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Original article

Association between PNPLA3rs738409 polymorphism decreased kidney function in postmenopausal type 2 diabetic women with or without non-alcoholic fatty liver disease

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ABSTRACT

Aim. – Evidence is emerging that PNPLA3 rs738409 polymorphism (the major genetic variant associated with susceptibility to non-alcoholic fatty liver disease [NAFLD]) is associated with chronic kidney disease (CKD) in non-diabetic individuals. Currently, little is known about this association in type 2 diabetic (T2DM) patients with and without NAFLD.

Methods. – We studied 101 Caucasian post-menopausal women with T2DM, consecutively attending our diabetes outpatient service during a 3-month period. Glomerular filtration rate ($eGFR_{CKD\text{-}EPI}$) was estimated using the CKD-Epidemiology Collaboration (CKD-EPI) equation, whilst albuminuria was measured with an immunonephelometric assay on morning spot urine samples. NAFLD was detected either by fatty liver index (FLI $\geq$ 60, n = 101) or by ultrasonography (n = 77). Genotyping was performed by TaqMan-Based RT-PCR system.

Results. – Eight patients had G/G, 41 G/C and 52 C/C PNPLA3 rs738409 genotypes, and 21 (20.8%) patients had CKD ($eGFR_{CKD\text{-}EPI}$ < 60 mL/min/1.73 m^2 or abnormal albuminuria). Compared to those with G/C or C/C genotypes, patients with G/G genotype had significantly lower $eGFR_{CKD\text{-}EPI}$ (63.7 $\pm$ 11 vs. 77.4 $\pm$ 17 vs. 81.9 $\pm$ 15 mL/min/1.73 m^2, P = 0.014) and higher prevalence of CKD (50% vs. 24.4% vs. 13.5%, P = 0.04). After adjustment for age, duration of diabetes, haemoglobin A_{1c}, HOMA-estimated insulin resistance, systolic blood pressure, hypertension treatment and FLI $\geq$ 60, rs738409 G/G genotype was independently associated with both lower $eGFR_{CKD\text{-}EPI}$ (β coefficient: −15.5, 95% CI −26.0 to −5.0, P = 0.004) and higher risk of CKD (adjusted-odds ratio 8.05, 95% CI 1.26–41.4, P = 0.03). Similar results were found when we adjusted for hepatic steatosis on ultrasography (instead of FLI $\geq$ 60).

Conclusion. – Regardless of the presence of NAFLD and common cardio-renal risk factors, in post-menopausal women with T2DM, the G/G genotype of rs738409 in the *PNPLA3* gene was strongly associated with lower $eGFR_{CKD\text{-}EPI}$ and higher prevalence of CKD.

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Abbreviations

ACR	albumin-to-creatinine ratio
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
CI	confidence interval
CKD	chronic kidney disease
CKD-EPI	chronic kidney disease epidemiology collaboration
CRP	C-reactive protein
eGFR	estimated glomerular filtration rate
FIB-4	fibrosis 4
FLI	fatty liver index
GGT	gamma-glutamyltransferase
HOMA-IR	homeostasis model assessment-insulin resistance
MDRD	modification of diet in renal disease
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
OR	odds ratio
PNPLA3	patatin-like phospholipase domain-containing protein 3
SNP	single nucleotide polymorphism
T2DM	type 2 diabetes mellitus

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease in many parts of the world, affecting up to 30% of adults in high-income countries [1]. The prevalence of NAFLD is much higher in people with type 2 diabetes mellitus (T2DM) (occurring in up to ~70% of these patients), who are a group of individuals at high-risk of developing the more severe histologic forms of NAFLD, such as non-alcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis [2]. It is now becoming increasingly clear that the clinical and economic burden of NAFLD does not only affect the risk of liver-related complications, but also affects the risk of developing cardiovascular disease and other extra-hepatic diseases that have a significant impact on healthcare expenditure [1,2]. Recently, a number of studies have shown that NAFLD is associated with an increased risk of both prevalent and incident chronic kidney disease (CKD) in patients with and without T2DM [3,4], independent of common cardio-renal risk factors. Identification of novel risk factors for CKD is clinically relevant, as CKD is a common pathologic condition worldwide that is associated with high morbidity, disability, mortality and healthcare costs [5,6,7].

Among the genetic factors that may influence the development and progression of NAFLD, the minor allele G of rs738409, *i.e.*, a non-synonymous single nucleotide polymorphism (SNP) in the *patatin-like phospholipase domain-containing protein 3* (*PNPLA3*) gene encoding an Ile148 Met change, has been recognized to be the major common genetic variant associated with susceptibility to NASH [8,9]. The *PNPLA3* gene is mostly expressed in the liver and has an acyl hydrolase activity [8,9]. In particular, the G allele of rs738409 is associated with the loss of hydrolyzing function of the protein, thus resulting in accumulation of lipid droplets into the hepatocytes [8,9]. Recently, some studies suggested that the presence of the G allele of rs738409 is significantly associated with lower estimated glomerular filtration rate (eGFR) and higher prevalence of CKD in nondiabetic middle-aged individuals, irrespective of the coexistence of NAFLD [10,11].

To our knowledge, it is currently uncertain whether such an association also occurs in patients with T2DM (i.e., a group of individuals at high-risk of developing both CKD and NAFLD). Therefore, the major aim of our cross-sectional study was to examine whether, and to what extent, *PNPLA3* rs738409 polymorphism (the major genetic variant associated with susceptibility to NASH) was associated with decreased kidney function in a sample of adult patients with T2DM (both with and without coexisting NAFLD).

Methods

Patients

In this exploratory analysis, we studied 101 post-menopausal Caucasian women with non-insulin treated T2DM, who consecutively attended our diabetes outpatient service during a 3-month period (from October to December 2017). We excluded all patients with:

- history of significant alcohol consumption (i.e., $>$ 20 grams of alcohol per day) and other known causes of chronic liver diseases (e.g., virus, drugs, autoimmunity and hemochromatosis);
- history of overt dysthyroidism, cirrhosis of any etiology, cancer and end-stage renal disease (defined as eGFR $<$ 15 mL/min/1.73 m^2 or chronic dialysis); and;
- treatment with hormone replacement therapy, steroids or anti-osteoporotic agents.

In this analysis, we included only post-menopausal women, because this study was primarily designed for exploring the association between NAFLD, bone mineral density and circulating levels of bone turnover biomarkers in post-menopausal women with non-insulin treated T2DM [12].

The local Ethics Committee approved the study protocol. All participants gave their written informed consent for participation in this research.

Clinical and laboratory data

Body mass index (BMI) was measured as kilograms divided by the square of height in meters. Waist circumference was measured at the midpoint between the lowest rib and the iliac crest. Blood pressure was measured with a standard sphygmomanometer after the patient had been seated quietly for at least 5 minutes. Patients were considered to have hypertension if their blood pressure was $\geq$ 140/90 mmHg or if they were taking any anti-hypertensive drug. Information on the type of menopause (physiological or surgical), smoking and use of medications was obtained from all patients via interviews during medical examinations.

Venous blood samples were collected in the morning after an overnight fast. Measurements of serum glucose, lipids, creatinine (measured using a Jaffé rate blanked and compensated assay), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), high-sensitivity C reactive protein (hs-CRP) and other biochemical blood parameters were obtained using standard laboratory procedures at the central Laboratory of our hospital. Haemoglobin A_{1c} (HbA_{1c}) was measured using the high-performance liquid chromatography analyzer Tosoh-G7 (Tosoh Bioscience Inc., Tokyo, Japan). Fasting insulin levels were measured using a chemiluminescent immuno-

assay (LIAISON, DiaSorin, Saluggia, Italy). Homeostasis model assessment (HOMA-IR) score was used for estimating insulin resistance.

GFR was estimated using both the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [13] and the four-variable Modification of Diet in Renal Disease (MDRD) study equation [14]. However, the CKD-EPI equation, which uses the same four variables as the MDRD study equation, has been shown to be more accurate than the MDRD study equation for estimating GFR in different populations, as well as for predicting the risk of mortality in patients with T2DM [15]. Urinary albumin excretion was assessed with an immunonephelometric assay (Beckman-Coulter IMMAGE; Beckman-Coulter Instruments, Fullerton, CA, USA) on a morning spot urine sample and expressed as the albumin-to-creatinine ratio (ACR); abnormal albuminuria was defined as a urinary ACR $\geq$ 30 mg/g creatinine.

In this study, chronic kidney disease (CKD) was defined as the presence of $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria. Pre-existing history of ischemic heart disease was defined as a documented history of myocardial infarction, angina pectoris or coronary revascularization procedures. Presence of diabetic retinopathy (diagnosed with fundoscopy after pupillary dilation) was also recorded.

Definition of NAFLD

In this study, we used two different methods for diagnosing NAFLD: the fatty liver index (FLI, which was available in the whole sample, $n = 101$) and liver ultrasonography (available in a subgroup of 77 women) after excluding individuals with other causes of liver diseases (as described in the exclusion criteria). In particular, we calculated the fatty liver index (FLI), i.e., a widely used marker of NAFLD [16], for identifying patients with NAFLD. In accordance with previous reports [17,18,19], a FLI $\geq$ 60 indicates individuals with hepatic steatosis on ultrasound. FLI was calculated as follows [19]: FLI = [$e^{0.953}$ $\times$ Ln(triglycerides) + 0.139 $\times$ BMI + 0.718 $\times$ Ln(GGT) + 0.053 $\times$ waist circumference – 15.745)] / [1 + $e^{0.953}$ $\times$ Ln(triglycerides) + 0.139 $\times$ BMI + 0.718 $\times$ Ln(gamma-glutamyltransferase) + 0.053 $\times$ waist circumference – 15.745] $\times$ 100. A liver ultrasonography was also conducted on a subgroup of women ($n = 77$; 76.2% of total) by a single expert physician (using an Esaote MyLab 70 ultrasound with a 4 MHz probe), who was blinded to the laboratory findings of participants. Hepatic steatosis was diagnosed according to specific ultrasonographic characteristics, such as diffuse hyper-echogenicity of the liver relative to kidneys, ultrasonographic beam attenuation, and poor visualization of intra-hepatic vessel borders and diaphragm [16,20]. Therefore, NAFLD was defined either by FLI $\geq$ 60 or by presence of hepatic steatosis on ultrasonography, separately, after exclusion of significant alcohol intake or other identifiable causes of liver disease. No large studies have validated the use of FLI in predicting hepatic fat content on imaging techniques in patients with T2DM, although the few published studies (performed in small series of patients) provided inconclusive results [21,22]. In our study, the Cohen's kappa coefficient for agreement between FLI $\geq$ 60 and ultrasonography for detecting NAFLD was relatively low ($k = 0.22$). Finally, we also used a validated cut-off of fibrosis-4 score (FIB-4 > 2.67) for non-invasively diagnosing the presence of advanced NAFLD fibrosis among individuals aged $\geq$ 65 years [16,23].

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA Blood Mini Kit (Qiagen, Germany). Genotyping of rs738409 in *PNPLA3* gene was carried out by a predesigned TaqMan probe (Applied Biosystem, California, USA), underling the I148 M substitution, according to manufacturers' protocol. Polymorphism genotyping was performed using 7900 HT Real Time PCR (Applied Biosystem, California, USA). Distribution of the genotype was in Hardy–Weinberg equilibrium and the call rate exceeded 99%. The allele G of rs738409 variant was present in 28% of chromosomes.

Statistical Analysis

Due to the exploratory (hypothesis-generating) design of the study, it was not possible to compute a sample size. Data are expressed as means $\pm$ SD, medians (inter-quartile ranges, IQR) or percentages. The *PNPLA3* rs738409 associations were assessed using an additive genetic model. The Fisher's exact test for categorical variables, the one-way ANOVA for normally distributed continuous variables and the Kruskal-Wallis test for non-normally distributed variables were used to test the differences in clinical and biochemical characteristics among patients stratified by *PNPLA3* rs738409 polymorphism (Table 1). The association between *PNPLA3* rs738409 variant and values of both $eGFR_{CKD\text{-}EPI}$ and $eGFR_{MDRD}$ (included as continuous measures, i.e. for each SD decrement) was tested using both an unadjusted linear regression model and the following two multivariable regression models:

- model 1 adjusted for age, duration of diabetes, HbA_{1c}, HOMA-IR score, systolic blood pressure, use of any anti-hypertensive drugs (i.e., beta-blockers, renin-angiotensin system inhibitors, calcium-channel antagonists or diuretics) and presence of NAFLD (as detected by FLI $\geq$ 60), and;
- model 2 further adjusted for smoking history, statin use, pre-existing ischemic heart disease and abnormal albuminuria (*i.e.*, urinary ACR $\geq$ 30 mg/g) (Table 2).

Moreover, we also tested the association between *PNPLA3* rs738409 variant and the risk of prevalent CKD (defined as $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria) by using both an unadjusted logistic regression model and an adjusted logistic regression model 1, including the same list of the aforementioned covariates (Table 3). In these multivariable regression models we did not additionally adjust for anthropometric parameters and serum triglyceride and liver enzyme levels, because these variables are already included in the FLI equation. In the subgroup of patients with available liver ultrasound data ($n = 77$), we also repeated the same multivariable linear and logistic regression models, including the presence of NAFLD (diagnosed with ultrasonography, instead of FLI $\geq$ 60) among the covariates (Tables S1 and S2; see supplementary materials associated with this article on line). Covariates included in all multivariable regression models were selected as potential confounding factors based on their biological plausibility. A *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using STATA software, version 14.2 (STATA, College Station, Texas, USA).

Results

Among the 101 post-menopausal elderly women with T2DM included in the study (mean $\pm$ SD: age 71.7 $\pm$ 9 years; BMI 29.5 $\pm$ 5 kg/m^2; HbA_{1c} 51 $\pm$ 8 mmol/mol), 8 (7.9%) women had G/G, 41 (40.6%) had G/C, and 52 (48.5%) had C/C *PNPLA3* rs738409 genotype, respectively. In addition, 21 (20.8%) of these women had CKD (defined as $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria). In the subgroup of 77 patients in whom data on liver ultrasonography were also available, 62 (80.5%) patients met the

Table 1
Main clinical and biochemical characteristics of post-menopausal women with non-insulin treated type 2 diabetes, stratified by *PNPLA3* rs738409 polymorphism.

	CC genotype (n = 52)	GC genotype (n = 41)	GG genotype (n = 8)	P-value
Age (years)	71.6 ± 9	71.4 ± 8	74.2 ± 8	0.68
Weight (kg)	74.4 ± 14	71.9 ± 11	76.2 ± 11	0.58
BMI (kg/m^2)	29.9 ± 5	30.4 ± 5	28.9 ± 5	0.55
Waist circumference (cm)	100.7 ± 14	97.0 ± 11	98.0 ± 6	0.36
Diabetes duration (years)	11.7 ± 9	13.6 ± 9	10.6 ± 8	0.51
Current smokers (%)	17.3	14.6	12.5	0.32
Systolic blood pressure (mmHg)	140 ± 17	138 ± 17	143 ± 13	0.68
Diastolic blood pressure (mmHg)	78 ± 10	74 ± 8	77 ± 8	0.18
White blood count (10^9/L)	7.2 ± 1.3	7.2 ± 2.3	7.2 ± 1.1	0.98
Haemoglobin (g/L)	131.2 ± 10	131.3 ± 9	132.2 ± 12	0.96
Platelet count (10^9/L)	251 ± 62	260 ± 54	262 ± 47	0.71
Total cholesterol (mmol/L)	4.08 ± 0.8	4.13 ± 0.9	4.26 ± 0.6	0.86
LDL cholesterol (mmol/L)	2.01 ± 0.6	2.10 ± 0.8	2.21 ± 0.5	0.66
HDL cholesterol (mmol/L)	1.56 ± 0.4	1.49 ± 0.3	1.44 ± 0.3	0.40
Triglycerides (mmol/L)	1.34 ± 0.5	1.32 ± 0.6	1.82 ± 0.6	0.10
Fasting glucose (mmol/L)	6.9 ± 1.1	7.4 ± 1.9	6.9 ± 1.1	0.23
Fasting insulin (mIU/L)	8.9 ± 6.2	9.3 ± 9.4	15.0 ± 7.9	0.12
HOMA-insulin resistance score	2.3 (1.4-3.3)	2.1 (1.1-3.7)	4.3 (2.7-6.7)	0.32
HbA_{1c} (mmol/mol Hb)	52.1 ± 7.7	51.6 ± 8.9	51.4 ± 6.2	0.95
C-reactive protein (mg/L)	2.1 (0.8-3.9)	1.1 (0.4-3.1)	1.7 (0.8-5.1)	0.90
Albumin (g/L)	44.2 ± 2.6	44.6 ± 2.4	44.4 ± 2.9	0.73
AST (IU/L)	24 ± 8	22 ± 6	28 ± 6	0.09
ALT (IU/L)	15 ± 7	12 ± 5	16 ± 9	0.12
GGT (IU/L)	25 ± 24	20 ± 18	29 ± 18	0.32
Albuminuria (mg/g)	3.3 (2.9–6.6)	3.6 (2.8–5.9)	4.9 (3.1–28)	0.46
Abnormal albuminuria (%)	7.7	8.0	28.6	0.09
CKD (%)	13.5	24.4	50.0	0.04
Hypertension (%)	80.7	87.8	87.5	0.63
Ischemic heart disease (%)	11.5	12.2	12.5	0.99
Diabetic retinopathy, any degree (%)	3.8	9.8	12.5	0.53
Metformin (%)	73.1	82.9	87.5	0.42
Sulfonylureas (%)	28.8	36.6	12.5	0.37
Pioglitazone (%)	5.8	2.4	0.0	0.60
DPP-4 inhibitors (%)	28.8	21.9	25.0	0.75
GLP-1 analogues (%)	11.5	14.6	0.0	0.50
SGLT-2 inhibitors (%)	5.8	7.3	0.0	0.72
Anti-platelet drugs (%)	46.1	48.8	50.0	0.96
Beta-blockers (%)	30.8	31.5	37.5	0.93
ARB/ACE-inhibitors (%)	63.5	73.2	75.0	0.56
Calcium-channel antagonists (%)	19.2	19.5	12.5	0.89
Diuretics (%)	25.0	48.8	37.5	0.08
Statins (%)	80.8	73.2	75.0	0.68
FIB-4 score > 2.67 (%)	17.3	9.9	25.0	0.40
FLI ≥ 60 (%)	46.2	39.0	62.5	0.45
Hepatic steatosis on US (%), n = 77	85.0	73.3	71.4	0.43
FLI ≥ 60 and/or hepatic steatosis on US (%), n = 77	85.0	85.7	76.7	0.64

Sample size, n = 101, unless where indicated. Data are expressed as means ± SD, medians or percentages. Differences between the groups were tested by the Fisher's exact test for categorical variables, the one-way ANOVA for normally distributed continuous variables, and the Kruskal-Wallis test for non-normally distributed variables.
CKD was defined as $eGFR_{CKD\text{-}EPI}$ < 60 mL/min/1.73 m^2 or abnormal albuminuria (urinary ACR ≥ 30 mg/g creatinine).
ACE: angiotensin-converting enzyme; ALT: alanine aminotransferase; ARB: angiotensin II receptor blocker; AST: aspartate aminotransferase; BMI: body mass index; CKD: chronic kidney disease; DPP-4: dipeptidyl peptidase-4; FIB-4: fibrosis-4 score; FLI: fatty liver index; GGT: gamma-glutamyltransferase; GLP-1: glucagon-like peptide-1; HOMA: homeostasis model assessment; SGLT-2: sodium/glucose cotransporter-2; US: ultrasonography.

diagnostic criteria for NAFLD. Among those with NAFLD, 36 patients had mild hepatic steatosis and 26 had moderate-to-severe steatosis on ultrasonography, respectively.

Table 1 shows the clinical and biochemical characteristics of participants, stratified by *PNPLA3* rs738409 polymorphism. Compared to patients with either G/C or C/C genotypes, those with G/G genotype had a significantly higher prevalence of CKD (50% vs. 24.4% *vs.* 13.5%) and also tended to have higher abnormal albuminuria. Age, BMI, waist circumference, duration of diabetes, smoking, blood pressure, complete blood count, HbA_{1c}, HOMA-IR score, plasma lipid profile, hs-CRP, albumin, liver enzymes, diabetic retinopathy, ischemic heart disease, as well as the use of oral hypoglycaemic agents, statins and anti-hypertensive drugs did not significantly differ among the three groups of patients. Additionally, the prevalence of hepatic steatosis (as detected either by FLI ≥ 60 or by ultrasonography) and advanced NAFLD fibrosis (as defined by FIB-4 score > 2.67) was comparable among the three patient groups.

Notably, as shown in Fig. 1, the values of both $eGFR_{CKD\text{-}EPI}$ and $eGFR_{CKD\text{-}EPI}$ decreased progressively in relation to the *PNPLA3* rs738409 variant (P = 0.014 and P = 0.025 for the trend by one-way ANOVA, respectively).

Table S3 (see supplementary materials associated with this article on line) shows the main clinical/biochemical data and *PNPLA3* rs738409 genotype of patients, stratified by both CKD and NAFLD (as detected by FLI ≥ 60). The four subgroups of patients significantly differed in terms of BMI, waist circumference, HOMA-insulin resistance, plasma lipid profile and GGT concentrations. Conversely, the percentage of individuals with the risk allele (G) of rs738409 was not significantly different among the patient groups.

As reported in Table 2, the risk allele (G) of rs738409 was significantly associated with lower $eGFR_{CKD\text{-}EPI}$ (β coefficient

Table 2
Multivariable linear regression analyses – Association between *PNPLA3* rs738409 genotypes and kidney function (expressed as 1-SD decrement in either $eGFR_{CKD\text{-}EPI}$ or $eGFR_{MDRD}$) in post-menopausal women with type 2 diabetes.

	B coefficient(s)	95% confidence interval	*P*-value
Adjusted model 1			
$eGFR_{CKD\text{-}EPI}$ for 1-SD decrement (i.e., 16 mL/min/1.73 m^2)			
PNPLA3 rs738409			
CC genotype	Ref.	Ref.	–
GC genotype	−5.08	−11.2 to −0.39	0.04
GG genotype	−15.2	−25.4 to −5.07	0.004
$eGFR_{MDRD}$ for 1-SD decrement (i.e., 20 mL/min/1.73 m^2)			
PNPLA3 rs738409			
CC genotype	Ref.	Ref.	–
GC genotype	−6.44	−14.3 to +1.39	0.11
GG genotype	−19.0	−33.7 to −4.27	0.01
Adjusted Model 2			
$eGFR_{CKD\text{-}EPI}$ for 1-SD decrement (i.e., 16 mL/min/1.73 m^2)			
PNPLA3 rs738409			
CC genotype	Ref.	Ref.	–
GC genotype	−5.67	−11.3 to −0.08	0.05
GG genotype	−15.5	−26.0 to −5.04	0.004
$eGFR_{MDRD}$ for 1-SD decrement (i.e., 20 mL/min/1.73 m^2)			
PNPLA3 rs738409			
CC genotype	Ref.	Ref.	–
GC genotype	−5.94	−14.1 to +2.19	0.15
GG genotype	−19.4	−34.7 to −4.19	0.01

Sample size, n = 101. Data are expressed as beta coefficients and 95% confidence intervals (CI) as tested by multivariable linear regression analysis. The continuous values of either $eGFR_{CKD\text{-}EPI}$ or $eGFR_{MDRD}$ were included as the dependent variable in these regression models. Ref.: reference category.
Multivariable linear regression model 1 was adjusted for age, duration of diabetes, HbA_{1c}, systolic blood pressure, HOMA-IR score, use of antihypertensive drugs and presence of NAFLD (as assessed by FLI ≥ 60). Model 2 was adjusted for the same set of covariates included in model 1 *plus* smoking history, statin use, prior ischemic heart disease and abnormal albuminuria (urinary ACR ≥ 30 mg/g).

−15.2, 95%CI −25.4 to −5.1, P = 0.004), even after adjustment for age, diabetes duration, HbA_{1c}, HOMA-IR score, systolic blood pressure, hypertension treatment and NAFLD (as assessed by FLI) (model 1). After further adjustment for smoking, statin use, history of ischemic heart disease and abnormal albuminuria (model 2), the results remained essentially unchanged, showing that the risk allele (G) of rs738409 was independently associated with lower $eGFR_{CKD\text{-}EPI}$ (β coefficient −15.5, 95%CI −26.0 to −5.0, P = 0.004). Other variables that were independently associated with lower $eGFR_{CKD\text{-}EPI}$ were older age (β coefficient −0.74, 95%CI −1.1 to −0.4, $P < 0.001$) and presence of NAFLD (β coefficient −9.50, 95%CI −15.7 to −3.3, P = 0.003). As also shown in the table, similar results were found even when we used the MDRD study equation (instead of the CKD-EPI equation) for estimating GFR.

Table 3 shows the association between the risk allele (G) of *PNPLA3* rs738409 and the presence of CKD (defined as $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria). In univariable logistic regression analysis (unadjusted model), the risk allele (G) of rs738409 was significantly associated with a ∼6.5-fold increased risk of prevalent CKD. Adjustment for age, diabetes duration, HbA_{1c}, HOMA-IR score, systolic blood pressure, hypertension treatment and presence of NAFLD (as assessed by FLI) did not substantially modify these results.

Interestingly, when we restricted the aforementioned multivariable linear and logistic regression analyses to the subgroup of patients (n = 77) with available liver ultrasound data (Tables S1-S2; see supplementary materials associated with this article on line), the presence of risk allele (G) of rs738409 in *PNPLA3* gene was confirmed to be independently associated with lower $eGFR_{CKD\text{-}EPI}$ (or lower $eGFR_{MDRD}$) and with higher prevalence of CKD, even after adjusting for NAFLD (as detected by ultrasonography) and other potential confounding factors.

Table 3
Logistic regression analyses – Association between *PNPLA3* rs738409 genotypes and risk of prevalent CKD in post-menopausal women with type 2 diabetes.

	Odds ratio(s)	95% confidence interval	*P*-value
Unadjusted model			
PNPLA3 rs738409			
CC genotype	Ref.	Ref.	–
GC genotype	2.07	0.71 – 6.03	0.18
GG genotype	6.43	1.29 – 31.8	0.02
Adjusted model			
PNPLA3 rs738409			
CC genotype	Ref.	Ref.	–
GC genotype	3.97	1.02 – 15.4	0.04
GG genotype	8.05	1.26 – 41.4	0.03

Sample size, n = 101. Data are expressed as odds ratios ± 95% confidence intervals (CI) as assessed by either univariate (unadjusted) or multivariable logistic regression analyses. Presence of CKD (defined as $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria) was included as the dependent variable in these regression models. Ref.: reference category.
All these multivariable regression models were adjusted for age, duration of diabetes, HbA1c, systolic blood pressure, HOMA-IR score, use of antihypertensive drugs and presence of NAFLD (as assessed by FLI ≥ 60).

Discussion

This is the first cross-sectional study aimed at examining the association of *PNPLA3* rs738409 polymorphism with kidney function and risk of prevalent CKD (defined as $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria) in patients with T2DM, a group of individuals at high risk for developing both CKD and NAFLD.

The main and novel findings of our study were that the presence of the risk allele (G) of rs738409 was significantly associated with lower values of both $eGFR_{CKD\text{-}EPI}$ and $eGFR_{MDRD}$, as well as with a greater risk of CKD (defined as $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria) in an outpatient sample of Caucasian post-menopausal women with T2DM. Notably, these associations were independent of age, duration of diabetes, HbA_{1c}, HOMA-IR score, smoking, systolic blood pressure, hypertension treatment, statin use, prior history of ischemic heart disease and NAFLD (as detected either by FLI or by ultrasonography). In addition, other risk factors that were independently associated with reduced kidney function were older age and coexisting NAFLD.

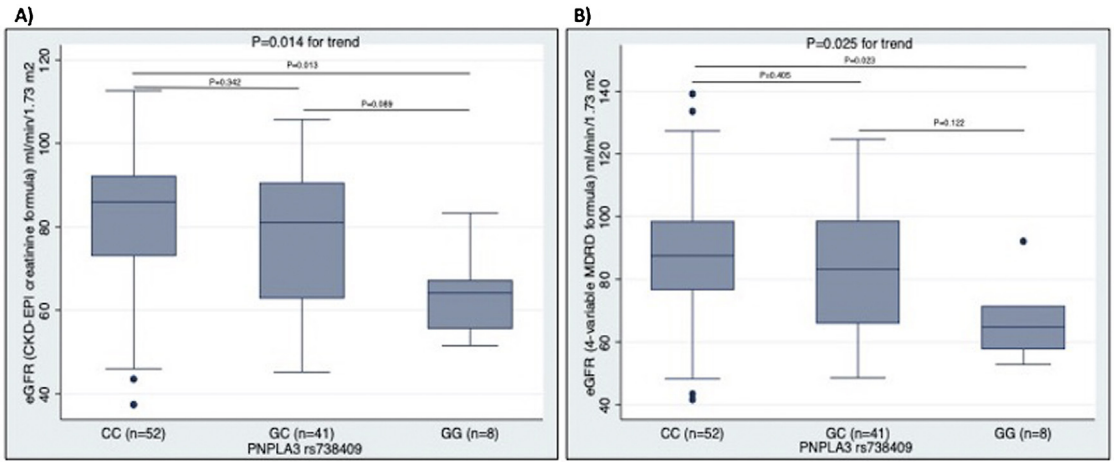

Fig. 1. Box plot of estimated glomerular filtration rate values [i.e., using the $eGFR_{CKD\text{-}EPI}$ (A) and $eGFR_{MDRD}$ equations (B)] of post-menopausal type 2 diabetic women, stratified by *PNPLA3* rs738409 polymorphism. *P*-values for the trend by one-way ANOVA. A post-hoc Tukey's HSD (Honestly Significant Difference) test was used for pairwise comparisons of means among the three groups of patients. The central rectangle spans the 1st quartile to the 3rd quartile (the inter-quartile range [IQR]). The segment inside the rectangle shows the median and "whiskers" above and below the box show the locations of 1.5 *x* IQR values.

This latter finding is supported by the results of multiple observational studies showing that the presence and severity of NAFLD is associated with an increased risk of both prevalent and incident CKD in patients with and without T2DM [3,4]. Unfortunately, we did not assess hepatic steatosis by ultrasound in all subjects and we did not attempt to grade accurately the severity of hepatic steatosis in those subjects in whom ultrasound was undertaken. That said, in the subgroup of 77 patients with available liver ultrasound data we failed to find an association between eGFR values and ultrasonographic hepatic steatosis. Therefore, we did not examine the association between increasing hepatic fat content and eGFR or albuminuria according to *PNPLA3* rs738409 genotype. However, in line with previous studies showing that hepatic fibrosis stage is the strongest predictor of all-cause death and cardiovascular events in individuals with NAFLD [1,2], it is reasonable to assume that patients with NASH and hepatic fibrosis are at substantially higher risk of incident CKD than patients with simple steatosis alone [3,4], since it is likely that the mechanisms linking hepatic fibrosis and increased cardiovascular events are similar to those linking hepatic fibrosis and CKD.

Our findings corroborate and expand the results of two recent studies, showing that *PNPLA3* rs738409 polymorphism was significantly associated with an increased risk of prevalent CKD in predominantly non-obese, non-diabetic individuals [10,11]. In a preliminary cross-sectional study of nearly 200 non-obese, non-diabetic middle-aged individuals (about a third of whom had biopsy-proven non-cirrhotic NAFLD), Musso et al. reported that the G allele of rs738409 was significantly associated with lower $eGFR_{CKD\text{-}EPI}$ values and with a higher prevalence of CKD both in patients with and in those without NAFLD [10]. However, it was unclear from this preliminary study (published as a research letter) whether these associations were also adjusted for established cardio-renal risk factors. Also, in an exploratory cross-sectional and longitudinal study, Oniki et al. [11] examined the relationship between *PNPLA3* genotype and kidney function decline in 740 Japanese elderly men and women (~10% with established diabetes), recruited during a health-screening program. These authors showed that the *PNPLA3* G/G genotype carriers had lower $eGFR_{CKD\text{-}EPI}$ values than those carrying the C/C genotype, independently of age, sex, BMI, diabetes, hypertension, dyslipidaemia and ultrasound-defined NAFLD. Notably, in a subgroup of nearly 350 non-obese men and women followed for a median period of 5 years, the authors also confirmed that the *PNPLA3* G/G genotype was associated with a significant decline in eGFR values [11]. However, the generalizability of the findings of this study to other ethnic groups remains untested.

That said, we believe that the evidence from this and the two aforementioned studies is of potential clinical importance for the management of both patients with and without T2DM. The combined data support the assertion that *PNPLA3* genotyping might be useful for identifying individuals who are more at risk of developing severe liver disease in NAFLD. This may be particularly important for individuals without established diabetes, since diabetes is a recognized risk factor that is causally implicated in the development of more severe liver disease in NAFLD. Additionally, *PNPLA3* genotyping might also be useful for identifying subjects with a higher risk of developing NAFLD-related extra-hepatic complications, such as CKD, through the promotion of specific prevention and treatment strategies for carriers of the *PNPLA3* rs738409 G/G genotype.

It is known that the *PNPLA3* gene encodes a trans-membrane polypeptide chain exhibiting triglyceride hydrolase activity, which is highly expressed on endoplasmic reticulum and lipid membranes of hepatocytes and adipocytes [24,25,26]. The rs738409 C > G SNP in the *PNPLA3* gene, encoding the isoleucine to methionine variant at protein position 148 (I148 M), impairs the phospholipase activity of the enzyme, and accumulates at the surface of lipid droplets, where it acquires the ability to alter triglyceride and phospholipid turnover, thus inducing hepatic fat accumulation [24–26]. To date, while the role of *PNPLA3* rs738409 gene variant in the development and progression of NAFLD has been established [8,9,27], the putative biological mechanisms underlying the association between the G allele of rs738409 and risk of CKD remain poorly known. As NAFLD is closely associated with an increased risk of CKD in both patients with and without T2DM [3,4], the most obvious explanation for our findings is that the association between the G allele of rs738409 and kidney dysfunction is simply an epiphenomenon of coexisting NAFLD. However, it should be noted that in our study the significant associations of the G allele of rs738409 with both lower eGFR and higher prevalence of CKD persisted even after adjustment for NAFLD (detected either by FLI or by ultrasonography) and other established cardio-renal risk factors (such as age, diabetes duration, HbA_{1c}, HOMA-IR score, smoking, hypertension, statin use, prior ischemic heart disease and abnormal albuminuria). Experimentally, there is evidence showing that PNPLA3 is also

expressed in sinusoidal pericytes (being this evidence stronger for hepatic stellate cells) that store retinol and regulate its release in response to multiple metabolic signals [28], and carriage of the *PNPLA3* risk variant is associated with an increased release of proinflammatory, pro-oxidant and profibrogenic factors [29]. In kidney, pericytes have also become an intensively studied cell population in renal biology and pathophysiology [30]. Renal pericytes are stromal cells that have been reported to play critical roles in angiogenesis, regulation of renal medullary and cortical blood flows, and may also serve as progenitors of interstitial myofibroblasts in kidney fibrogenesis [30]. Although it remains unclear whether PNPLA3 may be involved in the regulation of functions of renal pericytes, it is plausible to hypothesize that the 148 M *PNPLA3* risk allele (impairing the phospholipase activity of the enzyme) might favour an unbalanced activation of pericytes in the kidneys, thus promoting the development of both liver and kidney fibrosis [31]. Indeed, kidney fibrosis is considered as the underlying pathological process of CKD. It is a complex and progressive process, in which a number of different acquired and genetic factors and molecular mediators (including also various phospholipases, such as PLA2 and PLD4) are involved [30,32]. However, further mechanistic studies are needed to better elucidate the role of the I148 M *PNPLA3* variant on the development of kidney fibrosis.

Our study has some important limitations that should be mentioned. First, the cross-sectional design of the study limits our ability to establish the causality and temporality of the observed associations, although this limitation is mitigated by the fact that the genetic variants under study are inherited and, therefore, reverse causation does not apply. Second, it is possible that there may be a selection bias from including an outpatient sample of Italian post-menopausal women with non-insulin treated T2DM. Hence, our results might not be necessarily generalizable to other ethnic groups or to other patients with T2DM, not attending a diabetes outpatient service. Third, we did not perform either a liver biopsy, which is the reference method for diagnosing and staging NAFLD, or a magnetic resonance-proton density fat fraction imaging, which is an accurate, non-invasive method for measuring hepatic fat content [16]. However, we believe that it would have been hazardous to perform liver biopsies in these T2DM patients with fairly normal serum liver enzymes. Finally, the number of individuals with isolated abnormal albuminuria was relatively low, thus limiting our ability to examine the differential impact of *PNPLA3* rs738409 polymorphism on eGFR and albuminuria, separately.

Notwithstanding these limitations, our study has some important strengths, including the consecutive enrolment of the study population, the completeness of the database, the adjustment for multiple common risk factors for CKD, and the exclusion of patients with important comorbidities (e.g., advanced kidney disease, cirrhosis or cancer). We believe that including patients with these comorbidities might have confounded the interpretation of data.

In conclusion, the results of this exploratory, cross-sectional study suggest that the G allele of rs738409 is closely associated with decreasing kidney function and CKD (defined as $eGFR_{CKD\text{-}EPI} < 60$ mL/min/1.73 m^2 or abnormal albuminuria) in an outpatient sample of Caucasian post-menopausal women with T2DM, independent of the presence of NAFLD and other established cardio-renal risk factors. Future larger follow-up and mechanistic studies are needed to better understand the link between *PNPLA3* rs738409 polymorphism and risk of decreased kidney function and a possible interaction with hepatic fat content.

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Authors' contributions

AM and GT conceived and designed the study. CZ performed genotyping. AC performed liver ultrasonography. AM, ES and GL researched data and reviewed/edited the manuscript. AC, GZ, GL, LV, CDB, CM and EB contributed to discussion and reviewed/edited the manuscript. AM and GT analyzed the data and wrote the manuscript. AM and GT are the guarantors of this work and, as such, had full access to all the data of the study and take responsibility for the integrity and accuracy of data. All authors approved the final version of the manuscript.

Disclosure of interest

The authors declare that they have no competing interest.

Appendix A. Supplementary material

Supplementary materials (Table S1-S2-S3) associated with this article can be found at http://www.scincedirect.com.

Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.diabet.2019.01.011.

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