Sudden cardiac arrest: Studies on risk and outcome
Blom, Marieke

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Sudden cardiac arrest associated with use of a non-cardiac drug that reduces cardiac excitability: evidence from bench, bedside, and community

*These authors contributed equally

Non-cardiac drugs that impair cardiac repolarization (electrocardiographic QT-prolongation) are associated with increased sudden cardiac arrest (SCA) risk. Emerging evidence suggest that non-cardiac drugs that impair cardiac depolarization and excitability (electrocardiographic QRS-prolongation) also increase SCA risk. Nortriptyline, which blocks the SCN5A-encoded cardiac sodium channel, may exemplify such drugs. We aimed to study whether nortriptyline increase SCA risk, and to establish the underlying mechanisms.

We studied QRS-durations at rest/exercise in an index patient who experienced ventricular tachycardia during exercise while using nortriptyline, and compared them to those of 55 controls with/without nortriptyline and 24 controls with BrS without nortriptyline who carried an SCN5A mutation. We performed molecular-genetic (exon-trapping) and functional (patch-clamp) experiments to unravel the mechanisms of QRS-prolongation by nortriptyline and the SCN5A mutation found in the index patient. We conducted a prospective community-based study among 944 victims of ECG-documented SCA and 4354 matched controls to determine the risk for SCA associated with nortriptyline use.

Multiple mechanisms may act in concert to increase SCA risk during nortriptyline use. Pharmacological (nortriptyline), genetic (loss-of-function SCN5A mutation) and/or functional (sodium channel inactivation at fast heart rates) factors conspire to reduce cardiac sodium current and increase SCA risk. Nortriptyline use in the community was associated with 4.5-fold increased SCA risk (adjusted OR 4.5 [95%CI 1.1-19.5]), particularly when other sodium channel blocking factors were present.

Nortriptyline increases SCA risk in the general population, particularly in the presence of genetic and/or non-genetic factors that decrease cardiac excitability by blocking the cardiac sodium channel.
Introduction

Sudden cardiac arrest (SCA) accounts for 50% of cardiovascular deaths in Western societies. SCA is mostly caused by ventricular tachycardia/fibrillation (VT/VF), and is in ~50% of cases the first sign of heart disease, predominantly occurring out-of-hospital. Its survival rate is dismal (<10%). The best solution is to prevent SCA by recognizing causative pathophysiologic processes and identifying subjects at risk.

Intensive translational research on rare monogenic inherited arrhythmia syndromes (e.g., long QT syndrome) has established the role of cardiac ion channel dysfunction (e.g., caused by very low-frequency gene variants) in SCA risk. These insights may be applicable to understand the causes of SCA in common conditions. For instance, the risk for VT/VF and SCA may be increased by non-cardiac drugs (drugs for non-cardiac disease) that impair cardiac repolarization, thereby prolonging the QT-interval. In some patients, the genetic basis of this acquired long QT syndrome was established with the identification of rare gene variants in ion channels that control cardiac repolarization. In conjunction with drugs that block repolarizing currents, these variants impair repolarization, culminating in VT/VF.

Impaired cardiac depolarization and excitability may also cause VT/VF by facilitating re-entrant excitation. This possibility has so far received less recognition. Depolarization of the sarcolemma initiates the cardiac action potential, which triggers cardiac excitation (reflected on the ECG by the QRS-complex). Accordingly, impaired depolarization reduces cardiac excitability and causes QRS-prolongation. This may result from sodium current ($I_{Na}$) reduction due to sodium channel dysfunction, for example by mutations in SCN5A, the gene that encodes the major subunit of the cardiac sodium channel. SCN5A-related inheritable arrhythmia syndromes include Brugada syndrome (BrS) and cardiac conduction disease. $I_{Na}$ reduction may also be acquired by drugs, particularly in the presence of concomitant diseases which reduce cardiac excitability. This was shown in the CAST trial, where, class 1C cardiac anti-arrhythmic drugs (established cardiac sodium channel blockers) caused excess mortality in patients with increased susceptibility due to cardiac ischemia or heart failure. Accordingly, these drugs may also induce VT/VF in BrS patients.

Suspicion is mounting that non-cardiac depolarization-blocking drugs may also increase susceptibility to SCA. Small studies found that some antidepressant drugs (e.g., nortriptyline) may induce BrS ECG-pattern and SCA. These non-cardiac drugs can block the sodium channel, thereby increasing the risk for VT/VF, and SCA, particularly when other conditions that also reduce $I_{Na}$ (perhaps inherited) are present. To explore this new concept, we studied nortriptyline as an example of such drugs. The causative mechanisms were investigated in a patient with repeated syncope and VT during therapeutic nortriptyline use. We performed clinical, molecular-genetic, and functional studies, and identified multiple mechanisms, both genetic and non-genetic, that conspired to unleash the pro-arrhythmic potential of nortriptyline. Furthermore,
we conducted a prospective community-based study and determined the risk for SCA associated with nortriptyline use in the general population.

Methods

This study was approved by the local ethics committee, and conforms to the principles outlined in the Declaration of Helsinki. All patients gave informed consent for participation. An expanded Methods description is provided in the Supplemental Methods.

Clinical studies

Study groups

ECG analysis was performed in four groups: (1) index patient; (2) 35 healthy ECG-control men (age: 42±1yrs) without cardiac history who used no drugs; (3) nortriptyline group, including 20 otherwise healthy men (49±1yrs) treated with 25-50mg nortriptyline (n=7), 75-100mg nortriptyline (n=7), 150mg nortriptyline (n=6); (4) BrS group, including 24 men (43±2yrs) with BrS who carried an SCN5A mutation but used no nortriptyline.

ECG analysis

Twelve-lead ECG tracings were optically magnified to facilitate manual analysis. Heart rate and QRS-interval (leads V₅ or II) were analyzed. Within one individual, the mean QRS value of five beats was calculated. ECGs were analyzed at baseline in all groups. The index patient (group 1), healthy ECG-control men (group 2), and BrS patients (group 4) underwent a symptom limited treadmill test using the Bruce protocol. ECGs were analyzed at different time points during exercise and at peak exercise.

Molecular-genetic and functional studies

Mutation analysis

The coding and splice-site regions of SCN5A were analyzed in the index patient using standard methods. The presence of the identified mutation was tested in the parents of the patient and 1740 unrelated control individuals of European descent (DNA-controls). We searched for the presence of the mutation in the 1000Genomes database, the NHLBI Exome Variant Server, and the Genome of the Netherlands Project (GoNL).

cDNA analysis

Blood samples from the index patient and two control individuals of European descent (SCN5A-controls) were collected in PAXgene tubes. Total RNA was isolated from
blood lymphocytes. SCN5A transcripts were reverse-transcribed, PCR-amplified and sequenced.

Exon-trapping

A 622 base pairs SCN5A genomic fragment containing exon 27 and flanking intronic sequences was PCR-amplified using DNA from the index patient as template. Wild-type and mutant fragments were cloned and subjected to exon-trapping for interrogation of any abnormal splicing events in the mutant (Exon Trapping System kit; Life Technologies). Splicing products were investigated by PCR and sequencing.

Generation of expression construct

The megaprimer approach was used to generate mutant SCN5A cDNA in which the final 96 base pairs of exon 27 were deleted. Wild-type and mutant SCN5A cDNA in the pCGI plasmid backbone were used in electrophysiologic studies.

Cellular electrophysiology

Using the patch-clamp technique, sodium currents were measured in HEK-293 cells transfected with wild-type or mutant SCN5A constructs. Cardiac action potentials were measured in left ventricular myocytes isolated from healthy rabbit hearts. 18

Community-based study

Setting

We conducted a case-control study using the AmsteRdam REsuscitation STudies (ARREST) infrastructure. ARREST is an ongoing, prospective, community-based study that we designed to establish the genetic19 and clinical20 determinants of SCA in the general population with a particular emphasis on drug use. Accordingly, we prospectively collect complete medical and medication data of all patients who suffer out-of-hospital SCA with ECG-documented VT/VF in a contiguous region of the Netherlands (~2.4 million inhabitants, >95% coverage).19,20 Matched controls (by age, sex, and index [SCA] date) are randomly drawn from the general community, using a database of community pharmacies that contains demographic and complete medication data (>2 million individuals, PHARMO Record Linkage System).21 Since nearly all patients in the Netherlands are registered at a single community pharmacy, independent of prescriber, pharmacy records are essentially complete.22 Each case is matched to up to five PHARMO-controls. Data for the present study were collected from July 2005 to January 2008.
All medication prescriptions of ARREST-cases and PHARMO-controls during the year before the index date were identified from computerized databases of pharmacists. Use of medication was defined current if the index date fell within the prescribed duration or within a maximum of 10% after the prescribed duration (to deal with carry-over effects).

Additional factors impairing cardiac excitability in ARREST

For cases who used nortriptyline, we investigated whether the presence of additional factors that impair cardiac excitability by retrieving data from hospital of admission and/or general practitioner. The following factors known to impair cardiac excitability were assessed: other (than nortriptyline) drugs that block the cardiac sodium channel, exercise prior to SCA, fever, (acute) cardiac ischemia, and heart failure. The presence of SCN5A mutations was assessed in cases who used nortriptyline and of whom DNA was available.

Covariates and risk factors

For all cases and PHARMO-controls, the following risk factors for SCA were assessed: cardiac ischemia, heart failure, hypertension, diabetes mellitus, and hypercholesterolemia. Risk factors were derived from medication use. Use of QT prolonging drugs,
antiarrhythmic drugs, cardiac glycosides, diuretics, angiotensin converting enzyme-
inhibitors, angiotensin receptor-blocking agents, α-adrenoceptor blockers and calcium
channel blockers were considered covariates.

Statistical analysis

Data are mean±SEM. Group comparisons were made with Student’s t-test or Analysis
of Variance. Differences for the ECG parameters in baseline values were tested between
groups with Student’s t-test (Figures 5A and 5B). Differences for maximum upstroke
velocities were tested between groups with one-way repeated measures ANOVA (Figure
8C). The relative risk for VT/VF associated with nortriptyline use was estimated by
calculating the adjusted odds ratio with 95% confidence interval (OR [95%CI]) using
conditional logistic regression analysis. Covariates and risk factors that were univariately
associated with VT/VF (p<0.1) were included in the regression analyses if they changed
the point estimate of the association between nortriptyline use and VT/VF by >5%. All
analyses were performed using SPSS for Mac (version 16.0 for Mac, Chicago IL, USA).

Results

Clinical studies
Index patient

The index patient, a 35-year-old Caucasian man without cardiac history or family history
of sudden death, presented with repeated syncope during running (he had started training
Figure 3. Exercise test of the index patient after discontinuation of nortriptyline.

<table>
<thead>
<tr>
<th>73</th>
<th>137</th>
<th>162</th>
<th>187</th>
</tr>
</thead>
<tbody>
<tr>
<td>beats per minute</td>
<td>beats per minute</td>
<td>beats per minute</td>
<td>beats per minute</td>
</tr>
</tbody>
</table>

ECG traces of leads $V_1$ and $V_6$ from the index patient at different heart rates during exercise test after discontinuation of nortriptyline. The QRS-interval prolongs from 110ms at baseline to 143ms at the maximum heart rate of 187bpm, and the ST-segment in $V_1$-$V_6$ elevates from 0.06mV at baseline to 0.55mV at the maximum heart rate.

Figure 4. Ajmaline testing.

(A) ECG traces of lead $V_1$ and $V_6$ from the index patient at baseline. (B) ECG traces of lead $V_1$ and $V_6$ from the index patient after intravenous infusion of ajmaline. Ajmaline causes QRS-prolongation from 110ms to 179ms and ST-elevation from 0.17mV to 0.49mV; this fulfills the diagnostic criteria for BrS.
for a marathon). His history was unremarkable, except for depression for which he had used nortriptyline 150mg OD for one year. His resting ECG while on nortriptyline displayed sinus tachycardia (102 beats per minute [bpm]) with prolonged PR (223ms) and QRS (131ms) intervals and no further abnormalities. Physical examination, serum electrolytes, echocardiography, and coronary angiography were unremarkable. During exercise testing, his QRS-interval prolonged to 180ms and his ST-segment in V1-V2 elevated from 0.17mV to 0.58mV at the maximum heart rate of 180bpm (Figure 1). At this point, he developed polymorphic VT with syncope (Figure 2). He recovered spontaneously, and his ECG-parameters returned to pre-exercise levels. Nortriptyline was discontinued and exercise testing was repeated. This time, his resting ECG displayed sinus rhythm at a normal rate (73bpm) with PR (208ms) and QRS (110ms) intervals at the upper limit of normal. At the maximum heart rate of 187bpm, his QRS-interval prolonged to 143ms and his ST-segment in V1-V2, elevated from 0.06mV to 0.55mV (Figure 3), but no arrhythmia or syncope occurred. Because the ST-segment elevation during exercise was suggestive of BrS, he underwent drug challenge with the sodium-channel blocking antiarrhythmic drug ajmaline. Ajmaline caused QRS-prolongation from 110ms to 179ms and ST-elevation from 0.17mV to 0.49mV (Figure 4); this fulfilled the diagnostic criteria for BrS. This diagnosis was supported by the fact that echocardiography and exercise tests did not indicate alternative explanations for these typical ECG-changes, e.g. ischemic heart disease, heart failure or cardiomyopathy.
ECG analysis

In the absence of nortriptyline the baseline ECG of the index patient exhibited QRS-prolongation of similar magnitude as that of patients who used 150mg nortriptyline, or BrS patients. QRS duration was indeed significantly longer in patients who used 150mg nortriptyline and in BrS patients compared to ECG controls who used no nortriptyline (P<0.001 for both comparisons). Nortriptyline induced additional QRS prolongation in the index patient (Figure 5B). The exercise testing ECG in the index patient in the absence of nortriptyline evoked QRS-prolongation, similar to BrS patients, while exercise testing in the index patient during nortriptyline use induced extreme QRS-

Figure 6. Nucleotide change c.4719C>T in SCN5A and its effect on mRNA splicing.

(A) cytosine (C) to thymine (T) nucleotide change at 95 base pairs from 3’end of exon 27 of SCN5A. (B) SCN5A splicing product from peripheral lymphocytes of the index patient (patient) and two SCN5A-control individuals (control). The upper band corresponds with the normal splicing product containing the complete exon 27. The lower band corresponds with the abnormal splicing product lacking the terminal 96 base pairs of exon 27. (C) c.4719C>T mutation generates a sequence within exon 27 that more closely resembles the splice donor site consensus sequence than the natural intron 27 splice donor site.
prolongation when heart rates exceeded 150bpm, followed by VT (Figure 5C). QRS-duration exercise remained unchanged in ECG-controls.

Molecular-genetic studies

Mutation analysis revealed a cytosine to thymine nucleotide change at 95 base pairs from the 3’ end of exon 27 (c.4719C>T [NM_198056]; Figure 6A) in SCN5A, which was not found in 1740 unrelated individuals. This nucleotide change was found in his father, who tested positive for BrS during ajmaline-testing, but not his mother, who tested negative during ajmaline-testing. Other relatives declined investigation. The c.4719C>T mutation does not cause an amino acid substitution. Instead, it is expected
to cause aberrant mRNA splicing by creating a novel premature splice site through the generation of a sequence within exon 27 that more closely resembles the splice donor site consensus sequence than the natural splice donor site in intron 27 (Figure 6C). Analysis of SCN5A-transcripts from peripheral lymphocytes from the index patient identified an abnormal splicing product lacking the terminal 96 base pairs of exon 27, corresponding to abnormal splicing at the novel splice site generated by the mutation (Figure 6B-C). Only the normal splicing product containing the complete exon 27 was identified in two SCN5A-controls. To confirm the impact of the mutation on splicing, we constructed wild-type and mutant hybrid minigenes in an exon-trapping vector and analyzed the splicing products in HEK-293 cells. The wild-type minigene consistently generated wild-type transcripts, while the mutant minigene generated both wild-type transcripts and transcripts with the terminal 96 bases of exon 27 deleted (not shown).

**Functional studies**

Deletion of the terminal 96 bases of exon 27 causes in-frame deletion of amino acids 1574-1605 of the channel protein, thereby disrupting the transmembrane segments S2 and S3 of channel domain 4 (Figure 7). This may affect channel protein folding, intracellular trafficking, and/or function. HEK-293 cells expressing wild-type SCN5A constructs displayed typical inward sodium currents, but no currents could be recorded from cells expressing the construct in which the terminal 96 base pairs of exon 27 were deleted (Figure 8A). As $I_{Na}$, through functional cardiac sodium channels triggers the initial phase of the cardiac action potential, we measured the maximum upstroke velocity of this initial phase to probe sodium channel availability (Figure 8B). Exposure of ventricular myocytes to 1μM nortriptyline (equivalent to serum levels during chronic use of 150mg nortriptyline) slowed the maximum upstroke velocity, indicating decreased sodium channel availability. Repetitive stimulation of myocytes at increasing frequencies (to mimic increasing heart rates during exercise) progressively slowed maximum upstroke velocity even in the absence of nortriptyline. In the presence of nortriptyline, this slowing effect was larger (P<0.001; Figure 8C).

**Community-based studies**

To determine whether increased risk for SCA following nortriptyline use applies to the general population, we conducted a community-based study on the association of nortriptyline use and SCA. We identified 944 SCA cases with ECG-documented VT/VF in ARREST; these cases were matched to 4354 PHARMO-controls. The mean age was 65 years in cases (77% male), and 65 years in PHARMO-controls (77% male). To indicate that the data acquisition system was valid, the known risk factors for SCA were associated with SCA. Use of cardiovascular medication was also associated with increased risk for VT/VF, as was use of QT prolonging drugs, confirming the established pro-arrhythmic potential of these drugs (Table 1). Four cases (0.4%) and
Figure 8. Effects of mutation, nortriptyline and stimulation frequency on sodium channel availability.

(A) Representative current traces from HEK-293 cells expressing SCN5A (wild-type or c4719C>T), obtained by depolarizing cells from a holding potential of -90mV to 50mV for 300ms. (B) Upstroke of the initial phase of the action potential, obtained by stimulating a rabbit ventricular myocyte in the absence of nortriptyline. (C) Maximum upstroke velocities of the initial phase of the ventricular action potential at different stimulation frequencies without nortriptyline (blue) or with nortriptyline (red).
Table 1. Baseline characteristics, demographics and distribution of covariates

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (N = 944)</th>
<th>Controls (N = 4354)</th>
<th>OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>729 (77.2)</td>
<td>3374 (77.5)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>215 (22.8)</td>
<td>980 (22.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Age in years, mean (SD)</strong></td>
<td>64.8 (15.0)</td>
<td>64.7 (14.9)</td>
<td></td>
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<tr>
<td><strong>Co–morbidities#</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Cardiac ischemia</td>
<td>365 (38.7)</td>
<td>931 (21.4)</td>
<td>2.7 (2.3–3.2)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>415 (44.0)</td>
<td>1273 (29.2)</td>
<td>2.1 (1.8–2.4)</td>
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<td>Diabetes mellitus</td>
<td>163 (17.3)</td>
<td>428 (9.8)</td>
<td>2.0 (1.6–2.4)</td>
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<tr>
<td>Heart failure</td>
<td>142 (15.0)</td>
<td>148 (3.4)</td>
<td>5.4 (4.2–6.9)</td>
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<tr>
<td>Dyslipidemia</td>
<td>337 (35.7)</td>
<td>965 (22.2)</td>
<td>2.1 (1.8–2.4)</td>
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<td><strong>Concomitant medication</strong></td>
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<tr>
<td>QT prolonging drugs†</td>
<td>39 (4.1)</td>
<td>100 (2.3)</td>
<td>1.9 (1.3–2.7)</td>
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<tr>
<td>Antiarrhythmic drugs§</td>
<td>18 (1.9)</td>
<td>62 (1.4)</td>
<td>1.4 (0.8–2.3)</td>
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<tr>
<td>Cardiac glycosides</td>
<td>86 (9.1)</td>
<td>104 (2.4)</td>
<td>4.6 (3.3–6.2)</td>
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<td>Diuretics</td>
<td>359 (38.0)</td>
<td>906 (20.8)</td>
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<td>Angiotensin converting enzyme inhibitors</td>
<td>288 (30.5)</td>
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<td>Angiotensin receptor blocking drugs</td>
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<td>414 (9.5)</td>
<td>1.7 (1.4–2.1)</td>
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<td>β-blockers</td>
<td>335 (35.5)</td>
<td>894 (20.5)</td>
<td>2.3 (1.9–2.7)</td>
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<tr>
<td>Calcium channel blockers</td>
<td>163 (17.3)</td>
<td>427 (9.8)</td>
<td>2.0 (1.6–2.4)</td>
</tr>
</tbody>
</table>

Data are expressed as number (%) unless otherwise indicated; SD, Standard Deviation; *Odds Ratios matched for age, gender and index date; # Co-morbidities are based on medication use in the half year prior to index date. § List derived from www.Gipdatabank.nl, a list of available antiarrhythmic drugs in the Netherlands; † CERT List of QT prolonging drugs, www.azcert.org.

Table 2. Use of nortriptyline and risk for SCA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (n=944)</th>
<th>Controls (n=4354)</th>
<th>OR* (95% CI)</th>
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<tr>
<td>Non-use</td>
<td>940 (99.58)</td>
<td>4350 (99.91)</td>
<td>1.0 (reference)</td>
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<tr>
<td>Current use</td>
<td>4 (0.42)</td>
<td>4 (0.09)</td>
<td>4.6 (1.2–18.4) p=0.03</td>
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</tbody>
</table>

Data are expressed as number (%) unless otherwise indicated. *Odds Ratios matched for age, gender and index date. After evaluation of the risk factors, we found no relevant confounding.
four PHARMO-controls (0.1%) were current users of nortriptyline on the index date. Use of nortriptyline was associated with a 4.6-fold increased risk for SCA (OR 4.6 [1.2–18.4] p=0.03, Table 2). After evaluation of the risk factors, we found no relevant confounding. In support of a multiple-hit model, in which multiple sodium channel blocking factors add up to result in disease-causing sodium channel blockage, all cases who used nortriptyline at the time of SCA had one or more additional causes for sodium channel blockage, in addition to nortriptyline use (Supplemental Table 1). While one case had performed exercise immediately before SCA, we identified other concomitant causes for sodium-channel blockage: use of other cardiac sodium channel blocking drugs (lithium\textsuperscript{14}, ropinirole\textsuperscript{26}), ischemia and/or acute myocardial infarction\textsuperscript{27} and heart failure.\textsuperscript{10} DNA was available of two cases who used nortriptyline; no SCN5A mutations were found in these patients.

Discussion

We provide proof-of-concept from basic, clinical and epidemiologic studies that blockage of the cardiac sodium channel by therapeutic dosages of a non-cardiac drug (nortriptyline) may be critical and cause life-threatening arrhythmias. This applies in particular when other causes of cardiac sodium channel block are also present, e.g., genetic variants (loss-of-function mutation in SCN5A) and/or functional factors (reduction of sodium current by fast heart rates). Accordingly, use of nortriptyline in the general population is associated with a 4.6-fold increased risk for SCA. Future studies must establish whether these findings also apply to other non-cardiac drugs that block cardiac sodium channels. If so, these findings may serve as basis for development of new guidelines for the safe use of such drugs.

Nortriptyline was initially proposed as a anti-arrhythmic drug, because its cardiac actions resemble those of Class 1A and 1C antiarrhythmic drugs.\textsuperscript{28} However, later studies found that it might be associated with SCA,\textsuperscript{13,29} similar to Class 1A and 1C antiarrhythmic drugs.\textsuperscript{10} Nevertheless, it has remained unknown why nortriptyline evokes arrhythmias at therapeutic dosages in some patients, but not in all. Cellular studies revealed that nortriptyline blocks the cardiac sodium channel.\textsuperscript{30} Ventricular tachyarrhythmias may occur when \(I_{Na}\) is severely reduced.\textsuperscript{10} Yet, such reduction is normally only achieved with nortriptyline overdosage. Thus, when therapeutic nortriptyline dosages are used (as by the index patient), other predisposing factors are required to reduce \(I_{Na}\) sufficiently to provide a setting that is permissive for arrhythmia occurrence. Recognizing such factors is crucial to prevent arrhythmias in individuals who use nortriptyline. In the index patient, an SCN5A loss-of-function mutation and exercise-related fast heart rates acted in concert with nortriptyline to reduce \(I_{Na}\) to a critical level. The important role of fast heart rates in causing VT in the index patient was supported by the observations that VT and syncope occurred only during exercise,
while they had not occurred in the year prior to this analysis, when he had already used nortriptyline, but had not trained for the marathon. Our data are supported by a recent experimental study in isolated canine right ventricular wedge preparations showing that amitriptyline (another tricyclic antidepressant) unmasked BrS ECG pattern and facilitated the development of arrhythmias only when $I_{Na}$ was further counteracted by an increase in the repolarizing potassium current $I_{Ko}$, which opposes $I_{Na}$ during the initial phase of the cardiac action potential.$^{31}$

The c.4719C>T mutation abolished $I_{Na}$ completely (Figure 8A). In the index patient, who was heterozygous for this mutation, this is likely to result in 50% loss in the number of functional cardiac sodium channels (haploinsufficiency), explaining why his resting QRS-intervals in the absence of nortriptyline were prolonged to a similar extent as in ECG-controls who used 150mg nortriptyline (Figure 5A). Fast heart rates, as mimicked by stimulation of ventricular myocytes at high frequencies, decreased the availability of cardiac sodium channels because of their intrinsic opening and closing behavior (Figure 8C). Opening and closing result from transitions of channel domains between different conformational states. Sodium channels open (activate) during the initial phase of the cardiac action potential, and inactivate within milliseconds thereafter. Before they can re-open, channels need time to re-enter their original conformational state (recovery from inactivation) during the diastolic interval that ends at the beginning of the next heart beat. At faster heart rates, this interval becomes too short for full recovery, causing reduction in the fraction of channels available for opening.$^{32}$ The resulting $I_{Na}$ decrease is normally small, as reflected by the minimal reduction of the maximum upstroke velocity of ventricular action potentials at higher stimulation frequencies (Figure 8C), and the lack of change in QRS-duration in ECG-controls during exercise (Figure 5C). However, when $I_{Na}$ is already reduced at baseline (e.g., due to an SCN5A mutation), additional $I_{Na}$ reduction by fast heart rates may be sufficient to reduce cardiac excitability, as indicated by QRS-prolongation in BrS patients, including the index patient, during exercise (Figure 5C). In addition, fast heart rates allow nortriptyline to reduce the cardiac sodium current, because nortriptyline requires repetitive opening and closing of sodium channels to act as blocker (use-dependent block),$^{33}$ as it blocks these channels more effectively in their inactivated state. Thus, nortriptyline blocks cardiac sodium channels more potently at fast heart rates, when the channels enter their inactivated state more frequently. This was reflected by the extreme QRS-prolongation in the index patient when heart rates exceeded 150bpm during nortriptyline use (Figure 1 and Figure 5C), and by the reduction of the maximum upstroke velocity of ventricular action potentials in the presence of nortriptyline at higherstimulation frequencies (Figure 8C).

Having determined how nortriptyline may cause potentially lethal cardiac sodium channel block, we determined whether nortriptyline use increases SCA risk in the general population. We found that this risk is increased 4.6-fold, in agreement
with previous studies that linked established cardiac sodium channel blocking drugs (Class 1A and 1C antiarrhythmic drugs) to SCA.\textsuperscript{12-14} Consistent with the finding in the index patient that multiple factors may be required to cause critical sodium channel blockade and potentially lethal cardiac arrhythmias, we found that all four SCA cases who used nortriptyline in the community-based study had at least one additional cause of sodium channel blockade. These causes included concomitant use of other cardiac or non-cardiac drugs that block the cardiac sodium channel,\textsuperscript{34} ischemia,\textsuperscript{27} and heart failure.\textsuperscript{10} Although none of the genotyped cases who used nortriptyline had a mutation in \textit{SCN5A}, this does not rule out that genetic predisposition may have contributed to SCA. Mutations in genes which encode proteins that regulate intracellular trafficking and functional properties of sodium channels may also reduce $I_{Na}$ and render individuals more susceptible to VT/VF. Yet, these genes were not tested here.\textsuperscript{4} Of note, our finding that additional causes of sodium channel blockade may be required for nortriptyline to cause SCA is analogous to studies into the risk for SCA of QT-prolonging drugs. In those studies, various risk factors that are associated with QT-prolongation increase the risk for SCA upon use of QT-prolonging drugs.\textsuperscript{5}

Our findings that a very low-frequency (private) genetic variant, as found in the index patient, contributes to SCA risk provides support to the growing realization that susceptibility to complex disease might stem from rare variants acting in concert with common acquired risk factors. Furthermore, our findings stress the need of finding ways to uncover the possible disease-causing potential of mutations that are silent on the coding level.\textsuperscript{35}

In conclusion, we conducted comprehensive clinical, molecular-genetic, functional, and community-based studies on the association between nortriptyline use and lethal cardiac arrhythmias. We demonstrated that nortriptyline at therapeutic dosages may block the cardiac sodium channel to a critical level and cause life-threatening arrhythmias when other genetic and/or non-genetic causes of cardiac sodium channel block are also present. Accordingly, we found that nortriptyline use increases the risk for SCA 4.6-fold in the community. Based on these findings, we recommend clinicians to be extremely careful in prescribing nortriptyline to subjects with QRS-prolongation at baseline, subjects suspected of BrS, or subjects using other cardiac or non-cardiac drugs known to possess the ability to block the cardiac sodium channel.\textsuperscript{34} For the latter, the website “www.brugadadrugs.org”, which we have recently initiated to ensure worldwide availability of information on safe drug use in BrS patients, may be used. An exercise testing may serve as a tool to unveil a possible pre-existing deficit in conduction reserve before prescribing nortriptyline. However, future studies must establish which screening strategies are most effective to identify individuals at risk for excess mortality from the use of nortriptyline or other non-cardiac drugs that block the cardiac sodium channel.
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Conflict of interest statement: The authors declare that they have no conflict of interest.
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Supplemental Methods

Clinical studies

Mutation analysis

Informed consent was obtained from the index patient, and genomic DNA was extracted from peripheral blood lymphocytes. SCN5A exons and exon-intron boundaries were amplified by polymerase chain reaction (PCR). Mutation detection was performed by denaturing high performance liquid chromatography, followed by sequencing of fragments with an abnormal elution profile. The presence of the identified mutation was tested in the parents of the index patient and in 1740 unrelated control individuals of European descent (DNA-controls). We also looked up the presence of the mutation in the 1000Genomes database, the NHLBI Exome Variant Server and the Genome of the Netherlands Project (GoNL).

Molecular genetic and biophysical studies

cDNA analysis

Blood samples from the patient and two control Caucasian individuals (SCN5A-controls) were collected in PAXgene tubes. Total RNA was isolated from blood lymphocytes. SCN5A transcripts were reverse transcribed and PCR-amplified using primers located in exon 26 (5’TCCAAGAAGCCGAGACG3’; F) and 28 (5’ACATCATGAGGGCAAGG3’; R, also used for first-strand synthesis). Subsequently the PCR products were sequenced.

Exon-trapping

A 622 base pair SCN5A genomic fragment containing exon 27 and flanking intronic sequences (197 base pairs upstream and 154 base pairs downstream) was PCR-amplified using DNA from the index patient as template. PCR products were subsequently cloned into the pSPL3 exon-trapping vector of the Exon Trapping System kit (Life Technologies), and wild-type and mutant recombinant clones were identified by DNA sequencing. Wild-type and mutant pSPL3-exon 27 plasmids were transfected into human embryonic kidney (HEK-293) cells using EscortV transfection reagent (Sigma). Forty-eight hours post-transfection, cells were harvested and total RNA was isolated. Splicing products were reverse transcribed and PCR-amplified following the instructions of the manufacturer (Exon Trapping System kit; Life Technologies). PCR-products were analyzed using electrophoresis and DNA sequencing.

Generation of expression construct

The megaprimer approach was used to generate mutant SCN5A cDNA in which the final 96 base pairs of exon 27 were deleted. The megaprimer was synthesized by PCR using wild-type SCN5A cDNA as template and 5’CCATGAGAGCTGGGCTCCA3’ (F) and GATGATGTCCGAGAGCACAGTGCCTGTGAAGATGGCCACAA3’ (R) as primers. The megaprimer (258 base pairs) was gel-purified and used in a second PCR reaction with reverse primer 5’AGGGTGGGAAAGTGAGG3’ (R) and genomic DNA as template. This PCR product was cloned into the pSP64T-SCN5A construct via BstEII and BsaAI restriction sites. Subsequently, mutant SCN5A
cDNA was subcloned into the expression vector pCGI for bicistronic expression of the channel protein and GFP reporter.

Cellular electrophysiology

The effects of the mutation on sodium currents were assessed in HEK-293 cells transfected with 0.5μg of pCGI-SCN5A (wild-type or mutant) construct using Lipofectamine (Invitrogen), at 48 hours post-transfection. The effects of nortriptyline on cardiac ventricular action potentials were measured in left ventricular myocytes isolated from healthy New Zealand White rabbit hearts. Measurements were performed with the patch-clamp technique.27

Community-based study

Setting

We conducted a case-control study using the Amsterdam Resuscitation Studies (ARREST) infrastructure. ARREST is an ongoing prospective, community-based study aimed at establishing the genetic and clinical determinants of SCA in the general population in a contiguous region (urban and rural communities, ~2.4 million inhabitants) of the Netherlands. Data were retrieved in the study period July 2005 – January 2008. Details of the study design are provided elsewhere.28-30 In short, the ARREST research group prospectively collects data of all cardiopulmonary resuscitation efforts in collaboration with all Emergency Medical Services in the study region, using a mandatory multiple-source notification system (consisting of personnel of dispatch centers, ambulance services and all 14 area hospitals). ARREST is staffed 24 hours per day and 7 days per week. This ensures complete coverage of the study region and inclusion of >95% of all patients with out-of-hospital SCA.28-30 ARREST includes all such patients. A data collection infrastructure is used that records all out-of-hospital SCA parameters, from ambulance dispatch to discharge from the hospital or to death. Cases are included as follows. After each suspected out-of-hospital SCA, the dispatch center notifies the study center, providing information on the place and circumstances of SCA. Ambulance personnel are obliged by protocol to send the ECGs to the study center by modem directly after resuscitation, and to call the study center after every out-of-hospital SCA to provide extra information on the resuscitation (e.g., whether SCA was witnessed, whether basic life support was provided before arrival of ambulance personnel, whether the patient died at the resuscitation site or was transported to a hospital). If an automated external defibrillator (AED) was used before arrival of the ambulance personnel, the study center is notified by the dispatch center, ambulance personnel and/or the user of the AED (most AEDs in the study region carry a label with the request to notify the study center after the AED is used). ARREST personnel visit the resuscitation site upon notification to collect the AED ECG recording. ECG recordings from the ambulance monitor/defibrillator or AED are used to determine whether VT/VF occurred during SCA.

Definition of SCA and inclusion of cases and controls in ARREST

SCA in ARREST was defined as cardiac arrest due to cardiac causes in an out-of-hospital setting with ECG-documentation of VT/VF. Patients with VT/VF in whom cardiopulmonary resuscitation resulted in their survival were also classified as SCA. Cases with a non-cardiac cause of VT/VF (e.g., trauma, intoxication,
drowning, suicide) or without VT/VF (these patients typically had asystole or pulseless electrical activity) were excluded. Matched controls (by age, sex, and index date) were randomly drawn from the general population, using a database of community pharmacies (PHARMO Record Linkage System). This system includes the demographic details and complete medication histories from community pharmacies for >2 million community-dwelling residents. Each case was matched to up to five PHARMO-controls. The index date was defined as the date on which VT/VF occurred in cases. The controls were assigned the same index date as the patient to whom they had been matched.

Exposure definition of nortriptyline
All medication prescriptions of ARREST cases and PHARMO-controls during the year before the index date were identified from computerized databases of pharmacists. Because virtually all patients in the Netherlands are registered with a single community pharmacy, pharmacy records are essentially complete. Prescription durations were calculated by dividing the total number of units issued per prescription by the prescribed daily number of units. Use of medication was defined current if the index date fell within the prescribed duration or within a maximum of 10% after the prescribed duration (to deal with carry-over effects).

Additional factors impairing cardiac excitability in ARREST
For cases who used nortriptyline, it was investigated whether they had additional factors that impaired cardiac excitability by retrieving data from the hospital of admission and/or the general practitioner. The following established factors that impair cardiac excitability were assessed: other (than nortriptyline) cardiac or non-cardiac drugs that block the cardiac sodium-channel, exercise prior to SCA, fever (acute) cardiac ischemia, and heart failure. The presence of mutations in SCN5A was assessed in cases who used nortriptyline, of whom DNA was available.

Covariates and risk factors
For all cases and PHARMO-controls, the following established risk factors for SCA were assessed: cardiac ischemia, heart failure, hypertension, diabetes mellitus, and hypercholesterolemia. Risk factors were derived from medication use. Use of QT-prolonging drugs, antiarrhythmic drugs, cardiac glycosides, diuretics, angiotensin converting enzyme-inhibitors, angiotensin receptor-blocking agents, -blockers and calcium channel blockers were also considered as covariates.

Mutation analysis
Informed consent was obtained from cases who used nortriptyline on the index date, and SCN5A mutation analysis was conducted as in the index patient.

Statistical analysis
Data are mean±standard error of the mean (SEM). Group comparisons were made with Student’s t-test or Analysis of Variance, where appropriate. The relative risk for VT/VF associated with nortriptyline use was estimated by calculating the adjusted odds ratio with 95% confidence interval (OR [95%CI]) using
conditional logistic regression analysis. Covariates and risk factors which were univariately associated with VT/VF (at a p<0.1 level) were included in the regression analyses if they changed the point estimate of the association between nortriptyline use and VT/VF by >5%. All analyses were performed using SPSS (version 16.0 for Mac, Chicago IL, USA).