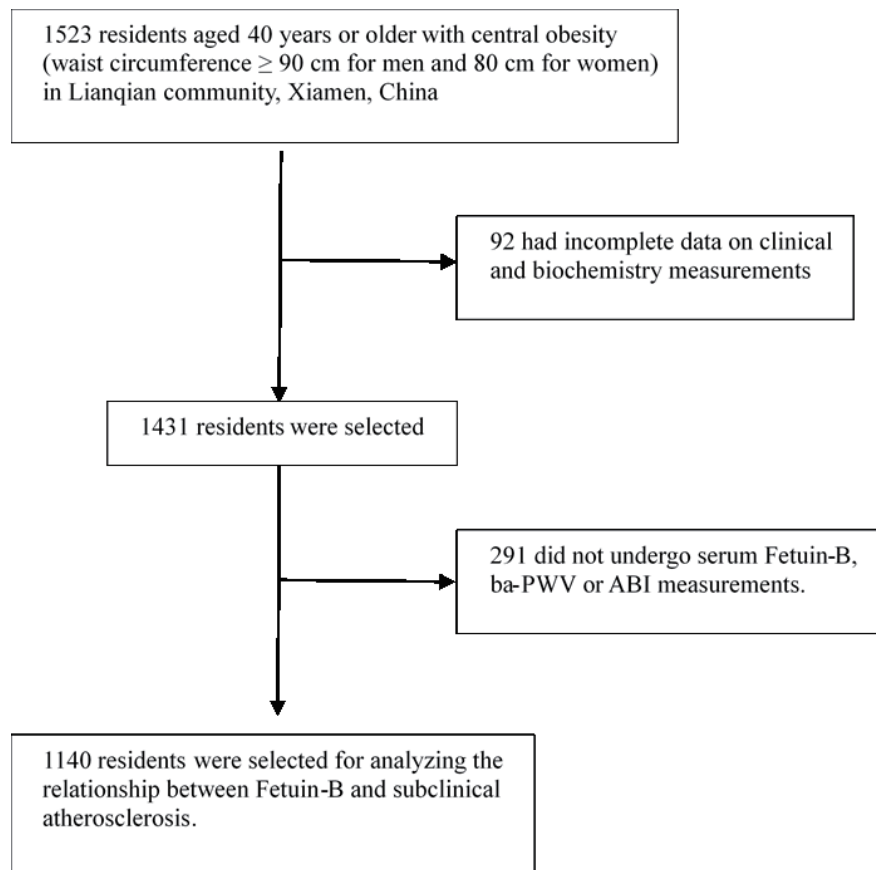
Fig. 1. Study subjects selection diagram

subject health status. Hepatic steatosis was diagnosed based on characteristic sonographic features, including hepatorenal echo contrast, liver parenchymal brightness, deep beam attenuation, and vessel blurring[13]. The definition of NAFLD was based on hepatic ultrasonography diagnosis of hepatic steatosis without excessive alcohol consumption, viral or autoimmune liver disease.

Selection and Genotyping of Tagging SNPs in FETUB Locus

Details on selection and genotyping of tagging SNPs in the FETUB locus have been described previously[10]. Briefly, a genomic area on human chromosome 3q27.3, encompassing the *FETUB* gene (17.18 kb, eight exons), as well as 5 and 3 kb of its 5'- and 3'-flanking regions, respectively (based on the International HapMap Project phase III data on the Chinese Han Beijing population (release #28 August 2010, accessed 24 February 2016)), was screened. Seven HapMap SNPs were present and showed the Hardy-Weinberg equilibrium. We focused on the common SNPs only and found 6 SNPs with minor allele frequencies (MAFs) ≥ 0.05 and were genotyped in ≥

50% of the HapMap individuals. Based on Tagger analysis using Haploview 4.2 software (http://hapmap.ncbi.nlm.nih.gov/), the following four SNPs were selected as tagging SNPs covering all the other common SNPs within the locus with an $r^2 > 0.8$ (100% coverage): rs4686434 (A/G), rs3733159 (T/G), rs1047115 (A/C) and rs6785067 (G/A) (**Supplementary Fig. 1**). Three of the four *FETUB* tagging SNPs (rs3733159, rs1047115, and rs6785067) obeyed the Hardy-Weinberg equilibrium ($p > 0.05$). The MAFs of four tested SNPs ranged from 4.1% to 46.7% (**Supplementary Table 1**). Results of the linkage disequilibrium (D', r^2) showed the observed genetic linkage between the tested SNPs was low or moderate (r^2 range: 0.05–0.45).

Subclinical Atherosclerosis Measurements

Brachial ankle pulse wave velocity (ba-PWV) and ABI, indices of subclinical atherosclerosis in the present study, were determined by an arteriosclerosis detection device, Colin VP-1000 (Model BP203RPE III, Omron Corporation, Japan) after subjects had rested for 10–15 minutes. For ba-PWV measurement, pulse waves were measured with cuffs placed on the

right/left upper arm and the right/left ankle simultaneously. Differences in the start times of pulse waves were corrected for distance. ABI was calculated by the higher values of systolic blood pressure (BP) in either dorsalis pedis or posterior tibial arterial divided by the higher brachial systolic BP[14]. The higher value of ba-PWV and lower value of ABI in either limb were used for further analysis[14, 15].

Statistical Analyses
Power Calculation
Power calculation for association of tested SNPs with high ABI (defined as the third tertile of ABI vs. the first and second tertiles of ABI) was conducted using Quanto (version 1.2.4). Among all of the subjects, 380 were defined as high ABI (the third tertile) and 760 were controls (the first and second tertiles). The significance value of 0.05 (two-sides) and log-additive genetic model were used. Under these assumptions, for *FETUB* rs3733159, of which the minor alleles may show increased likelihoods of high ABI and MAF >40%, power was sufficient (power > 0.8). Power for FETUB rs4686434, of which the minor allele may show decreased likelihoods of high ABI and MAF >20%, was approximately sufficient. But powers for rs1047115 and rs6785067 (both MAFs <10%) were not sufficient (power <0.8).

Skewness and kurtosis tests for normality of serum Fetuin-B level, ba-PWV, and ABI were conducted and found following approximation of normal distribution. Data was presented as the mean ± standard deviation for continuous variable or number and percentage for categorical variable. Differences between subjects (categorized by tertiles of ba-PWV, ABI and genotypes of *FETUB* tagging SNPs) were analyzed using one-way ANOVA for continuous variables and chi-square test for categorical variables. Hardy-Weinberg equilibrium was tested using the chi-square test (one degree of freedom). Linkage disequilibrium (D', r^2) between the tested SNPs was analyzed using MIDAS v1.0 (http://www.genes.org.uk/software/midas)[16].

Associations of the *FETUB* tagging SNPs genotypes with ba-PWV and ABI were evaluated by coding the genotype as subjects with 1 or 2 minor alleles v.s. those with 0 minor alleles. Multivariable linear regression was used to explore the independent associations of serum Fetuin-B and genotypes of *FETUB*, tagging SNPs with ba-PWV and ABI in different models with adjustment for potential confounders. In model 1, age and sex were adjusted for; in model 2, educational levels, smoking and drinking habits, and regular physical exercise plus model 1 were adjusted for; in model 3, waist, TC, HDL-cholesterol, and serum uric acid plus

model 2 were adjusted for. In model 4, NAFLD (yes vs. no) was further adjusted simultaneously. In model 5, insulin resistance (yes vs. no) was further adjusted.

In each model, Fetuin-B levels were presented per standard deviation (SD) increase, and genotypes were presented as subjects with 1 or 2 minor alleles v.s. those with 0 minor alleles. Based on screening four non-linked tagging SNPs for *FETUB* locus in parallel, a *p* value of 0.0125 was considered statistically significant according to Bonferroni correction for multiple comparisons within the four *FETUB* tagging SNPs. All statistical analyses were performed using Stata14.0 (StatCorp, College Station, TX).

Results
Characteristics of Subjects Stratified by Tertiles of ba-PWV and ABI
Among the 1,140 subjects, 802 (70.4%) were women and the mean ages (± SD) were 53.6 (± 6.9) years for all. **Table 1** shows the differences of characteristics stratified by tertiles of ba-PWV and ABI. In general, with increasing tertiles of ba-PWV, subjects were more likely to be male, older, and have significantly increased levels of waist, systolic and diastolic BP, triglyceride, TC, serum uric acid, HOMA-IR, and decreased level of HDL-C. Increased tertiles of ba-PWV were also significantly associated with higher prevalence rates of NAFLD (48.8%, 61.5%, and 70.5% for tertiles of 1-3, respectively, $p < 0.001$), insulin resistance (46.2%, 60.4%, and 71.6% for tertiles of 1-3, respectively, $p < 0.001$) and serum Fetuin-B (3.85 ± 1.39, 4.09 ± 1.40, and 4.27 ± 1.46 μg/ml, $p = 0.047$). Genotypes of the four tested *FETUB* tagging SNPs were not significantly associated with increasing tertiles of ba-PWV. Increased tertiles of ABI were significantly associated with male gender, old age, ever smoking and drinking, increased levels of systolic and diastolic BP, and serum uric acid. But there was no significant difference of prevalence of NAFLD, serum Fetuin-B level s, or genotypes of tested *FETUB* tagging SNPs among increasing tertiles of ABI.

Differences of ba-PWV and ABI Stratified by Genotypes of FETUB Tagging SNPs
Table 2 shows that all genotypes of the *FETUB* tagging SNPs were not significantly associated with levels of ba-PWV or ABI.

Associations of Serum Fetuin-B and FETUB SNPs with ba-PWV and ABI
Based on the multivariable linear regression analyses with adjustment for potential confounding fac-

Table 1. Characteristics of subjects by tertiles of brachial ankle pulse wave velocity and ankle-brachial index

	ba-PWV (cm/s)				ABI			
	Tertile 1	Tertile 2	Tertile 3		Tertile 1	Tertile 2	Tertile 3	
Variables	1239.5 ± 96.3	1481.2 ± 60.9	1853.3 ± 246.1	*P* value	1.00 ± 0.05	1.08 ± 0.02	1.16 ± 0.04	*P* value
N (%)	381 (33.4%)	379 (33.3%)	380 (33.3%)		423 (37.1%)	372 (32.6%)	345 (30.3%)	
Female (*n*, %)	286 (75.1%)	250 (66.0%)	266 (70.0%)	0.023*	317 (74.9%)	265 (71.2%)	220 (63.8%)	0.003[†]
Age (years)	50.4 ± 6.6	53.7 ± 6.5	56.6 ± 6.5	<0.001[‡]	52.2 ± 7.1	53.6 ± 6.9	55.1 ± 6.7	<0.001[‡]
Education, (*n*, %)				0.218				0.234
Illiteracy	98 (25.7%)	100 (26.4%)	123 (32.4%)		125 (29.6%)	103 (27.7%)	93 (27.0%)	
Elementary school	115 (30.2%)	112 (29.6%)	106 (27.9%)		136 (32.2%)	99 (26.6%)	98 (28.4%)	
Middle school	78 (20.5%)	95 (25.1%)	86 (22.6%)		85 (20.1%)	83 (22.3%)	91 (26.4%)	
High school or above	90 (23.6%)	72 (19.0%)	65 (17.1%)		77 (18.2%)	87 (23.3%)	63 (18.2%)	
Ever smoking (*n*, %)	93 (24.4%)	110 (29.0%)	84 (22.1%)	0.082	93 (22.0%)	86 (23.1%)	108 (31.3%)	0.007[†]
Ever drinking (*n*, %)	50 (13.1%)	71 (18.7%)	56 (14.7%)	0.089	59 (14.0%)	48 (12.9%)	70 (20.3%)	0.013*
Regular physical exercise (*n*, %)	123 (32.3%)	135 (35.6%)	121 (31.8%)	0.482	135 (31.9%)	120 (32.3%)	124 (35.9%)	0.442
Waist circumference (cm)	92.6 ± 7.4	94.3 ± 7.7	93.5 ± 6.1	0.005[†]	93.2 ± 7.6	93.4 ± 6.9	93.8 ± 6.7	0.525
Systolic blood pressure (mmHg)	121.4 ± 11.7	133.2 ± 13.1	146.4 ± 17.5	<0.001[‡]	130.9 ± 16.6	134.1 ± 18.1	136.6 ± 17.8	<0.001[‡]
Diastolic blood pressure (mmHg)	73.2 ± 8.5	80.3 ± 8.9	84.8 ± 11.2	<0.001[‡]	78.1 ± 10.4	80.0 ± 10.8	80.5 ± 11.0	0.006[†]
Triglyceride (mmol/L)	1.55 ± 1.03	1.99 ± 1.32	2.17 ± 1.43	<0.001[‡]	1.96 ± 1.39	1.79 ± 1.17	1.95 ± 1.30	0.132
Total cholesterol (mmol/L)	5.69 ± 1.04	5.95 ± 1.04	6.07 ± 1.13	<0.001[‡]	5.92 ± 1.10	5.88 ± 1.05	5.91 ± 1.09	0.849
HDL-cholesterol (mmol/L)	1.41 ± 0.30	1.36 ± 0.28	1.35 ± 0.29	0.016*	1.38 ± 0.31	1.37 ± 0.27	1.37 ± 0.30	0.876
Serum uric acid (μmol/L)	349.0 ± 95.9	372.8 ± 90.0	370.1 ± 95.1	<0.001[‡]	354.5 ± 91.9	364.7 ± 89.8	374.7 ± 100.6	0.012*
HOMA-IR (*10^{-6}mol*IU*L^{-2})	2.84 ± 1.65	3.67 ± 3.22	4.09 ± 2.82	<0.001[‡]	3.69 ± 2.74	3.34 ± 1.91	3.56 ± 3.28	0.184
Insulin resistance (*n*, %)	176 (46.2%)	229 (60.4%)	272 (71.6%)	<0.001[‡]	266 (62.9%)	218 (58.6%)	193 (55.9%)	0.140
NAFLD (*n*, %)	186 (48.8%)	233 (61.5%)	268 (70.5%)	<0.001[‡]	265 (62.7%)	222 (59.7%)	200 (58.0%)	0.404
Serum Fetuin-B (μg/ml)	3.85 ± 1.39	4.09 ± 1.40	4.27 ± 1.46	0.047*	4.19 ± 1.39	4.18 ± 1.45	4.06 ± 1.31	0.365
FETUB Genotypes								
rs4686434 AG/GG	162 (42.5%)	159 (42.0%)	154 (40.5%)	0.848	162 (38.3%)	168 (45.2%)	145 (42.0%)	0.145
AA	219 (57.5%)	220 (58.1%)	226 (59.5%)		261 (61.7%)	204 (54.8%)	200 (58.0%)	
rs3733159 TG/GG	267 (70.1%)	271 (71.5%)	281 (74.0%)	0.487	304 (71.9%)	276 (74.2%)	239 (69.3%)	0.343
TT	114 (29.9%)	108 (28.5%)	99 (26.0%)		119 (28.1%)	96 (25.8%)	106 (30.7%)	
rs1047115 AC/CC	80 (21.0%)	62 (16.4%)	70 (18.4%)	0.258	85 (20.1%)	66 (17.7%)	61 (17.7%)	0.607
AA	301 (79.0%)	317 (83.6%)	310 (81.6%)		338 (79.9%)	306 (82.3%)	284 (82.3%)	
rs6785067 GA/AA	30 (7.9%)	29 (7.7%)	31 (8.2%)	0.967	38 (9.0%)	29 (7.8%)	23 (6.7%)	0.494
GG	351 (92.1%)	350 (92.4%)	349 (91.8%)		385 (91.0%)	343 (92.2%)	322 (93.3%)	

*$p<0.05$, [†]$p<0.01$, [‡]$p<0.001$
All percentages are column percentage;
Abbreviations: ABI, Ankle-brachial index; ba-PWV, brachial ankle pulse wave velocity; HDL, high-density lipoprotein; NAFLD, non-alcoholic fatty liver disease.

tors in different models, **Table 3** shows the (standardized) regression coefficients (*β*) of serum Fetuin-B and genotypes of the tested *FETUB* tagging SNPs on ba-PWV and ABI. With adjustment for sex and age in model 1, higher serum Fetuin-B was significantly associated with increased ba-PWV, and the standardized *β* of per SD increase of serum Fetuin-B was 0.075 (*p*=0.008). With additional adjustments for educational levels, ever smoking, ever drinking and regular physical exercise habits in model 2, and further adjustment for waist, TC, HDL-cholesterol, and serum uric acid in model 3, the standardized *β* of per SD increase

of serum Fetuin-B for ba-PWV were 0.071 (*p*=0.011) and 0.057 (*p*=0.039), respectively. Even with further adjustment for NAFLD in model 4, both NAFLD and serum Fetuin-B level were still significantly associated with increased ba-PWV (the standardized *β*: 0.127 and 0.055, respectively; both *p*-values <0.05). All genotypes of the 4 *FETUB* tagging SNPs were not significantly associated with ba-PWV in all of the different models. In model 5, with further adjustment for insulin resistance, plus model 4, both NAFLD and insulin resistance were significantly and positively associated with ba-PWV (the standardized *β*: 0.086

Table 2. Differences of brachial ankle pulse wave velocity and ankle-brachial index by genotypes of FETUB tagging SNPs

	FETUB tagging SNPs					
	rs4686434			rs3733159		
Variables	AA	AG/GGP value	P value	TT	TG/GG	P value
ba-PWV (cm/s)	1529.2 ± 295.6	1517.9 ± 299.7	0.529	1515.9 ± 317.8	1527.8 ± 288.9	0.546
ABI	1.07 ± 0.07	1.08 ± 0.08	0.663	1.07 ± 0.08	1.07 ± 0.07	0.809

	FETUB tagging SNPs					
	rs1047115			rs6785067		
Variables	AA	AC/CC	P value	GG	GA/AA	P value
ba-PWV (cm/s)	1528.3 ± 298.2	1507.7 ± 292.8	0.364	1525.0 ± 297.4	1518.4 ± 296.5	0.839
ABI	1.07 ± 0.07	1.07 ± 0.08	0.459	1.07 ± 0.07	1.07 ± 0.08	0.890

*$p < 0.0125$
Abbreviations: ABI, Ankle-brachial index; ba-PWV, brachial ankle pulse wave velocity; IHTG, intrahepatic triglyceride; NAFLD, non-alcoholic fatty liver disease.

and 0.183, respectively; both *p*-values < 0.05), however the association between serum Fetuin-B and ba-PWV was not significant (the standardized β: 0.047, $p = 0.086$).

The multivariable linear regression analyses on ABI showed neither serum Fetuin-B levels nor genotypes of the tested *FETUB* tagging SNPs were significantly associated with ABI with adjustment for potential confounding factors in different models.

Discussion

In the present study of 1,140 community-living Chinese adults with central obesity, we found that increasing tertiles of ba-PWV were significantly associated with higher prevalence of NAFLD and serum Fetuin-B. After adjusting for traditional CVD risk factors, even NAFLD per se, we found serum Fetuin-B level was significantly and positively associated with ba-PWV. But there was no significant association between serum Fetuin-B and ABI. Furthermore, we found all genotypes of the four tested *FETUB* tagging SNPs were not significantly associated with either ba-PWV or ABI.

NAFLD has been consistently associated with metabolic/insulin resistance syndrome and, thus, has been proposed to predict atherosclerosis and CVD[1, 6-7], but factors linking NAFLD to atherosclerosis and CVD have not been fully understood. Fetuin-B was the second member of the fetuin family, the cystatin superfamily of cysteine protease inhibitor[17]. As a new kind of hepatokine, it is of interest to explore the role of Fetuin-B on the association linking NAFLD to ath-

erosclerosis and CVD. Meex and Zhu *et al.* found a significant association between Fetuin-B and liver steatosis[8, 18]. We recently found that serum Fetuin-B level was independently and positively correlated with intrahepatic triglyceride content[9]. In the present study based on the same cohort, we found that, with increasing tertiles of ba-PWV, which is an ideal indicator for assessing subclinical atherosclerosis and is widely used to assess cardiovascular risk in general populations, subjects were more likely to show higher prevalence rate of NAFLD and increased levels of serum Fetuin-B. And the positive association between serum Fetuin-B and ba-PWV sustained statistically significant with adjustment for traditional CVD risk factors in the multivariable linear regression analysis. We are probably the first, to the best of our knowledge, to report the positive association of serum Fetuin-B with ba-PWV. However, ABI, another non-invasive measurement of subclinical atherosclerosis[14], was not significantly associated with serum Fetuin-B in the present study.

Evidence about mechanisms underlying the association between Fetuin-B and atherosclerosis is not available at present. Meex found that Fetuin-B impaired insulin sensitivity in myotubes and hepatocytes, but they found Fetuin-B had no effect on pro-inflammatory signaling or cytokine release, and they concluded Fetuin-B might induce insulin resistance in a manner quite distinct from that of Fetuin-A[8]. Zhu and co-workers reported that serum Fetuin-B increased in subjects with NAFLD[18]. We previously found that subjects with the highest levels of serum Fetuin-B showed significantly higher NAFLD preva-

Table 3. Multivariable linear regression of serum Fetuin-B and genotypes of *FETUB* tagging SNPs on brachial ankle pulse wave velocity and ankle-brachial index

Variables	ba-PWV (cm/s)				ABI			
	Coefficient	SE	Standardized coefficient	*P* value	Coefficient	SE	Standardized coefficient	*P* value
All Sample (*N* = 1,140)								
Model 1								
Serum Fetuin-B[†]	22.359	8.349	0.075	0.008*	-0.00027	0.00225	-0.00365	0.903
FETUB genotypes								
rs4686434 (AG/GG v.s. AA)	-11.586	16.488	-0.019	0.482	0.00149	0.00442	0.00978	0.736
rs3733159 (TG/GG v.s. TT)	14.517	18.049	0.022	0.421	0.00175	0.00484	0.01047	0.718
rs1047115 (AC/CC v.s. AA)	-16.344	20.915	-0.021	0.435	-0.00297	0.00561	-0.01538	0.597
rs6785067 (GA/AA v.s. GG)	1.851	30.111	0.002	0.951	-0.00011	0.00807	-0.00038	0.990
Model 2								
Serum Fetuin-B[†]	21.250	8.309	0.071	0.011*	-0.00029	0.00225	-0.00383	0.898
FETUB genotypes								
rs4686434 (AG/GG v.s. AA)	-11.032	16.406	-0.018	0.501	0.00157	0.00443	0.01032	0.723
rs3733159 (TG/GG v.s. TT)	9.354	17.996	0.014	0.603	0.00178	0.00486	0.01067	0.714
rs1047115 (AC/CC v.s. AA)	-16.817	20.796	-0.022	0.419	-0.00301	0.00562	-0.01562	0.592
rs6785067 (GA/AA v.s. GG)	1.529	29.940	0.001	0.959	-0.00020	0.00808	-0.00072	0.980
Model 3								
Serum Fetuin-B[†]	17.050	8.247	0.057	0.039*	-0.00018	0.00226	-0.00238	0.937
FETUB genotypes								
rs4686434 (AG/GG v.s. AA)	-13.364	16.176	-0.022	0.409	0.00181	0.00443	0.01191	0.683
rs3733159 (TG/GG v.s. TT)	9.977	17.742	0.015	0.574	0.00197	0.00486	0.01182	0.685
rs1047115 (AC/CC v.s. AA)	-15.409	20.517	-0.020	0.453	-0.00349	0.00562	-0.01810	0.535
rs6785067 (GA/AA v.s. GG)	-3.975	29.558	-0.004	0.893	0.00005	0.00809	0.00018	0.995
Model 4								
NAFLD (yes v.s. no)	77.304	17.897	0.127	<0.001*	-0.01394	0.00493	-0.09094	0.005*
Serum Fetuin-B[†]	16.291	8.185	0.055	0.047*	-0.00004	0.00226	-0.00056	0.985
FETUB genotypes								
rs4686434 (AG/GG v.s. AA)	-13.688	16.048	-0.023	0.394	0.00187	0.00442	0.01229	0.672
rs3733159 (TG/GG v.s. TT)	10.053	17.602	0.015	0.568	0.00196	0.00484	0.01174	0.686
rs1047115 (AC/CC v.s. AA)	-13.408	20.362	-0.018	0.510	-0.00385	0.00560	-0.01997	0.492
rs6785067 (GA/AA v.s. GG)	-0.116	29.339	-0.001	0.997	-0.00064	0.00807	-0.00230	0.937
Model 5								
NAFLD (yes v.s. no)	52.051	18.010	0.086	0.004*	-0.01224	0.00505	-0.07979	0.016*
Insulin resistance (yes v.s. no)	110.714	17.118	0.183	<0.001*	-0.00749	0.00480	-0.04904	0.119
Serum Fetuin-B[†]	13.822	8.050	0.047	0.086	0.00013	0.00226	0.00167	0.956
FETUB genotypes								
rs4686434 (AG/GG v.s. AA)	-12.836	15.759	-0.021	0.416	0.00181	0.00441	0.01192	0.681
rs3733159 (TG/GG v.s. TT)	15.498	17.299	0.023	0.371	0.00160	0.00485	0.00960	0.741
rs1047115 (AC/CC v.s. AA)	-12.436	19.995	-0.016	0.534	-0.00392	0.00560	-0.02031	0.484
rs6785067 (GA/AA v.s. GG)	-7.570	28.830	-0.007	0.793	-0.00014	0.00807	-0.00052	0.986

*$p < 0.05$; [†]OR and 95%CI was impressed by per SD increase of serum Fetuin-B.
Model 1 was adjusted for sex and age;
Model 2 was adjusted for educational level, ever smoking, ever drinking and regular physical exercise + model 1;
Model 3 was adjusted for waist, total cholesterol, HDL-cholesterol and serum uric acid + model 2.
Model 4 was adjusted for NAFLD + model 3.
Model 5 was adjusted for insulin resistance + model 4.
Abbreviations: ABI, Ankle-brachial index; ba-PWV, brachial ankle pulse wave velocity; NAFLD, non-alcoholic fatty liver disease.

lence, and the highest IHTC content, and that elevated Fetuin-B level was independently associated with increased risk of insulin resistance[9]. Together with the present finding that NAFLD was significantly and positively associated with ba-PWV but the significant association between Fetuin-B and ba-PWV disappeared with further adjustment for insulin resistance, we therefore suspected that Fetuin-B might link NAFLD to atherosclerosis through insulin resistance. But future studies about other mechanisms, such as oxidative stress and endothelial dysfunction besides insulin resistance, through which Fetuin-B induces atherosclerosis should be conducted[4, 19-20].

The *FETUB* gene encodes the protein Fetuin-B. *FETUB* rs4686434 SNP is an intron variant, characterized by a A-to-G substitution. We previously found that *FETUB* rs4686434 G-allele carriage was significantly associated with lower serum Fetuin-B and decreased IHTG content[10]. But we did not find any significant association between genotypes of the four tested *FETUB* tagging SNPs and the indicators of subclinical atherosclerosis (ba-PWV and ABI) in the present study. One possible reason for the null association between the tested tagging SNPs, ba-PWV and ABI was due to the small sample size in the present study, therefore, future studies with relatively large sample size are warranted to verify our findings.

Since women had significantly higher serum Fetuin-B levels than men (4.37 ± 1.39 v.s. 3.64 ± 1.24 μg/ml, $p < 0.001$), we therefore conducted separate analyses for women and men about the independent association of serum Fetuin-B and common genetic variants in *FETUB* locus with ba-PWV and ABI. We actually found similar results (data not shown), but the power has decreased dramatically due to the much smaller sample size for the subgroup analyses.

Causes for the discrepancy that serum Fetuin-B was found to be positively associated with ba-PWV but not with ABI in the present study, were not clearly understood. ABI measurement provides one of the most practical means to assess the presence of atherosclerosis, and ba-PWV, which mainly reflects the left ventricular structure and function, is affected by not only central elastic arteries but also peripheral muscular ones[21]. In our previous publication, even in the present study, we both found that, compared to controls, subjects with NAFLD showed significantly increased ba-PWV levels but did not show it significantly decreased ABI[22]. It should be noted that all subjects in the present study were all centrally obese with much higher prevalence rates of NAFLD (51.5%) than the general adults, which may prevent us from finding significant association of NAFLD with ba-PWV and ABI in the obese adults.

We should acknowledge the following limitations in the present study. The first major limitation was that our subjects were all centrally obese with a relatively higher prevalence rate of NAFLD. Around 60% of our subjects were diagnosed as NAFLD, and we may, therefore, under-estimate the true associations of serum Fetuin-B with subclinical atherosclerosis (ba-PWV and ABI). Our findings could not be generalized into the common populations, especially for lean subjects. Another major limitation was that our sample size might not have sufficient power to find significant associations between these candidate variants of the *FETUB* locus and the two indicators of subclinical atherosclerosis (ba-PWV and ABI). Therefore, an independent cohort with larger sample size, especially from a prospective cohort study design, should be conducted to validate our findings in future. Third, NAFLD was determined by hepatic ultrasonography scanning, which was considered unreliable and difficult to use in obese subjects[23]. Fourth, we cannot preclude the possibility of reverse causality between serum Fetuin-B and atherosclerosis due to our cross-sectional study design. Fifth, although Meex, Zhu and we all found significant association of serum Fetuin-B with liver steatosis[8, 9, 18], Peter recently found serum Fetuin-B level did not correlate with liver fat content or insulin sensitivity[24], therefore, more studies should be conducted in different populations before the consistent findings between Fetuin-B and liver steatosis has been reached. Sixth, we should acknowledge that although serum Fetuin-B is mainly from liver, it may also be from other organs, as Denecke *et al.* suggested[25]. Seventh, ba-PWV and ABI may not be ideal indices for atherosclerosis, especially for those obese subjects. Last but not the least, a few hepatokines, such as FGF-21, Fetuin-A and selenoprotein P[4, 5], which have been associated with atherosclerosis and may confound our findings, have not been tested in the present study. But we believed this will not eliminate the association between Fetuin-B and ba-PWV, since we have adjusted for NAFLD per se in the multivariable linear regression analyses.

Conclusions

Factors linking NAFLD to atherosclerosis have not been understood clearly and there is no human study on the association of Fetuin-B with subclinical atherosclerosis. We are probably the first, to the best of our knowledge, to report the significantly positive association of serum Fetuin-B with ba-PWV in the obese Chinese adults. Together with our finding that common genetic variants in the *FETUB* locus were not significantly associated with atherosclerosis indica-

tors, our results may imply that serum Fetuin-B may not be causally associated with ba-PWV but may link NAFLD to atherosclerosis via inducing insulin resistance. Further studies about other mechanisms, such as oxidative stress and endothelial dysfunction, through which Fetuin-B may induce atherosclerosis, should be conducted in future.

Acknowledgments

Author Contributions

The study concept and design was framed by ZL and WL. The questionnaire was developed by WL, CH, ZL and XL. WL, DW, YL, ML, CL, XS and ZC collected data and created the tables. WL and ZL conducted the statistical data analysis and drafted the manuscript. XL and SY contributed to discussion and revision. All authors read and approved the final manuscript.

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Compliance with Ethical Standards
Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards.

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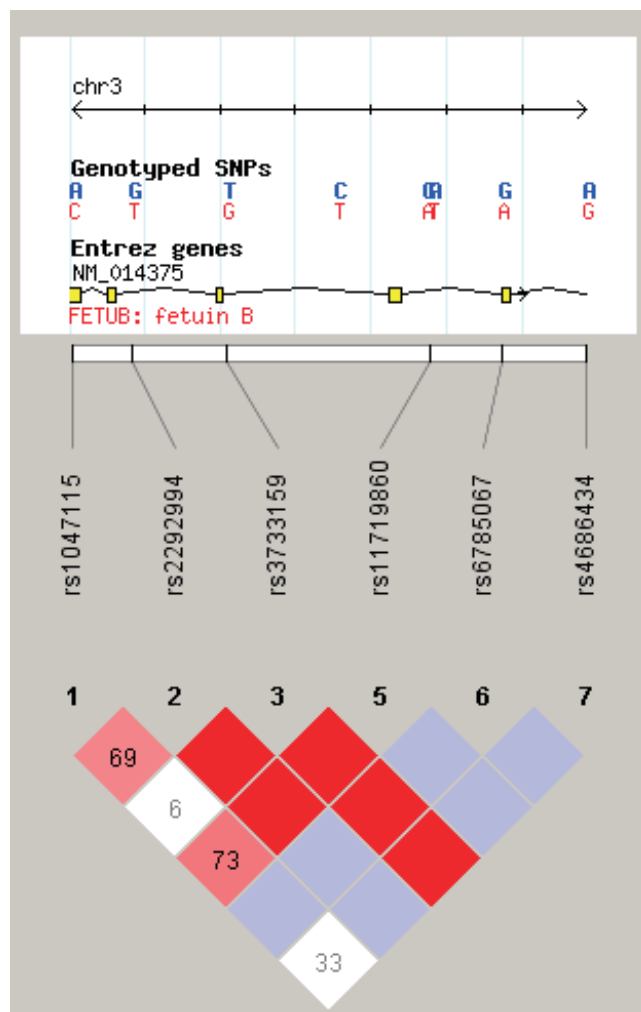
Supplementary Fig. 1. Tagging SNPs in *FETUB* gene

The *FETUB* gene consists of 8 exons and spans 17.18 kb from nucleotide position 186,635,828 to nucleotide position 186,653,008. The analyzed region additionally included 5 kb of the 5'-flanking region and 3 kb of the 3'-flanking region.

Supplementary Table 1. Genotypes of FETUB tagging SNPs

FETUB tagging SNPs	Genotype	*N*	MAF(%)	*P* value for HWE test
rs4686434 (A>G)	AA /AG /GG	665 /436 /39	22.5	0.002*
rs3733159 (T>G)	TT /TG /GG	321 /573 /246	46.7	0.745
rs1047115 (A>C)	AA /AC /CC	928 /201 /11	9.8	0.974
rs6785067 (G>A)	GG /GA /AA	1050 /87 /3	4.1	0.404

*$p < 0.05$

Abbreviations: HWE, Hardy-Weinberg equilibrium; MAF, Minor allele frequency.