Analysis for genetic modifiers of disease severity in patients with Long QT Syndrome Type 2

Kolder et al., *Genetic modifiers in LQT2*

Iris C.R.M. Kolder PhD[1,2]*, Michael W.T. Tanck PhD[2]*, Pieter G. Postema MD PhD[1]*, Julien Barc PhD[1,3-6], Moritz F. Sinner MD[7,8], Sven Zumhagen MD[9], Anja Husemann[9,10], Birgit Stallmeyer[9], Tamara T. Koopmann PhD[1], Nynke Hofman PhD[11], Arne Pfeufer MD[12-14], Peter Lichtner PhD,[13] Thomas Meitinger PhD[13,14], Britt M. Beckmann MD[7,8], Robert J. Myerburg MD[15-17], Nanette H. Bishopric MD[15-17], Dan M. Roden MD[18], Stefan Kääb MD, PhD[7,8], Arthur A.M. Wilde MD, PhD[1,3,19] Jean-Jacques Schott PhD[4-6,20,∫], Eric Schulze-Bahr MD [9,10,∫], Connie R. Bezzina PhD[1,3,∫]

*∫These authors contributed equally

1 Department of Clinical and Experimental Cardiology, Academic Medical Center, Amsterdam, The Netherlands.

2 Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, Amsterdam, The Netherlands.

3 ICIN (Netherlands Heart Institute), Utrecht, The Netherlands

4 Institut National de la Santé et de la Recherche Médicale (INSERM) Unité Mixte de Recherche (UMR) 1087, L'Institut du Thorax, Nantes, France.

5 Centre National de la Recherche Scientifique (CNRS) UMR 6291, Nantes, France.

6 Université de Nantes, Nantes, France.

7 Department of Medicine I, University Hospital Munich, Campus Grosshadern, Ludwig-Maximilians University, Munich, Germany.

8 Munich Heart Alliance, Munich, Germany.

9 Institute for Genetics of Heart Diseases, Department of Cardiovascular Medicine,, University Hospital Münster, Germany.

10 Interdisciplinary Centre for Clinical Research (IZKF) of the University of Münster, Germany.

11 Department of Clinical Genetics, Academic Medical Center, Amsterdam, The Netherlands.

12 Institute of Genetic Medicine, EURAC Research, Bolzano, Italy.

13 Institute of Human Genetics, Helmholtz Zentrum Munich, Neuherberg, Germany.

14 Institute of Human Genetics, Technische Universität München, Munich, Germany.

15 Department of Medicine, University of Miami Miller School of Medicine, Miami, Florida, USA.

16 Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, Florida, USA.

17 Hussmann Institute of Human Genomics, University of Miami Miller School of Medicine, Miami, Florida, USA.

18 Department of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, USA.

19 Princess Al-Jawhara Al-Brahim Centre of Excellence in Research of Hereditary Disorders, Jeddah, Kingdom of Saudi Arabia

20 Centre Hospitalier Universitaire (CHU) Nantes, L'Institut du Thorax, Service de Cardiologie, Nantes, France.

Corresponding author: Connie R. Bezzina, Department of Clinical and Experimental Cardiology, Room L2-108, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. C.R.Bezzina@amc.uva.nl

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Abstract

Background - Considerable interest exists in the identification of genetic modifiers of disease severity in the Long QT Syndrome (LQTS) as their identification may contribute to refinement of risk stratification.

Methods and Results – We searched for single nucleotide polymorphisms (SNPs) that modulate the QTc-interval and the occurrence of cardiac events in 639 patients harboring different mutations in *KCNH2*. We analyzed 1,201 SNPs in and around 18 candidate genes, and in another approach investigated 22 independent SNPs previously identified as modulators of the QTc-interval in genome-wide association studies (GWAS) in the general population.

In an analysis for quantitative effects on the QTc-interval, 3 independent SNPs at *NOS1AP* (rs10494366, $p=9.5\times10^{-8}$; rs12143842, $p=4.8\times10^{-7}$; rs2880058, $p=8.6\times10^{-7}$) were strongly associated with the QTc-interval with marked effects (>12ms/allele). Analysis of patients versus general population controls uncovered enrichment of QTc-prolonging alleles in patients for 2 SNPs, located respectively at *NOS1AP* (rs12029454, OR=1.85 [95% CI, 1.32-2.59], $p=3\times10^{-4}$) and *KCNQ1* (rs12576239; OR=1.84 [95% CI, 1.31-2.60], $p=5\times10^{-4}$). An analysis of the cumulative effect of the 6 *NOS1AP* SNPs by means of a multi-locus genetic risk score (GRS_{NOS1AP}) uncovered a strong linear relationship between GRS_{NOS1AP} and the QTc-interval ($p=4.2\times10^{-7}$). Furthermore, patients with a GRS_{NOS1AP} in the lowest quartile had a lower relative risk of cardiac events compared to patients in the other quartiles combined (p=0.039).

Conclusions – We uncovered unexpectedly large effects of *NOS1AP* SNPs on the QTc-interval and a trend for effects on risk of cardiac events. For the first time we linked common genetic variation at *KCNQ1* with risk for LQTS.

Introduction

The congenital Long QT Syndrome (LQTS) is a heritable disorder associated with QTc-interval prolongation on the electrocardiogram (ECG) and an increased risk of sudden cardiac death from torsade de pointes polymorphic ventricular tachycardia. Mutations in multiple genes, primarily encoding ion channel subunits have been identified in patients with the disorder. In around 75% of cases, the disease is caused by the inheritance of a mutation in either *KCNQ1* (LQT1), *KCNH2* (LQT2), or *SCN5A* (LQT3).[1]

Despite previous achievements in gene discovery, important issues in the clinical management LQTS patients remain. As for most Mendelian disorders, patient management is complicated by the variability in disease severity among mutation carriers.[2] Variability is observed both in the extent of the QTc-interval prolongation as well as in the occurrence of arrhythmic events. While some mutation carriers display a severely prolonged QTc-interval, the QTc-interval of others may be within the normal range. Similarly, not all patients suffer arrhythmic events. Established modulators of disease severity include sex, age, heart rate, intake of QTc-prolonging drugs, and affected gene and mutation location.[3–6] Furthermore, in ~10% of cases clinical disease severity can be explained by compound heterozygosity.[7] However, while additional genetic factors are also expected to play a role, these are largely unexplored.[8–10]

We here investigated the role of common genetic variants (minor-allele frequency, MAF >10%) in the form of SNPs in patients with LQT2. In one approach we conducted a comprehensive analysis of haplotype-tagging SNPs in 18 candidate genes. In a second approach we investigated the effect of SNPs that have over the last years been associated with the QTc-interval in GWAS conducted in the general population.[11–15]

Methods

LQT2 patients

The study population consisted of 639 individuals from 254 families of European descent, all harboring a mutation in *KCNH2*. Patients carrying >1 mutation in *KCNH2* or carrying a second mutation in another LQTS gene were excluded. These subjects were drawn from the LQTS registries of four European clinical centers: Amsterdam (The Netherlands), Münster (Germany), Munich (Germany), and Nantes (France). The Medical Ethical Committee at each center approved the study. All subjects or their guardians provided informed consent for genetic and clinical studies. Analyses were conducted in a set of 353 patients (Set 1), a non-overlapping set of 286 patients (Set 2), and in Set 1 and Set 2 combined. Patient Set 1 and Set 2 were drawn a few years apart of each other from the LQTS registries of the same four European academic centers. Routine clinical and ECG parameters were acquired at the time of patient enrollment in each of the registries (see **Data Supplement** for QTc-interval measurement). A first cardiac event was defined as a first unexplained syncope, a first documented ventricular tachycardia or a first aborted cardiac arrest. The observation period for cardiac events started at birth and lasted either to the initiation of anti-adrenergic therapy (β-blockers) or the date of the last medical visit (without anti-adrenergic therapy).

Selection of SNPs and genotyping

Candidate gene SNPs

Eighteen candidate genes (listed in **Supplementary Table 1**) were selected based on their involvement in cardiac arrhythmia syndromes or their role as functionally important subunits of these genes. Since at the time of assay design, the *NOS1AP* locus was already associated with the QTc-interval in GWAS, this gene was also included in the candidate gene study. SNPs for genotyping were selected from all HapMap SNPs available for the CEU population

within the genes and the 50 kb flanking regions. Tag-SNPs were selected using Tagger[16] employing the following criteria: pairwise only tagging with $r^2 \geq 0.8$ and a minor allele frequency (MAF) ≥ 10%. A total of 1424 SNPs were derived in this way for genotype analysis using an Illumina GoldenGate custom assay (**Data Supplement**).

The systematic analysis of haplotype-tagging SNPs in the 18 candidate genes was conducted in LQT2 patient Set 1 (n=353). SNPs found to be significantly associated with the QTc-interval in this analysis were subsequently investigated in LQT2 patent Set 2 (n=286).

SNPs from QTc-interval GWAS

We also investigated SNPs previously associated with the QTc-interval in GWAS conducted in the general population. Twenty-two independent SNPs were identified from the literature.[11–15] SNPs were pruned based on their extent of linkage disequilibrium (LD, $R^2<0.5$). SNPs thus selected were genotyped in patient Sets 1 and 2 combined (n=639; Set 1+ Set 2) using iPLEX Gold chemistry (**Data Supplement**).

Calculation of the Genotype Risk Scores

The genotypes from the 22 SNPs from GWAS studies were used to calculate an un-weighted multi-locus genetic risk score (GRS_{22}). In addition, a GRS based on the six *NOS1AP* SNPs was also generated (GRS_{NOS1AP}). The directionality of the effect of each SNP was based on the original publication[11,12,15,17,18]. For each QT-shortening allele, one point was subtracted from the score, whereas one point was added for each QT-prolonging allele. A negative GRS indicates an excess of QT-shortening alleles, whereas a positive GRS indicates an excess of QT-prolonging alleles.

Statistical analyses

QTc-interval data were normally distributed (Shapiro-Wilk statistic W>0.90) and are reported as mean ± standard deviation.

Effects of *KCNH2* mutation type and/or location, effects of covariates and effects of SNPs and the GRS on the QTc-interval were estimated using the linear mixed effect model function (lmekin). The model fit of the GRS_{NOS1AP} was compared to the GRS_{22} using Akaike's a information criterion (AIC). The effect of SNPs and the GRS on the secondary endpoint 'age at first cardiac event', were estimated using the Cox proportional hazards function (coxme). Both lmekin and coxme functions from the coxme package in R[19] and are correlated random effects models. The models allow for a per-patient random effect that are correlated based on a matrix containing the kinship coefficients for each pair of individuals. This way, dependency between some of the study subjects due to familial relatedness is taken into account. For each SNP-phenotype relationship, an additive genetic model was assumed.

The effects of the SNPs and the GRS on QTc-interval were adjusted for center, sex, age at ECG, proband status, β-blocker use at the time of the ECG, and mutation type and location, whereas the effects of the SNPs on age at first cardiac event were adjusted for center, sex and mutation type and location only. With respect to mutation type and location, mutations were classified into 5 different classes: (1) nonsense, frameshift (small indels or splice site mutations), large deletions and insertions, independent of location, (2) missense, N-terminal, (3) missense, transmembrane S1-S4, (4) missense, transmembrane S5-loop-S6, and (5) missense, C-terminal. The classes were treated in the models as an unordered factor. The annotation of mutation location was based on the Uniprot database (http://www.uniprot.org/uniprot/Q12809).

For the 22 SNPs from QTc-interval GWAS we also compared genotype counts between the probands from Set 1 and Set 2 combined (n=278), with those of 498 general population controls drawn from the Genome of the Netherlands project (GoNL).[20] For 20 of the 22 GWAS SNPs investigated, genotypes were available in all 498 GoNL controls. For the remaining two SNPs genotype information was available in 497 (rs4725982) and 472

(rs2074238) individuals, respectively. Genotype counts were compared using logistic regression assuming an additive genetic model. (No covariates were included due to lack of access to individual participant data for GoNL).

The SNAP tool (http://www.broadinstitute.org/mpg/snap/) was used to assess LD between SNPs using the CEU reference population. The significance thresholds applied and a statement on statistical power can be found in the **Data Supplement**.

Results

Study populations

The characteristics of the LQT2 patients studied are presented in **Table 1**. Patient Set 1 was comparable to patient Set 2 and only differed in the occurrence of cardiac events (p=0.039). Considering Sets 1 and 2 together, QTc-intervals differed significantly between probands (479 ± 50 ms) and relatives (460 ± 40 ms, $p=8\times10^{-7}$). Males and females had similar QTc-intervals (462 ± 44, males; 468 ± 43 ms, females; p=0.06). Beta-blocker use at the time of the ECG did not affect the QTc-interval (464 ± 43 in non-users; 469 ± 49 in users; p=0.32). The relatively low beta-blocker use at the time of ECG (~16%) most likely reflects the fact that the ECGs used in this study were ECGs acquired at enrollment.

Effects of *KCNH2* mutation type and location

Since the type and location of the *KCNH2* mutation may affect the extent of QTc-interval prolongation[6] we evaluated such effects in the patients in the current study. Considering Sets 1 and 2 combined, a total of 197 different *KCNH2* mutations were present among the 639 patients who originated from 254 different families. The number of patients per family ranged from 1 to 20. We grouped the nonsense, frameshift (small indels or splice site mutations) and large duplications/deletions as one category since these mutation types are all expected to

have a drastic effect on the protein structure and likely lead to haploinsufficiency. We detected no difference in extent of QTc-interval prolongation when this category of mutations was compared to missense mutations (p=0.13). We then classified the missense mutations according to the channel sub-domain in which they occurred (locations of missense mutations in the channel are represented in **Supplementary Figure 1)**; we found that carriers of a missense mutation in the transmembrane non-pore region (S1-S4) had on average a longer QTc-interval compared to individuals carrying a missense mutation in any of the other 3 locations, i.e. transmembrane pore region (S5-pore-S6), N-terminus or C-terminus ($p=2.0\times10^{-4}$, **Table 2**). When the 25 patients with S1-S4 region missense mutations were excluded (8 different mutations), patients with a missense mutation in the pore region (transmembrane S5-loop-S6) displayed a longer QTc-interval compared to patients with a non-pore missense mutation (N- or C-terminal, p=0.046) Missense mutations were primarily located at the N- and C-termini and the S5-pore-S6 region (**Supplementary Figure 1**).

Individual SNP effects

Candidate gene study

Following quality control, a total of 1,201 SNPs across the 18 candidate genes were left for analysis for effects on the QTc-interval as a quantitative variable in the 353 LQT2 patients of Set 1 (all association results are listed in the **Data Set file/Supplementary Table 2 available online**). Three SNPs passed the pre-set Bonferroni-corrected p-value threshold for association of $p<4.2\times10^{-5}$ (0.05/1202); these included a SNP at the *NOS1AP* locus (rs16847548), a SNP at *KCNH2* (rs956642) and a SNP at *CASQ2* (rs1935778; **Table 3**). The minor allele at both the *NOS1AP* and *CASQ2* loci was associated with a longer QTc-interval, while that at *KCNH2* was associated with a shorter QTc-interval. The absolute effect sizes per minor allele were >12 ms in all three cases.

The 3 SNPs that were significantly associated with the QTc-interval in Set 1 were subsequently tested in Set 2 (**Table 3**). In Set 2, only the *NOS1AP SNP* (rs16847548) displayed a significant association at the Bonferroni-corrected p-value threshold of $p<0.016$ (0.05/3). The direction of the effect was consistent with that found in Set 1, with the minor allele being associated with a longer QTc-interval. The other two SNPs (rs1935778, rs956642) showed a non-significant effect on QTc-interval in Set 2 (effect <0.5 ms). Combining the results improved the accuracy for all estimates, but the effects of rs1935778 and rs956642 were reduced with 40-50% (**Table 3**).

SNPs from QTc-interval GWAS

Analysis of SNP effects on the QTc-interval analyzed as a quantitative variable: Twenty-two SNPs previously found to associate with the QTc-interval in the general population were analyzed for modulatory effects on the QTc-interval as a quantitative variable in Sets 1 and 2 combined (**Table 4**). Three SNPs (rs10494366, rs12143842, rs2880058), all from the *NOS1AP* locus were found to associate with the QTc-interval at the pre-set Bonferroni-corrected p-value threshold of 2.27×10^{-3} (0.05/22). In all cases the minor allele was associated with a longer QTc-interval and the effect size per minor allele was >12 ms. Of note, rs16847548 which was found to associate with the QTc-interval in the candidate gene study (**Table 3**), is in LD with rs12143842 (R^2=0.88). Four SNPs displayed nominal statistical significance; these were rs12029454 and rs16857031 at *NOS1AP*, rs2074238 at *KCNQ1*, and rs17779747 at *KCNJ2*.

Case-control analysis: The 22 SNPs from QTc-interval GWAS were also investigated for association with LQTS status using a case-control design employing independent probands from Set 1 and 2 combined as cases, and general population individuals from the GoNL project as controls (**Table 5**).[20] In this analysis, 2 SNPs were significantly ($p<2.27\times10^{-3}$) associated with LQTS status and displayed the expected directionality of effect, that is, the

allele associated with a longer QTc-interval in the general population was the risk allele. The SNPs were located at *NOS1AP* (rs12029454) and *KCNQ1* (rs12576239), respectively. Another 7 SNPs displayed a nominal association.

Individual SNP effects on cardiac events

SNPs that displayed a significant or nominal association in any of the above analyses were LD-pruned (R^2<0.5) and assessed for association with cardiac events in Sets 1 and 2 combined (n=639). Since it has been previously suggested that SNP effects on risk of cardiac events might be more pronounced in patients with QTc<500 ms,[9] we also tested for association with cardiac events in this sub-group alone. Of the 12 SNPs tested (**Table 6**), none were associated with cardiac events after correction for multiple testing (0.05/12; $p<4.2\times10^{-3}$). However, three SNPs, two at *NOS1AP* and one at *KCNE1,* were nominally associated with cardiac events. In all three cases, the allele associated with a longer QTc-interval increased risk. The results differed somewhat when LQT2 patients with QTc-interval <500 ms were analyzed separately, with the effect of the *KCNE1* SNP no longer remaining (nominally) significant (**Table 6**). Re-analysis of the 3 SNPs by adding QTc-interval as an additional covariate in the model, resulted in lower and non-significant relative risks for all three (rs10494366, 1.15 [0.92 – 1.44]; rs12029454, 1.27 [1.00 – 1.62]; rs1805128, 1.26 [0.95 – 1.67]).

Genetic risk score

We finally tested the effect of the 22 SNPs from GWAS, and a subset of six *NOS1AP* SNPs thereof, in aggregate by first generating two multi-locus genetic risk scores (GRS_{22} and GRS_{NOS1AP}) per individual and then testing these GRS for association with QTc-interval and occurrence of cardiac events. This analysis was conducted in patients from Sets 1 and 2

combined. The GRS_{22}, that varied from -8 to 14 with a mean (± SD) of 3.0 ± 3.8, was strongly associated with the QTc-interval with an increase of 2.3 (S.E.: 0.50) ms per point increase in GRS_{22} ($p=4.3\times10^{-6}$; **Figure 1A**). There was a linear increase in QTc-interval with increasing GRS_{22}; patients with GRS_{22} in the 2nd, 3rd or 4th quartile had mean QTc-intervals that were, respectively, 7 (S.E. 5), 13 (S.E. 6) and 19 (S.E. 5) ms longer than individuals in the lowest GRS_{22} quartile. When the 6 *NOS1AP* SNPs were not included in the GRS calculation, the correlation between the GRS and the QTc-interval was no longer significant (p=0.15). The GRS_{NOS1AP}, consisting of the six *NOS1AP* SNPs only, showed a similar/better fit than the GRS_{22} (AIC_{GRS22}: 5199.2, $AIC_{GRSNOS1AP}$: 5194.9). The GRS_{NOS1AP}, varying from 0 to 11, was strongly associated with the QTc-interval with an increase of 3.5 (S.E.: 0.69) ms per point increase in GRS_{NOS1AP} ($p=4.2\times10^{-7}$; **Figure 1B**). Patients with GRS_{NOS1AP} in the 2nd, 3rd or 4th quartile had mean QTc-intervals that were, respectively, 14 (S.E. 5), 15 (S.E. 5) and 23 (S.E. 5) ms longer than individuals in the lowest GRS_{NOS1AP} quartile.

No associations were found between GRS_{22} or GRS_{NOS1AP} quartiles and the occurrence of a cardiac event, neither in the entire LQT2 patient sample (GRS22: p=0.192; GRS_{NOS1AP}: p=0.119; **Figure 1C, D, Supplemental Figure 2A, B**), nor in the subset of patients with a QTc-interval <500 ms (data not shown). The results did not differ when only patients with documented VT or aborted cardiac arrest/VF were considered (data not shown). While risk of a cardiac event did not increase linearly between quartiles, inspection of the data in Figure 1C, D and Supplemental Figure 2 suggested that individuals in the quartile with the lowest GRS (Q1) might be protected as opposed to individuals in any of the other 3 quartiles (Q2-4). A statistical comparison of the cumulative event-free survival in these two groups, that is Q1 versus Q2-Q4 uncovered a protective effect for patients in Q1 (GRS_{22} RR 0.67, 95% CI 0.46 – 0.98, p=0.041; GRS_{NOS1AP} RR 0.69, 95% CI 0.48 – 0.98, p=0.039; **Supplementary Figure 3**).

The QTc-interval was a strong predictor of cardiac events in patients with a QTc-interval in the highest quartile with a RR of 2.11 (95% C.I. 1.35-3.30) as compared to patients in the lowest QTc-interval quartile ($p=7.9\times10^{-7}$, **Supplementary Figure 4**).

Discussion

Considerable interest exists in the identification of genetic factors that modulate disease severity in the LQTS as the identification of such factors is expected to contribute to the refinement of risk stratification in the individual patient. However, studies aimed at the identification of these genetic factors are scarce.[8-10] In this study we undertook two approaches to identify common genetic variants that modulate the QTc-interval and the occurrence of cardiac events in a large set of patients with LQT2. In one approach we conducted an exploratory analysis of SNPs tagging common haplotypes within and around 18 candidate genes. In a second approach we investigated the role of 22 independent SNPs from 14 chromosomal loci that were previously identified as modulators of the QTc-interval in GWAS studies conducted in the general population. Our analysis confirms and extends on previous observations that common genetic variants at the *NOS1AP* locus modulate disease severity in the LQTS. We identified multiple SNPs at this locus displaying markedly large effects on the QTc-interval among LQT2 patients and/or enrichment of the QTc-prolonging allele in LQT2 patients versus general population controls. Additionally, two *NOS1AP* SNPs also appeared to impact on the risk of cardiac events. Similar effects on the QTc-interval and risk of cardiac events were observed when the *NOS1AP* SNPs were considered in aggregate as a GRS. Our data also implicates for the first time common genetic variation at *KCNQ1* as a risk factor for LQTS.

NOS1AP

Genome-wide association studies conducted in the general population have consistently shown that SNPs at the *NOS1AP* locus exert the strongest influence of any of the common genetic variation known to influence the QTc-interval.[11,12,15,17] In this study, SNPs at *NOS1AP* have similarly emerged as the strongest modifiers of the QTc-interval and possible modifiers of cardiac events among LQT2 patients, both when considered as single variants, as well as when considered cumulatively as a GRS. Of the six independent signals (R^2<0.4) that we tested at this locus, three (rs10494366, rs12143842 and rs2880058) displayed highly significant associations with the QTc-interval; one of these (rs10494366) also displayed a suggestive association with the occurrence of cardiac events. Besides these, rs12029454 was significantly enriched in LQT2 probands versus controls and displayed a suggestive association with both the QTc-interval and cardiac events.

Three studies have previously investigated the role of *NOS1AP* SNPs as modulators of disease severity in LQTS. One study investigated *NOS1AP* SNPs in 135 carriers of the founder mutation *KCNQ1*-A341V and identified rs4657139 (in high LD with our rs2880058) and rs16847548 (in high LD with our rs12143842) as modifiers of the QTc-interval and risk of cardiac events.[8] A second study analyzed *NOS1AP* SNPs in 901 LQTS patients of different genetic subtypes (primarily LQT1-3).[9] This study also identified rs4657139 and rs16847548 as modifiers of the QTc-interval, and detected effects on cardiac events for rs4657139 and rs10494366. A third study tested *NOS1AP* SNPs in 112 phenotypically discordant (one clinically affected and one not) patient duos carrying the same mutation in either *KCNQ1* or *KCNH2* and identified a suggestive association between rs12029454 and the QTc-interval.[10]

Keeping in mind that these three studies and ours are for several reasons not directly comparable (e.g. different sizes of the patient study sample which impacts on the statistical power, different study design, patients studied harbor mutation in different LQTS gene, and

the fact that not all studies investigated every independent signal linked thus far to the QTc-interval in the general population), in aggregate their findings allow us to start drawing some conclusions concerning the role of *NOS1AP* SNPs in modulation of disease severity in the LQTS. It is obviously clear that common genetic variation at this locus also modulates the QTc-interval in patients with the LQTS, with some individual SNPs (such as rs12143842 and rs2880058) now displaying highly convincing associations with the QTc-interval in the majority of the studies. Another observation emerging from these studies is that the effect of *NOS1AP* SNPs on the QTc-interval is larger in LQTS patients as compared to that observed in the general population in previous GWAS. In our analysis for example, each T-allele at rs12143842 increased the QTc-interval by an average of 13.2 ms while its effect in a large sample of the general population was of 3.15 ms.[11] We also detected similarly large effects for rs10494366 and rs2880058 (see **Table 4**). Effect sizes of 7 and 8 ms were observed for rs4657139 and rs16847548 respectively, in the study of Tomas and co-workers.[9] The larger effect sizes among LQTS patients are likely due to the sensitized genetic background of these patients: they are all carriers of a rare genetic variant with a putatively large deleterious effect on repolarization reserve which in turn may make the repolarization process more permissive to the effect of common genetic variation. This observation brings forward the possibility that further genetic studies in LQTS patients may uncover QTc-modulating genetic variants that would otherwise remain unidentified in GWAS conducted in the general population due to the small effect size in the latter.

However, while strong associations have been laid in LQTS patients between *NOS1AP* SNPs and the QTc-interval, this cannot be said of the effect of the same SNPs on the risk of cardiac events. In our study, while 2 out of the 6 *NOS1AP* SNPs we tested were nominally associated with risk of cardiac events, none, even those displaying very robust effects on the QTc-interval, displayed association p-values for cardiac events that exceeded the Bonferroni-

corrected threshold for multiple testing. Notwithstanding, considering the fact that QTc-modulating *NOS1AP* SNPs have already been implicated in modulation of risk for cardiac events in two studies[8,9], one could argue that the Bonferroni correction we applied is too harsh. Of the 2 SNPs that showed a nominal association with cardiac events in our study, rs10494366 was previously associated with risk of cardiac events by Tomas and co-workers.[9] *NOS1AP* encodes a nitric oxide synthase adapter protein. Functional studies have suggested that it regulates action potential duration of cardiomyocytes via calcium and potassium currents.[21] The *NOS1AP* SNPs that impact on the QTc-interval are located in the non-coding regions of the gene and if their effect on the QTc-interval indeed occurs through *NOS1AP*, it is then likely that this occurs through modulation of the level of NOS1AP transcript abundance and consequently protein levels.

SNPs at other loci

In our analysis of the 22 SNPs from GWAS for quantitative effects on the QTc-interval, besides the SNPs at *NOS1AP* discussed above, no additional SNPs passed the Bonferroni-corrected significance threshold. Two SNPs (rs2074238 at *KCNQ1*, and, rs17779747 at *KCNJ2*) however displayed a nominal association with a direction of effect consistent with that found previously in the general population.[11,12] The T-allele at rs2074238 is associated with a shorter QTc-interval. Of note, this SNP was recently reported to be associated with a shorter QTc-interval and decreased risk of symptoms in the study of Duchatelet et al.[10] We detected no effect of this SNP on cardiac events in the LQT2 patients studied here. The study by Duchatelet et al. however detected larger effects for this SNP, both on the QTc-interval as well as cardiac events, as compared to our study, and while our study was sufficiently powered to detect those effects, it was underpowered to uncover an association with the small effects we detected.

Besides our quantitative trait analysis of the 22 SNPs from GWAS with the QTc-interval, we additionally investigated their association with LQTS syndrome status in a case-control

association analysis of the LQT2 probands versus individuals from the general population (**Table 5**). This additional analysis uncovered two significant associations with the expected direction of effect (i.e. the QTc-interval prolonging allele being enriched among the cases versus the controls) highlighting the potential utility of this approach as recently also demonstrated by us for the Brugada Syndrome.[22] Our current analysis, for the first time, linked rs12576239 at *KCNQ1* with susceptibility to the LQTS.

Genetic Risk scores

We considered for the first time the combined effect of all 22 SNPs linked to the QTc-interval by constructing a genetic risk score for each individual (GRS_{22}) and relating it to the QTc-interval and occurrence of cardiac events. We demonstrated a significant positive linear relationship between GRS_{22} and the QTc-interval. The correlation between GRS_{22} and the QTc-interval however appears to be largely driven by the effect of the 6 *NOS1AP* SNPs as the association between the GRS and the QTc-interval did not remain significant when these SNPs were removed from the GRS calculation. A GRS based on the 6 *NOS1AP* SNPs only (GRS_{NOS1AP}), showed a similar predictive value for QTc to that of the GRS_{22}. No significant (linear) relation was found between either GRS_{22} or GRS_{NOS1AP} and the risk for cardiac events, but patients with scores in the first quartile had significantly less events than the patients in the other three quartiles combined. The latter observation will require further investigation in additional patients.

Effect on cardiac events

Our single SNP analysis did not uncover significant associations between any of the investigated SNPs and the occurrence of cardiac events. Furthermore, our GRS analyses did not reveal a linear relationship between the GRS and risk of cardiac events. On the one hand,

when one considers the fact that the SNPs tested are candidates with a strong *a priori* probability of being involved, one could argue that in the single SNP analysis, our correction for multiple testing might be too conservative. On the other hand, one can posit that while the QTc-interval is governed by an appreciable genetic component, the precipitation of arrhythmias in the LQTS may be heavily influenced by other factors such as environmental triggers that vary largely across patients. In any case, the low relative risk associated with these variants currently precludes their immediate clinical utility for arrhythmia risk stratification.

Many SNPs previously shown to affect the QTc-interval in the general population were silent with respect to their effect on the QTc-interval in our analysis. Some investigators have argued that the effect of SNPs in LQTS patients is dwarfed by the large effect of the primary mutation (so called 'ceiling effect').[8] While this seems a plausible explanation, it is unclear why *NOS1AP* SNPs are not affected by this phenomenon. One possibility could be the larger effect size among LQTS patients of *NOS1AP* SNPs as compared to the others, which would argue for investigation of the non-associating SNPs in larger patient sets.

Candidate gene study

Besides SNPs from QTc-interval GWAS we also systematically investigated the effect of haplotype-tagging SNPs in 18 candidate genes in LQT2 patient Set 1 (**Table 3**). Besides rs16847548 in *NOS1AP* this analysis uncovered two associations, at *CASQ2* (rs1935778) and *KCNH2* (rs956642), respectively. Neither of the latter two SNPs was however validated in patient Set 2. Although these two SNPs may merit further investigation in additional samples, these signals may represent a false positive association. One could argue that our correction for multiple testing in Set 1 may be too stringent and that true associations may exist above the Bonferroni-corrected p-value threshold we employed as the 18 genes were selected based

on their high *a priori* probability for modulating the QTc-interval. Nevertheless, we preferred to apply stringent criteria for the most reliable findings with the current data.

Study limitations

In the current study, we limited genetic heterogeneity by considering only LQTS patients with a *KCNH2* genetic defect. Nevertheless, although we accounted for this in the statistical analysis, some confounding may remain as a consequence of the variability in the severity of the haploinsufficient defect and/or the biophysical defect associated with the different *KCNH2* mutations among the patients. Considering the fact that the LQTS is a rare disorder we have here studied a substantial number of patients. However, the patient set may yet be considered modest for the study of common genetic variants with small effects. The effect of SNPs that we describe here may be different in the setting of other LQTS genetic subtypes. Furthermore, SNP effects may be allele-dependent as we previously demonstrated for SNPs in the 3' UTR of the *KCNQ1* gene.[23] The design of the current study precludes the analysis for such effects.

Conclusions

Our comprehensive analysis demonstrates that among SNPs previously linked to the QTc-interval in the general population, *NOS1AP* SNPs are the strongest modulators of the QTc-interval in patients with LQT2. The effect of these SNPs in LQT2 patients is markedly larger then that observed in the general population. Our study also uncovered common genetic variation at *KCNQ1* as a risk factor for LQTS.

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Disclosures

None

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Table 1. **Characteristics of the LQT2 patients studied**

	LQT2 patients Set 1 n = 353	**LQT2 patients Set 2** n = 286	**LQT2 patients Set 1 + Set 2** n = 639
Female	208 (59%)	157 (55%)	365 (57%)
Proband	86 (24%)	88 (31%)	174 (27%)
Median (IQR) age at ECG (years)	30 (28)	27 (31)	29 (30)
β-blocker use at time of ECG	60 (17%)	44 (15%)	104 (16%)
Mean (±SD) QTc-interval (ms)	467 ± 43	463 ± 44	465 ± 44
Cardiac event	126 (36%)	76 (27%)	202 (32%)
Median (IQR) follow-up (years)	26 (30)	27 (33)	26 (32)

Table 2. Effect of *KCNH2* mutation type and location on the QTc-interval

Mutation type and location	Patient Set 1 n=353	QTc-interval (ms)	Patient Set 1 + Set 2 n=639	QTc-interval (ms)
nonsense, frameshift, large deletions and insertions, all locations	150 (42%)	466 ± 40	277 (43%)	463 ± 40
missense, N-terminus	77 (22%)	460 ± 49	150 (23%)	458 ± 47
missense, transmembrane S1-S4	11 (3%)	522 ± 48	25 (4%)	496 ± 55
missense, transmembrane S5-loop-S6	86 (24%)	474 ± 40	132 (20%)	474 ± 40
missense, C-terminus	29 (8%)	455 ± 32	55 (9%)	462 ± 46

Table 3. SNPs from the candidate gene study that were associated with the QTc-interval

SNP	Chr	Candidate gene	Major allele	Minor allele	MAF	Effect on QTc-interval in Set 1* (n=353) β±SE (ms)	†P-value	Effect on QTc-interval in Set 2* (n=286) β±SE (ms)	‡P-value	Effect on QTc-interval in Set 1 + Set 2* (n=639) β±SE (ms)	P-value
rs16847548	1	*NOS1AP*	A	G	0.255	16.9±3.5	1.0×10^{-6}	10.1±3.8	**0.007**	13.2 ± 2.6	4.8×10^{-7}
rs1935778	1	*CASQ2*	A	G	0.419	12.4±2.9	2.1×10^{-5}	0.5±3.6	0.894	7.6 ± 2.4	0.001
rs956642	7	*KCNH2*	A	G	0.405	-14.8±3.1	1.3×10^{-6}	0.3±3.7	0.942	-7.1±2.4	0.003

Chr: chromosome, MAF: minor allele frequency.

* The coded allele is the minor allele in all cases.

† SNPs passing the discovery-phase Bonferroni corrected p-value threshold ($p<4.2\times10^{-5}$) are listed.

‡ The P-value for the SNP passing the replication-phase Bonferroni corrected p-value threshold ($p<0.016$) is depicted in bold.

Table 4. Effects of SNPs previously associated with the QTc-interval in the general population, in LQT2 Sets 1 and 2 combined

SNP from GWAS (n=22)	Chromosome	Closest gene	Major allele	*Minor allele	*Effect on QTc-interval (n=639)	
					β±SE (ms)	†P value
rs10494366	1q23.3	*NOS1AP*	T	G (↑)	14.1±2.6	**9.5×10^{-8}**
rs12029454	1q23.3	*NOS1AP*	G	A (↑)	8.4±3.1	0.007
§rs12143842	1q23.3	*NOS1AP*	C	T (↑)	13.2±2.6	**4.8×10^{-7}**
rs16857031	1q23.3	*NOS1AP*	C	G (↑)	6.8±3.3	0.043
rs2880058	1q23.3	*NOS1AP*	A	G (↑)	12.2±2.5	**8.6×10^{-7}**
rs4657178	1q23.3	*NOS1AP*	C	T (↑)	1.5±2.7	0.595
rs10919071	1q24.2	*ATP1B1*	A	G (↓)	3.4±3.5	0.335
rs37062	16q21	*CNOT1*	A	G (↓)	-1.8±2.7	0.518
rs1805128	21q22.12	*KCNE1*	G	A (↑)	4.7±4.7	0.309
rs2968863	7q36.1	*KCNH2*	G	A (↓)	4.0±3.0	0.175
rs4725982	7q36.1	*KCNH2*	C	T (↑)	1.8±3.1	0.552
rs12576239	11p15.5	*KCNQ1*	C	T (↑)	3.3±3.3	0.318
rs2074238	11p15.5	*KCNQ1*	C	T (↓)	-10.0±5.1	0.049
rs2074518	17q11.2-q12	*LIG3*	G	A (↓)	-0.7±2.4	0.765
rs8049607	16p13.13	*LITAF*	C	T (↑)	0.9±2.4	0.702
rs846111	1p36.31	*RNF207*	C	G (↓)	-0.2±2.9	0.954
rs12053903	3p22.2	*SCN5A*	T	C (↓)	-2.8±2.7	0.297
rs3825214	12q24.21	*TBX5*	A	G (↑)	0.5±3.1	0.865
rs17779747	17q24.3	*KCNJ2*	G	T (↓)	-7.0±2.6	0.007
rs2478333	13q13	*SUCLA2*	C	A (↑)	-1.4±2.6	0.586
rs11970286	6q22	*PLN*	C	T (↑)	-0.3±2.5	0.900
rs12210810	6q22	*PLN*	G	C (↓)	0.9±6.7	0.894

*The coded allele is the minor allele in all cases. The direction of effect found in genome-wide association studies conducted in the general population, are denoted in parenthesis; ↑, increase in QTc-interval, ↓, decrease in QTc-interval.

†P-values for SNPs passing the Bonferroni corrected p-value threshold ($p<2.3\times10^{-3}$) are depicted in bold.

§In LD with rs16847548 from candidate gene study ($R^2=0.88$); see Table 3.
GWAS, genome-wide association study.

Table 5. Case-control analysis of SNPs previously associated with the QTc-interval in the general population, in probands from LQT2 Sets 1 and 2 combined

SNP from GWAS	Closest gene	Coded allele	Frequency coded allele (cases /controls)	OR (95% CI)	P-value	Concordance with SNP effect on QTc-interval
rs10494366	*NOS1AP*	G	0.43/0.34	1.35 [1.03-1.76]	0.028	yes
rs12029454	*NOS1AP*	A	0.21/0.13	1.85 [1.32-2.59]	**0.0003**	**yes**
rs12143842	*NOS1AP*	T	0.31/0.23	1.49 [1.13-1.96]	0.005	yes
rs16857031	*NOS1AP*	G	0.17/0.14	1.25 [0.89-1.76]	0.20	
rs2880058	*NOS1AP*	G	0.40/0.32	1.39 [1.08-1.80]	0.014	yes
rs4657178	*NOS1AP*	T	0.31/0.23	1.45 [1.10-1.93]	0.009	yes
rs10919071	*ATP1B1*	G	0.12/0.11	1.20 [0.82–1.77]	0.35	
rs37062	*CNOT1*	G	0.25/0.24	1.04 [0.79-1.37]	0.77	
rs1805128	*KCNE1*	A	0.04/0.02	1.95 [1.06–3.57]	0.03	yes
rs2968863	*KCNH2*	A	0.22/0.24	0.89 [0.65-1.21]	0.45	
rs4725982	*KCNH2*	T	0.20/0.20	1.00 [0.74-1.34]	0.98	
rs12576239	*KCNQ1*	T	0.20/0.12	1.84 [1.31–2.60]	**0.0005**	**yes**
rs2074238	*KCNQ1*	T	0.06/0.08	0.77 [0.49-1.21]	0.26	
rs2074518	*LIG3*	A	0.46/0.46	0.97 [0.75-1.25]	0.82	
rs8049607	*LITAF*	T	0.46/0.53	0.77 [0.60-0.98]	0.03	yes
rs846111	*RNF207*	G	0.28/0.31	0.86 [0.65–1.13]	0.27	
rs12053903	*SCN5A*	C	0.32/0.35	0.88 [0.68-1.14]	0.31	
rs3825214	*TBX5*	G	0.22/0.21	1.09 [0.81-1.47]	0.58	
rs17779747	*KCNJ2*	T	0.36/0.33	1.14 [0.87-1.50]	0.34	
rs2478333	*Intergenic*	A	0.41/0.33	1.43 [1.11-1.86]	0.007	yes
rs11970286	*PLN*	T	0.46/0.45	1.08 [0.83-1.40]	0.56	
rs12210810	*PLN*	C	0.03/0.06	0.56 [0.29-1.10]	0.09	

Table 6. Effect of SNPs on event-free survival in Sets 1 and 2 combined

SNP	Closest gene	Major allele	Minor allele	*Effect on event-free survival		*Effect on event-free survival in patients with QTc<500ms	
				RR [95% CI]	†P value	RR [95% CI]	†P value
rs10494366	*NOS1AP*	T	G	1.30 [1.04 - 1.61]	*0.020*	1.35 [1.04 - 1.77]	*0.027*
rs12029454	*NOS1AP*	G	A	1.37 [1.08 - 1.74]	*0.011*	1.45 [1.07 - 1.95]	*0.015*
rs12143842	*NOS1AP*	C	T	1.14 [0.91 - 1.42]	0.246	1.13 [0.86 - 1.48]	0.373
rs16857031	*NOS1AP*	C	G	1.03 [0.78 - 1.35]	0.855	0.93 [0.66 - 1.32]	0.694
rs4657178	*NOS1AP*	C	T	1.22 [0.97-1.53]	0.08	1.32 [1.00-1.74]	0.06
rs2880058	*NOS1AP*	A	G	1.16 [0.94 - 1.44]	0.167	1.22 [0.95 - 1.57]	0.118
rs1805128	*KCNE1*	G	A	1.33 [1.01 – 1.76]	*0.044*	1.29 [0.89 - 1.85]	0.174
rs2074238	*KCNQ1*	C	T	0.83 [0.53 - 1.31]	0.422	0.78 [0.46 - 1.32]	0.356
rs12576239	*KCNQ1*	C	T	1.13 [0.86-1.48]	0.38	1.02 [0.74-1.42]	0.89
rs17779747	*KCNJ2*	G	T	1.14 [0.92 - 1.42]	0.221	1.21 [0.94 - 1.55]	0.137
rs8049607	*LITAF*	C	T	0.91 [0.74-1.14]	0.42	0.93 [0.73-1.20]	0.59
rs2478333	*intergenic*	C	A	1.04 [0.83-1.31]	0.73	1.05 [0.82-1.34]	0.70

*The coded allele is the minor allele.

† P values for nominally-associating SNPs are displayed in italics.

Figure legends

Figure 1. (A, B) Association between the Genetic Risk Scores GRS_{22} and GRS_{NOS1AP} and the QTc-interval in LQT2 patient Set 1 and Set 2 combined (n=639; GRS_{22}: $p=4.3\times10^{-6}$; GRS_{NOS1AP}: $p=4.2\times10^{-7}$). **(C, D)** Analysis of the relation between the Genetic Risk Scores GRS_{22} and GRS_{NOS1AP} and event-free survival in LQT2 patient Set 1 and Set 2 combined (n=639; GRS_{22}: p=0.192, GRS_{NOS1AP}: p=0.119). Q1 is the quartile with the lowest genetic risk score.

Figure 1A

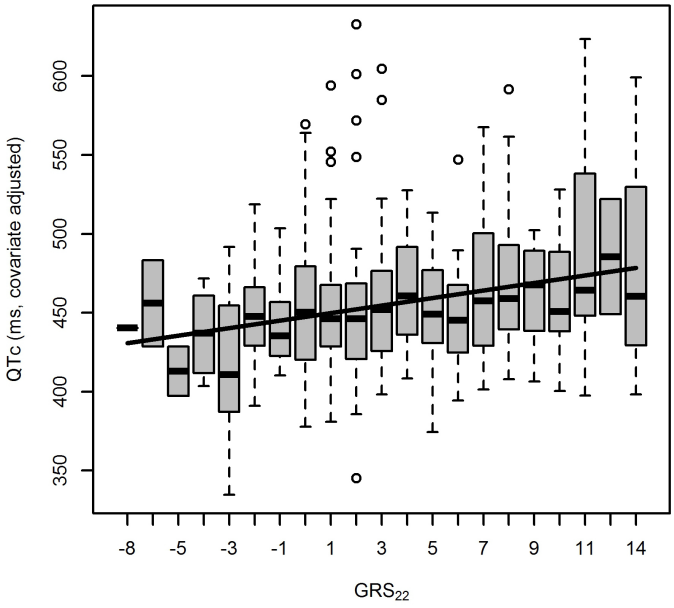

QTc (ms, covariate adjusted)
350
400
450
500
550
600
-8
-5
-3
-1
1
3
5
7
9
11
14
GRS22

Figure 1B

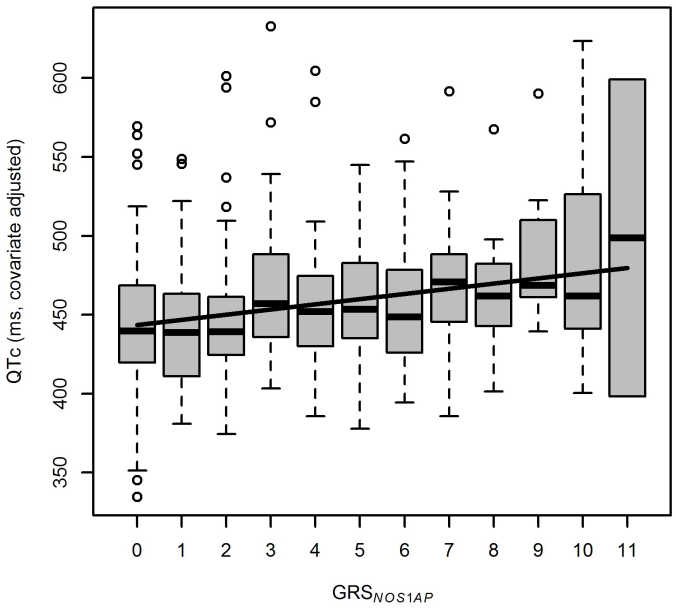

QTc (ms, covariate adjusted)
350
400
450
500
550
600
0
1
2
3
4
5
6
7
8
9
10
11
GRS$_{NOS1AP}$

Figure 1C

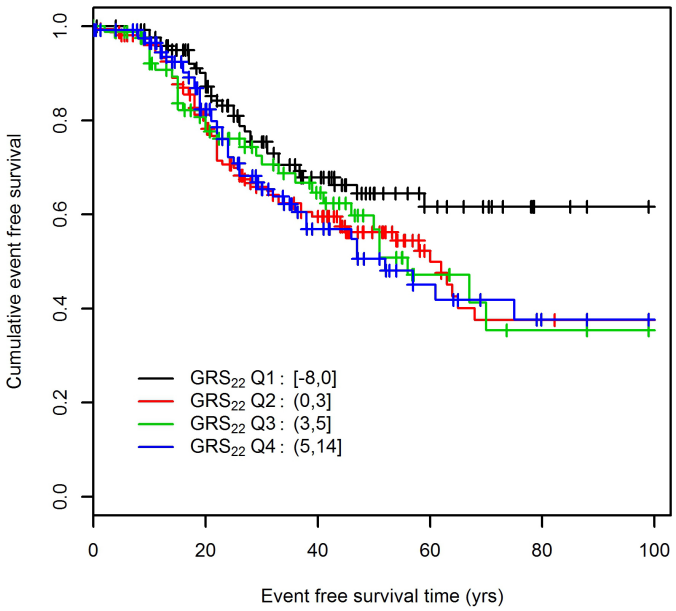
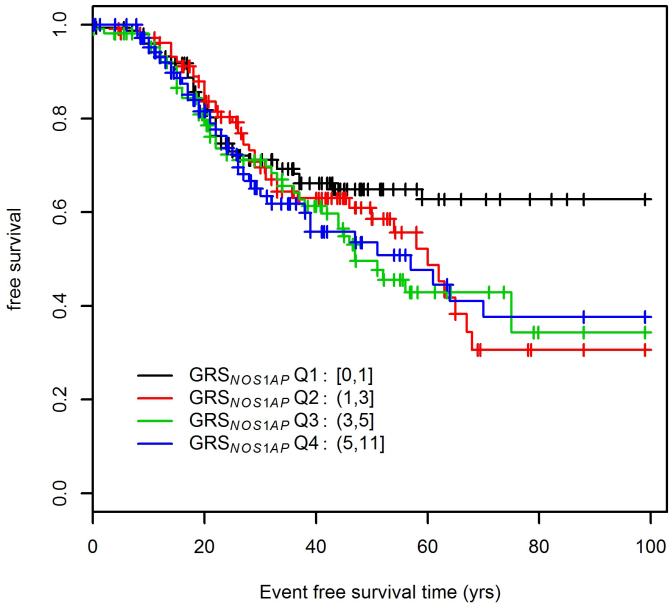

Cumulative event free survival
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
Event free survival time (yrs)
GRS22 Q1 : [-8,0]
GRS22 Q2 : (0,3]
GRS22 Q3 : (3,5]
GRS22 Q4 : (5,14]

Figure 1D


free survival
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Event free survival time (yrs)
GRS$_{NOS1AP}$ Q1 : [0,1]
GRS$_{NOS1AP}$ Q2 : (1,3]
GRS$_{NOS1AP}$ Q3 : (3,5]
GRS$_{NOS1AP}$ Q4 : (5,11]