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

Zhibin Li¹, Chunmei He², Yongwen Liu³, Dongmei Wang⁴, Mingzhu Lin⁵, Changqin Liu⁵, ⁶, Xiulin Shi⁵, Zheng Chen⁶, Xuejun Li⁵, Shuyu Yang² and Weihua Li⁷

Zhibin Li and Chunmei He contributed equally to this work.

¹Epidemiology Research Unit, The First Affiliated Hospital of Xiamen University, Xiamen, China
²Xiamen Diabetes Institute, The First Affiliated Hospital of Xiamen University, Xiamen, China
³Department of Nursing, The First Affiliated Hospital of Xiamen University, Xiamen, China
⁴School of Medicine, Xiamen University of Xiamen, China
⁵Department of Endocrinology and Diabetes, The First Affiliated Hospital of Xiamen University, Xiamen, China
⁶Department of Endocrinology and Diabetes, the Teaching Hospital of Fujian Medical University, Xiamen, China
⁷Department of Cardiology, The First Affiliated Hospital of Xiamen University, Xiamen, China

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). Multi-variable 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 risk1-3. 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 cross-talk4, 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\textsuperscript{6, 7}. Meex RC \textit{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\textsuperscript{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\textsuperscript{9}.

Fetuin-B is encoded by the \textit{FETUB} gene, which is located in the human chromosome 3q27.3 with eight exons. The \textit{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 \textit{FETUB} rs4686434 had lower levels of serum Fetuin-B and decreased IHTG content than their controls\textsuperscript{10}. We further found a significant joint effect between \textit{FETUB} rs4686434 and patatin-like phospholipase domain-containing-3 (\textit{PNPLA3}) rs738409, another genetic variant associated with NAFLD in multi-ethnic populations in genome-wide association studies\textsuperscript{11}, on IHTG content\textsuperscript{10}. Our findings suggest that the genetic variant on \textit{FETUB} rs4686434 might influence hepatic triglyceride accumulation. Our and others’ findings suggest that serum Fetuin-B levels and genetic variants on \textit{FETUB} rs4686434 might influence hepatic triglyceride accumulation.

Available evidence has documented that both NAFLD and insulin resistance are closely associated with atherosclerosis \textsuperscript{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 \textit{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 \textit{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\textsuperscript{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 \textit{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 electrochemiluminescence 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 ≥ 2.6 \* 10^{-6} mol/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%\textsuperscript{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
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).

**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

---

Figure 1: Study subjects selection diagram

1523 residents aged 40 years or older with central obesity (waist circumference ≥ 90 cm for men and 80 cm for women) in Lianqian community, Xiamen, China

- 92 had incomplete data on clinical and biochemistry measurements
- 1431 residents were selected
- 291 did not undergo serum Fetuin-B, ba-PWV or ABI measurements.
- 1140 residents were selected for analyzing the relationship between Fetuin-B and subclinical atherosclerosis.
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. The higher value of ba-PWV and lower value of ABI in either limb were used for further analysis.

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’, r²) between the tested SNPs was analyzed using MIDAS v1.0 (http://www.genes.org.uk/software/midas).

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 vs. 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 vs. 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

| Variables                        | ba-PWV (cm/s) | ABI              |
|----------------------------------|---------------|------------------|
|                                  | Tertile 1     | Tertile 2        | Tertile 3        |
| Age (years)                      | 50.4±6.6      | 53.7±6.5         | 56.6±6.5         |
| Education, (n, %)                |               |                  | ˂0.001*          |
| Illiteracy                       | 98 (25.7%)    | 100 (26.4%)      | 123 (32.4%)      |
| Elementary school                | 115 (30.2%)   | 112 (29.6%)      | 106 (27.9%)      |
| Middle school                    | 78 (20.5%)    | 95 (25.1%)       | 86 (22.6%)       |
| High school or above             | 90 (23.6%)    | 72 (19.0%)       | 65 (17.1%)       |
| Ever smoking, (n, %)             | 93 (24.4%)    | 110 (29.0%)      | 84 (22.1%)       |
| Ever drinking, (n, %)            | 50 (13.1%)    | 71 (18.7%)       | 56 (14.7%)       |
| Regular physical exercise (n, %) | 123 (32.3%)   | 135 (35.6%)      | 121 (31.8%)      |
| Waist circumference (cm)         | 92.6±7.4      | 94.3±7.7         | 93.5±6.1         |
| Systolic blood pressure (mmHg)   | 121.4±1.17    | 133.2±13.1       | 146.4±17.5       |
| Diastolic blood pressure (mmHg)  | 73.2±8.5      | 80.3±8.9         | 84.8±11.2        |
| Triglyceride (mmol/L)            | 1.55±1.03     | 1.99±1.32        | 2.17±1.43        |
| Total cholesterol (mmol/L)       | 5.69±1.04     | 5.95±1.04        | 6.07±1.13        |
| HDL-cholesterol (mmol/L)         | 1.41±0.30     | 1.36±0.28        | 1.35±0.29        |
| Serum uric acid (mg/dL)          | 349.0±95.9    | 372.8±90.0       | 370.1±95.1       |
| HOMA-IR (mU/L)                   | 2.84±1.65     | 3.67±3.22        | 4.09±2.82        |
| Insulin resistance, (n, %)       | 176 (46.2%)   | 229 (60.4%)      | 272 (71.6%)      |
| NAFLD (n, %)                     | 186 (48.8%)   | 233 (61.5%)      | 268 (70.5%)      |
| Serum Fetuin-B (µg/ml)           | 3.85±1.39     | 4.09±1.40        | 4.27±1.46        |

**FETUB Genotypes**

| rs4686434 | rs3733159 | rs1047115 | rs6785067 |
|-----------|-----------|-----------|-----------|
| AG/GG     | TG/GG     | AC/CC     | GA/AA     |
| 162 (42.5%) | 267 (70.1%) | 80 (21.0%) | 30 (7.9%) |
| 159 (40.2%) | 271 (71.5%) | 62 (16.4%) | 29 (7.7%) |
| 154 (40.5%) | 278 (74.0%) | 70 (18.4%) | 31 (8.2%) |
| 162 (38.3%) | 304 (71.9%) | 85 (20.1%) | 38 (9.0%)  |
| 168 (45.2%) | 276 (74.2%) | 66 (17.7%) | 29 (7.8%)  |
| 145 (42.0%) | 239 (69.3%) | 61 (17.7%) | 23 (6.7%)  |
| 0.848      | 0.487     | 0.258     | 0.967     |
| 0.415      | 0.343     | 0.607     | 0.494     |

All genotypes 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

| Variables   | FETUB tagging SNPs |   |   |   |   |
|-------------|--------------------|---|---|---|---|
|             | rs4686434          |   |   |   |   |
|             | AA                 |   |   |   |   |
| ba-PWV (cm/s) | 1529.2 ± 295.6     |   |   |   |   |
| ABI         | 1.07 ± 0.07        |   |   |   |   |
|             | AG/GGP value       |   |   |   |   |
|             | 1517.9 ± 299.7     |   |   |   |   |
|             | 1.08 ± 0.08        |   |   |   |   |
|             | P value            |   |   |   |   |
|             | 0.529              |   |   |   |   |
|             | TT                 |   |   |   |   |
| ba-PWV (cm/s) | 1515.9 ± 317.8     |   |   |   |   |
| ABI         | 1.07 ± 0.08        |   |   |   |   |
|             | TG/GG              |   |   |   |   |
| ba-PWV (cm/s) | 1527.8 ± 288.9     |   |   |   |   |
| ABI         | 1.07 ± 0.07        |   |   |   |   |
|             | P value            |   |   |   |   |
|             | 0.546              |   |   |   |   |
|             | rs3733159          |   |   |   |   |
|             | AA                 |   |   |   |   |
| ba-PWV (cm/s) | 1528.3 ± 298.2     |   |   |   |   |
| ABI         | 1.07 ± 0.07        |   |   |   |   |
|             | AC/CC              |   |   |   |   |
| ba-PWV (cm/s) | 1507.7 ± 292.8     |   |   |   |   |
| ABI         | 1.07 ± 0.08        |   |   |   |   |
|             | P value            |   |   |   |   |
|             | 0.364              |   |   |   |   |
|             | TT                 |   |   |   |   |
| ba-PWV (cm/s) | 1525.0 ± 297.4     |   |   |   |   |
| ABI         | 1.07 ± 0.07        |   |   |   |   |
|             | GG                 |   |   |   |   |
| ba-PWV (cm/s) | 1518.4 ± 296.5     |   |   |   |   |
| ABI         | 1.07 ± 0.08        |   |   |   |   |
|             | P value            |   |   |   |   |
|             | 0.839              |   |   |   |   |
|             | rs1047115          |   |   |   |   |
|             | AA                 |   |   |   |   |
| ba-PWV (cm/s) | 1525.8 ± 288.9     |   |   |   |   |
| ABI         | 1.07 ± 0.07        |   |   |   |   |
|             | AC/CC              |   |   |   |   |
| ba-PWV (cm/s) | 1518.4 ± 296.5     |   |   |   |   |
| ABI         | 1.07 ± 0.08        |   |   |   |   |
|             | P value            |   |   |   |   |
|             | 0.809              |   |   |   |   |
|             | rs6785067          |   |   |   |   |
|             | AA                 |   |   |   |   |
| ba-PWV (cm/s) | 1525.8 ± 288.9     |   |   |   |   |
| ABI         | 1.07 ± 0.07        |   |   |   |   |
|             | AC/CC              |   |   |   |   |
| ba-PWV (cm/s) | 1518.4 ± 296.5     |   |   |   |   |
| ABI         | 1.07 ± 0.08        |   |   |   |   |
|             | P value            |   |   |   |   |
|             | 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 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, 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. As a new kind of hepatokine, it is of interest to explore the role of Fetuin-B on the association linking NAFLD to atherosclerosis and CVD. Meex and Zhu et al. found a significant association between Fetuin-B and liver steatosis. We recently found that serum Fetuin-B level was independently and positively correlated with intrahepatic triglyceride content. 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, 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. Zhu and co-workers reported that serum Fetuin-B increased in subjects with NAFLD. 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 | rs6785067 (GA/AA v.s. GG) | -11.586 | 16.488 | -0.019 | 0.482 | 0.00149 | 0.00442 | 0.00978 | 0.736 |
| Model 2   | Serum Fetuin-B | 21.250 | 8.309 | 0.071 | 0.011* | -0.00029 | 0.00225 | -0.00383 | 0.898 |
| FETUB genotypes | rs6785067 (GA/AA v.s. GG) | -11.032 | 16.406 | -0.018 | 0.501 | 0.00157 | 0.00443 | 0.01032 | 0.723 |
| Model 3   | Serum Fetuin-B | 17.050 | 8.247 | 0.057 | 0.039* | -0.00018 | 0.00226 | -0.00238 | 0.937 |
| FETUB genotypes | rs6785067 (GA/AA v.s. GG) | -13.364 | 16.176 | -0.022 | 0.409 | 0.00181 | 0.00443 | 0.01191 | 0.683 |
| 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 | rs6785067 (GA/AA v.s. GG) | -13.408 | 20.362 | -0.020 | 0.453 | -0.00349 | 0.00562 | -0.01810 | 0.535 |
| 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 | 0.0013 | 0.00226 | 0.00167 | 0.956 |
| FETUB genotypes | rs6785067 (GA/AA v.s. GG) | -12.836 | 15.759 | -0.021 | 0.416 | 0.00181 | 0.00441 | 0.01192 | 0.681 |

*p<0.05; 1 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 1;
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.
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. 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, we should acknowledge that although serum Fetuin-B is mainly from liver, it may also be from other organs, as Denecke et al. suggested. 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, 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
This study was completed with the assistance of the Lianqian community health service center, Xiamen, China. And we are grateful to all the subjects for their participation.

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.

Funding Information
ZL was founded by the National Key R&D Program of China grant no: 2017YFC0907100, CH was founded by Natural Science Foundation of Fujian Province grant no: 2015J01559, CL was founded by Natural Science Foundation of China grant no: 81870611 & Xiamen Municipal Bureau of Science and Technology grant no: 3502Z20154010, ML was founded by Xiamen Municipal Bureau of Science and Technology grant no: 3502Z20164008.

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.

References
1) Targher G, Day CP, Bonora E: Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. N Engl J Med, 2010; 363: 1341-1350
2) Targher G, Byrne CD: Clinical Review: Nonalcoholic fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications. J Clin Endocrinol Metab, 2013; 98: 483-495
3) Oni ET, Agatston AS, Blaha MJ, Fialkow J, Cury R, Sposito A, Erbel R, Blankstein R, Feldman T, Al-Mallah MH, Santos RD, Budoff MJ, Nasir K: A systematic review: burden and severity of subclinical cardiovascular disease among those with nonalcoholic fatty liver; should we care? Atherosclerosis, 2013; 230: 258-267
4) Luo J, Xu L, Li J, Zhao S: Nonalcoholic fatty liver disease as a potential risk factor of cardiovascular disease. Eur J Gastroenterol Hepatol, 2015; 2: 193-199
5) Yoo HJ, Choi KM: Hepatokines as a link between obesity and cardiovascular diseases. Diabetes Metab J, 2015; 39: 10-15
6) Weikert C, Stefan N, Schulze MB, Pischon T, Berger K, Joost HG, Häring HU, Boeing H, Fritsche A: Plasma fetuin-a levels and the risk of myocardial infarction and ischemic stroke. Circulation, 2008; 118: 2555-2562
7) Dogru T, Genc H, Tapan S, Aslan F, Ercin CN, Ors F, Kara M, Sertoglu E, Karsioglu Y, Bagci S, Kurt I, Sonmez A: Plasma fetuin-A is associated with endothelial dysfunction and subclinical atherosclerosis in subjects with nonalcoholic fatty liver disease. Clin Endocrinol (Oxf), 2013; 78: 712-717
8) Meex RC, Hoy AJ, Morris A, Brown RD, Lo JC, Burke M, Goode RJ, Kingwell BA, Kraakman MJ, Febbraio MA, Greve JW, Rensen SS, Mollol MP, Lancaster GI, Bruce CR, Wattr MJ: Fetuin B is a secreted hepatocyte factor linking steatosis to impaired glucose metabolism. Cell Metab, 2015; 22: 1078-1089
9) Wang D, Liu Y, Liu S, Lin L, Liu C, Shi X, Chen Z, Lin M, Yang S, Li Z, Li X: Serum fetuin-B is positively associated with intrahepatic triglyceride content and increases the risk of insulin resistance in obese Chinese adults: a cross-sectional study. J Diabetes, 2018; 10: 581-588
10) Li Z, Lin M, Liu C, Chen Z, Wang D, Shi X, Yang S, Li X: The rs4686434 variant in the FETUB locus is associated with intrahepatic triglyceride content in obese Chinese adults. J Diabetes, 2018; 10: 916-925. doi: 10.1111/1753-0407.12774
11) Romeo S, Kozlitina J, Xing C, Pertzsemidias A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH: Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet, 2008; 40: 1461-1465
12) Bornfeldt KE, Tabas I: Insulin resistance, hyperglycemia, and atherosclerosis. Cell Metab, 2011; 14: 575-585
13) Fan JG: Chinese Liver Disease Association: Guidelines for management of nonalcoholic fatty liver disease: an updated and revised edition. Zhonghua Gan Zang Bing Za Zhi, 2010; 18: 163-166
14) Aboyans V, Criqui MH, Abraham P, Allison MA, Creager MA, Diehm C, Fowkes FG, Hiatt WR, Jonsson B, Lacroix P, Marin B, McDermott MM, Norgren L, Pande RL,
Association of Fetuin-B with Subclinical Atherosclerosis

Preux PM, Stoffers HE, Treat-Jacobson D; American Heart Association Council on Peripheral Vascular Disease; Council on Epidemiology and Prevention; Council on Clinical Cardiology; Council on Cardiovascular Nursing; Council on Cardiovascular Radiology and Intervention, and Council on Cardiovascular Surgery and Anesthesia: Measurement and interpretation of the ankle-brachial index: a scientific statement from the American Heart Association. Circulation, 2012; 126: 2890-2909

15) Lehmann ED: Clinical value of aortic pulse-wave velocity measurement. Lancet, 1999; 354: 528-529

16) Gaunt TR, Rodríguez S, Zapata C, Day IN: MIDAS: software for analysis and visualisation of interallelic disequilibrium between multiallelic markers. BMC Bioinformatics, 2006; 7: 227

17) Olivier E, Soury E, Ruminy P, Husson A, Parmentier F, Daveau M, Salier JP: Fetuin-B, a second member of the fetuin family in mammals. Biochem J, 2000; 350 Pt 2: 589-597

18) Zhu J, Wan X, Wang Y, Zhu K, Li C, Yu C, Li Y: Serum fetuin B level increased in subjects of nonalcoholic fatty liver disease: a case-control study. Endocrine, 2017; 56: 208-211

19) Stefan N, Haring HU: The role of hepatokines in metabolism. Nature reviews Endocrinology, 2013; 9: 144-152

20) Kotronen A, Juurinen L, Tiikkainen M, Vehkavaara S, Yki-Jarvinen H: Increased liver fat impaired impaired insulin clearance, and hepatic and adipose tissue insulin resistance in type 2 diabetes. Gastroenterology, 2008; 135: 122-130

21) Yu WC, Chuang SY, Lin YP, Chen CH: Brachial-ankle vs carotid-femoral pulse wave velocity as a determinant of cardiovascular structure and function. J Hum Hypertens, 2008; 22: 24-31

22) Liu Y, Liu C, Shi X, Lin M, Yan B, Zeng X, Chen N, Lu S, Liu S, Yang S, Li X, Li Z: Correlations of nonalcoholic fatty liver disease and serum uric acid with subclinical atherosclerosis in obese Chinese adults. Journal of Diabetes, 2017; 9: 586-595

23) Mottin CC, Moretto M, Padoin AV, Swarowsky AM, Toneto MG, Glock L, Repetto G: The role of ultrasound in the diagnosis of hepatic steatosis in morbidly obese patients. Obesity surgery, 2004; 14: 635-637

24) Peter A, Kovařová M, Staiger H, Machann J, Schick F, Königsrainer A, Königsrainer I, Schleicher E, Fritsche A, Haring HU, Stefan N: The hepatokines fetuin-A and fetuin-B are upregulated in the state of hepatic steatosis and may differently impact on glucose homeostasis in humans. Am J Physiol Endocrinol Metab, 2018; 314: E266-E273. doi: 10.1152/ajpendo.00262.2017

25) Denecke B, Gruber S, Schafer C, Heiss A, Wolte M, Jahn-Dechent W: Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. The Biochemical journal, 2003; 376: 135-145
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 /111 | 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.

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.