

Effects of *PPM1K* rs1440581 and rs7678928 on serum branched-chain amino acid levels and risk of cardiovascular disease

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ABSTRACT

Objective: This study aimed to investigate the effects of *PPM1K* rs1440581 and rs7678928 single nucleotide polymorphisms (SNPs) on the serum branched-chain amino acids (BCAAs) levels and cardiovascular disease (CVD) risk.

Methods: Anthropometric and biochemical examinations were performed at baseline and the end of 4 years in 234 individuals who were randomly recruited from the *Diabetes Prevention Programme in Huai'an* and received lifestyle intervention and follow up for 4 years. Serum BCAAs (leucine, isoleucine and valine (Val)) levels were measured by hydrophilic interaction chromatography-tandem mass spectrometric method and the *PPM1K* rs1440581 and rs7678928 were detected by high-throughput SNP genotyping at baseline. The associations of rs1440581 and rs7678928 with serum BCAA levels and risk for CVD after 4 years were further evaluated.

Results: The distribution frequencies of *PPM1K* rs1440581 and rs7678928 met the Hardy-Weinberg equilibrium ($p > .05$). The baseline serum levels of Val ($p = .022$) and total BCAAs ($p = .026$) in subjects with rs1440581 CC genotype were higher than in those with TT genotype. There were no significant differences in the serum levels of BCAAs among subjects with different genotypes of rs7678928. After 4-year follow-up, the subjects with rs1440581 CC genotype had higher systolic blood pressure (SBP) ($p = .027$), diastolic blood pressure (DBP) ($p = .019$), triglycerides (TGs) ($p = .019$) and lower high-density lipoprotein cholesterol (HDL-c) ($p = .008$) than those with TT genotype, and had higher AST level than those with TT ($p = .030$) or TC ($p = .003$) genotype; the subjects with rs7678928 TT genotype had higher SBP ($p = .039$) and DBP ($p = .019$) and lower HDL-c than those with CC ($p = .017$) genotype. Lifestyle intervention had little influence on the serum levels of fasting plasma glucose (FPG), TG, HDL-c, alanine aminotransferase (ALT), AST and creatinine (CREA) in subjects with rs1440581 CC genotype or rs7678928 TT genotype ($p > .05$). The incidences of CVD and non-alcoholic fatty liver disease (NAFLD) in subjects with rs1440581 CC genotype were higher than in those with TT genotype; the incidence of CVD in subjects with rs7678928 TT genotype was higher than in those with CC ($p < .05$) genotype.

Conclusions: Allele C of *PPM1K* rs1440581 was associated with elevated serum Val, total BCAAs and CVD risks. rs1440581 CC genotype may be a better marker than baseline serum BCAAs in predicting the risk for CVD.

Trial registration: *Diabetes Prevention Programme in Huai'an* of Huai'an Second People's Hospital, ChiCTR-TRC-14005029.

KEY MESSAGE

1. Allele C of *PPM1K* rs1440581 was relevant to elevated serum Val and total BCAAs.
2. *PPM1K* rs1440581 CC and rs7678928 TT genotypes were associated with CVD risk.
3. *PPM1K* rs1440581 CC genotype carriers were more likely to have liver injury and develop NAFLD.





Abbreviations: AAA: aromatic amino acid; AC: abdominal circumference; ADA: American Diabetes Association; ALT: alanine aminotransferase; ANOVA: analysis of variance; AST: aspartate aminotransferase; BCAA: branched chain amino acid; BCKA: branched chain α -ketoacid; BCKDC: branched chain α -ketoacid dehydrogenase complex; BCKDK: branched chain α -ketoacid dehydrogenase kinase; BMI: body mass index; BP: blood pressure; BUN: blood urea nitrogen;

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CREA: creatinine; CT: computed tomography; CTA: computed tomography angiography; CV: cardiovascular; CVD: cardiovascular disease; DBP: diastolic blood pressure; ECG: electrocardiograph; FPG: fasting plasma glucose; GWAS: genome-wide association study; HbA1c: haemoglobin A1c; HDL-c: high-density lipoprotein cholesterol; HFF: hepatic fat fraction; Hi-SNP: high-throughput SNP; Ile: isoleucine; IVUS: intravascular ultrasound; LDL-c: low-density lipoprotein cholesterol; Leu: leucine; MSUD: maple diabetes; NAFLD: non-alcoholic fatty liver disease; Phe: phenylalanine; PPM1K/PP2CM: protein phosphatase 1K; SBP: systolic blood pressure; SD: standard deviation; SNP: single nucleotide polymorphism; T2DM: type 2 diabetes mellitus; TBIL: total bilirubin; TC: total cholesterol; TGs: triglycerides; TP: total protein; Tyr: tyrosine; UA: uric acid; Val: valine; WHO: World Health Organization

Background

Branched-chain amino acids (BCAAs; including leucine (Leu), isoleucine (Ile) and valine (Val)) are essential amino acids that cannot be synthesized *de novo*. They are involved in the regulation of cell growth, neurotransmitter synthesis, carbohydrate utilization and lipid metabolism [1]. Some observational studies have shown that the elevated serum levels of BCAAs are associated with metabolic dysfunction [2]. Our previous cross-sectional study also indicated that the elevated serum BCAA levels were related to dyslipidaemia and positively associated with the risk for cardiovascular disease (CVD) [3,4]. However, whether the causative relationship between elevated serum BCAA levels and CVD remains unclear.

Branched chain α -ketoacid dehydrogenase complex (BCKDC) is the key enzyme that catalyses the first irreversible step in the BCAA metabolism, and the phosphorylation of branched chain α -ketoacid dehydrogenase kinase (BCKDK) can inhibit the activity of BCKDC. Conversely, BCKDC is activated by the mitochondrial isoform of PP2C domain-containing protein phosphatase 1K (PPM1K, also known as PP2CM) [5]. A genome-wide association study (GWAS) in Caucasians found that a marker (rs1440581) at 4q22 of *PPM1K* was associated with both Fischer's ratio (ratio of BCAA levels to the concentrations of phenylalanine (Phe) plus tyrosine (Tyr)) and Val [6]. In addition, Mendelian randomization analysis in 16,596 Caucasians showed that the *PPM1K* rs1440581 and rs7678928 single nucleotide polymorphisms (SNPs) were associated with serum Leu and Val levels, and rs7678928 SNP was associated with serum Ile level. Both SNPs were found to reach the genome-wide level of significance [7]. However, no study has been conducted to investigate the correlations between *PPM1K* SNPs and serum BCAA levels in Chinese population. It is also unclear whether the rs1440581 and rs7678928 SNPs affect the CVD risk in the Chinese population.

In the present study, two *PPM1K* SNPs (rs1440581 and rs7678928) were detected in the middle-aged and elderly Chinese Han people, and their associations

with the serum BCAA levels were investigated. After 4-year follow-up, the associations between two SNPs and risk for CVD were further evaluated.

Methods

Study population and baseline data collection

A total of 234 subjects (age: 40–79 years) were from the *Diabetes Prevention Programme in Huai'an* and received routine health examinations between August 2014 and September 2014. All the subjects were randomly selected from participants in a previous cross-sectional study [4].

The anthropometric measurements (height, weight, abdominal circumference (AC)) were performed and the body mass index (BMI) was calculated as the subject's weight (kg) divided by height squared (m^2). The demographics (age, gender and race), lifestyle (history of smoking and drinking), history of diseases and medications were collected using standard questionnaires and interviews by trained investigators. In addition, fasting blood samples were collected for the measurement of serum BCAAs and other biochemical examinations. The blood pressure (BP) was also recorded. The exclusion criteria were as follows: (1) coronary heart disease, including myocardial infarction and angina pectoris; (2) previously diagnosed kidney diseases, including nephritis, autoimmune or drug-induced kidney disease, renal failure, or kidney transplant with dialysis treatment; (3) previously diagnosed serious hepatic diseases, including fatty liver, liver cirrhosis and autoimmune hepatitis; (4) peripheral arterial sclerosis disease; (5) malignant diseases.

Subjects received lifestyle intervention and were followed up for 4 years. Subjects without SNPs genotypes (three of rs1440581, three of rs7678928) and those without biochemical examinations during follow-up period ($n = 22$) were excluded. At the end of study, 209 subjects completed this study. The inclusion of these subjects is shown in [Figure 1](#).

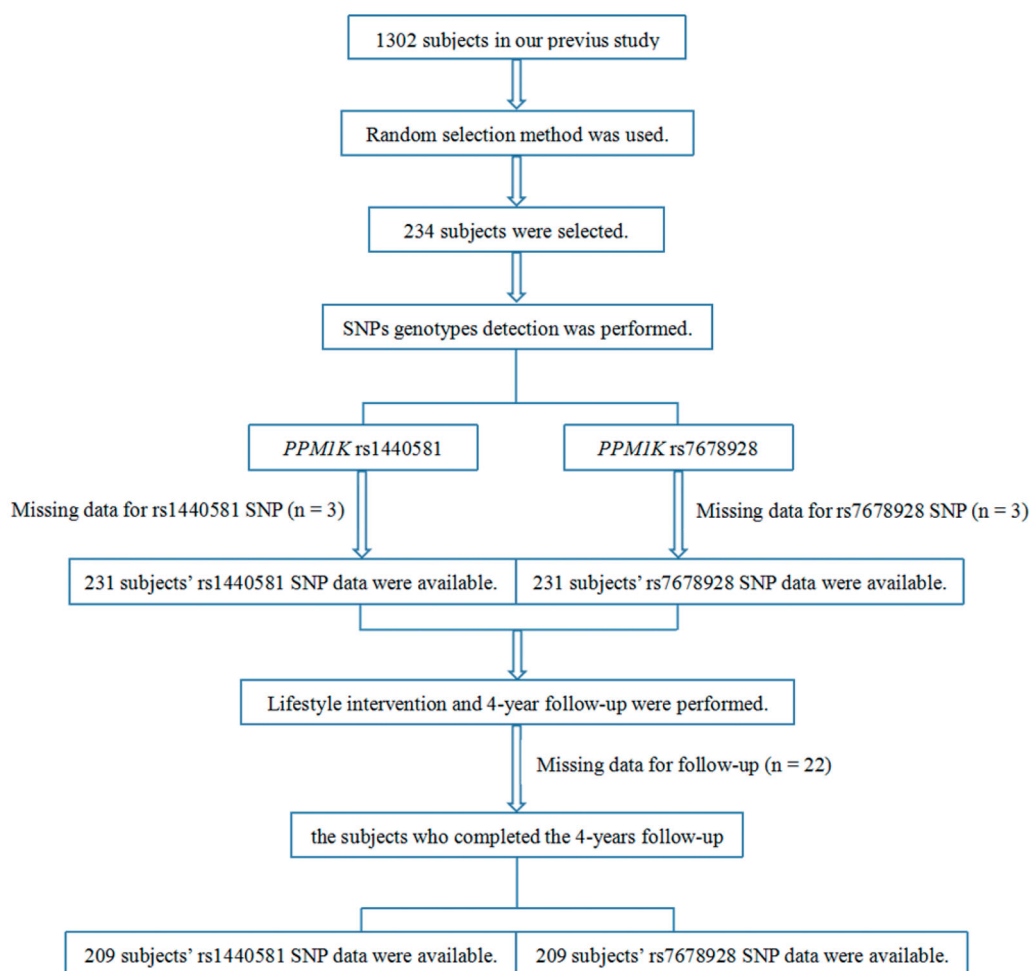


Figure 1. Schematic process of subject recruitment.

Table 1. The sequences of primers used in PCR.

Primer	Sequence (5'–3')	
rs1440581	TATTAGACTCCAACATAGACACCC	CTACTCCAGGTATATAGCCCAAAG
rs7678928	CTAAGATGAAGTTGTTTGACCTTC	CAGAGTCCAGTCACCAAGTACAGC

Sample size

One hundred subjects were randomly selected in a pilot study and received SNPs detection. The sample size was computed by using PASS software 11 (NCSS, LLC, Kaysville, UT). In the one-way analysis of variance (ANOVA) model, power (1-beta) was 0.90, alpha was 0.05, the ratio of heterozygosity to homozygosity was 1:2, and the mean and standard deviation (SD) of serum BCAA levels in each genotype group in the pilot study were input. Finally, 234 subjects were included according to the maximum requirements output of different BCAAs in each SNP.

DNA extraction and SNP genotyping

At the baseline, fasting blood samples were collected from all subjects and stored at -80°C for DNA extraction.

Genotypes detection was performed in Shanghai Biowing Co., Ltd. (Shanghai, China) (www.biowing.com.cn) by high-throughput SNP (Hi-SNP) genotyping [8], which is based on multiplex PCR coupled with next generation sequencing. Specific primers designed by using Primer 3 software (<http://frodo.wi.mit.edu>) (Table 1).

Target genes were initially amplified with $10\mu\text{L}$ of PCR reaction containing $2\mu\text{L}$ of human genomic DNA, 0.5U of Hot Start DNA Polymerase (Rendu Biotechnology, Shanghai, China), $1\mu\text{L}$ of PCR buffer (Rendu Biotechnology, Shanghai, China), $0.8\mu\text{L}$ of dNTPs, $1\mu\text{L}$ of MgSO_4 , $3.2\mu\text{L}$ of ddH_2O and $2\mu\text{L}$ of each primer in the first round PCR. The following conditions were used for PCR: 95°C for 15 min, four cycles of 94°C for 30 s, 60°C for 10 min and 72°C for 30 s, and 20 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s. The second round PCR ($20\mu\text{L}$) was

performed using 10 μ L of first round PCR products, 0.5 U of Hot Start DNA Polymerase, 2 μ L of PCR buffer, 0.8 μ L of dNTPs, 1 μ L of $MgSO_4$, 3.6 μ L of ddH₂O and 3.6 μ L of Barcode. The following conditions were used for PCR: 95 °C for 15 min, five cycles of 94 °C for 30 s, 60 °C for 4 min and 72 °C for 30 s, and 10 cycles of 94 °C for 30 s, 65 °C for 1 min and 72 °C for 30 s. The bridge PCR products were sequenced on the Illumina X-10 (Illumina Technologies Corporation, San Diego, CA) sequencing platform, and the operation flow was carried out according to the standard SOP.

Lifestyle intervention and follow-up data collection

All subjects received health education and lifestyle instruction such as dietary and exercise interventions. The specific intervention measures were reported in *The Da Qing IGT and Diabetes Study* [9]. In general, dietary intervention was conducted as follows: (1) subjects with BMI <25 kg/m² were prescribed with a diet containing 25–30 kcal/kg body weight calorie, 55–65% carbohydrate, 10–15% protein and 25–30% fat, and encouraged to consume more vegetables, control alcohol intake and reduce their monosaccharose intake; (2) subjects with BMI \geq 25 kg/m² were encouraged to reduce calorie intake so as to gradually lose weight at a rate of 0.5–1.0 kg/month until they achieved a BMI of 23 kg/m². Exercise intervention was administered as follows: subjects were educated and encouraged to increase the physical exercise by at least 1 U/day and by 2 U/day (if possible) for subjects younger than 50 years and having no evidence of CVD or arthritis. Medication and medical advice were given if necessary. Subjects received free health examinations once yearly. According to the results of examinations, specialists and nurses designed standard treatment plans.

After 4-year follow-up, detailed information was collected by the trained investigators about lifestyle (history of smoking and drinking), history of diseases (hypertension, diabetes, non-alcoholic fatty liver disease (NAFLD), CVD, etc.) and medication as reported in previous study [4]. All subjects were fasted for more than 8 h, and the venous blood samples were collected between 07:00 and 09:00 in the morning for the detection of fasting plasma glucose (FPG), triglycerides (TGs), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL),

creatinine (CREA) and uric acid (UA). As mentioned previously, SBP and DBP were also measured.

Incident cases ascertainment and definitions

Hypertension was defined as systolic blood pressure (SBP) \geq 140 mmHg, diastolic blood pressure (DBP) \geq 90 mmHg or use of antihypertensive medication within 2 weeks. Type 2 diabetes mellitus (T2DM) was diagnosed according to the 2012 American Diabetes Association (ADA) criteria as follows: FPG \geq 126 mg/dL (7.0 mmol/L) or 2-h plasma glucose in the 75-g oral glucose tolerance test (OGTT) \geq 200 mg/dL (11.1 mmol/L) or HbA1c \geq 6.5% [10]. Subjects were diagnosed with NAFLD by abdominal ultrasonography and the alcohol consumption less than 40 g/day for men or less than 20 g/day for women [11].

CVD were defined as follows: (1) cardiac death; (2) heart failure; (3) fatal and non-fatal myocardial infarction (including asymptomatic myocardial infarction); (4) fatal and non-fatal stroke; (5) leg amputation above the ankle; (6) any revascularization of the coronary, leg or carotid arteries; (7) angina confirmed by new electrocardiograph (ECG) abnormalities; (8) asymptomatic myocardial ischaemia (occult coronary heart disease) confirmed by computed tomography (CT), computed tomography angiography (CTA), intravascular ultrasound (IVUS) or other auxiliary examinations; (9) intermittent claudication with an ankle-brachial index less than 0.90.

Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (Statistical Product and Service Solutions Inc., Chicago, IL). Quantitative data are presented as mean \pm SD. One-way ANOVA was used for comparisons among groups. Bonferroni's method was used for comparisons between two groups. Categorical variables are presented as numbers (%) and were compared among groups with the Chi-square test. Student's *t*-test was applied to compare the data before and after lifestyle intervention. Multiple logistic regression analysis was used to investigate the odds ratio (OR) for CVD in carriers of different genotypes in three models. Model 1 was unadjusted. Model 2 was adjusted for baseline clinical characteristics (gender, age, BMI, AC, BP, FPG, TG, TC, LDL-c, HDL-c, ALT, AST and CREA) and history of smoking and drinking; model 3 was further adjusted for history of hypertension and diabetes medication, changes in TG, TC and HDL-c in the follow-up period and prevalence of hypertension

Table 2. Baseline characteristics of subjects according to SNP genotypes.

Variable	rs1440581			<i>p</i>	rs7678928			<i>p</i>
	TT	TC	CC		CC	CT	TT	
<i>n</i> (%)	66 (28.6)	116 (50.2)	49 (21.2)	–	86 (37.2)	115 (49.8)	30 (13.0)	–
Age (years)	56.4±5.1	56.1±4.4	56.69±4.5	.762	55.8±5.0	56.7±4.4	56.1±4.5	.431
Male, <i>n</i> (%)	19 (28.8)	31 (26.7)	11 (22.4)	.743	22 (25.6)	31 (27.0)	8 (26.7)	.976
History of smoking, <i>n</i> (%)	8 (12.1)	16 (13.8)	3 (6.1)	.371	8 (9.3)	17 (14.8)	0 (0)	.057
History of drinking, <i>n</i> (%)	8 (12.1)	12 (10.3)	6 (12.2)	.908	6 (7.0)	16 (13.9)	3 (10.0)	.290
History of hypertension medication, <i>n</i> (%)	13 (19.7)	32 (27.6)	18 (36.7)	.127	31 (36.0)	21 (18.3)	10 (33.3)	.013*
History of diabetes medication, <i>n</i> (%)	6 (9.1)	14 (12.1)	8 (16.3)	.501	9 (10.5)	15 (13.0)	4 (13.3)	.838
BMI (kg/m ²)	24.54±4.53	24.38±3.60	24.48±3.02	.959	24.39±4.10	24.25±3.74	25.33±2.64	.372
AC (cm)	83.13±9.78	82.53±8.24	82.52±8.81	.895	82.17±9.04	82.17±8.42	85.67±9.15	.127
SBP (mmHg)	140±21	137±18	145±22	.100	139±21	139±18	144±23	.381
DBP (mmHg)	83±14	83±15	86±13	.360	83±13	84±15	88±14	.277
FPG (mmol/L)	5.87±1.96	5.93±1.83	6.43±2.64	.286	5.9±1.96	6.04±2.02	6.21±2.56	.815
HbA1c (%)	5.91±1.00	6.11±1.18	6.13±1.47	.489	5.91±0.99	6.13±1.25	6.16±1.48	.390
TG (mmol/L)	1.99±0.86	1.99±1.27	2.13±1.23	.769	1.99±0.97	1.99±1.21	2.18±1.40	.705
TC (mmol/L)	5.16±0.91	5.18±0.72	5.09±1.02	.834	5.18±0.83	5.18±0.85	5.00±0.95	.585
LDL-c (mmol/L)	2.78±0.71	2.77±0.58	2.61±0.69	.320	2.77±0.66	2.74±0.61	2.65±0.73	.692
HDL-c (mmol/L)	1.40±0.61	1.33±0.44	1.43±0.55	.455	1.44±0.73	1.30±0.27	1.39±0.48	.166
ALT (U/L)	26.02±13.36	25.71±14.60	27.94±18.22	.678	25.03±12.59	27.45±17.27	25.43±12.28	.504
AST (U/L)	22.42±7.10	22.59±9.42	22.84±8.44	.968	22.28±6.99	23.05±9.39	21.83±9.47	.713
TBIL (μmol/L)	12.73±7.14	11.89±5.28	14.15±15.21	.319	12.21±6.13	12.86±11.06	12.75±4.81	.869
CREA (μmol/L)	66.59±13.52	67.36±13.95	67.87±14.70	.882	67.10±14.21	67.54±14.04	66.20±14.70	.897
BUN (mmol/L)	4.77±1.15	5.04±1.12	5.02±1.34	.298	4.77±1.24	5.15±1.14	4.84±1.22	.076
UA (μmol/L)	269.15±70.80	270.2±84.65	284.72±70.57	.494	272.07±75.93	273.81±82.49	273.34±70.97	.988
Phe (μg/mL)	19.35±4.65	19.28±3.77	20.77±6.60	.236	19.19±4.36	19.86±5.20	18.94±3.92	.492
Tyr (μg/mL)	15.22±3.32	15.56±2.89	15.67±3.64	.702	15.20±3.11	15.55±3.23	15.49±3.25	.742

BMI: body mass index; AC: abdomen circumference; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; HbA1c: haemoglobin A1C; TG: triglyceride; TC: total cholesterol; LDL-c: low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TP: total protein; TBIL: total bilirubin; CREA: creatinine; BUN: blood urea nitrogen; UA: uric acid; Phe: phenylalanine; Tyr: tyrosine.

Data are presented as the mean ±SD or number (%), as appropriate. Comparisons among three groups were done using one-way ANOVA. Bonferroni's method was used for comparisons between two groups.

**p* < .05.

and diabetes after follow-up based on model 2. A value of *p* < .05 was considered statistically significant.

Results

Baseline characteristics

A total of 234 subjects were included in this study. After excluding those lacking SNP (three of rs1440581, three of rs7678928), 231 subjects were included for further analysis of baseline characteristics (Table 2). The frequencies of TT, TC and CC genotypes in rs1440581 were 28.6%, 50.2% and 21.2%, respectively; the frequencies of allele T and C were 53.7% and 46.3%, respectively. All alleles and genotypes of rs1440581 met Hardy-Weinberg equilibrium (*p* = .882, $\chi^2=0.022$). The frequencies of CC, CT and TT genotypes in rs7678928 were 37.2%, 49.8% and 13.0%, respectively; the frequencies of allele C and T were 62.1% and 37.9%, respectively. All alleles and genotypes of SNP rs7678928 met Hardy-Weinberg equilibrium (*p* = .379, $\chi^2=0.773$). The average age of subjects whose rs1440581 SNP data were available was 56.33 ± 4.63 years, and 26.4% was males in these subjects. No significant differences were found in the baseline characteristics among SNP rs1440581

genotypes (*p* > .05). The average age of subjects whose rs7678928 SNP data were available was 56.26 ± 4.67 years, and 26.4% was males in these subjects. Except for the history of hypertension medication (*p* = .013), there were no significant differences in the baseline characteristics among SNP rs7678928 genotypes (*p* > .05).

Associations of rs1440581 and rs7678928 with baseline serum BCAA levels and Fischer's ratio

Baseline serum BCAA levels were compared according to two SNPs genotypes. The average baseline serum Val level of subjects with TT, TC and CC genotype in rs1440581 was 30.77 ± 6.31 μg/mL, 32.24 ± 7.27 μg/mL and 34.43 ± 8.33 μg/mL, respectively, while the average serum total BCAA level was 70.32 ± 13.83 μg/mL, 72.57 ± 14.59 μg/mL and 78.00 ± 15.56 μg/mL, respectively. Results showed that the levels of serum Val (*p* = .022) and total BCAAs (*p* = .026) in subjects with CC genotype in rs1440581 were significantly higher than in those with TT genotype (Figure 2). There were no significant differences in the serum BCAA level among genotypes of rs7678928 (*p* > .05) (Figure 2). No marked differences were observed in Fischer's ratio among different SNPs genotypes (*p* > .05).

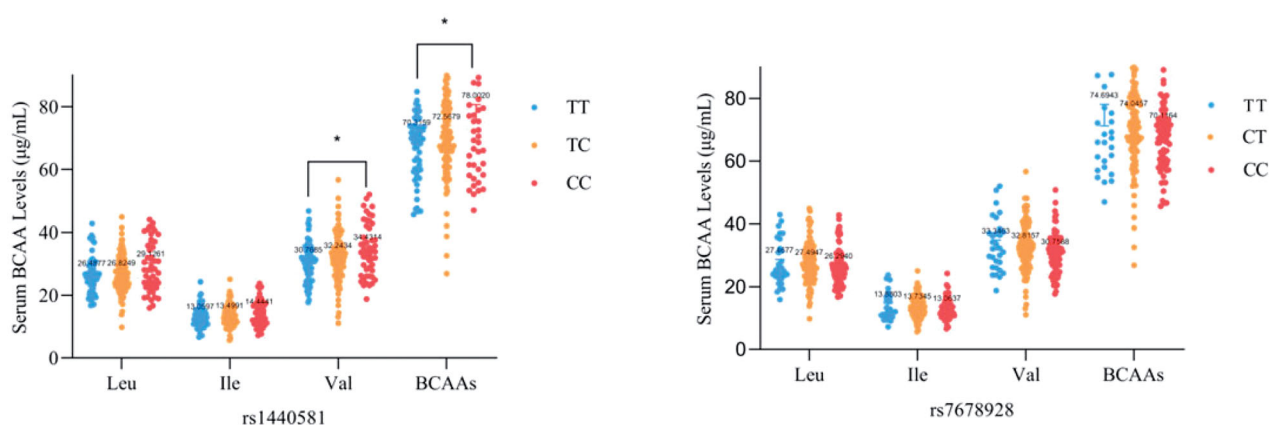


Figure 2. Serum BCAA levels of subjects according to SNPs genotypes. Comparisons among three groups were done using one-way ANOVA. Bonferroni's method was used for comparison between two groups. * $p < .05$. Leu: leucine; Ile: isoleucine; Val: valine; BCAA: branched amino acid.

Table 3. CV characteristics of subjects according to SNP genotypes after 4-year follow-up.

Variable	rs1440581			p	rs7678928			p
	TT	TC	CC		CC	CT	TT	
n	59	106	44	–	80	103	26	–
SBP (mmHg)	132 ± 20	137 ± 18	143 ± 27	.056	133 ± 20	136 ± 19	145 ± 29	.105
DBP (mmHg)	82 ± 11	85 ± 11	88 ± 15	.021*	83 ± 11	84 ± 11	91 ± 16	.022*
FPG (mmol/L)	5.12 ± 0.80	5.21 ± 1.31	5.50 ± 1.87	.393	5.13 ± 0.87	5.30 ± 1.59	5.32 ± 1.46	.672
TG (mmol/L)	1.34 ± 0.40	1.42 ± 0.60	1.66 ± 0.76	.025*	1.43 ± 0.52	1.43 ± 0.64	1.59 ± 0.68	.438
TC (mmol/L)	5.13 ± 0.90	4.95 ± 1.01	5.19 ± 1.04	.290	5.03 ± 1.00	5.08 ± 0.98	5.14 ± 1.13	.878
LDL-c (mmol/L)	3.06 ± 0.87	2.99 ± 0.81	3.36 ± 0.97	.062	2.99 ± 0.92	3.10 ± 0.79	3.36 ± 1.00	.166
HDL-c (mmol/L)	1.73 ± 0.37	1.62 ± 0.36	1.52 ± 0.28	.010*	1.69 ± 0.38	1.62 ± 0.34	1.47 ± 0.27	.020*
ALT (U/L)	19.14 ± 8.82	19.00 ± 10.23	21.72 ± 11.94	.310	20.56 ± 11.37	18.37 ± 9.22	21.26 ± 10.30	.240
AST (U/L)	19.21 ± 5.07	18.61 ± 6.47	23.02 ± 11.07	.013*	19.87 ± 6.25	19.23 ± 8.14	22.13 ± 8.85	.221
TBIL (µmol/L)	14.35 ± 7.97	12.67 ± 6.14	13.65 ± 4.54	.260	14.40 ± 7.90	12.44 ± 4.77	14.28 ± 6.71	.095
CREA (µmol/L)	57.23 ± 13.51	55.31 ± 13.86	60.33 ± 13.84	.127	57.19 ± 14.17	56.12 ± 13.58	58.88 ± 14.53	.643
UA (µmol/L)	273.56 ± 75.96	256.47 ± 74.35	262.21 ± 78.98	.383	261.97 ± 76.03	258.70 ± 69.63	253.27 ± 81.97	.546

SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; TG: triglyceride; TC: total cholesterol; LDL-c: low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin; CREA: creatinine; UA: uric acid.

Data are presented as mean ± SD. Comparisons among three groups were done using one-way ANOVA. Bonferroni's method was used for comparison between two groups.

* $p < .05$.

Associations of rs1440581 and rs7678928 with CV characteristics after 4-year follow-up

We excluded the subjects without biochemical examinations during 4-year follow-up period ($n = 22$). Then, 209 subjects were included for the analysis (Table 3). Results showed there were statistical differences in the DBP ($p = .021$), TG ($p = .025$), HDL-c ($p = .010$) and AST ($p = .013$) among genotypes in rs1440581 after follow-up. In addition, the subjects with rs1440581 CC genotype had higher SBP ($p = .027$), DBP ($p = .019$) and TG ($p = .019$) and lower HDL-c ($p = .008$) than those with TT genotype, and had higher AST level than those with TT ($p = .030$) or TC ($p = .003$) genotype. The DBP ($p = .022$) and HDL-c ($p = .020$) were significantly different among genotypes of rs7678928. Moreover, subjects with rs7678928 TT genotype had higher SBP

($p = .039$) and DBP ($p = .019$), and lower HDL-c than those with CC genotype ($p = .017$).

Characteristics of subjects with rs1440581 and rs7678928 before and after 4-year follow-up

Furthermore, the characteristics of subjects before and after lifestyle intervention were analysed. Results showed the serum FPG, TG, HDL-c, ALT, AST and CREA were improved significantly after 4 years ($p < .001$) (Table 4). In subjects with different *PPM1K* rs1440581 genotypes, the serum FPG, TG, ALT, AST and CREA were significantly improved ($p < .05$) except in CC genotype carriers; the serum LDL-c of TC and CC genotypes carriers increased ($p < .05$), whereas it remained unchanged in the TT genotype carriers; serum HDL-c

of all genotypes carriers were improved, while the improvement in the T-allele carriers was more evident than in the C-allele carriers ($p < .05$). Except for rs7678928 TT genotype carriers, the serum FPG, TG, HDL-c, ALT, AST and CREA of subjects with CT and CC genotypes were significantly improved ($p < .05$); the improvement of serum HDL-c was more evident in the C-allele carriers than in the T-allele carriers ($p < .05$). In

Table 4. Characteristics of subjects before and after lifestyle intervention.

Variable	Baseline	After intervention	<i>t</i>	<i>p</i>
FPG (mmol/L)	5.98 ± 2.05	5.24 ± 1.33	-4.518	<.001*
TG (mmol/L)	1.96 ± 1.02	1.45 ± 0.59	-5.965	<.001*
HDL-c (mmol/L)	1.39 ± 0.53	1.62 ± 0.35	5.605	<.001*
ALT (U/L)	26.17 ± 15.18	19.62 ± 10.19	-5.122	<.001*
AST (U/L)	22.61 ± 8.59	19.80 ± 7.54	-3.361	<.001*
CREA (μmol/L)	67.05 ± 14.15	56.83 ± 13.80	-7.533	<.001*

FPG: fasting plasma glucose; TG: triglyceride; HDL-c: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CREA: creatinine.

Data are presented as mean ± SD. Student's *t*-test was used for comparing the data before and after the intervention.

* $p < .05$.

addition, the SBP of rs7678928 CC genotype carriers was also improved significantly (Table 5).

Associations of rs1440581 and rs7678928 with CVD, T2DM and NAFLD after 4-year follow-up

We further analysed the incidences of CVD and other chronic metabolic diseases after follow-up. The incidences of CVD, NAFLD and T2DM increased gradually in the order of TT, TC and CC genotype in rs1440581. The subjects with CC genotype in rs1440581 had significantly higher incidences of CVD ($p = .046$) and NAFLD ($p = .039$) than those with TT genotype (Figure 3). The same trend was also noted in the incidences of CVD and T2DM in the order of CC, CT and TT genotype in rs7678928. *PPM1K* rs7678928 TT genotype carriers had a higher incidence of CVD than CC genotype carriers ($p = .049$) (Figure 3). Unfortunately, there was no significant difference in the prevalence of T2DM among SNPs genotypes ($p > .05$).

Table 5. Changes in biochemical indexes after intervention according to SNP genotypes.

Variable	rs1440581			<i>p</i>	rs7678928			<i>p</i>
	TT	TC	CC		CC	CT	TT	
<i>n</i>	59	106	44	-	80	103	26	-
ΔSBP (mmHg)	-5.83 ± 28.15	-2.51 ± 25.64	-0.36 ± 40.66	.694	-6.60 ± 26.94*	-2.86 ± 29.30	1.54 ± 41.71	.549
ΔDBP (mmHg)	-1.71 ± 16.48	2.04 ± 19.82	1.86 ± 22.97	.474	-0.40 ± 18.52	0.72 ± 19.40	4.23 ± 24.65	.585
ΔFPG (mmol/L)	-0.82 ± 1.82*	-0.84 ± 2.52*	-0.32 ± 2.72	.446	-0.84 ± 2.02*	-0.81 ± 2.76*	-0.28 ± 2.01	.561
ΔTG (mmol/L)	-0.53 ± 1.08*	-0.63 ± 1.45*	-0.27 ± 0.93	.275	-0.44 ± 1.16*	-0.66 ± 1.42*	-0.27 ± 0.72	.273
ΔTC (mmol/L)	0.12 ± 1.29	-0.22 ± 1.31	-0.19 ± 1.19	.250	0.09 ± 1.33	-0.26 ± 1.29*	-0.10 ± 1.14	.186
ΔLDL-c (mmol/L)	0.18 ± 1.15	0.37 ± 0.93*	0.54 ± 1.13*	.205	0.29 ± 1.15*	0.33 ± 0.94*	0.63 ± 1.16*	.336
ΔHDL-c (mmol/L)	0.46 ± 0.46*	0.15 ± 0.75*	0.15 ± 0.36*	.001*	0.39 ± 0.50*	0.16 ± 0.74*	0.08 ± 0.26	.002*
ΔALT (U/L)	-7.91 ± 21.37*	-7.53 ± 17.89*	-1.90 ± 15.85	.188	-6.30 ± 21.56*	-7.93 ± 16.81*	-1.47 ± 13.92	.281
ΔAST (U/L)	-4.28 ± 12.45*	-4.39 ± 10.75*	2.36 ± 13.62	.005*	-3.10 ± 12.66*	-3.68 ± 12.00*	2.59 ± 10.20	.058
ΔTBIL (μmol/L)	1.28 ± 16.30	0.46 ± 7.36	2.31 ± 7.61	.619	1.27 ± 14.51	0.42 ± 6.97	3.48 ± 8.69	.421
ΔCREA (μmol/L)	-13.35 ± 18.76*	-11.45 ± 20.62*	-3.20 ± 18.18	.024*	-10.81 ± 19.42*	-11.01 ± 19.75*	-3.36 ± 20.37	.189
ΔUA (μmol/L)	-0.91 ± 97.31	-21.54 ± 105.41*	5.37 ± 95.08	.240	-3.49 ± 105.60	-17.67 ± 99.49	0.81 ± 109.73	.557

SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; TG: triglyceride; TC: total cholesterol; LDL-c: low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin; CREA: creatinine; UA: uric acid.

Data are presented as mean ± SD. Comparisons among three groups were made using one-way ANOVA. Bonferroni's method was used for comparison between two groups.

* $p < .05$.

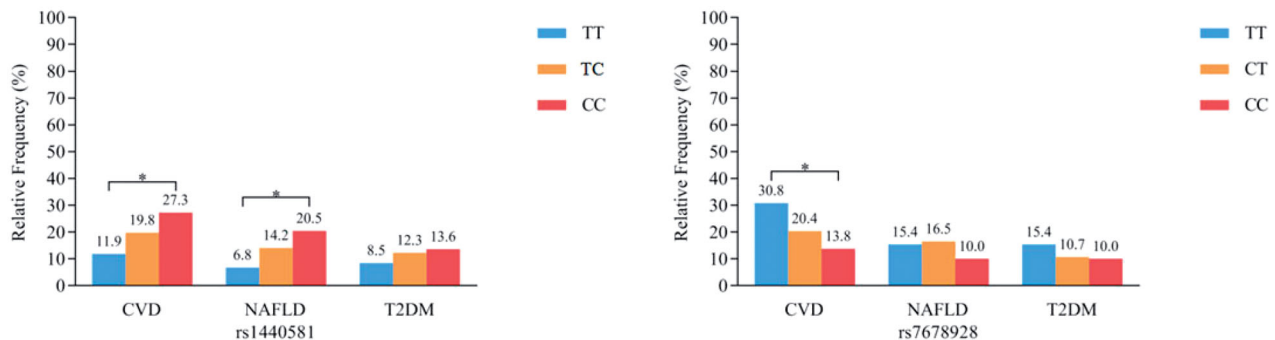


Figure 3. Incidences of CVD, NAFLD and T2DM of subjects according to SNP genotypes after 4-year follow-up. Comparisons among three groups were done using one-way ANOVA. * $p < .05$. CVD: cardiovascular disease; NAFLD: non-alcoholic fatty liver disease; T2DM: type 2 diabetes.

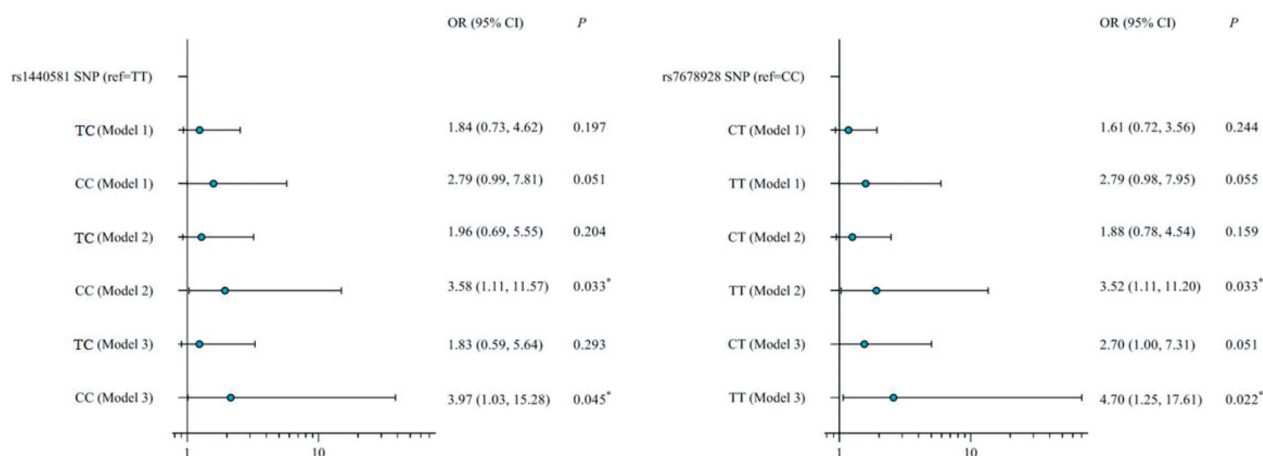


Figure 4. Association of rs1440581 and rs7678928 with CVD after 4-year follow-up. Data are ORs and 95% confidence interval (95% CI) from multivariate logistic regression models. There was no adjustment in the model 1. In the model 2, adjustment was done for baseline clinical characteristics (gender, age, BMI, AC, BP, FPG, TG, TC, LDL-c, HDL-c, ALT, AST, CREA) and history of smoking and drinking; In the model 3, adjustment was done for the history of hypertension and diabetes medication, changes in TG, TC and HDL-c over the follow-up period and prevalence of hypertension and diabetes after follow-up period based on model 2. * $p < .05$.

Multiple logistic regression analyses showed CC genotypes in rs1440581 was associated with increased risk of CVD (OR = 2.79, 95% confidence interval (CI) [0.99–7.81], $p = .51$) without adjustment. The association was more significant after adjustment in the model 2 (OR = 3.58, 95% CI [1.11–11.57], $p = .033$) and model 3 (OR = 3.97, 95% CI [1.03–15.28], $p = .045$) (Figure 4). Similarly, TT genotype in rs7678928 was also associated with increased risk of CVD after adjustment in the model 2 (OR = 3.52, 95% CI [1.11–11.20], $p = .033$) and model 3 (OR = 4.70, 95% CI [1.25–17.61], $p = .022$) (Figure 4).

Discussion

PPM1K is a kind of branched chain α -ketoacid (BCKA) dehydrogenase phosphatase and acts importantly in the metabolism of BCAAs by dephosphorylating BCKDHA. Mutations in *PPM1K* can increase the circulating BCAA levels and lead to an intermittent form of maple diabetes (MSUD) [5]. A GWAS study in Caucasians shows that a variant rs1440581 located upstream of *PPM1K* is a top SNP affecting circulating BCAA levels, while rs7678928 is also associated with Ile level [7]. As far as we know, few studies have been conducted to investigate the associations of rs1440581 and rs7678928 with serum BCAA levels in Chinese population.

Our study showed that the serum Val and total BCAA levels in subjects with CC genotype in rs1440581 were higher than in those with TT genotype, which was consistent with findings from the

study in Caucasians [6]. This indicates that the C-allele of rs1440581 may be responsible for the increased of circulating BCAA levels in the Chinese population. The rs1440581 polymorphism seems to effect *PPM1K* expression in Chinese, lowering *PPM1K* activity in C-allele carriers, and therefore serum BCAAs accumulate by affecting the BCKDC activity. However, unlike the previous studies of Kettunen et al. [6] and Lotta et al. [7], Leu level and Fischer's ratio slightly increased in the rs1440581 C-allele carriers as compared to those in T-allele carriers in our study. Considering that Fischer's ratio is affected by the concentration of serum aromatic amino acids (AAAs), further investigation is needed to determine whether this difference is accidental.

Our previous cross-sectional study showed that the baseline serum BCAA levels were positively related to TG level and CVD risk [3,4]. The conclusion was subsequently confirmed in different races [12,13]. Further investigation is needed to determine if the rs1440581 and rs7678928 polymorphisms have a causal relationship with the CVD risk. Therefore, the subjects of a previous study were randomly selected to explore the relationship between *PPM1K* SNPs (rs1440581 and rs7678928) and CVD risk in this longitudinal perspective study. After 4-year follow-up, results showed that the SBP, DBP, serum TG and AST in rs1440581 CC genotype carriers were significantly higher than in those with TT genotype, and these are important risk factors of CVD [14–16], whereas the serum HDL-c was lower than in the subjects with TT genotype. The biochemical parameters of these individuals, such as

serum TG, HDL-c, ALT, AST and CREA, were all improved significantly after 4-year lifestyle intervention. Further longitudinal comparison among genotypes found that the serum TG, ALT, AST and CREA in rs1440581 CC genotype carriers showed no significant decrease, and the improvement of serum HDL-c in C-allele carriers was less significant than in T-allele carriers. These findings suggest that the CC genotype in rs1440581 seems to be a high risk factor of CVD. Furthermore, after 4-year follow-up, the incidence of CVD in rs1440581 CC genotype carriers (27.3%) was significantly higher than in TT genotype carriers (11.9%).

In addition, blood glucose is also an independent risk factor of CVD. *PPM1K* is a susceptibility gene that can affect the risk for T2DM [17]. A recent study from Shanghai Rui-Jin Hospital investigated rs1440581 as a genetic marker of BCAAs and found that rs1440581 was significantly associated with the risk of incident T2DM in Chinese [18]. Unfortunately, the incidence of T2DM showed an increasing trend in the order of TT, CT and CC genotype in rs1440581 although there was no significant difference in our study. This might be explained as that the follow-up time was still shorter and the sample size was smaller. The intervention might also delay the progression of T2DM. However, serum FPG did not decrease significantly after intervention in only CC genotype carriers which indicates that rs1440581 polymorphism may play a role in glucose metabolism. Of course, more studies with longer follow up period are needed to confirm the relationship of *PPM1K* rs1440581 and rs7678928 with the incidence of T2DM.

In brief, CC genotype in rs1440581 not only causes a higher baseline BCAA concentrations, but also affects the longitudinal improvement of traditional CVD risk factors and subsequent CVD. The mechanism is not clear. *PPM1K* is essential for cell survival and healthy development with the highest expression level in the heart and brain [19]. As reported in a previous study [20], reactive oxygen species (ROS) were significantly elevated in the *PPM1K*-deficient cells, the *PPM1K*-deficient mitochondria were more susceptible to calcium-induced permeability transition pore opening in the absence of external BCKA/BCAA challenge, and *PPM1K* as a mitochondrial matrix phosphatase may regulate cardiomyocyte function and viability via modulating ROS and permeability transition pore. However, more studies are needed to investigate the relationships of *PPM1K* rs1440581 and rs7678928 SNPs with CVD.

Few studies have examined the association of rs7678928 with CVD risk. Except for TT genotype carriers, the serum FPG, TG, ALT, AST and CREA

significantly decreased, while serum HDL-c increased. Moreover, the improvement of serum HDL-C in C-allele carriers was more evident than in T-allele carriers. In other words, the individuals carrying rs7678928 TT genotype may benefit less from the improvement of CVD risk factors than those without this genotype when they receive same intervention. In addition, the incidence of CVD after 4 years follow-up in TT genotype carriers was higher than in CC genotype carriers. Thus, rs7678928 TT genotype may be another risk factor for CVD. Due to the absence of evidence that rs7678928 polymorphism affects baseline circulating BCAA levels and the subsequent serum BCAA levels were not dynamically investigated, more studies are needed to determine whether rs7678928 polymorphism contributes to the differences in the CVD risk among genotypes.

The study of White et al. showed that *PPM1K* regulates liver glucose and lipid metabolism via regulation of the crucial *de novo* lipogenic enzyme ATP citrate lyase [21]. In our study, C-allele in rs1440581 increased baseline BCAA levels and serum AST level after 4-year follow-up. After the intervention, serum ALT and AST decreased significantly except in the CC genotype carriers. Thus, we speculate that CC genotype in rs1440581 may also be used as a predictor of liver injury. The incidence of NAFLD in rs1440581 CC genotype carriers (20.5%) was also higher than in TT genotype carriers (6.8%). Supportably, there is evidence showing that the serum Val and Ile are higher in patients with NAFLD, and the higher baseline Val level predicts subsequent increase of hepatic fat fraction (HFF) [22], which has been confirmed in mice [23]. Further investigation is needed to confirm the relationship between rs1440581 SNP and NAFLD in studies with extended follow-up time.

Although the serum BCAA levels were not dynamically monitored, our prospective cohort study for the first time assessed the relationships of *PPM1K* SNPs (rs1440581 and rs7678928) with BCAA levels and subsequent CVD risk in middle-aged and elderly Chinese Han population. In the future, we will expand the sample size and extend the follow-up time to further investigate other metabolic diseases among subjects with different SNPs genotypes after intervention. In addition, the results of our study are needed to be further confirmed in different populations.

Conclusions

The CC genotype in *PPM1K* rs1440581 is associated with the increased baseline serum Val and total BCAA

levels. According to Mendelian randomization theory [24], rs1440581 CC genotype may be a better marker of CVD risk.

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Ethics approval and consent to participate

This study was part of the *Diabetes prevention programme in Huai'an* (ChiCTR-TRC-14005029) and was approved by the Huai'an Second People's Hospital Ethics Committee of the XuZhou Medical University (Huai'an City, Jiangsu Province, China). All subjects understood the purpose and procedures of the study and gave their consent to participate in this study.

Consent for publication

Consent to publish has been obtained from the participant to report individual patient data.

Disclosure statement

The authors declare that they have no competing interests.

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Data availability statement

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Authors contributions

ZYL and WH were the major contributors in writing the original draft, funding acquisition and validation. WH and ZYL gave the conceptualization. ZYL was the major contributor in writing, review and editing the manuscript. ZYL also contributed to visualization. WNY participated in overall supervision. HRH was in charge of data curation. ZYL and SRW provided formal analysis. Investigation was mainly completed by XQW and YWL. XQ was responsible for the main part of methodology. JL and SYL were in charge of software. HWZ provided resources. WNY contributed to project administration. All authors read and approved the final manuscript.

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