Metabolic syndrome but not genetic polymorphisms known to induce NAFLD predicts increased total mortality in subjects with NAFLD (OPERA Study)

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Abstract

Metabolic syndrome (MetS) and genetic polymorphisms PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738 are known inductors of non-alcoholic fatty liver disease (NAFLD). However, knowledge about how these affect the mortality of subjects with NAFLD is scarce. Therefore, we investigated the impact of MetS, PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738 on overall and cardiovascular disease (CVD) specific mortality among subjects with or without NAFLD. NAFLD diagnosis was based on liver ultrasound at the baseline. After this and other comprehensive examinations, 958 middle-aged Finns, 249 with NAFLD, were followed for 21 years. The mortality data was gathered from the National Death Registry. After multiple adjustments, the NAFLD individuals with MetS had increased risk of overall mortality as compared to the NAFLD subjects without MetS (2.054 (1.011–4.173, $p=0.046$)). However, PNPLA3 rs738409 (1.049 (0.650–1.692, $p=0.844$)), TM6SF2 rs58542926 (0.721 (0.369–1.411, $p=0.340$)) or MBOAT7 rs641738 (0.885 (0.543–1.439, $p=0.621$)) did not affect the overall mortality. MetS was also a marker of increased risk of CVD mortality (15% vs. 2%, $p=0.013$) while genetic polymorphisms did not affect CVD mortality. In conclusion, MetS, but not the gene polymorphisms studied, predicts increased overall and CVD-specific mortality among NAFLD subjects.

Key words:

non-alcoholic fatty liver disease, cardiovascular disease, mortality, metabolic syndrome, PNPLA3, TM6SF2, MBOAT7

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as accumulation of hepatic fat exceeding 5% of liver weight without excess alcohol drinking or other secondary causes [1]. With an estimated global prevalence of about 25% [2] it is one of the leading causes of liver injury worldwide [1-3]. NAFLD is a continuum covering simple hepatosteatorosis and the more progressive forms, non-alcoholic steatohepatitis (NASH) and fibrosis, predisposing to liver cirrhosis and hepatocellular carcinoma [1]. NAFLD is also a risk factor for extrahepatic complications, such as type 2 diabetes (T2D), cardiovascular diseases (CVDs) and atrial fibrillation [1,4,5]. It has been estimated that only about 9% of biopsy-verified NAFLD subjects will die of hepatic reasons and up to about 40% due to CVDs [6,7].

Several environmental and genetic factors modify the nature of NAFLD [8]. The most studied genetic variants known to affect the risk of NAFLD and its progression are the rs738409 variant of Patatin-like phospholipase domain-containing 3 gene (PNPLA3), the Transmembrane 6 superfamily member 2 (TM6SF2) rs58542926, and the Membrane-bound O-acyltransferase domain containing 7 (MBOAT7) rs641738 [9-12]. The PNPLA3 rs738409 loses the normal wild-type gene function of hydrolytic activity on triglycerides and glycerolipids, thus exposing to steatorosis, NASH and liver fibrosis [12-17]. Although fibrosis is associated with increased risk of all-cause mortality in NAFLD patients [18], PNPLA3 rs738409 did not affect the mortality of subjects with hepatosteatorosis and was associated with reduced mortality in female subjects without hepatosteatorosis [19]. TM6SF2 is disturbed by the rs58542926 variant, making it more unstable, affecting its function in lipid metabolism and resulting in intrahepatic fat accumulation [16,20,21], and possibly even more progressive NAFLD stages [11,17]. The MBOAT7 rs641738 variant is known to reduce the expression of the transferase which alters the acyl remodeling of

phosphatidylinositol in the liver, leading to association with all NAFLD spectrum stages [9], or at least liver fibrosis [16].

The metabolic syndrome (MetS), a cluster of CVD and T2D risk factors including impaired fasting glucose, hypertension, dyslipidemia and central obesity, has also been indicated as a risk factor in NAFLD patients based on two studies. According to the study of Younossi et al. [22] in NAFLD patients, MetS increased the risk of overall, liver- and CVD-specific mortality compared to those without MetS. Similar results were reported for CVD events and new-onset T2D by our group [23]. To the best of our knowledge, the study of Younossi et al. [22] is the only one showing the effect of MetS on the total mortality of NAFLD subjects, and only the effect of PNPLA3 rs738409 variant on mortality has been reported [19] while the impact of other gene variants is unknown. Therefore, we investigated the impact of these gene variants and MetS on overall and CVD mortality in a prospective middle-aged cohort in the Oulu Project of Elucidating the Risk of Atherosclerosis (OPERA) study (n=958), with a mean follow-up time of 21.0 years.

Materials and Methods

The OPERA study was originally launched to study the risk factors of atherosclerotic diseases [24]. A total of 300 men and 300 women aged 40–59 years and randomly selected from the Register of the Social Insurance Institute for the reimbursement of hypertension medication were invited to the study (original study group: ‘hypertensives’). After randomization of subjects from the National Health Register (including all inhabitants), their 1:1 age- and sex-matched controls without known hypertension were also invited (original study group: ‘controls’). Thus, 1,200 subjects were invited to the study. The invitation was accepted by 1,045 subjects (87.1%) including 520 men (261 from the hypertension group and 259 from the control group) and 525 women (258 from the hypertension group and 267 from the control group) [24].

At the time of enrollment in 1990–1993, comprehensive laboratory tests after an overnight fast, standardized health questionnaires and clinical examinations, including the assessment of hepatic steatosis by liver-kidney contrast on ultrasonography [25] by an experienced radiologist, were performed at the research laboratory of the Department of Internal Medicine, University of Oulu. MetS was defined by the criteria of the International Diabetes Federation, where abdominal obesity has a central role [26]. Homeostasis model assessment (HOMA-IR), a surrogate for insulin resistance, was calculated by the formula: $\text{HOMA-IR} = \text{fS-insulin (mU/L)} \times \text{fP-glucose (mmol/L)} / 22.5$ [27]. Systolic and diastolic blood pressure was measured from the right arm in a sitting position after an overnight fast and after 10 to 15 minutes’ rest using an automatic oscillometric blood pressure recorder (Dinamap, Critikon Ltd, Tampa, FL, USA). Three measurements were made at one-minute intervals, and the means of the last two were reported.

Subjects with excess alcohol drinking, i.e. ≥ 210 g a week in men or ≥ 140 g a week in women [1], (n=63 in men and n=13 in women) or missing hepatic steatosis data (n=17, of which 11 without excess alcohol drinking) were excluded from the present study. Due to the very low prevalence of hepatitis B and C in Finland, the presence of these viruses was not examined which is an established practice in the Finnish NAFLD studies [2]. Thus, the final number of study participants was 958. PNPLA3 rs738409, TM6SF2 rs58542926, and MBOAT7 rs641738 were successfully genotyped for 948 subjects, 937 subjects and 935 subjects, respectively. The subjects were subgrouped to carriers of the risk allele (heterozygous or homozygous) or non-carriers of the risk allele (homozygous). In the total cohort, there were 249 subjects (26.0%) with NAFLD.

Mortality data was acquired from the National Death Registry. CVD-related deaths were defined as coronary artery disease (CAD) or stroke (subarachnoidal bleeding excluded) as a cause of death. CAD included I20–I25, I46, R96, R98 [ICD-10]/410–414, 798 (not 7980A) [ICD-8/9] as the underlying cause of death or immediate cause of death and I21 or I22 [ICD-10]/410 [ICD-8/9] as first to third contributing cause of death. Stroke included I61, I63 (not I636), I64 [ICD-10]/431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9]/431 (except 43101, 43191) 433, 434, 436 [ICD-8] as the main diagnosis (symptom or cause), as the first or second side diagnosis (symptom or cause), as the third side diagnosis (ICD-8/9 only), as an underlying cause of death or immediate cause of death, or as a first to third contributing cause of death.

The study subjects gave written informed consent for the use of their clinical records. The Ethics Committee of the Northern Ostrobothnia Hospital District (Oulu, Finland) approved the study (48/2009).

Genotyping

DNA was extracted from human nucleated cells from frozen plasma using high-salt method. All single nucleotide polymorphism analyses (PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738) were performed by TaqMan assays (Life Technologies, Carlsbad, CA, USA).

Statistics

Statistical analyses were performed by using IBM SPSS Statistics for Windows version 24 (Armonk, NY, USA) and GraphPad Prism, version 7.0 (GraphPad Software Inc., CA, USA). Chi-Square test was used for categorical variables and analysis of variance (ANOVA) for continuous variables for the statistical significance of differences. The Cox hazards model was used for the predictive value for overall mortality and Chi-Square for the statistical significances of differences in CVD-specific mortality. The p-value <0.05 was considered statistically significant.

Results

In the total cohort, MetS was present in 350 (37%) individuals, including 186 (53%) subjects with NAFLD, whereas there were 608 (63%) subjects without MetS, 63 (10%) of them with NAFLD ($p<0.001$). The prevalence of PNPLA3 rs738409 ($p=0.025$), TM6SF2 rs58542926 ($p=0.002$) and MBOAT7 rs641738 ($p=0.044$) was greater among NAFLD subjects than among subjects without NAFLD (Table 1).

The baseline characteristics in subjects by MetS and NAFLD status are depicted in Table 2.

In the total cohort, there were 191 (55%) men and 79 (23%) diabetic subjects among individuals with MetS, whereas among those without MetS, the number of men was 259 (43%) and that of diabetics was 18 (3%) ($p<0.001$ for both). The subjects with MetS had higher body mass index (BMI), systolic and diastolic blood pressure, alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), left ventricular mass index (LVMI), low-density lipoprotein (LDL) cholesterol, triglycerides and HOMA-IR but lower high-density lipoprotein (HDL) cholesterol than subjects without MetS ($p=0.001$ for LDL cholesterol; for the others, $p<0.001$). Supplementary Table 1 shows the counts and the statistics for these variables.

The main baseline characteristics of the study participants by the presence or absence of each gene polymorphisms and with or without NAFLD are provided in Supplementary Table 2 and 3, respectively. As the tables show, the subjects with NAFLD and with PNPLA3 rs738409 had lower BMI than NAFLD subjects without this gene variant ($p=0.027$). Moreover, GGT was higher among MBOAT rs641738 carriers as compared to the MBOAT rs641738 non-carriers in the NAFLD group ($p=0.019$). In the non-NAFLD group, PNPLA3 rs738409 tended to imply lower systolic blood pressure ($p=0.033$) but higher HDL cholesterol ($p=0.033$).

In the total cohort at baseline, there were no statistically significant differences in the variables in PNPLA3 rs738409 carriers as compared to non-carriers, while BMI was higher (28.6kg/m² vs. 27.6kg/m², p=0.032) and LDL cholesterol lower (3.3mmol/L vs. 3.6mmol/L, p=0.014) in the carriers of TM6SF2 rs58542926 than in those without this risk allele, and the presence of MBOAT7 rs641738 was associated with higher ALT and GGT (for ALT 32U/L vs. 29U/L, p=0.021; for GGT 45U/L vs. 37U/L, p=0.021).

Fig. 1 shows that the subjects with MetS had increased risk for total mortality as compared to the subjects without MetS irrespective of whether NAFLD was present or not. The statistical significance of the differences remained in all three subgroups even after multiple corrections (age, BMI, gender, smoking, original OPERA study group) in the Cox hazards model (the corrected p-values for subjects with NAFLD, subjects without NAFLD and both groups combined were p=0.046, p=0.027 and p=0.001, respectively). Notably, the presence of NAFLD did not affect the death rates as compared to the absence of NAFLD (p=0.326 in subjects with MetS and p=0.955 in subjects without MetS).

The overall mortality among the carriers of PNPLA3 rs738409, TM6SF2 rs58542926 or MBOAT7 rs641738 as compared to the non-carriers of these gene variants was not statistically significantly different for any of the gene variants (Table 3).

The Hazards Ratios for the risk of overall death by the number of gene polymorphisms are shown in Fig. 2. As the figure shows, gene polymorphisms, however prevalent, did not increase the risk of death. Rather, the gene polymorphisms could be seen as offering some protection against all-cause mortality, although statistical significance, even after multiple adjustments (age, sex, BMI, original study group and smoking), was gained only in total cohort (n=933) among those with one risk gene polymorphism (p=0.039). When the study participants were re-grouped to those without any genetic polymorphism (n=167) and those with 1–3 gene polymorphisms (n=766), the latter group was

protected from overall mortality (0.677, CI95% 0.488–0.940, $p=0.020$), but after multiple adjustments (see above), the protection attenuated and the statistical significant of differences was lost (0.723, CI95% 0.520–1.005, $p=0.054$). This was also the result when comparing those with one or two gene polymorphisms to the non-carriers ($p=0.025$; after multiple adjustments, $p=0.069$). The categorical re-grouping (individuals having 1–3 gene polymorphisms vs. individuals who did not have any polymorphism) showed non-significant differences within the NAFLD group ($p=0.105$) or non-NAFLD group ($p=0.090$; other data not shown).

The CVD-specific death rates did not significantly depend on PNPLA3 rs738409 or MBOAT7 rs641738 status (Fig. 3). There was a numerical difference in CVD-specific death rates in subjects with and without TM6SF2 rs58542926 alleles, indicating protective effect of variant genotype, but the difference did not reach statistical significance. However, as in the case of overall mortality, MetS at baseline was a marker of increased risk of CVD death during the follow-up. The same results are also provided in Supplementary Table 4 as numerical data.

Discussion

The present study shows that the presence of MetS predicts higher risk of CVD mortality and overall mortality in subjects with NAFLD as well as in subjects without NAFLD as compared to subjects without MetS. However, although PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738 are associated with increased prevalence of NAFLD [9-12,28], the present study shows that these gene variants do not predict increased overall mortality among middle-aged subjects with or without NAFLD in a long-term follow-up.

Despite the increased hepatic fat accumulation, PNPLA3 rs738409 and TM6SF2 rs58542926 are not known to be associated with insulin resistance due to the distinct nature of the accumulated lipid content [28-30]. The intrahepatic lipid composition with PNPLA3 rs738409 is predominantly shown to consist of polyunsaturated triacylglycerols and not metabolically harmful ceramides or diacylglycerols, which along with saturated or monounsaturated triacylglycerols and fatty acids are abundant in obesity-related NAFLD [29,30]. Likewise, TM6SF2 rs58542926 enriches the hepatic lipidome with triglycerides, cholesteryl-esters and polyunsaturated fatty acids, but diminishes its phosphatidylcholine levels [31]. Neutral lipids such as triglycerides and esters do not induce insulin resistance [32], and polyunsaturated fatty acids are also considered protective against liver injury and CVDs [33,34]. The mechanism how MBOAT7 rs641738 alters the hepatic lipidome is not yet well-elucidated, but according to one report, it only alters the composition of liver phosphatidylinositols [35] which regulate cell membranes and signal transduction mechanisms [35,36]. Even though there are divergent reports about the association between MBOAT7 rs641738 and insulin resistance [9,37], the present study may suggest that MBOAT rs641738 does not induce insulin resistance, based on unaffected HOMA-IR and diabetes prevalence at baseline. It is also of note that none of these gene variants are related to obesity-related adipose tissue inflammation,

which also has a central role in the development of insulin resistance [28,38]. Indeed, obesity-related NAFLD and insulin resistance are tightly and bidirectionally associated [39]. While obesity-related NAFLD is also an epiphenomenon of the fat accumulation of other ectopic sites, such as the heart [40], which is also a risk factor for heart-associated mortality [41,42], ectopic fat accumulation has not been reported in genetic NAFLD. Furthermore, the potent role of dysbiotic gut microbiota in obese NAFLD subjects [43,44] on insulin resistance induction [45,46] should not be ignored.

A convincing report with a large cohort showed that PNPLA3 rs738409 and – more clearly – TM6SF2 rs58542926 conferred some protection against CAD, whereas MBOAT7 rs641738 had a neutral effect on it [47]. Other studies have also reported similar findings for TM6SF2 rs58542926 [10,48], whereas contradictory results have been presented about the association between PNPLA3 rs738409 and atherosclerosis [49-51]. Worth mentioning is also the Copenhagen Study, consisting a large cohort of the Danish general population, which showed no association between PNPLA3 rs738409 or TM6SF2 rs58542926 and CAD [52]. Although MBOAT7 rs641738 is shown to increase not only the risk of NAFLD but also its severity [35], there are no prior reports of how it affects overall, CVD- or liver-specific mortality. To our knowledge, this is also the first report of the neutral effect of MBOAT7 rs641738 on overall and CVD-specific mortality.

The core of all NAFLD management is individual risk stratification: those at the greatest risk of NAFLD complications need to be aggressively treated while others can be offered guidance on how to avoid future risk accumulation [53]. The present study suggests that the three above-mentioned gene polymorphisms do not predict increased overall mortality in a long-term follow-up among middle-aged individuals. The individuals with genetic predisposition to NAFLD may be considered to have a disease constrained to the liver without increased risk of CVDs or metabolic abnormalities [16,28]. The present study is in line with the earlier reports with respect to the role of MetS in deteriorating the prognosis in NAFLD [22,23]. Thus, the control of cardiometabolic risk factors

remains a cornerstone of NAFLD management. Indeed, a recently published cohort study of 18 million Europeans demonstrated that after adjustments for traditional CVD risk factors, NAFLD is not associated with an increased risk of CVDs [54].

We recognize that as a post-hoc study our study may in some parts lack sufficient power to show statistical significance of difference between the groups. For instance, with a larger number of study participants, TM6SF2 rs58542926 might have been shown to protect against CVD-related deaths, as suggested by some earlier studies [48,55], or the protective trend in overall mortality by the number of gene polymorphisms might have become statistically significant. In addition, the cohort included a number of hypertensive individuals. However, the prevalence of hypertension among the study participants (51%) was similar to the hypertension prevalence in Finnish community-based studies in the 1990s [56]. Thus, the OPERA cohort can be considered to be quite close to a community-based cohort. Finally, although ultrasound is often used as the first-line method in NAFLD assessment in the clinical practice, it is relatively insensitive and provides only qualitative assessment that does not allow a reliable detection of fibrosis and cirrhosis [1,57].

In summary, our study shows that metabolic syndrome, but not PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738, increases the risk of overall and cardiovascular-specific mortality among middle-aged individuals irrespective of the presence of NAFLD. However, more studies with larger study cohorts are needed to confirm the findings.

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Disclosure statement

The authors report no conflicts of interest.

	NAFLD+ n=249	NAFLD– n=709	Total cohort n=958	p-value
PNPLA3 rs738409 (homozygotes)	104/245, 42% (8%)	269/703, 38% (4%)	373/948, 39% (5%)	0.025
TM6SF2 rs58542926 (homozygotes)	45/243, 19% (2%)	74/694, 11% (0.3%)	119/937, 13% (1%)	0.002
MBOAT7 rs641738 (homozygotes)	158/244, 65% (19%)	443/691, 64% (13%)	601/935, 64% (15%)	0.044

Table 1. The prevalence of genetic polymorphisms. Successful genotyping for NAFLD+, n=243–245; for NAFLD-, n=691–703; for total cohort, n=935–948. The statistical significance of difference between NAFLD- and NAFLD+ was calculated by Chi-Square.

	NAFLD+, MetS+ (n=186)	NAFLD+, MetS- (n=63)	p-value	NAFLD-, MetS+ (n=164)	NAFLD-, MetS- (n=545)	p-value
age (years)	52 ± 6	51 ± 6	0.175	51 ± 6	51 ± 6	0.693
gender (female) n (%)	83 (45%)	22 (35%)	0.178	76 (46%)	327 (60%)	0.002
PNPLA3 rs738409	72 (39%)	32 (52%)	0.091	60 (37%)	209 (39%)	0.613
TM6SF2 rs58542926	30 (16%)	15 (25%)	0.158	15 (9%)	59 (11%)	0.472
MBOAT7 rs641738	114 (63%)	44 (71%)	0.236	110 (67%)	333 (63%)	0.304
Smoking (pack years)	11 ± 14	12 ± 14	0.563	12 ± 16	7 ± 11	<0.001
Diabetics, n (%)	62 (33%)	7 (11%)	0.001	17 (10%)	11 (2%)	<0.001
HOMA-IR	6.4 ± 5.1	2.7 ± 1.7	<0.001	3.2 ± 2.4	1.8 ± 1.3	<0.001
BMI (kg/m ²)	32.1 ± 4.6	28.2 ± 4.8	<0.001	29.9 ± 3.8	25.4 ± 3.3	<0.001
Systolic blood pressure (mmHg)	154 ± 20	151 ± 20	0.355	152 ± 21	144 ± 22	<0.001
Diastolic blood pressure (mmHg)	93 ± 10	91 ± 12	0.328	91 ± 11	86 ± 12	<0.001
LVMI (g/m ²)	139 ± 39	136 ± 37	0.629	137 ± 37	125 ± 36	0.001

GFR (ml/min)	83 ± 16	87 ± 15	0.090	83 ± 14	84 ± 16	0.579
ALT (U/L)	48 ± 27	39 ± 21	0.021	32 ± 25	24 ± 10	<0.001
GGT (U/L)	72 ± 97	46 ± 41	0.036	45 ± 39	30 ± 26	<0.001
LDL (mmol/L)	3.7 ± 1.0	3.4 ± 1.0	0.130	3.7 ± 0.9	3.4 ± 0.9	0.010
HDL (mmol/L)	1.1 ± 0.3	1.4 ± 0.4	<0.001	1.1 ± 0.3	1.5 ± 0.4	<0.001
Triglycerides (mmol/L)	2.3 ± 1.3	1.4 ± 0.5	<0.001	2.1 ± 1.2	1.1 ± 0.5	<0.001

Table 2. The baseline characteristics of study participants by the presence or absence of NAFLD and MetS. For subjects with NAFLD, n=243–249, except for LVMI (n=245). For subjects without NAFLD, n=703–709, except for LVMI (n=655), TM6SF2 rs58542926 (n=694) and MBOAT7 rs641738 (n=691). The categorical variables are presented as absolute count (with %) and continuous variables as mean values ± standard deviation. Abbreviations: ALT, Alanine Aminotransferase; BMI, Body Mass Index; GFR, Glomerulus Filtration Rate by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation; GGT, Gamma-Glutamyltransferase; HDL, High-Density Lipoprotein; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; LDL, Low-Density Lipoprotein; LVMI, Left Ventricular Mass Index; MetS, Metabolic Syndrome.

Gene variant	Non-alcoholic fatty liver disease	Number of risk allele carriers of all subjects	Death rate in subjects with risk allele	Death rate in subjects without risk allele	Hazard ratio (risk allele carrier vs. non-carrier)	CI95%	p-value
PNPLA3 rs738409	yes	104/245 (42%)	29/104 (28%)	40/141 (28%)	1.049	0.650-1.692	0.844
	no	269/703 (38%)	52/269 (19%)	81/434 (19%)	1.032	0.729-1.462	0.858
	combined	373/948 (39%)	81/373 (22%)	121/575 (21%)	1.050	0.792-1.391	0.736
TM6SF2 rs58542926	yes	45/243 (19%)	10/45 (22%)	58/198 (29%)	0.721	0.369-1.411	0.340
	no	74/694 (11%)	10/74 (14%)	121/620 (20%)	0.668	0.351-1.273	0.220
	combined	119/937 (13%)	20/119 (17%)	179/818 (22%)	0.741	0.467-1.177	0.204
MBOAT7 rs641738	yes	158/244 (65%)	43/158 (27%)	26/86 (30%)	0.885	0.543-1.439	0.621
	no	443/691 (64%)	76/443 (17%)	55/248 (22%)	0.758	0.536-1.073	0.118
	combined	601/935 (64%)	119/601 (20%)	81/334 (24%)	0.800	0.603-1.061	0.121

Table 3. The Hazard Ratios for overall mortality by the carriers of PNPLA3 rs738409, TM6SF2 rs58542926 or MBOAT7 rs641738 compared to the non-carriers of these gene variants. The Cox Hazards model was used.

Figure Legends

Fig. 1. Overall mortality by the presence of MetS and NAFLD. The Cox hazards model was used. MetS, Metabolic Syndrome; NAFLD, Non-Alcoholic Fatty Liver Disease.

Fig. 2. The Hazards Ratios for overall mortality by the number of gene polymorphisms (PNPLA3 rs738409, TM6SF2 rs58542926 and MBOAT7 rs641738). NAFLD, non-alcoholic fatty liver disease.

Fig. 3. CVD mortality by the presence or absence of PNPLA3 rs738409, TM6SF2 rs58542926, MBOAT7 rs641738 or MetS. The statistical significance of differences was measured using Chi-Square test. A. Subjects with NAFLD (n=243–245 for gene polymorphisms, n=249 for MetS), B. Subjects without NAFLD (n=691–703 for gene polymorphisms, n=709 for MetS) and C. Total cohort (n=935–948 for gene polymorphisms, n=958 for MetS). Abbreviation: CVD, cardiovascular disease.

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**Overall mortality
without Mets**

Hazard Ratios

**Overall mortality
with MetS**

10/63, 16% (NAFLD+)

NAFLD+

60/186, 32% (NAFLD+)

88/545, 16% (NAFLD-)

NAFLD-

45/164, 27% (NAFLD-)

98/608, 16% (total cohort)

Total cohort

105/350, 30% (total cohort)

Hazard Ratio with 95% confidence interval

The Hazard Ratios with 95% CI with MetS vs. without MetS were: 2.186 (1.118-4.271, $p=0.022$) and after adjustments, 2.054 (1.011-4.173, $p=0.046$) for subjects with NAFLD; 1.818 (1.269-2.604, $p=0.001$) and after adjustments, 1.616 (1.056-2.473, $p=0.027$) subjects without NAFLD; 2.005 (1.522-2.640, $p<0.001$) and after adjustments, 1.718 (1.231-2.399, $p=0.001$) for total cohort. The multiple adjustments were made for age, sex, BMI, smoking and the original study group.

The Hazard Ratios with 95% CI were 0.551 (0.296-1.025; p=0.060) for 1 risk genotype, 0.756 (0.408-1.400; p=0.374) for 2 risk genotypes and 0.485 (0.112-2.102; p=0.344) for 3 risk genotypes.

The Hazard Ratios with 95% CI were 0.703 (0.457-1.082; p=0.109) for 1 risk genotype, 0.739 (0.449-1.216; p=0.234) for 2 risk genotypes and 0.384 (0.092-1.607; p=0.190) for 3 risk genotypes.

The Hazard Ratios with 95% CI were 0.641 (0.451-0.912; p=0.013. After adjustments for age, sex, BMI, smoking and original study group, 0.686 (0.480-0.981; p=0.039) for 1 risk genotype, 0.774 (0.526-1.138; p=0.195) for 2 risk genotypes and 0.447 (0.161-1.240; p=0.083) for 3 risk genotypes.

A NAFLD+

B NAFLD-

C Total cohort

	Subjects with MetS (n=350)	Subjects without MetS (n=608)	p-value
BMI (kg/m ²)	31.1 ± 4.4	25.7 ± 3.5	<0.001
Smoking (pack years)	11 ± 15	7 ± 12	<0.001
HOMA (Ummol/L ²)	4.9 ± 4.4	1.9 ± 1.4	<0.001
Systolic blood pressure (mmHg)	153 ± 20	144 ± 22	<0.001
Diastolic blood pressure (mmHg)	92 ± 10	87 ± 13	<0.001
ALT (U/L)	41 ± 27	25 ± 13	<0.001
GGT (U/L)	60 ± 76	32 ± 28	<0.001
LVMI (g/m ²)	138 ± 38	126 ± 37	<0.001
LDL cholesterol (mmol/L)	3.7 ± 0.9	3.4 ± 0.9	0.001
HDL cholesterol (mmol/L)	1.1 ± 0.3	1.5 ± 0.4	<0.001
Triglycerides (mmol/L)	2.2 ± 1.3	1.2 ± 0.5	<0.001

Supplementary Table 1. The variables with statistically significant differences in the total cohort by the presence or absence of metabolic syndrome. The categorical variables are presented as absolute numbers (with %) and continuous variables as mean values ± standard deviation. Abbreviations: ALT, Alanine Aminotransferase; BMI, Body Mass Index; GGT, Gamma-Glutamyltransferase; HDL, High-Density Lipoprotein; HOMA, Homeostatic Model Assessment of

Insulin Resistance; LDL, Low-Density Lipoprotein; LVMI, Left Ventricular Mass Index; MetS, Metabolic Syndrome.

	PNPLA3 risk allele carrier (n=104)	PNPLA3 no risk allele carrier (n=141)	p- value	TM6SF2 risk allele carrier (n=45)	TM6SF2 no risk allele carrier (n=198)	p- value	MBOAT7 risk allele carrier (n=158)	MBOAT7 no risk allele carrier (n=86)	p- value
age (years)	53 ± 6	51 ± 6	0.088	51 ± 6	52 ± 6	0.075	52 ± 6	53 ± 5	0.212
gender (female) n (%)	45 (43%)	60 (43%)	0.911	22 (49%)	82 (41%)	0.360	67 (42%)	38 (44%)	0.788
Smoking (pack years)	11 ± 15	12 ± 14	0.677	10 ± 12	12 ± 15	0.365	10 ± 13	13 ± 16	0.173
MetS	72 (69%)	111 (79%)	0.091	30 (67%)	152 (77%)	0.158	114 (72%)	68 (79%)	0.236
Diabetics, n (%)	27 (26%)	41 (29%)	0.590	12 (27%)	55 (28%)	0.880	39 (25%)	28 (33%)	0.188
HOMA (Ummol/(L ²))	5.3 ± 5.6	5.6 ± 4.1	0.725	6.2 ± 5.5	5.3 ± 4.6	0.297	5.3 ± 4.5	5.8 ± 5.4	0.360
BMI (kg/m ²)	30.4 ± 4.7	31.8 ± 5.0	0.027	31.6 ± 5.6	31.2 ± 4.8	0.670	31.3 ± 5.1	31.0 ± 4.7	0.655
Systolic blood pressure (mmHg)	153 ± 21	153 ± 20	0.943	154 ± 23	153 ± 20	0.668	153 ± 20	153 ± 21	0.978
Diastolic	92 ± 11	92 ± 10	0.802	93 ± 12	92 ± 10	0.570	93 ± 11	91 ± 10	0.412

blood pressure (mmHg)									
LVMI (g/m ²)	134 ± 40	141 ± 38	0.210	128 ± 36	141 ± 39	0.076	138 ± 37	138 ± 43	0.970
GFR (ml/min)	84 ± 15	84 ± 16	0.836	86 ± 16	84 ± 15	0.322	84 ± 16	84 ± 16	0.982
ALT (U/L)	48 ± 30	44 ± 23	0.198	44 ± 19	46 ± 27	0.641	47 ± 29	42 ± 19	0.115
GGT (U/L)	73 ± 102	60 ± 75	0.262	44 ± 27	67 ± 84	0.071	75 ± 106	48 ± 25	0.019
LDL (mmol/L)	3.5 ± 1.0	3.7 ± 1.0	0.242	3.4 ± 1.1	3.7 ± 0.9	0.052	3.5 ± 1.0	3.7 ± 1.0	0.217
HDL (mmol/L)	1.2 ± 0.3	1.2 ± 0.4	0.936	1.2 ± 0.3	1.2 ± 0.3	0.897	1.2 ± 0.3	1.2 ± 0.4	0.978
Triglycerides (mmol/L)	2.1 ± 1.3	2.1 ± 1.2	0.757	2.1 ± 1.4	2.1 ± 1.2	0.829	2.1 ± 1.2	2.1 ± 1.4	0.920

Supplementary Table 2. The baseline characteristics of study participants with NAFLD by the presence or absence of each gene polymorphism. For PNPLA3 rs738409, n=242–245 (for LVMI, n=210); TM6SF2 rs58542926, n=240–243 (for LVMI, n=210); MBOAT7 rs641738, n=241–244 (for LVMI, n=209). The categorical variables are presented as absolute numbers (with %) and continuous variables as mean values ± standard deviation. Abbreviations: ALT, Alanine Aminotransferase; BMI, Body Mass Index; GFR, Glomerulus Filtration Rate by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation; GGT, Gamma-Glutamyltransferase; HDL, High-Density Lipoprotein; HOMA, Homeostatic Model Assessment of Insulin Resistance; LVMI, Left Ventricular Mass Index; MetS, Metabolic Syndrome.

	PNPLA3 risk allele carrier (n=269)	PNPLA3 no risk allele carrier (n=434)	p- value	TM6SF2 risk allele carrier (n=74)	TM6SF2 no risk allele carrier (n=620)	p- value	MBOAT7 risk allele carrier (n=443)	MBOAT7 no risk allele carrier (n=248)	p- value
age (years)	51 ± 6	51 ± 6	0.271	51 ± 5	51 ± 6	0.751	51 ± 6	52 ± 6	0.143
gender (female) n (%)	156 (58%)	246 (57%)	0.397	41 (55%)	358 (58%)	0.701	259 (58%)	140 (56%)	0.607
Smoking (pack years)	8 ± 12	8 ± 13	0.650	7 ± 13	8 ± 13	0.431	8 ± 13	8 ± 13	0.904
MetS	60 (22%)	104 (24%)	0.613	15 (20%)	149 (24%)	0.472	110 (25%)	53 (21%)	0.304
Diabetics, n (%)	9 (3%)	19 (4%)	0.319	2 (3%)	26 (4%)	0.538	15 (3%)	12 (5%)	0.344
HOMA (Ummol/(L ²))	2.1 ± 2.0	2.2 ± 1.6	0.418	1.9 ± 1.1	2.2 ± 1.8	0.314	2.1 ± 1.7	2.2 ± 1.9	0.509
BMI (kg/m ²)	26.3 ± 3.9	26.6 ± 3.9	0.342	26.8 ± 4.0	26.4 ± 3.9	0.491	26.6 ± 3.9	26.2 ± 3.8	0.217
Systolic blood pressure (mmHg)	143 ± 21	147 ± 22	0.033	145 ± 21	146 ± 22	0.650	145 ± 22	147 ± 23	0.164
Diastolic	88 ± 12	87 ± 13	0.345	87 ± 14	87 ± 12	0.760	87 ± 12	88 ± 13	0.399

blood pressure (mmHg)									
LVMI (g/m ²)	126 ± 38	128 ± 36	0.490	130 ± 33	127 ± 37	0.499	126 ± 36	129 ± 38	0.431
GFR-Epi (ml/min)	85 ± 15	83 ± 15	0.265	85 ± 16	84 ± 15	0.467	84 ± 15	84 ± 15	0.990
ALT (U/L)	26 ± 12	26 ± 17	0.619	24 ± 9	26 ± 16	0.354	27 ± 17	24 ± 11	0.058
GGT (U/L)	34 ± 35	33 ± 26	0.586	34 ± 30	34 ± 30	0.913	34 ± 29	33 ± 31	0.532
LDL (mmol/L)	3.5 ± 1.0	3.5 ± 0.9	0.592	3.3 ± 0.8	3.5 ± 0.9	0.068	3.5 ± 0.9	3.5 ± 1.0	0.738
HDL (mmol/L)	1.44 ± 0.38	1.38 ± 0.37	0.033	1.4 ± 0.4	1.4 ± 0.4	0.448	1.4 ± 0.4	1.4 ± 0.4	0.703
Triglycerides (mmol/L)	1.3 ± 0.7	1.4 ± 0.9	0.397	1.4 ± 1.4	1.4 ± 0.7	0.441	1.4 ± 0.9	1.3 ± 0.6	0.096

Supplementary Table 3. The baseline characteristics of study participants without NAFLD by the presence or absence of each gene polymorphism. For PNPLA3 rs738409, n=701–703 (for LVMI, n=648); TM6SF2 rs58542926, n=692-694 (for LVMI, n=640); MBOAT7 rs641738, n=689–691 (for LVMI, n=637). The categorical variables are presented as absolute numbers (with %) and continuous variables as mean values ± standard deviation. Abbreviations: ALT, Alanine Aminotransferase; BMI, Body Mass Index; GFR, Glomerulus Filtration Rate by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation; GGT, Gamma-Glutamyltransferase; HDL, High-Density Lipoprotein; HOMA, Homeostatic Model Assessment of Insulin Resistance; LDL, Low-Density Lipoprotein; LVMI, Left Ventricular Mass Index; MetS, Metabolic Syndrome.

	Non- alcoholic fatty liver disease	CVD death rate in subjects with risk allele or MetS	CVD death rate in subjects without risk allele or MetS	p-value
PNPLA3 rs738409	yes (n=245)	11/104 (11%)	17/141 (12%)	0.932
	no (n=703)	14/269 (5%)	23/434 (5%)	0.972
	combined (n=948)	25/373 (7%)	40/575 (7%)	0.944
TM6SF2 rs58542926	yes (n=243)	3/45 (7%)	25/198 (13%)	0.518
	no (n=694)	1/74 (1%)	36/620 (6%)	0.196
	combined (n=937)	4/119 (3%)	61/818 (7%)	0.215
MBOAT7 rs641738	yes (n=244)	17/158 (11%)	11/86 (13%)	0.772
	no (n=691)	23/443 (5%)	14/248 (6%)	0.244
	combined (n=935)	40/601 (7%)	25/334 (7%)	0.219
MetS	yes (n=249)	27/186 (15%)	1/63 (2%)	0.013
	no (n=709)	15/164 (9%)	22/545 (4%)	0.004

	combined (n=958)	42/350 (12%)	23/608 (4%)	<0.001
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Supplementary Table 4. CVD mortality by the presence or absence of PNPLA3 rs738409, TM6SF2 rs58542926, MBOAT7 rs641738 or MetS. The statistical significance of differences was measured using Chi-Square test. Abbreviations: CVD, Cardiovascular Disease; MetS, Metabolic Syndrome.