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The Dual and Opposite Role of the *TM6SF2*-rs58542926 Variant in Protecting Against Cardiovascular Disease and Conferring Risk for Nonalcoholic Fatty Liver: A Meta-analysis

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The aim of this work was to estimate the strength of the effect of the *TM6SF2* E167K (rs58542926 C/T) variant on blood lipid traits and nonalcoholic fatty liver disease (NAFLD) across different populations. We performed a systematic review by a meta-analysis; literature searches identified 10 studies. The rs58542926 exerts a significant role in modulating lipid traits, including total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and NAFLD. However, this influence on lipids and NAFLD is opposite between genotypes in the dominant model of inheritance. Pooled estimates of random effects in 101,326 individuals showed that carriers of the minor T allele (EK+KK individuals), compared with subjects homozygous for the ancestral C allele (EE genotype), are protected from cardiovascular disease (CVD), showing lower levels of TC, LDL-C, and TG; the differences in mean ± standard error (mg/dL) are −8.38 ± 1.56, −3.7 ± 0.9, and −9.4 ± 2.1, respectively. The rs58542926 variant was not associated with high-density lipoprotein cholesterol in a large sample (n = 91,937). In contrast, carriers of the T allele showed a moderate effect on the risk of NAFLD (odds ratio: 2.13; 95% confidence interval: 1.36–3.30; *P* = 0.0009; n = 3273) and approximately ∼2.2% higher lipid fat content when compared with homozygous EE (n = 3,413). *Conclusions:* The rs58542926 appears to be an important modifier of blood lipid traits in different populations. As a challenge for personalized medicine, the C-allele, which has an overall frequency as high as 93%, is associated with higher blood lipids, whereas the T allele confers risk for NAFLD; in turn, CVD and NAFLD are strongly related outcomes. Although the variant confers protection against CVD at the expense of an increased risk of NAFLD, it does not explain the link between these two complex diseases. (HEPATOLOGY 2015;62:1742-1756)

Cardiovascular (CV) disease (CVD) is acknowledged as a leading cause of death globally, with an estimated population attributable fraction for mortality of approximately 40.6%.[1] A high proportion of CVD risk is attributed to circulating blood lipids,[2] which have a moderate heritability.[3] Moreover, individuals diagnosed with metabolic syndrome (MetS)—which cluster multiple CVD risk factors,

Abbreviations: CI, confidence intervalo; CV, cardiovascular; CVD, cardiovascular disease; GWAS, genome-wide association studies; HDL-C, high-density lipoprotein cholesterol; H-MRS, hydrogen magnetic resonance spectroscopy; HWE, Hardy-Weinberg equilibrium; LDL-C, low-density lipoprotein cholesterol; MAF, minor allele frequency; MetS, metabolic syndrome; MI, myocardial infarction; MS, mass spectrometry; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; SD, standard deviation; SE, standard error; SNP, single-nucleotide polymorphism; TC, total cholesterol; TG, triglycerides; US, ultrasonographic.

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including lipid traits, type 2 diabetes, and obesity—are at a significant risk of CV events and death.[4,5] Empirical evidence indicates that MetS more strongly predicts CV and coronary heart disease, as well as overall mortality, than its individual components.[5] Notably, nonalcoholic fatty liver disease (NAFLD), which is a chronic metabolic liver disease with global prevalence,[6] has long been associated with all the components of MetS[7] and CVD.[8-12] In fact, NAFLD is regarded as the hepatic manifestation of MetS.

Both CVD and NAFLD, as with many other complex disorders, are modulated by the interaction between the genetic component, which is polygenic, and environmental insults. The genetic component of lipid traits has been explored in many existing studies. More specifically, major advances have been made in recent years by genome-wide association studies (GWAS) that successfully identified more than 100 gene regions for plasma lipids, almost half of which also influence CVD.[13-16] Recent efforts to refine the known loci or find new genes influencing lipid traits have led to the simultaneous discovery of a nonsynonymous variant (rs58542926) located in *TM6SF2* (transmembrane 6 superfamily member 2) gene that was associated not only with blood lipid levels—including serum total cholesterol (TC),[17,18] low-density lipoprotein cholesterol (LDL-C),[17,19] and triglycerides (TG)[17,19]—but also myocardial infarction (MI) risk.[17] Remarkably, scientists from the Dallas Heart Study that employed an exome-wide approach to elucidate variants involved in liver fat accumulation also reported that rs58542926 is associated with fatty liver.[19]

Further replication candidate gene association studies showed that rs58542926 is associated with TC.[20-23] However, the association with LDL-C was not successfully replicated in several other studies.[20,21,23] Indeed, there are remarkable differences not only in the magnitude of the effect, but also the significance of the association, even in the larger GWAS. For instance, in the study conducted by Holmen et al., serum TG levels were significantly associated with the variant in the discovery stage ($P = 3.7 \times 10^{-7}$), but not in the replication stage ($P = 0.38$).[17]

On the other hand, whereas the rs58542926 variant seems not to influence high-density lipoprotein cholesterol (HDL-C) levels, the association was not uniformly explored in either major GWAS or in candidate gene association studies.

Likewise, association with NAFLD or liver fat content was replicated by some,[20,21,23-25] but not all studies.[22,24] In fact, in one of these investigations, the investigators reported an association with liver fibrosis, but not histological degree of steatosis.[24]

The rs58542926 C/T variant, located in chromosome 19:19268740, is a missense variant in which the nucleotide change from C to T in the forward strand results in an amino acid substitution from glutamate (E) to lysine (K) at position 167 of the protein (E167K, p.Glu167Lys). Functional *in vitro* and experimental studies showed that *TM6SF2* is a key regulator of liver fat metabolism with opposing effects on the secretion of TG-rich lipoproteins and hepatic lipid droplet content.[19,26] Results yielded by human studies indicated that the variant is associated with decreased gene and protein expression in the liver of affected patients.[21] Thus, the available evidence, when examined in conjunction, suggests that the variant might regulate liver transcript and protein expression in an allele-specific manner in an animal model,[19] as well as in humans.[21]

Surprisingly, rs58542926, which has a relatively low minor allele frequency (MAF) not only in the Caucasian population (0.09), but globally (0.07) (http://browser.1000genomes.org/), presents a clinical paradox, given that the C (Glu167) ancestral allele was shown to be associated with higher levels of blood lipids whereas the T allele (Lys167) was associated with NAFLD, as recently highlighted.[21]

In view of the background knowledge mentioned earlier, our primary aim was to examine the evidence provided in the available literature in order to estimate the strength of the effect of rs58542926 on both circulating lipid traits and NAFLD across different populations. In addition, we assessed whether associations are consistent across studies in magnitude and direction for all explored traits.

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Materials and Methods

Data Sources and Study Selection. In order to identify relevant published studies, electronic searches of PubMed at the National Library of Medicine (http://ncbi.nlm.nih.gov/entrez/query), EMBASE and the Science Citation Index databases, and Google Scholar were done for the query "TM6SF2" all fields and "rs58542926, gene or variants or polymorphism or alleles." Moreover, the sources cited in the retrieved articles were also checked and the PubMed link "related articles" was used to identify additional articles. The literature search included all pertinent studies (without any country restrictions) published until May 2015 for which an abstract or article was available for review. This strategy resulted in 20 sources, for which abstracts were first reviewed. The authors (S.S. and C.J.P.) conducted this initial assessment independently, either to determine the eligibility criteria or examine the appropriateness of the source with respect to the research issue. If these criteria were met, the article was retrieved in its entirety. There were no discrepancies in this process; more details on data collection can be found in Supporting Fig 1.

We followed the appropriate methods for conducting meta-analysis of genetic association studies, as stipulated in The Human Genome Epidemiology Network (HuGENet) guidelines (http://www.cdc.gov/genomics/hugenet/participate.htm).

Inclusion and Exclusion Criteria for Data Source Selection. Inclusion criteria for the meta-analysis were: candidate gene association studies, either population-based or hospital-based case-control, and GWAS concerning the relationship between the *TM6SF2* rs58542926 variant, and (1) blood lipid traits (TC, LDL-C, HDL-C, and TG) and (2) fatty liver or liver fat content, in which information about the number of subjects in each category was given, sufficient data to calculate outcomes were available, data were expressed in a meaningful manner (details are provided later), and genotyping was performed by a validated molecular method.

Exclusion criteria were: duplicate publications, redundant information on genotyped subjects included in more than one study, and unpublished articles. For lipid traits, studies were excluded if biochemical determinations were performed by nonstandard methods. In the case of NAFLD, secondary causes of steatosis, including alcohol abuse, hepatitis B and hepatitis C virus infection, and the use of drugs known to precipitate steatosis were also set as exclusion criteria.

Because the number of homozygous subjects for the T allele is either null in candidate association studies or small in the larger GWAS, we decided to compare the homozygous for the C (Glu167) allele (EE genotype) versus carriers of the T allele (Lys167), namely, heterozygous EK+ homozygous KK genotypes. This strategy followed the guidelines for reporting systematic review and meta-analysis of gene disease association studies (http://www.cdc.gov/genomics/hugenet/participate.htm). Furthermore, the reviewed literature revealed consensus among authors, indicating that the chosen model should express genotype risk in a meaningful way according to some biological background.[27] Thus, for each phenotype, we evaluated the association results stratified by ethnicity.

Data Collection. For each study, information concerning the following participant characteristics was collected: demographic information (age, sex, and ethnicity); lipid traits measured by any standard analytical method (we analyzed the variables after converting them to uniform units: (mg/dL), variables expressed as mean ± standard deviation (SD) (standard error [SE] was converted to SD); fatty liver disease as a disease trait evaluated either using hydrogen magnetic resonance spectroscopy (H-MRS), liver ultrasonographic (US) examination indicative of fatty infiltration, or liver biopsy; and liver fat content quantified by H-MRS or liver biopsy.

In GWAS studies,[17,19] the authors reported lipid values in mean mg/dL ± SE/SD; the meta-analysis was performed on the differences in means. Odds ratio (OR) for fatty liver was calculated by taking healthy control subjects as the reference group.

Data on explored phenotypes were extracted for EE and EK+KK genotypes, and analyses were based on comparing genotype groups without any further adjustment for confounding factors.

Statistical Analysis. Complete details are provided in the Supporting Information. All calculations were performed using the Comprehensive Meta-Analysis computer program (Biostat, Inc., Englewood, NJ).

Results

We evaluated 10 studies that were identified using the search strategy described in Supporting Fig 1. and met the previously noted inclusion criteria. Characteristics of studies included in the meta-analysis of lipid traits[17,19-22,28] (n = 6) are shown in Table 1, whereas those included in the meta-analysis of fatty liver or liver fat content[19,21-25,28,29] (n = 8) are summarized in Table 2. One study that reported lipid traits[23] was excluded

Table 1. Characteristics of the Studies on the Association Between the Nonsynonymous (p.Glu167Lys) rs58542926 C/T Variant of *TM6SF2* and Blood Lipids Traits (Total Sample Size: 101,326 Individuals)

First Author, Year, Ref.	Population Ethnicity (Country)	Study Design and Sample Size (N)	Features and Subjects' Characteristics	Genotyping Method and HWE*	Comments, Concerns, and Bias	Lipid Trait	Age of the Subjects	Female (%)
Holmen, 2014[17]	Norwegian, Norway	Genome-wide coding variation to identify novel lipid genes Population based N = 10,309 N S1 = 5,643 N S2 = 4,666	S1: Nord-Trøndelag Health Study (the HUNT study): subjects diagnosed MI (primary phenotype) and healthy controls without CVD, collected during second survey S2: Tromsø Study: hospital-diagnosed MI collected with six surveys (Tromsø 4).	Illumina HumanExome Beadchip arrays Genotypes were tested and found to be in HWE.	Population stratification unlikely: in this study 100% Norwegian participants Variant passed genome-wide significance ($P < 5 \times 10^{-8}$). Biochemical determinations conducted on nonfasting blood samples in 16% of HUNT participants; 25% of Tromsø Study participants ≥4 hours fasting before blood draw Information on lipid-lowering medication was not available.	No. LDL-C (FE) No. TG No. TC	Adult HUNT study (≥20 years) Tromsø Study (≥25 years)	35
Dongiovanni, 2015[20]	European descendent; mixed: Italy, Finland, and Sweden	Candidate-gene association study Hospital-based N = 3,020 N HB = 1,201 (Italy and Finland) N MO = 1,819 (Sweden)	HB: Patients with NAFLD MO: SOS Study: Morbid obese subjects enrolled for bariatric surgery	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Population stratification untested by the authors	TC HDL-C TG LDL-C (FE)	Adult A: 42 ± 6 B: 49 ± 7 Children A: 10 ± 3 (n = 142)	A: 48 B: 70
Kozlitina A, 2014[19]	Mixed Hispanics/ African Americans/ European Americans USA	Exome-wide association study of liver fat content Population based N = 4,587 (Hispanics: N = 745 African Americans N = 2,365 European Americans N = 1,354)	DHS: Dallas Heart Study: individuals recruited for exploration of CV risk factors and fatty liver	Illumina Infinium HumanExome BeadChip (A) Genotypes were tested and filtered to be in HWE.	Population stratification was computed by ancestry markers. Variant passed genome-wide significance ($P < 5 \times 10^{-8}$).	LDL-C (FE) HDL-C TG	Adult 46 ± 11	57
Kozlitina B, C, 2014[19]	B: Dallas Biobank (European Americans) C: Danish population; Copenhagen study	Replication on results of exome-wide association study on liver fat content Population based	B: Preventive medicine at the Cooper Clinic in Dallas, Texas C: The Copenhagen General Population	Taqman assay (B and C) Genotypes were tested and found to be in HWE.	Population stratification unlikely: (B) 100% participants European Americans; (C) all	LDL-C (FE) HDL-C TG	B: 53 ± 11 C: 58 ± 10	B: 35 C: 55

Table 1. *Continued*

First Author, Year, Ref.	Population Ethnicity (Country)	Study Design and Sample Size (N)	Features and Subjects' Characteristics	Genotyping Method and HWE*	Comments, Concerns, and Bias	Lipid Trait	Age of the Subjects	Female (%)
	USA/Sweden	N B = 7,585 N C = 73,532	Study (CGPS) and the Copenhagen City Heart Study (CCHS)		participants were of Danish descent. C: Included individuals known to be on self-reported lipid-lowering medication			
Sookoian, 2015[21]	European descendent (Caucasian)*: Argentina	Candidate-gene association study Hospital based N = 361	Patients with NAFLD	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Population stratification tested and negative.	TC HDL-C LDL-C TG	Adult 50 ± 12	62
Wong, 2014[22]	Chinese: China	Candidate-gene association study Population-based N = 922	Subjects assessed for noninvasive screening of fatty liver and liver fibrosis	TaqMan 5′-nuclease assays Authors did not report HWE testing, but our calculations show that all genotype frequencies were in HWE.	Population stratification untested but unlikely given the subjects were restricted to Chinese	TC HDL-C LDL-C	Adult 46 ± 10	58
Grandone, 2015[28]	Italy	Candidate-gene association study Hospital-based N = 1,010	Obese children >95th percentile recruited to explore the association between lipid traits and hepatic steatosis	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Population stratification untested by the authors	TC, LDL-G, TG, HDL-C	Pediatric 4-16 years	50

Lipid traits measured by enzymatic colorimetric method.

*The authors reported lipid values in mg/dL ± SE; the meta-analysis was performed on the differences in means.

Abbreviations: FE, estimated using the Friedewald equation; S1, stage 1; S2, stage 2; NA, not available.

Table 2. Characteristics of the Studies on the Association Between the Nonsynonymous (p.Glu167Lys) rs58542926 C/T Variant of *TM6SF2* and NAFLD (Total Sample Size: 5,537 Individuals)

First Author, Year, Ref.	Population Ethnicity (Country)	Study Design and Sample Size (N)	Genotyping Method and HWE	Comments, Concerns, and Bias	Features and Subjects Characteristics	Liver Biopsy (N)	Age of the Subjects (Mean ± SD, Years)	Female Sex (%)
Kozlitina A, 2014[19]	GWASmixed* (Hispanics/African Americans/European Americans) USA	Population-based study on hepatic lipid content N = 2,733	Illumina Infinium HumanExome BeadChip Genotypes were tested and found to be in HWE.	Each participant completed a detailed staff-administered survey, including questions about medical history and medication use (alcohol intake might be misclassified). Population stratification was computed ancestry markers. Variant passed genome-wide significance ($P < 5 \times 10^{-8}$).	Hepatic steatosis measured by H-MRS	NA	Adult A: 46 ± 11	57
Sookoian, 2015[21]	Candidate-gene association study European descendent (Caucasian)* Argentina	Hospital-based case control on NAFLD N = 361 Controls N = 132 Cases N = 226	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Population stratification tested and negative Controls selected from the same source of cases	Hepatic steatosis measured by liver US in controls and LB in cases	226	Adult 50 ± 12	62
Zhou, 2015[23]	Candidate-gene association study Finnish (Finland)	Hospital-based based on metabolic studies for lipidomic analysis N = 300	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Population stratification untested but unlikely given the subjects were restricted to Finninsh Controls selected from the same source of cases	Hepatic steatosis measured by H-MRS	NA	Adult 48 ± 1	37
Wong, 2014[22]	Chinese (China)	Candidate-gene association study Population based N = 922	Subjects assessed for noninvasive screening of fatty liver and liver fibrosis	TaqMan 5′-nuclease assays Authors did not report HWE testing, but our calculations show that all genotype frequencies were in HWE.	Population stratification untested but unlikely given the subjects were restricted to Chinese.	NA	Adult 46 ± 10	58
Wang 2015[25]	Candidate-gene association study Chinese (China)	Population based N = 733	TaqMan 5′-nuclease assays Authors did not report HWE testing, but our calculations show that all genotype frequencies were in HWE among controls.	Population stratification untested but unlikely given the subjects were restricted to Finnish Controls selected from the same source of cases	Hepatic steatosis diagnosed by liver US	NA	Adult 45 ± 3	40

Table 2. *Continued*

First Author, Year, Ref.	Population Ethnicity (Country)	Study Design and Sample Size (N)	Genotyping Method and HWE	Comments, Concerns, and Bias	Features and Subjects Characteristics	Liver Biopsy (N)	Age of the Subjects (Mean ± SD, Years)	Female Sex (%)
Liu, 2014[24]	Candidate-gene association study European Caucasian (United Kingdom) (Discovery cohort)	Mixed: population based (controls: data from 1000 Genomes and cases: hospital based). N = 728 Controls: 349 Cases: 379	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Controls are composed of genotyping from the 1000 Genomes, which has population stratification; therefore, they may not be representative of the source population. Population stratification untested in cases but unlikely given the subjects were restricted to UK (discovery phase)	Controls: Health status is unknown; this could introduce bias. Patients: Hepatic steatosis diagnosed by LB	349	Adult (controls: unknown, cases: 51.5 ± 12)	Controls: Age for controls unknown; this could introduce bias. Cases: 42
Grandone, 2015[28]	Candidate-gene association study (Italy)	Hospital based N = 532	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Fatty liver assessed by US in a subsample randomly selected from the entire cohort (n = 1,010)	Obese children, no control group	NA	Pediatric 4-16 (range) years	50
Mancina M, 2015[29]	Candidate-gene association study (Italy)	Hospital based N = 151	TaqMan 5′-nuclease assays Genotypes were tested and found to be in HWE.	Helsinki study: fatty liver assessed by H-MR. Milan study: fatty liver assessed by LB	Helsinki study: healthy subjects ofboth sexes recruited at Helsinki University Central Hospital	NA	Adult 46 ± 10	63
					Milan study: obese patients who underwent bariatric surgery	63	39 ± 8	66

*Self-reported ethnicity.

Abbreviations: LB, liver biopsy; NA, not available.

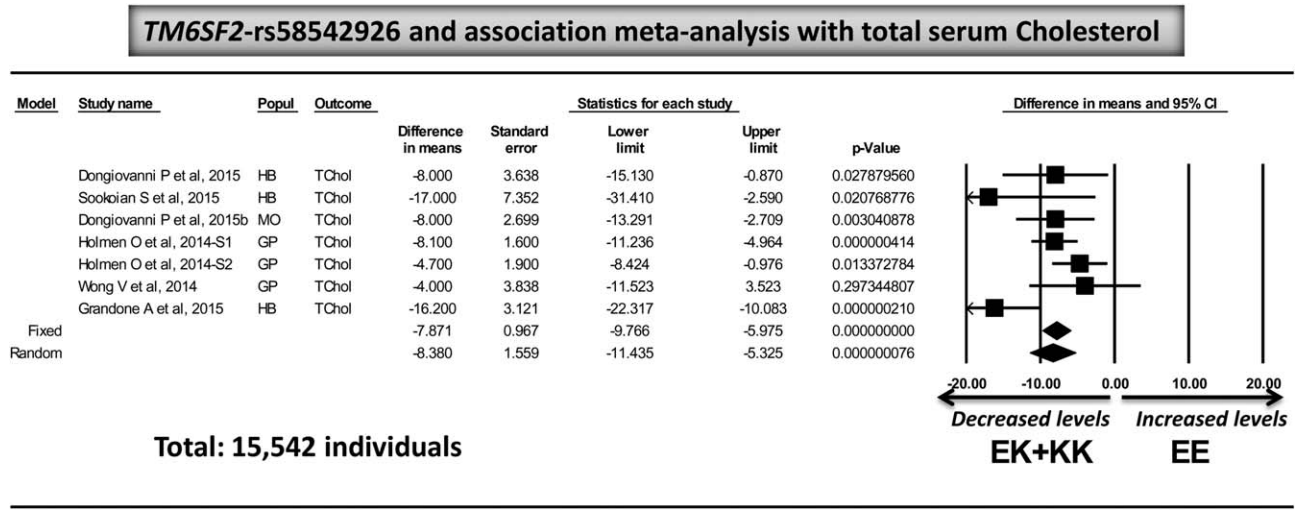

Fig. 1. Forest plot of rs58542926 variant (homozygous EE vs. EK+KK) and total cholesterol levels, and random and fixed effect models. The effect indicates the difference in means ± SE and the corresponding lower and upper limits.

General footnote: The first author of the study and the year of publication are shown after the citation name; Dongiovanni, 2015 HB: stands for hospital-based study on NAFLD; Dongiovanni, 2015 b: SOS Study (Swedish Obese Subjects); Kozlitina, 2014 a: Dallas Heart Study; Kozlitina, 2014 b: Dallas Biobank; Kozlitina, 2014 c: Danish population Copenhagen Study; Holmen, 2014 S1: Nord-Trøndelag Health Study (the HUNT study); Holmen, 2014 S2: Tromsø Study; Mancina, 2015 a: subjects recruited at Helsinki University Central Hospital; Mancina, 2015 b: obese patients from Milan.

Popul indicates design features; GP, general population; HB, hospital based; MO, morbid obese subjects. In the graph, filled squares stand for the effect of individual studies, and filled diamonds express combined fixed and random effects.

from the lipids meta-analysis because lipids were measured by a nonstandard method (mass spectrometry; MS) and lipid identification was based on an internal spectral library or on *de novo* identification using tandem MS. Because this approach differs significantly from those employed in the remaining studies, and the results were expressed as median (range), exclusion was fully justified. Another study that measured both lipid traits and liver fat content[29] was only included in NAFLD meta-analysis because data on circulating lipids were included in a previous study[20] and that might cause duplicate analysis. Finally, data regarding TC from a large GWAS on blood lipids[18] were excluded because the results were not expressed in a meaningful manner to perform uniform comparisons and our efforts to have access to the raw data were unsuccessful; this decision was made after contacting the authors.[18]

All the remaining studies scored well in terms of adequate descriptions of selection criteria and reference test, blind assessment of the reference test, and the availability of clinical data. Major concerns or putative bias that were detected in the studies are summarized in Tables 1 and 2.

Study Characteristics. Six studies included in the meta-analysis were hospital-based case-control[20,21,23,24,28,29] and the other four were population-based[17,19,22,25] studies. There were nine studies involving adults[17,19-25,29] and only one including children.[28]

Genotyping for rs58542926 was carried out using Taqman assay in all candidate association studies[20-25,28,29] and by specific Illumina arrays (Illumina, San Diego, CA)[17,19] (details are provided in Tables 1 and 2) in the remaining two GWAS studies.

Distribution of genotypes was in Hardy-Weinberg equilibrium (HWE), as stated by the authors; whenever this information was not clearly disclosed, calculations were performed according to the genotype frequencies reported in the article.[22,25]

Lipid Traits. Associations for blood lipid traits were extracted from six studies that disclosed extractable data about total TC,[17,20-22,28] LDL- C,[17,19-22,28] HDL-C,[19-22,28] and TG.[17,19-21,28] This analysis included 101,326 individuals of both sexes, as shown in Table 1. Mean values of blood lipid traits according to the genotype model are shown in Supporting Table 1.

TC was significantly associated with rs58542926 variant (fixed model: $P = 1 \times 10^{-9}$; random model: $P = 7.6 \times 10^{-8}$; Fig. 1) in five homogeneous studies ($P = 0.052$; I^2, 52), including 15,542 individuals, without evidence of publication bias ($P = 0.5$). The magnitude of the effect revealed a difference in means of -8.38 ± 1.559 mg/dL in the random model.

Meta-regression analysis did not reveal any correlation between the male proportion in the studied populations and the effect of rs58542926 variant on serum total TC (slope, 6.8; $P = 0.36$), suggesting that sexual

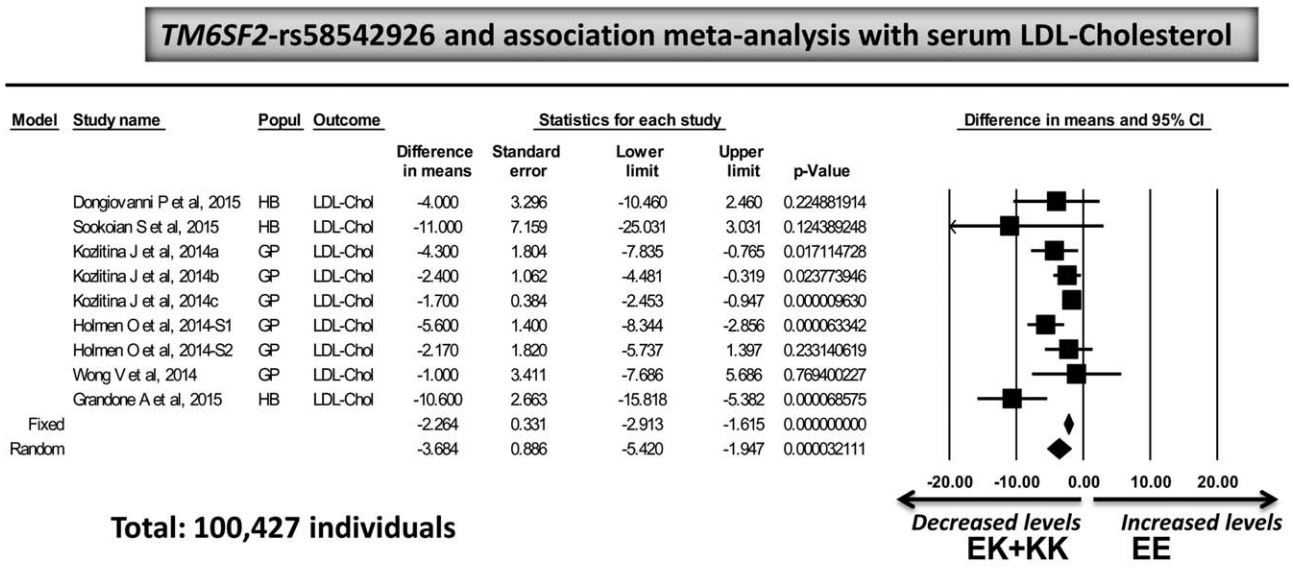

Fig. 2. Forest plot of rs58542926 variant (homozygous EE vs. EK+KK) and LDL-C levels and random and fixed effect models. The effect indicates the difference in means ± SE and the corresponding lower and upper limits.

dimorphism might not be involved in the effect of the single-nucleotide polymorphism (SNP) on this variable. Nevertheless, a significant correlation was observed between the effect of rs58542926 variant and ethnicity (Supporting Fig. 1A), suggesting that the influence of the variant would be stronger among Caucasian (slope, 1.95; $P = 0.02$).

Joined analysis of results from The Global Lipids Genetics Consortium that included 92,605 individuals[18] with data of present meta-analysis also showed significant results (n = 108,147; random model: $P = 6 \times 10^{-9}$); complete details are provided in Supporting Fig. 1B).

Likewise, although with a modest random effect (differences in means: -3.684 ± 0.886 mg/dL) LDL-C was significantly associated with the variant (fixed, $P = 1 \times 10^{-9}$; random, $P = 0.000032$; Fig. 2) in six heterogeneous studies ($P = 0.008$; I^2, 61.6), including 100,427 individuals, without evidence of publication bias ($P = 0.6$). The studies were stratified by age, and heterogeneity was successfully solved when the study including a pediatric population[28] was removed from the analysis ($P = 0.14$; I^2, 35.6); results on LDL-C stratified by ethnicity are shown in Supporting Fig. 2.

In contrast, no significant association was found for HDL-C in four homogeneous studies including 91,937 individuals (Supporting Fig. 3).

Finally, TG serum levels were significantly associated with rs58542926 variant (fixed, $P = 1 \times 10^{-9}$; random, $P = 0.0000095$; Fig. 3) in five heterogeneous studies ($P = 0.007$; I^2, 61.87), including 91,017 individuals; without evidence of publication bias ($P = 0.12$). To address potential sources of heterogeneity, the examined studies were stratified by ethnicity; however, the heterogeneity remained significant and was specifically restricted to Scandinavian ($P = 0.001$; I^2, 81.6), but not to Caucasian ($P = 0.129$; I^2, 33.6) population, as shown in Supporting Fig. 4 Likewise, heterogeneity still remained significant after stratifying the studies by age.

Nevertheless, the effect estimate seems to be robust (observed difference in means was between -8.133 and -10.436 mg/dL; $P = 0.000009$) because similar and significant results still remained after excluding one study at a time (Supporting Fig. 5.). In the random model, the estimated effect is even higher (differences in means ± SE: -9.429 ± 2.129 mg/dL).

Once again, metaregression analysis did not reveal any correlation between the male proportion in the studied populations and the effect of rs58542926 on serum TG (slope, 11.36; $P = 0.2$).

Fatty Liver Disease and Liver Fat Content. The analysis of fatty liver disease included 5,537 individuals. More specifically, pertinent data were extracted from five studies,[21,22,24,25,28] including 3,273 individuals, that reported genotype counts in cases of NAFLD (disease cases) and controls, or from four studies,[19,21,23,29] including 3413 subjects, that reported extractable information on liver fat content. The key characteristics of these studies are summarized in Table 2.

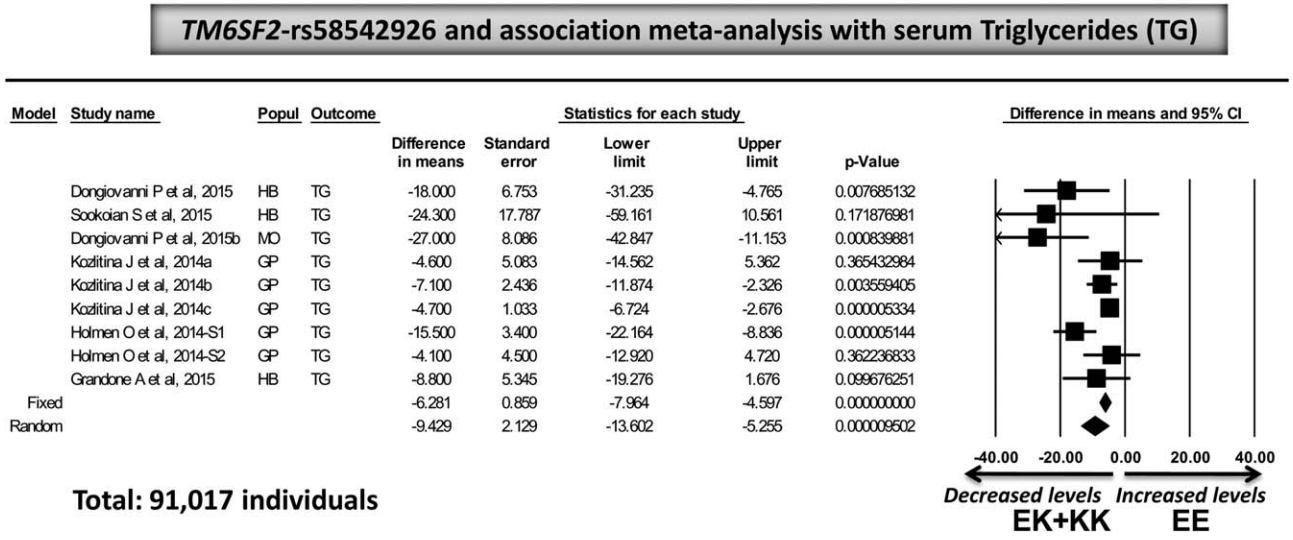

Fig. 3. Forest plot of rs58542926 variant (homozygous EE vs. EK+KK) and serum TG levels and random and fixed effect models. The effect indicates the difference in means ± SE and the corresponding lower and upper limits.

The rs58542926 variant was significantly associated with NAFLD in either the fixed ($P = 0.0000025$) or the random ($P = 0.0009$) model (Fig. 4) in five heterogeneous studies, indicating that carriers of the T allele show a moderate effect on the risk of NAFLD (OR, 2.13; 95% confidence interval [CI]: 1.36-3.30; $P = 0.0009$; n = 3273); heterogeneity among studies, as assessed by the Q statistic: $P = 0.004$; I^2, 73.6. The studies were stratified by age, and heterogeneity was successfully solved when the pediatric study[28] was removed from the analysis ($P = 0.296$; I^2, 18.95; Supporting Fig. 6. The results of the Begg and Mazumdar's rank correlation test ($P = 0.42$) indicated absence of publication bias.

Moreover, the comparison between cases and controls showed that carriers of the T allele, although with a modest effect, have a significant increase in liver fat content (quantitative trait) either in random effect or fixed models ($P = 1 \times 10^{-9}$; Fig. 4). Finally, neither heterogeneity, as assessed by the Q statistic ($P = 0.78$; I^2, 0), nor publication bias ($P = 0.115$), among studies could be observed. Results of liver fat content stratified by ethnicity are shown in Supporting Fig. 6.

Discussion

Two premises link CVD and NAFLD: the level of major blood lipids is strongly linked to CVD risk, and the liver plays a critical physiological role in the production and secretion of lipoproteins, cholesterol, and TG. Abnormalities in the kinetics of lipids between compartments, including packaging lipids efficiently—particularly in tissues involved in its metabolism, such as the liver—result in abnormal levels in circulation, thus posing an incremental risk of CVD. One of the better examples known to support this premise is familial hypercholesterolemia.

Summary of the Main Findings. In this study, we aimed to explore the role of a missense variant of *TM6SF2*, rs58542926—a gene the function of which was unknown until the past year—in the modulation of two highly related phenotypes: CVD and NAFLD. Of note, the results of this well-powered meta-analysis—by summarizing the amount of evidence, degree of replication, and absence of publication bias—show that the rs58542926 variant exerts a significant influence not only on the modulation of circulating lipid traits, but also liver fat accumulation. However, this influence is opposite between genotypes in the dominant model of inheritance. In fact, in carriers of the T allele, our results confirm an inverse effect relationship between the lower levels of blood lipids and the risk of developing NAFLD.

The results of pooled estimates showed that carriers of the minor T allele (EK+KK individuals), compared with subjects homozygous for the ancestral C allele (EE genotype), are protected from CVD, showing lower levels of TC, LDL-C, and TG. More specifically, the mean for TC is 8.38 ± 1.559, LDL-C is 3.684 ± 0.886, and TG is 9.429 ± 2.129 (SE, mg/dL) lower in EK+KK individuals compared with subjects carrying the EE genotype. This estimation was yielded by the random model and seems to be justified, owing to the variation

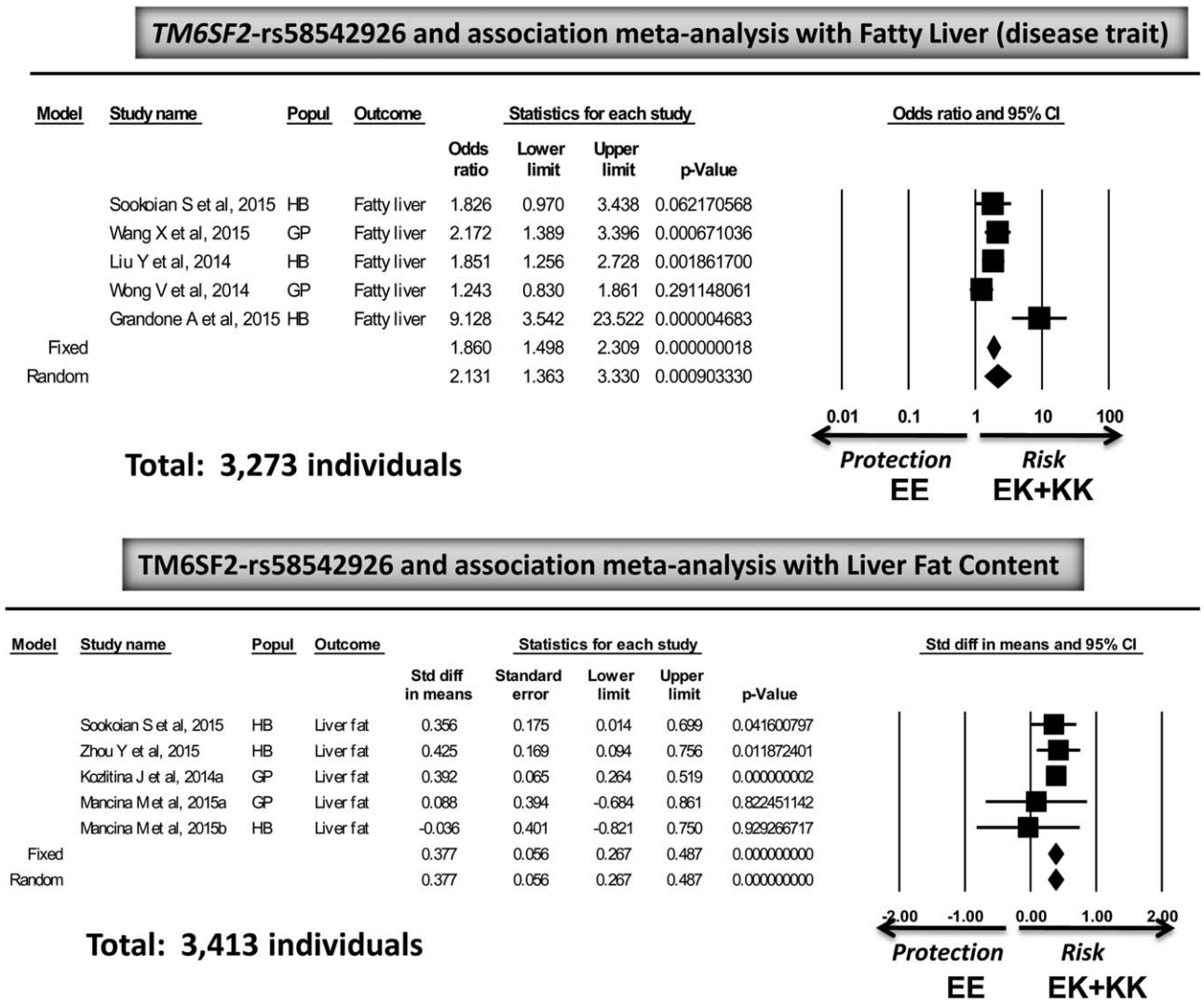

Fig. 4. Forest plot of rs58542925 variant and NAFLD. Upper: Forest plot of rs58542926 (homozygous EE vs. EK+KK) and fatty liver (as dichotomic variable) and random and fixed effect models. The effect indicates Odds Ratios and the corresponding 95% CI limits. Lower: Forest plot of rs58542926 (homozygous EE vs. EK+KK) and liver fat content (as continuous variable) and random and fixed effect models. The effect indicates the standardized mean difference (D) and the corresponding lower and upper limits.

of the effect according to ethnicities that may be, in part, a result of a different MAF among populations. Metaregression showed a lack of association between the effect of rs58542926 variant on lipid traits and male sex. Interestingly, rs58542926 was not associated with HDL-C in a large sample of pooled subjects (n = 91,937), suggesting that *TM6SF2* is not involved in the biology of this lipoprotein. This finding is reasonable, given that HDL-C particles are characterized by a process of synthesis, assembly, and trafficking that is different from LDL-C- and TG-carrying lipoproteins.

In contrast, carriers of the EK+KK genotype have 2.13-fold higher risk of developing NAFLD and show approximately 2.2% higher hepatic fat content when compared with carriers of the EE genotype. This effect was explored in a sample smaller than that employed for assessing lipid traits. Nonetheless, it was reproduced consistently across studies. Even assuming an additive model of inheritance for the rs58542926 variant, the estimated effect in homozygous KK subjects would be still small (~4.4%); unfortunately, this model could not be tested because information on homozygous for the T allele was not included in the original articles or the sample size was too small to be considered.

It is important to highlight that the effect of the rs58542926 variant on NAFLD is modest compared to the larger effect of *PNPLA3*-rs738409—so far, the major determinant of fatty liver disease susceptibility, accounting for approximately 5% of the disease variance. For instance, subjects homozygous for the risk G

allele of rs738409 have a 3.26-fold greater risk of developing NAFLD when compared with individuals homozygous for the wild-type allele.[30]

Limitations and Quality of the Evidence. On the whole, the results of this meta-analysis show no evidence of publication bias, and the overall quality and methodology of studies was high.

Potential flaws that might affect the results could be lack of fasting among participants of major GWAS[17] or inclusion of subjects known to be on lipid-lowering medication.[17,19] Nevertheless, it is unlikely that this would have impacted on the study findings, invalidating the magnitude or even the presence of the observed effect, given that previous reports showed that estimation of the risk of CVD associated with lipid levels is equally strong in subjects who did not fast as in those who did.[2] Another potential flaw in these studies could be the presence of population stratification. However, given that almost all studies controlled for this variable, as shown in Tables 1 and 2, this does not seem to be an issue.

A note of caution should be added, given that the presence of heterogeneity may potentially restrict the interpretation of the pooled risk estimates, in particular, concerning the association of the variant with LDL-C and TG. Heterogeneity in a meta-analysis is mostly produced by differences in study design and background characteristics of the subjects. However, the random effect model, where heterogeneity is no longer the main issue,[31,32] yielded a significant result pertaining to the association with TG. Although heterogeneity was addressed statistically by applying the random effect model, and was justified by genetic variability within the population examined, as already mentioned, we aimed to further investigate its potential sources where possible. Thus, the complete data set was utilized for the investigation of heterogeneity by sensitivity analysis. Although we could not identify any clear methodological discrepancy that could account for heterogeneity, the stratified analysis by ethnicity indicated that the major component of the heterogeneity might be attributed to studies that included Scandinavian subjects. Nevertheless, the consequent sensitivity analysis revealed that, across all the studies, the pooled risk estimates reflected similar tendencies, indicating that the effect estimate is robust. Finally, in the case of LDL-C and fatty liver, the heterogeneity was clearly explained by the study including obese children.[28]

Finally, because we did not have access to the raw data, the results (rs58542926 effects) presented here are slightly different from those in the original studies. In addition, one of the larger studies on blood lipids[17] calculated the variant effects' assuming an additive, but not a dominant, model; nevertheless, the difference in the estimated effect may be small given the low frequency of the KK genotype.

Unfortunately, results on TC from a large GWAS had to be excluded because the effect was not expressed in appropriate units to be included in the joined analysis[18]; nevertheless, the reported effect is in the same direction of the effect observed in our meta-analysis (Supporting Fig. 1B). To increase the quality of the summarized evidence, we strongly recommend the inclusion—either in candidate gene association studies or in GWAS—of all the data in standard format (complete record of all of the traits by genotypes and genotype counts in cases and controls).

Potential Public Health Impact and Other Implications of the Results. The primary analysis of our study was focused on the effect of the minor T allele that confers protection from CVD at the expense of an increased risk of NAFLD. This analysis, which is also the strategy of GWAS, is based on the allele frequency (the frequency at which the less abundant—or minor—allele is present in the population); minor alleles confer risk more often than protection,[33] and their effects are usually modest. Remarkably, the dual role in protecting against a disease, but conferring risk for another, is not only distinctive of rs58542926 variant, but also uncommon. Hence, the results of our meta-analysis could be also interpreted in light of the "ancestral susceptibility model,"[34] which assumes that the allele found in chimpanzee (or in our closely related species) is the ancestral one; the alternative allele is often regarded as a "derived allele."[34] In this model, homozygous EE might be at risk of CVD and could be protected from NAFLD and the opposite is true for the K carriers (Fig. 5). What might be the clinical implications of this model? If we consider the increase in the level of blood lipids observed in this meta-analysis, the EE genotype was associated with a relatively significant effect on blood levels of TC (approximately 4% above an upper limit of 200 mg/dL), LDL-C (approximately 2%-3% above an upper limit of 150 mg/dL), and TG (approximately 6.3% above an upper limit of 150 mg/dL) for a single variant influencing a polygenic trait. This effect on lipid traits is not negligible for a single variant compared with the rare and inherited forms of hypercholesterolemia, which are caused by a single mutation. For instance, the increase in TC levels for the familiar (inherited monogenic) hypercholesterolemia caused by a loss-of-function mutation in LDL-receptor gene (*LDLR*) or by a mutation in the gene encoding apolipoprotein B (*APOB*) is in the order of 103 and 54 mg/dL, respectively, with

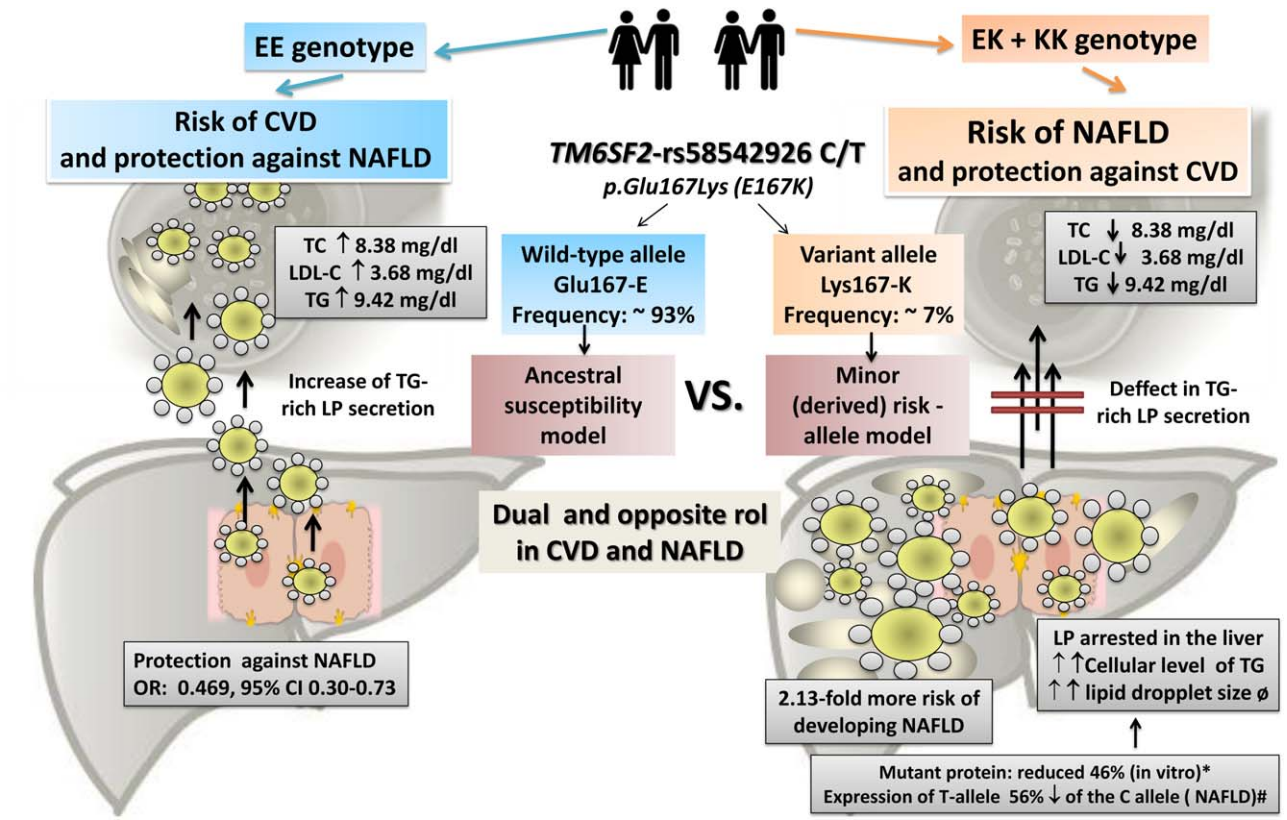

Fig. 5. Graphical summary picture on the clinical meaning of the *TM6SF2*-rs58542926 variant: the minor (derived)-risk allele model versus the ancestral-susceptibility model. The graph summarizes the main results of our meta-analysis on the dual and opposite role of *TM6SF2*-rs58542926 in protecting against CVD and conferring risk for NAFLD. In addition, the graph illustrates the paradigm of the putative effect of rs58542926 based on the ancestral-susceptibility model, which suggests that the increased level of blood lipids might be clinically relevant. The evidence from GWAS studies showed that ~43% the disease-associated alleles are, in fact, the ancestral alleles.[42] Changes in environment and lifestyle associated with modern societies would have shifted the role of the ancestral C allele from an "energy regulator" in times of unpredictable food supply to a nonadvantageous trait (increased blood lipid levels) in times of food overabundance and "atherogenic" diets. Hence, the ancestral allele would no longer confer a selective advantage, but would modulate a potentially detrimental phenotype in concert with other variants in different loci determined by the genetic architecture of this complex trait. In addition, the summary graph illustrates current knowledge on the role of TM6SF2 gene and protein function on lipid metabolism in the liver and its impact on the modulation of blood lipid traits. *Data extracted from reference 19, ** reference 17, ø reference 26, and # reference 21. Allelic frequencies were extracted from the 1000 Genomes database (http://browser.1000genomes.org/) and represent overall allele frequency. Abbreviations: CHD, coronary heart disease; LP, lipoprotein; TG-rich LP, triglyceride-rich lipoprotein.

around 30 mg/dL attributed to mutations in the *STAP1* gene.[35] Nevertheless, it remains unknown whether the increase in the level of blood lipids observed in carriers of the EE genotype can be translated in terms of CVD risk and also whether this change has any real impact on public health.

In addition, the observed increase of 9.6 mg/dL in TG levels associated with the EE genotype might have clinical decision-making implications. For example, (1) efficacy of currently used medications in lowering lipids and lipoproteins in patients with hypertriglyceridemia is in the range of 10%-20%, using standard doses,[36] and (2) TG lowering by pharmacological intervention is directly proportional to the baseline TG level.[38]

Moreover, results of mendelian randomization studies, which combine epidemiological data with genetics, have consistently shown the impact of gene variants that influence lipid traits on the predisposition to developing CVD, particularly MI.[38] In fact, these studies support a causal effect of blood lipids on CVD risk and show that minor changes in lipid parameters can become quite significant over time in vulnerable subjects.

Another important issue to consider is the relatively low (~7%) frequency of the protective T allele for CVD and the remarkable high frequency of the "major" C allele, which is as high as 93% in the population (based on the data sourced from 1000genomes.org/).

This issue suggests that, in order to develop personalized medicine, many challenges need to be overcome. The biological function of TM6SF2 gene/protein and the functional impact of rs58542926 on the modulation of lipid metabolism and lipoprotein kinetics between

compartments clearly support the observed outcomes. However, the presence of opposite clinical effects for an SNP depending on the presence of the variant or the wild-type allele on two highly related phenotypes (CVD and NAFLD) presents a dilemma for both patients and practitioners, who thus require pragmatic guidance of genetic risk prediction and sound evidence-based recommendations. At the same time, existing evidence suggests that, in order to improve both CVD and NAFLD outcomes, the synthesis of cholesterol and TG by the liver should be decreased. However, the data analyzed here indicate that TM6SF2 is probably not a good target because the design of a pharmacological agent for lowering blood lipids based on this protein to reduce the risk of MI, myocardial infarction may be probably successful but it would also increase the risk for NAFLD.

Implications for the Uunderstanding of CV and Liver Disease. The results yielded by this meta-analysis support the biological importance and plausibility of the role of *TM6SF2* on both CVD and NAFLD. *TM6SF2*-rs58542926 was initially mapped in a multigene locus (*NCAN/CILP2/PBX4/TM6SF2*), along with other variants in moderate linkage disequilibrium, for instance, rs2228603 in neurocan (*NCAN*) (pairwise r^2: 0.798; 1000genomes: phase_1_CEU) that initially masked the discovery of the casual variant associated with lipids- and liver metabolic-associated traits.[39] Given the lack of a putative biological involvement of neurocan in lipid biology and liver metabolism of lipoproteins, the variant in *NCAN* locus is now regarded as a spurious signal for association.

Furthermore, available mechanistic evidence relevant to the association showed that TM6SF2: (1) acts by promoting very-low-density lipoprotein secretion[19,26]; (2) influences hepatic lipid droplet metabolism by modulation of lipid droplet area and size (26), showing that inhibition of *TM6SF2* function leads to a marked increase in lipid droplet area[26]; and (3) is highly expressed in normal liver, but decreased in patients with NAFLD (reduced expression of liver TM6SF2 was associated with a high degree of steatosis).[21] Moreover, *in vitro* levels of mutant 167K protein were reduced by 46%.[19] Finally, allelic-specific expression analysis of complementary DNA isolated from human liver tissue of patients with NAFLD confirmed that expression levels of rs58542926-T are approximately 56% of that of the C allele.[21] Taken together, this evidence indicates that the variant seems to be associated with a decreased function. A summary of the biological and functional relevance of TM6SF2 gene/protein and E167K variant is depicted in Fig. 5, showing the paradox that a variant in a gene may induce both risk and protection for two related outcomes, such as CVD and NAFLD, making personalized medicine more challenging.

In conclusion, although *TM6SF2*-rs58542926 T allele confers protection against CVD at the expense of higher risk for NAFLD, it does not explain the link between these two complex diseases. In fact, the association between NAFLD and CVD includes not only the influence of a genetic background in a vulnerable subject, but also, among other factors, overabundance of proinflammatory mediators, endothelial dysfunction, and deregulation of mediators of atherogenesis.[9,11,40,41]

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