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SLC22A3 is associated with lipoprotein (a) concentration and cardiovascular disease in familial hypercholesterolemia

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ABSTRACT

Background and aims: Several clinical and genetic factors have been shown to modulate the cardiovascular risk in subjects affected by familial hypercholesterolemia (FH). Genome wide association studies (GWAS) in the general population have identified several single nucleotide polymorphisms (SNPs) significantly associated with the risk of cardiovascular disease (CVD). This include the rs2048327 variant in the *SLC22A3* gene. However, the effect of this SNP in FH subjects is unknown. The objectives of this study are to investigate the association between rs2048327 and the prevalence of CVD as well as with the concentration of lipoprotein (a) (Lp (a)), in a cohort of genetically-confirmed heterozygous FH patients.

Methods: An enzyme-linked immunoassay kit was used to assess the Lp (a) concentration, whereas an exome chip genotyping method was used to impute the rs2048327 genotype.

Results: The cohort comprised 287 non-carriers (TT), 305 heterozygous carriers (TC) and 76 homozygous carriers of the rs2048327 variant. In a model corrected for traditional cardiovascular risk factors, rs2048327 was significantly associated with Lp (a) level (median value of 12, 16 and 29 mg/dL in TT, TC and CC carriers, respectively, $p < .0001$). In a model corrected for cardiovascular risk factors and Lp(a) value, carrying the C allele was associated with a 2-fold increased risk of CVD (OR 1.96, 95%CI 1.21–3.19, $p = .007$).

Conclusions: In this study, we demonstrated that the rs2048327 SNP of the *SLC22A3* gene was significantly associated with Lp(a) as well as with CVD events in FH subjects. Further studies are required in order to investigate the mechanisms behind these associations.

1. Introduction

Cardiovascular disease (CVD) is the most-common cause of death worldwide, representing 31% of all global deaths [1]. The susceptibility of an individual to develop CVD depends on modifiable and non-modifiable risk factors such as age, sex, hypercholesterolemia, hypertension, diabetes, smoking, physical inactivity, diet, stress, abdominal obesity, as well as genetic [2]. Familial hypercholesterolemia (FH), a relatively frequent (1:250) autosomal codominant disease, is an example of a severe monogenic condition predisposing to CVD. Indeed, FH is associated with extremely high lifelong levels of low-density lipoprotein cholesterol (LDL-C) and therefore with a very high risk of premature atherosclerosis and CVD events. It has been estimated that as many as 5% of myocardial infarctions (MI) in patients under the age of 60 and 20% under the age of 45 are caused by FH [3]. However, despite the high CVD risk associated with this severe disease, numerous studies have shown that it is still possible to stratify the risk using clinical or genetic variables [4]. Several large genome wide association studies (GWAS) have identified rs2048327 in the solute carrier family 22 (*SLC22A3*) gene as being associated with lipid traits or with cardiovascular disease risk in the general population [5,6].

The *SLC22A3* gene in chromosome 6 codes for the organic cation

Abbreviation: BMI, Body mass index; CAD, coronary artery disease; CI, confidence interval; CVD, cardiovascular disease; EMT, extraneuronal monoamine transporter; FH, familial hypercholesterolemia; GWAS, genome wide association studies; HDL-C, high-density lipoprotein cholesterol; Hmz, homozygous; Htz, heterozygous; IRCM, Montreal Clinical Research Institute; LDL-C, low-density lipoprotein cholesterol; LDLR, LDL receptor; Lp(a), lipoprotein (a); MAF, minor allele frequency; MI, myocardial infarctions; OCT3, organic cation transporter 3; odds ratio, OR; SLC22A3, solute carrier family 22; SNPs, single nucleotide polymorphisms

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transporter 3 (OCT3) protein (originally known as extraneuronal monoamine transporter (EMT)). This transporter is expressed in various tissues and has been shown to mediate the bidirectional transport of numerous endogenous molecules, drugs and xenobiotics across the cell membrane [7]. The expression of OCT3 has been shown to be modulated by genetic variants as well as cholestasis, but would be independent of age and sex [8]. SNPs in the *SLC22A3* gene, including the rs2048327, have been associated with diverse conditions or lipid traits such as lipoprotein (a) (Lp(a)) concentration [9–11], LDL-C level [6], obsessive–compulsive disorder [12], prostate cancer [13] and colorectal cancer [14]. The association between SNPs in the *SLC22A3* gene and CVD has been investigated in several studies, but remains controversial. Indeed, some studies reported a significant association between the rs2048327 SNP in the *SLC22A3* gene alone or in combination with other polymorphisms in the *SLC22A3-LPAL2-LPA* gene cluster and CVD [9,15–17], whereas other authors did not [18–20]. However, to date, this association has never been investigated in FH patients.

The objectives of the present study are therefore to investigate the association between a SNP in the *SLC22A3* gene, the rs2048327, and the prevalence of CVD as well as the Lp(a) concentration in a cohort of genetically-confirmed heterozygous FH patients.

2. Materials and methods

2.1. Study population and data collection

We recruited a cohort of 668 genetically confirmed heterozygous adult FH patients from the lipid clinic of the Montreal Clinical Research Institute (IRCM). In short, among the 2654 patients with a Dutch Lipid Criteria score ≥ 3 (possible, probable or definite FH) in the retrospective IRCM database, 1267 underwent genetic testing. From these, 725 patients fulfilled the genetic diagnosis for FH. We excluded 55 patients under 18 years of age and 2 homozygous patients (See **Supplemental Fig. 1**). Medical records were reviewed to establish extensive phenotypic data at baseline (the first visit at the lipid clinic). All patients were Caucasian and of French-Canadian origin. DNA samples were thus screened for the presence of classical French-Canadian mutations in the LDL receptor (*LDLR*): deletion > 15 kb of the promoter and exon 1, deletion > 5 kb of exons 2 and 3, W66G (exon 3), E207K (exon 4), Y468X (exon 10) and C646Y (exon 14). CVD events were defined as follow: the presence of self-reported angina, myocardial infarction, coronary angioplasty, coronary bypass surgery, claudication, peripheral angioplasty, peripheral arterial surgery, transient ischemic attack, stroke, and carotid endarterectomy. All events were further validated by the physician by requesting copies of relevant tests and progress notes of emergency visits and hospitalizations. To determine untreated lipid levels, all patients were asked to stop their cholesterol lowering medication 4 weeks before their first visit at the lipid clinic. All patients gave written informed consent, approved by the IRCM ethics institutional review board. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. Molecular analysis

Blood samples were collected after a 12-h overnight fast. Lipid parameters were measured by standard laboratory techniques and performed at the laboratory of the IRCM lipid clinic. LDL-C was measured using the gold standard method (ultracentrifugation) [21]. Lipoprotein (a) concentration (total particle mass (mg/dL)) was measured using a commercial ELISA kit (Macra EIA Kit; Strategic Diagnostics Industries, Newark, NJ). The conversion factor to transform Lp(a) from mg/dL to g/L (SI unit) is 0.01. For *LDLR* mutation analysis, DNA was extracted from peripheral blood leukocytes according to standard protocols. The method for genotypes determination is described in details elsewhere [22].

The rs2048327 genotype in the *SLC22A3* gene was obtained via an exome chip genotyping method (Illumina HumanCoreExome-24 version 1-0A microarray) as previously described [23]. The rs2048327 SNP was imputed with an excellent imputation quality (0.998).

2.3. Statistical analyses

The IBM SPSS Statistics version 25 (IBM Corp, Armonk, NY) was used for statistical analysis. Depending on the nature of the data, results are presented either as mean +/− standard deviation, medians (interquartile range (Q1-Q3)) or n (percent). We used a cut off of 0.05 for statistical significance. All reported *p* values were 2-sided.

Two variables were abnormally distributed and were therefore log-transformed before statistical analysis (triglycerides and Lp(a)). Differences in continuous variables between the three groups were assessed by ANOVA, whereas a Chi2 was used for categorical variables. The Tukey test was used for multiple comparison testing. The strength of association between the rs2048327 and Lp(a) value was assessed by linear regression, whereas the association between this SNP and the prevalence of CVD events was determined by logistic regression. Dominant models (TT = 0, TC = 1 and CC = 1), additive models (TT = 0, TC = 1, CC = 2) and recessive models (TT = 0, TC = 0 and CC = 1) were tested. For the rs2048327 variant, T is the ancestral allele and C represents the minor allele (referring to the forward [positive] strand).

3. Results

3.1. Description of the study cohort according to the rs2048327 genotype and the CVD status

The subjects' characteristics according to the rs2048327 genotype are presented in Table 1. A total of 668 genetically-confirmed adult heterozygous FH patients are included in the present study. Most subjects (69%) carried a null *LDLR* mutation, whereas the rest of the cohort carried a defective *LDLR* mutation. All subjects were of French Canadian origin. Of those, 287 were non-carriers (TT), whereas 381 were carriers of the *SLC22A3* SNP (305 heterozygous carriers (TC) and 76 homozygous carriers (CC)). The minor allele frequency (MAF) for allele C was 0.34. Genotype distribution was in Hardy–Weinberg equilibrium ($p < .0001$). In brief, there was no statistical difference between the three genotype groups concerning all studied CVD risk factors (sex, age, hypertension, smoking, diabetes, body mass index (BMI), prior statin use, total cholesterol, triglycerides, LDL-C, high-density lipoprotein cholesterol (HDL−C), non-HDL-C, apolipoprotein B and type of *LDLR* mutation).

3.2. Lp(a) concentration according to the rs2048327 genotype

The median value of Lp(a) concentration (median (Q1-Q3) (g/L)) was 0.12 (0.05–0.28) in the non-carriers (TT), 0.16 (0.06–0.37) in the heterozygous carriers (TC) and 0.29 (0.10–0.60) in the homozygous carriers (CC) of the rs2048327. These values were significantly different between the three groups ($p < .0001$) and the significant differences were observed between TT and CC, as well as between TC and CC groups (Fig. 1). As presented in Table 2, all statistical models of regression (dominant, additive or recessive) were significant for the association between rs2048327 genotype and Lp(a) concentration, regardless if the model was uncorrected or corrected for all traditional cardiovascular risk factors.

3.3. CVD prevalence according to the rs2048327 genotype

According to a dominant model (carriers (TC and CC) vs non-carriers (TT)), the rs2048327 was significantly ($p < .05$) associated with the prevalence of CVD events, regardless if the model was uncorrected (OR 1.40), corrected for all traditional cardiovascular risk factors (OR

Table 1
Subjects' characteristics according to *SLC22 A3* genotype.

Variables	Rs2048327 genotype				
	Reference	Non-carriers	Htz carriers	Hmz carriers	*p* value
		TT	TC	CC	
		***N* = 287**	***N* = 305**	***N* = 76**	
Sex	Male (%)	127 (44%)	124 (41%)	36 (47%)	0.48
Age	(year)	41 ± 14	40 ± 14	38 ± 13	0.25
Hypertension	Yes (%)	48 (17%)	58 (19%)	17 (22%)	0.49
Smoking	Ever (%)	174 (61%)	207 (68%)	51 (67%)	0.16
Diabetes	Yes (%)	11 (4%)	9 (3%)	2 (3%)	0.79
BMI	(kg/m^2)	24.9 ± 4.7	25.1 ± 5.0	24.3 ± 3.8	0.39
Prior statin use	Yes (%)	213 (74%)	229 (75%)	56 (74%)	0.96
Total cholesterol	(mmol/L)	9.45 ± 1.77	9.35 ± 1.70	9.13 ± 1.52	0.36
	(mg/dL)	365 ± 68	362 ± 66	353 ± 59	
Triglycerides	(mmol/L)	1.39 (1.08–1.98)	1.47 (1.04–2.01)	1.39 (1.04–1.96)	0.54
	(mg/dL)	123 (96–175)	130 (92–178)	123 (92–174)	
LDL-C	(mmol/L)	7.37 ± 1.53	7.41 ± 1.49	7.25 ± 1.38	0.71
	(mg/dL)	285 ± 59	287 ± 58	280 ± 53	
HDL-C	(mmol/L)	1.04 ± 0.31	1.01 ± 0.30	1.04 ± 0.35	0.64
	(mg/dL)	40 ± 12	39 ± 12	40 ± 14	
Non-HDL-C	(mmol/L)	8.42 ± 1.80	8.39 ± 1.68	8.18 ± 1.50	0.56
	(mg/dL)	326 ± 70	324 ± 65	316 ± 58	
Apolipoprotein B	(g/L)	2.24 ± 0.49	2.26 ± 0.51	2.17 ± 0.44	0.46
Type of *LDLR* mutation	Null (%)	197 (69%)	213 (70%)	52 (68%)	0.94

Data for continuous normally distributed variables are expressed as mean ± standard deviation. Continuous logarithmic variables (triglycerides, lipoprotein (a)) are expressed as median (interquartile range). Categorical variables are expressed as frequency (n (%)).
BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; Hmz: homozygous; Htz: heterozygous; LDL-C, low-density lipoprotein cholesterol; LDLR: LDL receptor.

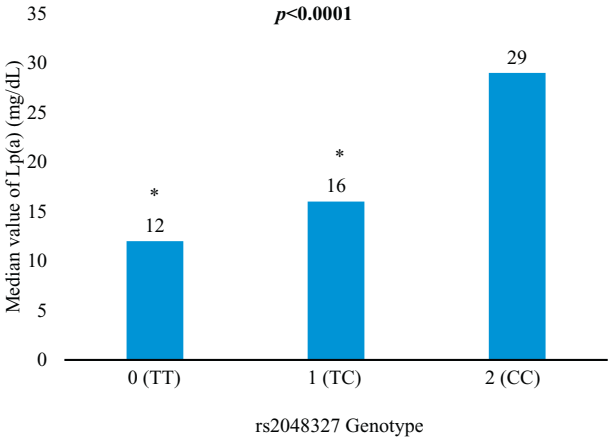

Fig. 1. Lp(a) according to the rs2048327 genotype. Bold type indicates *p* value < .05. *p* value for ANOVA between the three genotype groups. * Significantly different from the CC group. Lp(a): lipoprotein (a). 1.00 mg/dL of Lp(a) = 0.01 g/L.

1.95) or corrected for all traditional cardiovascular risk factors and Lp (a) concentration (OR 1.96). This association remained significant even when only the MI events were studied (**Supplemental Table 1**). In an additive model, the association between the rs2048327 and CVD events was significant only in the corrected models ($p < .05$). There was no significant association in recessive models. These results are presented in Table 2. The prevalence of CVD events in the non-carriers (TT), heterozygous carriers (TC) and homozygous carriers (CC) of the rs2048327 variant is 30%, 38% and 36%, respectively (Fig. 2).

4. Discussion

It has been estimated that around 20% of coronary artery disease (CAD) heritability would be explained by GWAS significant loci in the general population [24]. We now know that this is also true for FH cohorts, in which several SNPs have been shown to modulate the CVD risk [4].

In the present FH Caucasian cohort, the MAF of rs2048327 was 0.34, which is consistent with previous findings in the general population, in which authors reported a MAF between 0.34 and 0.37 in Caucasian populations [11,16,19].

This is the first study to investigate the association between a variant in the *SLC22A3* gene and the prevalence of CVD in a cohort of FH patients. We demonstrated that carrying one or two copies of the C allele of the rs2048327 SNP in the *SLC22A3* gene was significantly associated with a ~ 2-fold increased risk of prevalent CVD in this population. This association was stronger in the dominant model than in the additive model. In a large-scale association study, a small but significant association between the rs2048327 and CAD was reported, with a 9% increased risk for carriers of allele C (OR 1.09) [17]. In the pioneer genome-wide haplotype study of Trégouët et al., the authors showed that the combination of 4 SNPs in the *SLC22A3-LPAL2-LPA* gene cluster (that included the rs2048327) was strongly associated with CAD. However, when the rs2048327 was studied alone, the association with CAD did not reach statistical significance ($P = 3.14 \times 10^{-11}$) [15]. In this manuscript, the authors suggested that the effect of this locus on CAD risk would be mediated by an effect on Lp(a) level [15]. Indeed, in the GeneBank study, the TT genotype of rs2048327 was associated with a mean Lp(a) value of 24 mg/dL, whereas this value increases to 41 in TC carriers and 55 in CC carriers ($P = 6.2 \times 10^{-37}$) [9].

In our study, the presence of the risk allele C of the rs2048327 was also significantly associated with a higher Lp(a) level. Furthermore, carrying two C allele had a stronger effect on Lp(a) level than carrying a single C allele. Accordingly, the additive model was superior to the dominant or the recessive models for the association between the SNP and Lp(a) level. However, when the association between the rs2048327 and CVD was corrected for the Lp(a) level in addition to the other cardiovascular risk factors, the association with CVD remained significant.

Table 2
CVD prevalence and Lp(a) value according to the rs2048327 genotype of the *SLC22A3* gene.

Prediction of prevalent CVD	OR	95% CI	*p* value
CVD-dominant model			
Uncorrected	1.40	1.01–1.95	**0.04**
Corrected for all [a]	1.95	1.26–3.02	**0.003**
Corrected for all [a] + Lp(a)	1.96	1.21–3.19	**0.007**
CVD – additive model			
Uncorrected	1.22	0.96–1.55	0.10
Corrected for all [a]	1.52	1.11–2.09	**0.009**
Corrected for all [a] + Lp(a)	1.53	1.07–2.18	**0.02**
CVD – recessive model			
Uncorrected	1.06	0.65–1.75	0.81
Corrected for all [a]	1.30	0.68–2.49	0.43
Corrected for all [a] + Lp(a)	1.27	0.61–2.64	0.52

Prediction of Lp(a) value	β	*p* value
Lp(a) – dominant model		
Uncorrected	0.134	**0.001**
Corrected for all [a]	0.149	**0.001**
Lp(a) – additive model		
Uncorrected	0.183	< **0.0001**
Corrected for all [a]	0.205	< **0.0001**
Lp(a) – recessive model		
Uncorrected	0.175	< **0.0001**
Corrected for all [a]	0.199	< **0.0001**

Bold type indicates *p* value < .05.
CI: confidence interval; CVD: cardiovascular disease; Lp(a): lipoprotein (a); OR: odds ratio.
[a] All includes age, sex, body mass index, hypertension, diabetes, smoking, prior statin use, HDL-C, LDL-C and triglycerides.

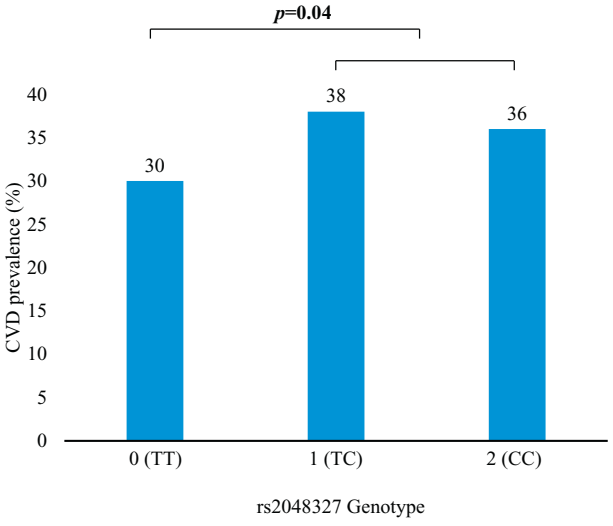

Fig. 2. CVD prevalence according to the rs2048327 genotype. *p* value for uncorrected analysis (dominant model). Bold type indicates *p* value < .05. CVD: cardiovascular disease.

Therefore, the impact of carrying the risk allele C on the CVD risk appears to be partly explained by the increase in Lp(a) level, but other mechanisms may contribute to the risk. The rs2048327 is an intronic SNP (non-coding section) and the exact mechanism by which this locus increase the CVD risk remains unknown. The present results suggest that the effect of this variant on CVD risk would be stronger in FH patients than in the general population in which the effect size of this SNP is relatively small.

The strengths of the present study include the genetic homogeneity of the cohort, where 100% of the FH subjects carried a classical French Canadian mutation, which drastically limits the number of FH-causing mutations found in our cohort. On the other hand, we can not generalize these results to patients carrying other FH-causing mutations in other genes (such as *APOB* or *PCSK9*). Furthermore, we acknowledge that the retrospective design of the study as well as the young age of the patients (40 years of age), which could have underestimated the effect of carrying the risk allele C on the CVD risk. However, the Mendelian randomisation nature of this study, where the effect of the SNP is present since birth, eliminates the possibility of a reverse causation and controls for confounding factors. Indeed, we observed no imbalance in the clinical characteristics among the three genotype groups.

5. Conclusions

In conclusion, this genetic association study is a replication of previously published findings on the association between rs2048327 SNP in the *SLC22A3* gene and CVD risk. The novelty of this study is the nature of the cohort, which comprised only genetically-confirmed FH patients. We demonstrated that carrying the risk allele C was associated with an increased Lp(a) level, as well as with an increased risk of cardiovascular disease in this FH cohort. Further studies are required to understand the mechanisms by which this intronic variant affects the CVD risk.

Conflict of interest

A.B. received research grants from Merck Frosst, Amgen, Sanofi, Astra Zeneca and the Fondation Leducq. He has participated in clinical research protocols from Pfizer, Regeneron Pharmaceuticals Inc., The Medecines Company, Amgen, Acasti Pharma Inc., Novartis, Sanofi,

Ionis Pharmaceuticals, Inc., Astra Zeneca, Akcea and Merck Frosst. He has served on advisory boards and received honoraria for symposia from Amgen, Akcea and Sanofi.

S.B. has participated in clinical research protocols from Akcea, The Medecines Company and Sanofi. She has served on advisory boards for Novo Nordisk, Merck Frosst, Valeant Pharmaceuticals, Eli Lilly and Amgen and received honoraria for symposia from Sanofi-aventis, Merck Frosst, Amgen, Akcea, Novo Nordisk, Valeant Pharmaceuticals and Boehringer Ingelheim.

M.P. has nothing to declare.

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Author contributions

The authors' contributions were as follows: All authors contributed to the discussion, analysis and interpretation of data and have reviewed the article for the intellectual content. M.P performed statistical analysis and has drafted the manuscript. All authors have approved the final article. A.B. had primary responsibility for final content.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.02.008.

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