

# **PNPLA3 I148M gene variant and Chronic Kidney Disease in Type 2 Diabetic Patients With NAFLD: Clinical and Experimental Findings**

**Short title:** PNPLA3 and decreased kidney function

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## **ABBREVIATION LIST**

ACR, albumin-to-creatinine ratio  
ALT, alanine aminotransferase  
AST, aspartate aminotransferase  
BMI, body mass index  
CKD-EPI, chronic kidney disease Epidemiology Collaboration  
CKD, chronic kidney disease  
e-GFR, estimated glomerular filtration rate  
LSM, liver stiffness measurement  
MDRD, Modification of Diet in Renal Disease  
mRNA, messenger RNA  
NAFLD, nonalcoholic fatty liver disease  
PNPLA3, patatin-like phospholipase domain-containing protein 3  
HOMA-IR, homeostasis model assessment-insulin resistance  
hs-CRP, high sensitivity C-reactive protein  
GGT, gamma-glutamyltransferase  
T2DM, type 2 diabetes mellitus

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## ABSTRACT

**Background & Aims:** Emerging evidence suggests an association between patatin-like phospholipase domain-containing protein-3 (*PNPLA3*) rs738409 (I148M protein variant), and risk of chronic kidney disease (CKD), but the mechanisms underpinning this association are poorly understood.

**Methods:** We studied 157 patients with type 2 diabetes (T2DM) who underwent ultrasonography and vibration-controlled transient elastography for diagnosing nonalcoholic fatty liver disease (NAFLD). CKD was defined as estimated glomerular filtration rate (e-GFR)  $<60$  ml/min/1.73m<sup>2</sup> and/or abnormal albuminuria. We surveyed *PNPLA3* mRNA expression in human tissues, using the liver as a positive control, and also measured *PNPLA3* mRNA and protein expression levels in human cell lines represented in the kidney and the liver.

**Results:** 112 patients had NAFLD and 43 had CKD. Patients homozygous for the I148M variant (n=11) had lower e-GFR levels ( $60.6 \pm 11.7$  vs.  $77.8 \pm 15.9$  vs.  $83.5 \pm 16.5$  ml/min/1.73m<sup>2</sup>,  $p=0.0001$ ) and higher prevalence of CKD ( $63.6\%$  vs.  $24.2\%$  vs.  $25.0\%$ ,  $p=0.028$ ), compared to those with I/M (n=66) and I/I (n=80) *PNPLA3* genotype. The association of I148M homozygosity with lower e-GFR levels ( $p<0.0001$ ) and higher risk of CKD (adjusted-odds ratio 6.65; 95%CI 1.65-26.8,  $p=0.008$ ) was independent of liver disease severity (as detected by liver stiffness  $\geq 7$ kPa) and other risk factors. *PNPLA3* mRNA expression was greatest in liver and renal cortex, and podocytes showed high *PNPLA3* mRNA and protein levels, comparable to that of hepatocytes and hepatic stellate cells, respectively.

**Conclusions:** The *PNPLA3* I148M variant was associated with CKD, independently of common renal risk factors and severity of NAFLD. *PNPLA3* expression levels were particularly high in renal podocytes.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and chronic kidney disease (CKD) are two worldwide public health problems affecting up to nearly 25% (NAFLD) and up to nearly 15% (CKD) of adults in Western countries (1-4). Both diseases are progressive chronic conditions that represent a spectrum of diseases extending from relatively mild disease, with only modest changes in function, to severe debilitating disease with end-stage organ damage, necessitating either dialysis or organ transplantation in order to sustain life. The worldwide rise in the number of individuals with CKD and consequent kidney failure necessitating renal replacement therapy is threatening to reach epidemic proportions over the next decade (3,4). Importantly, the presence of CKD not only increases the risk of developing end-stage renal disease but also the risk of developing cardiovascular disease and other comorbidities (5). For this reason, the early identification of acquired and genetic risk factors for CKD is crucial in clinical practice.

Several susceptibility gene variants predisposing to NAFLD have been identified in different populations (6-8). Among the genetic factors that influence the development and progression of NAFLD, the rs738409 C>G single nucleotide polymorphism in the *patatin-like phospholipase domain-containing protein-3 (PNPLA3)* gene, encoding for the p.Ile148Met change (I148M), is the single genetic variant accounting for the largest proportion of NAFLD risk in the population (6-8).

A large body of evidence supports the existence of an association between the presence and severity of NAFLD and increased prevalence and incidence of CKD, independent of obesity, type 2 diabetes mellitus (T2DM), hypertension and other common cardio-metabolic risk

factors (9-16). Emerging evidence suggests that the *PNPLA3* I148M variant is also associated with decreasing levels of estimated glomerular filtration rate (e-GFR) or presence of CKD, irrespective of common renal risk factors and presence or severity of NAFLD in both adults and children (17-23).

Presently, however, there are very few studies that have examined the association between the *PNPLA3* I148M variant and decreased kidney function among patients with T2DM (19). This is important because patients with T2DM represent a group of individuals, who are at high risk of developing both CKD and the more severe forms of NAFLD (that also includes hepatocellular carcinoma) (1-3). In addition, the putative mechanisms underpinning the association between the *PNPLA3* I148M variant and risk of kidney damage are poorly understood. In particular, it is not known whether the increased predisposition to kidney damage in carriers of the I148M variant is mediated through NAFLD or by other pleiotropic effects, including a possible direct role in the kidney. Emerging evidence suggests that renal podocytes accumulate lipid droplets, and this process is associated with NLRP3 inflammasome activation and is involved in the pathophysiology of diabetic nephropathy (24).

Thus, the major aims of our clinical and experimental study were firstly to investigate the association between the *PNPLA3* I148M variant and risk of CKD in an outpatient sample of T2DM individuals without known liver diseases. Having demonstrated an association between CKD and *PNPLA3* I148M, our second aim was to examine whether *PNPLA3* gene and protein expression are detectable in the kidney. Specifically, for this second aim, we assessed *PNPLA3* mRNA and protein levels in various human tissues, performing experiments in human cell lines that included both kidney and liver cell populations.

## **MATERIALS AND METHODS**

### *Patients*

We enrolled 157 Caucasian patients with T2DM, who consecutively attended our secondary care diabetes service over a 6-month period, and who accepted to undergo liver ultrasonography and vibration-controlled transient elastography for diagnosing and staging NAFLD. Of these patients, 77 women with T2DM have been included in a previously published study in which we investigated relationships between *PNPLA3* polymorphism and CKD in post-menopausal women (19). We excluded all patients with: (a) history of significant alcohol consumption and other known causes of chronic liver diseases (*e.g.*, virus, drugs, autoimmunity and hemochromatosis); (b) cirrhosis of any aetiology, active cancer and end-stage kidney disease (defined as e-GFR <15 ml/min/1.73 m<sup>2</sup> or dialysis); (c) chronic use of potentially hepatotoxic drugs, including steroids, tamoxifen, amiodarone, methotrexate or treatment with hormone replacement therapy (only for women); and (d) treatment with insulin. We excluded patients on insulin treatment (*i.e.*, an established risk factor for osteoporosis and bone fractures), because this study was primarily designed for exploring the association between NAFLD and bone turnover biomarkers (25). According to the technical limitations of the vibration-controlled transient elastography, patients with congestive heart failure or free abdominal fluid were also excluded from the study.

The study protocol was approved by the local Ethics Committee of the Hospital Trust of Verona. All patients 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. Patients were considered to have hypertension if their blood pressure was  $\geq 140/90$  mmHg or if they were taking any anti-hypertensive agents.

Venous blood samples were collected in the morning after an overnight fast. Complete blood count, liver enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma-glutamyltransferase [GGT]), creatinine (measured using a Jaffé rate blanked and compensated assay), high-sensitivity C reactive protein (hs-CRP) and other biochemical parameters were measured by standard laboratory procedures at the Central Laboratory of our hospital, using the relative reference techniques on Roche Cobas® 8000 (Roche Diagnostics, Basel, Switzerland). Low-density lipoprotein (LDL)-cholesterol was calculated using the Friedewald's equation. Hemoglobin A1c (HbA1c) was measured using the high-performance liquid chromatography analyzer Tosoh-G7 (Tosoh Bioscience Inc., Tokyo, Japan). Fasting insulin was measured using a chemiluminescent immunoassay (LIAISON, DiaSorin, Saluggia, Italy). Homeostasis model assessment (HOMA-IR) score was used for estimating insulin resistance.

e-GFR was estimated using both the CKD-Epidemiology Collaboration (CKD-EPI) equation (26) and the four-variable Modification of Diet in Renal Disease (MDRD) study equation (27).

Albuminuria was measured with an immuno-nephelometric 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 urinary ACR  $\geq 30$  mg/g creatinine. Timed or 24-h urine collections are more burdensome and

add little to prediction or accuracy of albuminuria assessment compared with spot urinary ACR (28). In this study, CKD was defined as the presence of e-GFR <60 ml/min/1.73 m<sup>2</sup> and/or urinary ACR ≥30 mg/g creatinine (28).

Presence of ischemic heart disease was defined as a documented history of myocardial infarction, angina or coronary revascularization procedures. Presence of diabetic retinopathy (diagnosed with fundoscopy after pupillary dilation) was also recorded in all patients.

#### *Ultrasonography and liver stiffness measurement*

A trained physician, who was blinded to participants' clinical and biochemical details, performed both ultrasonography and liver stiffness measurement (LSM) in all patients after at least 8 hours of fasting.

Hepatic steatosis was diagnosed by ultrasonography (using an Esaote MyLab 70 ultrasound with a 4 MHz probe) according to specific ultrasonographic characteristics, such as diffuse hyperechogenicity of the liver relative to kidneys, ultrasonographic beam attenuation, and poor visualization of intra-hepatic vessel borders and diaphragm. The ultrasonographic severity of hepatic steatosis was expressed with a semi-quantitative scale (i.e., mild, moderate or severe) (19).

LSMs were performed using vibration-controlled transient elastography (FibroScan® Echosens, Paris, France) with the M probe. Further details of the technical examination procedures have been described elsewhere (29,30). In brief, each LSM measurement was considered adequate when including at least 10 valid measurements for each patient, with a success rate >60% and



variability of measurements (IQR/M) <30% of the median. The presence of significant liver fibrosis was defined by the presence of LSM  $\geq 7$  kPa (that corresponds to the Kleiner fibrosis stage F $\geq 2$  on liver histology) (30). Unfortunately, we did not have the FibroScan<sup>®</sup> XL probe. Although the FibroScan<sup>®</sup> XL probe reduces transient elastography failure and facilitates reliable LSM in severely obese patients, both the M and XL probes have comparable accuracy for diagnosing significant liver fibrosis (31). The applicability and accuracy of the FibroScan<sup>®</sup> M probe to identify significant fibrosis is excellent in patients with overweight or grade 1 obesity (BMI  $\leq 35$  kg/m<sup>2</sup>) (32). In this study only 11 (7%) patients had a BMI >35 kg/m<sup>2</sup>. Our FibroScan<sup>®</sup> device was not equipped with the controlled attenuation parameter (CAP) for measuring hepatic steatosis. However, Carvalhana *et al.* reported a good correlation (Spearman's  $\rho=0.75$ ,  $p<0.0001$ ) between ultrasound and CAP when comparing the diagnostic accuracy of these two techniques for detecting hepatic steatosis (33).

#### *Genetic analysis*

In all participants genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Germany). Genotyping of rs738409 in *patatin-like phospholipase domain-containing protein-3 (PNPLA3)* gene (I148M variant) was carried out by a predesigned TaqMan probe (Applied Biosystem, California, USA), according to manufacturer's protocol. Single nucleotide polymorphism (SNP) genotyping was performed using 7900 HT Real-Time PCR (Applied Biosystem, California, USA). The call rate exceeded 99% for both SNPs and genotypes' distributions were in Hardy-Weinberg equilibrium. The minor allele frequency of *PNPLA3* rs738409 was 28%.

#### *Gene expression in tissues*

*PNPLA3* gene expression data were retrieved from the BioGPS database across 262 normal human organs and tissues, where they were evaluated by the U133plus2 Affymetrix microarray (<http://biogps.org/#goto=genereport&id=80339>). The values are represented as Z-scores that were produced by the Gene Expression Barcode method (34). According to this standardization method, a Z-score >5 indicates that an annotated gene is expressed at significant level in a specific tissue.

### *Cell cultures*

We selected human cell lines as models of cell types represented both in the liver (as a positive control) and in the kidney to assess *PNPLA3* expression. HepG2 (hepatoma cells), LX2 (hepatic stellate cells), and conditionally immortalized human podocyte cell lines (35,36) were cultured in DMEM (Life Technologies, USA) supplemented with 10% FBS (Life Technologies, USA), 1% L-glutamine (Life Technologies, USA) and 1% penicillin streptomycin (Life Technologies, USA). HK2 (kidney tubular cells) were cultured in DMEM F12 (Life Technologies, USA) supplemented with 10% FBS (Life Technologies, USA), 1% L-Glutamine (Life Technologies, USA) and 1% penicillin streptomycin (Life Technologies, USA). Human umbilical vein endothelial cells (HUVEC), as a model for endothelial cells expressed in renal glomeruli, were cultured in Endothelial Cell Growth Medium–2 (EGM2) (Lonza, Switzerland).

### *Gene expression evaluation*

Total RNA was extracted from cells using the Trizol reagent (Life Technologies, USA) and messenger RNA (mRNA) was retro-transcribed by VILO IV Superscript Kit with ezDNase (Life Technologies, USA). The mRNA expression levels of *PNPLA3* were determined using TaqMan probes (Life Technologies, USA) according to the manufacturer's protocol. Experiments were

assayed in triplicate and repeated twice. *PNPLA3* gene expression was normalized for beta-actin, as the reference gene.

### *Immunoblotting*

Cell lines were lysed and proteins were separated by electrophoresis and then transferred to nitrocellulose membranes for Western blotting. The following antibodies were used: anti-*PNPLA3* (ab81874, Abcam, UK) and anti- $\beta$  actin (Cell Signaling, USA). Protein expression levels were quantified using ImageJ Software (<https://imagej.nih.gov/ij/download.html>).

### *Statistical analysis*

Data are expressed as means $\pm$ SD, medians (interquartile ranges [IQRs]) or percentages. The Fisher's exact test was used for categorical variables, the one-way analysis of variance (ANOVA) was used for normally distributed continuous variables and the Kruskal-Wallis test was used for non-normally distributed variables (*i.e.*, diabetes duration, fasting insulin, HOMA-IR score, hs-CRP and GGT levels), to investigate the differences in clinical and biochemical characteristics among patients with T2DM, stratified by the *PNPLA3* rs738409 (I148M). The association between the *PNPLA3* I148M variant and levels of e-GFR<sub>CKD-EPI</sub> (included as a continuous measure, *i.e.*, for each 1 ml/min/1.73 m<sup>2</sup> decrement) was tested using both an unadjusted linear regression model and a multivariable linear regression model adjusted for diabetes duration, HbA1c, hypertension (*i.e.*, blood pressure  $\geq$ 140/90 mmHg or use of any anti-hypertensive drugs), presence of significant fibrosis (*i.e.*, LSM  $\geq$ 7 kPa on FibroScan<sup>®</sup>) and abnormal albuminuria. We also tested the association between the *PNPLA3* I148M variant and the risk of having CKD (included as a dichotomous variable, *i.e.*, defined as e-GFR<sub>CKD-EPI</sub> <60 mL/min/1.73 m<sup>2</sup> and/or abnormal albuminuria) by using an unadjusted

logistic regression model and a multivariable logistic regression model that included the same list of the aforementioned covariates (*except* for abnormal albuminuria that was included in the CKD definition). The impact of the *PNPLA3* I148M variant on levels of e-GFR and risk of CKD was assessed using additive genetic models. Covariates included in the above-mentioned multivariable regression models were selected based on their significance in univariable analyses, or based on their biological plausibility as potential confounding factors. However, in these models we did not include sex and age among the covariates, as these two variables were included in the equation used for estimating e-GFR (and, consequently, also in the CKD definition).

With regards to the experimental part of our study, HepG2 hepatocytes were used as the reference standard to report relative expression. The unpaired Student's *t*-test was used to test differences in *PNPLA3* mRNA and protein levels.

All statistical tests were two-sided, and *p*-values <0.05 (two-tailed) were considered to be statistically significant. Statistical analyses were performed using STATA software, version 14.2 (STATA, College Station, TX).

## **RESULTS**

### *Association between PNPLA3 I148M variant, reduced e-GFR levels and CKD*

Overall, 157 Caucasian patients with T2DM (mean age 69±9 years; 51% men; mean BMI 28.4±5 kg/m<sup>2</sup>; mean HbA1c 6.9±0.7%) without known liver diseases were included in the study. 43 (27.4%) patients had CKD, 112 (71.3%) had hepatic steatosis on ultrasonography and

30 (19.1%) had LSM  $\geq$ 7 kPa on vibration-controlled transient elastography. In addition, 11 (7%) of these patients had M/M (*i.e.*, homozygous carriers of the I148M variant), 66 (42.1%) had I/M (heterozygous carriers of the I148M variant), and 80 (50.9%) had I/I *PNPLA3* rs738409 genotype (homozygous carriers of the wild-type 148I allele), respectively.

The clinical and biochemical characteristics of patients stratified by *PNPLA3* I148M variant are listed in **Table 1**. Patients homozygous for the I148M variant had significantly higher serum levels of triglycerides, GGT and creatinine than those who were not. In addition, among patients homozygous for the I148M variant there was a higher proportion of individuals with e-GFR<sub>CKD-EPI</sub> <60 ml/min/1.73 m<sup>2</sup>, as well as a higher proportion of individuals with CKD (defined as e-GFR<sub>CKD-EPI</sub> <60 ml/min/1.73 m<sup>2</sup> and/or abnormal albuminuria).

Age, sex, adiposity measures (including patients with BMI >35 kg/m<sup>2</sup>), duration of diabetes, smoking, blood pressure, white blood cell count, platelet count, hemoglobin, serum transaminases, lipids (except for triglycerides), fasting glucose, insulin, HbA1c, HOMA-IR score, hs-CRP, and proportion of abnormal albuminuria, hypertension, ischemic heart disease and diabetic retinopathy, as well as use of anti-hyperglycemic agents, anti-hypertensive drugs, anti-platelets or statins did not significantly differ among the three groups of patients. In addition, the proportion of hepatic steatosis on ultrasonography and significant fibrosis on FibroScan<sup>®</sup> was also comparable among the three patient groups.

Notably, as shown in **Fig. 1**, the values of both e-GFR<sub>CKD-EPI</sub> (panel A) and e-GFR<sub>MDRD</sub> (panel B) decreased significantly across the *PNPLA3* genotype, with the lowest e-GFR values among patients homozygous for the I148M variant.

As reported in **Table 2**, the risk allele of rs738409, encoding for the I148M protein variant, was significantly associated with lower e-GFR<sub>CKD-EPI</sub> levels both in the unadjusted linear regression model ( $\beta$  coefficient -22.9, 95% CI -33.5 to -12.3,  $p < 0.0001$ ) and in the adjusted regression model adjusting for diabetes duration, HbA1c, hypertension, abnormal albuminuria, and presence of significant liver fibrosis ( $\beta$  coefficient -23.2, 95% CI -33.2 to -13.2,  $p < 0.0001$ ). The additive effect of each 148M allele corresponded to a decrement of nearly 9 ml/min/1.73 m<sup>2</sup> in e-GFR<sub>CKD-EPI</sub> levels ( $\beta$  coefficient -8.71, 95% CI -12.7 to -4.7). In this latter adjusted model, other variables that were independently associated with lower e-GFR<sub>CKD-EPI</sub> levels were longer diabetes duration ( $\beta$  coefficient -0.47, 95% CI -0.76 to -0.19,  $p = 0.001$ ) and abnormal albuminuria ( $\beta$  coefficient -6.87, 95% CI -13.2 to -0.51,  $p = 0.035$ ).

When we additionally adjusted the above-mentioned regression models for use of statins and glucagon-like peptide-1 receptor agonists (GLP-1RAs) or sodium-glucose cotransporter 2 (SGLT-2) inhibitors (*i.e.*, two classes of antihyperglycemic drugs that have been reported to improve renal outcomes in large randomized controlled trials in T2DM), the risk allele of rs738409 remained significantly associated with lower e-GFR<sub>CKD-EPI</sub> levels (adjusted  $\beta$  coefficient -23.3, 95% CI -33.4 to -13.1,  $p < 0.0001$ ). Almost identical results were also observed when we used the MDRD study equation, instead of the CKD-EPI equation, for estimating e-GFR (data not shown).

**Table 3** provides the association between the *PNPLA3* I148M variant and risk of having CKD, and the effect of adjustment for established renal risk factors and potential confounders. In univariable regression analysis (unadjusted model), the homozygosity for the I148M variant

was associated with an approximate five-fold increased risk of having CKD (unadjusted-odds ratio 5.27, 95% CI 1.39-19.8,  $p=0.014$ ). Notably, adjustment for duration of diabetes, HbA1c, hypertension, and presence of significant liver fibrosis did not alter these results.

#### *PNPLA3 is highly expressed in renal podocytes*

To assess whether the increased susceptibility to kidney damage in carriers of the risk allele (G) of rs738409 could be due to a direct effect of the *PNPLA3* I148M variant on the kidney, we have also assessed *PNPLA3* mRNA expression in human cells from different tissues (see **Fig. 2A** for the liver and kidney expression and **Supplementary Table 1** for the whole set of tissues available). Notably, we found that the liver and the kidney were the two organs that expressed highest *PNPLA3* mRNA levels (**Fig. 2A**). Additionally, in the kidney the *PNPLA3* mRNA expression was significantly higher in the cortex than in the medulla.

We next examined the relative expression levels of *PNPLA3* in human hepatic and renal cells. In particular, we tested hepatocytes (HepG2), hepatic stellate cells (LX2) and, for the kidney, SV40-immortalized podocytes and HK2 tubular cells. As the alteration of the endothelial barrier in the glomeruli has a key role in the pathogenesis of diabetic nephropathy, we also quantified *PNPLA3* expression in HUVEC cells. These data showed that *PNPLA3* mRNA levels were only slightly lower in renal podocytes than in HepG2 cells, which expressed *PNPLA3* most, but mRNA levels were higher in podocytes than in LX2, HK2 and HUVEC cells (**Fig. 2B**). The high level of *PNPLA3* mRNA expression variant in podocytes was not accounted for by carriage of the *PNPLA3* I148M. Indeed, whilst HepG2 and LX2 cells were homozygous for the mutant I148M allele, renal podocytes and HK2 cells were homozygous for the wild-type 148I allele (**Fig. 2B**).

Finally, we quantified *PNPLA3* protein levels in the different cell lines. Remarkably, renal podocytes showed higher levels of *PNPLA3* protein compared to HepG2, HK2 and HUVEC cells, with levels that were comparable to those of LX2 cells (**Fig. 2C and 2D**).

## DISCUSSION

The main and novel findings of our clinical and experimental study were firstly, the carriage of the *PNPLA3* rs738409 polymorphism, encoding for the I148M protein variant (*i.e.*, the major genetic variant associated with susceptibility to NAFLD development and progression), was closely associated with decreasing e-GFR levels and an increased prevalence of CKD in patients with T2DM. Patients were not known to have liver disease and these associations were independent of duration of diabetes, glycaemic control, hypertension and presence of significant fibrosis on FibroScan<sup>®</sup>. Secondly, our data show that *PNPLA3* mRNA levels were greatest in the liver and in the kidney (in particular in the cortical region), when compared to those in other organ tissues. Thirdly, among the major cellular types of the kidney, podocytes were characterized by higher *PNPLA3* mRNA and protein expression levels than tubular cells and other renal cell types. Furthermore, these expression levels were comparable to those of cells, namely hepatocytes and hepatic stellate cells, which mediate the impact of the I148M variant on liver disease.

In this study, the prevalence of hepatic steatosis and raised liver stiffness was not different across *PNPLA3* I148M genotypes. This finding seems to be at variance with previous studies



showing a significant association between *PNPLA3* I148M gene variant and risk of NAFLD progression, especially in cohorts of patients with biopsy-confirmed NAFLD (1,2,6,37). In our patients with T2DM who were not selected for the presence of liver diseases, as expected we found a relatively low frequency of individuals with homozygosity for the 148M variant (*i.e.*, only 7% of these T2DM patients had M/M genotype, a frequency comparable to that of the Italian general population [37]), despite the presence of a high prevalence of NAFLD (*i.e.*, 71.3% had NAFLD on ultrasonography and nearly 20% had LSM  $\geq$ 7 kPa on FibroScan®). Thus, it is possible to hypothesize that we did not have sufficient power to detect the adverse effects of the *PNPLA3* I148M gene variant *per se* on both the development of NAFLD, which was already high due to T2DM itself, and its progression to NASH and advanced fibrosis, which is not a frequent event in individuals not selected for the presence of liver damage. In fact, patients with T2DM have a myriad of other non-genetic, acquired risk factors (such as obesity, insulin resistance and other metabolic syndrome features) that play important roles in the development and progression of NAFLD (1,2).

Collectively, the results of our study corroborate recent observations showing that *regardless* of established renal risk factors and the presence of NAFLD, the *PNPLA3* I148M variant was strongly associated with decreasing e-GFR levels, abnormal albuminuria and higher prevalence of CKD in both adults and children (17-23). For instance, our group recently showed that carriage of the I148M variant was independently associated with decreasing e-GFR levels and increasing urinary protein excretion in 142 overweight non-diabetic children with biopsy-proven NAFLD (21). Similarly, Sun *et al.* reported that homozygosity for the I148M variant was independently associated with an increased prevalence of CKD and abnormal albuminuria or higher levels of urinary neutrophil gelatinase-associated lipocalin (*i.e.*, a

marker of renal tubular injury) in 217 Chinese adults with biopsy-proven NAFLD (20). Moreover, in a cross-sectional study of 77 post-menopausal T2DM women, Mantovani *et al.* reported that homozygosity for the 148M variant was independently associated with lower levels of e-GFR<sub>CKD-EPI</sub> and higher risk of having CKD (19). Finally, Oniki *et al.* reported that the *PNPLA3* I148M variant was significantly associated with a decline in kidney function over a mean follow-up of ~5 years in Japanese elderly individuals (18).

*PNPLA3* is highly expressed on the lipid droplets of hepatocytes and it is involved in the development and progression of NAFLD (6-8,38). Specifically, the I148M allele of the *PNPLA3* gene impairs the phospholipase activity of the enzyme and promotes liver fat accumulation, by accumulating at the surface of lipid droplets, where it interacts with comparative gene identification-58 (CGI-58), which is an essential cofactor for the adipose triglyceride lipase, thereby impairing hepatic lipid remodeling (39). However, accumulating evidence indicates that *PNPLA3* is highly expressed in hepatic stellate cells, *i.e.*, the liver pericytes representing the main body storage of retinoids, which regulate blood flow in liver sinusoids in physiological conditions (40). In response to liver damage, hepatic stellate cells are activated from a quiescent to a pro-fibrotic state (41,42). This process is associated with the release of retinol from intracellular lipid droplets, a process that requires *PNPLA3*, whereas the I148M variant impairs this process, thus resulting in a pro-inflammatory and pro-fibrotic liver phenotype (43-45). To date, while the role of the *PNPLA3* I148M variant in the development and progression of NAFLD is beginning to be unraveled, the mechanisms underpinning the association between the I148M allele and the decline in e-GFR levels are poorly understood and remain largely speculative. A plausible explanation for this finding is that this association is mediated by the presence and severity of NAFLD. However, the results of our study and

those of other recently published studies suggest that the association between the *PNPLA3* I148M variant and decreased kidney function was independent of the shared renal/metabolic risk factors and the presence of hepatic steatosis and severity of liver fibrosis (18-22).

Although we could not rule out that the impact of the *PNPLA3* I148M variant on kidney damage is mediated by quantitative or qualitative alterations of hepatic fat content leading to the release of inflammatory mediators, it is therefore possible to hypothesize that the *PNPLA3* I148M variant might exert adverse effects on the kidney (19-21,40). To test this hypothesis, we have assessed *PNPLA3* mRNA and protein expression levels in various human tissues and performed experiments using human cell lines representing the major renal cell populations. We included both renal podocytes that are mainly found in the glomeruli of the renal cortex and HK2 cells that are an immortalized proximal tubule epithelial cell line from normal human kidney. Across the human tissues studied, we found that the *PNPLA3* mRNA expression levels were highest in the liver and in the renal cortex. In keeping with these data, among the major cellular types of the kidney, we found that podocytes were characterized by higher *PNPLA3* mRNA and, in particular, protein expression levels, compared to renal tubular cells and other cell types (including hepatocytes). We hypothesize that different post-translational protein regulation may account for the different ratios between *PNPLA3* mRNA and protein levels across cell types under our experimental conditions. However, it is important to note that the *PNPLA3* protein expression in podocytes was comparable to that of hepatic stellate cells, which are known to express this protein at the highest level in the liver (6-8,38). Interestingly, kidney damage leads to the activation of renal podocytes and pericytes, which, similarly to hepatic stellate cells, are stromal cells partially covering capillary walls that play a key role both in angiogenesis and in regulating renal medullary and cortical blood flows, thus promoting kidney fibrogenesis and glomerulosclerosis (46,47). Thus, our findings are

consistent with the hypothesis that carriage of the *PNPLA3* I148M variant adversely affects kidney function, independent of the presence of significant hepatic fibrosis, possibly through a direct effect on renal podocytes. Although it is known that serum retinol concentrations are increased in patients with CKD (48), scant data are currently available on the regulation of lipid and retinol metabolism in renal podocytes (46,47). However, recent data suggest that podocytes can accumulate lipid droplets, and that this process is associated with activation of the inflammasome and is involved in the pathogenesis of glomerulopathy associated with T2DM and obesity (24,49-51). Consistently with decreased retinol bioavailability in pericytes carrying the *PNPLA3* I148M variant (43-45), it has been reported that a highly selective agonist for retinoic acid receptor  $\beta$ 2 (*i.e.*, AC261) was able to ameliorate diabetic nephropathy in an experimental mouse model (52). However, further mechanistic studies are needed to examine the role of *PNPLA3* and the impact of the I148M variant on retinol and lipid metabolism in the kidney, as well as on the inflammatory and fibrogenic responses to injury in renal podocytes.

If confirmed in future studies, our observations may have important clinical implications, because they supports the assertion that *PNPLA3* genotyping might be useful not only for identifying subjects with greater susceptibility to NAFLD development and progression, but also for identifying those patients with NAFLD who are at higher risk of developing CKD, thus facilitating the implementation of specific prevention programs and treatment strategies for CKD among the carriers of the *PNPLA3* I148M variant.

Our study has some other important limitations that should be mentioned. First, the cross-sectional design of the study limits establishing temporal or causal relationships between the *PNPLA3* I148M variant and risk of CKD. However, this limitation is mitigated by the fact that

this genetic variant is inherited and, therefore, reverse causation does not apply. Second, although we did not perform a liver biopsy, the diagnosis of NAFLD was performed by ultrasonography and vibration-controlled transient elastography, which are two imaging methods widely used in routine clinical practice (53). Third, although we did not directly measure GFR (by plasma iohexol disappearance), we used the two most widely accepted serum creatinine-based equations for estimating GFR in adults (25,26). Fourth, our patient cohort comprised exclusively Caucasian individuals with non-insulin-treated T2DM without known liver diseases, attending a diabetes outpatient service. Thus, results cannot be necessarily extrapolated to the general population of patients with T2DM or other ethnic groups. Finally, we cannot definitely exclude residual confounding as a result of unmeasured risk factors.

Despite these limitations, our study has also important strengths, including the relatively large sample size, the consecutive enrollment of the study population, the completeness of the database, the adjustment for established renal risk factors, the use of vibration-controlled transient elastography for measuring liver stiffness, as well as the exclusion of patients with important comorbidities (*e.g.*, advanced kidney disease, cirrhosis or active cancer). In addition, this is the first study that has examined the *PNPLA3* mRNA and protein expression levels in human cell lines representing the major renal cell populations.

In conclusion, the results of this study show that in patients with T2DM, the *PNPLA3* I148M variant was associated with decreasing e-GFR levels and higher risk of having CKD. These associations were independent of established renal risk factors, diabetes-related variables and the severity of NAFLD. Our findings also show that *PNPLA3* was expressed at high levels in the

renal cortex, mainly in podocytes. However, more research is needed to better understand the role of the *PNPLA3* I148M variant in the development and progression of kidney damage and the function of the I148M mutant protein in the kidney.

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**Table 1.** Main clinical and biochemical characteristics of patients with non-insulin-treated T2DM, stratified by *PNPLA3* I148M variant.

	I/I genotype (n=80)	I/M genotype (n=66)	M/M genotype (n=11)	P value
Age (years)	69 ± 10	70 ± 7	69 ± 13	0.94
Sex (men) (%)	50.0	54.6	36.4	0.51
Weight (kg)	78 ± 16	78 ± 14	80 ± 11	0.94
BMI (kg/m <sup>2</sup> )	28.8 ± 5	27.8 ± 4	29.0 ± 5	0.35
BMI >35 kg/m <sup>2</sup> (%)	7.5	4.6	18.2	0.23
Waist circumference (cm)	101 ± 14	99 ± 11	97 ± 11	0.41
Diabetes duration (years)	9 (6-19)	11.5 (7-18)	10 (7-15)	0.89
Current smokers (%)	15.0	10.6	9.1	0.75
Systolic blood pressure (mmHg)	134 ± 17	133 ± 16	145 ± 17	0.08
Diastolic blood pressure (mmHg)	76 ± 9	76 ± 9	79 ± 8	0.62
White blood count (10 <sup>9</sup> /l)	7.2 ± 1.5	7.1 ± 1.7	7.7 ± 3.1	0.59
Haemoglobin (g/l)	137 ± 13	139 ± 13	136 ± 12	0.53
Platelet count (10 <sup>9</sup> /l)	233 ± 58	239 ± 59	250 ± 65	0.62
Total cholesterol (mmol/l)	3.9 ± 0.8	4.1 ± 0.9	4.2 ± 0.9	0.41
LDL cholesterol (mmol/l)	1.9 ± 0.7	2.1 ± 0.8	2.2 ± 0.8	0.06
HDL cholesterol (mmol/l)	1.4 ± 0.4	1.3 ± 0.3	1.4 ± 0.3	0.60
Triglycerides (mmol/l)	1.5 ± 0.8	1.3 ± 0.6	1.8 ± 0.9	<b>0.048</b>
Fasting glucose (mmol/l)	7.1 ± 1.4	7.2 ± 1.6	7.3 ± 1.5	0.92
Fasting insulin (mIU/l)	6.9 (3.3-10.2)	5.9 (3.6-9.4)	13.4 (3.7-16)	0.16
HOMA-IR score	2.1 (1.0-3.4)	1.9 (1.1-2.9)	4.3 (1.3-7.2)	0.14
HbA1c (%)	6.9 ± 0.7	6.9 ± 0.7	7.2 ± 0.7	0.42
hs-CRP (mg/l)	1.2 (0.6-2.9)	1.4 (0.5-3.7)	1.0 (0.7-2.5)	0.84
Albumin (g/l)	47 ± 4	47 ± 3	46 ± 4	0.75
AST (IU/l)	25 ± 9	23 ± 7	28 ± 8	0.16
ALT (IU/l)	16 ± 8	13 ± 6	16 ± 8	0.11
GGT (IU/l)	22 (15-36)	16 (13-26)	27 (17-44)	<b>0.023</b>
Creatinine (umol/l)	72.6 ± 20	79.1 ± 22	98.2 ± 31	<b>0.001</b>
CKD (%)	25.0	24.2	63.6	<b>0.028</b>
e-GFR <sub>CKD-EPI</sub> <60 ml/min/1.73 m <sup>2</sup> (%)	8.7	12.1	60.0	<b>0.001</b>
Abnormal albuminuria (%)	22.5	13.6	27.3	0.30
Hypertension (%)	82.5	78.8	72.7	0.60
Ischemic heart disease (%)	18.7	7.6	9.1	0.13
Diabetic retinopathy, any degree (%)	11.6	12.5	10.0	0.92
Metformin users (%)	83.7	86.4	100.0	0.45
Sulfonylurea users (%)	31.2	24.2	18.2	0.56
Pioglitazone users (%)	8.7	9.1	9.1	0.99
DPP-4 inhibitor users (%)	28.7	21.2	18.2	0.57
GLP-1 analogue users (%)	12.5	27.3	18.2	0.06
SGLT-2 inhibitor users (%)	13.7	9.1	0.0	0.39
Anti-platelet users (%)	50.0	42.4	45.4	0.65
Beta-blocker users (%)	34.6	27.3	27.3	0.61
ARB/ACE-inhibitor users (%)	69.2	54.6	72.7	0.18
Calcium-channel antagonist users (%)	28.2	18.2	9.1	0.23
Diuretic users (%)	29.5	34.8	18.2	0.59
Statin users (%)	82.3	75.8	63.6	0.28
Hepatic steatosis on ultrasound (%)	75.0	66.7	72.7	0.55
LSM ≥7 kPa on FibroScan® (%)	20.0	18.2	18.2	0.95

Sample size, n=157. Data are expressed as means ± SD, medians or percentages. Differences among the three groups were tested by the Fischer'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 (i.e., duration of diabetes, HOMA-IR score, fasting insulin, GGT and hs-CRP concentrations).

**Abbreviations:** 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; GGT: gamma-glutamyltransferase; GLP-1: glucagon-like peptide-1; HOMA-IR: homeostasis model assessment-insulin resistance; hs-CRP, high sensitivity C-reactive protein; LSM, liver stiffness measurement; SGLT-2: sodium/glucose cotransporter-2.

**Note:** CKD was defined as e-GFR<sub>CKD-EPI</sub> <60 ml/min/1.73 m<sup>2</sup> and/or abnormal albuminuria; abnormal albuminuria was defined as spot urinary ACR ≥30 mg/g; hypertension was defined as blood pressure ≥140/90 mmHg or use of any anti-hypertensive drugs.

**Table 2.** Linear Regression Analysis – Effect of *PNPLA3* I148M variant on e-GFR<sub>CKD-EPI</sub> levels in patients with T2DM.

	<b>B coefficient(s)</b>	<b>95% CI(s)</b>	<b>Standardized B coefficient(s)</b>	<b>P value</b>
<b>Unadjusted linear regression model</b>				
<b>e-GFR<sub>CKD-EPI</sub> for 1 ml/min/1.73 m<sup>2</sup> decrement</b>				
<i>PNPLA3</i> rs738409				
I/I genotype	<i>Ref.</i>	<i>Ref.</i>	-	-
I/M genotype	-5.61	-10.8 to -0.36	-0.16	0.026
M/M genotype	-22.9	-33.5 to -12.3	-0.33	<0.0001
<b>e-GFR<sub>CKD-EPI</sub> for 1 ml/min/1.73 m<sup>2</sup> decrement</b>				
<i>PNPLA3</i> rs738409	-8.49	-12.6 to -4.34	-0.31	<0.0001
<b>Adjusted linear regression model *</b>				
<b>e-GFR<sub>CKD-EPI</sub> for 1 ml/min/1.73 m<sup>2</sup> decrement</b>				
<i>PNPLA3</i> rs738409				
I/I genotype	<i>Ref.</i>	<i>Ref.</i>	-	-
I/M genotype	-5.77	-10.8 to -0.72	-0.17	0.025
M/M genotype	-23.2	-33.2 to -13.2	-0.34	<0.0001
<b>e-GFR<sub>CKD-EPI</sub> for 1 ml/min/1.73 m<sup>2</sup> decrement</b>				
<i>PNPLA3</i> rs738409	-8.71	-12.7 to -4.70	-0.32	<0.0001

Sample size, *n*=157. Data are expressed as beta coefficients and 95% confidence intervals (CI) as tested by linear regression analysis. The continuous values of e-GFR (for every 1 ml/min/1.73 m<sup>2</sup> decrement) were included as the dependent variable in all linear regression models. The impact of *PNPLA3* rs738409, encoding for the I148M protein variant, on levels of e-GFR was assessed using an additive genetic model. Ref. = reference category.

\*This multivariable linear regression model was adjusted, along with *PNPLA3* rs738409 genotypes, for the following covariates: duration of diabetes, HbA1c, hypertension (i.e., blood pressure ≥140/90 mmHg or use of any anti-hypertensive drugs), presence of LSM ≥7 kPa on FibroScan® and abnormal albuminuria (i.e., spot urinary ACR ≥30 mg/g creatinine).

**Table 3.** Logistic Regression Analysis – Effect of *PNPLA3* I148M variant on risk of prevalent CKD in patients with T2DM.

	Odds Ratio(s)	95% CI(s)	P value
<b>Unadjusted logistic regression model</b>			
<b>CKD (yes vs. no)</b>			
<i>PNPLA3</i> rs738409			
I/I genotype	<i>Ref.</i>	<i>Ref.</i>	-
I/M genotype	0.96	0.45 – 2.04	0.92
M/M genotype	5.27	1.39 – 19.8	0.014
<b>Adjusted logistic regression model *</b>			
<b>CKD (yes vs. no)</b>			
<i>PNPLA3</i> rs738409			
I/I genotype	<i>Ref.</i>	<i>Ref.</i>	-
I/M genotype	0.99	0.45 – 2.14	0.96
M/M genotype	6.65	1.65 – 26.8	0.008

Sample size, *n*=157. Data are expressed as odds ratio(s) and 95% confidence intervals (CI) as tested by logistic regression analysis. The presence of CKD was included as the dependent variable in all logistic regression models. The impact of *PNPLA3* rs738409, encoding for the I148M protein variant, on risk of CKD was assessed using an additive genetic model. Ref. = reference category.

\*This multivariable logistic regression model was adjusted, along with *PNPLA3* rs738409 genotype, for the following covariates: duration of diabetes, HbA1c, hypertension (blood pressure  $\geq$ 140/90 mmHg or use of any anti-hypertensive drugs) and presence of LSM  $\geq$ 7 kPa on FibroScan®.

## FIGURE LEGENDS

**Fig. 1.** Box plot of e-GFR<sub>CKD-EPI</sub> (*panel A*) and e-GFR<sub>MDRD</sub> (*panel B*) values of 157 patients with T2DM, stratified by *PNPLA3* I148M variant. *P*-values for the trend by the Kruskal-Wallis test. The central rectangle spans the first quartile to the third quartile (the IQR). The segment inside the rectangle shows the median, and the whiskers above and below the box indicate the locations of 1.5 x IQR values.

**Fig. 2.** *PNPLA3* rs738409 expression in human organs and tissues. *Panel A:* Z-score values of *PNPLA3* expression in tissues. The dash line indicates the threshold above which a gene is considered expressed. A Z-score >5 suggests that an annotated gene is expressed in a specific tissue. Other tissues refer to the mean of Z-scores of tissues that do not include kidney and the liver. *Panel B:* Genotype and *PNPLA3* mRNA expression level in HepG2 hepatocytes, LX2 (hepatic stellate cells) and conditionally immortalized human renal podocytes, HK2 (renal tubular cells) and Human Umbilical Vein Endothelial cells (HUVEC). *Panel C:* *PNPLA3* protein expression in HepG2 hepatocytes, LX2 (hepatic stellate cells) and conditionally immortalized human renal podocytes, HK2 (tubular cells) and Human Umbilical Vein Endothelial cells (HUVEC). *Panel D:* *PNPLA3* protein quantification in HepG2 hepatocytes, LX2 (hepatic stellate cells) and conditionally immortalized human renal podocytes, HK2 (tubular cells) and Human Umbilical Vein Endothelial cells (HUVEC).

**NB:** in all panels above: (\*) represents the statistical significance of values normalized to HepG2 hepatocyte control, whereas (•) represents the statistical significance of values normalized to podocyte control.