**NOS1AP Is a Genetic Modifier of the Long-QT Syndrome**

Lia Crotti, MD, PhD; Maria Cristina Monti, PhD; Roberto Insolia, BSc; Anna Peljto, MS; Althea Goosen, BSc; Paul A. Brink, MD; David A. Greenberg, PhD; Peter J. Schwartz, MD*; Alfred L. George, Jr, MD*

**Background**—In congenital long-QT syndrome (LQTS), a genetically heterogeneous disorder that predisposes to sudden cardiac death, genetic factors other than the primary mutation may modify the probability of life-threatening events. Recent evidence indicates that common variants in *NOS1AP* are associated with the QT-interval duration in the general population.

**Methods and Results**—We tested the hypothesis that common variants in *NOS1AP* modify the risk of clinical manifestations and the degree of QT-interval prolongation in a South African LQTS population (500 subjects, 205 mutation carriers) segregating a founder mutation in *KCNQ1* (A341V) using a family-based association analysis. *NOS1AP* variants were significantly associated with the occurrence of symptoms (rs4657139, \( P = 0.019 \); rs16847548, \( P = 0.003 \)), with clinical severity, as manifested by a greater probability for cardiac arrest and sudden death (rs4657139, \( P = 0.028\); rs16847548, \( P = 0.014 \)), and with greater likelihood of having a QT interval in the top 40% of values among all mutation carriers (rs4657139, \( P = 0.03 \); rs16847548, \( P = 0.03 \)).

**Conclusions**—These findings indicate that *NOS1AP*, a gene first identified as affecting the QTc interval in a general population, also influences sudden death risk in subjects with LQTS. The association of *NOS1AP* genetic variants with risk for life-threatening arrhythmias suggests that this gene is a genetic modifier of LQTS, and this knowledge may be clinically useful for risk stratification for patients with this disease, after validation in other LQTS populations. ([Circulation. 2009;120:1657-1663.])

**Key Words:** arrhythmia ■ genetics ■ KCNQ1 protein, human ■ long-QT syndrome ■ nitric oxide synthase

The congenital long-QT syndrome (LQTS) is an inherited disorder of abnormal myocardial repolarization in which there is a high risk for potentially lethal cardiac arrhythmias. The disorder is caused by mutations in several genes, most of which encode ion channel subunits involved in the regulation of the cardiac action potential. The most common form of LQTS (LQT1) is caused by mutations in *KCNQ1*, a gene encoding the pore-forming subunit of potassium channels responsible for the slow cardiac delayed rectifier current. In many families, LQTS exhibits incomplete penetrance and variable expressivity, which suggest the existence of factors other than the primary mutation that can modify the probability of symptoms. Identification of genetic modifiers of LQTS would lead to improved risk stratification among mutation carriers and could also provide information about the risk for life-threatening arrhythmias in more common conditions, such as acute myocardial infarction and congestive heart failure.

**Clinical Perspective on p 1663**

A prolonged QT interval is a surrogate measurement of prolonged ventricular repolarization and is a widely recognized subclinical marker for increased risk of life-threatening cardiac arrhythmia in congenital and acquired forms of LQTS and after a myocardial infarction. A recent genome-wide association study identified genetic variation in *NOS1AP*, which encodes a nitric oxide synthase adaptor protein, as a contributor to QT-interval duration in the general population. Although the absolute quantitative effect of *NOS1AP* variants on the QT interval in healthy subjects was small, explaining up to 1.5% of QT interval variation, the replication of this finding in several distinct populations demonstrated that the
association is robust.10–18 Further analyses have found an association between NOS1AP and risk for sudden death in a general population19 and increased cardiovascular mortality in users of calcium channel blockers.20 Whether genetic variation in NOS1AP contributes to the risk of sudden death in congenital LQTS is not known.

We tested the hypothesis that NOS1AP is a genetic modifier of LQTS in a South African population segregating the KCNQ1-A341V mutation and exhibiting variable disease expression among mutation carriers.21 This population is particularly well suited for testing genetic modifier hypotheses because all at-risk subjects share the same disease-causing mutation, a feature that offers advantages over using LQTS populations having heterogeneous mutations in multiple different genes, a factor known to confer varying levels of arrhythmia risk.22–24

Methods

Study Population

We studied a LQT1 South African founder population of mixed Dutch and French Huguenot origin harboring a mutation in KCNQ1 (A341V).21 Cardiac events were defined as syncope (fainting spells with transient but complete loss of consciousness), aborted cardiac arrest (requiring resuscitation), and sudden cardiac death. Mutation carriers were classified as either symptomatic or asymptomatic. Symptomatic subjects were mutation carriers who experienced at least 1 cardiac event; to be defined as asymptomatic, a mutation carrier had to be at least 15 years old and not treated with a β-blocker or calcium channel blocker. In addition to symptomatic mutation carriers, the study population also included mutation carriers with transient but complete loss of consciousness, aborted cardiac arrest (requiring resuscitation), and sudden cardiac death. Mutation carriers were classified by the severity of their clinical manifestations into 2 groups: those with a severe form of the disease (cardiac arrest or sudden cardiac death) and those with a milder form of the disease (symptomatic patients with no cardiac arrest/sudden cardiac death). Baseline ECGs recorded in the absence of β-blocker therapy were coded and subsequently analyzed by 1 investigator (L.C.) blinded to genotype. Baseline heart rate and duration of the QT and RR intervals in leads II and V1 were measured from resting 12-lead ECGs. To allow QT values to be compared among subjects, the QT interval was corrected for heart rate (QTc) with the use of the Bazett formula.

All probands and family members provided written informed consent for clinical and genetic testing. Protocols were approved by the ethical review boards of Tygerberg Hospital of Stellenbosch University and the University of Pavia and by the Vanderbilt University institutional review board. Approved consent forms were provided in English or Afrikaans as appropriate.

Genotyping

Genotyping of index cases and family members for the A341V mutation was described previously.21 The NOS1AP variants rs4657139, rs16847548, rs12567209, rs10494366, and rs6683968 were genotyped with the use of the TaqMan assay (ABI Prism 7900HT; Applied Biosystems, Foster City, Calif). Three of these variants (rs10494366, rs4657139, rs6683968)9,11,25 were among the first to be tested for association with QT interval in general populations, whereas the remaining 2 variants (rs16847548, rs12567209) were associated with sudden cardiac death in a community-based study.21 The Figure, panel A, provides the minor allele frequency for each of the tested polymorphisms in our population and in the Western European ancestry sample of the HapMap Project.

Statistical Analysis

Data are reported as mean and SD for continuous variables; whenever the distribution was skewed, median, interquartile range, or quintiles were reported. Differences in baseline characteristics among groups of subjects were assessed with either a t test or χ2 test. Two-sided P values <0.05 were considered statistically significant. Association analyses were performed with the use of the pedigree disequilibrium test (PDT), which allows the use of related trios and discordant sib pairs from extended pedigrees to identify associations of disease and marker.20 Extended pedigrees are ideally suited for analysis by PDT, and the program is robust to any nonindependence among pedigrees.27 In the original description of this founder population, there were 22 extended pedigrees, including up to 5 generations that could be genealogically linked. Because PDT can only handle up to 3-generation families, we subdivided the founder population into a series of 49 nonoverlapping 3-generation pedigrees. Triads were then defined as informative nuclear families in which there is at least 1 affected child, both parents genotyped at the marker, and at least 1 heterozygous parent. Discordant sib pairs were also informative if they had at least 1 affected and 1 unaffected sibling with different marker genotypes, with or without parental genotype data. Informative extended pedigrees contained at least 1 informative nuclear family and/or discordant sibship. Affectedness status of subjects was dependent on the phenotype of interest: symptomatic mutation carriers, symptomatic mutation carriers with a severe form of the disease, or mutation carriers with a prolonged QTc.

Because we examined association with up to 5 variants, we applied a correction for multiple testing based on the spectral decomposition of matrices of the pairwise correlation coefficient (r) between variants.28,29 This method estimates the effective number of independent markers (Mv41) by taking account of the intermarker linkage disequilibrium (LD); the test criteria are then adjusted by the Bonferroni correction as though there were Mv41 independent tests. Using this approach, we determined that there were 4 effectively

<table>
<thead>
<tr>
<th>Variant</th>
<th>Alleles</th>
<th>MAF</th>
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<td>A</td>
<td>0.31 (0.29)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>rs16847548</td>
<td>C</td>
<td>0.20 (0.13)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>rs12567209</td>
<td>A</td>
<td>0.05 (0.12)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>rs10494366</td>
<td>G</td>
<td>0.32 (0.37)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>rs6683968</td>
<td>T</td>
<td>0.31 (0.30)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td></td>
</tr>
</tbody>
</table>

Figure. Variants and LD in NOS1AP. A, Minor allele frequencies (MAF) for each NOS1AP variant observed in the study population and in the Western European ancestry sample of the HapMap Project (numbers in parentheses). The minor allele is listed on top. B, Pairwise LD between 5 NOS1AP variants determined with the use of HapMap data for white Europeans. The value within each diamond represents the pairwise correlation between variants (measured as r) defined by the top left and top right sides of the diamond. The approximate locations of NOS1AP exons 1 and 2 are shown as black squares.
independent tests among the 5 genotyped NOS1AP variants. Therefore, in the initial PDT association analysis between symptoms and NOS1AP genotype, we used a corrected α-level of 0.0125 (0.05/4) as the threshold for statistical significance.

We also performed an empirical calculation of the type I error level, which is not dependent on any explicit or hidden statistical assumptions of the PDT method. With the use of an extensive computer simulation facility developed by 1 of the authors (P.D.-power, D.A.G.), a computer simulation randomly assigned marker genotypes to the exact family structures of the families in the data set. Marker and disease loci were simulated to be biallelic, and the loci were in linkage equilibrium. The relationship of the markers to the disease locus represented the null hypothesis (that is, there was no association between the disease and the marker). We simulated 10,000 such data sets and showed that the false-positive rate followed a χ² distribution. Thus, the particular characteristics of this data set represented no unusual or confounding problems to the PDT.

Statistical calculations were performed with the use of STATA 10 (StataCorp, College Station, Tex) and the PDT software (http://www.chg.duke.edu/research/pdt.html). LD across NOS1AP was evaluated with the use of Haploview (http://www.broad.mit.edu/mpg/haploview) with the HapMap data for this region (http://www.hapmap.org). The r² correlation coefficient (Figure, panel B) and the normalized disequilibrium coefficient (D’) were used as a measure of LD.30

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Study Population
We studied a South African LQT1 (KCNQ1-A341V mutation) population that consisted of 500 family members, of whom 205 were mutation carriers, 228 were noncarriers, and 67 were not genetically tested. For this study, DNA samples were available for 255 subjects. There was no sex bias (female subjects, 47%; male subjects, 53%). Among the 205 mutation carriers, there were 174 subjects that had a clearly defined phenotype status. Thirty mutation carriers were classified as asymptomatic and were older than 15 years and not treated with β-adrenergic receptor antagonists (β-blockers); 9 other subjects without symptoms were too young (age <15 years) to be classified.21 Among the 165 subjects with a defined and classifiable phenotype, 135 had symptoms (82%) (syncope with transient but complete loss of consciousness, aborted cardiac arrest requiring resuscitation, or sudden cardiac death), with a median age at first cardiac event of 6 years (interquartile range, 4 to 10). Among the 135 symptomatic subjects, 56 suffered cardiac arrest and/or sudden cardiac death, and the remaining 79 symptomatic mutation carriers had only syncope. These findings are consistent with the unusual severity of this particular mutation as demonstrated by our prior analysis of 21 unrelated families from 8 different countries all carrying the KCNQ1-A341V mutation.31

One hundred nine mutation carriers and 101 noncarriers with a resting ECG recorded in the absence of β-blocker therapy were analyzed for differences in QTc interval. Baseline QTc was longer in mutation carriers than in noncarriers (487±44 versus 402±23 ms; P<0.001), with no significant differences in mean age at the time of ECG recording or distribution of male and female subjects between the 2 groups. Despite sharing the same genetic defect, mutation carriers exhibited a wide range of QTc (397 to 676 ms).

Association Between NOS1AP and Clinical Manifestations
NOS1AP variants were genotyped in 255 individuals (143 mutation carriers, including 135 with a classifiable phenotype and 8 subjects younger than 15 years, and 112 noncarriers) grouped into 49 three-generation pedigrees derived from the founder population. We analyzed the association between symptoms and NOS1AP genotype using the PDT in 30 informative pedigrees including 29 informative triads and 102 informative discordant sib pairs that were selected by the PDT software. Two NOS1AP variants exhibited differential transmission when evaluated for association with the occurrence of cardiac symptoms (rs4657139, PDT P=0.019; rs16847548, PDT P=0.003; Table 1). After correction for multiple hypothesis testing (see Methods), only the minor allele of rs16847548 (C allele) remained significantly associated with an increased risk of cardiac events. However, these 2 variants (rs4657139, rs16847548) were only 6 kb apart and are in LD (D’=1; r²=0.36) among white subjects of Western European ancestry genotyped by the HapMap project. Three other NOS1AP variants (rs12567206, rs10494366, rs6683968) were not significantly associated with symptoms in the South African LQTS population. The unassociated variants were either distant from the other 2 markers in NOS1AP (rs10494366, rs6683968) or exhibited very low minor allele frequency (rs12567206; Figure). The 2 markers with high r² had minor allele frequency values of 31% and 20% (Figure).

We further tested whether the NOS1AP risk alleles at rs4657139 and rs16847548 were associated with the occurrence of severe cardiac events (cardiac arrest, sudden death) among symptomatic KCNQ1-A341V mutation carriers. In this analysis, rs4657139 and rs16847548 were both significantly associated with the risk of life-threatening events (rs4657139, PDT P=0.028; rs16847548, PDT P=0.014), suggesting that NOS1AP variants modify risk for life-threatening cardiac events in this Afrikaner LQTS population. We cannot compute a relative risk for life-threatening events caused by the presence of the risk allele because mutation carriers are related, which produces a risk that is biased upward. However, an odds ratio can be considered the upper bound of the risk calculated with the use of unrelated symptomatic subjects. With that caveat, mutation carriers with at least 1 copy of the minor allele at rs16847548 or rs4657139 have a 1.4 (95% confidence interval, 0.76 to 2.6) or 1.8 times (95% confidence interval, 1.1 to 3.3) greater chance of having life-threatening events than the mutation carriers without the minor allele, respectively.

Association Between NOS1AP and QT Interval
We also tested for association between the 2 NOS1AP variants that were associated with symptoms and the QTc. We examined allele sharing between 2 groups of KCNQ1-A341V mutation carriers defined by the upper and lower 40% of QTc values. We did not consider the central quintile in this analysis to avoid the inclusion of a confounding “gray area.” Therefore, this analysis only included mutation carriers with QTc ≤472 ms or QTc >492 ms as measured by a resting ECG in the absence of β-blockers (n=118) to avoid the
confounding effects of treatment. Among 21 informative pedigrees included in this analysis, there were 14 informative triads and 49 informative discordant sib pairs.

Minor alleles of the 2 NOS1AP variants associated with symptoms were significantly associated with a QTc $>$492 ms in the population (rs4657139, PDT $P=0.03$; rs16847548, PDT $P=0.03$; Table 2), which is consistent with the effect of these variants on QTc observed in healthy populations.$^9$–$^{18}$ Importantly, QTc prolongation associated with NOS1AP was observed in subjects despite an already markedly prolonged QTc interval.

## Discussion

The main finding of our study is that common NOS1AP variants are modifiers of the clinical severity of congenital LQTS and are associated with a greater chance of having a more prolonged QT interval in mutation carriers. This is the first evidence, demonstrated in subjects sharing the same mutation, that NOS1AP variants are associated with a greater risk for cardiac arrest and sudden death in LQTS. These findings may contribute to the refinement of individual risk stratification in LQTS and help to prompt consideration of new mechanistic hypotheses of arrhythmia susceptibility in this disease.

### Table 1. PDT for the Association Between NOS1AP Variants and Symptoms in a South African LQTS Population

<table>
<thead>
<tr>
<th>SNP and Allele</th>
<th>Triads (Parental Contribution)</th>
<th>Discordant Sib Pairs</th>
<th>PDT Statistic</th>
<th>$P$</th>
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<td></td>
<td>Transmitted</td>
<td>Not Transmitted</td>
<td>Affected</td>
<td>Unaffected</td>
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<td>Association with any symptoms</td>
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<tr>
<td>n</td>
<td>58</td>
<td>58</td>
<td>56</td>
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<td></td>
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<tr>
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<td>39</td>
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<td>95</td>
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</tr>
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<td>C</td>
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<td>25</td>
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*No. of subjects is shown in parentheses.

### Table 2. PDT for the Association Between NOS1AP Variants and QTc Interval (QTc $\geq$493 ms vs QTc $\leq$472 ms) in KCNQ1-A341V Mutation Carriers

<table>
<thead>
<tr>
<th>SNP and Allele</th>
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<th>PDT Statistic</th>
<th>$P$</th>
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<tr>
<td></td>
<td>Transmitted $n=28^*$</td>
<td>Not Transmitted $n=28$</td>
<td>QTc $&gt;492$ $n=25$</td>
<td>QTc $\leq472$ $n=37$</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>13</td>
<td>6</td>
<td>19</td>
<td>22</td>
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<tr>
<td>T</td>
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<tr>
<td>C</td>
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<td>1</td>
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NOS1AP and the Clinical Manifestations of LQTS

Inherited arrhythmia susceptibility, such as in LQTS, is a known cause of sudden cardiac death, especially in young adults and children. Accurate risk stratification is critically important for effective utilization of preventive strategies, but even among subjects found to carry the same LQTS mutation, the probability of life-threatening cardiac events can vary considerably. This clinical heterogeneity can be explained in rare cases by compound heterozygosity, but common genetic factors other than the primary disease-causing mutation are also likely modifiers of arrhythmic risk. Defining genetic modifiers of LQTS could have a significant impact on the accuracy of individual risk stratification.

We had the opportunity to test NOS1AP as a candidate LQTS modifier gene in a large group of subjects carrying the same mutation as the underlying cause for arrhythmia susceptibility. This unique study design eliminates the confounding effects of genetic and allelic heterogeneity that is present when a study involves multiple different disease-causing mutations that are known to carry widely different arrhythmic risk.

We specifically studied an LQT1 founder population harboring a mutation in KCNQ1 (A341V) that exhibits a wide range of QTc values and clinical manifestations. The novel finding is that the minor allele at common NOS1AP variant rs16847548 is associated with the risk of cardiac events and, importantly, with the occurrence of life-threatening events. These findings are in agreement with the association of rs16847548 with the risk of sudden cardiac death demonstrated in a general population of white Americans.

NOS1AP and the QT Interval in LQTS

We also observed an association between the minor allele of 2 NOS1AP variants (rs4657139 and rs16847548) with the probability of having QTc duration in the top 40% of all QTc values among mutation carriers. Although this observation may not seem surprising at first glance given the prior associations with QT duration in general populations, we regarded this finding as somewhat unexpected. Whereas a modest effect on QT duration was detectable in very large populations having mean QT values within a normal range, we were not certain that an association of NOS1AP with QT could be detected in an LQTS population with a mean QTc value close to 500 ms because of a predicted “ceiling effect” in which the contribution of the underlying mutation to QT interval duration might dwarf any minor effect of NOS1AP variation. This is why we were impressed by the fact that, even with a small sample size and analysis of QTc as a categorical variable, the association of rs4657139 and rs16847548 with QTc could be demonstrated in our LQTS population.

Potential Biological Influence of NOS1AP Genetic Variants

There is scant information on the biological influence of NOS1AP genetic variation on function or expression of the gene and the manner in which this relates to effects on the QT interval or risk for cardiac events. Because NOS1AP variants associated with the QT interval are located in noncoding regions of the gene, the presumption is that transcriptional influences exerted by cis-acting elements may differ among alleles. Work from laboratories investigating genetic associations between NOS1AP and schizophrenia has elucidated potential transcriptional effects of certain common variants with the use of in vitro reporter-gene experiments. Specifically, the A allele of 1 variant (rs12742393) located in the second intron enhances binding of a presumed nuclear transcription factor and drives greater transcriptional activity of the NOS1AP promoter in human neural cell lines. Similar studies with the use of cardiac tissue have not been published.

Although the potential transcriptional effects of NOS1AP variants on gene expression in heart are not known, Chang et al found that overexpression of the NOS1AP gene product (CAPON) in isolated guinea pig myocardies causes attenuation of L-type calcium current, a slight increase in rapid delayed rectifier current (I\text{Kr}), and shortening of action potentials. These observations suggest plausible cellular mechanisms that might explain our findings in this study. For example, if we postulate that genetic variants in NOS1AP impair expression and lead to lower levels of CAPON, then, based on the study by Chang et al, we might expect increased L-type calcium current with associated arrhythmogenic consequences. Furthermore, because calcium current is enhanced by sympathetic activation, a greater effect would be anticipated in conditions associated with augmented catecholamine release such as physical or emotional stress, the predominant clinical circumstances associated with lethal arrhythmic episodes in LQT1.

Study Limitations

By studying this highly unique founder population, we take advantage of genetic homogeneity, which is essential for assessing the contribution of potential modifiers. However, the limitation of this approach is that the feasibility of performing a comparable replication study is extremely low. Whether our findings made in this founder population will apply to LQTS mutation carriers in other populations remains to be determined. Because of the restricted size of our study population, the statistical power of the data was insufficient to test all known NOS1AP variants previously associated with variation of QT duration or an unlimited number of other candidate variants. Furthermore, a much larger population would have been required to examine effects of NOS1AP variants on the QT interval analyzed as a continuous variable. Ascertainment bias could have influenced our results because subjects carrying both KCNQ1-A341V and the NOS1AP risk allele have a greater probability of sudden death. However, this potential bias would have actually diminished our chances of observing a significant association. This suggests conceptually that our findings are robust to any selection bias imposed by the greater risk of death in such carriers.

Conclusion

We have demonstrated a significant association between common variants in NOS1AP and the clinical severity of LQTS with special reference to life-threatening arrhythmias. The association of NOS1AP genetic variants with risk for life-threatening arrhythmias points to NOS1AP as a genetic modifier of LQTS, and this knowledge should become
clinically useful for risk stratification after validation in other LQTS populations.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Congenital long-QT syndrome (LQTS) is a type of heritable primary cardiac arrhythmia susceptibility disease and a known cause of sudden death, especially in young adults and children. Among those found to carry the same mutation, the probability of life-threatening cardiac events can vary considerably, leading to the hypothesis that genetic factors other than the primary disease-causing mutation may modify arrhythmic risk in LQTS. Common genetic variants in *NOS1AP* are associated with the QT-interval duration in the general population, and in this study, we tested whether *NOS1AP* variants modify the risk of clinical manifestations and the degree of QT-interval prolongation in members of a large South African LQTS population all carrying the same mutation (*KCNQ1*-A341V). We found that the minor alleles of 2 *NOS1AP* variants were associated with increased risk of life-threatening events and with the probability of having a rate-corrected QT interval in the upper 40th percentile (>492 ms) of values in the study population. These observations indicate that *NOS1AP* is a genetic modifier of LQTS, and this knowledge should become clinically useful for risk stratification after validation in other LQTS populations.
**NOS1AP Is a Genetic Modifier of the Long-QT Syndrome**

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