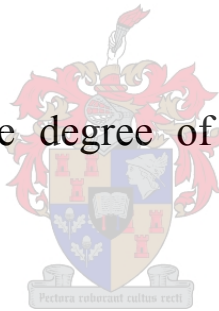


An investigation of the clinical profile and extent of Long QT Syndrome (LQTS) associated with the KCNQ1-A341V mutation in South Africa and with the KCNH2-A1116V mutation in an Italian family and the role that autonomic nervous system (ANS) activity and genetics play in clinical variability.

Lia Crotti

Dissertation presented for the degree of Doctor in Philosophy at the University of Stellenbosch



Promoter: Prof. Paul A. Brink

External Promoter: Prof. Peter J. Schwartz

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I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and I have not previously in its entirety or in part submitted it at any University for a degree.

Signature:

A handwritten signature in black ink, appearing to be 'K. O. Oki', written in a cursive style.

Date: 23rd July 2007

ENGLISH SUMMARY

Background

Although great progress has been made in defining genes conferring the majority of genetic risk in Long QT Syndrome (LQTS) patients, there remains a substantial challenge to explain the widely observed variability in disease expression and phenotype severity, even among family members, sharing the same mutation. Identifying clinical and genetic variables capable of influencing/predicting the clinical phenotype of LQTS patients would allow a more accurate risk stratification, important for determining prognosis, selecting patients for the most appropriate therapy, and counseling asymptomatic mutation carriers (MCs).

To address these questions an Italian LQT2 family and a South African Founder LQT1 population have been used.

Methods and Results

Italian LQT2 family. The proband, a 44-yr-old white woman, presented with ventricular fibrillation and cardiac arrest. Intermittent QT prolongation was subsequently observed and LQT2 was diagnosed following the identification of a missense *KCNH2* mutation (A1116V). The proband also carried the common *KCNH2* polymorphism K897T on the non-mutant allele. Relatives who carried A1116V without K897T were asymptomatic but some exhibited transient mild QTc prolongation suggesting latent disease. Expression studies in Chinese Hamster Ovary (CHO) cells, demonstrated that the presence of *KCNH2*-K897T is predicted to exaggerate the *IKr* reduction caused by the A1116V mutation. These data explain why symptomatic LQTS occurred only in the proband carrying both alleles.

South African LQT1 population. The study population involved 320 subjects, 166 MCs and 154 non mutation carriers (NMCs). Off β -blocker therapy, MCs had a wide range of QTc values (406-676 ms) and a QTc>500 ms was associated with increased risk for cardiac events (OR=4.22; 95%CI 1.12-15.80; p=0.033). We also found that MCs with a heart rate <73 bpm were at significantly lower risk (OR=0.23; 95%CI 0.06-0.86; p=0.035). In a subgroup of patients Baroreflex Sensitivity

(BRS) was determined both in presence and absence of β -blocker therapy. BRS, analyzed in subjects in the 2nd and 3rd age quartiles (age 26-47) to avoid the influence of age, was lower among asymptomatic than symptomatic MCs (11.8 ± 3.5 vs 20.1 ± 10.9 ms/mmHg, $p<0.05$). A BRS in the lower tertile carried a lower risk of cardiac events (OR 0.13, 95%CI 0.02-0.96; $p<0.05$). This study also unexpectedly determined that KCNQ1-A341V was associated with greater risk than that reported for large databases of LQT1 patients: A341V MCs were more symptomatic by age 40 (79% vs 30%) and became symptomatic earlier (7 ± 4 vs 13 ± 9 years), both $p<0.001$. Accordingly, functional studies of KCNQ1-A341V in CHO cells with KCNE1, identified a dominant negative effect of the mutation on wild-type channels.

Conclusion

Our findings indicate that risk stratification for LQTS patients must be more individually tailored and may have to take into account the specific mutation and probably additional clinical and genetic variables capable of influencing/predicting the clinical phenotype of LQTS patients. As a matter of fact, we have provided evidence that a common *KCNH2* polymorphism may modify the clinical expression of a latent LQT2 mutation and the availability of an extended kindred with a common mutation allowed us to highlight that KCNQ1-A341V is associated with an unusually severe clinical phenotype and to identify two autonomic markers, HR and BRS, as novel risk factors.

AFRIKAANSE OPSOMMING

Agtergrond

Alhoewel groot vooruitgang gemaak is in herkenning van die bydrae wat enkelgene maak tot risiko in pasiënte met die Lang QT Sindroom (LQTS), is 'n beduidende uitdaging om die waargenome veranderlikheid in siekte-uitdrukking en graad van aantasting, selfs onder lede van dieselfde familie wat dieselfde mutasie deel, te verklaar. Die identifikasie van kliniese en genetiese veranderlikes wat die vermoë het om die kliniese fenotipe van pasiënt met LQTS te beïnvloed/voorspel sal 'n meer akkurate risiko stratifikasie toelaat. Hierdie is belangrik vir die bepaling van prognose, keuse van pasiënte vir die beste toepaslike behandeling, en berading van asimptomaties mutasiedraers (MCs). Om die vrae aan te spreek is 'n Italiaanse LQT2 familie en 'n Suid-Afrikaanse stigtersbevolking gebruik.

Metodes en Resultate

Italiaanse LQT2 familie. Die indeksgeval is 'n 44 jaar-oue wit vrou wie met ventrikulêre fibrillasie en hartstilstand voorgedoen het. Intermitterende QT verlenging is later waargeneem (maksimum QTc 530 ms.) en LQT2 word diagnoseer na identifikasie van 'n KCNH2 mutasie (A1116V). Die indeksgeval dra ook 'n algemene KCNH2 polimorfisme K897T op die nie-mutant allel. Familielede wat die A1116V sonder K897T dra was nie simptome nie, maar sommige vertoon voorbygaande geringe verlenging van QTc wat latente siekte suggereer. Geenuitdrukkingstudies wat in Chinese hamster ovariële (CHO) selle uitgevoer is, demonstreer dat die teenwoordigheid van KCNH2-K897T voorspel dat 'n vergrote IKr afname sal plaasvind as gevolg van die A1116V mutasie. Hierdie gegewens verklaar waarom simptomaties LQTS slegs in die indeksgeval, wie beide allele dra, voorkom.

Suid-Afrikaanse LQT1 stigterbevolking. Die studiepopulasie het 320 persone betrek, 166 mutasiedraers (MCs) en 154 nie-mutasiedraers (NMCs). Van β -blokker behandeling af het MCs 'n

wye strek van QTc waardes (406-676ms) en 12% van individue had 'n normale QTc (\leq 440ms). 'n QTc >500 was assosieerd met 'n verhoogde risiko vir hartverwante gebeurtenisse (OR=4.22; 95%CI 1.12-15.80; $p=0.033$). Ons het ook bevind dat MCs met 'n hartspoed <73 slae per minuut 'n beduidende laer risiko gehad het (OR=0.23; 95%CI 0.06-0.86; $p=0.035$). In 'n subgroep van pasiënte is baroreseptorsensitiwiteit (BRS) bepaal beide in die teenwoordigheid en in die afwesigheid van β -blokker behandeling. BRS, soos ontleed in studiepersone in die 2^{de} and 3^{de} ouderdomskwartiele om die invloed van ouderdom te vermy, was laer onder asimptomatiese dan simptomatiese MCs (11.8 ± 3.5 vs 20.1 ± 10.9 ms/mmHg, $p<0.05$). 'n BRS in die laer tertiel had 'n laer risiko van kardiaale gebeurtenisse gehad (OR 0.13, 95%CI 0.02-0.96; $p<0.05$). Die studie het ook onverwags gevind dat KCNQ1-A341V 'n groter risiko het dan die wat rapporteer is vir groot databasisse van LQT1 pasiënte: A341V MCs meer simptomaties by ouderdom 40 (79% versus 30%) en word vroeër simptomaties (7 ± 4 jare gevind versus 13 ± 9), beide $p<0.001$. Diensooreenkomstig, is die funksionele studies van KCNQ1-A341V in CHO selle met KCNE1 uitgevoer en word 'n dominant-negatiewe ten opsigte van die mutasie op wilde-tipe kanale demonstreer.

Gevolgtrekking

Ons bevindings wys daarop dat die risikostratifikasië van pasiënte met LQTS meer individu-spesifiek gemaak moet word en dat mens moontlik die spesifieke mutasie en waarskynlik ekstra kliniese en genetiese veranderlikes in berekening moet bring om die kliniese fenotipe te voorspel. In werklikheid, het ons bewys gelewer dat 'n algemene polimorfisme KCNH2 kliniese uitdrukking van latente LQT2 kan wysig. Verder het die beskikbaarheid van 'n uitgebreide familieboom wat 'n mutasie deel ons toegelaat om aan te toon dat KCNQ1-A341V met 'n ongewoon erge kliniese fenotipe assosieer is en om twee autonome merkers, hartspoed en BRS, as voorheen nie-beskrewe risikofakore, te identifiseer.

NOTES:

Part of the data presented in this thesis have been published in two different articles:

1. **Crotti L**, Lundquist AL, Insolia R, Pedrazzini M, Ferrandi C, De Ferrari GM, Vicentini A, Yang P, Roden DM, George AL Jr, Schwartz PJ. A common HERG polymorphism, K897T, acts as a genetic modifier of the Congenital Long QT Syndrome. *Circulation* 2005; 112:1251-1258.
2. Brink PA, **Crotti L**, Corfield V, Goosen A, Durrheim G, Hedley P, Heradien M, Geldenhuys G, Vanoli E, Bacchini S, Spazzolini C, Lundquist AL, Roden DM, George AL, Schwartz PJ. Phenotypic variability and unusual clinical severity of congenital Long QT Syndrome in a Founder Population. *Circulation* 2005; 112: 2602-2610.

Candidate's personal involvement in the different experiments reported:

Clinical Phenotyping:

- I have personally collected all the clinical information of the Italian LQTS family, that I am currently following in the “Molecular Cardiology Ambulatory” in the University Hospital “Policlinico San Matteo” in Pavia, under the direction of Prof. Peter Schwartz.
- I have actively participated in the collection of all the clinical and autonomic data of the South African families together with Prof. Paul Brink, Prof. Emilio Vanoli and Ms Althea Goosen, sometime with the participation of additional South African or Italian personnel.
- I also created a database in Filemaker Pro and with the help of Ms Althea Goosen all the clinical paper-files of the South African patients, written in Afrikaans, were converted in electronic files written in English and were enriched with the clinical information collected during the visit of a number of these patients. This database was the starting point for most of the analyses that we have performed and that we are currently performing. To perform

this part of the project I went to Cape Town three times a year (for three weeks each time) for a period of 4 years.

- **Molecular investigation:** I have personally performed the molecular screening of our Italian Family in the “Molecular Cardiology Laboratories”, in the University Hospital “Policlinico San Matteo” in Pavia, under the direction of Prof. Peter Schwartz. Nowadays there are three molecular biologists working in the laboratory; therefore I don’t do the work personally anymore, but the work I describe in this thesis was performed by myself. The Haplotype work on the South Africa families was performed by the South African group under the direction of Prof. Valerie Corfield and Prof. Paul Brink.
- **Functional Study:** the biophysical characterization of the *KCNH2* variants and of the KCNQ1-A341V variant was performed in the Department of Genetic Medicine at the University of Vanderbilt, Nashville, Tennessee (USA), under the direction of Prof. Alfred L. George. I spent three months working in his laboratories and I personally performed site-directed mutagenesis and cloning both the wild-type and the mutant alleles into pIRES-EGFP and pIRES-DsRed for use in co-expression study. I also transfected the plasmid DNA in CHO cells. The patch clamp work, was performed by Mr. Andrew Lundquist, under Prof. Alfred L. George supervision.

ABBREVIATIONS LIST

BB	β -blocker
BRS	Baroreflex sensitivity
CHO	Chinese Hamster Ovary cells
CI	Confidence Interval
ECG	Electrocardiogram
EGFP	Enhanced Green Fluorescent Protein
HR	Heart Rate
ICD	Internal Cardioverter Defibrillator
IQR	Interquartile Range
IRES	Internal Ribosomal Entry Site
IT	Italian
LQTS	Long QT Syndrome
LQT1	Long QT Syndrome type 1 (disease-causing mutation on <i>KCNQ1</i>)
LQT2	Long QT Syndrome type 2 (disease-causing mutation on <i>KCNH2</i>)
MCs	Mutation Carriers
NMCs	Non Mutation Carriers
OR	Odd Ratio
PCR	Polymerase Chain Reaction
Pts	Patients
QTc	QT corrected for heart rate by using Bazett's formula
SA	South African
SD	Standard Deviation

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INTRODUCTION

The congenital long QT syndrome (LQTS) is an inherited disorder characterized by prolongation of the QT interval and an increased risk for life-threatening ventricular arrhythmias (1,2). The disease is genetically heterogeneous caused by mutations in one of several genes including *KCNQ1*, *KCNH2*, *KCNE1* and *KCNE2* encoding potassium channel subunits, the cardiac sodium channel gene *SCN5A*, the L-type calcium channel gene *CACNA1C* (3,4), *CAV3* and *SCNB4* (5,6).

There are several reasons for the current widespread interest in LQTS. One is represented by the dramatic manifestations of the disease, namely syncopal episodes which often result in cardiac arrest and sudden death and usually occur in conditions of either physical or emotional stress in otherwise healthy young individuals, mostly children and teenagers. Another reason is that, while LQTS is a disease with a very high mortality rate among untreated patients, very effective therapies are available; this makes the existence of symptomatic and undiagnosed or misdiagnosed patients unacceptable and inexcusable. Finally, the identification of genes associated with the congenital long QT syndrome (LQTS) has had a major impact on understanding the molecular basis for ventricular arrhythmias and sudden cardiac death (7). The impressive correlation between specific mutations and critical alterations in the ionic control of ventricular repolarization makes this syndrome a unique paradigm which allows to correlate genotype and phenotype, thus providing a direct bridge between molecular biology and clinical cardiology in the area of sudden cardiac death.

Although great progress has been made in defining individual genes conferring the majority of genetic risk in LQTS patients and in elucidating complex genotype-phenotype correlations (8-10), there remains a substantial challenge to explain the widely observed variability in disease expression and phenotype severity. As a matter of fact, members of the same family that share the same mutation may have varying degrees of QT prolongation and widely different phenotypes,

ranging from no symptoms to sudden death and the underlying mechanisms of this phenotypic variability are still unknown. **Identifying clinical and genetic variables capable of influencing/predicting the clinical phenotype of LQTS patients would allow a more accurate risk stratification, important for determining prognosis, selecting patients for the most appropriate therapy, and counseling asymptomatic mutation carriers (MCs).**

The main objective of this study is to test our hypothesis (11) that, among LQTS patients, clinical severity is modified – in addition to already known factors such as gender and QT interval duration – by the autonomic nervous system and by genetic variants that could contribute to a greater or lesser propensity to respond to stimuli with release of catecholamines and/or by variants on the genes causing LQTS that might increase the loss of repolarizing potassium currents and further prolong the QT interval in some patients, thereby further increasing their risk for cardiac events. In the present study we will focus on the two main genetic subgroups, LQT1 and LQT2, which together account for 90% all LQTS.

We have previously shown (12) that most cardiac events (96%) in the LQT1 subgroup are triggered by either physical exercise (68%) or by emotional stress (28%). Both of these conditions involve a rapid or sudden increase in catecholamines release. The underlying predisposing mechanism is represented by a reduction in I_{Ks} current, the hallmark of the LQT1 subgroup. The LQT2 subgroup has a unique position because even though the normal I_{Ks} current prevents events during exercise, these patients are exquisitely sensitive to startle, especially auditory stimuli as a telephone ring or an alarm clock (12). For these patients the most dangerous trigger is represented by an unexpected startle while at rest; hence, another specific relation with sudden release of norepinephrine. Thus, it is especially in the LQT1 subgroup – but also, in a different way, in the LQT2 subgroup - in which variability in sympathetic activation or in its effects on the ventricular myocytes is more likely to determine outcome, that one can expect genetic alterations or abnormalities in the sympathetic control of cardiac function to play a "modifier" role on the clinical expression of LQTS. These considerations provide the rationale for the exploration of the

"adrenergic cascade", upstream and downstream, to assess the presence of functional polymorphisms that might modify either the amount of norepinephrine (and/or of epinephrine) released at the ventricular level or the sensitivity of the adrenergic receptors to catecholamines.

Experimental (13) and clinical studies (14) carried out by our group have shown that, in the presence of an arrhythmogenic substrate such as a healed myocardial infarction, a major risk factor for sudden arrhythmic death is represented by "autonomic imbalance". This term indicates an alteration in the balance between sympathetic and vagal activity, such as an increase of sympathetic and/or decrease in vagal activity. The latter is often the primary determinant of this arrhythmogenic imbalance that is identified clinically by a reduction in Baroreflex Sensitivity (BRS), a marker of the ability to reflexly increase vagal activity [a factor that, by antagonizing sympathetic activity, has a protective action against ventricular fibrillation (14)]. There is growing evidence that BRS has a wide distribution in normal individuals (13-15) and that it is genetically determined to a strong degree (16-17). The direct link with the present project is represented by the fact that genetic alterations leading to a lower-than-normal BRS will result in more norepinephrine being released under stress. Individuals genetically predisposed toward lower values of BRS will respond to physical or emotional stress with more NE acting on the heart, partly because of reduced vagal antagonism. If they have an arrhythmogenic substrate, myocardial infarction or LQTS, they will be at higher risk for life-threatening arrhythmias. This is what we have already demonstrated for myocardial infarction (14) and what we will test in the present study.

Thus, as recently proposed (11), in the search for genes that modify the propensity for life-threatening arrhythmias, there is a strong rationale to screen LQT1 and LQT2 patients for polymorphisms which might affect sympathetic function, baroreflex sensitivity or the degree of potassium channels activation during sympathetic activation. Also the duration of the QT interval is an important risk stratifier (8). As polymorphisms on the genes known to cause LQTS could further affect QT duration, this is an additional area worth exploring.

To address these questions an Italian LQT2 family and a South African Founder LQT1 population have been used, providing new genetic and clinical insights into the comprehension of disease-expression variability.

METHODS

Study Populations

Italian LQT2 Family (IT-A1116V)

A 44-year old white Italian female presented to IRCCS Policlinico San Matteo for clinical care following cardiac arrest due to ventricular fibrillation. After informed consent was obtained using a protocol approved by the Ethics Review Board of the Policlinico San Matteo, Pavia, blood was collected from the proband and members of her extended family for isolation of DNA. The proband and the family members were evaluated with basal electrocardiogram (ECG), hyperventilation test, exercise stress test, echocardiogram and 12-lead ECG 24 hour Holter Recording. Clinical and genetic data were recorded on specific forms and included demographic information, personal and family history of disease, symptoms and therapy. Data were subsequently stored in the database of the “Cardiac Arrhythmogenic Diseases Outpatient Clinic” of the Policlinico San Matteo, Pavia.

South African LQT1 Founder Population (SA-A341V)

A cohort of individuals (18) harboring an identical LQTS-causative mutation in KCNQ1 (A341V) was investigated for the possibility of a founder effect. Starting from probands, family trees were constructed and ancestral relationships were researched through genealogical studies. To exclude the possibility that the mutation arose independently on more than one occasion, which would be contrary to the founder hypothesis, haplotype data on the genomic segment encompassing KCNQ1 were also used to confirm likely lines of descent of the shared A341V mutation from the founding couple.

Clinical and genetic data concerning mutation-carriers (MCs) and first degree relatives were recorded on designed forms and included demographic information, personal and family history of disease, symptoms and therapy. 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 patients were MCs who experienced at least one episode of syncope or cardiac arrest, whereas asymptomatic MCs were those individuals without these events. Unexpected sudden death that occurred before age 40 without a known cause was categorized as related to LQTS (19) and was assumed to have occurred in MCs. Data were stored in a relational database developed jointly by authors from the University of Stellenbosch and from the University of Pavia.

All probands and family members provided written informed consent for clinical and genetic evaluations. Protocols were approved by the Ethical Review Boards of the Tygerberg Hospital of Stellenbosch University, Vanderbilt University, and the University of Pavia. Approved consent forms were provided in English or Afrikaans as appropriate.

Genotyping/Haplotyping

Italian LQT2 Family (IT-A1116V)

Genomic DNA was extracted from peripheral blood leukocytes using standard methods and diluted to 50ng/μl. All coding exons of *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* were amplified by polymerase chain reaction (PCR) using previously published primer pairs (20-24) or additional primers with annealing temperatures of 53-69 °C. Amplicons were screened for sequence variants using denaturing high performance liquid chromatography (DHPLC) analysis performed on two different automated DNA fragment analysis systems (Wave™ models 1100 and 3500HT, Transgenomic, San Jose, CA). Elution profiles were compared with normal control samples included in each analysis. Products exhibiting divergent chromatographic profiles were enzymatically purified (ExoSAP-IT; Amersham Bioscience, Piscataway, NJ, USA) and sequenced (Big-Dye Terminator v1.1 cycle sequencing kit; Applied Biosystems) with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

South African LQT1 Founder Population (SA-A341V)

Peripheral blood was collected from all index cases and family members entered into the study. Genomic DNA was extracted from lymphocytes or Epstein-Barr virus-transformed cell lines as previously described (25).

Polymerase chain reaction (PCR)-based detection of the A341V founder mutation, which results in the loss of a *HhaI* restriction enzyme site in KCNQ1 exon 7, was used to genotype members of the LQTS families. Exon 7 was PCR amplified, by using primers X7F and X7R (26) to generate a 190 bp amplicon for restriction digestion.

Selected informative members of each LQTS-affected family were genotyped at eight linked microsatellite loci (D11S4046, D11S1318, D11S4088, D11S4146, D11S4181, D11S1871, D11S1760, D11S1323) that span the KCNQ1 region. Haplotypes were determined by studying family structures and by using Mendelian rules of inheritance.

Phenotypic Assessment

Italian LQT2 Family (IT-A1116V)

Baseline ECG, hyperventilation test, exercise stress test, echocardiogram and 12-lead ECG 24 hours Holter Recording were performed in the proband and in her family members. Duration of the QT and RR intervals in leads II and V3 were measured from the different ECGs available. To allow QT values to be compared among subjects, the QT interval was corrected for heart rate (QTc) by using Bazett's formula

South African LQT1 Founder Population (SA-A341V)

A baseline ECG recorded in the absence of β -blocker therapy was available for 131/154 (85%) non-carriers and for 93/166 (56%) MCs. All ECGs were coded and the most recent one not on therapy was subsequently analyzed blinded to genotype.

Baseline heart rate (HR) and duration of the QT and RR intervals in leads II and V3 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) by using Bazett's formula. For comparisons of heart rate and QTc between symptomatic and asymptomatic patients, we used only ECGs recorded after age 15. This was done because the heart rate of healthy individuals is significantly greater before age 15 (27) and because absence of cardiac events before age 15 does not predict that an LQTS proband will remain asymptomatic throughout life. Thus, 7 patients with an ECG taken before age 15 were not included in this analysis, which therefore is limited to 86 patients.

Eighty-three subjects accepted to perform an "Autonomic Evaluation Study". Accordingly, all subjects were either hospitalized or stayed in a hotel close to Tygerberg Hospital for 4 days to perform the study both on and off beta-blocker therapy. Plasma concentrations of propranolol and atenolol were determined by a sensitive and specific method using high-performance liquid chromatography (HPLC) with fluorometric detection, modified by Braza AJ et al (28). Plasma levels below 20 ng/mL were considered as "non therapeutic".

After environmental acclimation, while the subjects were resting comfortably, ECG, blood pressure, and respiratory activity were simultaneously recorded for a 10-minute period, digitized and stored by a PC based software system (Bioengineering Service Montescano, Italy). BRS was determined (at least two determinations per subject) by the phenylephrine method, relating a transient increase in blood pressure (20-30 mmHg) induced by bolus injections of phenylephrine (2-3 $\mu\text{g}/\text{kg}$, as necessary) to the resultant lengthening of the RR interval (29). The slope of a best-fit regression line defined BRS. Beat-to-beat RR interval and blood pressure (BP) were continuously recorded (FINAPRES, Ohmeda) and then digitally converted using a desktop computer. BRS was calculated by two experienced investigators (Prof Emilio Vanoli and Dr. Maria Teresa La Rovere) independently and blind to each other's measurements. BRS was determined in 75 individuals from this founder population. Age has an important influence on BRS (29) and, indeed, in the 75 subjects under study whose age range was 16-65 years there was a significant ($p < 0.001$) negative correlation between age and BRS ($r = 0.67$). Accordingly, we focused our analysis on the 2nd and 3rd age quartiles that included 38 subjects (age 26-47) and represented the middle 50% of the tested

population. In this group the correlation with age was not significant (see Results). This approach allowed a reliable assessment of BRS while controlling for the age effect.

Statistical Analysis

Comparisons of groups identified on the basis of the clinical characteristics and genotype were performed in univariate analysis. Student's t-test or Mann-Whitney U test, as appropriate, were used for continuous variables and Fisher's exact test was used for categorical variables. QTc duration and heart rate in MCs were divided in tertiles and the upper tertile of each variable, 500 ms and 73 bpm respectively, was used as a clinical risk factor. To determine the association of the selected clinical variables with the occurrence of cardiac events, odds ratios (OR) for unadjusted data and their 95% confidence intervals (95% CI) were calculated.

The change in heart rate and in BRS values produced by BB was evaluated by the paired-samples t test procedure or by the Wilcoxon signed rank test. To estimate the risk of cardiac events associated with predetermined values of BRS, this variable was dichotomized at the first tertile of its distribution in the populations under study. Odds ratios (ORs) with 95%CI was estimated by logistic regression.

Time to the first cardiac event (syncope, cardiac arrest or sudden cardiac death) before initiation of β -blocker therapy and before age 40, was determined by Kaplan-Meier cumulative estimates. The South African A341V population (SA-A341V) was compared with the largest published data set on 355 LQT1 patients (30), referred here to as the LQT1 database. The age-related probability of surviving a first cardiac event was described by QTc, gender, and by the specific genetic defect.

The contribution of heart rate, QTc duration and gender to the risk of experiencing a cardiac event was determined by a logistic regression model where the presence or absence of a clinical history of cardiac events was used as the dependent variable. Odds ratios and 95% CI were computed while controlling for the covariates introduced in the model.

Data are reported as mean and standard deviation (SD) for continuous variables; whenever the distribution was skewed, median and interquartile range (IQR) were reported. Two-sided p-values <0.05 were considered statistically significant. Statistical calculations were performed by using SPSS software (version 12).

Site-Directed Mutagenesis

Italian LQT2 Family (IT-A1116V)

The two alleles identified in this study, A1116V and K897T, were constructed in a recombinant HERG cDNA using PCR site-directed mutagenesis (31). The final constructs were assembled in bicistronic mammalian expression plasmids (pIRES2-EGFP, pIRES2-DsRed, BD Biosciences-Clontech, Palo Alto, CA) in tandem with an internal ribosomal entry site (IRES) and either enhanced green fluorescent protein (EGFP) or DsRed for use as indicators of successful transfection. All constructs were sequenced to verify the mutation and to exclude polymerase errors. Wild-type HERG and the variant alleles were subcloned into pIRES2-EGFP and pIRES2-DsRed for use in co-expression experiments.

South African LQT1 Founder Population (SA-A341V)

Three mutations (A341V, G314S, 543 del/ins) were constructed in a recombinant human KCNQ1 cDNA by using polymerase chain reaction mutagenesis (primer sequences available on request). G314S produces a severe *in vitro* phenotype caused by a strong dominant negative effect, while 543 del/ins displays no dominant negative effect (32,33). All constructs were assembled in the bicistronic pIRES2-EGFP vector (BD Biosciences/Clontech, Palo Alto, CA) for mammalian cell expression experiments then verified by restriction mapping and DNA sequencing. Expression of KCNQ1 is driven by the CMV immediate-early promoter and enables simultaneous expression of enhanced green fluorescent protein from the same plasmid to mark transfected cells.

Cell Culture and Electrophysiology

Italian LQT2 Family (IT-A1116V)

Chinese hamster ovary cells (CHO-K1, ATCC) were grown at 37°C in 5% CO₂ in F-12 nutrient mixture medium supplemented with 10% fetal bovine serum (FBS, ATLANTA Biologicals, Norcross, GA, USA), 2 mM L-glutamine, and penicillin (50 units/ml)- streptomycin (50 µg/ml). Unless otherwise stated, all tissue culture media was obtained from Life Technologies (Grand Island, NY, USA). Cells were transiently transfected using Fugene-6 (Roche Diagnostics, Indianapolis, IN) with 3 µg plasmid DNA. In co-expression experiments, cells were co-transfected with 3 µg of each plasmid. Following transfection (48-72 hours), fluorescent cells were selected by epifluorescence microscopy (green for single transfections, yellow for co-transfections) for use in whole-cell patch clamp recording experiments. Non-transfected CHO cells grown under these conditions did not exhibit measurable endogenous potassium currents with the recording conditions employed for this study.

Whole-cell currents were measured in the broken-patch, whole-cell configuration of the patch clamp technique (34) using an Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA, USA). The bath solution consisted of (in mM): NaCl 145, KCl 4, MgCl₂ 1, CaCl₂ 1.8, glucose 10, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid) 10, adjusted to pH 7.35 with NaOH, ~275 mosmol/kg. The pipette solution consisted of (in mM): KCl 110, ATP-K₂ 5, MgCl₂ 2, EDTA (ethylenediaminetetraacetic acid) 10, HEPES 10, adjusted to pH 7.2 with KOH, ~265 mosmol/kg. The pipette solution was diluted 7-10% with distilled water to prevent activation of swelling-activated currents. Patch pipettes were pulled from thick-wall borosilicate glass (World Precision Instruments, Inc., Sarasota, FL, USA) with a multistage P-97 Flaming-Brown micropipette puller (Sutter Instruments Co., San Rafael, CA, USA) and fire-polished. Pipette resistance was 1-4 M., and as reference electrode, a 1-2% agar-bridge with composition similar to the bath solution was utilized. Whole-cell current traces were filtered at 5 kHz and acquired at 1-2 kHz. All chemicals were purchased from SIGMA Chemicals (St. Louis, MO, USA).

The holding potential was -80 mV and whole-cell currents were measured from -80 to $+70$ mV (in 10 mV steps) 1990 ms (activation) and 2200 ms (tail currents) after the start of the voltage pulse. The access resistance and apparent membrane capacitance were estimated as described by Lindau and Neher (35). Pulse generation, data collection and analyses were done with Clampex 8.1 (Axon Instruments, Inc.). Statistical comparisons were made using Student's t-test and significance was assumed for $P < 0.05$.

South African LQT1 Founder Population (SA-A341V)

Mutant KCNQ1 plasmids were expressed in a Chinese hamster ovary cell line (CHO-K1, CRL 9618, American Type Culture Collection, Rockville, MD, USA) stably expressing wildtype KCNQ1 and its ancillary subunit KCNE1 to generate the repolarizing current I_{Ks} , as previously described (36). This I_{Ks} cell line was generated by stable integration of a bicistronic KCNE1-IRES2-KCNQ1 cassette by using targeted homologous recombination mediated by *Flp* recombinase. This approach enabled uniform expression of KCNQ1 and KCNE1 from a single genomic locus and resulted in a consistent level of current in all cells. Cells were grown at 37°C in 5% CO_2 in F-12 nutrient mixture medium supplemented with 10% fetal bovine serum (FBS, ATLANTA Biologicals, Norcross, GA, USA), 2 mM L-glutamine, penicillin (50 units/ml)-streptomycin (50 $\mu\text{g/ml}$), and 600 $\mu\text{g/ml}$ hygromycin. All tissue culture media was obtained from Life Technologies (Grand Island, NY, USA). Cells were transiently transfected by using Fugene-6 (Roche Diagnostics Corp, Indianapolis, IN). Following transfection (48-72 hours), fluorescent cells were selected by epifluorescence microscopy for use in whole-cell patch clamp recording experiments.

Whole-cell currents were measured in the whole-cell configuration of the patch clamp technique (37) by using an Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA). The bath solution consisted of (in mM): NaCl 132, KCl 4.8, MgCl_2 1.2, CaCl_2 2, glucose 5, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) 10, pH=7.4, ~ 275 mosmol/kg. The pipette solution consisted of (in mM): K-aspartate 110, ATP- K_2 5, MgCl_2 1, EGTA (ethylene glycol-bis- $[\beta$ -aminoethyl ether]) 11, HEPES 10, MgCl_2 1, pH=7.3, ~ 265 mosmol/kg. The pipette solution was

diluted 7-10% with distilled water to prevent activation of swelling-activated currents. Patch pipettes were pulled from thick-wall borosilicate glass (World Precision Instruments, Inc., Sarasota, FL) with a multistage P-97 Flaming-Brown micropipette puller (Sutter Instruments Co., San Rafael, CA) and fire-polished. Pipette resistance was 1-4 M Ω , and as reference electrode, a 1-2% agar-bridge with composition similar to the bath solution was utilized. Whole-cell current traces were filtered at 5 kHz and acquired at 1-2 kHz. All chemicals were purchased from SIGMA Chemicals (St. Louis, MO).

The holding potential was -80 mV and whole-cell currents were measured from -80 to $+60$ mV (in 10 mV steps) 1990 ms after the start of the voltage pulse. The access resistance and apparent membrane capacitance were estimated as described by Lindau and Neher (38). Pulse generation, data collection and analyses were done with Clampex 8.1 (Axon Instruments, Inc.) and SigmaPlot 7.0 software suites.

RESULTS: Italian LQT2 Family (IT-A1116V)

Clinical Phenotype

The proband had palpitations associated with pre-syncopal episodes since age 20, and a cardiac arrest due to ventricular fibrillation at age 44. The patient had no exposure to medications with pro-arrhythmic effects. No specific trigger for the episode of cardiac arrest was identified, as she was quietly sitting in the car while her husband was driving. Subsequent investigations including brain and chest CT scans, echocardiography, coronary angiography, cardiac magnetic resonance imaging, a standard clinical electrophysiological study and flecainide challenge test were all normal. Shortly after cardiac arrest serum potassium was 3.5 mEq/L, but was subsequently found to be in the normal range. A surface resting ECG was normal with a QTc of 425 msec. A diagnosis of idiopathic ventricular fibrillation was made, prompting implantation of an internal defibrillator (ICD) and initiation of beta-blocker therapy.

During a follow-up 12-lead 24-hour ECG recording, periods of bifid T waves in leads V3-V5 and prolonged QTc (maximum QTc 530 ms) were observed (Fig. 1). During most of the recording, however, the morphology and QTc duration were normal indicating that the patient had a labile QT interval. An exercise stress test showed a normal rate adaptation of ventricular repolarization: in basal condition, at a heart rate of 77 bpm, QTc was 450 ms and did not increase at peak exercise when heart rate reached 111 bpm. A subsequent ECG showed a basal QTc of 470 ms with negative T-waves in lead V3-V4, but a prolonged QTc up to 510 ms in V3 during a hyperventilation test (Fig. 2). Family history was negative for syncope or sudden cardiac death, although one sibling of the proband was a stillborn.

Identification of a Novel *KCNH2* Mutation

We screened DNA from the proband for mutations in all coding exons of *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* using DHPLC. An abnormal elution profile was identified in one fragment encompassing *KCNH2* exon 15 amplified from the proband, but this was not observed in

130 control Caucasian individuals. Sequence analysis revealed a heterozygous missense mutation that results in an alanine to valine substitution at position 1116 (designated A1116V, Fig. 3A). This amino acid lies within the distal carboxyl-terminus of the encoded protein (HERG) and is highly conserved among homologous sequences of several species including human, mouse, rat and dog (Fig. 3B). A second *KCNH2* variant (K897T) was identified in the proband. This is a common polymorphism with an estimated prevalence in Caucasian populations up to 33% (39-41).

Segregation of *KCNH2* Variants

Segregation of A1116V and K897T in the proband's family indicates that the two variants are located on separate *KCNH2* alleles (Fig. 4). Mutation screening revealed that A1116V, but not K897T, was present in a brother and in his two children. All A1116V carriers other than the proband were asymptomatic with normal baseline QT intervals (Fig. 5). Twelve-lead 24-hour Holter recordings were completely normal in the brother (Fig. 5B) and showed rare transient episodes of mild QTc prolongation in a 22-year-old niece (maximum QTc 480 ms, Fig. 5C). A 9-year-old nephew had a borderline normal QTc during most of the recording, but brief periods of biphasic or notched T waves in leads V3-V4 and prolonged QTc in all leads were observed (Fig. 5D). However, the QTc was always less than 500 ms in the nephew and QTc prolongation occurred only during periods of increased heart rate. The hyperventilation test was normal in the brother but induced QT prolongation in both niece (maximum QTc 490 ms) and nephew (maximum QTc 480 ms). The hyperventilation test is a useful clinical tool able to increase the sensitivity of standard ECG in detecting mutation carriers (42). The K897T polymorphism was also identified in the proband's son and in one of her sisters. Both of these K897T carriers were asymptomatic with normal QTc intervals by standard ECG and during the hyperventilation test. One of the proband's brothers refused medical contact. The genetic status of her parents could not be ascertained because they were deceased.

Biophysical characterization of *KCNH2* variants

We studied the functional features of both variants separately and together using whole-cell patch clamp recording of recombinant HERG heterologously expressed in cultured CHO-K1 cells. Functional characterization of A1116V revealed significantly reduced activating and tail current densities at positive potentials when compared to wildtype HERG (WT-HERG) (Fig. 6A-C, $P < 0.05$). Similarly, expression of K897T generated current densities that were significantly less than WT-HERG at potentials greater than +10 mV (Fig. 6B,C, $P < 0.05$). Tail current density was 50% lower in cells expressing A1116V compared to cells expressing WT-HERG, but only 25% lower in cells expressing K897T (Fig. 6C).

Comparing the two variants to one another, activating and tail current densities for A1116V were significantly less than K897T at positive potentials (Fig. 6B,C, $P < 0.05$). A significant shift in the voltage-dependence of activation was also observed for A1116V ($V_{1/2}$, -5.1 ± 2.4 mV) compared to WT-HERG ($V_{1/2}$, $+1.1 \pm 1.7$ mV; Fig. 6D, $P < 0.05$) but there was no significant shift observed for K897T. More detailed analyses of kinetic properties demonstrated that A1116V exhibits slower recovery from inactivation, whereas the time course and voltage-dependencies of inactivation and deactivation were indistinguishable among the three alleles. We conclude that both A1116V and K897T cause mild channel dysfunction, but A1116V has greater functional impairments.

Because the proband carried both A1116V and K897T on separate alleles, we examined the effects of co-expressing both variants in the same cells. To demonstrate expression of both constructs in a single cell, we coupled expression of the variant HERG alleles to either EGFP or DsRed fluorescent proteins using bicistronic IRES vectors, and identified co-expressing cells by yellow fluorescence (combined EGFP and DsRed). Coexpression of either A1116V or K897T with WT-HERG did not significantly alter the magnitude of activating currents (Fig. 7A). By contrast, cells co-expressing A1116V and K897T together exhibited significantly lower current density compared to WT-HERG alone at potentials greater than -10 mV. Current density at positive

potentials was also lower in cells co-expressing both variant alleles compared with co-expression of A1116V or K897T with WT-HERG (Fig. 7A). Tail current density was significantly lower in cells co-expressing A1116V and K897T compared to WT-HERG alone, co-expression of K897T and WT-HERG, and co-expression of A1116V and WT-HERG at positive potentials (Fig. 7B). There was no significant shift in the voltage-dependence of activation observed in any of these experiments (Fig. 7C). Based upon these findings, we determined that the K897T polymorphism exaggerates the reduction in *IKr* caused by A1116V.

FIGURES: Italian LQT2 Family (IT-A1116V)

Figure 1: ECG recordings from the proband. The morphology and QTc duration were normal during most of the recording (A); however, there were also transient episodes of bifid T waves in leads V3-V5 (B) and prolonged QTc (maximum QTc 530 ms) (C).

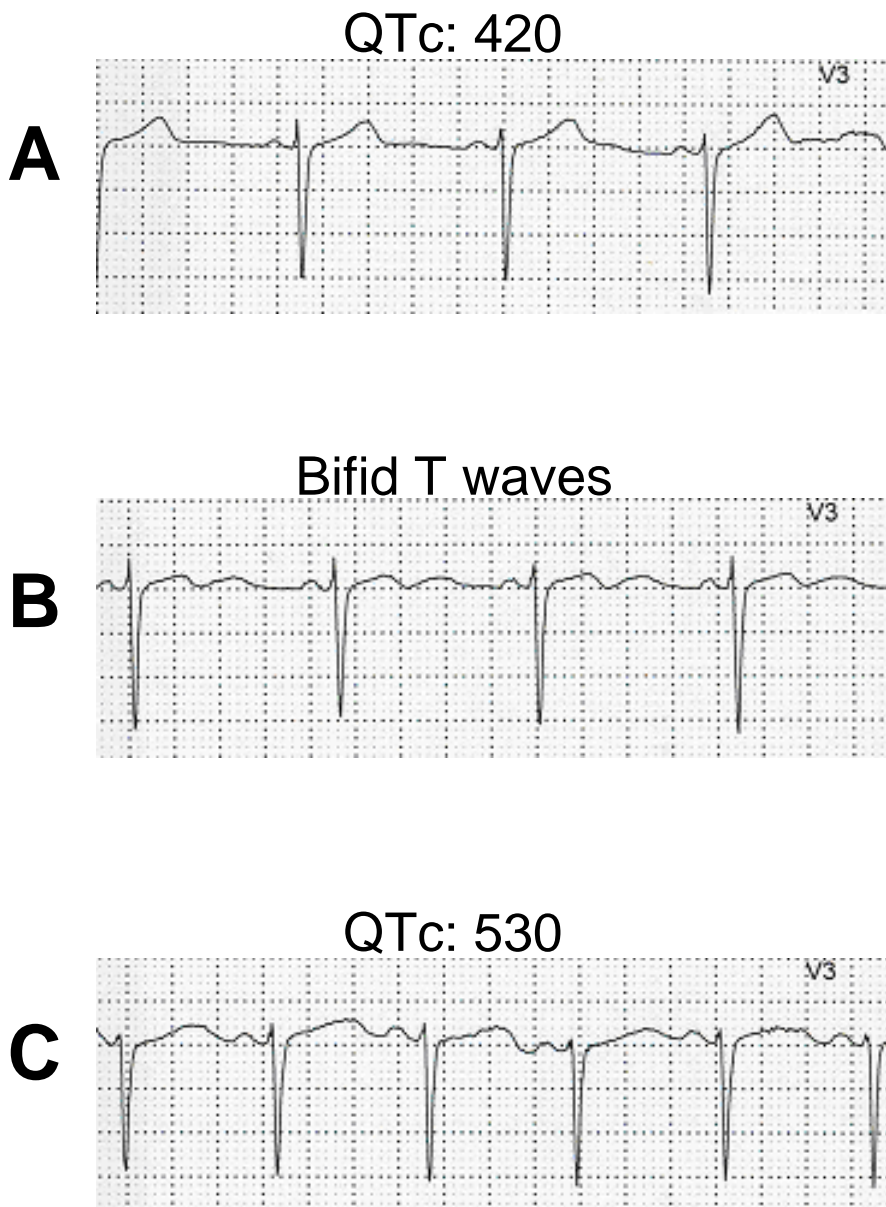
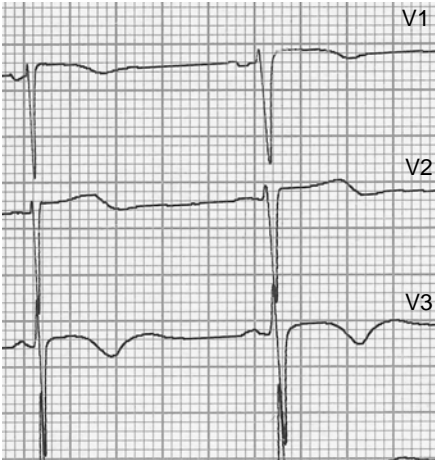


Figure 2. Hyperventilation test in the proband. The baseline ECG showed a QTc of 470 msec with negative T waves in V3-V4; During hyperventilation QTc prolonged to 510 ms in V3 and the repolarization abnormalities were magnified. QTc represents the mean value of five consecutive beats.

Basal



60" hyperventilation test

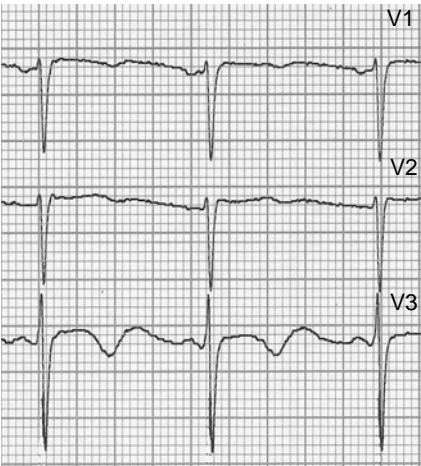
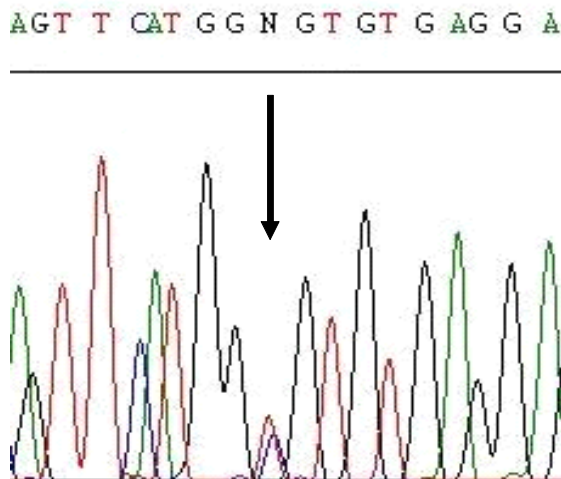


Figure 3. Identification of *KCNH2*-A1116V. (A) DNA sequence chromatogram showing a heterozygous mutation (C to T transition) resulting in an alanine to valine substitution at codon 1116. (B) Alignment of HERG amino acid sequence from various species illustrating that alanine at position 1116 is highly conserved.

A



B

	1116
	↓
Human	LSQVSQFM <u>A</u> CEELPPGA
Mouse	LSQVSQFV <u>A</u> FEELPAGA
Rat	LSQVSQFV <u>A</u> FEELPAGA
Dog	LSQVSQFM <u>A</u> FEELPPGA

Figure 4. Segregation of A1116V and K897T in the LQTS pedigree. Analysis of the pedigree indicates that the two variants exist on separate *KCNH2* alleles. Family history was negative for syncopal events or sudden cardiac death, though one of the proband's siblings was stillborn in the 9th month of pregnancy.

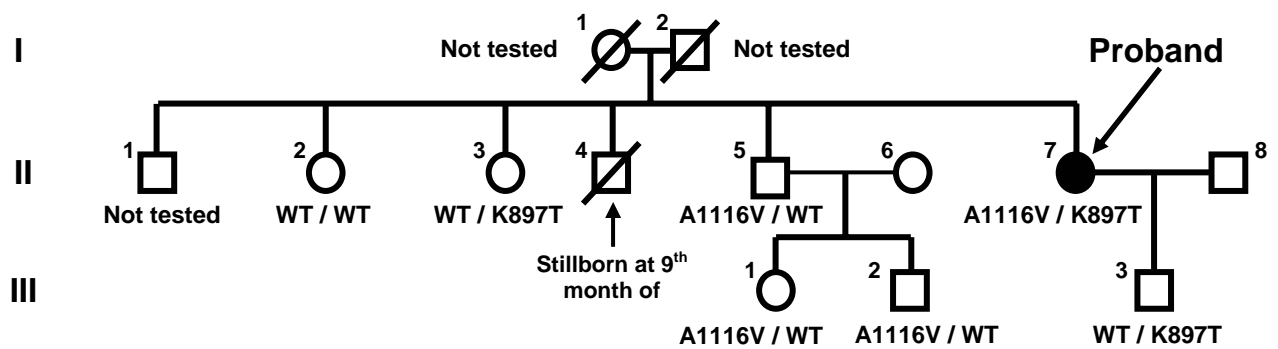


Figure 5. Characteristics of asymptomatic *KCNH2*-A1116V carriers. (A) Partial family tree from Figure 3. The A1116V mutation, but not K897T, was identified in one brother and his two children. (B) Normal QTc in the brother recorded during 12-lead 24-hour ECG monitoring. (C) The 22-year-old niece had a normal baseline QTc, but during a 24-hour ECG recording, rare transient episodes of mild QTc prolongation were observed (maximum QTc 480 ms). (D) The 9-year-old nephew had a normal baseline QTc, but during 24-hour ECG recording, periods of biphasic or notched T waves in leads V3-V4 and prolonged QTc were observed. However, QTc was always less than 500 ms and prolongation occurred only during increased heart rate.

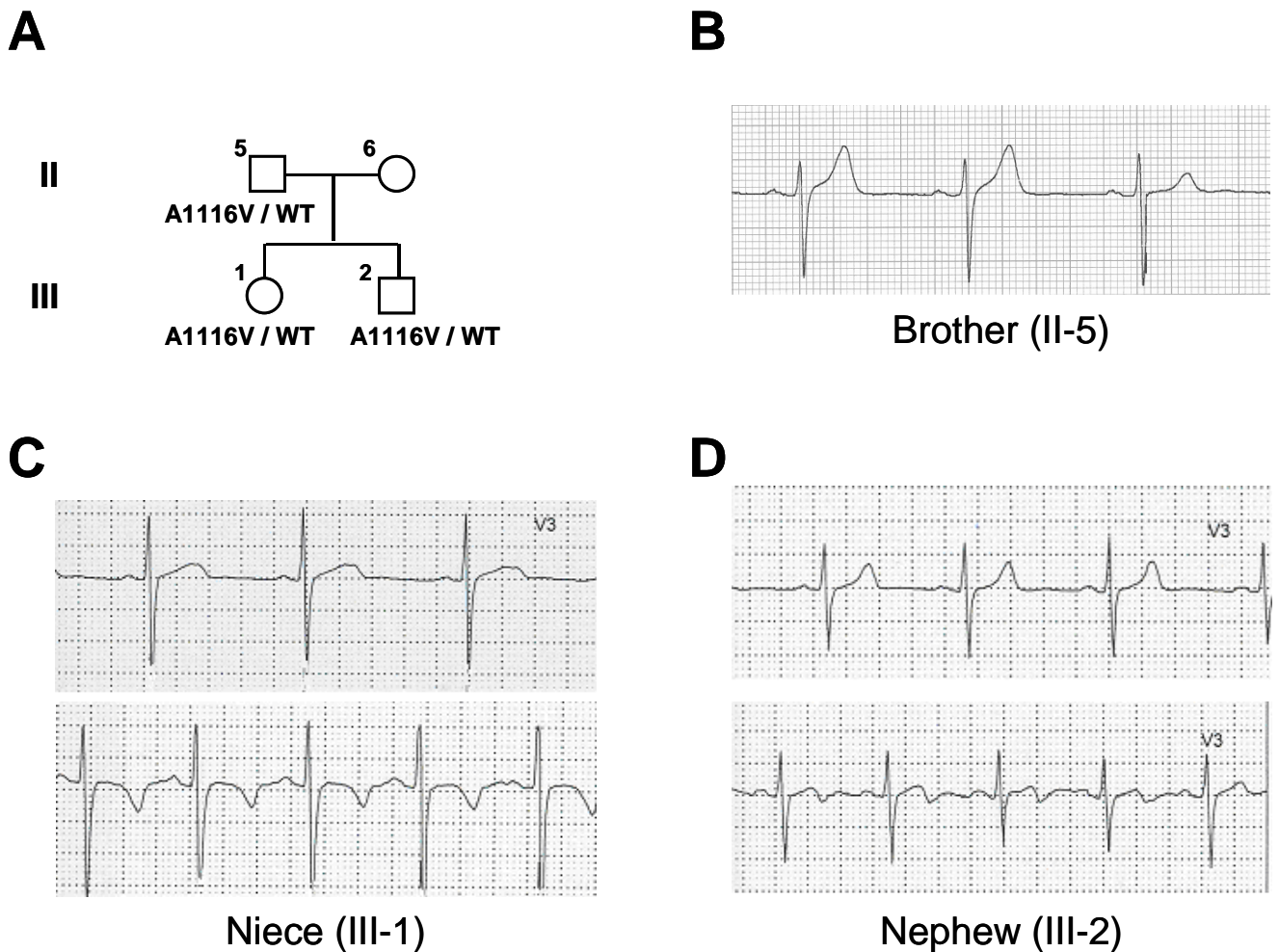


Figure 6. Functional characterization of A1116V and K897T HERG variants. (A) Representative traces illustrating potassium currents observed in CHO cells transiently transfected with WT-HERG or A1116V (horizontal and vertical scale bars represent 1000 ms and 225 pA, respectively). (B) Current-voltage relationship for potassium current densities (normalized to membrane capacitance) measured in CHO cells expressing WT-HERG (WT, solid circles, $N = 9$), K897T (open circles, $N = 9$), or A1116V (solid squares, $N = 8$). (C) Current-voltage relationship for amplitude of peak tail current densities following repolarization to -50 mV for WT-HERG (WT, solid circles, $N = 9$), K897T (open circles, $N = 9$), or A1116V (solid squares, $N = 8$). (D) Normalized current-voltage relationship for peak tail current densities for WT-HERG (WT, solid circles, $N = 9$), K897T (open circles, $N = 9$), or A1116V (solid squares, $N = 8$). Data were recorded at test potentials ranging from -80 to $+70$ mV stepped in 10 mV increments from the holding potential of -80 mV for 2000 ms, followed by repolarization to -50 mV for 2000 ms. Data are shown as means \pm SEM.

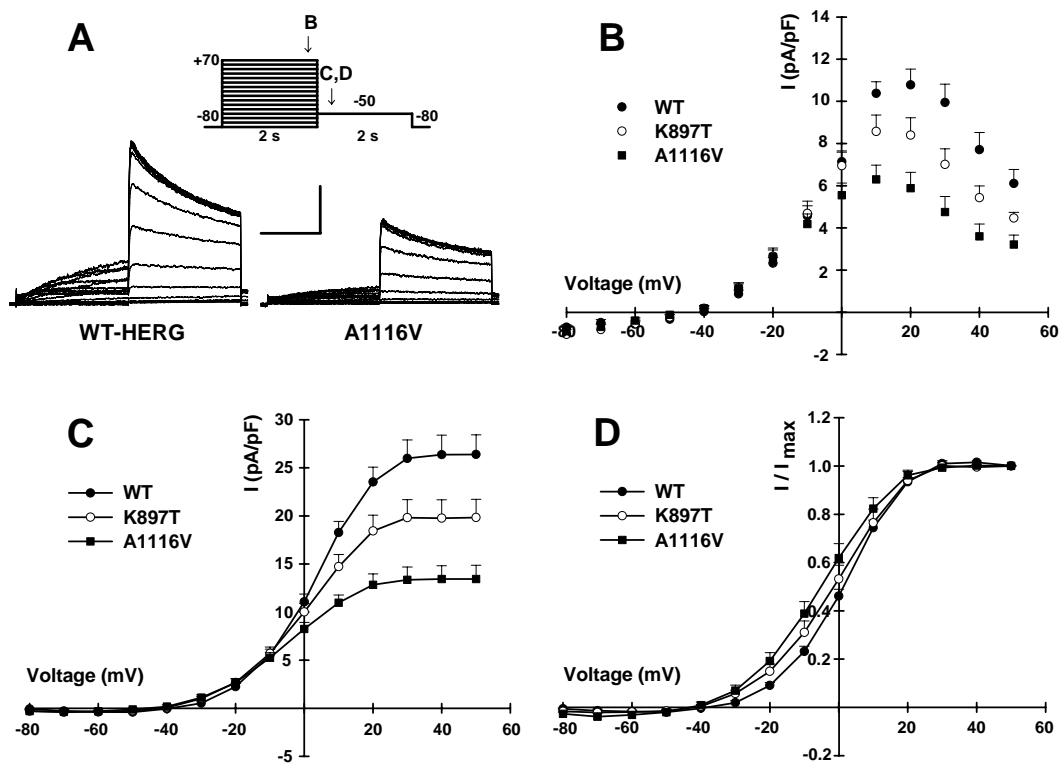
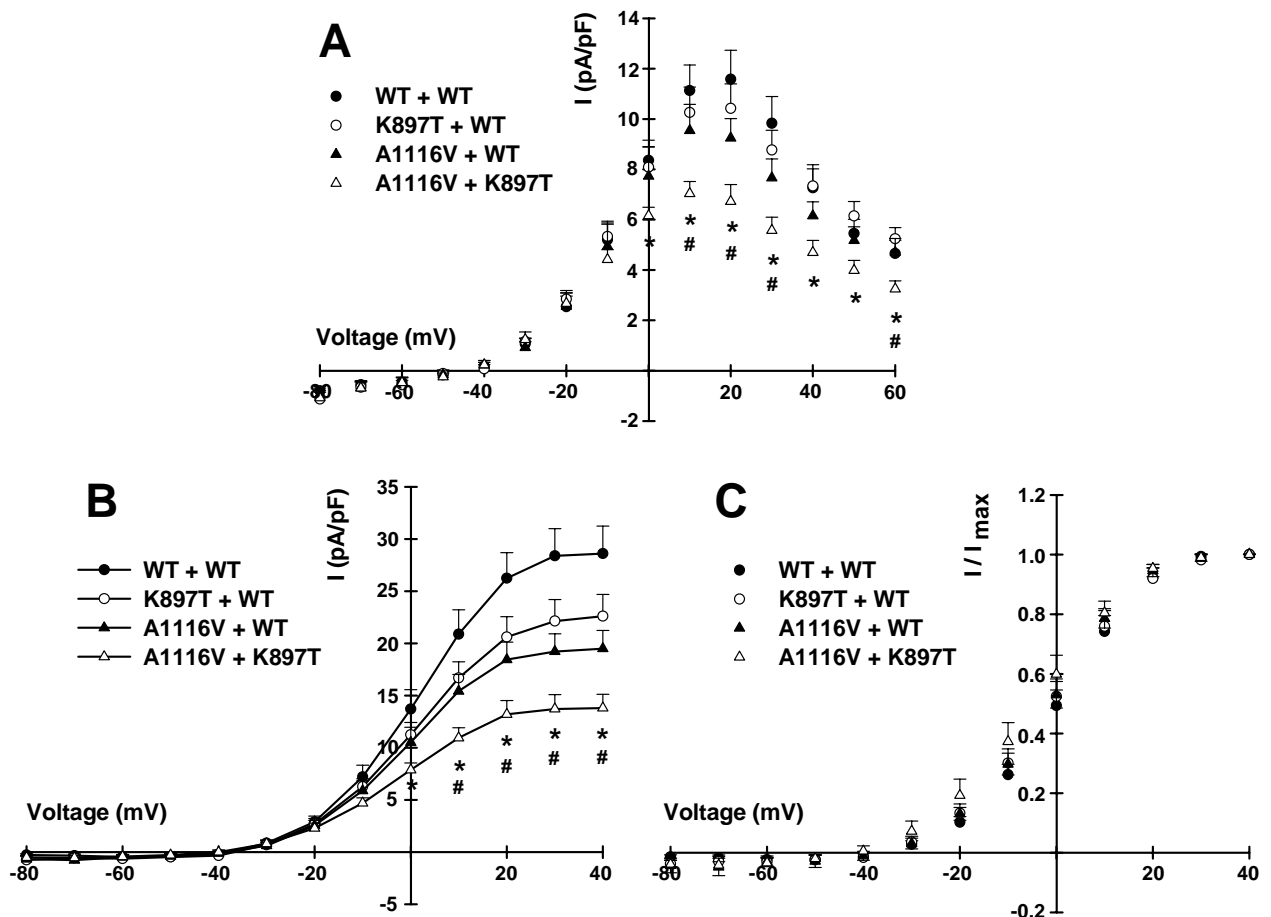


Figure 7. Co-expression of HERG variants. Plasmids encoding WT-HERG, A1116V or K897T were expressed in tandem with either EGFP or DsRed. In each experiment, the allele listed first in the inset legend is coupled to EGFP, while the second is coupled to DsRed. **(A)** Current-voltage relationship for potassium currents (normalized to membrane capacitance) measured in CHO cells co-expressing WT-HERG + WT-HERG (closed circles, $N = 9$), K897T + WT-HERG (open circles, $N = 9$), A1116V + WT-HERG (closed triangles, $N = 10$), or A1116V + K897T (open triangles, $N = 10$). **(B)** Current-voltage relationship for peak tail current densities after repolarization to -50 mV. **(C)** Normalized current-voltage relationship for peak tail current densities. Data are shown as means \pm SEM. Significant differences between A1116V + K897T and WT-HERG + WT-HERG are denoted by *, while significant differences between A1116V + K897T and A1116V + WT-HERG are denoted by # ($P < 0.05$).



RESULTS: South African LQT1 Founder Population (SA-A341V)

Family Ascertainment, Genealogy, and Genotyping

A LQTS founder population (SA-A341V) consisting of 22 apparently unrelated kindreds was ascertained in South Africa. All index cases could be traced to a single founding couple, of mixed Dutch and French Huguenot origin, who married in approximately 1730 (Fig. 8). The disease associated haplotypes of index cases strongly support the founder hypothesis (Fig. 8).

Of 345 individuals in the study population, 166 were mutation-carriers, 154 were non-carriers and 25 were not genetically tested (Fig. 9).

Clinical Phenotypes

Among the 166 MCs, females (54%) and males were similarly represented. According to definitions reported in the Method Session (page 5 and 6), 131 (79%) were symptomatic with a median age at first cardiac event of 6 years (IQR 4-10), and 23 (14%) suffered sudden cardiac death before age 20. The 26 patients here defined as asymptomatic were those older than 15 years with no events. Nine other patients without events were too young (age <15) to be designated as asymptomatic (43)

Among the 166 MCs, a basal ECG without beta-blocker therapy was available in 92, while 74 had no ECG data. The two subgroups were no significant different in terms of gender (females: 59% in patients with an ECG, vs 47% in patients without an ECG; $p=0.16$), cardiac events (Symptomatic MCs: 74% vs 85%, $p=0.09$) and age at first event (7.39 ± 4 vs 7.13 ± 4 years; $p=0.7$); however, none of the 23 sudden cardiac death had an ECG available. Eighty-six MCs and 102 non-carriers, with a basal ECG recorded after age 15 (Fig. 9), were analyzed for differences in QTc interval, heart rate and symptoms. Baseline QTc was longer in MCs than in non-carriers (487 ± 45 vs 401 ± 25 ms, $p<0.001$; Fig. 10). Despite sharing the same genetic defect, mutation-carriers as a group exhibited a wide range of QTc values (406-676 ms) with 12% of individuals having a normal QTc (≤ 440 ms). QTc was longer among symptomatic as compared with asymptomatic MCs (493 ± 48 vs

468±31 ms, $p=0.026$; Fig. 11). A $QTc \geq 500$ ms was associated with an increased risk of experiencing cardiac events (OR=4.22; 95% CI 1.12-15.80; $p=0.033$).

Because I_{Ks} magnitude is rate-dependent, we examined the role of HR as a predictor of events. Baseline HR was not different between MCs and non-carriers (69±12 vs 70±11 bpm). The baseline heart rate of symptomatic MCs was also very similar to that of non-carriers (71±11 vs 70±11 bpm). By contrast, asymptomatic carriers had a significantly lower heart rate compared to symptomatic individuals (65±13 vs 71±11 bpm, $p=0.026$). Mutation carriers in the lowest 2 tertiles, defined by HR <73 bpm, were at lower risk for cardiac events compared to those with in the highest tertile, HR ≥73 bpm (OR=0.23; 95% CI, 0.06-0.86; $p=0.035$). There was no correlation between age and HR. We performed multivariate analyses to determine the clinical variables (HR, QTc, gender) best predicting risk for cardiac events in the SA-A341V population. We considered HR and QTc as categorical variables with the same cut-off used in univariate analysis. Both $QTc \geq 500$ ms (OR=4.98; 95% CI, 1.21-20.55; $p=0.026$) and $HR \geq 73$ (OR=4.11; 95% CI, 1.03-16.44; $p=0.046$) were found to be significant risk factors for experiencing cardiac events after controlling for other covariates included in the model. Gender was not an independent risk factor in our analysis.

Results from both univariate and multivariate analyses identify HR and QTc as important factors in determining disease expression in this population. Figure 12 presents the distribution of symptomatic and asymptomatic MCs among four quadrants defined by cut-off values of HR and QTc. The smallest proportion of symptomatic subjects was found in the quadrant defined by HR <73 bpm and $QTc < 500$ ms. In this subgroup, the risk of cardiac events was significantly lower than for all other subjects combined (OR=0.19; 95% CI, 0.06-0.59; $p=0.005$). However, there was still a significant risk of cardiac events in this subgroup as most subjects (60%) represented in this quadrant were symptomatic. Interestingly, the impact of HR in risk-stratification was stronger in the subgroup of patients with a $QTc < 500$ ms compared to that with a $QTc \geq 500$ ms. Indeed, there was a

linearly increasing proportion of symptomatic mutation-carriers from the lower to the upper tertile of heart rate representing an incremental risk (OR=2.5; 95% CI, 1.11-5.62; p=0.026) (Fig. 13).

Baroreflex Sensitivity and Risk for Cardiac Events

As explained in the Methods the analysis of BRS was limited to subjects in the two middle age quartiles (26-47 years) to avoid the influence of age (29). Indeed, within this group (n= 38) there was no correlation between BRS and age ($r = -0.17$, NS). Furthermore, even subdividing this group according to the median value (36 years) there was no difference in BRS across the two subgroups.

The mean value of BRS in the entire group of 38 subjects was 16.7 ± 8.8 ms/mmHg. As expected by a distribution of autonomic parameters independent of the LQTS mutation, the BRS values between MCs (n = 22) and non-MCs (n = 16) were not different (17.1 ± 9.7 vs 16.3 ± 7.6 , NS) (Fig. 14A). By contrast, a significant difference emerged when the analysis compared the asymptomatic (n = 8) to the symptomatic (n = 14) MCs (11.8 ± 3.5 vs 20.1 ± 10.9 ; p = 0.04) (Fig. 14B). A BRS ≤ 12 ms/mmHg, which corresponds to the first tertile of the distribution among all the 38 subjects, carried a significantly lower risk of suffering cardiac events compared to a BRS in the upper two tertiles (OR 0.13, 95%CI 0.02-0.96, p < 0.05) (Fig. 14B). Indeed, among MCs with a BRS > 12 ms/mmHg, 10 of 12 (83%) subjects were symptomatic. BRS values in the first tertile, which represents the lower end of the spectrum of normal values among these South African families, will be referred to as “relatively low”.

When we assessed whether the level of BRS was associated with differential risk for arrhythmic events in the 15 patients with a QTc ≤ 500 ms we found that none of the MCs in the first tertile (BRS ≤ 12 ms/mmHg) had cardiac events whereas 80% of those with a BRS >12 ms/mmHg were symptomatic (p < 0.01) (Fig. 15).

The subgroup of MCs with BRS values without beta-blocker therapy is representative of the entire population of MCs, as there are no significant difference in terms of gender (females: 57% in

patients with BRS, vs 52% in patients without BRS; $p=0.6$), cardiac events (Symptomatic MCs: 74% vs 81%, $p=0.4$) and age at first event (7.16 ± 3.7 vs 7.31 ± 4 years; $p=0.84$).

In 22 of these 38 subjects we assessed BRS both on and off BB and observed that treatment significantly increased BRS by 5 ± 8 ms/mmHg ($p < 0.05$). Importantly, this effect was not uniform across the three subgroups (non-MCs, symptomatic and asymptomatic MCs). Indeed, while a trend for greater BRS during BB was evident in both symptomatic MCs and non-MCs (4.5 ± 7 and 9 ± 11 ms/mmHg, respectively) it was practically absent in asymptomatic subjects (0.83 ± 7 ms/mmHg) (Fig. 16). Among MCs on BB treatment and with a BRS above the lower tertile (14 ms/mmHg), 7 of 8 (87.5%) subjects had symptoms, a similar proportion to that observed off BB (83% with symptoms, among those with a BRS above the lower tertile).

Clinical Severity in the South African LQTS Population

As our ascertainment revealed relatively few asymptomatic patients in the SA-A341V population, and 14% incidence of sudden death before age 40, we considered the possibility that the KCNQ1-A341V mutation segregating in these families might be associated with a greater incidence of cardiac events compared to that reported for LQT1 subjects in general. To test this hypothesis, we compared clinical severity between the SA-A341V and LQT1 populations (30). In our population, the availability of information for 161 KCNQ1-A341V MCs, including 126 who were symptomatic, allowed us to analyze the cumulative event-free survival (Kaplan-Meier analysis) before the institution of β -blocker therapy and before age 40. Five MCs were not included in the analysis because time to first event was not available.

Compared to the LQT1 population, the SA-A341V group exhibited a more severe form of the disease. The SA-A341V carriers became symptomatic earlier than LQT1 (7 ± 4 vs 13 ± 9 years, $p<0.001$), with a 79% incidence of a first cardiac event by age 40 compared with 30% observed for LQT1 ($p<0.001$). As shown in Fig. 17, the cumulative probability of suffering a first cardiac

episode before initiating β -blocker therapy and before age 40 was significantly greater ($p<0.0001$) for the SA-A341V population, in which the event-free survival is 20% by age 15 as compared with 80% for the LQT1 database.

Our findings on the clinical severity of the SA-A341V phenotype could be partly explained by the significantly lower prevalence of MCs with a $QTc<440$ ms in this population as compared to the LQT1 database (12% vs 36%, $p<0.001$). This in turn can account for the longer QTc measured in MCs in our population (487 ± 45 vs 466 ± 44 ms, $p<0.001$). Because of this concern, we performed separate Kaplan-Meier estimates of cardiac event free survival for groups distinguished by QTc . Furthermore, to avoid the confounding role of patients with $QTc<440$ ms, we restricted our analysis to subjects with $QTc\geq 500$ ms. We also constructed Kaplan-Meier curves separately by gender and observed that they were similar for males and females. As the time course of events is much more rapid for LQT1 males, who have their events earlier in time, and as in our population the number of males available for this analysis was small we have chosen not to combine data for males and females and to present in Fig. 18 only the data based on the larger and more homogenous female group ($n=24$). These analyses support the finding that the clinical severity of LQTS observed in the SA-A341V population is significantly greater than LQT1 in general.

To further support the finding, we compared our SA-A341V population also to the one reported by Zareba et al (19) even though it included only 112 patients from 10 families (Fig.19). This was done because Zareba's population had an average QTc very similar to that of the SA-A341V patients (490 ± 43 vs 487 ± 45 ms). The Kaplan-Meier curves remained significantly different ($p<0.01$) as significant were the difference of patients with a first event by age 15 (55% vs 80%, $p<0.001$) and the difference in mortality (2% vs 14%, $p<0.001$). This conclusively demonstrates the unusual clinical severity associated with A341V.

Functional characterization of KCNQ1-A341V

Previous studies have characterized the KCNQ1-A341V mutation in *Xenopus* oocytes (44-46) or COS7 cells (47). These studies reported that this allele is a simple loss-of-function mutation that does not exhibit a dominant-negative effect on WT KCNQ1 suggesting that it may cause less severe disease. However, oocytes express an endogenous *Xenopus* KCNQ1 (48) and multiple KCNE accessory subunits (49). Accordingly, and in light of our observation that South African LQTS families segregating KCNQ1-A341V have a severe clinical phenotype, we reassessed the functional consequences of this allele in a cultured mammalian cell system. In these experiments, we recognized the need to verify co-expression of WT and mutant KCNQ1 along with the KCNE1 accessory subunit, a potential confounding variable for interpreting previously-reported COS7 cell data (47). Therefore, we transiently transfected KCNQ1-A341V-IRES-EGFP into CHO cells stably expressing consistent levels of WT KCNQ1 with KCNE1 (stable I_{Ks} cell line). We then performed whole-cell patch clamp recording on cells exhibiting green fluorescence i.e. cells coexpressing both A341V and both subunits for I_{Ks} (Fig. 20). Cells transiently transfected with the empty pIRES2-EGFP expression vector exhibited slowly activating outward current consistent with I_{Ks} . Co-expressing KCNQ1-A341V in these cells reduced the magnitude of I_{Ks} by approximately 50%. By comparison, co-expression of a recessive LQTS mutant (543-del/ins) had no effect on I_{Ks} amplitude, whereas a strong dominant LQTS mutation (G314S) suppressed current by approximately 70% at positive voltages. These results demonstrate that KCNQ1-A341V exerts dominant suppression of I_{Ks} to an extent slightly less than a strong dominant mutation but behaves in a manner distinct from a pure loss-of-function allele.

FIGURES: South African LQT1 Founder Population (SA-A341V)

Figure 8: Lines of descent of the KCNQ1-A341V mutation from a common founder couple P are shown. The haplotype that segregates with the mutation is consistent with a common origin of the mutation and with minimal recombination over 10 generations. Genealogical information for pedigree 170, in which the common haplotype segregates and for pedigree 180, a single individual, could not be found. As phase could not be determined for the latter, maternal and paternal alleles at D11S4046 and D11S1318 are shown. Haplotypes were constructed from the results of the combination of alleles inherited at D11S4046, D11S1318, A341V, D11S4088, D11S4146, D11S4181, D11S1871, D11S1760, D11S1323 in the order telomere to centromere. Common haplotypes are bordered. Index cases are shown as diamonds to preserve anonymity. Circles denote females and squares males in the line of descent. Ped=pedigree. Year of birth is shown below individuals. The letters P, Q, T refer to couples in the first two generations from which the mutation descended.

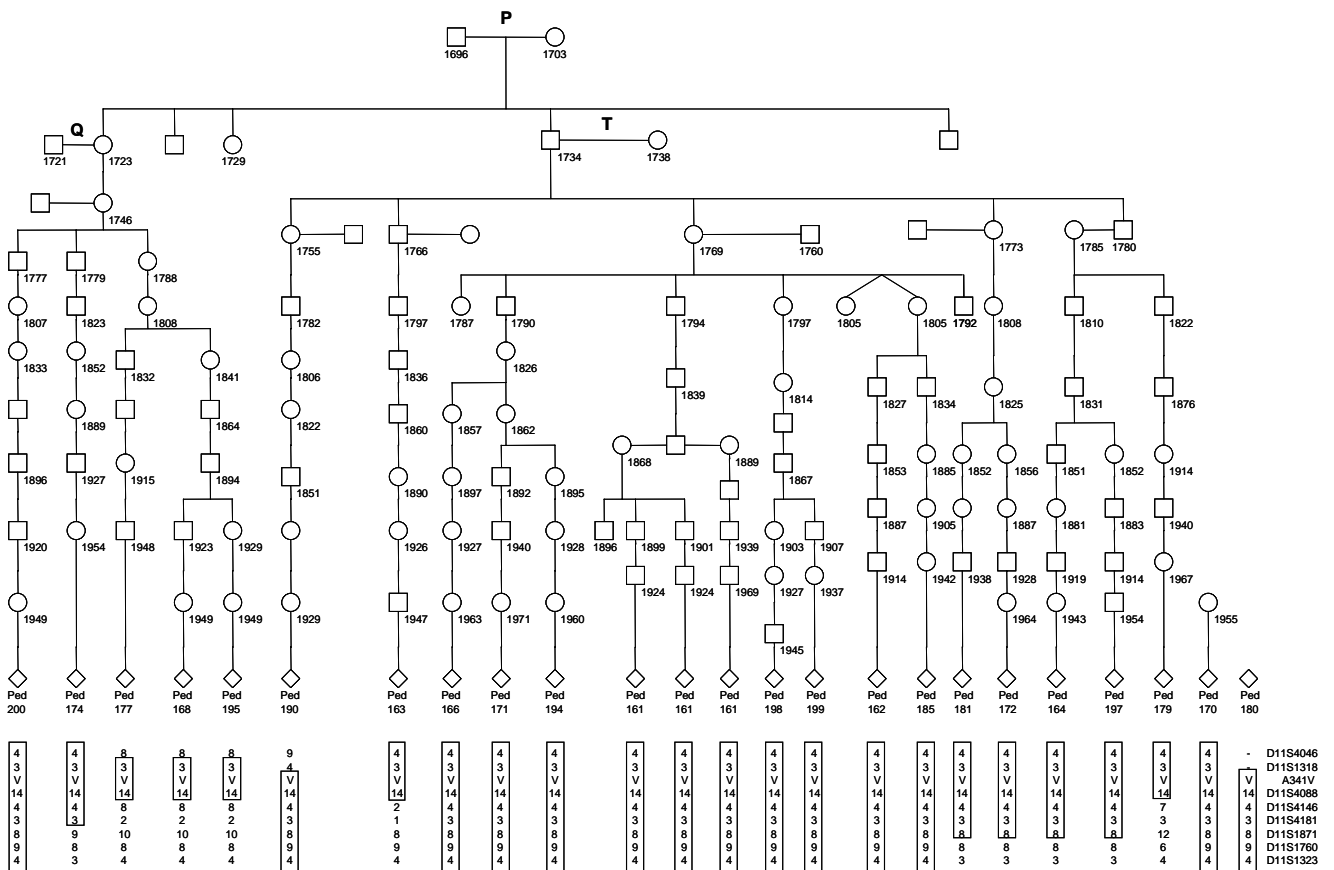


Figure 9: Flow-chart with the number of subjects, divided by subgroups in the study population. An ECG off BB therapy was available in 93 MCs, of these 86 were recorded after 15 years of age and were used for ECG analysis. Appropriate data for survival analysis were available for 161/166 MCs; of these, an ECG off β -blocker therapy was available in 89.

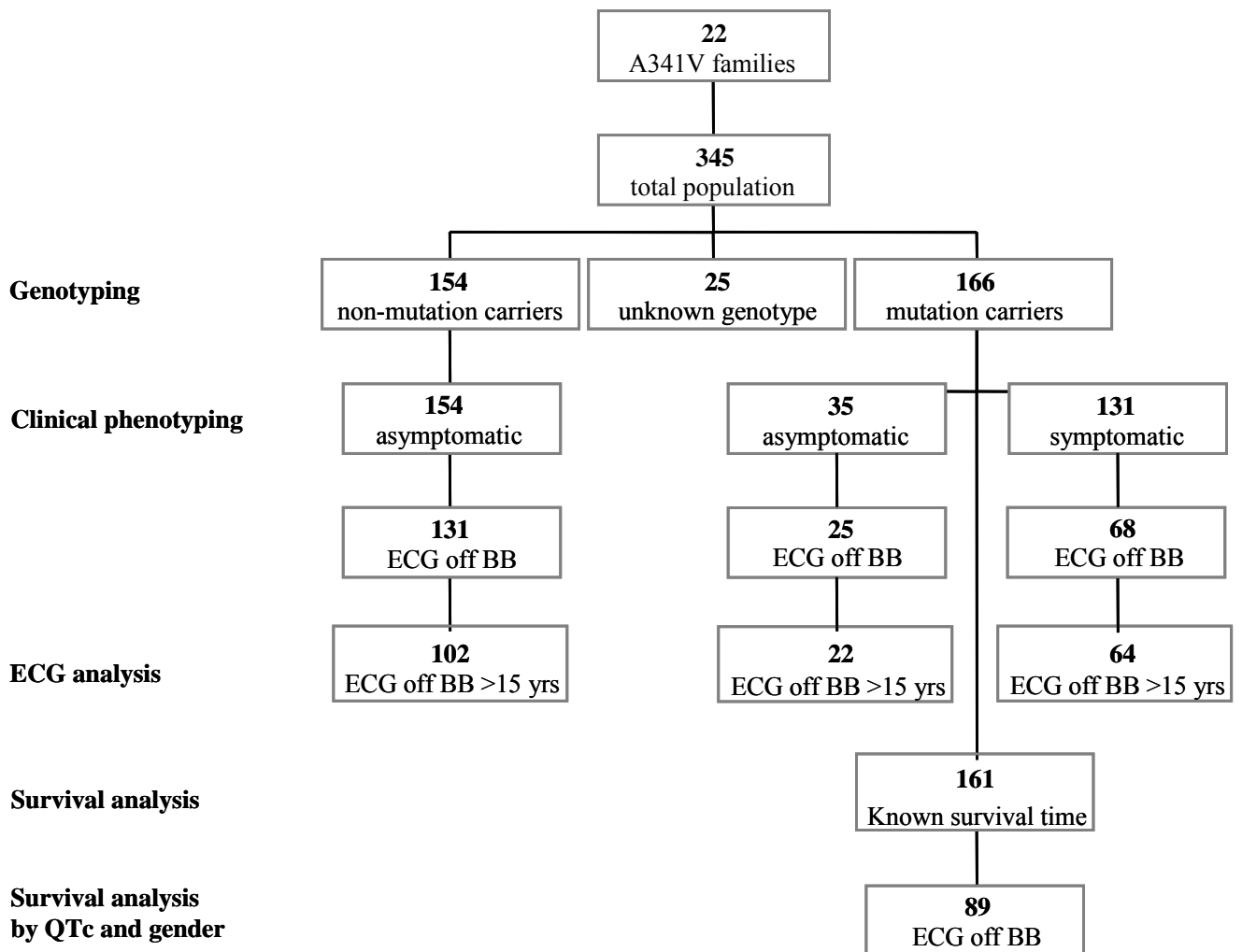


Figure 10: Basal QTc in mutation and non-mutation carriers. The long horizontal line represents the upper limit of normal values for men (440 ms). The short horizontal lines represent the mean. In two MCs and in three NMCs the QTc was not measurable because of the presence of bundle branch block.

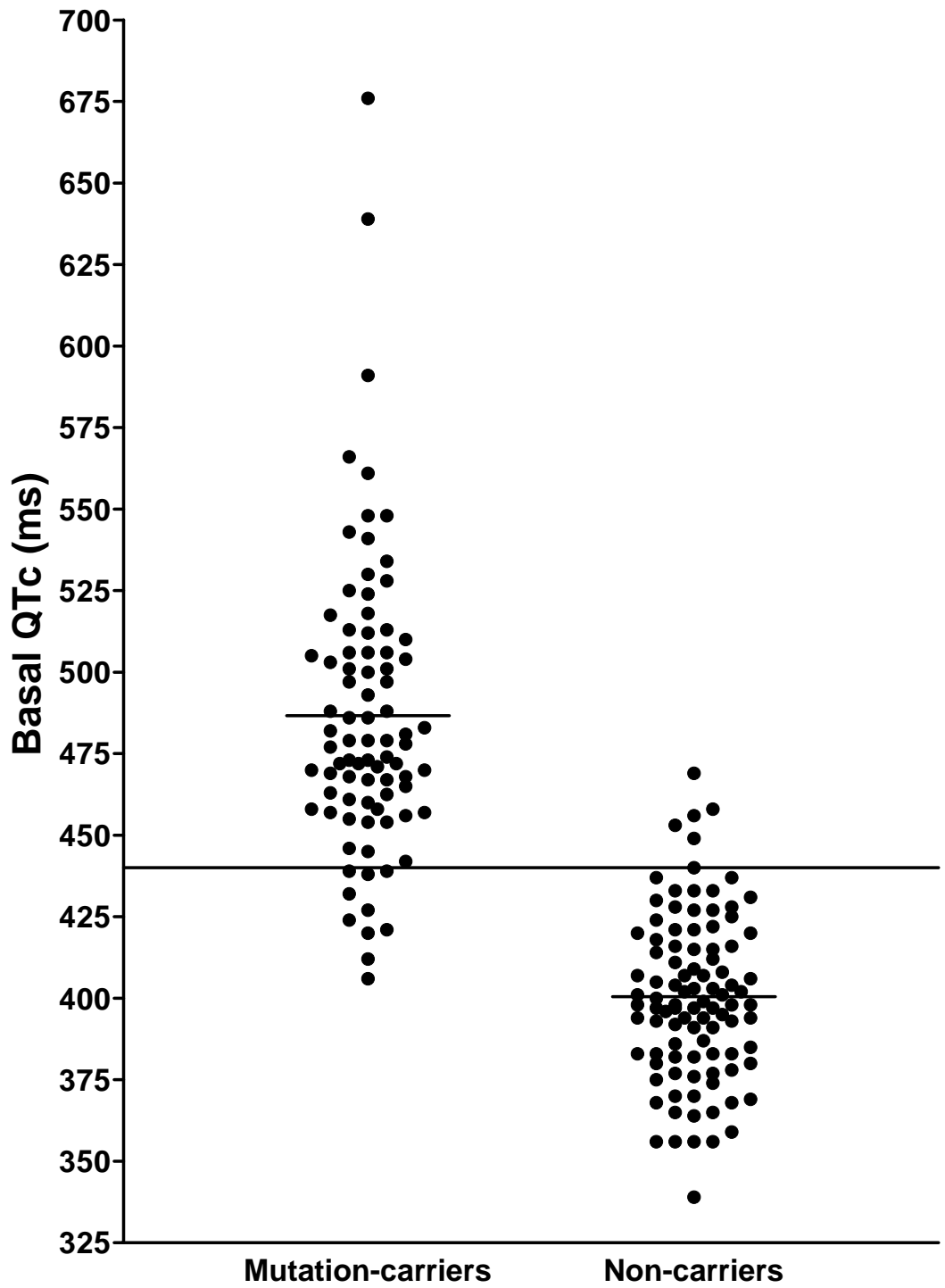


Figure 11: Basal QTc in symptomatic and asymptomatic mutation carriers. The long horizontal line represents the upper limit of normal values for men (440 ms). The short horizontal lines represent the mean.

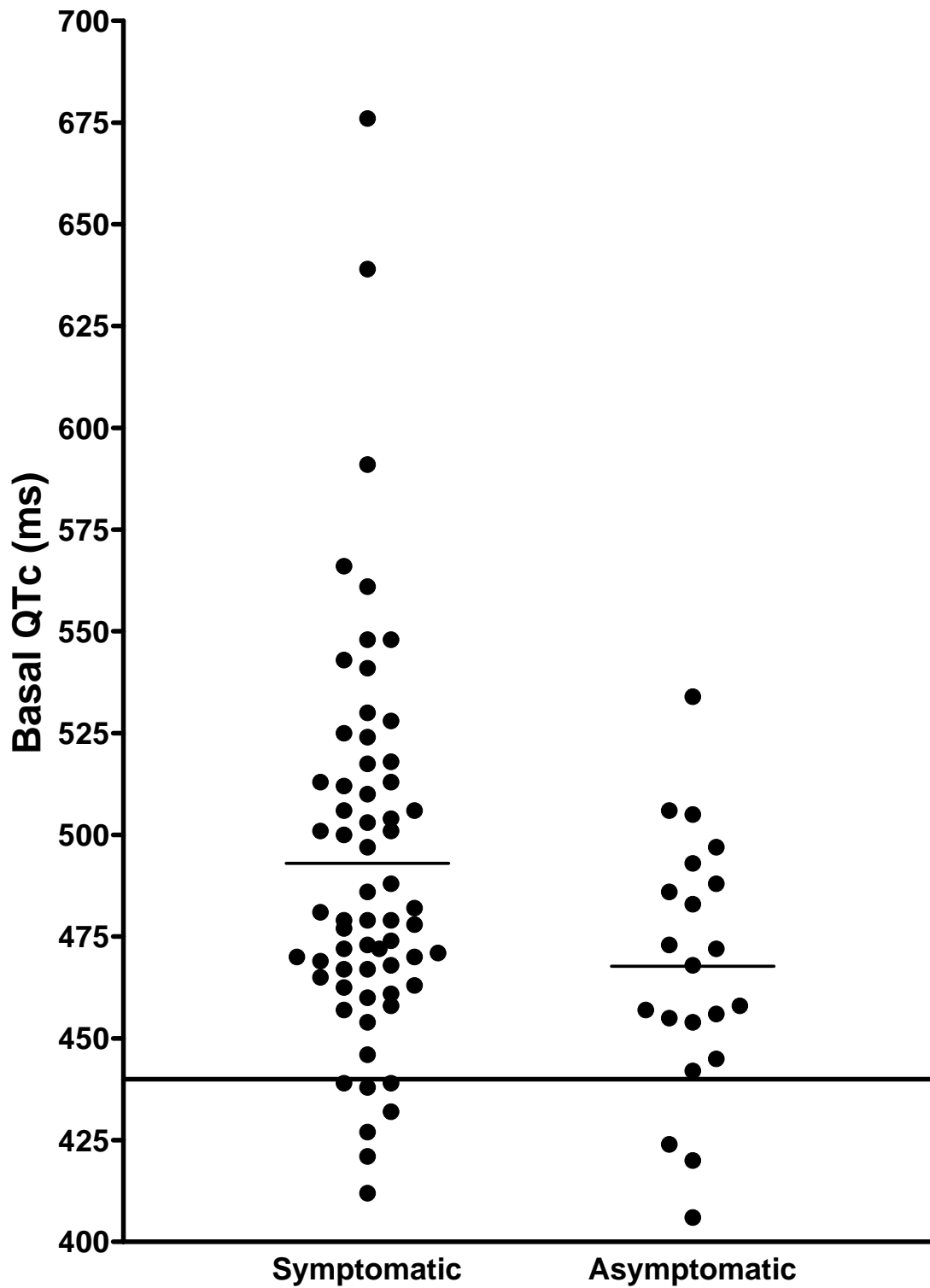


Figure 12: Distribution of mutation-carriers according to symptoms, HR and QTc. The cut-off at 500 ms and at 73 bpm represent the upper tertile.

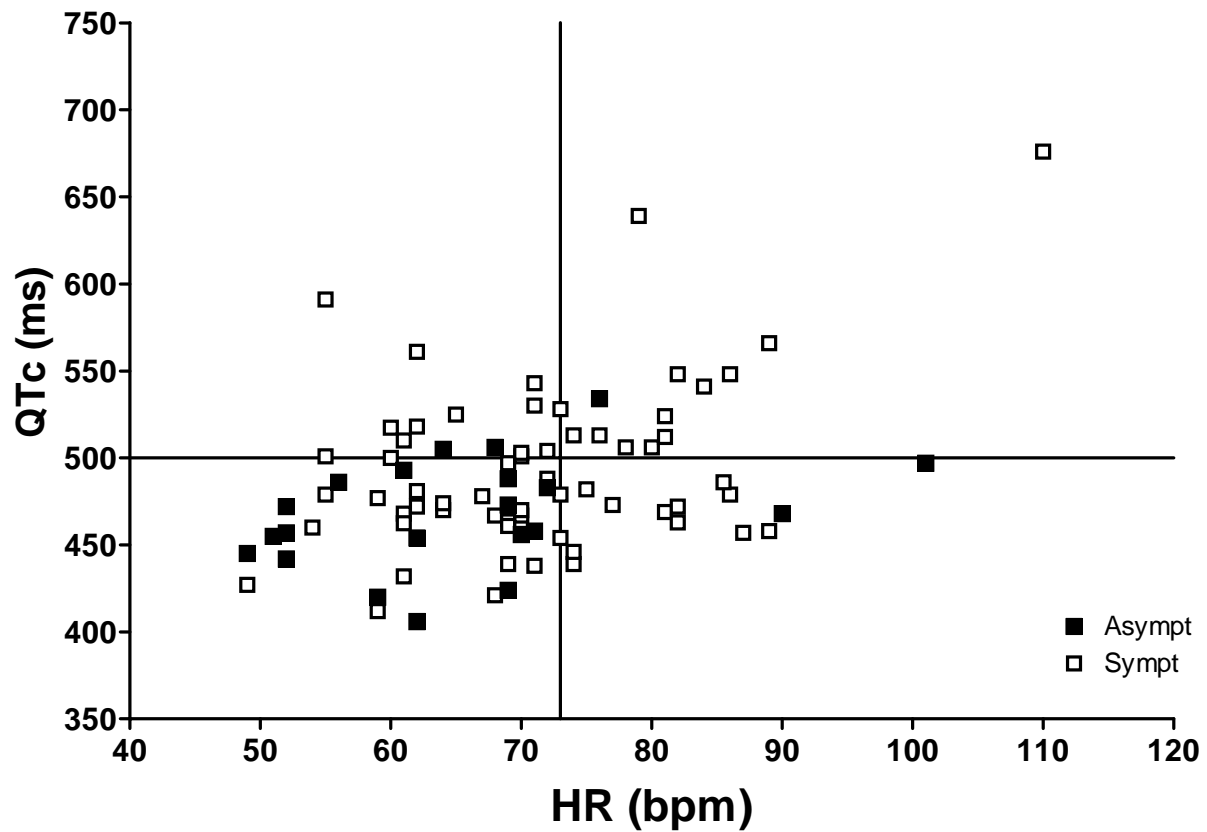


Figure 13: Percentage of symptomatic patients with a basal QTc < 500 ms in each tertile of heart rate. The cut-off at 500 ms and at 73 bpm represent the upper tertile.

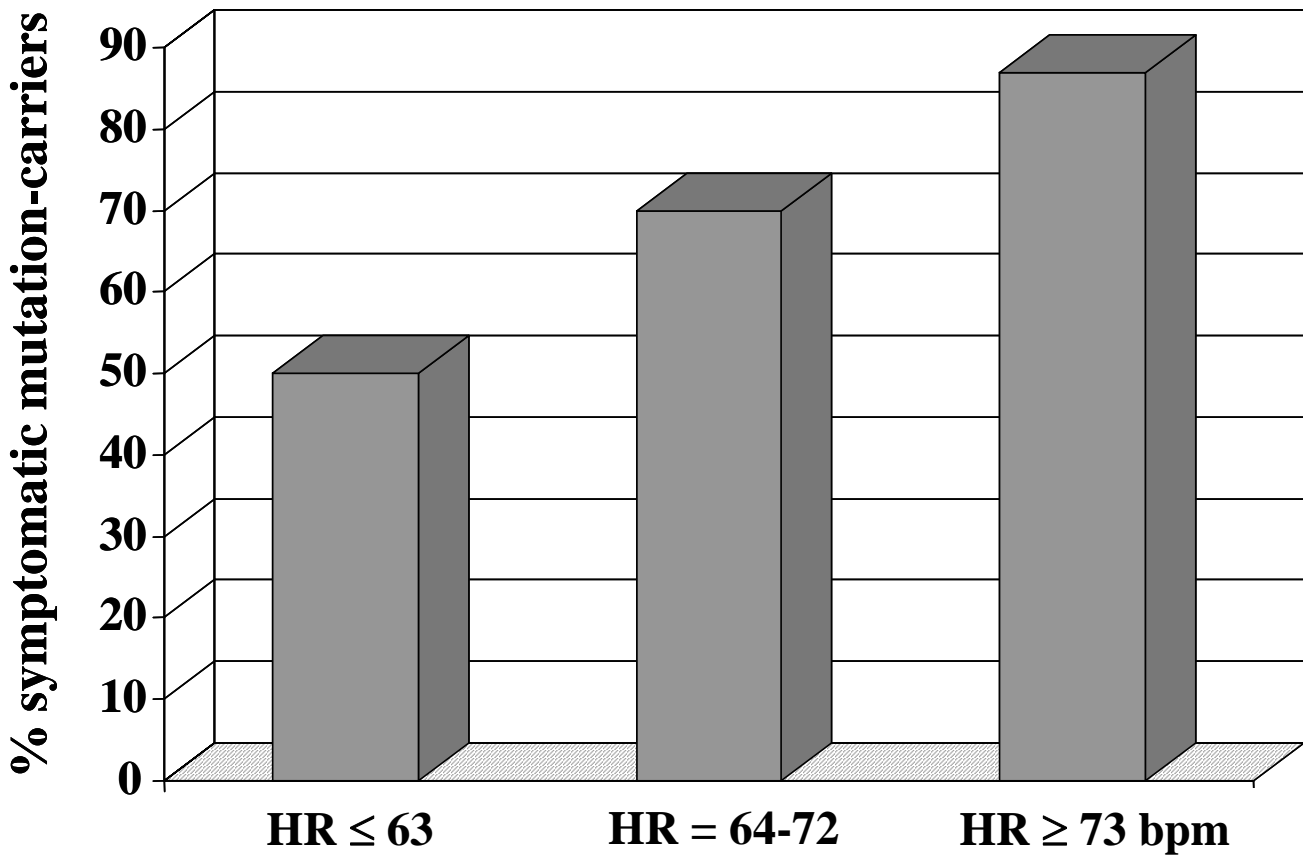


Figure 14: BRS values off-BB in different groups. BRS values off-BB in the entire group under study aged 26-47 years divided in MCs and in non-MCs (4A), and in symptomatic and asymptomatic MCs (4B). Mean and standard deviation are shown for each subgroup. The dashed horizontal line represents the lower tertile of BRS values.

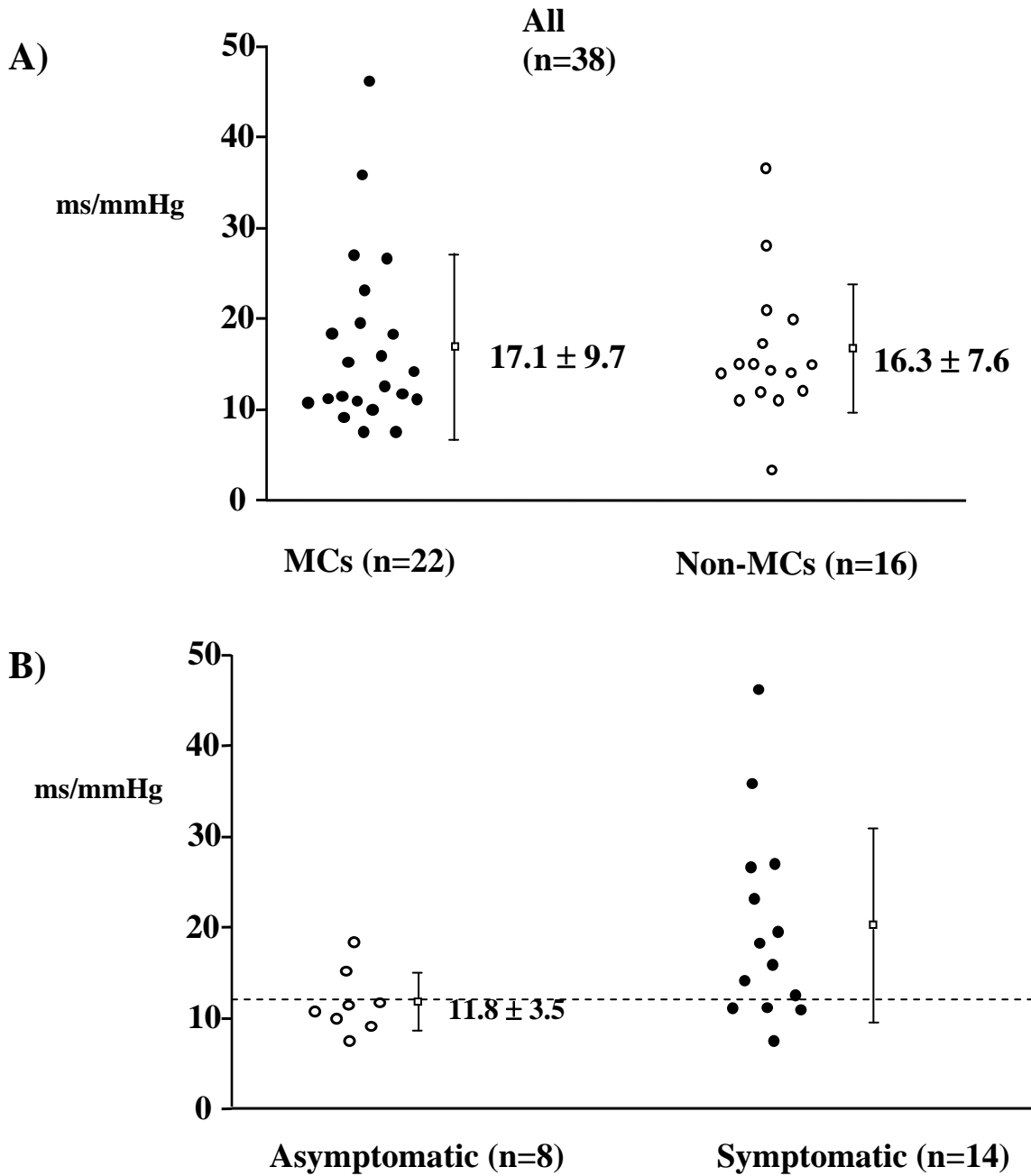


Figure 15: Distribution of mutation-carriers according to symptoms, BRS and QTc. The cut-off at 500 ms of QTc represents the upper tertile. The cut-off at 12 msec/mmHg of BRS represents the lower tertile.

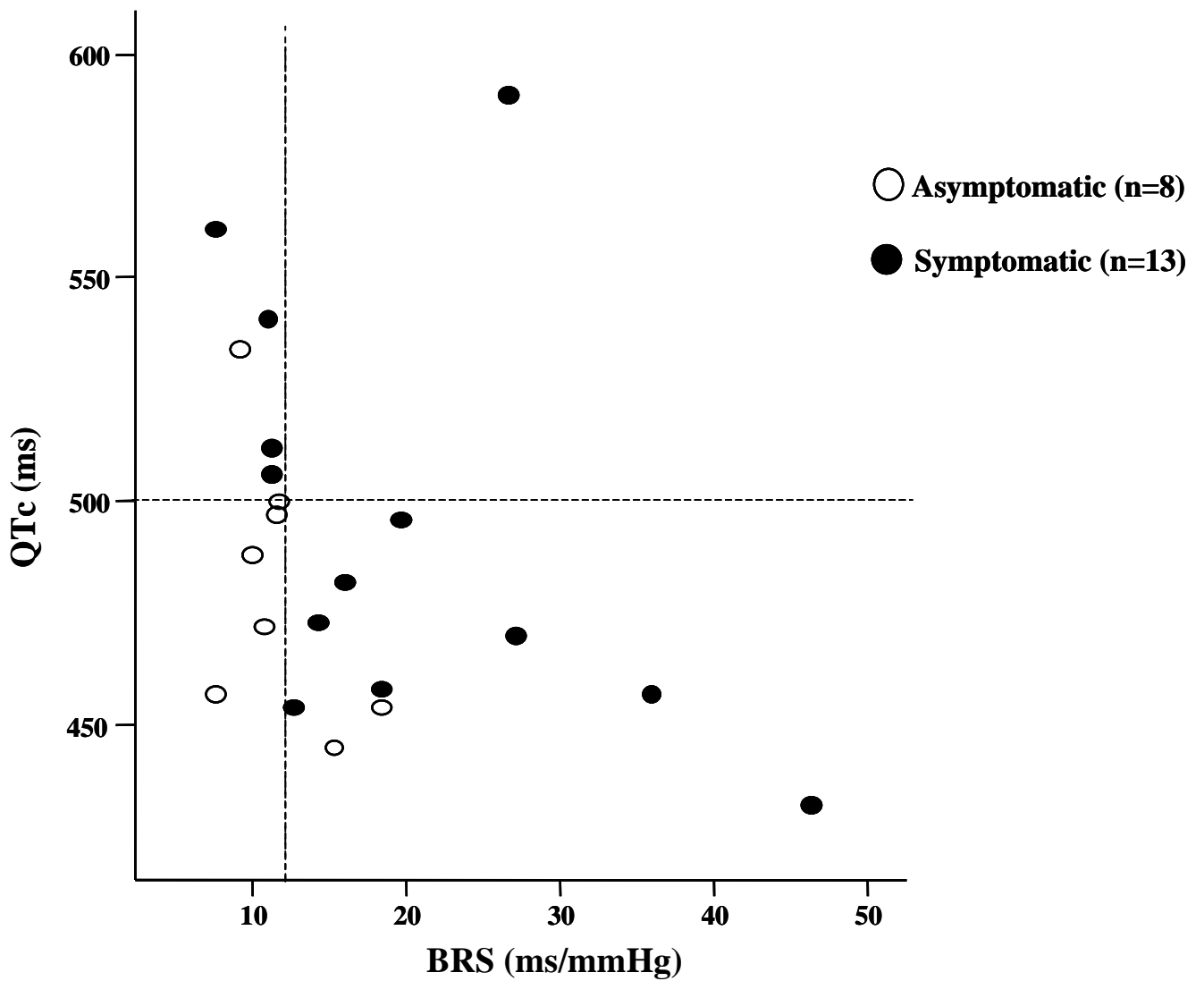


Figure 16: Effect of BB therapy on BRS. The increase in BRS during BB therapy was evident in symptomatic MCs, but it was practically absent in asymptomatic MCs.

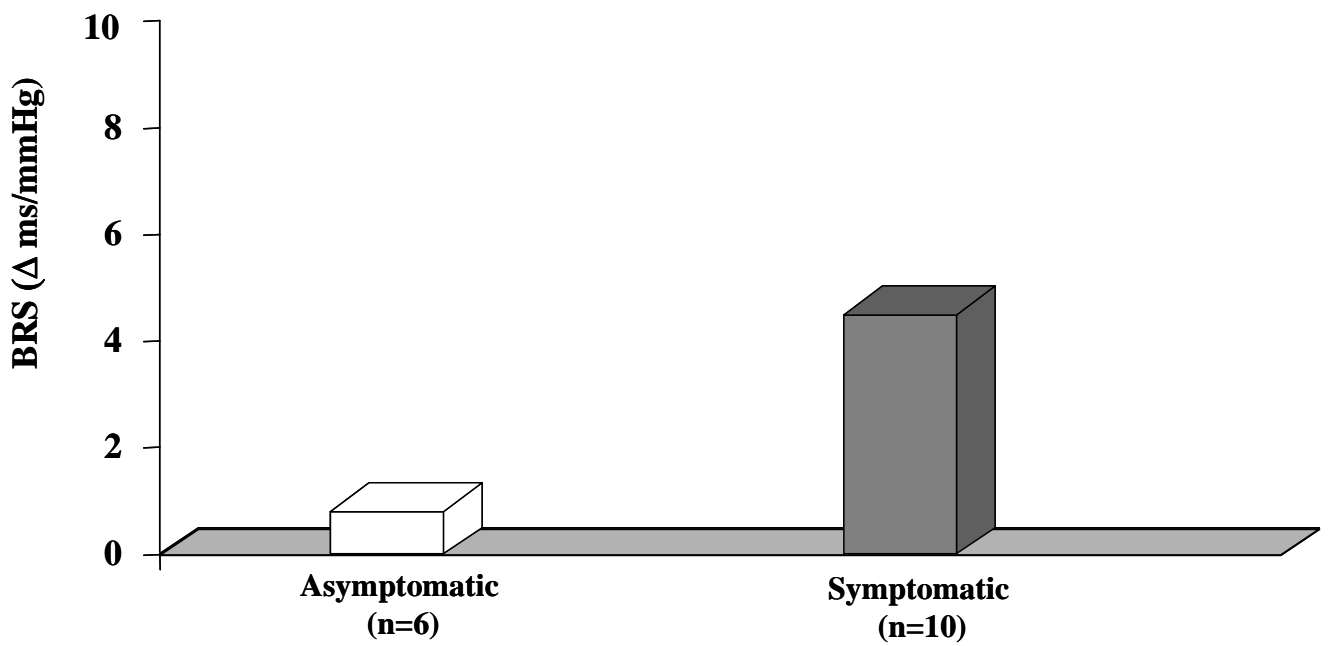


Figure 17: Kaplan-Meier curves of event-free survival in the LQT1 database (from ref. 30) population and in the SA-A341V population. The numbers under the curves represent subjects at risk.

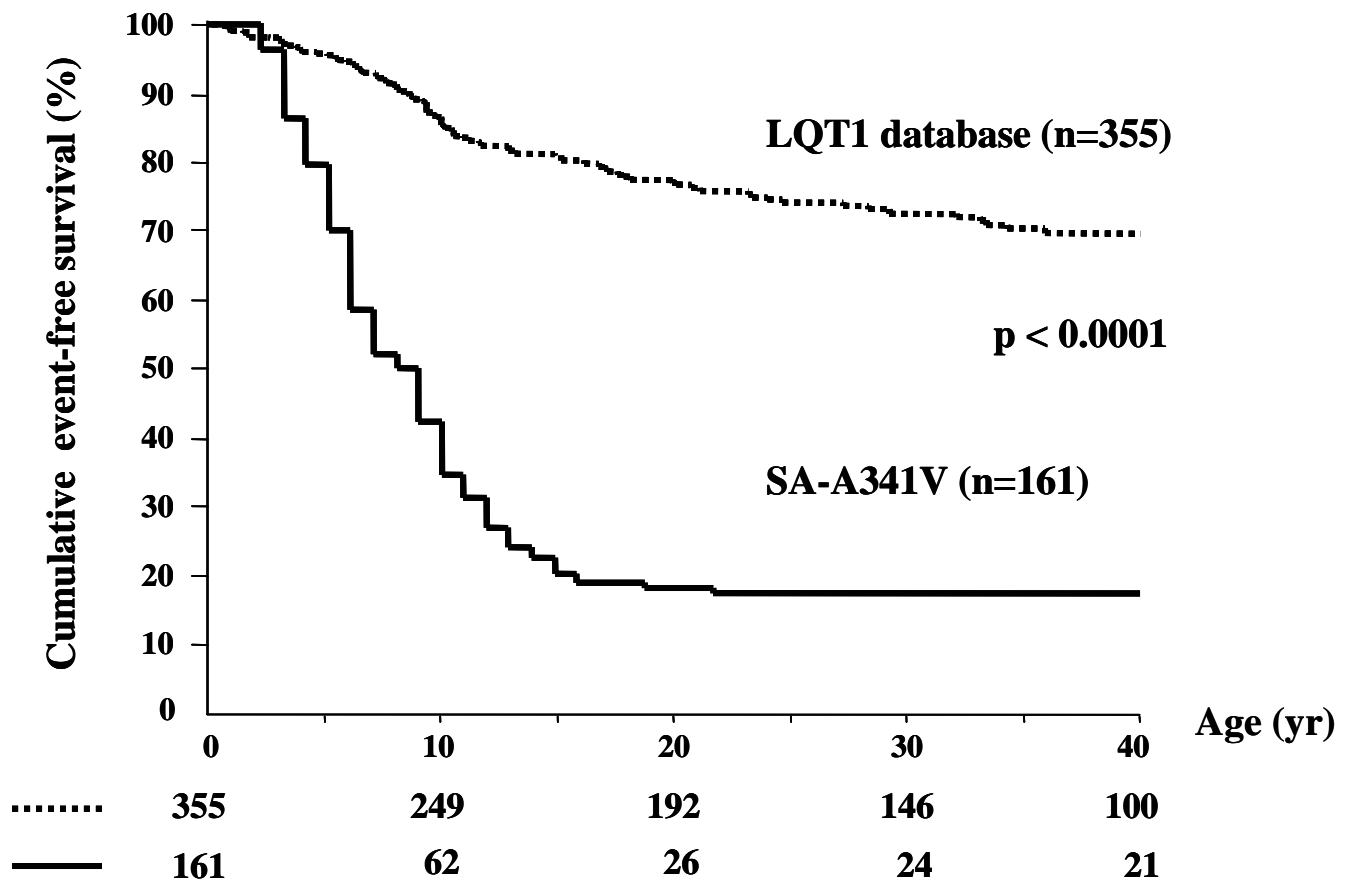


Figure 18: Kaplan-Meier curves of event-free survival among females with a QTc \geq 500 ms from the LQT1 database (from ref. 30) and from the SA-A341V population.

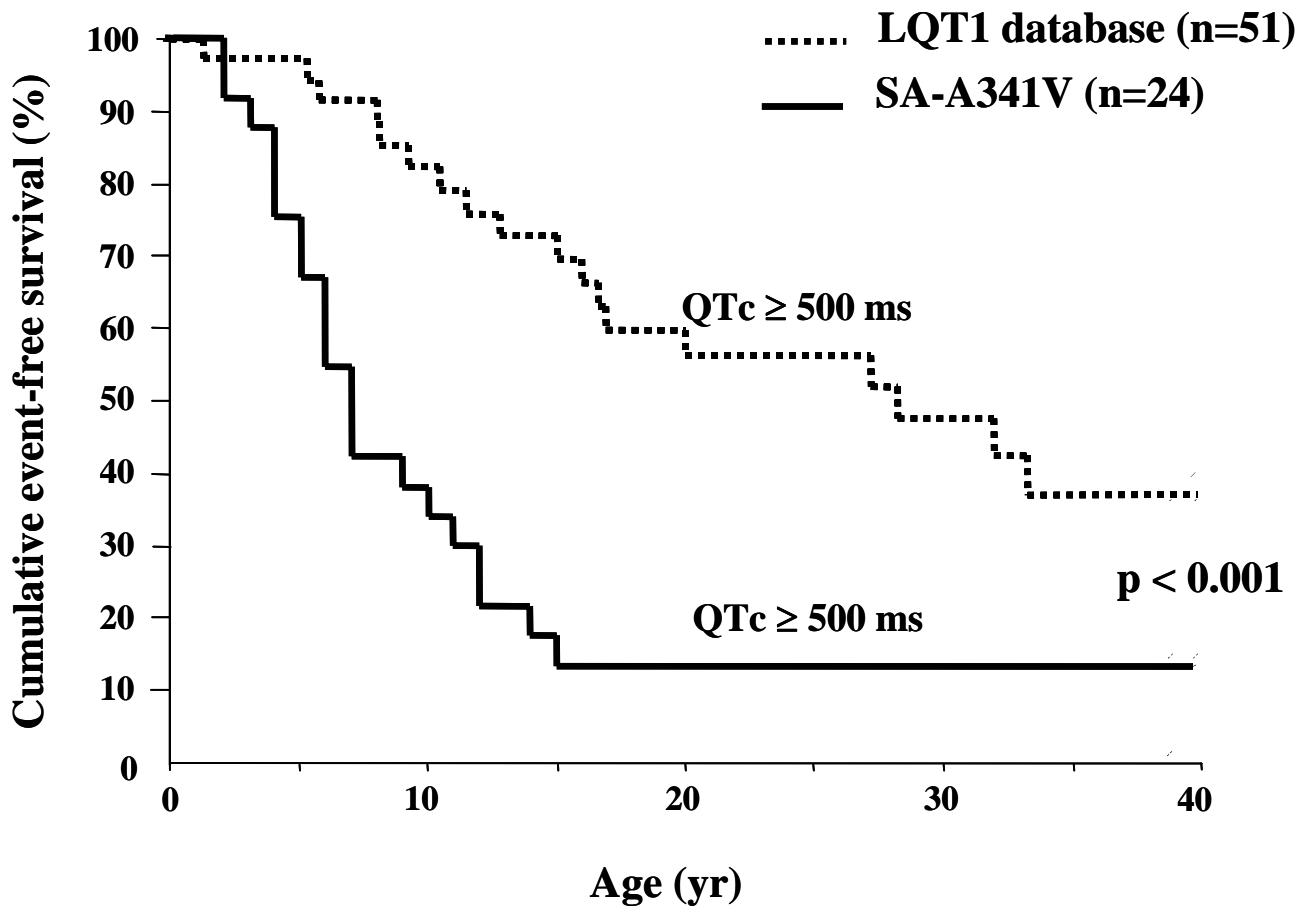


Figure 19: Comparison between the SA-A341V and the LQT1 population as reported by Zareba et al (19). The Kaplan Meier estimate of the cumulative probability of a cardiac event in the LQT1 group was obtained by reconstructing the original curve based on the information reported in the paper by Zareba et. al. Numbers above curves represent the cumulative probability of a cardiac event at 5, 10, 15 and 40 years for both curves. Numbers above the Zareba's curve and the corresponding number of patients at risk (bottom) were obtained by reconstructing the curve.

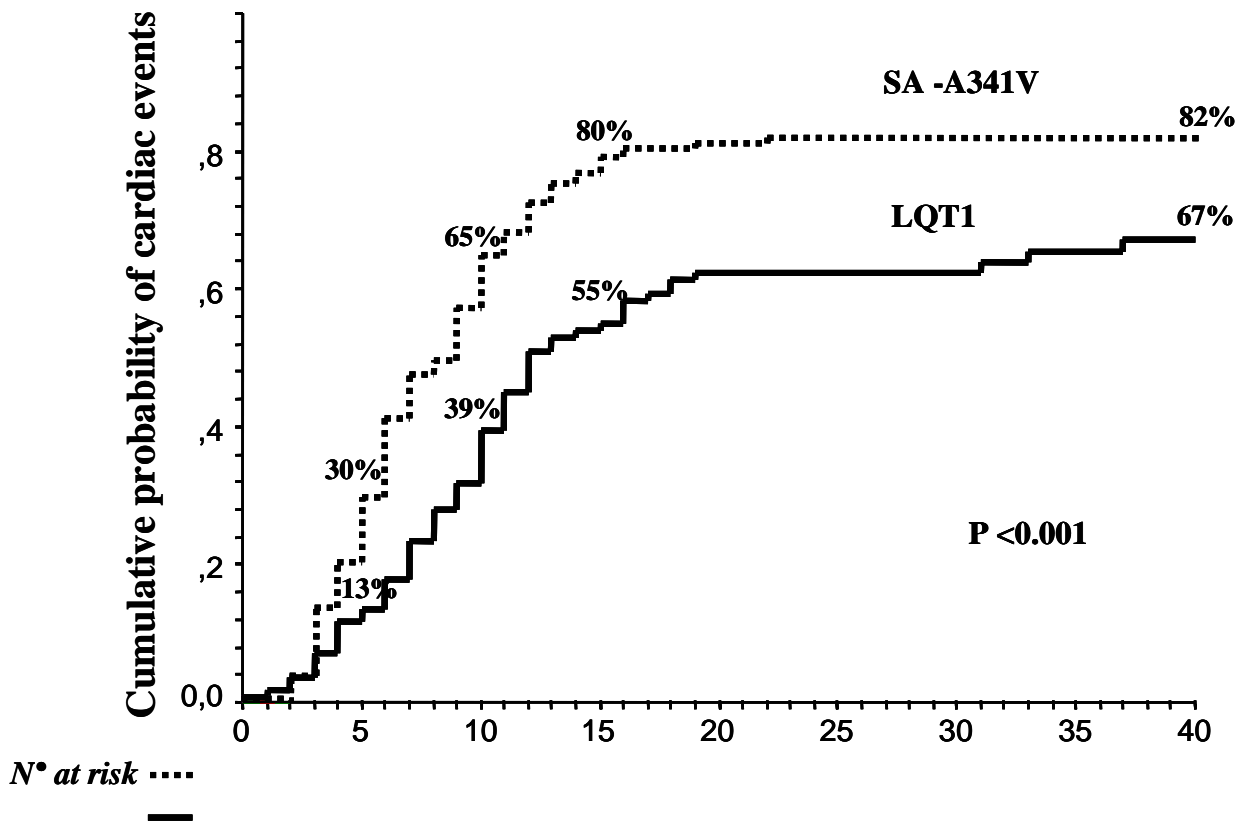
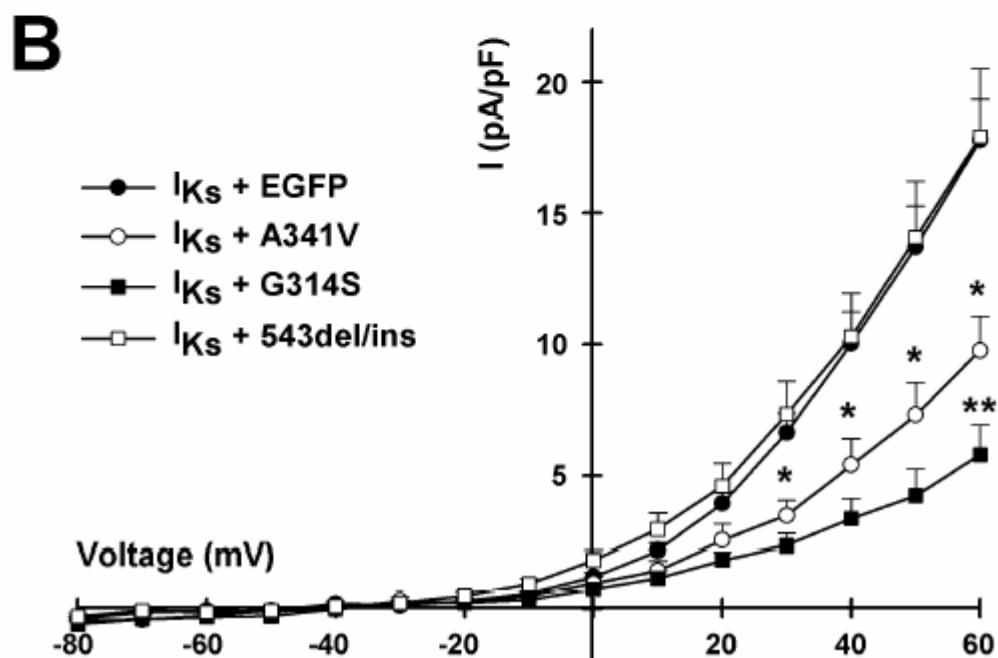
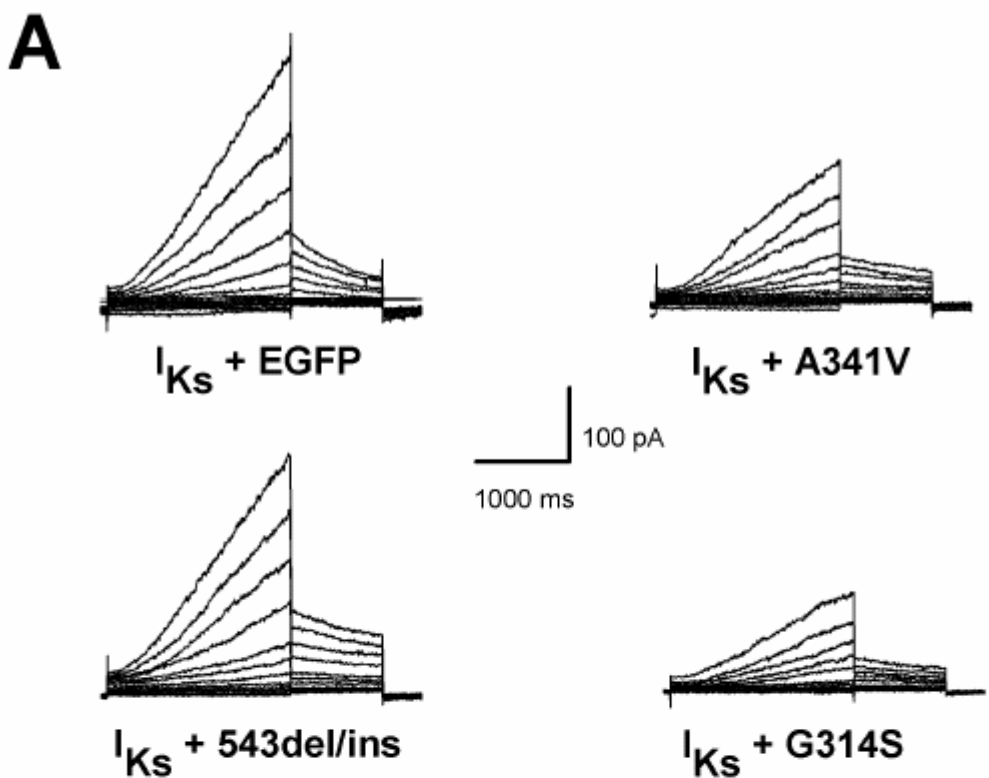


Figure 20: Functional characterization of KCNQ1-A341V. Whole-cell patch clamp recording of heterologously expressed KCNQ1 mutations in stable I_{Ks} cells. Representative current records obtained from I_{Ks} cells co-expressing vector alone (EGFP), A341V, 543del/ins or G314S (17A). Current-voltage relationships for mutant KCNQ1 channels expressed in I_{Ks} cells. Current is normalized to cell capacitance. Statistical differences ($P < 0.05$) in current densities between I_{Ks} + EGFP and I_{Ks} + A341V are indicated by single asterisks. Statistical difference ($P < 0.05$) between I_{Ks} + G314S and I_{Ks} + A341V is indicated by a double asterisk (20B).



DISCUSSION: *Italian LQT2 Family (IT-A1116V)*

The findings on the Italian LQT2 family provide clinical, molecular and *in vitro* electrophysiological evidence that a very common *KCNH2* polymorphism can act as a genetic modifier of the clinical severity of the long QT syndrome.

The genetic basis of symptomatic and apparently sporadic LQTS in a middle-aged woman was determined to be a novel heterozygous missense mutation (A1116V) in *KCNH2* co-segregating with the common *KCNH2*-K897T variant on the opposite allele. Other family members carrying only one of these variants were asymptomatic but some showed evidence of subclinical disease associated with A1116V.

Biophysical characterization revealed that the degree of functional HERG impairment in the heterozygous condition for A1116V is relatively mild compared to other LQTS mutations causing the full phenotypic expression of LQTS. This is consistent with the concept that A1116V is associated with a latent form of LQTS. However, when co-expressed with K897T, a more substantial reduction in HERG activity was observed. These findings are concordant with the clinical observation that symptomatic LQTS occurred only in the proband carrying both variant alleles. Because K897T is a common polymorphism and by itself has not been associated with LQTS, we propose that this allele acts as a genetic modifier to promote the clinical expression of the disease caused by A1116V.

Genetic Modifiers of LQTS

Inherited disorders are said to exhibit incomplete penetrance when less than 100% of mutation carriers exhibit disease manifestations. This is very common in autosomal dominant LQTS and current wisdom posits the existence of modifier genes to partly explain this phenomenon. Despite widespread acceptance of this notion, there have been very few specific genetic modifiers identified in LQTS or other inherited arrhythmia syndromes. Ye and colleagues reported that H558R, a common *SCN5A* polymorphism present in 20-30% of Caucasians, modifies

the *in vitro* activity of the *SCN5A*-M1766L mutation when present together (50). These studies provided tenable mechanisms for intramolecular complementation but the practical significance of the data was limited by the absence of genetic evidence that the two variants occurred on the same allele in LQTS subjects. Homozygosity for *SCN5A*-H558R has also been shown to provoke conduction system disease associated with the mutation *SCN5A*-T512I in a young child (51). A related example involves the observation of atrial standstill associated with *SCN5A*-D1275N in a Dutch family cosegregating a common connexin-40 promoter haplotype (52). As discussed earlier, coinheritance of *KCNQ1* mutations with the common variant *KCNE1*-D85N predisposes to greater degrees of QT prolongation and more severe symptoms in two of the families reported by Westenskow *et al.* (53). Inherited predisposition to hypokalemia caused by renal salt wasting has also been observed as an unusual form of genetic modification of LQTS associated with a *KCNQ1* mutation (54).

Genetic factors other than the primary mutation may worsen the severity of LQTS or, as illustrated by the case we present here, expose latent disease. In the situation where a modifier allele unmasks an otherwise subclinical form of LQTS, the correct diagnosis is extremely difficult without the support of genetic testing and segregation analysis of the extended pedigree. Even identification of the novel A1116V mutation did not suffice in explaining the occurrence of LQTS in the proband we report here. Although the occurrence of ventricular fibrillation in the absence of physical exercise is consistent with the current knowledge regarding genotype-phenotype correlation in LQT2 (12,55), the location of the A1116V mutation (C-terminus) is usually associated with a mild or benign form of LQTS (56,57) rather than the presenting symptom of cardiac arrest observed in this case. Only when the co-inheritance of the common polymorphism K897T was considered did the genetic data coincide with clinical observations.

The electrophysiologic characterization of A1116V and K897T, alone and in combination, provided additional evidence to support the hypothesis that co-inheritance of both variants is necessary for clinically overt disease. A1116V produces a significant, but less dramatic, reduction

in *IKr* than other LQTS-associated *KCNH2* mutations. This is consistent with individuals carrying only A1116V exhibiting either a normal QTc or transient signs of modest QT prolongation. K897T also reduced *IKr*, but to a lesser degree than A1116V and only at a voltage of 10 mV or greater. Co-expression of the two variants led to a significantly greater reduction of *IKr* compared to that produced by each individual allele. The reduction in *IKr* caused by the co-expression of A1116V and K897T was evident at voltages that are likely relevant to the membrane potential during the action potential plateau. This explains why the actual loss of repolarizing current was of sufficient magnitude to produce, even transiently, major QT prolongations and electrical instability in the proband. The functional studies reported here were performed in the absence of accessory subunits such as KCNE2. Recent experimental evidence has questioned whether KCNE2 acts as an obligate β subunit for *KCNH2* (58) and other investigations have demonstrated that this protein has very restricted cardiac expression (59,60). It is conceivable that co-expression of KCNE2 might influence the functional behavior of the *KCNH2* mutant but the physiological relevance would be uncertain.

Clinical and Functional Significance of *KCNH2*-K897T

The *KCNH2*-K897T variant has previously attracted the interest of several investigators, but there has been inconsistent evidence for association of this allele with a clinical phenotype. This is a common SNP in Caucasians with a minor allele frequency of 24% (40) and 33% of the general population is heterozygous (32). The proportion of other ethnic groups that are heterozygous for K897T is lower (7-8% in Asian, Black and Hispanic populations) (39).

This variant may have an impact on the QT interval. Pietila and colleagues (61) found that K897T was associated with longer QT intervals in middle-aged females. Similarly, Paavonen *et al.* (41) reported on a group of LQT1 patients all carrying the same *KCNQ1* mutation (G589D) who exhibited longer QT intervals upon exercise if they also carried the K897T allele. By contrast, Bezzina *et al.* (40) reported that QTc was shorter among control subjects homozygous for K897T.

Although the latter study surveyed a large population ($N = 1382$), which increases the statistical power, the actual differences in QTc were rather small (10 ms).

Similarly, previous heterologous expression studies to determine the functional impact of K897T are not in complete agreement. Anson *et al.* (62), using human embryonic kidney cells, observed several differences between K897T and WT-HERG channels. K897T channels activated at more negative potentials, inactivated and recovered from inactivation faster compared to WT channels. These authors proposed that the small decreases (10-30%) of current observed for K897T channels could cause a subtle increase in action potential duration. Using similar methodology, Paavonen and colleagues (41) reported a smaller current density, slower inactivation, and reduced expression of the K897T variant compared to wild type. The authors speculate that these changes could lead to increased arrhythmia susceptibility in settings of reduced repolarization. By contrast, Bezzina *et al.* (40) concluded that K897T might decrease action potential duration slightly leading to accelerated repolarization and shorter QT intervals based upon computer simulations incorporating their own electrophysiological observations. Finally, Scherer *et al.* (63) found no difference between K897T and WT-HERG when experiments were performed in *Xenopus* oocytes.

Our findings are consistent with the majority of the studies described above, particularly in that expression of the K897T variant resulted in slightly lower current density compared to WT-HERG. Similarly to several of the previous studies, our work utilized cultured mammalian cells, however we used bicistronic IRES plasmids that enabled greater certainty in co-expression experiments. The small decreases in current density that we observed for K897T in both the homozygous and heterozygous states are not expected to cause disease alone, but may accentuate the effects of reduced repolarization reserve in certain settings such as QT-prolonging drugs or co-inheritance of a LQTS mutation.

Implications for Risk Stratification in LQTS

This study contributes to the “proof of concept” that common polymorphisms can act as genetic modifiers of the clinical severity in familial arrhythmogenic disorders. The novel and unexpected finding is that the clinical manifestations of LQTS can be exacerbated also by a polymorphism with a very high prevalence (30%) in the general population.

The importance of this concept should not be underestimated. Clearly, other SNPs will be identified that are able to either increase or decrease the arrhythmic risk of patients affected by LQTS or other inherited arrhythmia syndromes. Furthermore, the present observation contributes to the search for modifier genes capable of modulating the risk for sudden death in more prevalent cardiac diseases (64). Relevant examples are acute myocardial infarction and congestive heart failure, two conditions burdened by a significant number of unexpected sudden cardiac deaths occurring either during the first few minutes of an ischemic episode or in patients with only modest degrees of pump failure. From a practical point of view, risk stratification of patients affected by LQTS (30) will have to consider the presence or absence of other SNPs, as well as K897T, as they are demonstrated to act as genetic modifiers. Molecular genetics integrated with clinical cardiology is progressively offering new clues for a better management of patients at risk for sudden death.

DISCUSSION: South African LQT1 Founder Population (SA-A341V)

In the present study we analyzed a population of LQT1 patients, established in South Africa more than three centuries ago and sharing a mutation identical by descent caused by a founder effect, to begin defining factors capable of explaining phenotypic variability in this disease. We identified two autonomic markers, heart rate and baroreflex sensitivity, as novel factors determining risk for cardiac events in this LQT1 population. We also provided unexpected evidence for the unusual clinical severity manifested by carriers of the KCNQ1-A341V mutation in this population, and further demonstrated, contrary to previous reports, that this mutation exerts dominant-negative effects on heterologously expressed I_{Ks} . Our findings have several conceptual and clinical implications.

Phenotypic Heterogeneity in a LQTS Founder Population

The concept that some patients might have LQTS and also a normal QT interval (phenotypic heterogeneity) was originally proposed in 1980 (65). Support for this hypothesis emerged with the observation that within the International LQTS Registry (7) approximately 5-6% of family members with a QTc < 440 ms suffered cardiac events (66). In 1992, Vincent *et al.* (67) reported that in 3 LQTS families linked to chromosome 11 there was overlap in QTc duration in the range of 440-460 ms between MCs and non-carriers, and that 6% of carriers had a QTc < 440 ms. The relationship between clinical heterogeneity and the phenomenon of low penetrance was demonstrated by Priori *et al.* (68) in 1999 by showing that asymptomatic MCs with normal QT intervals can be identified in LQTS families.

The observation of incomplete penetrance in LQTS impacts the estimation of disease prevalence, fosters the notion that a reduced “repolarization reserve” (69) might create a substrate predisposing to drug-induced Torsades-de-Pointes, and defines a clinically important subset of LQTS patients as “silent mutation carriers” that have a latent form of the syndrome. Explaining incomplete penetrance in LQTS is a great challenge that will require new clinical resources and

insights into disease behavior. We believe that the South African LQTS founder population is such a resource and has great potential for addressing important unanswered questions about this disease.

Our present data further illustrate phenotypic heterogeneity in LQTS within a large population of related LQTS individuals carrying the same primary mutation. Given the expectation that all individuals carrying KCNQ1-A341V should have similar reductions in I_{Ks} , our findings point to the existence within this South African population of additional genetic or environmental influences affecting duration of the QT interval and the probability of cardiac events. Common variants in cardiac ion channel genes similar to or including those recently described by Pfeufer *et al.* (70) are excellent candidate modifiers. In this regard, we have demonstrated in our IT-A1116V family, that a very common KCNH2 polymorphism (K897T) can exaggerate loss of repolarizing current produced by a LQTS mutation and unmask a severe clinical phenotype (71). Equally plausible are modifier genes having no obvious link to myocyte electrophysiology.

Heart Rate as a Risk Factor in LQTS

The repolarizing current I_{Ks} activates during increased heart rate and is essential for QT interval adaptation during tachycardia. Without this adaptive response the progressive reduction in the RR interval could lead to ventricular activation during the vulnerable period of the T wave (72) increasing the probability of ventricular fibrillation. This helps to explain why 79% of the lethal arrhythmic episodes in LQT1 patients, with mutations impairing I_{Ks} , occur during exercise (12). This is in striking contrast to the observation that most lethal episodes for LQT2 and LQT3 patients occur during startle reactions and at rest or sleep, respectively (12).

Adrenergic activation, as well as fast heart rates (even without adrenergic activation), lead to accumulation of I_{Ks} (73). In LQT1 patients, I_{Ks} accumulation is impaired caused by reduced function of channels containing a mutant subunit and may contribute to reduced adaptation of action potential duration during increased heart rate, the well-known “failure to shorten” the QT interval on exercise characteristic of LQT1. This phenomenon also facilitates the occurrence of electrical

alternans, which often manifests on the surface ECG as T wave alternans, a phenomenon described first in an LQT1 patient under emotional stress (74) and recognized as a marker of cardiac electrical instability often preceding onset of Torsades-de-Pointes.

The critical role of I_{Ks} in QT adaptation led us to explore if the propensity to develop cardiac events in the SA-A341V population was related to heart rate. We found that while symptomatic MCs had values almost identical to those of the non-carriers, the asymptomatic carriers had a significantly lower heart rate at rest. Indeed, a resting heart rate <73 bpm significantly lowered the probability of having arrhythmic events. Thus, a lower heart rate - even with the caution necessary when dealing with relatively small differences- appears to be a protective factor for patients with mutations affecting I_{Ks} . This novel finding may partially explain the high efficacy of β -blocker therapy for LQT1 patients. Indeed, our two recent studies showed that in 157 (75) and in 187 (76) LQT1 patients the long term combined incidence of cardiac arrest and sudden death was only 1.2% and 1.1%, respectively. Thus, it appears that β -blockers are effective in LQT1 patients because they act not only on the triggers (12) but also on the substrate by modifying heart rate.

On the other hand, while patients with a resting heart rate ≥ 73 bpm were all at higher risk for cardiac events (OR=4.22, 95% CI 1.13-15.81; $p=0.033$), within the patients with a QTc <500 ms there was a linear correlation between risk and heart rate level. This is practically important because it suggests that when the substrate is weaker the arrhythmogenic role of faster rate becomes predominant whereas it is somewhat less important in the presence of a major arrhythmogenic substrate (QTc ≥ 500 ms). This finding, to be accepted within the caution appropriate for extrapolations from a single ECG tracing, contributes to the novel concept, originated by this study, that heart rate plays a significant modulating role on the risk for cardiac events and that this arrhythmogenic role is accentuated in the presence of a moderate - but not excessive - QT prolongation.

Lower Baroreflex sensitivity correlates with reduced arrhythmia risk

In the present study we also tested the hypothesis (77) that the different clinical severity observed among family members carrying the same LQT1 mutation might be due to inborn or acquired variations in the autonomic control of heart rate. To reduce the possibility of chance observations we studied members of a founder population all carrying the same mutation (*KCNQ1*-A341V), focused on the age group in which autonomic responses were not influenced by age (2nd and 3rd age quartiles), and, given the potentially confounding role of BB, performed the study both on and off treatment.

Our hypothesis was that a reduced or relatively low BRS would have been associated with greater arrhythmic risk. This was the pattern that predicts increased arrhythmic and mortality risk after a myocardial infarction (14,78-80). Contrary to our expectations, we actually found that lower BRS values were associated with reduced risk. There is, however, a plausible biological explanation for this finding. At variance with patients affected by ischemic heart disease who are at risk when there is insufficient vagal activity to antagonize the arrhythmogenic sympathetic activity (81), LQT1 subjects are at greatest risk for arrhythmias whenever heart rate changes too rapidly. When heart rate increases quickly, reduced *IKs* prevents the necessary QT adaptation (QT shortening) and a new ventricular depolarization may encroach the vulnerable phase of the T wave (72), thus initiating ventricular tachycardia or fibrillation. When heart rate decreases quickly, the sudden RR lengthening may increase the amplitude of early after-depolarizations and initiate Torsades-de-Pointes through triggered activity

Indeed, those MCs who have a BRS value in the lower tertile (<12 ms/mmHg), had a markedly reduced risk of being symptomatic with an odds ratio of 0.13. In addition, when the analysis was restricted to the patients with a less severe arrhythmogenic substrate ($QTc \leq 500$ ms) the probability of being symptomatic was dependent on the level of BRS. In addition to the fact that none of the MCs in the lower tertile for BRS had symptoms, it is worth noting (Fig. 15) that the only two asymptomatic patients with a BRS > 12 ms/mmHg also had borderline QTc prolongation

(445 and 454 ms). This suggests that within these South African families when the substrate is not overwhelmingly arrhythmogenic ($QTc \leq 500$ ms) there are two distinct patterns observed in asymptomatic MCs: relatively low BRS even with a prolonged QTc (490-500 ms) or $BRS > 12$ ms/mmHg and a near normal QTc . The reduced arrhythmia risk associated with lower BRS values persisted in the absence of BB even despite the small sample size.

Taken altogether, these data provide evidence that the individual autonomic make-up, as represented by BRS, effectively contributes to modulate arrhythmic risk in patients with the *KCNQ1*-A341V mutation. Indeed, in this population a “blunted” autonomic response, as revealed by relatively low BRS, is a protective factor and may possibly hold true in other LQT1 subjects.

Clinical Severity in SA-A341V

In studying this founder population, we made the serendipitous finding that *KCNQ1*-A341V is associated with a very high incidence of cardiac events. Previously, the only suggestion that different mutations could carry a different risk for cardiac events was limited to their location in the predicted topology of the gene product, and most of the interest focused on whether or not the mutations were located within or outside the pore region. Donger et al. (82) suggested that *KCNQ1* C-terminal mutations are associated with a *forme fruste* of LQTS and Moss et al. (57) provided evidence for a more malignant clinical course associated with *KCNH2* mutations in the pore region. Subsequently, Zareba et al. (83) reported that among LQT1 patients no differences in the risk for cardiac events were observed between those with transmembrane mutations and those with C-terminal mutations. By contrast, Shimizu et al. (84) found that LQT1 patients with transmembrane mutations were at higher risk for cardiac events.

In the present study, we found that *KCNQ1*-A341V is associated with an unusually severe clinical phenotype in the South African population. This conclusion is supported by multiple lines of evidence. There is a striking, and highly significant, difference in the percentage of symptomatic patients and in Kaplan-Meier survival curves between SA-A341V patients and LQT1 database

patients with a diversity of mutations. Furthermore, when the analysis was limited to the more homogeneous group of females with a $QTc \geq 500$ ms, the large difference in the probability of experiencing a cardiac event was still observed. Finally and importantly, cardiac mortality is particularly high in these patients as 23 of 166 (14%) died suddenly.

Given the importance of this finding, we compared our SA-A341V population also to the one reported by Zareba et al (19) even though it included only 112 patients from 10 families. This was done because Zareba's population had an average QTc very similar to that of the SA-A341V patients (490 ± 43 vs 487 ± 45 ms). The Kaplan-Meier curves remained significantly different ($p < 0.01$) as significant were the difference of patients with a first event by age 15 (55% vs 80%, $p < 0.001$) and the difference in mortality (2% vs 14%, $p < 0.001$). This conclusively demonstrates the unusual clinical severity associated with A341V. Our data also confirm the major prognostic importance of a $QTc \geq 500$ ms, but in a more genetically uniform population compared to previous studies (30,85).

Dominant Suppression of I_{Ks} by KCNQ1-A341V

Previous studies examining the functional consequences of KCNQ1 mutations associated with LQTS have revealed a spectrum of channel dysfunction. Consistent with the autosomal dominant inheritance of Romano-Ward syndrome, many KCNQ1 mutations exert dominant-negative effects on the WT channel in heterologous expression systems, whereas mutations associated with the Jervell and Lange-Nielsen syndrome are typically pure loss-of-function alleles. Prior characterization of KCNQ1-A341V in oocytes (44-46) or COS7 cells (47) demonstrated little or no dominant activity of this mutation suggesting that it may be associated with a milder form of the disease. However, our survey of the phenotype associated with this allele indicates that it confers a more severe clinical picture (Figs. 17-20). We reassessed the functional properties of this mutation by using a mammalian cell system that insures co-expression of both WT and mutant channels with the KCNE1 accessory subunit. This system avoids the potential confounding influence of endogenous KCNQ1 and KCNE channel subunits that exist in *Xenopus* oocytes and the

uncertainties associated with transient transfection of multiple separate plasmids in COS7 cells (47). Our findings indicate that KCNQ1-A341V exerts a dominant-negative effect on I_{Ks} and therefore is not a pure loss-of-function mutation. This is more consistent with an allele associated with a severe clinical phenotype.

CONCLUSIONS AND CLINICAL IMPLICATIONS

The present data amplify the evidence for phenotypic heterogeneity in LQTS and indicate that even within populations sharing an identical mutation due to a founder effect there is a wide spectrum of clinical manifestations.

Our findings also indicate that risk stratification for LQTS patients must be more individually tailored and may have to take into account the specific mutation (i.e. KCNQ1-A341V was associated with a more severe phenotype compared to other LQT1 mutations) and probably additional clinical (i.e. HR, BRS) and genetic variables (i.e. KCNH2-K897T and other SNPs) capable of influence/predict the clinical phenotype of LQTS patients.

The study of the Italian LQT2 family gave the first clinical, genetic and electrophysiological evidence indicating that a very common non-synonymous SNP (30% carrier frequency among Caucasians) can act as a genetic modifier of the clinical expression of a LQTS mutation having low penetrance. This further validates the concept of genetic modification of LQTS by a common variant and carries important practical implications, as similar mechanism may contribute to the risk for sudden cardiac death in more prevalent diseases.

The study of the South African founder LQT1 population allowed us to identify heart rate and BRS, two autonomic markers, as novel risk factors. It is also evident that this South African LQTS population represents a useful human disease model for the identification and study of modifier genes.

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