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Genetic variation in *NOS1AP* is associated with sudden cardiac death: evidence from the Rotterdam Study

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Common variation within the nitric oxide-1 synthase activator protein (*NOS1AP*) locus is strongly related to QT interval, a sudden cardiac death (SCD) risk factor. A recent report describes common variation in *NOS1AP* associated with SCD in a US population of European ancestry. The objective of the current study was to obtain additional evidence by investigating the association between *NOS1AP* variants and SCD in the prospective population-based Rotterdam Study. The study population consisted of 5974 European ancestry subjects, aged 55 years and older, genotyped on Illumina arrays. SCD was defined according to European Society of Cardiology guidelines. Smoking, body mass index, diabetes mellitus, hypertension, heart failure and myocardial infarction were used as covariates in Cox proportional hazard models. Results were combined with reported evidence using inverse-variance weighted meta-analysis. Two hundred and eight (109 witnessed) cases of SCD occurred during a mean follow-up of 10.4 years. Within the Rotterdam Study alone, no significant associations were observed. Upon pooling of results with existing data, we observed strengthening of existing evidence for rs16847549 (US data HR = 1.31, *P* = 0.0024; Rotterdam Study HR = 1.18, *P* = 0.16; joint HR = 1.26, *P* = 0.0011). When the case definition in the Rotterdam Study was restricted to witnessed SCD, association of rs16847549 with SCD became stronger (joint *P* = 0.00019) and additionally the association between rs12567209 and SCD gained significance (US data HR = 0.57, *P* = 0.0035; Rotterdam Study HR = 0.69, *P* = 0.23; joint HR = 0.60, *P* = 0.0018). In conclusion, this study provided additional evidence for association between genetic variation within *NOS1AP* and SCD. The mechanism by which this effect is exerted remains to be elucidated.

INTRODUCTION

Sudden cardiac death (SCD) is one of the major causes of cardiovascular mortality in developed countries and mostly occurs in individuals unrecognized to be at risk (1). Familial aggregation of SCD, independent of other risk factors, suggests a genetic role in SCD risk (2–5). By the use of genome-wide association studies (GWAS), it is possible to search for common variants associated with complex traits. Owing to the small size of existing SCD collections, the statistical power to study this trait through GWA studies is limited. The focus has been on intermediate quantitative measures predictive of SCD. One of the quantitative predictors of arrhythmogenesis is the electrocardiographic (ECG) QT interval duration, a non-invasive measure of ventricular repolarization. Variation of the QT interval in the general

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Table 1. Baseline characteristics of the Rotterdam Study population

	Genotyped sample[a]		All SCD cases		Witnessed SCD cases	
	Men, $n = 2427$ (40.6%)	Women, $n = 3547$ (59.4%)	Men, $n = 104$ (50%)	Women, $n=104$ (50%)	Men, $n = 67$ (61.5%)	Women, $n = 42$ (38.5%)
Age, years, mean (SD)	68.1 (8.2)	70.3 (9.6)	71.5 (7.5)	74.0 (7.7)	70.1 (6.9)	72.7 (8.2)
Follow-up time, years, mean (SD)	10.0 (3.8)	10.6 (3.7)	6.4 (3.7)	7.7 (3.7)	6.4 (3.5)	6.7 (3.6)
Current smoking, *n* (%)	716 (29.5)	623 (17.6)	28 (26.9)	14 (13.5)	18 (26.9)	5 (11.9)
Past smoking, *n* (%)	1474 (60.7)	951 (26.8)	69 (66.4)	35 (33.7)	44 (65.7)	19 (45.2)
Body mass index, kg/m^2, mean (SD)	25.7 (3.0)	26.7 (4.1)	25.4 (3.0)	27.2 (4.0)	25.4 (3.0)	27.1 (4.2)
Hypertension, *n* (%)	714 (29.4)	1283 (36.2)	49 (47.1)	57 (54.8)	31 (46.3)	25 (59.5)
Diabetes mellitus, *n* (%)	248 (10.2)	383 (10.8)	13 (12.5)	23 (22.1)	11 (16.4)	13 (31.0)
Myocardial infarction, *n* (%)	412 (17.0)	284 (8.0)	42 (40.4)	16 (15.4)	26 (38.8)	6 (14.3)
Heart failure, *n* (%)	74 (3.0)	120 (3.4)	17 (16.3)	6 (5.8)	10 (14.9)	1 (2.4)

[a]Includes all SCD cases.

population is ~35% heritable and prolonged QT intervals are associated with increased cardiovascular morbidity and mortality, including SCD (6–8). Using GWAS, a common variant (rs10494366) in the nitric oxide synthase-1 adaptor protein (*NOS1AP*) gene was identified and reproducibly associated with QT interval duration (9–13). Previously, we have reported a fine mapping effort for this association in the Rotterdam study, which refined the signal of association to rs12143842 (14). This SNP was consistently the strongest associated variant in recent GWAS reports on QT interval (15–17).

Recently, two SNPs with very low linkage disequilibrium (rs16847548 and rs12567209) at the *NOS1AP* locus were reported to be independently associated with SCD risk in a large US community-based population study of European ancestry (18), but to date no replication has been reported. The rs12143842 common variant is in considerable linkage disequilibrium with rs16847548 (HapMap CEU $r^2 = 0.82$) but not rs12567209 (HapMap CEU $r^2 = 0.027$). The goal of the present study was to test for association of rs12143842 or its recently reported proxy rs16847548 and the second independent variant rs12567209 with SCD in the Rotterdam Study, to attempt to validate these associations.

RESULTS

Study subjects and genotyping

Baseline characteristics for the study population, consisting of all genotyped Rotterdam Study participants ($n = 5974$), and SCD cases are summarized in Table 1. During a mean of 10.4 years of follow-up, 208 SCDs, 109 of which witnessed, were identified. Successful genotype calls were made in 99.7% (rs12143842) and 99.8% (rs12567209) of the subjects, respectively. The genotype frequencies are in Hardy–Weinberg equilibrium for both rs12143842 (T-allele frequency=24.0%) and rs12567209 (A-allele frequency=14.9%) with $P = 0.72$ and $P = 0.85$, respectively. The imputed genotype for rs16847548 was available for all subjects. The imputation quality of this SNP was very good with an observed/expected variance ratio of 0.993.

For secondary analyses exploring the effect of a differential outcome definition on established risk factors, the study population consisted of 5661 subjects from the Rotterdam study, all with available QT measurements and without a left or right bundle branch block, atrial fibrillation and a QRS duration <120 ms, to minimize QT measurement errors. During a mean follow-up of 10.8 years, 154 cases of SCD were identified, 85 of which witnessed.

NOS1AP variants association with SCD in the Rotterdam study

The proportional hazards assumption for a constant hazard ratio (HR) over time was met. After adjustment for age and sex, rs12143842, rs16847548 and rs12567209 variants showed non-significant trends for association with SCD under an additive genetic model in the Rotterdam Study alone (Table 2). Since rs16847548 is in linkage disequilibrium with rs12143842 (HapMaP CEU $r^2 = 0.82$), the results are very similar for these SNPs (Table 3). For simplicity in subsequent analyses, we show results for the genotyped rs12143842 SNP only. Upon inclusion of both rs12567209 and rs12143842 in the same model, in addition to age and sex, the HR for rs12567209 changed from 0.85 [95% confidence interval (CI) 0.57–1.26] to 0.86 (95% CI 0.57–1.28) and for rs12143842 from 1.18 (95% CI 0.95–1.47) to 1.17 (95% CI 0.93–1.45). When QT interval, heart rate (RR interval) and QRS duration were subsequently added as continuous variables in the model, the HRs for rs12567209 and rs12143842 were 0.74 (95% CI 0.47–1.17) and 1.06 (95% CI 0.82–1.35), respectively, (Table 2).

To compare our results with the results from Kao *et al*. (18), QT interval was also modeled as a categorical variable (quintiles). This showed similar results compared with analysis including the linear QT variable for both rs12567209 (HR = 0.73; 95% CI 0.46–1.16) and rs12143842 (HR = 1.07; 95% CI 0.84–1.36). Additional adjustment for myocardial infarction, diabetes, heart failure, hypertension, smoking and body mass index did not change the effect estimates (Table 2).

To account for potential measurement errors of the QT interval, additional analyses were performed with exclusion of those subjects who showed a left or right bundle branch block, QRS duration over 120 ms or had prevalent atrial fibrillation. This did not change the results substantially (Table 2).

Table 2. Association results for rs12567209 and rs12143842 with all and witnessed SCD in the Rotterdam Study

	Cases, *n*	rs12567209 GG	HR (95% CI) per additional A-allele	rs12143842 CC	HR (95% CI) per additional T-allele
All SCD, model 1[a]	208	1.00 (ref)	0.85 (0.57–1.26) $P = 0.42$	1.00 (ref)	1.18 (0.95–1.47) ($P = 0.13$)
All SCD, model 2[b]	208	1.00 (ref)	0.86 (0.57–1.28) $P = 0.18$	1.00 (ref)	1.17 (0.93–1.45) $P = 0.18$
All SCD, model 3[c]	178	1.00 (ref)	0.74 (0.47–1.17) $P = 0.20$	1.00 (ref)	1.06 (0.82–1.35) $P = 0.66$
All SCD, model 4[d]	175	1.00 (ref)	0.74 (0.46–1.18) $P = 0.21$	1.00 (ref)	1.11 (0.87–1.43) $P = 0.40$
All SCD, model 5[e]	126	1.00 (ref)	0.77 (0.45–1.34) $P = 0.36$	1.00 (ref)	1.14 (0.86–1.52) $P = 0.37$
Witnessed SCD, model 1[a]	109	1.00 (ref)	0.69 (0.37–1.27) $P = 0.23$	1.00 (ref)	1.42 (1.06–1.89) $P = 0.018$
Witnessed SCD, model 2[b]	109	1.00 (ref)	0.73 (0.40–1.35) $P = 0.27$)	1.00 (ref)	1.39 (1.04–1.86) $P = 0.027$
Witnessed SCD, model 3[c]	99	1.00 (ref)	0.72 (0.38–1.37) $P = 0.31$	1.00 (ref)	1.27 (0.93–1.74) $P = 0.14$
Witnessed SCD, model 4[d]	95	1.00 (ref)	0.67 (0.34–1.33) $P = 0.25$	1.00 (ref)	1.34 (0.97–1.85) $P = 0.08$
Witnessed SCD, model 5[e]	66	1.00 (ref)	0.79 (0.36–1.72) $P = 0.55$	1.00 (ref)	1.36 (0.93–1.99) $P = 0.11$

[a]Model 1: adjusted for age and sex.
[b]Model 2: Model 1+alternate SNP.
[c]Model 3: Model 2+QRS, QT and RR interval.
[d]Model 4: Model 3+hypertension at baseline and time dependent, body mass index, diabetes mellitus, heart failure and myocardial infarction.
[e]Model 5: Model 4, after exclusion of subjects with left or right bundle branch block or QRS duration over 120 ms.

Table 3. HRs for classical SCD risk factors by phenotype definition in the total Rotterdam Study population with QT interval measurements and without bundle branch block, atrial fibrillation or QRS duration over 120 ms ($n = 5661$)

	Cases, *n*	All SCD, HR (95% CI)	Cases, *n*	Witnessed SCD, HR (95% CI)
QT quintile 1	36	1.00 (ref)	16	1.00 (ref)
QT quintile 2	38	1.38 (0.85–2.25)	21	2.01 (1.00–4.03)
QT quintile 3	34	1.48 (0.86–2.56)	21	2.64 (1.24–5.64)
QT quintile 4	46	2.20 (1.18–4.09)	27	4.42 (1.87–10.44)
Male	75	1.00 (ref)	50	1.00 (ref)
Female	79	0.43 (0.31–0.60)	35	0.29 (0.19–0.46)

Presented are HRs per QT interval quintile adjusted for age, sex and RR interval (inverse heart rate) and HR for sex adjusted for age.

Secondary analyses

Since some non-differential misclassification was expected in non-witnessed SCD, analyses were repeated using the restricted witnessed SCD case definition. The common variants studied here and established SCD risk factors were analyzed to assess the effect of the more stringent case definition. Stronger effects were observed for established SCD risk factors including QT interval and sex when using this restricted definition despite the reduction in case sample size (Table 3). When the genetic variants were tested for association with witnessed SCD, significant associations were observed for rs12143842 (and the correlated SNP rs16847548). Under the assumption of an additive genetic model, the HR per additional T-allele of rs12143842 was 1.42 (95% CI 1.06–1.89). Effect estimates for rs12567209 moved away from the null, but did not reach statistical significance, with an observed HR of 0.69 (95% CI 0.34–1.27).

Meta-analyses

The findings of this study were combined with recent results from Kao *et al*. (Table 4). With the addition of Rotterdam Study results, the evidence for association with rs16847548 strengthened, irrespective of the outcome definition used (original HR = 1.31, $P = 0.0024$; joint HR = 1.26, $P = 0.0011$ for all SCD and HR = 1.33, $P = 0.00019$ for witnessed SCD). For rs12537209, the significance level only increased when we used the restricted witnessed SCD definition (original HR = 0.57, $P = 0.0035$; joint HR = 0.69, $P = 0.0075$ for all SCD and HR = 0.60, $P = 0.0018$, for witnessed SCD).

DISCUSSION

Main finding

In the present study, additional evidence is presented that common variation at the *NOS1AP* locus is associated with increased SCD risk. Adjustment for potential confounders, such as cardiovascular co-morbidities or risk factors did not change the results. Overall in the Rotterdam Study sample, no significant effects for association between common variants and SCD were observed. However, when we restricted the case definition to only witnessed SCD, significant effects for rs12143842 and its proxy rs16847549 were seen despite the reduced number of cases.

Combination of the results with recently published data from the Cardiovascular Health Study and Atherosclerosis Risk in Communities study by Kao *et al*. (18) resulted in a strengthened overall significance of the association between rs16847548 and SCD, regardless of the phenotype definition. For rs12567209, the incremental effect on significance was dependent on the outcome definition. When a stricter outcome definition was used, this resulted in strengthened statistical significance.

NOS1AP and SCD

The mechanism by which *NOS1AP* influences QT interval and/or SCD risk remains to be fully elucidated. *NOS1AP* encodes the CAPON protein that is expressed in the heart and interacts with nitric oxide synthase-1 (NOS1) enhancing nitric oxide (NO) production (19). NOS1 and NO play an important role in regulating cardiac physiology such as

Table 4. Joint analysis of the Rotterdam Study results and ARIC plus CHS results (18)

	rs12567209		rs16847548			
	Allelic		Dominant		Allelic	
ARIC+ CHS						
HR	0.57		0.53		1.31	
Ln(HR)	−0.562		−0.635		0.270	
SE	0.192		0.201		0.089	
P-value	0.0035		0.0015		0.0024	
RS	All	Witnessed	All	Witnessed	All	Witnessed
HR	0.85	0.69	0.80	0.70	1.18	1.40
Ln(HR)	−0.165	−0.378	−0.229	−0.364	0.165	0.339
SE	0.204	0.311	0.217	0.318	0.117	0.153
P-value	0.42	0.23	0.29	0.25	0.16	0.027
Joint						
HR	0.69	0.60	0.64	0.57	1.26	1.33
Ln(HR)	−0.374	−0.511	−0.448	−0.558	0.232	0.287
SE	0.140	0.164	0.147	0.170	0.071	0.077
P-value	0.0074	**0.0018**	0.0024	**0.0010**	**0.0011**	**0.00019**

All results are based on sex and age adjusted allelic models. *P*-values in bold reflect increased significance with the addition of RS results. ARIC, Atherosclerosis Risk in Communities study; CHS, Cardiovascular Health Study; Ln(HR), natural log of the hazard ratio=beta estimate from Cox regression model; SE, standard error of the regression coefficient; RS, Rotterdam Study.

calcium turnover and adrenergic response (20,21). Overexpression of CAPON leads to the inhibition of the L-type calcium channels and an increase in the delayed rectifier potassium current resulting in hastening of the action potential (19). It remains to be proven whether the influence of common variation in *NOS1AP* on this physiological mechanism is also the mechanism for the increased risk of SCD. In the original report by Kao *et al*., association of the common variants with SCD was independent of the effect on QT interval duration. We observed for rs12143842, a proxy for the variant reported by Kao *et al*. rs16847548, that the effect on SCD was attenuated upon inclusion of QT interval in the model. For rs12567209 (which has not been associated with QT interval duration), adjustment for QT interval resulted in a slightly stronger effect of the common variant on SCD; however, no significant results were observed in the Rotterdam study sample alone. From the existing data, it is difficult to say whether these genetic variants exert their effect on SCD through modulation of the QT interval or via other mechanisms. Both in the presented and previously described samples, the QT interval was measured well before the event. Additionally, the common variants might very well not be the causal SNPs. As indicated by Kao *et al*., the effect measures of these SNPs on QT interval and SCD might differ due to unequal linkage to the causal SNP, leaving residual association between the SNP and SCD after adjustment for QT interval. The fact that we observed attenuation of the effect on SCD for rs12143842 could be due to stronger correlation between this variant and the causal SNP.

Methodological considerations

Advantages of our study are the large community-based sample size, prospective ascertainment of risk factors and active surveillance for SCD events over a relatively long duration of follow-up. Extensive information was available with respect to SCD events, facilitating careful case adjudication. The homogeneity of the Rotterdam Study population and quality control procedures identifying population outliers result in a low chance of population substructure underlying the findings. In prior GWAS analysis of QT interval, no indication of population stratification was observed in the Rotterdam Study (15).

A limitation is the possibility of alternative causes of abrupt death, especially at increasing age. Specifically, for an unwitnessed abrupt death alternative causes of sudden death might lead to misclassification of the outcome due to less extensive information for adjudication. Since case ascertainment was blinded to genotype, this misclassification is non-differential and would lead to bias towards the null hypothesis of finding no association. Therefore, a reduction in misclassification of the outcome by excluding unwitnessed SCD could explain the observation of stronger associations for witnessed SCD. This is supported by the behavior of risk estimates for classically established SCD risk factors. Finally, with the available cases, the power for replicating the findings for rs16847548 and rs12567209 was 68 and 92%, respectively, under the assumption there is no case misclassification within the Rotterdam Study.

Conclusion

This study provided additional evidence for an association between common genetic variation within *NOS1AP* and SCD. The strength of association depended on the strictness of the phenotype definition used. The mechanism through which this effect is exerted remains to be elucidated.

MATERIALS AND METHODS

Setting and design

The Rotterdam Study is a population-based cohort study of chronic diseases in the elderly, which started in 1990. All inhabitants of Ommoord, a Rotterdam suburb in the Netherlands, aged $\geq$55 years ($n = 10\,278$) were invited to

participate. Of them, 78% ($n = 7983$) gave their written informed consent. Baseline examinations took place from March 1990 through July 1993. Follow-up examinations are carried out periodically. Participants are continuously monitored for major events through automated linkage with general practitioner's files. Clinical characteristics, including smoking, body mass index, diabetes mellitus, hypertension, heart failure and myocardial infarction were ascertained as previously described (8,22–25). Detailed information on design, objectives and methods has been described elsewhere (26,27). The Medical Ethics Committee of the Erasmus Medical Center approved the study and all participants gave written informed consent.

Adjudication of SCD

The ascertainment of SCD cases in the Rotterdam Study was performed according to European Society of Cardiology guidelines (28) and has been described previously (8). SCD cases were defined as a witnessed natural death attributable to cardiac causes, heralded by abrupt loss of consciousness, within 1 h of onset of acute symptoms or as an unwitnessed, unexpected death of a person seen in a stable medical condition within 24 h before death without evidence of a non-cardiac cause (28).

QT, QRS and RR interval measurements

As described in earlier studies of ECG parameters in the Rotterdam Study (8), we used a 10 s resting 12-lead ECG (average 8–10 beats), which was recorded on an ACTA ECG (ESAOTE, Florence, Italy) at a sampling frequency of 500 Hz and stored digitally. All ECGs were processed by the Modular ECG analysis system (MEANS), which has been evaluated extensively, to obtain QT, QRS and RR duration measurements (29–32). MEANS also determines the presence of right or left bundle branch block. For the current analyses, we used measurements from baseline ECG recordings.

Genotyping of *NOS1AP* common variants

Genotyping, quality control and imputation procedures in the Rotterdam Study have been previously described (15). The Rotterdam Study is a homogenous European sample and quality control procedures removed population outliers from the GWAS dataset. Briefly, of all 7983 participants, 5974 subjects of European descent were successfully genotyped on the Infinium II HumanHap550K Genotyping BeadChip® version 3 (Illumina) as part of a large population-based project on genetics of complex traits and diseases. In addition to the direct genotypes generated, we have access to ~2.5 million autosomal genotypes that were imputed based on linkage disequilibrium patterns observed in HapMap CEU reference samples (Utah residents of Northern and Western European descent) using Mach 1.0.15 (33). Imputed was the genotype dosage, a value between 0 and 2, that reflects the expected number of alleles.

For all subjects, direct genotype information of rs12143842 and rs12567209 was extracted from the dataset. For rs16847548, the imputed genotype dosage was retrieved. The metric used to reflect imputation quality is the observed over expected variance of the allele frequency (34). This ratio ranges between 0 and 1, where a value of 1 reflects perfect imputation. No other genotypes were extracted and tested for an association with SCD.

Association analysis

Genotype frequencies of genotyped SNPs were tested for Hardy–Weinberg equilibrium by calculating exact *P*-values. HRs for time to SCD from baseline were estimated with Cox proportional hazards models. Cox proportional hazards analyses were performed with SPSS for Windows, 15.0 (SPSS Inc., Chicago, IL, USA). The proportional hazards assumption was assessed using log-minus-log plots. Overall, additive genetic models were used but for rs12567209 a dominant model was additionally fitted due to the lower minor allele frequency, and thus the small number of homozygous variant carriers. In addition to the genotype, known risk factors such as gender, smoking and hypertension at baseline and time-dependent age, body mass index, diabetes mellitus, heart failure and myocardial infarction were included in the model. To assess if the SNPs exert their effect on SCD through modulation of the heart rate corrected QT interval, both QT interval and heart rate measured at baseline were included in the model.

The primary outcome definition included both witnessed and unwitnessed SCD. Since potential random misclassification of the outcome was expected in unwitnessed cases, additional analyses describing the association between the genetic variants and SCD were performed using a restricted definition, only including witnessed SCD. This was regarded as a secondary outcome. We additionally estimated the effect of QT interval and gender, established SCD risk factors, using both the primary (all) and secondary (witnessed) outcome definition. Larger effects are expected when using a stricter case definition due to a reduction in non-differential misclassification.

Since the presented sample was smaller than the sample in the original report describing the association between these SNPs and SCD (18), non-significant findings in the current analysis might be due to a lack of power. Nevertheless, this sample may add valuable evidence for association by combination with earlier results. Hereto, results were combined with the results from the literature using inverse variance weighted meta-analyses methods and the overall association evidence is presented.

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Conflict of Interest statement. None declared.

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