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Xiaoqian Gao

Tianjin Medical University

Ruiqin Hou

Peking University People's Hospital

Xin Li

Tianjin Medical University

Hui-Huan Luo

Tianjin Medical University

Zhong-Ze Fang (✉ fangzhongze@tmu.edu.cn)

Tianjin Medical University <https://orcid.org/0000-0003-1113-9262>

Research

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The association between leucine and diabetic nephropathy (DN) in different gender: A cross-sectional study in Chinese patients with type 2 diabetes

Xiaoqian Gao^{1, #}, Ruiqin Hou^{3, #}, Xin Li¹, Hui-Huan Luo¹, Zhong-Ze Fang^{1, 2, *}

¹Department of Toxicology and Sanitary Chemistry, School of Public Health, Tianjin Medical University, Tianjin, China;

²Tianjin Key Laboratory of Environment, Nutrition and Public Health, Tianjin, China;

³ Department of Blood Transfusion, Peking University People's Hospital, Beijing, China.

These two authors equally contributed to this work.

*** Correspondence to:**

Zhong-Ze Fang, Department of Toxicology and Sanitary Chemistry, School of Public Health, Tianjin Medical University, Tianjin, 300070, China;

E-mail: fangzhongze@tmu.edu.cn.

Abstract

Background: This study aimed to evaluate how leucine are associated with diabetic nephropathy (DN) in type 2 diabetes(T2D) patients and the gender difference of this association.

Methods: We retrieved 1,032 consecutive patients with T2D from the same tertiary care center and extracted clinical information from electronic medical record. Plasma

leucine was measured by liquid chromatography-mass spectrometer. Restricted cubic spline (RCS) was conducted to examine potential non-linear relationship between leucine with DN and stratified leucine into categories. Logistic regression was used to obtain odds ratio (OR) and confidence interval (CI). Additive interaction was used to estimate the interaction effect between leucine and gender for DN.

Results: The results showed a negative linear correlation between leucine and DN. After stratifying all patients by gender, this relationship only remained significant in women (OR:0.57, CI:0.41-0.79), and the relationship was still significant in additive interaction analysis.

Conclusion: In conclusion, diabetes patients with high levels of leucine have a lower risk of developing diabetic nephropathy only in female.

Keywords: leucine, diabetic nephropathy (DN), metabolism, type 2 diabetes (T2D)

Background

Diabetic nephropathy (DN) which is one of common complications of diabetes, greatly increases mortality and medical expenses in type 2 diabetes (T2D) patients(1,2). DN increased enormous societal burden, as it amplifies the risk of other diabetes complications including cardiovascular disease, heart failure, infections(3–7). Since DN is a kind of progressive disease, it is important to search for new approaches that can effectively forecast and prevent the onset of DN. However, noninvasive available markers for accurate prediction and diagnoses of DN in diabetic

patients are lacking now(8). It is worth noting that many studies have found the burden of diabetes is different in different genders(9). For example, women have higher mortality rate for diabetes-related deaths, including DN(10). One may speculate that there is maybe something different in disease pathways and predictors of DN between men and women.

With the development of technologies which allow the high-throughput profiling of metabolic status from a blood specimen (metabolomics), more and more researches focused on exploring whether metabolite profiles affect the onset and development of DN via combining epidemiology and metabolomics (11–14)

Insufficient insulin secretion which is a risk factor of DN has been found to be associated with plasma amino acids(15). Different from other amino acids, leucine, one of branched-chain amino acids (BCAAs), is catabolized in skeletal muscle which is important organ for the regulation of blood glucoses(16,17). Recently, leucine has been reported to attenuate DN progression with improving insulin sensitivity and decreasing adiposity in HFD-fed animal(18,19). However, the specific mechanism on the regulation of leucine towards DN still unclear. At present, there are some population studies expounding the relationship between leucine and diabetes, but there is still a lack of population evidence about the effect of leucine on the risk of DN(20).

In this study, we established a cross-sectional study in a Chinese population, and aimed to 1) evaluate association between plasm leucine and risk of DN; 2) examine whether this association is affected by gender.

67

68 **Methods**

69 **Study population and settings**

70 The First Affiliated Hospital of Liaoning Medical University (FAHLMU) is a
71 comprehensive tertiary care center serving 3.1 million people, in Jinzhou, Liaoning
72 Province, China. 1898 T2D patients were diagnosed, among which, 866 patients were
73 excluded due to the ages under 18 years old or lacked height, weight and blood
74 pressure. Based on these exclusion criteria, a total of 1031 research subjects were
75 included. The diagnosis of diabetes was based on the standard published by WHO in
76 1999 or treated with antidiabetic drugs(21). The Ethics Committee for Clinical
77 Research of FAHLMU approved the ethics of the study, and informed consent was
78 waived due to the retrospective nature of the study, which is consistent with the
79 Declaration of Helsinki.

80

81 **Data collection and definitions**

82 The data retrieved from the electronic medical records for both groups contained
83 demographic and anthropometric information, as well as current clinical factors,
84 medications and complications of diabetes. Demographic included gender, current
85 status of smoking and alcohol consumption. Anthropometric measurements yielded
86 information included height, weight, systolic blood pressure (SBP) and diastolic blood
87 pressure (DBP). Duration of diabetes and diabetic nephropathy were recorded.
88 Clinical parameters contained glycosylated hemoglobin((HbA1c), triglyceride (TG),

high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), urinary creatinine (UA), serum creatinine (SCR). Details of medication use were documented, including oral anti-diabetic drugs, insulin, and lipid lowering drugs, statins.

In hospitals, anthropometric indices were measured by using standardized procedures. Participants were allowed to wear light clothes and no shoes. Height and weight were measured to the nearest 0.5 cm and 0.1 kg respectively. Blood pressure was measured behind the right arm of an adult cuff using a standard mercury sphygmomanometer and post-measurement at an appropriate size, after a 10 minutes rest in a sitting position. Age was calculated from the date of birth to the date of hospitalization or medical examination, and was calculated in years. The body mass index (BMI) was calculated as the ratio of weight(kg) to squared height(meters) classifying overweight and obesity according to the criteria recommended by the National Health Commission in China(22). The diagnostic criteria for diabetic nephropathy was based on the national guidelines for the prevention and management of diabetes at the basic level(23).

Laboratory assay

Dried blood spots were used in the assay of metabolomics, which were prepared from capillary whole blood through 8-h fasting. We measured the metabolites by direct infusion mass spectrometry technology equipped with the AB Sciex 4000 QTrap system (AB Sciex, Framingham, MA, USA). High-purity water and acetonitrile were

purchased from Thermo Fisher (Waltham, MA, USA), and utilized as diluting agent and mobile phase. 1-Butanol and acetyl chloride were obtained from Sigma-Aldrich (St Louis, MO, USA). Isotope-labeled internal standard samples of amino acids (NSK-A) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA), while standard samples of the leucine were purchased from Chrom Systems (Grafelfing, Germany). In brief, 8.5 mL of venous blood was drawn from each participant at 08:00 to 09.30 hours in the morning after 8-h fasting. Laboratory tests were carried out at a special diagnostic laboratory. The level of lipid profiles was analyzed by an automatic biochemistry analyzer (Hitachi 7150, Tokyo, Japan). We also assayed the level of HDL-C and LDL-C by the selective solubilization method (Determiner L-HDL, LDL test kit; Kyowa Medex, Tokyo, Japan).

Statistical Analysis

Data with the normal distribution was represented by the mean $\pm$ standard deviation (SD), or use the median (interquartile range). Categorical variable was in numbers (percentage). Whether there was a difference between DN group and non-DN group were tested separately in male and female. The continuous variable was judged by student's t-test or separate variance estimation t-test or Wilcoxon-W test when appropriate; Categorical variable was analyzed by chi-square test.

Binary logistic regression model was established to obtain the odds ratio (OR) and their 95% confidence intervals (CI). Traditional risk factors for type 2 diabetes patients with DN were adjusted using a structured adjustment scheme. We obtained

unadjusted OR values and the OR after adjusted age, gender, BMI (<18.5, 18.5-24.0, 24.0-28.0, >28.0 kg/m²), duration of diabetes, smoking, drinking, SBP, DBP, TG, LDL-C, HDL-C, HbA1c, UA, SCR, insulin, statins. Restricted cubic splines curve (RCS) is a smoothing curve that can provide more intuitive relationship curve. We chose 4 knots in RCS as suggested by Harrell. We have used it to obtain cutoffs for metabolites related to the risk of developing diabetes (24). We selected a cut-off point by visual checking of the curve where the odds of DN changed.

We repeated logistic regression analysis in males and females respectively to obtain OR values. Additive interaction analysis was used to verify the relationship between gender (male or female) and leucine (in 2 groups by RCS cutoff) for DN. We calculated the relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP) and synergy index (S) to estimate additive interactions(25). RERI>0, AP>0 or S>1 indicates a significant additive interaction(25). To avoid the bias caused by non-incident DN. We exclude the patients who with duration of DN >2 years to check the changes of the effect sizes of leucine for risk of DN.

A P < 0.05 was considered as statistically significant. All analyses were performed using R version 3.6 and SAS version 9.4 (Institute Inc., Cary, North Carolina, USA).

Results

Characteristics of the study population

The mean age and BMI of 1031 participants were 57.2 years (SD:13.8) and 25.3

kg/m² (SD:3.9). Of them, 46.8% were female. There was a total of 188 patients with DN and 92 of them are women. **Table 1** summarized the selected characteristics of DN and controls by sex. In women, cases tended to be older, had higher BMI and longer duration of diabetes, higher SBP, HbA1c, HDL-C, LDL-C, and SCR, and were less likely to use insulin and statins than patients without DN. In men, patients with DN had longer duration of T2D, higher SCR, UA and were less likely to use insulin than controls.

The relationship between diabetic nephropathy and leucine

The slope of RCS curve has a process from small to large and then small, which reaches its maximum at about 175μmol/L. Among all patients, leucine level of 84.8% patient were below 175μmol/L. (**Figure 1**)

In univariable regression, leucine was inversely associated with the risk of DN (OR: 0.8, 95% CI:0.67, 0.95). After further adjustment for traditional risk factors, the negative association was strengthened in multivariable analysis (OR:0.76, 95%CI: 0.62, 0.92). (**Table 2**)

Interaction between leucine and gender

Leucine was negative associated with the risk of DN in diabetes patients in the female population (OR: 0.51, 95%CI: 0.41, 0.79) while the relationship was not significant in male(**table3**). In female the risk of DN was decrease rapidly until around 175μmol/L of leucine and then started to relatively flat afterwards. In male, the association

between BMI and mortality disappeared (**Figure 2**). Using 175 as a cutoff value of leucine, leucine(<175 or ≥ 175 $\mu\text{mol/L}$) and gender(male or female) had a significant additive interaction for DN (AP: 0.90, 95%CI: 0.18–1.62; RERI: 0.60, 95%CI: 0.08–1.12; and S: 0.36, 95% CI: 0.11– 1.20).

Sensitivity analysis

After excluding 10 patients who had DN for more than 2 years, the effect size was larger. In the multivariable analysis, the OR value became 0.74 (0.61, 0.91), and the OR value in the female population changed to 0.56 (CI: 0.40, 0.77).

Discussion

In this study, we found that high plasma level of leucine was associated with decreased odds of DN in female T2D. Female with lower plasma leucine had three-fold more risk of DN than those who had higher. However, the association between leucine and risk of DN was not significant in male. We also found that additive interaction between sex and leucine for DN risk was significant to prove this association.

Although there are no population trials to compare results. The inverse association was consistent with many animal studies. Previous study has shown that leucine supplementation could alleviate early DN damage (19). There are some related experiments that can indirectly explore the effect of leucine on diabetic nephropathy. A study found that the supplement of leucine in diet can active the

AMPK(26), which was considered as a newly identified regulator of renal hypertrophy in diabetes(26,27). Higher plasma leucine has been found to reverse the disorder of tricarboxylic acid (TCA) cycle metabolism and carnitine metabolic disorders in mice with DN(28,29). Hyperlipidemia is also a common cause of DN, and the supplementation of leucine in animal tests can reduce blood lipid(27). By increasing energy consumption, leucine could reduce the likelihood of obesity and increase insulin sensitivity, which can reduce the risk of DN(1,30,31).

Some mechanistic studies suggested that leucine is a potent activator of mTORC1 which control the protein synthesis(32). Leucine directly binds Sestrin2, and then Sestrin2 releases GATOR2 which is a positive regulator of mTORC1 and insulin secretion(16,33–37). After activation, mTORC1 promotes protein synthesis and controls autophagy by phosphorylating a series of targets. Leucine also activates mTORC1 via other mechanisms, such as initiation of the colocalization of the lysosome with mTORC1(38). This is a possible speculation why leucine influence insulin secretion and then further affect the risk of DN. In other studies, it was found that the supplement of lacto-leucine during lactation take the change of insulin levels in the offspring which were affected by gender(28). This difference may be caused by that the activation of the mTOR pathway and the ribosomal kinase S6K1 showed a higher expression in female (39,40). The possibility of this difference was further confirmed in our population study. The negative correlation between leucine and DN was found only in women. The mechanism by which leucine protects DN need further clarify until now.

Our finding had important clinical and research implications: 1) The discovery of potential novel markers, leucine, will be helpful in diagnosis and disease prediction; 2) As an essential amino acid, leucine has only dietary sources(28). Noticeable, protein are often restricted in diabetic patients which possibly cause a deficiency of leucine for patient(1). This result may give clinical suggestion that When taking a protein-restricted diet, consider separate amino acid supplements; 3) Male mice were often used in animal experiments, which also causes the potential relationships between leucine and DN may be erased by gender selection. And it reminds us to consider exploring in more mechanism research and animal experiments to determine whether disease pathways can differ and if therapeutic targeting strategies are equally effective in both sexes.

The present study has some limitations. Firstly, the cross-sectional study can only prove the statistical association between plasma leucine and DN, but not the exact causal relationship. Due to the lack of research in population, this study provides clues and direction to the further relevant study. Secondly, due to the lack of time and energy, some of the influencing factors were not investigated, such as diet, which may have an impact on results. Nevertheless, we can indirectly adjust the bias of those factor through BMI. Thirdly, our subjects were hospitalized with T2D, who may have more severe diabetes and nephropathy complications. We try to exclude patients with diabetic nephropathy for more than 2 years in the sensitivity analysis. Results showed that the OR value reduced, and the results were still very significant in the total study population and female population. Therefore, the report of this study may have a low

estimate of the protective effect of leucine on diabetic nephropathy.

Conclusion

This study shows that leucine was inversely association with the risk of DN in women with T2D. Considering the shortcoming of cross-sectional studies, more studies with stronger causal justification and in other populations are needed for further confirmation.

Perspectives and significance

Gender differences in the effect of leucine on occurrence and development of diabetic nephropathy have been researched for some animal experiments and mechanism research. Our population research validates the results of before studies and provide some scientific and clinical significance. In previous animal experiments, male animals were often used, which may affect the discovery of this relationships.

Our exploration provided clues for explore possible predictors to improving the accuracy of clinical diabetic nephropathy prediction and diagnosis. And due to the nature of essential amino acids, the intervention of leucine can be taken by simple oral means. If the results replicate in population randomized controlled trial, we have reason to believe that improvement of plasma level of leucine in female patients could be used as a method to protect and control DN.

Ethics approval and consent to participate

The Ethics Committee for Clinical Research of LMUFAH approved the ethics of the study

Consent for publication

Informed consent was waived by the above ethics committee due to the retrospective nature of the study.

Availability of data and materials

Data are available on request to the corresponding author.

Competing interests

All other authors have no conflicts of interest to declare.

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Authors' contributions

Zhong-Ze Fang conceived the project, designed experiments. Xiaoqian Gao wrote the

manuscript and analyzed data. Hui-Huan Luo collect the information and contributed to the writing of this manuscript. Xin Li contributed to the data interpretation. Ruiqin Hou gave critical comments on the first draft. All authors edited the final version of the manuscript. All authors read and approved the final manuscript.

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FIGURE1|Odds ratio curves of leucine for diabetic nephropathy in Chinese type 2 diabetic patients.

The black curve was derived from univariable analysis. The blue one was derived from multivariate analysis that adjusted for age, gender, body mass index, duration of diabetes, smoking, drinking, systolic blood pressure, diastolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and triglyceride, glycosylated hemoglobin, urinary creatinine, serum creatinine, insulin, statins(i.e., the odds ratio for diabetic nephropathy was 1).

FIGURE2|Odds ratio curves of leucine for diabetic nephropathy in Chinese type 2 diabetic patients in different gender.

The black curve was derived from univariable analysis, the blue one derived from

440 multivariate analysis that adjusted for age, gender, body mass index, duration of
441 diabetes, smoking, drinking, systolic blood pressure, diastolic blood pressure,
442 low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride,
443 glycosylated hemoglobin, urinary creatinine, serum creatinine , insulin, statins(i.e., the
444 odds ratio for diabetic nephropathy was 1).

Table1 Clinical and biochemical characteristics of participants according to the occurrence of diabetic nephropathy.

Variables	Women		<i>p</i> ^a	Men		<i>p</i> ^a
	Non-DN (391)	DN (92)		Non-DN (452)	DN (96)	
	Mean/number (SD or %)	Mean/number (SD or %)		Mean/number (SD or %)	Mean/number (SD or %)	
Age (years)	59.16±12.81	60.54±9.8	<0.01	54.75±14.81	57.72±14.33	0.074
Weight (kg)	63.64±10.56	67.11±13.21	0.007	75.72±12.79	75.43±11.04	0.84
Height (cm)	160.00 (156.00, 163.00)	160.00 (158.00, 165.00)	0.235	172.00 (170.00, 175.00)	172.00 (170.00, 175.00)	0.616
BMI (kg/m ²)	24.83±3.83	25.87±4.36	0.024	25.53±3.83	25.54±3.41	0.977
BMI<18.5	70(17.9%)	24(26.1%)		104(23.01%)	23(23.96%)	
BMI≥18.5and<24.0	150(38.3%)	32(34.8%)		211(46.68%)	37(38.54%)	
BMI≥24.0and<28.0	162(41.3%)	34(37.0%)		122(26.99%)	35(36.46%)	
BMI≥28.0	9(2.3%)	2(2.2%)		15(3.32%)	1(1.04%)	
Smokingyes	25 (6.4%)	8 (8.7%)	0.577	243 (53.8%)	55 (57.3%)	0.604
Drinkingyes	13 (3.3%)	2 (2.2%)	0.811	221 (48.9%)	54 (56.2%)	0.231
Duration of diabetes (years)	6.97±7.44	8.93±7.71	0.024	3.00 (0.00, 10.00)	10.00 (2.75, 14.25)	<0.001
SBP (mmHg)	140.00 (122.00, 155.00)	149.50 (129.50, 174.00)	0.001	138.71±22.46	142.1±22.42	0.179
DBP (mmHg)	80.79±13.52	83.23±12.59	0.116	83.79±13.3	82.25±14.82	0.312
HbA1c (%)	7.53±3.22	8.64±2.68	0.002	7.66±3.07	8.24±3.11	0.096
Triglyceride (mmol/L)	1.22 (0.82, 2.08)	1.52 (1.02, 2.31)	0.089	1.22 (0.82, 2.00)	1.31 (0.89, 1.97)	0.561
HDL-C (mmol/L)	0.9±0.45	1.07±0.52	0.001	0.84 (0.45, 1.06)	0.85 (0.50, 1.07)	0.265
LDL-C (mmol/L)	2.42±1.24	2.79±1.31	0.013	2.25 (1.13, 2.95)	2.42 (1.27, 3.04)	0.505
SCR (μmol/L)	53.51 (43.41, 70.95)	57.55 (45.59, 119.00)	0.028	68.72 (58.00, 91.10)	87.78 (64.41, 314.17)	<0.001
UA (μmol/L)	307.00 (238.00, 445.50)	340.60 (264.25, 532.35)	0.073	345.00 (274.75, 499.00)	391.50 (326.00, 778.00)	0.004

Leucine (μmol/L)	124.87±43.17	112.69±31.61	<0.001	143.18±49.85	137.24±46.56	<0.001
<175μmol/L	349(79.9%)	42(93.33%)		352(77.87%)	100(22.12%)	
diabetic medications						
Acarbose	129 (33.0%)	43 (46.7%)	0.018	150 (33.2%)	42 (43.8%)	0.064
Metformin	131 (33.5%)	41 (44.6%)	0.061	155 (34.3%)	31 (32.3%)	0.797
Insulin	275 (70.3%)	85 (92.4%)	<0.001	321 (71.0%)	90 (93.8%)	<0.001
Statins	131 (33.5%)	45 (48.9%)	0.008	152 (33.6%)	41 (42.7%)	0.116

Data are mean (standard deviation), median (interquartile range) or n (%). BMI, body mass index. SBP, systolic blood pressure. DBP, diastolic blood pressure. HbA1c, glycated hemoglobin. HDL-C, high- density lipoprotein cholesterol. LDL-C, low-density lipoprotein cholesterol. UA, uric acid. SCR, serum creatinine.

^a Based on the t-test, Wilcoxon rank-sum test or χ^2 test as appropriate.

1 **Table2** Odds ratio of leucine for the risk of diabetic nephropathy.

	OR (95%CI)	<i>p</i>
Univariable model		
Leu, per $\mu\text{mol/L}$	0.8 (0.67,0.95)	0.012
<175 $\mu\text{mol/L}$	reference	
$\geq 175 \mu\text{mol/L}$	0.59 (0.36,0.97)	0.036
Multivariable model1		
Leu, per $\mu\text{mol/L}$	0.8 (0.67,0.96)	0.016
<175 $\mu\text{mol/L}$	reference	
$\geq 175 \mu\text{mol/L}$	0.61 (0.37,1.01)	0.045
Multivariable model2		
Leu, per $\mu\text{mol/L}$	0.76 (0.63,0.92)	0.006
<175 $\mu\text{mol/L}$	reference	
$\geq 175 \mu\text{mol/L}$	0.56 (0.33,0.94)	0.044
Multivariable model3		
Leu, per $\mu\text{mol/L}$	0.76 (0.62,0.92)	0.006
<175 $\mu\text{mol/L}$	reference	
$\geq 175 \mu\text{mol/L}$	0.58 (0.34,0.99)	0.038

2 Multivariable Model 1 was adjusted for age, gender, body mass index, duration of
3 diabetes, smoking, drinking.

4 Multivariable Model 2 was adjusted for variables in Model 1 and concentrations of
5 systolic blood pressure, diastolic blood pressure, triglyceride, low-density lipoprotein
6 cholesterol, high-density lipoprotein cholesterol, glycosylated hemoglobin.

7 Multivariable Model 3 was adjusted for variables in Model 2 and concentrations of
8 urinary creatinine, serum creatinine, insulin, statins.

9

10 **Table3** Odds ratio of leucine for the risk of diabetic nephropathy.

	female(n=483)		male(n=548)	
	OR(95%CI)	<i>p</i>	OR(95%CI)	<i>p</i>
Univariable model				
Leu, per $\mu\text{mol/L}$	0.71(0.55,0.93)	0.008	0.88(0.7,1.11)	0.274
<175 $\mu\text{mol/L}$	reference		reference	
$\geq 175 \mu\text{mol/L}$	0.28(0.08,0.92)	0.037	0.76(0.43,0.34)	0.329
Multivariable model1				

Leu, per $\mu\text{mol/L}$	0.7(0.53,0.91)	0.009	0.90(0.71,1.14)	0.380
<175 $\mu\text{mol/L}$	reference		reference	
$\geq 175 \mu\text{mol/L}$	0.29(0.09,0.98)	0.045	0.78(0.44,1.38)	0.394
Multivariable model2				
Leu, per $\mu\text{mol/L}$	0.59(0.43,0.81)	0.001	0.90(0.71,1.12)	0.399
<175 $\mu\text{mol/L}$	reference		reference	
$\geq 175 \mu\text{mol/L}$	0.26 (0.07,0.87)	0.030	0.75(0.42,1.36)	0.345
Multivariable model3				
Leu, per $\mu\text{mol/L}$	0.57(0.41,0.79)	<0.001	0.92(0.71,1.18)	0.493
<175 $\mu\text{mol/L}$	reference		reference	
$\geq 175 \mu\text{mol/L}$	0.24(0.07,0.83)	0.024	0.81(0.44,1.48)	0.489

- 11 Multivariable Model 1 was adjusted for age, gender, body mass index, duration of
12 diabetes, smoking, drinking.
- 13 Multivariable Model 2 was adjusted for variables in Model 1 and concentrations of
14 systolic blood pressure, diastolic blood pressure, triglyceride, low-density lipoprotein
15 cholesterol, high-density lipoprotein cholesterol, glycosylated hemoglobin.
- 16 Multivariable Model 3 was adjusted for variables in Model 2 and concentrations of
17 urinary creatinine, serum creatinine, insulin, statins.

Figures

Figure 1

Figure 1

Figure 2

Figure 2