Archives of Cardiovascular Disease (2019) 112, 124—134

Available online at ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com/en

CLINICAL RESEARCH

Common *p2y*$_{13}$ polymorphisms are associated with plasma inhibitory factor 1 and lipoprotein(a) concentrations, heart rate and body fat mass: The GENES study

Association entre des polymorphismes génétiques fréquents de p2y$_{13}$ *et les taux plasmatiques de l'inhibiteur IF1 et de lipoprotéine (a), la fréquence cardiaque et la masse grasse corporelle : résultats de l'étude GENES*

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Received 24 April 2018; accepted 10 September 2018
Available online 29 December 2018

Abbreviations: ADP:, adenosine diphosphate; ATP:, adenosine triphosphate; ATP:, aseadenosine triphosphatase; apoA-I:, apolipoprotein A-I; CAD:, coronary artery disease; DNA:, deoxyribonucleic acid; HDL:, high-density lipoprotein; HDL-C:, high-density lipoprotein cholesterol; IF1:, inhibitory factor 1; Lp(a):, lipoprotein(a); LpA-I:, lipoprotein A-I; Nt:, nucleotide; PCR:, polymerase chain reaction; RCT:, reverse cholesterol transport.

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https://doi.org/10.1016/j.acvd.2018.09.003

KEYWORDS
Coronary artery disease;
High density lipoprotein;
Lp(a);
LpA-I;
Purinergic receptor

Summary
Background. — The P2Y$_{13}$ purinergic receptor regulates hepatic high-density lipoprotein uptake and biliary sterol secretion; it acts downstream of the membrane ecto-F1-adenosine triphosphatase, which generates extracellular adenosine diphosphate that selectively activates P2Y$_{13}$, resulting in high-density lipoprotein endocytosis. Previous studies have shown that the serum concentration of the F1-adenosine triphosphatase inhibitor inhibitory factor 1 is negatively associated with cardiovascular risk.
Aim. — To evaluate whether *p2y$_{13}$* genetic variants affect cardiovascular risk.
Methods. — Direct sequencing of the *p2y$_{13}$* coding and flanking regions was performed in a subcohort of 168 men aged 45—74 years with stable coronary artery disease and 173 control subjects from the GENES study. The two most frequent mutations, rs3732757 and rs1466684, were genotyped in 767 patients with coronary artery disease and 789 control subjects, and their association with cardiovascular risk markers was analysed.
Results. — Carriers of the rs3732757 261 T and rs1466684 557G alleles represented 9% and 27.5% of the entire population, respectively. The allele frequencies were identical in patients with coronary artery disease and control subjects. The presence of 261 T was associated with higher concentrations of plasma lipoprotein A-I and inhibitory factor 1, increased fat mass and a lower heart rate. Moreover, the proportion of patients with coronary artery disease with a pejorative systolic ankle – brachial index was lower in carriers of the 261 T allele. In both populations, the 557G allele was associated with increased concentrations of lipoprotein(a), and an allele dose effect was observed.
Conclusions. — Two frequent *p2y13* variants are associated with specific bioclinical markers of cardiovascular risk. Although neither one of these variants appears to be related to the development of atherosclerotic disease, they may modulate the risk of additional cardiovascular complications.

MOTS CLÉS
Maladie de l'artère coronaire ;
Lipoprotéines de haute densité ;
Lipoprotéine (a) ;
Polymorphisme ;
récepteur purinergique

Résumé
Contexte. — Le récepteur purinergique P2Y$_{13}$ régule l'endocytose hépatique des HDL et la sécrétion des stérols biliaires. Il agit en aval de l'ecto-F1-ATPase membranaire dont l'activité génère de l'ADP extracellulaire qui stimule le récepteur P2Y$_{13}$ puis l'endocytose HDL. Des études antérieures ont montré que le taux sérique de l'inhibiteur de la F1-ATPase, IF1, est négativement associé au risque cardiovasculaire.
Objectif. — Évaluer si des variants génétiques de *p2y$_{13}$* affectent le risque cardiovasculaire.
Méthodes. — Le séquençage direct de la partie codante de *p2y$_{13}$* a été réalisé dans une sous-cohorte de 168 patients coronariens stables, âgés de 45 à 74 ans, avec une coronaropathie stable (CAD) et chez 173 sujets témoins issus de l'étude GENES. Les deux mutations les plus fréquentes, rs3732757 et rs1466684, ont été génotypées dans 767 patients coronariens et 789 témoins de la cohorte, et leur association avec des marqueurs de risque cardiovasculaire a été analysée.
Résultats. — Les porteurs des allèles rs3732757 261 T et rs1466684 557G représentent respectivement 9 % et 27,5 % de l'ensemble de la population. Les fréquences alléliques sont identiques chez les patients atteints de coronaropathie et les sujets témoins. La présence de 261 T est associée à des niveaux plasmatiques plus élevés de LpA-I et IF1, à une augmentation de la masse grasse et à une fréquence cardiaque abaissée. De plus, la proportion de patients atteints de coronaropathie avec un index bras – cheville péjoratif était plus faible chez les porteurs d'allèles 261 T. Dans les deux populations, l'allèle 557G était associé à des taux accrus de Lp (a), et un effet dose d'allèle a été observé.
Conclusions. — Deux fréquents variants de *p2y$_{13}$* sont associés à des marqueurs biocliniques spécifiques du risque cardiovasculaire. Bien qu'aucun de ces variants ne semble être liée au développement de la maladie athéromateuse, ils pourraient moduler le risque de complications cardiovasculaires supplémentaires.

Background

P2Y receptors are G protein-coupled receptors that are activated by extracellular nucleotides, such as adenosine diphosphate (ADP), adenosine triphosphate (ATP) and uridine triphosphate [1,2]. Among them, $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors are preferentially activated by ADP. $P2Y_1$ and $P2Y_{12}$ receptors mediate ADP-induced platelet aggregation [1]. The $P2Y_{13}$ receptor, while not present in blood platelets, is expressed in leukocytes and red blood cells, as well as in a variety of tissues, such as spleen, brain, liver, bone marrow, pancreas and placenta [3,4]. The *p2y*$_{13}$ gene is located on chromosome 3 (3q24), in close proximity to *p2y*$_{12}$, with which it shares 48% nucleotide sequence homology [3]. Like the $P2Y_{12}$ receptor, $P2Y_{13}$ is a $G\alpha i$-coupled receptor [4]; its roles have been investigated in various tissues and cell types. In red blood cells, $P2Y_{13}$ activation inhibits the release of extracellular ATP [5]. The $P2Y_{13}$ receptor appears to play a pivotal role in the differentiation of mesenchymal stem cells, enhancing an osteogenic phenotype while suppressing adipocyte differentiation [6]. In the liver, several studies have documented the role of the $P2Y_{13}$ receptor in stimulation of the uptake of high-density lipoprotein (HDL) particles and regulation of the last steps of reverse cholesterol transport (RCT) [7—9]. In this function, $P2Y_{13}$ acts in a coordinated sequence, downstream of the membrane ecto-F1-adenosine triphosphatase (ecto-F1-ATPase), an enzymatic complex analogous to mitochondrial ATP synthase, which, when present on the plasma membrane, generates extracellular ADP by ATP hydrolysis. Moreover, apolipoprotein A-I (apoA-I), the major protein component of HDL, binds with high affinity to ecto-F1-ATPase and stimulates its activity [10].

Various experimental approaches in *p2y*$_{13}$ knock-out animals, and the use of pharmacological agonists of the receptor, have consistently shown that $P2Y_{13}$ stimulates HDL uptake in liver, biliary sterol secretion and RCT [7,9,11—13]. Concordantly, in dyslipidaemic murine models, pharmacological activation of P2Y13 has been shown to lower atherosclerosis development, whereas $P2Y_{13}$ deficiency has been associated with greater atherosclerosis [12,14].

In the search for a biomarker for the F1-ATPase/$P2Y_{13}$ pathway that could be used for clinical investigation, we first demonstrated the presence, in human serum, of inhibitory factor 1 (IF1), the main regulator of F1-ATPase, and we evaluated its potential as a new marker of cardiovascular risk. In mitochondria, under hypoxic and acidic conditions, ATP synthase switches to ATP hydrolytic activity, and IF1 is then recruited to limit hydrolysis and thus preserve cellular ATP pools. Interestingly, IF1 has also been found in serum and measured using a specific immunoassay [15]. The IF1 concentration was found to be 20% lower in patients with coronary artery disease (CAD) compared with in matched control subjects. Subsequently, in a prospective study of patients with CAD with follow-up for 11 years, the IF1 serum concentration was found to be inversely related to mortality from cardiovascular issues and all causes [16,17]. Hence, serum IF1 appears to be inversely related to cardiovascular risk.

The P2Y13 receptor, as a downstream partner in this cascade, may be subject to genetic variations affecting its activity and/or expression. Thus, variants of the *p2y*$_{13}$ sequence ma be associated with altered cardiovascular risk. In a previous study, Amisten et al. reported that the $P2Y_{13}$ single-nucleotide polymorphism rs1466684, present in about 30% of the population, was not associated with myocardial infarction or classical cardiovascular risk factors [18]. In the present study, we have again investigated *p2y*$_{13}$ genetic variants, by direct sequencing of its two exons and adjacent non-coding regions, in the context of a large case-control study of CAD. Aside from the above-mentioned single-nucleotide polymorphism, we found a synonymous substitution present in 9% of the population. Associations were studied between these two major *p2y*$_{13}$ polymorphisms and an extended panel of clinical and biological markers related to cardiovascular risk and heart function.

Methods

Study sample

The Génétique et Environnement en Europe du Sud (GENES) study is a case-control study designed to assess the role of genetic, biological and environmental determinants in the occurrence of CAD. All of the participants signed an informed consent form, and the study protocol was approved by the local ethics committee (CCPPRB, Toulouse/Sud-Ouest, file #1-99-48). The study protocol was conducted according to the principles of the Declaration of Helsinki.

Briefly, as reported previously [19], the patients with CAD were men with stable CAD, aged 45—74 years, living in south-western France; they were recruited between 2001 and 2004 after admission to the Department of Cardiology of Toulouse University Hospital. Stable CAD was defined as a history of acute coronary syndrome, a history of coronary artery revascularization, documented myocardial ischaemia, stable angina or the presence at coronary angiography of a coronary stenosis of 50% or more. The control subjects were men aged 45—74 years of age selected from the general population using electoral rolls. Stratification into decadal age groups was employed to match the age distribution between the control subjects and the patients with CAD.

Data collection

As reported previously [19], the control subjects and the patients with CAD underwent a medical examination at the same health centre during the same period, including clinical and anthropometric measurements, and completed a health-related questionnaire. Blood was collected after an overnight fast; the blood sample collection was reported to the French Ministry of Research and the Regional Health Agency under number DC-2008-403 #1. Biological and biochemical variables were measured as described [15,19].

p2y$_{13}$ sequencing and genotyping

Genomic deoxyribonucleic acid (DNA) was isolated from ethylenediaminetetraacetic acid (EDTA)-treated blood samples using silica columns (NucleoSpin® Extract II; Macherey-Nagel, Düren, Germany). $P2RY_{13}$ is a 3.4 kb gene composed of two exons encoding 354 amino acids. Sequencing of

Table 1 Point mutations found in the coding sequence of *p2y$_{13}$*.

Nucleotide substitution and position (transcript allele change)	Protein: amino acid change and position	Reference
G/T Nt261 (ATC → ATA)	I → I (I80I)	rs3732757
A/G Nt277 (TTG → CTG)	L → L (L86L)	rs139399025
G/T Nt359 (GCT→GAT)	A → D (A113D)	rs144128158
A/G Nt557 (ATG → ACG)	M → T (M179 T)	rs1466684
T/C Nt889 (AGA → GGA)	R → G (R290G)	rs61736003

Nt: nucleotide. Analysis was carried out on 168 patients with coronary artery disease and 173 control subjects taken from the general population.

the coding sequence of P2RY$_{13}$ was first performed in a subpopulation of the GENES cohort (173 control subjects and 168 patients with CAD). The initial polymerase chain reaction (PCR) was performed using two primers flanking the two exons of the gene (forward primer, AGCAAATGTCCCAAAGGTCT; reverse primer, GGCCATTTGTATCCTGTTGC). Briefly, 5 μL of genomic DNA was used with 1 μL of each Invitrogen™ primer (10 μmol/L; ThermoFisher Scientific, Carlsbad, CA, USA), 0.25 μL of Applied Biosystems™ AmpliTaq® Gold (5 U/μL; ThermoFisher Scientific), 4 μL of magnesium chloride (25 μmol/L), 1 μL of deoxyribonucleotide triphosphate (Promega, Madison, WI, USA) and the addition of ultrapure water to 50 μL. After 40 amplification cycles, amplicons were purified on the NucleoSpin® Extract II columns according to the manufacturer's instructions. Sequencing was performed on an Applied Biosystems™ 3130 Genetic Analyzer (ThermoFisher Scientific) (GeT-Purpan Genomic Platform, Toulouse, France) in sense and antisense directions, by mixing 20—40 ng of genomic DNA, 3.2 μL of primers (1 μmol/L each; forward primers: AGCAAATGTCCCAAAGGTCT, CACCTGTGAATGAGGCAGAA, AGGGCTCATAGCCTTTGACA; reverse primers: TTGCATCACTGTGGTGTTCA, GGCCATTTGTATCCTGTTGC), 0.8 μL of Applied Biosystems™ BigDye® Terminator version 3.1 (ThermoFisher Scientific), 1.5 μL buffer and the addition of ultrapure water to 10 μL. Sequences were analysed using SeqScape® software, version 2.5 (ThermoFisher Scientific).

In a second phase, genotyping of rs3732757 (I80I) and rs1466684 (M179 T) was performed in 789 control subjects and 767 patients with CAD for whom complete data were available. Genotyping was carried out with 20 ng of genomic DNA, 7.5 μL of TaqMan® Universal PCR Master Mix (ThermoFisher Scientific), 0.375 μL of Applied Biosystems™ TaqMan® probes (ThermoFisher Scientific) and 2.125 μL of water. Reactions and readings were performed using a LightCycler® 480 (Roche Life Science, Indianapolis, IN, USA).

Statistical analyses

The data are presented as percentages for qualitative variables and means with standard deviations for quantitative variables. Allele frequencies were estimated using the gene-counting method, and an exact test was performed to identify departures from the Hardy-Weinberg equilibrium [20]. A general linear model approach to the two-way analysis of variance was used to test for differences between patients with CAD and control subjects, and between the different genotypes and their interactions for each clinical or biological variable analysed. The Shapiro-Wilks and Levene tests were used to test the normality of the distribution of the residuals and the homogeneity of the variances for each clinical and biological variable, respectively. When basic assumptions for analysis of variance were not satisfied, data were log transformed. Interactions between case-control status and genotype on levels of clinical or biological variables were tested by introducing a product term case-control status x genotype in the model (test of homogeneity of slopes). Because of the relatively small number of subjects with the TT genotype (*p2y$_{13}$*), heterozygotes and homozygotes for the T allele were pooled in these analyses. Analyses were two-tailed and $P < 0.05$ was considered to be significant.

Results

Mutations observed in the *p2y$_{13}$* sequence

Direct sequencing of the *p2y$_{13}$* coding and flanking regions was performed for 168 patients with CAD and 173 control subjects taken from the GENES study (Table 1 and Fig. 1). Three rare mutations were observed in the coding sequence, found in 1—3 subjects who were either patients with CAD or control subjects: rs139399025 (protein L86L, transmembrane domain II), rs144128158 (protein A113D, extracellular loop I) and rs61736003 (protein R290G, extracellular loop III); they were all in a heterozygous state and not associated with any particular phenotype. Two other mutations were found more frequently in the two populations: rs3732757, a G/T synonymous substitution at nucleotide (Nt) 261 (protein I80I, transmembrane domain II), and rs1466684, an A/G substitution at Nt557, resulting in a missense mutation (protein M179 T, transmembrane domain IV). It should be noted that the M179 T substitution, as numbered in the whole protein sequence, is identical to the M158 T mutation that has been reported, as numbered in the mature protein, after cleavage of the propeptide [18]. These two most frequent mutations were then genotyped for patients with CAD ($n = 754$ for Nt261 I80I; $n = 767$ for Nt557 M179 T) and the control subjects ($n = 789$) from the GENES cohort. We found that the minor allele frequencies were somewhat different from those reported in databases: 0.045 for the Nt261 T-allele (versus 0.06 in Ensembl Genom) and 0.152 for the Nt557

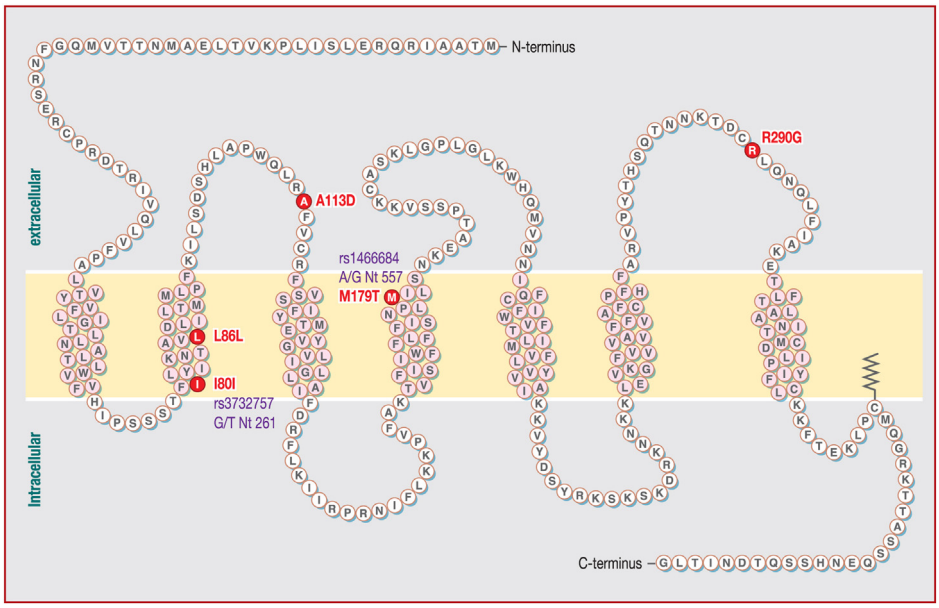

Figure 1. Location of the different mutations found in the coding sequence of $p2y_{13}$. The two main polymorphisms studied, rs3732757 and rs1466684, are highlighted by a yellow background.

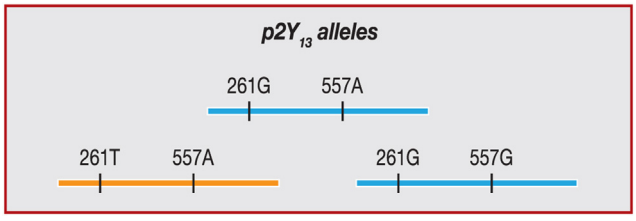

Figure 2. Common polymorphisms rs3732757 (Nt261) and rs1466684 (Nt557) in the $p2y_{13}$ coding sequence and associated alleles.

G-allele (versus 0.105 in Ensembl Genom). These frequencies were similar in the control subjects and the patients with CAD. Furthermore, the statistical analyses showed that the study population did not differ from the Hardy-Weinberg equilibrium for those two variations ($P = 0.13$ for the A557G mutation; $P = 0.52$ for the G261 T mutation). Surprisingly, the two substitutions appeared to be in strong linkage disequilibrium, as the Nt261 T minor allele was always found to be associated with the Nt557A major allele. Thus, these two polymorphisms defined three alleles (and not four)—261G/557A, 261 T/557A and 261G/557G—leading to six different genotypes (Fig. 2).

Characteristics of patients with CAD and control subjects according to $p2y_{13}$-G261 T genotype

As evidenced by the comparison between the patients with CAD and the control subjects (Table 2), patients with CAD displayed characteristics of metabolic syndrome, with an increased waist circumference, raised blood pressure, elevated glycaemia level and elevated insulin and triglyceride concentrations, but low concentrations of HDL markers: HDL cholesterol (HDL-C), apoA-I and lipoprotein A-I (LpA-I), a particular HDL subfraction containing apoA-I but not apoA-II. Concentrations of lipoprotein(a) (Lp[a]) and C-reactive protein were also found to be higher in patients with CAD than in control subjects, as was the proportion of fat mass in body composition. It should be noted that the concentrations of total cholesterol, low-density lipoprotein cholesterol and apolipoprotein B were found to be lower in patients with CAD than in control subjects, reflecting the large use of statins following a first coronary event. In our cohort, 58.4% of the patients with CAD were treated with statins versus 17.7% of control subjects. As reported previously [17], circulating concentrations of IF1, the main regulator of F1-ATPase, were found to be lower in patients with CAD than in control subjects.

Regarding the Nt261 genotype, the complete genotype was available for 754 patients with CAD and 789 control subjects. Carriers of the T-allele represented about 9% of the entire population. The presence of 261 T was associated with higher concentrations of LpA-I, and HDL-C followed a similar (although statistically non-significant) trend ($P = 0.09$). Plasma IF1 concentrations were higher in the carriers of 261 T, and their fat mass was increased (+5% relative to the 261G/G genotype). By contrast, the heart rate was found to be lower (−2.2 beats/min on average) in carriers of 261 T. It is noteworthy that these differences were observed in both populations: patients with CAD and control subjects taken from the general population. The association between Nt261 genotype and LpA-I concentrations remained significant after multiple adjustments for age, triglycerides and body mass index ($P = 0.031$). The association between the 261 T allele and IF1 remained statistically significant after multiple adjustments for age, heart rate and HDL-C ($P = 0.039$). Likewise, the association between Nt261 T and heart rate was not modified after adjustment for age, IF1 and HDL-C ($P = 0.041$). Regarding the association between Nt261 T and fat mass, this lost significance after adjustment for body mass index ($P = 0.066$), and even more so for

Table 2 Characteristics of patients with coronary artery disease and control subjects, according to the *p2y*$_{13}$ Nt261 genotype (rs3732757).

Nt261 genotype	Patients with CAD ($n = 754$)		Control subjects ($n = 789$)		P (patients/controls)[a]	P (genotype)[a]
	G/G ($n = 687$; 91.1%)	G/T and T/T ($n = 66$ and 1; 8.9%)	G/G ($n = 720$; 91.2%)	G/T and T/T ($n = 68$ and 1; 8.8%)		
Total cholesterol (g/L)	2.01 ± 0.44	1.95 ± 0.43	2.23 ± 0.38	2.32 ± 0.36	0.001	0.74
HDL-C (g/L)	0.43 ± 0.12	0.44 ± 015	0.55 ± 0.13	0.58 ± 0.15	0.001	0.09
LDL-C (g/L)	1.25 ± 0.39	1.18 ± 0.35	1.44 ± 0.33	1.50 ± 0.35	0.001	0.89
Triglycerides (g/L)[b]	1.70 ± 1.00	1.61 ± 0.88	1.20 ± 0.71	1.29 ± 1.14	0.001	0.71
ApoA-I (g/L)	1.24 ± 0.23	1.27 ± 0.24	1.52 ± 0.24	1.56 ± 0.28	0.001	0.12
LpA-I (g/L)	0.47 ± 0.15	0.49 ± 0.16	0.56 ± 0.17	0.60 ± 0.20	0.001	0.03
ApoB (g/L)	1.03 ± 0.25	1.00 ± 0.25	1.06 ± 0.22	1.10 ± 0.23	0.01	0.97
Lp(a) (g/L)[b]	0.46 ± 0.45	0.49 ± 0.39	0.29 ± 0.36	0.27 ± 0.40	0.001	0.79
IF1 (mg/L)	0.43 ± 0.13	0.47 ± 0.15	0.53 ± 0.15	0.56 ± 0.17	0.001	0.01
hs-CRP (mg/L)[b]	13.5 ± 23.0	11.8 ± 16.4	3.1 ± 6.1	3.2 ± 5.9	0.001	0.51
Glucose (mmol/L)	6.00 ± 2.14	5.75 ± 1.66	5.45 ± 1.11	5.30 ± 0.67	0.001	0.18
Insulin (IU/L)[b]	15.8 ± 19.9	19.6 ± 37.5	10.0 ± 7.0	10.1 ± 8.7	0.001	0.40
BMI (kg/m^2)	27.4 ± 3.9	27.5 ± 5.1	26.7 ± 3.3	27.0 ± 4.5	0.09	0.61
Waist (cm)	98.9 ± 10.6	99.9 ± 13.7	95.1 ± 9.5	96.3 ± 10.5	0.001	0.22
Fat mass (%)	27.9 ± 5.3	29.3 ± 5.9	26.1 ± 5.1	27.3 ± 5.2	0.001	0.02
SBP (mmHg)	139 ± 21	143 ± 21	137 ± 16	136 ± 15	0.003	0.32
Heart rate (beats/min)	64.2 ± 12.1	62.2 ± 9.0	63.7 ± 9.3	61.4 ± 9.7	0.47	0.03
Ankle – brachial index ≤ 0.9 (%)	34.7	22.4				0.05
LVEF < 50% (%)	27.9	17.9				0.09

Data are expressed as mean ± standard deviation or number. ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; IF1: inhibitory factor 1; LDL-C: low-density lipoprotein cholesterol; Lp(a): lipoprotein(a); LpA-I: lipoprotein A-I; SPB: systolic blood pressure; LVEF: left ventricular ejection fraction.

[a] Statistical significance was determined for the difference between patients with coronary artery disease and control subjects, and regarding the Nt261 genotype effect.

[b] Test done on log-transformed data.

waist circumference ($P = 0.210$), which is not surprising, as abdominal adiposity is highly correlated to fat mass.

Clinical explorations were carried out among the patients with CAD only, and the effects of the Nt261 genotype were assessed: the proportion of patients with an adverse systolic ankle – brachial index (≤ 0.9) was lower in carriers of the 261 T minor allele (22% versus 35% in the 261G/G genotype; $P < 0.05$). A similar (although statistically non-significant) trend was observed for the proportion of patients with a low (< 50%) left ventricular ejection fraction (18% versus 28%; $P = 0.09$). Lastly, no differences in angiographic severity scores were recorded based on the Nt261 genotype (data not shown).

The *p2y$_{13}$*-A557G polymorphism is associated with plasma LP(a) concentrations

Regarding the Nt557 genotype, 27.5% of patients with CAD and control subjects were found to be carriers of the 557G minor allele. Neither one of these populations diverged from the Hardy-Weinberg equilibrium for this variation. This polymorphism was found not to be associated with most of the clinical and biological variables investigated, except for Lp(a). The average Lp(a) concentration was 0.29 g/L in control subjects and 0.46 g/L in patients with CAD ($P = 0.001$; data not shown). In both populations, the presence of the 557G variation was associated with increased concentrations of Lp(a) ($P = 0.008$), and an allele dose effect was observed (Table 3). In the control subjects, 557G/G homozygosity was associated with a doubling of the Lp(a) concentration compared with the most frequent 557A/A genotype. Association of the 557G variant with elevated Lp(a) remained significant ($P = 0.014$) after adjustments for major serum lipids: total cholesterol, HDL-C and triglycerides. Because of its skewed distribution, Lp(a) was also analysed from both sides of a threshold value of 0.5 g/L, as higher concentrations are considered to confer an increased cardiovascular risk. In patients with CAD, an Lp(a) concentration > 0.5 g/L was found in 35% of carriers of 557A/A, 40% of carriers of 557A/G and 42% of carriers of 557G/G; among control subjects, the corresponding figures were 20%, 22% and 45% (not shown). The association of the Nt557 genotype with Lp(a) distribution was statistically significant ($P = 0.04$).

Because Nt261 and Nt557 are in strong linkage disequilibrium, defining three observed alleles (Fig. 2), analyses were resumed considering the six resulting genotypes (three homozygotes and three heterozygotes, not shown). However, this approach did not provide more information than considering the two substitutions separately, as described above.

Discussion

Over the last decade, studies from our group have demonstrated that $P2Y_{13}$, as an ADP receptor acting in coordination with membrane-bound F1-ATPase, is a key partner of RCT, promoting HDL hepatic uptake and secretion of bile sterols [7,9,11–13]. In this study, based on a large case-control study of CAD, we observed that common polymorphisms of the $P2Y_{13}$ gene are associated with selected bioclinical variables affecting cardiovascular risk.

About 9% of the subjects, from both of the populations, were carriers of the G261 T substitution. Although this is a synonymous mutation, it was associated with significant differences in biological and clinical features. It is therefore tempting to speculate that this variant might affect cellular expression of the $P2Y_{13}$ protein. Indeed, synonymous mutations may be associated with changes in messenger ribonucleic acid (mRNA) stability, resulting in changes in protein synthesis [21]. Although purely speculative at this stage, we hypothesize that there is lower expression of the protein encoded by the 261 T variant on the basis of two observations. First, this variation was associated with higher concentrations of LpA-I, a HDL subfraction containing apoA-I, but not apoA-II, predominant in large-sized HDL [22]. In the metabolic cycle of HDL, large HDL particles, following remodelling in the plasma compartment, are taken up by the liver, which initiates the last steps of RCT [23]. Increased LpA-I might thus reflect a delayed hepatic uptake of HDL particles, a process mediated by the F1-ATPase/$P2Y_{13}$ pathway. Second, it has been demonstrated that $P2Y_{13}$ directs the differentiation of mesenchymal stem cells into osteoblasts, to the detriment of the adipocyte lineage [6]. Concordantly, $P2Y_{13}$ deficiency favours the differentiation of adipocytes, the cellular expression of peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and an increase in bone fat mass [6]. Although these observations were made on bone marrow stem cells, the role of P2Y13 in mitigating the adipocyte differentiation programme might be a more general function of this receptor [24]. In the present study, we observed a 5% relative increase in body fat mass in carriers of 261 T, which might be concordant with lower expression of $P2Y_{13}$. In an attempt to monitor expression of the $P2Y_{13}$ protein as encoded by different gene variants, preliminary experiments were performed, by transfecting complementary DNA (cDNA) corresponding to the different allelic forms of $P2Y_{13}$ as defined above. Unfortunately, as a result of its targeting the proteasome, the $P2Y_{13}$ protein is expressed at very low levels [25], thus hampering comparison of $P2Y_{13}$ protein content.

In our two study populations, the 261 T variation was also found to be negatively associated with heart rate and positively associated with the plasma IF1 concentration. This observed relationship with IF1 concentrations lends further support to the notion that F1-ATPase and $P2Y_{13}$ act in close coordination; it suggests that a gene variant that appears to modulate expression of $P2Y_{13}$ exerts a feed-back effect on IF1, the main regulator of F1-ATPase, an upstream partner in this pathway. As suggested previously [16], serum IF1 might originate partly from cardiomyocyte mitochondria. Under hypoxic and acidic conditions, mitochondrial ATP synthase becomes an ATP hydrolytic enzyme, and IF1 is then recruited to the enzymatic complex, inhibiting its activity and preventing collapse of cellular ATP concentrations. Accordingly, it has been shown that hypoxia-inducible factor 1α can upregulate IF1 transcription [26]. Conversely, under normoxic conditions, mitochondrial ATP synthase would function to synthesize ATP, and IF1, not recruited to the complex, might be released into the extracellular medium. Thus, serum IF1 may partly reflect myocardial function. Interestingly, serum IF1 has been found to be correlated with heart rate and left ventricular ejection fraction in patients with coronary heart disease [16]. Large amounts of ATP (eg., in the

Table 3 Characteristics of patients with coronary artery disease and control subjects, according to the *p2y*$_{13}$ Nt557 genotype (rs1466684).

	Patients with CAD ($n=767$)			Control subjects ($n=789$)			P (patients/controls)[a]	P (genotype)[a]
Nt557 genotype	A/A ($n=557$; 72.6%)	A/G ($n=186$; 24.3%)	G/G ($n=24$; 3.1%)	A/A ($n=559$; 72.1%)	A/G ($n=200$; 25.4%)	G/G ($n=20$; 2.5%)		
Total cholesterol (g/L)	1.99 ± 0.42	2.05 ± 0.46	2.06 ± 0.60	2.25 ± 0.37	2.21 ± 0.40	2.37 ± 0.32	0.001	0.29
HDL-C (g/L)	0.43 ± 0.12	0.44 ± 0.13	0.44 ± 0.15	0.55 ± 0.13	0.56 ± 0.13	0.52 ± 0.10	0.001	0.31
LDL-C (g/L)	1.24 ± 0.37	1.27 ± 0.40	1.32 ± 0.46	1.45 ± 0.33	1.43 ± 0.33	1.61 ± 0.30	0.001	0.08
Triglycerides (g/L)[b]	1.70 ± 1.00	1.71 ± 0.98	1.75 ± 1.34	1.23 ± 0.81	1.16 ± 0.63	1.17 ± 0.48	0.001	0.86
ApoA-I (g/L)	1.23 ± 0.22	1.27 ± 0.23	1.25 ± 0.26	1.52 ± 0.25	1.53 ± 0.24	1.45 ± 0.18	0.001	0.18
LpA-I (g/L)	0.47 ± 0.15	0.48 ± 0.16	0.48 ± 0.14	0.55 ± 0.17	0.58 ± 0.18	0.51 ± 0.19	0.001	0.15
ApoB (g/L)	1.02 ± 0.25	1.05 ± 0.25	1.03 ± 0.32	1.07 ± 0.22	1.06 ± 0.22	1.13 ± 0.21	0.03	0.59
Lp(a) (g/L)[b]	0.44 ± 0.43	0.51 ± 0.47	0.54 ± 0.52	0.28 ± 0.34	0.29 ± 0.35	0.56 ± 0.74	0.001	0.008
IF1 (mg/L)	0.43 ± 0.13	0.45 ± 0.13	0.37 ± 0.12	0.53 ± 0.15	0.53 ± 0.16	0.53 ± 0.10	0.001	0.24
hs-CRP (mg/L)[b]	12.9 ± 21.8	13.4 ± 22.8	22.2 ± 30.9	3.0 ± 4.8	3.1 ± 5.9	2.9 ± 4.3	0.001	0.19
Glucose (mmol/L)	5.95 ± 2.12	6.08 ± 1.96	5.45 ± 0.95	5.49 ± 1.16	5.38 ± 0.84	5.07 ± 0.54	0.006	0.18
Insulin (IU/L)[b]	14.9 ± 21.8	14.9 ± 22.9	21.0 ± 18.7	9.9 ± 7.9	10.1 ± 6.9	8.1 ± 3.0	0.001	0.57
BMI (kg/m2)	27.3 ± 4.0	27.7 ± 4.3	27.7 ± 2.8	26.8 ± 3.6	26.8 ± 3.4	25.6 ± 2.7	0.06	0.19
Waist (cm)	98.9 ± 10.7	99.1 ± 11.5	100.5 ± 10.1	95.3 ± 9.9	95.6 ± 9.2	92.1 ± 7.9	0.001	0.76
Fat mass (%)	28.0 ± 5.4	28.0 ± 5.3	27.8 ± 4.9	26.4 ± 5.0	26.1 ± 5.4	24.2 ± 3.8	0.001	0.34
SBP (mmHg)	139.2 ± 20.8	140.1 ± 19.8	140.6 ± 23.2	137.1 ± 16.4	137.1 ± 15.0	134.2 ± 12.0	0.006	0.89
Heart rate (beats/min)	63.9 ± 12.5	64.6 ± 11.1	65.3 ± 8.3	63.4 ± 9.4	63.8 ± 9.1	63.4 ± 9.1	0.35	0.61
Ankle – brachial index ≤ 0.9 (%)	32.5	39.8	45.8					0.10
LVEF < 50% (%)	26.9	25.3	29.2					0.87

Data are expressed as mean ± standard deviation or number. ApoA-I: apolipoprotein A-I; ApoB: apolipoprotein B; BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high-sensitivity C-reactive protein; IF1: inhibitory factor 1; LDL-C: low-density lipoprotein cholesterol; Lp(a): lipoprotein(a); LpA-I: lipoprotein A-I; SPB: systolic blood pressure; LVEF: left ventricular ejection fraction.

[a] Statistical significance was determined for the difference between patients with coronary artery disease and control subjects, and regarding the Nt557 genotype effect.

[b] Test done on log-transformed data.

μM range) can be released in the vascular compartment, from erythrocytes and endothelial cells, particularly under hypoxia or in response to shear stress [27,28]. Extracellular ATP promotes vasodilatation, through interaction with P2Y2 and P2X4 receptors [29,30]. ATP effects would be partly endothelium-dependent and associated with the production of nitric oxide. Interestingly, in red blood cells, ADP acting on Gi-coupled $P2Y_{13}$ would temper ATP secretion, which is a cyclic adenosine monophosphate (cAMP)-dependent process [31]. $P2Y_{13}$ is a purinergic receptor highly expressed in red blood cells [5]. Hence, if the 261 T variant leads to a lower level of $P2Y_{13}$ expression, this would alleviate down-regulation of ATP secretion by red blood cells. Sustained ATP secretion would favour vasodilatation and the supply of oxygen to the myocardium, as reflected by increased release of IF1. An alternative hypothesis regarding the higher IF1 concentration in carriers of 261 T is that increased apoA-I, as reflected by LpA-I concentrations, would displace IF1 binding from membrane F1-ATPase.

In terms of cardiovascular risk, the *p2y*$_{13}$-261 T variant apparently does not interfere with the development of atherogenic disease, as the carriage frequency was identical in patients with CAD and control subjects. Interestingly, heart rate also did not differ between these two populations. As pointed out above, $P2Y_{13}$ plays a major role in the last steps of RCT, an antiatherogenic process [14]. Thus, the *p2y*$_{13}$-261 T variant might be associated with a somewhat lower expression of $P2Y_{13}$, but not to an extent that would compromise RCT. Conversely, lower heart rate, elevated systolic ankle – brachial index and conserved left ventricular ejection fraction are predictive of better outcomes in patients with CAD [16]. Ankle – brachial index is a non-invasive index, which generally reflects the progression of peripheral artery disease; in patients with CAD, it is may also be used as a prognostic marker of further complications [32,33]. Thus, the *p2y*$_{13}$-261 T variant, being associated with increased myocardial performance, might confer a good prognosis in the aftermath of an acute coronary syndrome. The supposedly lower risk of complications in carriers of *p2y*$_{13}$-261 T might be somewhat offset by the increase in fat mass observed in these subjects. However, this latter increase was not associated with an enhanced waist circumference, which limits its impact on cardiovascular risk.

Concerning the most frequent A557G polymorphism, previous reports of large case-control and population studies have not shown an association with acute myocardial infarction, diabetes or classical cardiovascular risk factors [18]. While our own data fully agree with these previous observations, we have extended the panel of biological markers, and found a strong and consistent association between the *p2y*$_{13}$-557G variant and increased Lp(a) concentrations. An elevated concentration of Lp(a) is now recognized to be an independent risk factor for CAD [34]. Large variations in Lp(a) plasma concentrations—from 0.01 to 2 g/L—are observed in the general population. Interestingly, in a recent large Mendelian randomization study, based on Lp(a) genetic scores, a variation of 0.1 g/L in Lp(a) concentration was found to be associated with a 5.8% change in the risk of CAD [35]. Concentrations of Lp(a) are mostly genetically determined, and are largely dependent on the number of kringle IV-2 repeats present in the protein and the corresponding gene [36], the latter located on chromosome 6. Our present data indicate that *p2y*$_{13}$ is a modulator gene for Lp(a) concentration. One first hypothesis is that *p2y*$_{13}$-A557G polymorphism might be in linkage disequilibrium with another gene polymorphism affecting Lp(a) concentrations. The study by Amisten et al. [18] demonstrated that the *p2y*$_{13}$-557G variant is in strong linkage disequilibrium with the H2 allele of the *p2y*$_{12}$ gene. $P2Y_{12}$ is an ADP receptor playing a pivotal role in platelet activation. Determination of common polymorphisms has led to the identification of two *p2y*$_{12}$ haplotypes (H1 and H2), with a frequency of 0.14 for the latter, close to the value of 0.15 observed for the Nt557 minor allele in the present study. Carriers of the H2 haplotype display maximal platelet aggregation in response to ADP [37]. Concordantly, they are more frequent among patients with peripheral artery disease [38] or CAD [39] compared with control subjects. Thus, the *p2y*$_{12}$-H2 haplotype, associated with the *p2y*$_{13}$-557G allele, would confer increased platelet reactivity and, further, hypercoagulation. As a result of partial homology with plasminogen, Lp(a) can bind with high affinity to fibrin, a process still amplified after exposure to plasmin [40,41]. Although purely speculative, we may hypothesize that, in a condition of hypercoagulability, binding of Lp(a) to fibrin or fibrin degradation products would be increased, so that the metabolic clearance of Lp(a) might be delayed. Alternatively, a more direct impact of *p2y*$_{13}$-Nt557 polymorphism on Lp(a) metabolism might be considered. In previous studies, we demonstrated that, when activated, $P2Y_{13}$, which itself is not a lipoprotein receptor, leads to cytoskeletal reorganization that promotes endocytosis of HDL particles [42]. It cannot be excluded that such a process might also facilitate Lp(a) endocytosis, through different candidate receptors [43–45], and that the $P2Y_{13}$ 179 T isoform (as encoded by the Nt557G variant), might be less effective at stimulating endocytosis. Whatever the underlying mechanisms, carriers of the *p2y*$_{13}$-557G/*p2y*$_{12}$-H2 allele should be at increased risk of developing atherothrombotic lesions. Indeed, Lp(a) competes with plasminogen for binding to fibrin, impairing its activation to plasmin. Thus elevated Lp(a) may impair fibrinolysis [40]. Moreover, Lp(a) binding to fibrin deposits may bring about cholesterol accumulation at sites of vascular injury.

Conclusions

Two frequent polymorphisms of the *p2y*$_{13}$ gene were found to be associated with specific bioclinical markers of cardiovascular risk. Neither of these appears to be related to the development of atherosclerotic disease, as allele frequencies were identical in patients with CAD and in control subjects; however, they might modulate the risk of further cardiovascular complications. The most frequent A557G mutation, which leads to an amino acid substitution, should confer an increased risk of thrombosis, with elevated Lp(a) and an association with increased platelet aggregation. By contrast, the synonymous G261 T variation, which affects 9% of the population, is associated with improved myocardial function, and thus should have a better prognostic value as regards further complications. Interestingly, despite their proximity in the *p2y*$_{13}$ gene, these two variations were associated with distinct sets of biological markers, which might

reflect different molecular impacts of these variations. As discussed above, the 261 T variant might be associated with a lower cellular expression of the P2Y$_{13}$ receptor. Concerning the most common 557G variation, its impact on coagulation/fibrinolysis should be considered as part of the phenotype associated with the *p2y$_{12}$*-H2 haplotype. Moreover, the fact that the two variations were not present simultaneously in a subject's *p2y$_{13}$* genotype strengthens the hypothesis of allele-specific effects. In conclusion, determination of *p2y$_{13}$* genotype may contribute to prognostic information in patients at risk of cardiovascular diseases.

Source of funding

This study was supported by the French National Research Agency (#ANR-16-CE18-0014-01), la Région Midi-Pyrénées (RBIO No.14054337) and La Fédération Française de Cardiologie.

Acknowledgments

The authors extend their appreciation to the participants in the GENES study.

Disclosure of interest

The authors declare that they have no competing interest.

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