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Identification and Characterization of Gene Variants Associated with Cardiac Repolarization (QT Interval)

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Summary

The QT interval from the ECG is a representation of the cardiac ventricular repolarization process during systole at the body surface. QT interval varies as a function of age, sex, heart rate, underlying heart disease and drugs. Prolongation or shortening of the QT interval can lead to cardiac arrhythmias, which are associated with an increase in cardiovascular morbidity and mortality. Under a genetic view the QT interval is a complex genetic trait with a heritability of 30% to 50%.

The aim of this thesis was to undertake a molecular genetic approach to identify novel genes and gene variants underlying variation in QT interval duration. By candidate gene and genome-wide association studies common gene variants underlying QT interval variation in the general population were identified. Initially in a candidate gene association study approach the detection of association between common gene variants in cardiac potassium channel genes and QT interval was demonstrated to be feasible. We showed that common variants in known Long-QT-disease genes modify the QT interval: Not only was the association of the previously published KCNH2-K897T variant confirmed, we also detected novel variants prolonging the QT interval in KCNQ1 intron 1 (rs727092) and in the promoter of KCNE1 (rs727957). Another nonsynonymous variant, KCNE1-G38S (rs1805127), previously published by another group, was not replicated and could be rejected as a major modifier in European populations.

In a genome-wide association study (GWAS) the NOS1AP/CAPON gene was shown to harbour the strongest common genetic variant modifying QT in the human genome (rs10494366). This finding was confirmed in two different populations.

Overall the work conducted in this thesis shows that common QT modifying variants exist in the human genome, which explain in part the relatively high heritability of the trait and can be identified by candidate gene based or genome-wide association studies.

Zusammenfassung

Das QT-Intervall aus dem EKG repräsentiert den Spannungsverlauf des Repolarisierungsprozesses des Herzventrikels während der Systole an der Körperoberfläche. Es ist abhängig von Alter, Geschlecht, Herzfrequenz, Grunderkrankungen des Herzens und Medikamenteneinnahme. Eine Verlängerung oder Verkürzung des QT-Intervalls kann zu Herzrhythmusstörungen führen, die mit einer Erhöhung der Morbidität und Mortalität assoziiert sind. Aus genetischer Sicht stellt das QT-Intervall ein komplexes genetisches Merkmal dar, das eine Erblichkeit von mindestens 30% aufweist.

Das Ziel dieser Doktorarbeit war die Durchführung eines molekularbiologischen Ansatzes zur Identifizierung neuer Gene und Genvarianten, die mit Veränderungen des QT-Intervalls assoziiert sind. Mit einer Kandidatengen- und einer genomweiten Assoziationsstudie wurden häufige Genvarianten, die in Zusammenhang mit einer Änderung des QT Intervalls stehen, in der Normalbevölkerung identifiziert. Zunächst wurde in einer Assoziationsstudie mit Kandidatengenen die Assoziation von häufigen Genvarianten im Gen für kardiale Kaliumkanäle als plausibel nachgewiesen. Diese Arbeit zeigt, dass häufige Varianten in bekannten Long-QT-disease Genen das QT Intervall modifizieren: Dabei wurde nicht nur die im Vorfeld publizierte Assoziation der KCNH2-K897T Variante bestätigt, wir entdeckten zusätzlich neue Varianten in Intron 1 von KCNQ1 (rs727092) und im Promotor von KCNE1 (rs727957), die den QT Intervall verlängern. Eine andere im Vorfeld publizierte nicht synonyme Variante, KCNE1 (rs1805127) konnte nicht repliziert werden.

Anschließend wurde in einer genomweiten Assoziationsstudie (GWAS) gezeigt, dass das NOS1AP/CAPON Gen die stärkste häufige Genvariante (rs10494366) im menschlichen Genom beherbergt, die das QT Intervall modifiziert. Dieses Ergebnis wurde in mehreren unabhängigen Populationen bestätigt.

Insgesamt zeigt diese Doktorarbeit, dass häufige, QT modifizierende Varianten im menschlichen Genom existieren, was die relativ hohe Erblichkeit dieses Merkmales zum Teil erklärt, und dass diese Varianten mittels kandidatengenbasierter oder genomweiter Assoziationsstudien identifiziert werden können.

List of original publications

Paper I:

Pfeufer A, Jalilzadeh S, Perz S, Mueller JC, Hinterseer M, Illig T, Akyol M, Huth C, Schopfer-Wendels A, Kuch B, Steinbeck G, Holle R, Nabauer M, Wichmann HE, Meitinger T, Kaab S. **Common variants in myocardial ion channel genes modify the QT interval in the general population: results from the KORA study**. Circ Res. 2005; 96:693-701

Paper II:

Arking DE, Pfeufer A, Post W, Kao WH, Newton-Cheh C, Ikeda M, West K, Kashuk C, Akyol M, Perz S, Jalilzadeh S, Illig T, Gieger C, Guo CY, Larson MG, Wichmann HE, Marban E, O'Donnell CJ, Hirschhorn JN, Kaab S, Spooner PM, Meitinger T, Chakravarti A. **A common genetic variant in the NOS1 regulator NOS1AP modulates cardiac repolarization**. Nat Genet. 2006; 38:644-51.

Paper III:

Akyol M, Prucha H, Sinner M, Balkau B, Eschwege E, Marre M, Tichet J, Meitinger T, Guichiney P, Kääb S, Pfeufer A. **Replication of association in the NOS1AP region to general population QT interval in the D.E.S.I.R. study.** Manuscript in preparation, 2008.

Paper IV:

Akyol M, Jalilzadeh S, Sinner MF, Perz S, Beckmann BM, Gieger C, Illig T, Wichmann HE, Meitinger T, Kaab S, Pfeufer A. **The common non-synonymous variant G38S of the KCNE1-(minK)-gene is not associated to QT interval in Central European Caucasians: results from the KORA study**. Eur Heart J. 2007; 28:305-9

Abbreviations

AD	autosomal dominant inheritance
AF	atrial fibrillation
LQTS	long QT syndrome
AR	autosomal recessive inheritance
ARVD	arrhythmogenic right ventricular dysplasia
AS	Andersen syndrome
AV Node	atrioventricular node
BS	Brugada syndrome
CACNA1C(CaV1.2)	calcium channel, L type, alpha-1C subunit
CAP	cardiac action potential
cDNA	complementary DNA
CPVT1	catecholaminergic polymorphic ventricular tachycardia type 1
DNA	deoxyribonucleic acid
ECG	electrocardiogram
KCNH2 (HERG)	potassium channel, voltage-gated, subfamily H, member 2
ICa-L or ICa-T	L- or T-type calcium current
IK1	inward potassium current
IKp	potassium plateau current
IKr or IKs	rapidly or slowly activated delayed rectifier potassium current.
IKur	ultra rapid potassium current
INa	sodium current.
INa/Ca	Na+/Ca2+ exchanger
ITo	1 and 2 transient outward potassium current type 1 and 2
JLNS	Jervell and Lange Nielsen syndrome
KCNE1	potassium channel, voltage-gated, subfamily E, member 1
KCNE2	potassium channel, voltage-gated, subfamily E, member 2
KCNJ2 (Kir2.1)	potassium channel, voltage-gated, subfamily J, member 2
KCNJ12	potassium channel, voltage-gated, subfamily J, member 12
KCNQ1	potassium channel, voltage-gated, subfamily Q, member 1
LQTS	long QT Syndrome
ms	milliseconds

mV	millivolt
OMIM	NCBI database- online mendelian inheritance in man
PCR	polymerase chain reaction
QTc_RAS	QT interval corrected for heart rate, age and sex
RNA	ribonucleic acid
RR	heart rate interval
RWS	Romano-Ward syndrome
RYR1	ryanodine receptor type 1
RYR2	ryanodine receptor type 2
SA Node	sinoatrial node
SCN5A	sodium channel, voltage-gated, member 5, alpha-subunit
SQTS	short QT syndrome
SSS	sick sinus syndrome
wt	wild-type
$\chi 2$	chi-square test

In addition, standard one-letter abbreviations are used for nucleotides and amino acids.

Table of Contents **Page**

Summary ii

Zusammenfassung iii

List of original publications iv

Abbreviations v

Table of contents vii

1. Introduction

1.1. Electrical Activity of the Heart 1

1.1.1. The Electrocardiogram 2

1.1.2. Ion Channels Underlying The Cardiac Action Potential 4

1.2. Cardiac Ion Channels 6

1.2.1. Voltage Gated Sodium Channels 10

1.2.2. Voltage Gated Calcium Channels 11

1.2.3. Voltage Gated Potassium Channels 12

1.2.4. Other Related Genes 17

1.3. Monogenic Long QT Syndrome 18

1.4. Molecular Genetics of Common Sequence Variants 20

2. Present Investigations 22

2.1. **Paper I:** Common Gene Variants in Candidate Genes 23

2.1.1. Study Population 23

2.1.2. Genotyping, Determination of Haplotype Blocks, Haplotypes and Genotype Phenotype Association Tests 24

2.1.3. Results and Discussion 24

2.2. **Paper II:** Identifying and Validating New Genetic Variants Influencing QT Interval 26

2.2.1. Study Population 27

2.2.2. Genome-Wide Scan, SNP Genotyping, SNP Selection for Stage II and III Genotyping 28

2.2.3. Results and Discussion 30

2.3. **Paper III:** Validation of significant SNPs from Genome-Wide Association Study in The DESIR Study 33

2.3.1. Study Population 33

2.3.2. Genotyping and Association Study 33

2.3.3. Results and Discussion 34

2.4. **Paper IV:** Replication of Glycine 38 Serine (G38S) Variant of the KCNE-Gene in the KORA Population 34

2.4.1. Study Population 34

2.4.2. Genotyping and Association Study 34

2.4.3. Results and Discussion 35

3. Conclusion 36

4. References 38

5. Acknowledgements 54

6. Published papers 55

7. Lebenslauf

1. Introduction

1.1. Electrical Activity of The Heart

The heart is a rhythmic electromechanical pump, pumping blood through the blood vessels of body. Electrical impulses control the mechanical activity of the heart. The electrical activity in the form of the action potential is the signal that initiates and coordinates cardiac contractility and pumping. These impulses are intrinsic to the heart (Baumgarten, 2004).

Normally myocardial electrical activity is initiated by an electrical pulse from specialized pacemaker cells that are located in the sinoatrial (SA) node and than propagated through the atria to the atrioventricular (AV) node. Following a brief pause in the AV node, excitation spreads in the conducting Purkinje fibers to the apex of the heart and into the working, ventricular myocardium (Figure1, Figure 3) (Jeanne et al., 2005).

The normal electrical activity in the heart is controlled by the movement of ions through specialised channels in the membranes of cardiac cells. The autonomic nervous system plays an essential role in regulating cardiac function and many of its effects are mediated via sympathetic and parasympathetic influences regulating the activity of these ion channels (McCorry et al., 2007).

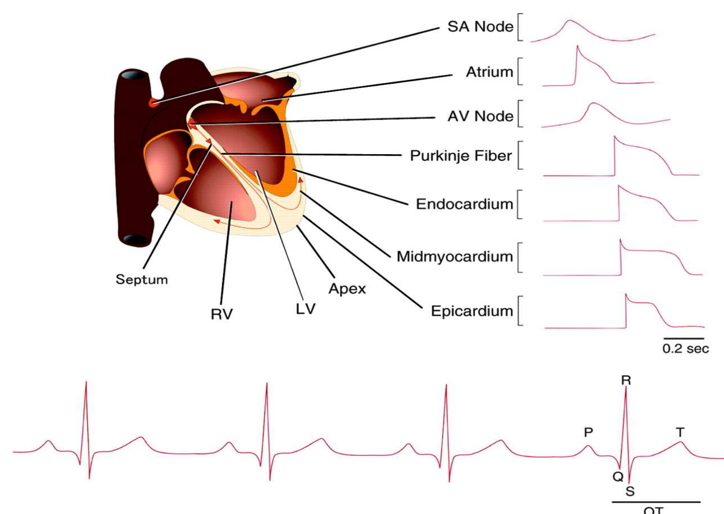

Figure 1. Electrical activity in the myocardium. Top: schematic of a human heart with illustration of typical action potential waveforms recorded in different regions. Bottom: schematic of a surface Electrocardiogram; three sequential beats are displayed. (Jeanne et al., 2005)

1.1.1. The Electrocardiogram.

The electrocardiogram (ECG or EKG) is a diagnostic tool that measures and records the electrical activity of the heart (Figure 2, Figure 3). ECG is used to measure the rate and regularity of heartbeats as well as the size and position of the chambers, the presence of any damage to the heart, and the effects of drugs or devices used to regulate the heart.

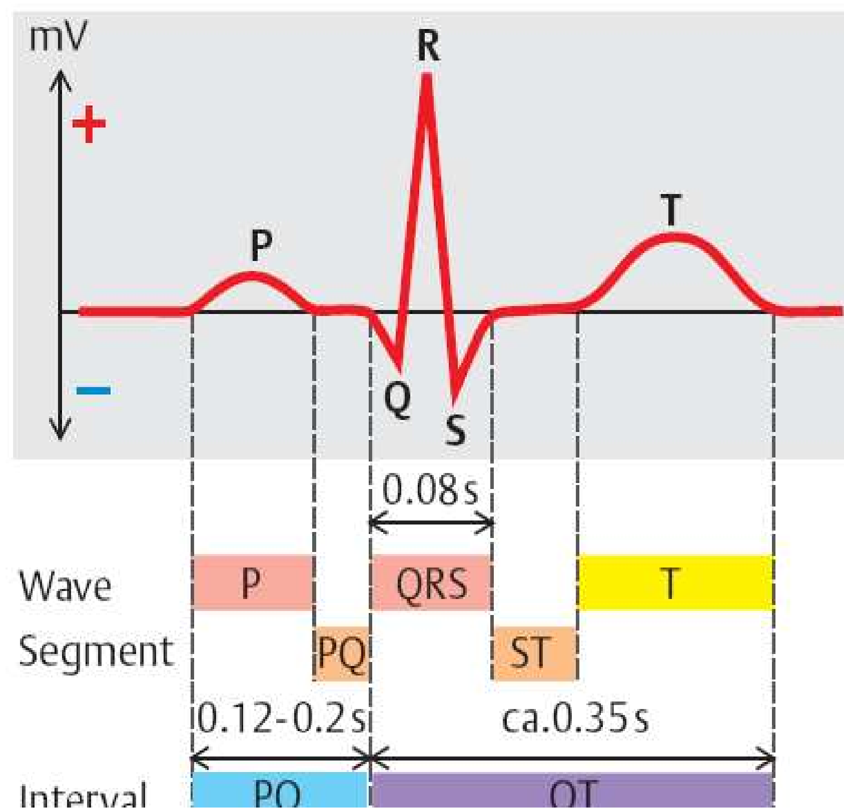

Figure 2. ECG (Despopoluos A., Silbernagl S. (**Colour Atlas of Physiology, 2003)**

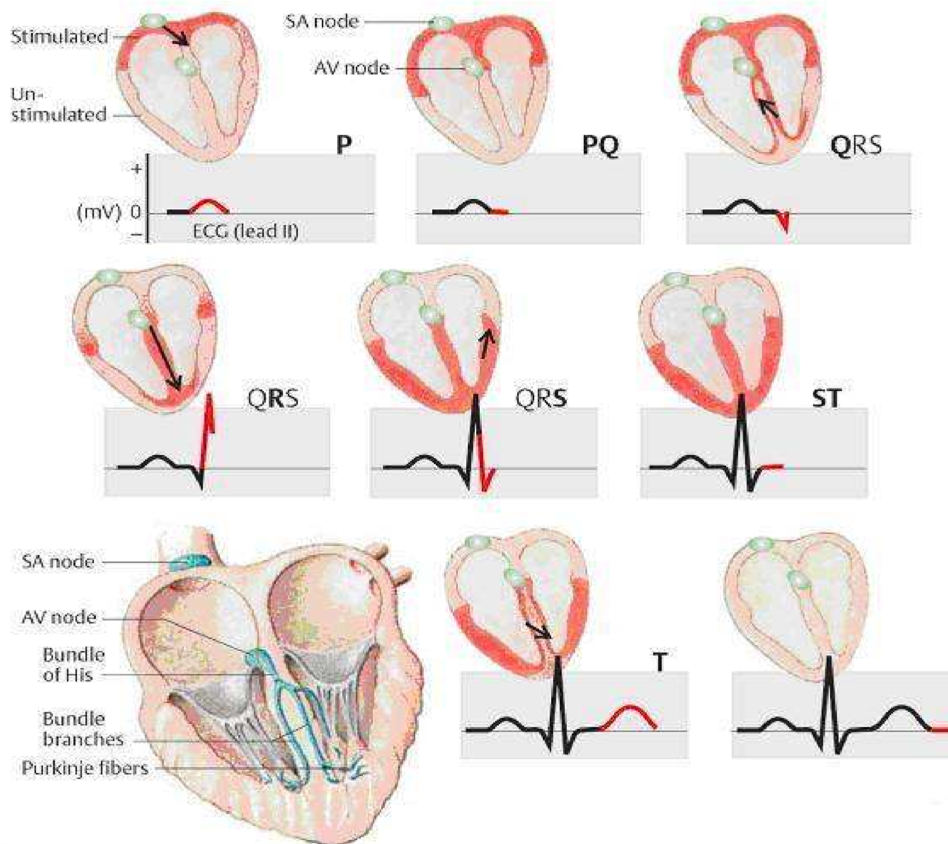

Figure3. Cardiac impuls conduction (Despopoluos A., Silbernagl S. **Colour Atlas of Physiology, 2003).**

P wave

The P wave represents the depolarization which spreads from the SA node throughout the atria. The duration is 80-100 ms. The brief isoelectric period after the P wave represents the time in which the impulse is passing within the AV node and the bundle of His. Repolarization of the atria normally cannot be visualized on the ECG since it tends to be masked by the QRS complex (Havmoller et al., 2007).

QRS complex

The QRS complex represents ventricular depolarization. The duration of the QRS complex is normally 60 to 120 miliseconds. This relatively short duration indicates that ventricular depolarization normally occurs rapidly. If the QRS complex is prolonged (> 120 miliseconds), conduction is impaired within the ventricles.

Prolongation of QRS (≥120 ms) occurs in 14% to 47% of heart failure (HF) patients. Left bundle branch block is far more common than right bundle branch block. Left-sided intraventricular conduction delay is associated with more advanced myocardial disease, worse left ventricular (LV) function, poorer prognosis, and a higher all-cause mortality rate compared with narrow QRS complex (Kashani et al., 2005).

ST segment

The ST segment represents the period from the end of ventricular depolarization to the beginning of ventricular repolarization. The ST segment connects the QRS complex and the T wave and has duration of 80 to 120 ms. Elevation ST segment is important in the diagnosis of ventricular hypoxia due to ischemia as well as to the diagnosis of a history of myocardial infarction (Kligfield et al., 2006).

T wave:

The T wave reflects the ventricular repolarization. The wave is rounded and positive under physiological conditions. Due to electrolyte imbalance, hyperventilation, CNS disease, ischemia or myocardial infarction the T wave can become inverted, peaked or flattened, prolonged or shortened.
The Q-T interval represents the time for both ventricular depolarization and repolarization. This interval can range from 200 to 400 milliseconds depending upon heart rate. At high heart rates, ventricular action potentials shorten in duration, which decreases the Q-T interval. In practice, the Q-T interval is expressed as a "corrected Q-T (**QTc**)" (Nearing et al., 2003). Several corrections formulas have been published, for example the formula of Bazett (Bazett et al., 1920) using a square root correction and the Framingham formula using a multivariate linear regression approach (Sagie et al., 1992).

1.1.2. Ion Currents Underlying The Cardiac Action Potential

The standard model used to understand the cardiac action potential is the action potential of the ventricular myocyte. The action potential has 5 phases (Figure 4) numbered from 0 to 4.

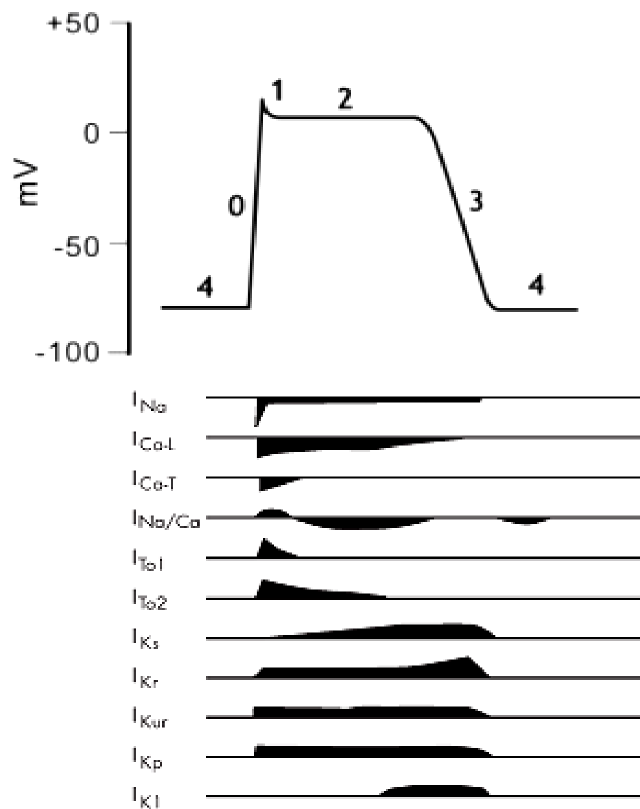

Figure 4. Action potential waveforms and underlying ionic currents in adult human (Roden et al., 2000).

Phase 4

Phase 4 is the resting membrane potential. The resting membrane potential is negative (about -90 mV) because potassium channels are open (K^+ conductance is high). Phase 4 is associated with K^+ currents, in which positive potassium ions are leaving the cell to keep up the negative membrane potential. At the same time, fast sodium channels and (L-type) slow calcium channels are closed.

Phase 0

Phase 0 is the upstroke or rapid depolarization phase when these cells are rapidly depolarized to a threshold voltage of about -70 mV; the rapid depolarization is caused by a transient increase in fast Na^+-channel conductance through fast sodium channels. This increases the inward directed, depolarizing Na^+ currents (I_{Na}) that are responsible for the generation of these -fast-response- action potentials. At the same time sodium channels open, gK^+ and outward directed K^+ currents fall as potassium channels close. These two conductance changes move the membrane potential to positive values.

Phase 1

Phase 1 is the early rapid repolarization, inactivation of the fast Na+ channels. The transient net outward current causing the small downward deflection of the action potential is due to the movement of K+ and Cl- ions, carried by the I_{to1} and I_{to2} currents, respectively.

Phase 2

Phase 2 is plateau phase of the cardiac action potential sustained by a balance between inward movement of Ca2+ through L-type calcium channels and outward movement of K+ through the slow delayed rectifier potassium channels, IKs. The sodium-calcium exchanger current, INa, Ca and the sodium/potassium pump current, INa, K also play minor roles during phase 2.

Phase 3

Phase 3 is the repolarization phase which ends the action potential. During phase 3, the L-type Ca2+ channels close, while the slow delayed rectifier (IKs) K+ channels are still open (Fig. 2). This ensures a net outward current, corresponding to negative change in membrane potential, thus allowing more types of K+ channels to open. These are primarily the rapid delayed rectifier K+ channels (IKr) and the inwardly rectifying K+ current, IK1. This net outward, positive current is responsible for the cell to repolarize. The delayed rectifier K+ channels close when the membrane potential is restored to about -80 to -85 mV, while IK1 remains conducting throughout phase 4, contributing to set the resting membrane potential (Shih, 1994) (Tan et al., 1995) .

1.2. Cardiac Ion Channels

Ion channels form pores in the cell membrane (Table 1). Through these pores ions flow across the membrane and depolarize or hyperpolarize the cell. Channels are macromolecular protein complexes within the lipid membrane. They are divided into distinct protein units called subunits. Each subunit has a specific function and is encoded by a different gene (Demolombe et al., 2005).

Channels can be classified into 3 types; non-gated, directly gated and second messenger gated channels. Among the directly gated channels are voltage gated (Na

(+), K (+), Ca (2+), Cl (-)) and ligand gated (ACh, Glutamate, GABA, Glycine) channels.

At the molecular level the coordinated cardiac activity requires ion currents, ion channels, structural proteins, and gap junctions responsible for the transmission of electrical and mechanical impulses across the cardiac myocytes. Research into the structure, function, and pathophysiology of ion channels has helped to clarify the roles of various ionic currents in both electrical activity and electromechanical coupling (Roden et el., 2002). Myocardial action potentials reflect the sequential activation and inactivation of inward (Na^+ and Ca^{2+}) and outward (K^+) current carrying ion channels (Table 1). In different regions of the heart, action potential waveforms are distinct, owing to differences in Na^+, and K^+ channel expression, and these differences contribute to the normal, unidirectional propagation of activity and to the generation of normal cardiac rhythms. Changes in channel functioning, resulting from inherited or acquired disease, affect action potential repolarization and can lead to the generation of life-threatening arrhythmias.

Table1. Genes encoding cardiac ion channel α β subunits (Roden et al., 2002)

	α Subunits		β Subunits	
Current	**Gene**	**Human chromosomal location**	**Gene**	**Human chromosomal location**
Inward currents				
I_{Na}	SCN5A	3p21	β_1 (*SCN1B*)	19q13.1-q13.2
			β_2 (*SCN2B*)	11q23
I_{Ca-L}	$\alpha_1 C$ (*CACNL1A1*)	12pter-p13.2	β_1 (*CACNB1*)	17q21-q22
			β_2 (*CACNB2*)	10p12
			$\alpha_2\delta$ (*CACNA2D1*)	7q21-22
I_{Ca-T}	$\alpha_1 H$ (*CACNA1H*)	16p13.3		
Outward currents				
I_{Ks}	*KvLQT1* (*KCNQ1*)	11p15.5	*minK/IsK* (*KCNE1*)	21q22.12
I_{Kr}	*HERG* (*KCNH2*)	7q36-q36	*minK/IsK* (*KCNE1*)	21q22.12
			MiRP1 (*KCNE2*)	21q22.12
I_{Kur}	*Kv1.5* (*KCNA5*)		*Kvβ1* (*KCNAB1*)	3q26.1
			Kvβ2 (*KCNAB2 ??*)	1p36.3
I_{K1}	*Kir2.1* (*KCNJ2*)	17q		
	Kir2.2 (KCNJ12)	17p11.1		
I_{K-Ach}	*GIRK1, Kir3.1* (*KCNJ3*)	2q24.1		
	+ *GIRK4, Kir3.4* (*KCNJ5*)	11q24		
I_{K-ATP}	*Kir6.2, BIR* (*KCNJ11*)	11p15.1	*SUR2* (*ABCC9*)	12p12.1
I_{TO}	*Kv4.3* (*KCND3*)	1p13.2		
	Kv1.4 (*KCNA4*)	11p14		
I_f, I_h	*BCNG2, HCN2*	19p13.3		
(pacemaker current)	HCN4	15q24-q25		
I_{Kp}	*TWIK1*(*KCNK1*)	1q42-q43		
	CFTR (*ABCC7*)	7q31.2		
	KvLQT1 (*KCNQ1*)	11p15.5	*MiRP1* (*KCNE2*)	21q22.12

Although expression of a single gene, encoding a pore-forming α subunit, is often sufficient to generate an ion current, recapitulation of all the physiologic features of a current in myocytes frequently requires function modifying accessory β subunits (Table 1). The generation of ion currents in cardiac myocytes requires coordinated function not only of α and β subunits, but also of multiple other gene products that determine such functions as trafficking, phosphorylation and dephosphorylation, posttranslational modification, assembly, and targeting and anchoring to specific subcellular domains. The mechanisms underlying these aspects of channel physiology are only now being delineated; moving information about individual gene products to a molecular view of cardiac physiology that incorporates multiple cell types, the extracellular milieu, and cell-cell communication continues to be a major experimental challenge (Roden et al., 2002).

Familial single-gene disorders such as long QT and Brugada syndromes, though rare, provide the opportunity to study a disease in which a single abnormal protein is responsible for arrhythmogenecity (Table 2). Genetic discoveries have also allowed new insights into how genes interact with the damaged heart muscle, drugs, or environment to trigger acquired arrhythmias (Roberts et al., 2003).

Table2. Inherited Voltage gated ion channels and heart disorders

Channel Gene	GenomicLocus	Disease	Inheritance	Reference
Sodium Channels				
SCN5A	3p21	LQT3	AD	Wang et al., 1995
		Brugada Syndrome	AD	Chen et al., 1998
		Progressive and Idiopathic cardiac Conduction defect	AD	Schott et al., 1999
Calcium Channels				
CACNA1C	12p13.3	Timothy Syndrome	AD	Splawski et al., 2004
RYR2*	1p42-43	Arrhytnogenic Polymorphic Ventricular tachycardia	AD	Tiso et al., 2001
		Catecholaminercig Polymorphic Ventricular tachycardia	AD	Laitinen et al., 2001
Potassium Channels				
KCNH2 (HERG)	7p35-36	LQT2	AD	Brugada et al., 2004
		Short QT Syndrome	AD	Curran et al., 1995
KCNE1	21q21.1-22.2	LQT5	AD	Splawski et al., 2004
		JLN2	AR	Tyson et al., 1997
KCNE2	21q21.1-22.2	LQT6	AD	Abbott et al., 1999
		Atrial Fibrillation	AD	Yang et al., 2004
KCNJ2	17p23	Anderson Syndrome	AD	Plaster et al., 2001
		LQT7	AD	Tristani et al., 2002
KCNQ1	11p15.5	LQT1	AD	Tyson et al., 1997
		JLN1	AR	Wang et al., 1996
		Short QT Syndrome	AD	Bellocq et al., 2004
		Atrial Fibrillation	AD	Chen et al., 2003

*RYR2 coding for intracellular calcium release channels in sarcoplasmic reticulum.

1.2.1. Voltage Gated Sodium Channels

These channels open and close in response to membrane potential. This family is largely responsible for action potential creation and propagation. The pore-forming α subunits consist of four homologous repeat domains (I-IV) each comprising six transmembrane segments (S1-S6) for a total of 24 transmembrane segments (Eduardo et al., 1998). The members of this family also coassemble with auxiliary β subunits, each spanning the membrane once. Both α and β subunits are extensively glycosylated. Only the principal (α) subunit is required for function (Jeanne et al., 2005).

SCN5A

SCN5A is associated with LQT3. The gene located on chromosome 3p21–24 is the cardiac sodium channel gene (George et al., 1995). It encodes a large protein of 2,016 amino acids with a putative structure of four homologous domains (DI-DIV), each of which contains six membrane-spanning segments from S1 to S6 (Gellens et al., 1992). The sodium current INa generated by this channel is responsible for the depolarization phase of the cardiac action potential (CAP) and contributes some current at the plateau phase. LQT-causing mutations in SCN5A act through a gain-of-function mechanism, in which the mutant channel functions, but with altered properties.

Romano-Ward syndrome (RWS) is called long QT syndrome type 3 (LQT3). RWS is inherited in an autosomal dominant pattern. Mutated SCN5A proteins stay open longer than usual, which allows sodium ions to continue flowing into cardiac muscle cells abnormally. This delay in channel closure disrupts the heart's normal rhythm, leading to the irregular heartbeat characteristic of Romano-Ward syndrome (Wang et al., 1995).

Cardiac conduction disease (CD) is a heart condition that increases the risk of fainting (syncope) and sudden death. This condition is caused by SCN5A mutations that lead to a partial or total loss of sodium channel function, reducing or stopping the flow of sodium ions into cardiac muscle cells. Cardiac conduction disease is inherited in an autosomal dominant pattern, which means one altered copy of the SCN5A gene in each cell is sufficient to cause the disorder (Tan et al., 2001).

Sick sinus syndrome (SSS) is a disorder of the sinoatrial node, which is a group of specialized cells in the heart that function as a natural pacemaker. The sinoatrial node cannot effectively regulate the heartbeat in people with this condition, leading to an abnormally slow heart rhythm (bradycardia) and an increased risk of dizziness and fainting. Mutations in the SCN5A gene that cause sick sinus syndrome produce nonfunctional sodium channels or abnormal channels that cannot transport ions properly. SSS is inherited in an autosomal recessive pattern (Benson et al., 2003).
SIDS is a major cause of death in babies younger than one year. It is characterized by sudden and unexplained death, usually during sleep. Although the cause of SIDS is often unknown, researchers have identified mutations in the SCN5A gene in some cases of this condition. Other genetic and environmental factors, many of which have not been identified, also play a part in determining the risk of SIDS (Plant et al., 2006).
Certain drugs, including medications used to treat arrhythmias, infections, seizures, and psychotic disorders, can lead to acquired long QT syndrome, increases the risk of cardiac arrest and sudden death in some people. A small percentage of cases of acquired long QT syndrome occur in people who have an underlying mutation in the SCN5A gene (Splawski et al., 2002).

1.2.2. Voltage-Gated Calcium Channels

Calcium channels open and close according to the membrane potential. This family contains 10 members, though these members are known to coassemble with $\alpha_2\delta$, β, and γ subunits. These channels play an important role in both linking muscle excitation. The α subunits have an overall structural resemblance to those of the sodium channels and are equally large. The α_1 subunit pore is the primary subunit necessary for channel functioning in the high voltage-gated calcium channels, and consists of the characteristic four homologous I-IV domains containing six transmembrane α-helices each. The α_1 subunit forms the Ca^{2+} selective pore which contains voltage sensing machinery (Jones et al., 1998).

CACNA1C

Gene map locus 12p13.3 Voltage-dependent calcium channels are made up of 4 repeated domains (I through IV) that each contain at least 6 membrane-spanning regions (S1 through S6), and the 4 domains are connected by linkers of variable length (Perez-Reyes et al., 1990).

Voltage-sensitive calcium channels mediate the entry of calcium ions into excitable cells and are also involved in a variety of calcium-dependent processes, including muscle contraction, hormone or neurotransmitter release, gene expression, cell motility, cell division and cell death. The isoform alpha-1C gives rise to L-type calcium currents. L-type calcium channels belong to the -high-voltage activated-group Calcium channels containing the alpha-1C subunit play an important role in excitation-contraction coupling in the heart (Tsien et al., 1991).

Timothy syndrome (TS) is characterized by multi organ dysfunction, including lethal arrhythmias, webbing of fingers and toes, congenital heart disease, immune deficiency, intermittent hypoglycaemia, cognitive abnormalities, and autism (Splawski et al., 2004). AV block had been previously reported in long QT syndrome and results from prolonged ventricular repolarization (Reichenbach et al., 1992, Marks et al., 1995).

Antzelevitch Wolpert syndrome (AWS) is characterized by shortened QT interval. Loss-of-function missense mutations in CACNA1C and CACNB2 encoding the alpha1- and beta2b-subunits of the L-type calcium channel are associated with a familial sudden cardiac death syndrome in which a Brugada syndrome phenotype is combined with shorter-than-normal QT intervals (Antzelevitch et al., 2007).

1.2.3. Voltage gated Potassium channels

Cardiac K+ selective currents carry outward currents in the physiological range of potential. They act either to set the resting potential near the K+ equilibrium potential. The long duration of the CAP is necessary to control and to prevent. Potassium currents are involved in nearly all phases of CAP. The pore-forming subunits of potassium channels have a homo- or heterotetrameric arrangement. Four subunits are arranged around a central pore. All potassium channel subunits have a distinctive pore-loop structure that lines the top of the pore and is responsible for potassium

selectivity (Synders et al., 1999).

KCNH2 (HERG)

KCNH2 gene located on chromosome 7q35–36 (Wang et al., 1996), which encodes a cardiac potassium channel core–forming subunit with six transmembrane segments (Curran et al., 1995). This channel ordinarily generates the rapidly activating delayed rectifier potassium current (IKr) in the heart. LQT-associated mutations in KCNH2 act through either a loss-of-function or a dominant-negative mechanism. The IKr potassium current is a major current at the repolarization phase of CAP. KCNH2 mutations reduce the repolarizing cardiac IKr current, thus causing prolongation of the CAP duration, leading to prolongation of the QT interval on ECG.

Short QT syndrome (SQTS). Mutations potentially increase the channels' activity. By allowing more potassium ions to flow out of cardiac muscle cells at a critical time during the heart beat (Hong et al., 2005, Schimpf et al. 2005, Hong et al., 2005).

Certain drugs, including medications used to treat arrhythmias (Sanguinetti et al. 1995), infections, seizures, and psychotic disorders, can lead to an abnormal heart rhythm in some people. This drug-induced heart condition, which is known as acquired long QT syndrome, increases the risk of cardiac arrest and sudden death. A small percentage of cases of acquired long QT syndrome occur in people who have an underlying mutation in the KCNH2 gene (Yang et al., 2002).

KCNQ1 (KVLQT1) and KCNE1 (MinK)

KVLQT1, the LQT1 gene located on chromosome 11p15.5 (Sanguinetti et al. 1996), is the first novel gene cloned for LQT. This gene encodes a potassium channel alpha-subunit with a conserved potassium-selective pore-signature sequence flanked by six transmembrane-spanning segments (S1 to S6). People who are heterozygous for mutations in KCNQ1 are at risk for autosomal dominant LQT (Wang et al., 1996). The KCNE1 gene is located on 21q22.1-q22.2 (McPherson et al., 1991, Chevillard et al., 1993). Homozygous or compound heterozygous mutations in KCNE1 cause autosomal recessive LQT. KCNE1 interacts with KCNQ1 and KCNH2. People who have mutations in both of their KCNQ1 alleles have a more severe disease, autosomal recessive LQT with congenital deafness. Mutations in KCNQ1, when heterozygous,

cause loss of channel function, altered channel gating, and/or a dominant-negative effect in which the mutant form of the channel subunit interferes with the function of the normal form. The KCNQ1 protein normally functions by interacting with KCNE1, a short potassium channel subunit with a mere 130 amino acids and only one transmembrane-spanning segment. The physical interaction between KCNQ1 and KCNE1 produces the slowly activating potassium current (IKs) in cardiac myocytes. Various ion currents such as IKs are responsible for the different phases of the cardiac action potential (CAP). The time course of one CAP can be divided into five phases. The IKs potassium current acts at the repolarization phase (phase 3). KCNQ1 mutations reduce the repolarizing cardiac IKs current, causing delayed repolarization and prolongation of the CAP duration, and prolongation of the QT interval on ECG (Nerbonne et al., 2005).

Atrial Fibrillation (AF) Changes in the KCNQ1 gene are an uncommon cause of an abnormal heart rhythm. The altered ion transport disrupts the heart's normal rhythm, resulting in AF (Chen et al., 2003).

JLNS Affected people typically have mutations in both copies of the KCNQ1 gene in each cell. Most of these changes lead to the production of a short, nonfunctional version of the KCNQ1 protein that cannot be used to assemble potassium channels. Other mutations alter a small number of amino acids in this protein, which alters the normal structure and function of the channels. At least one KCNQ1 mutation prevents PIP2 from effectively binding to and activating the channels, disrupting the normal flow of potassium ions across the cell membrane. An inability of cells in the inner ear and cardiac muscle to properly transport potassium ions leads to the hearing loss and arrhythmia found in JLNS (Mathew et al., 2001).

RWS Changes in the KCNQ1 gene are the most common cause of RWS. People with RWS usually have a mutation in only one copy of the KCNQ1 gene in each cell. Mutations allow the protein to form channels, but reduce the channels' ability to transport potassium ions out of cells. A small number of mutations disrupt the binding of PIP2 to the channels, which interferes with the normal flow of potassium ions across the cell membrane. A disruption in the flow of potassium ions in cardiac muscle results in the irregular heartbeat characteristic of Romano-Ward syndrome (Chena S. et al., 2003).

SQTS A mutation in the KCNQ1 gene has been associated with another heart condition called short QT syndrome. This mutation disrupts the usual function of ion

channels made with the KCNQ1 protein, increasing the channels' activity (Bellocq C et al., 2004).

Other disorders associated with the KCNQ1 gene. Mutations in the KCNQ1 gene are responsible for several other heart rhythm abnormalities including sudden infant death syndrome (SIDS) and acquired long QT syndrome. SIDS is a major cause of death in babies younger than one year. It is characterized by sudden and unexplained death, usually during sleep. Although the cause of SIDS is often unknown, some mutations identified in the KCNQ1 gene in a few cases of this condition. Other genetic and environmental factors, many of which have not been identified, also play a part in determining the risk of SIDS (Arnestad M et al., 2007).

Certain drugs, including medications used to treat arrhythmias, infections, seizures, and psychotic disorders, can lead to acquired long QT syndrome, increases the risk of cardiac arrest and sudden death in some people. A small percentage of cases of acquired long QT syndrome occur in people who have an underlying mutation in the KCNQ1 gene (Novotný et al., 2006, De Bruin et al., 2006).

KCNE2 (MiRP1)

KCNE2 is located on chromosome 21q22.1. The KCNE2 gene encodes the MiRP1 protein (minK-related peptide 1), an integral component of the IKr potassium channel. This gene has high homology with KCNE1; their close linkage (70 kb apart on chromosome 21q22) suggests that one arose as a result of gene duplication of the other. Mutations in KCNE2 cause LQT6. MiRP1 is a small integral membrane subunit that assembles with HERG to form IKr. Recently, MiRP1 was shown to interact with KVLQT1, resulting in a great change of the amplitude and gating properties of the KVLQT1 current (Jiang et al., 2004, Abbott et al., 1999).

RWS. Mutations in the KCNE2 gene have been identified in people with RWS. Mutations in the KCNE2 protein, which alters the protein's ability to regulate potassium channels in cardiac muscle cells. The channels open more slowly and close more rapidly than usual, decreasing the flow of potassium ions out of these cells. This disruption in ion transport causes an arrhythmia that increases the risk of fainting (syncope) and sudden death (Splawski et al., 2000, Millat et al., 2006, Abbott et al., 1999).

Familial atrial fibrillation (FAF) A mutation in the KCNE2 gene is associated with

rare cases of an abnormal heart rhythm called familial atrial fibrillation other disorders (Yang et al., 2004).

Some mutations in KCNE2 and KCNQ1 genes can cause acquired long QT syndrome and increase the risk of cardiac arrest and sudden death because of using medications used to treat arrhythmias, infections, seizures, and psychotic disorders (Novotný et al., 2006, De Bruin et al., 2006).

KCNC1

KCNC1 gene is located on chromosome 11p15. High amounts of Kv3.1 current decreased the timing accuracy of action potentials but enabled neurons to follow high-frequency stimuli. In a quiet environment (Ried et al., 1993), Kv3.1b was basally phosphorylated by protein kinase C in rat brainstem neurons. Kv3.1b was rapidly dephosphorylated in response to high-frequency auditory or synaptic stimulation, producing an increase in Kv3.1 current that facilitated high-frequency spiking (Song et al., 2005). the prolonged QT syndrome maps to this same region and because of pathophysiologic plausibility, mutations in the KCNC1 gene should be sought in that disorder (Grissmer et al., 1992).

KCNJ2

The inward rectifier K (+) channel Kir2.1 mediates the potassium I(K1) current in the heart. It is encoded by KCNJ2 gene that has been linked to Andersen syndrome. The KCNJ2 gene is located on 17q23.1-q24.2 (Derst et al., 2001). Recently, strong evidences has been provided that Kir2.1 channels were associated with mouse atrial fibrillation (AF), therefore we hypothesized that KCNJ2 was associated with familial AF (Plaster et al., 2001).

Andersen-Tawil syndrome (ATS). Mutations in the KCNJ2 gene lead to the production of a nonfunctional potassium channel. Some mutations change the shape of the channel so it cannot transport potassium ions, while other mutations prevent the channels from being inserted correctly into the cell membrane. Many KCNJ2 mutations prevent PIP2 from effectively binding to and activating potassium channels. If the KCNJ2 protein is unable to bind to PIP2, the channels remain closed and potassium ions are unable to flow into the cell. A loss of channel function in

skeletal and cardiac muscle cells disrupts the normal flow of potassium ions into these cells, resulting in episodes of muscle weakness (periodic paralysis) and an irregular heart rhythm. It is not known how mutations in the KCNJ2 gene contribute to developmental abnormalities of the head, face, and limbs often found in ATS (Tristani-Firouzi et al., 2002, Donaldson et al., 2003).

SQTS A KCNJ2 mutation has also been identified in one family with a heart condition called short QT syndrome (Priori et al., 2005).

FAF Mutations in the KCNJ2 gene are associated with rare cases of FAF. In cardiac muscle cells, this mutation appears to increase the flow of potassium ions through the channel formed by the KCNJ2 protein. The enhanced ion transport may alter the heart's normal rhythm (Ellinor et al., 2006).

1.2.4. Other Related Genes

RYR2

The RYR2 gene maps to 1q42.1-q43 (Mattei et al., 1994). Defects in RYR2 are the cause of familial arrhythmogenic right ventricular dysplasia 2 also known as arrhythmogenic right ventricular cardiomyopathy 2 (familial arrhythmogenic right ventricular dysplasia) is an autosomal dominant disease characterized by partial degeneration of the myocardium of the right ventricle, electrical instability, and sudden death. It is clinically defined by electrocardiographic and angiographic criteria: pathologic findings are the replacement of ventricular myocardium with fatty and fibrous elements which preferentially involve the right ventricular wall Defects in RYR2 are the cause of an autosomal dominant form of stress-induced polymorphic ventricular tachycardia also known as catecholaminergic polymorphic ventricular tachycardia. Stress-induced polymorphic ventricular tachycardia is a genetic arrhythmogenic disorder characterized by stress-induced, bidirectional ventricular tachycardia that may degenerate into cardiac arrest and cause sudden death (George et al., 2003). Defects in RYR2 are a cause of familial polymorphic ventricular tachycardia. This is an autosomal - dominant, inherited disease with a relatively early onset and a mortality rate of approximately 30% by the age of 30 years (Laitinen et al., 2001). Phenotypically, it is characterized by salvoes of bidirectional and

polymorphic ventricular tachycardia's in response to vigorous exercise, with no structural evidence of myocardial disease (Lehnart et al., 2004).

ANK2

ANK2 located on chromosome 4q25–27 (Tse et al., 1991), encodes ankyrin-B; Ankyrins bind a number of ion motive proteins essential to normal cardiac electrophysiology, including the Na-Ca exchanger; inositol 1,4,5-triphosphate receptor; Na-K ATPase; and the voltage-dependent sodium channel (NaV1.5) By the very nature of the role of ankyrins in excitation and contraction in cardiac myocytes, it is understandable that variants in ankyrin-B originally described in a family with congenital LQTS1 (Schott et al., 1995) would exhibit electrical phenotypes and Mutations in the ankyrin-B gene (ANK2) cause type 4 long-QT syndrome. Other electrophysiological abnormalities such as conduction block, idiopathic ventricular fibrillation, and catecholaminergic ventricular tachycardia have been included under the rubric of ankyrin-B syndrome (Priori et al., 2001). A mutation in the ankyrin binding domain of NaV1.5 has been associated with defective ankyrin-G binding and Brugada syndrome (Tomaselli et al., 2007). Dysfunction in ankyrin-B is linked with fatal arrhythmia in humans.

RWS It is unclear whether these mutations in ANK2 cause RWS or lead to another heart condition with some of the same signs and symptoms. Most often, mutations in the ANK2 gene lead to abnormalities of the heart's natural pacemaker (the sinoatrial node), a heart rate that is slower than normal (bradycardia), a disruption in the rhythm of the heart's lower chambers (ventricular arrhythmia), and an increased risk of fainting (syncope) and sudden death. Other symptoms, including seizures, dizziness, and migraine headaches, also have been reported in people with ANK2 mutations (Mohler et al., 2003, Mohler et al., 2004).

1.3. Monogenic Long-QT Syndrome

Long-QT syndrome (LQTS) is a cardiovascular disorder characterized by an impaired cardiac repolarization leading to a prolonged QT interval on the surface ECG. The clinical features of LQTS result from episodic ventricular tachyarrhythmia's, such as torsade de pointes and ventricular fibrillation (Schwartz et al., 1975, Moss et al.,

1991). LQTS clinical symptoms includes syncopes, seizures, and sudden death, usually in young, otherwise healthy individuals (Jervell et al., 1957, Ward, 1964). Two inherited forms and an acquired form of LQTS exist. The more common form, Romano-Ward syndrome is inherited as an autosomal dominant trait with variable penetrance is not associated with syndromic abnormalities and.(Romano et al. 1963, Ward, 1964). Jervell and Lange-Nielsen syndrome is characterized by the presence of deafness, a phenotypic abnormality inherited as an autosomal recessive trait (Table 3)(Jervell A et al., 1957, (Splawski et al., 2000). The estimated population prevalence of arrythmias up to 1 in 5.000. The Long QT Syndrome is the most frequent monogenic arrhythmia syndrome (Bezzina et al., 2005). Many external factors (medications, abnormal electrolyte balance, and medical conditions) can prolong the QT interval and cause acquired LQTS (Roden et al.,, 2005). The majority of LQTS families in which the disease has an identifiable cause. Approximately 75% of all cases have mutations in ion channels involved in the cardiac myocyte action potential (Splawski et al., 2000). LQTS results from either loss-of-function mutation in potassium channel genes, thus delaying repolarization, or gain of function mutations in sodium and calcium channel genes, thus sustaining depolarizing current (Table3) (Vincent, 1998, Newton-Cheh et al., 2007).

Table3. Nomenclature, gene names, and proteins associated with LQTS (adapted from Moss, 2005).

Disease	Gene (historical name)	Protein
LQT1	KCNQ1 (KVLQT1)	IKsK+ channel α subunit
LQT2	KCNH2 (HERG)	IKrK+ channel α subunit
LQT3	SCN5A	INaNa+ channel α subunit
LQT4	ANK2 (ANKB)	Ankyrin-B
LQT5	KCNE1	(minK) IKsK+ channel β subunit
LQT6	KCNE2	(MiRP1) IKrK+ channel β subunit
LQT7	KCNJ2	IKr2.1K+ channel α subunit

1.4. Molecular Genetics of Common Sequence Variants

Association studies compare the allele frequency of a polymorphic marker or sets of markers (haplotype) in unrelated patients and healthy controls drawn in a general population to identify markers with frequencies that differ significantly between two groups (Risch et al., 2000, Carlson, 2003 et al., Carlsonet al., 2004). Genetic associations arise because human populations share common ancestry (Kruglyak, 1999).

Association studies are sufficiently powerful only for common causal variants. The threshold for 'common' depends on sample and effect sizes as well as marker frequencies (Zondervan et al., 2004) the minor-allele frequency might need to be above 5% (Balding, 2006). Genetic association studies can be classified into several types; for example, as follows:

Candidate gene studies Most of the candidate genes were selected for further study either based on the results of previous linkage studies or on the basis of other evidence that they might affect disease risk.

Regional mapping studies Mapping methods developed to localize functional polymorphisms within large candidate regions or candidate genes, identified from previous linkage and/or association studies. Regional association mapping methods utilize linkage disequilibrium of alleles at high-density marker SNPs with the functional polymorphism, generated as the result of shared ancestry of individuals within the population (Morris, 2007).

Genome-wide association (GWA) studies. The main goal of the GWA approach is to identify novel loci, genes and gene variants involved in the physiology of a studied trait. The main advantages of GWA are that a GWA does not need a prior hypothesis about specific candidate genes and therefore new and unsuspected regions can be identified. It has been identified that not only coding SNPs but also nearby noncoding variants that might be equally or more strongly associated with the disease. In addition, with the dense genotyping chips containing sets of hundreds of thousands of single nucleotide polymorphisms (SNPs) available today, coverage of a large fraction of the human genome is provided. Large numbers of well-characterized samples have been assembled for many common traits and diseases and GWAS has been successfully applied (Barrett, 2006, Wellcome Trust Case Control Consortium, 2007).

The GWA phase is only the fist step of the complete work towards the identification of novel associated gene variants. The other steps are validation studies with a subset of SNPs associated in the GWA experiment, fine mapping and LD analysis of the associated region, independent replication and functional studies (Balding, 2006).

The first GWA was proposed a decade ago by Risch (Risch et al., 1996). The first GWAS performed and published in 2002 by Ozaki, examined myocardial infarction. It was hypothesis free and used 100k SNPs chip on the genome (Ozaki et al., 2002). In 2008 one million SNP chips are being launched and genotype accuracies have improved (Kingsmore et al., 2007). It may generate a number of new insights about the nature of individual differences (Lawrence et al., 2005). GWAS will soon become a standard experimental procedure, and could open new frontiers in our understanding and treatment of diseases (Hirschhorn et al., 2005).

2. Present Investigations

In this thesis several scientific goals were pursued. These and my personal contribution to them are as outlined:

1. To demonstrate the feasibility of association studies under the Common Disease Common Variant paradigm, the influence of common gene variants of 4 candidate cardiac ion channel genes on QT interval was investigated in a pilot study. A linkage disequilibrium based SNP association study was performed and 174 SNPs from candidate genes were screened in 689 individuals from the KORA study. Significant findings were confirmed in 3277 individuals. (**Paper I**)
In this study I, performed large parts of the SNP genotyping in the first and second stages and participated in the statistical analysis.

2. A common genetic variant influencing QT interval through a genome-wide association study was identified in 200 subjects at the extremes of the population-based QT interval distribution of 3966 subjects from the KORA study. Significant findings followed up in two independed samples in 2646 subjects from Germany and 1805 individuals from US Framingham Heart Study. (**Paper II**)
In this study I, participated in the study design, the statistical analysis and SNP selection for the second stage, performed large parts of this SNP genotyping and wrote parts of the manuscript.

3. The significant SNP from the genome-wide association study was replicated in the DESIR study from France. (**Paper III**)
In this study I, participated in the statistical analysis and wrote parts of the manuscript.

4. The previously published candidate gene association between QT interval and common non-synonymous Gly 38 Ser variant of the KCNE gene coding for the MinK potassium subunit was investigated and negatively replicated in Central Europeans from Germany (**Paper IV**).
In this study I, performed the SNP genotyping, participated in the statistical analysis and in the writing of the manuscript.

2.1. Common Gene Variants in Candidate Genes (Paper I)

We selected 4 candidate genes, namely KCNQ1, KCNH2, KCNE1 and KCNE2. Mutations in these genes are known to cause long-QT syndrome. We used a two step SNP genotyping design; in the first step, we genotyped selected 270 SNPs from the selected genes in the 689 individuals from the KORA study. Afterwards we validated the informative SNPs in the confirmatory sample of 3277 individuals from the same survey.

2.1.1. Study Population

For screening we used the KORA S4 study, which represents the general population living in or near Augsburg, Germany. Briefly, for KORA S4, 4,261 probands were studied between 1999 and 2001. 12-lead resting ECGs were recorded using digital systems. We adjusted QT for known covariates -heart rate, sex, and age- was called QTc_RAS (Rate, Age, and Sex). Correction factors were determined separately for each sex.

With the correction factors derived from the total sample of 3966 individuals, the formulas for

QTc_RAS were determined for males:

(1) QTc_RAS=QT-[0.152*(RR-1000 ms)]-[0.318 ms/year*(age-60 years)]

and for females:

(2) QTc_RAS=QT-[0.154*(RR-1000 ms)]]-[0.207 ms/year*(age-60 years)]-4.58 ms, where RR denotes RR interval in milliseconds.

We reinvestigated 880 persons specifically for cardiovascular diseases and we used 689 of them to screen. We used 3277 different individuals from the total study to confirm the positive genetic associations.

2.1.2. Genotyping, Determination of Haplotype Blocks, Haplotypes and Genotype Phenotype Association Tests

We selected 270 SNPs distributed in and around these 4 candidate genes (KCNQ1, KCNE1, KCNH, KCNE2). SNPs were chosen from the public dbSNP database. 19 SNPs were selected from exons or intron/exon boundaries. 251 SNPs were selected from introns and regions upstream and downstream of the coding region (criterion of equidistant spacing of 1 SNP every 5 kb). DNA was extracted from EDTA anticoagulated blood using a salting out procedure. 26 SNP genotypes were determined using PCR, primer extension, and MALDI-TOF mass spectrometry in a 384-well format (Sequenom). LD measures (D, *r*2) and haplotypes were determined with Haploview software. Haplotype block boundaries were defined based on the confidence interval of the D. Haplotype-phenotype association analysis based on sliding window haplotypes was performed using the haplotype trend regression test. SNPs were tested for association by linear regression analysis using QTc_RAS as the dependent variable. Significance levels were determined for both the one-degree and the two-degree of freedom test. In the the one-degree f freedom test, the independent variable was derived by transforming SNP genotypes (AA, Aa, aa) to a relational scale by counting the number of minor alleles (0, 1, 2) assuming a strictly codominant model with identical trait increases between genotypes. To determine combined effects, we counted the number of significant genotypic changes in each person to give a QT-prolongation score and performed ANOVA analysis using the score as the independent variable. To investigate if associated SNP-markers had also been identified in a categorical trait analysis, we analyzed groups of individuals with extreme QTc_RAS values in both and individual sexes in a case control–like design using the Cochran-Armitage test for trend.

2.1.3. Results and Discussion

After genotyping we found that for 33 SNPs the genotyping assays were not functional and had call rates below 0.8. In addition, 36 SNPs were monomorphic. 174 SNPs were genotyped successfully. The average call rate was 0.953 and the average minor allele frequencies were 0.258 (mean) and 0.251 (median). In the screening

sample, 34 of these SNPs showed association to QTc_RAS in the 1df-test, 18 of these being also significant in the 2df test.

We genotyped 13 nonredundant SNPs in the confirmation sample plus one additional SNP that tags another frequent haplotype in the block of an associated marker sample of 3277 individuals from the same survey.

Gene	***KCNQ1***	***KCNH2***	***KCNE1-KCNE2***
Genomic region	11p15.5-p15.4	7q36	21q22.-11q22.12
Genotyped region			
Start	2 406 312	149 970 666	34 630 685
End	2 856 274	150 090 991	34 803 884
Length of genotyped region	450 kb	120 kb	173 kb
Length of gene	404 kb	33 kb	13kb-7 kb
Exons in gene model	16	15	3- 2
SNP assays setup	131	90	49
Successfully genotyped SNPs for association in the screening sample	81	59	34
Genomic density of successfully genotyped SNP assay	1 SNP/5.6 kb	1SNP/2.0 kb	..1 SNP/5.1
Average call rate of successfully genotyped SNPs	95.4%	94.4%	96.6%
SNPs significantly associated with QTc_RAS in the screening sample	11	16	7
SNPs genotyped in the confirmation sample	5	5 (6)*	3
SNPs significantly associated with QTc_RAS in the confirmation sample	1	1 (2)*	1**
No. of haplotype blocks in the entire genotyped region	12	7	9
No. of haplotype blocks between Start and Stop codon of the gene	11	4	3-1
No. of SNP-markers not in LD blocks in the entire genotyped region	4	5	4

Table 4. KCNE1 and KCNE2 genes are adjacent on Chr. 21 and treated as one region. Start of each gene is given as the position of the first known start of mRNA transcription. All positions are taken from human genome assembly hg16. *In the KCNH2 gene, one of five SNPs was significantly associated to QTc_RAS in the two-step design. One additional associated SNP was identified by tagging the third haplotype in the associated block. **This SNP upstream of the KCNE1 gene only showed significant association in the confirmation step when no adjustment for multiple testing was performed.

Association was confirmed for four SNPs. First, we detected a previously undescribed QTL in intron 1 of the KCNQ1 gene. It contains two major haplotypes with frequencies of 0.570 and 0.379 that can be tagged by rs757092. This SNP showed association in both subsamples, the rare G-allele being associated to a QTc_RAS prolongation of +1.7 ms in heterozygotes and +3.3 ms in homozygotes. Second in the KCNH2 gene the previously known effect of SNP KCNH2-K897T (rs1805123) on the QT interval was confirmed: The rare 897T-allele was associated with a shortening of QTc_RAS of -1.9 ms in heterozygotes and -3.5 ms in homozygotes (0.36% of variance; *P*=0.0006). The effect was stronger in females. The K897T variant resides on a large haplotype block extending over 60 kb from exon 3 to 30 kb 3) of the gene, in which four haplotypes with allele frequencies above 0.05 exist among which KCNH2-K897T tags haplotype h2 (Hf=0.205). Third, typing the confirmation sample with a SNP tagging haplotype h3 (Hf=0.195) revealed a second effect in KCNH2: The rare A-allele of SNP rs3815459 was associated with a prolongation of QTc_RAS of +1.5 ms in heterozygotes and +4.5 ms in homozygotes (0.35% of variance; *P*=0.0004). Fourth, SNP rs727957 in the *KCNE1*-gene region showed a positive association to QTc_RAS in both the screening (*P*=0.0081) and the confirmation sample (*P*=0.0498), but did not exceed the adjusted significance level. In the total sample, the rare T-allele of the marker was associated with a prolongation of QTc_RAS of +1.0 ms in heterozygotes and +4.5 ms in homozygotes (0.23% of variance *P*=0.0051).

All four effects were acting independently of each other. In a additive model they together explained 0.95% of variation in corrected QT interval. This paper showed that common variants modifying QT could be detected and that their effects were additive to incrementally explain parts of the heritable fraction of QT interval.

2.2. Identifying and Validating New Genetic Variants influencing QT Interval (Paper II)

In this paper we performed a Genome Wide Association Study using a multi-stage study design. In stage I we only genotyped women from the extreme ends of the QT interval distribution. This strategy was designed to avoid the heterogeneity due to sex in the QT interval and because women have a lower prevalence of cardiovascular disease, which could confound QT interval measurement. We selected 100 women

from each extreme of the QT interval distribution (High-Low) in the KORA S4 study. This selection corresponds to QT intervals below the 7.5 percentile (385.7 ± 7.7 ms) or above the 92.5 percentile (444.8 ± 3.6 ms). These samples were genotyped using Affymetrix Centurion arrays.

In stage II, the first replication step, we included an additional 400 females having QTc_RAS below the 15th (n =200) or above the 85th percentile (n = 200). All 600 women who had their QTc_RAS means ~ s.d. apart and a mean trait difference of 45.5 ms were genotyped for SNPs that passed stage I criteria and flanking SNPs.

In stage III, we genotyped anonymous SNPs significant at P < 0.005 and candidate gene SNPs with P < 0.01 in stage II in the remaining 3,366 subjects of both genders. For the validation we used KORA F3 (2,646 individuals) and Framingham Heart Study (1,805 individuals) (Figure 4).

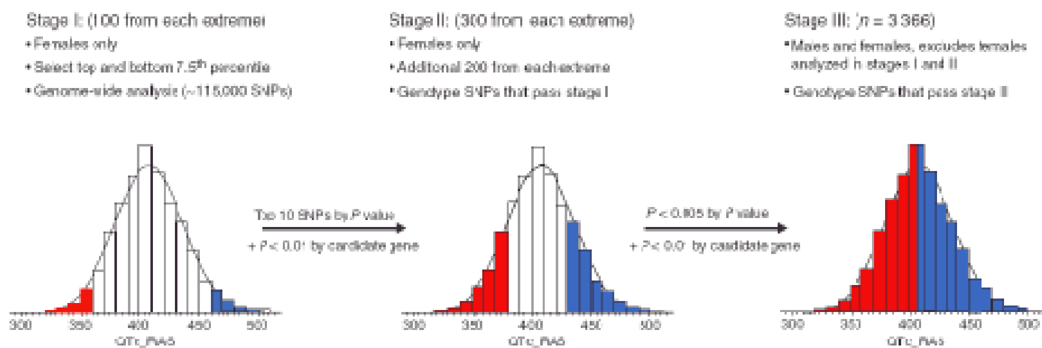

Figure 4. Genome wide association study of the QT interval. In stage I, genome wide genotyping was performed 100 females from each extreme of QTc_RAS. Two analysis approach were taken a genome wide scan and a candidate gene scan. In stage II, an additional 200 femnales from each extreme were genotyped, and the combined stage I and stageII sampes were analysed. In stage III. All samples not typed in stages I and II were genotyped, including both males and females, and both combined analysis were performed (Arking et al., 2006).

2.2.1. Study Population

We again used the KORA S4 study as we did in the candidate gene study. Additionally we used KORA F3 study in the replication phases after stage III. For this analysis, we excluded subjects with atrial fibrillation, pacemaker implant and/or pregnancy. We included 3,966 subjects from S4 for the three-stage genome-wide study, and 2,646 subjects from F3 were used for an independent replication. (KORA S3 study population, 4,856 subjects were studied between 1994 and 1995. In 2003

and 2004, 2,974 participants from S3 returned for follow-up (KORA F3). For this analysis, we excluded subjects with atrial fibrillation, pacemaker implant and/or pregnancy).

The Framingham heart study (FHS), the second replication sample, is a prospective epidemiologic study established in 1948 to evaluate potential risk factors for coronary heart disease. In 1971, 5,124 subjects were entered into the Framingham Offspring Study, including children or spouses of the children of the original cohort. We measured the QT interval in electrocardiograms (ECG) from offspring participants of the Framingham Heart Study examined between 1971 and 1975 who were free of atrial fibrillation or QT-influencing medication and survived to provide DNA collected during 1995–1998. This community-based cohort is predominantly of European ancestry (mean age 36.9 years; 51.4% women).

2.2.2. Genome-Wide Scan, SNP Genotyping, SNP Selection for Stage II and III Genotyping

Genome-wide assays and SNP genotyping: Stage I genome-wide analysis was performed using Affymetrix oligonucleotide arrays containing 115,571 SNPs. Genotypes were determined using the software tool GDAS3.0, with a setting of 0.05 for both homozygous and heterozygous genotype calls. Fourteen (7%) arrays with <85% overall genotyping call rates (across all SNPs) as well as 9,616 (8.3%) SNPs with overall genotyping call rates of <85% (across samples) were removed from the data set because their accuracy was 99.5%, as determined by extensive internal validation of repeat samples. We also removed 17,367 SNPs with minor allele frequency (MAF) <2.5%, as they would have no power under any study design. These procedures left us with 186/200 subjects (93.0%) and 88,548/115,571 SNPs (76.6%) for analysis. Additional genotyping was performed in S4, F3 and FHS using either TaqMan Assays on Demand or Assays by Design (Applied Biosystems) or primer extension MALDI-TOF genotyping technology (Autoflex HT, Sequenom).

Testing for population stratification. All SNPs were tested for Hardy-Weinberg equilibrium. FST for each SNP, with no missing data in the stage I analysis (11,431 SNPs), was calculated as the complement of the observed to expected heterozygosity.

SNP selection for stage II and III genotyping. SNPs representing the ten most significant loci from the genome-wide screen and P <0.01 from the candidate genes were selected for follow-up in stage II (Figure 5). For loci with multiple SNPs in high linkage disequilibrium (LD) (r^2>0.4), only the most significant SNP was selected. Flanking SNPs were chosen from the International HapMap project. LD was measured (r^2) for each HapMap SNP within 500 kb of the target SNP, and one was chosen on each side of the target SNP with an r^2 value between 0.4 and 0.8 and MAF≤0.2. In those cases where there was no flanking SNP within the r^2 limits, the closest SNP with MAF ≤0.2 was chosen. The SNPs showing P < 0.005 and P < 0.01 from the stage II genome-wide and candidate gene analyses, respectively, were selected for stage III genotyping.

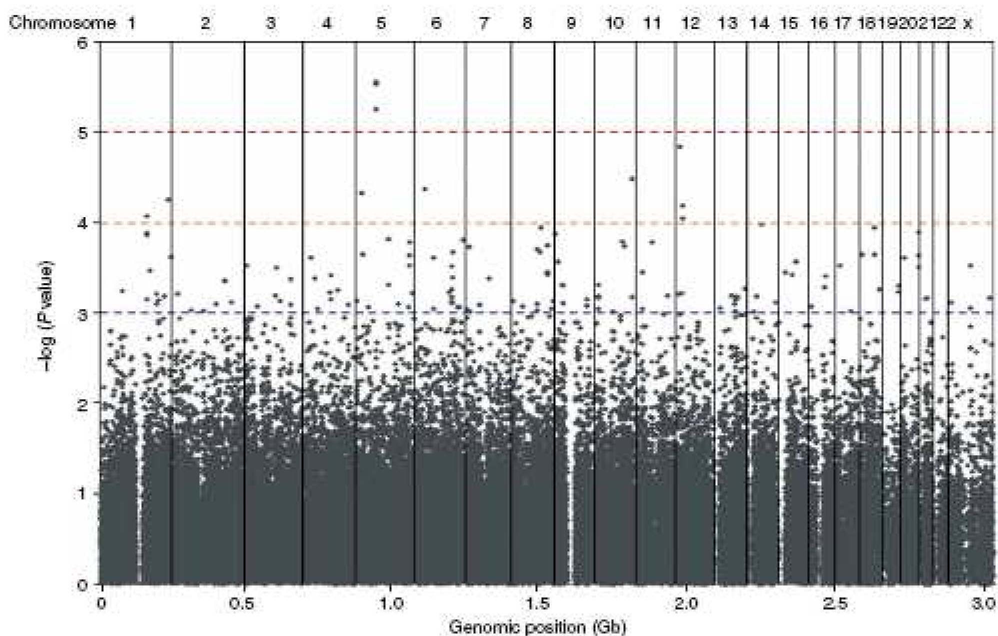

Figure 5. Genome-wide significance of QTc_RAS. The analysis compared 100 females from eachextreme of QTc_RAS (stage I). The x-axis is genomic position, and the y-axis is the negative base-10 logarithm of the P value. None of the SNPs showed genome-wide significance, but the top ten positives had P < 10^{-4} (Arking et al.,2006).

Statistical analyses of genetic effects: Stage I analysis of 200 women, including 100 from each extreme of QTc_RAS, was performed using a truncated measure analysis of variance (ANOVA) under additive, dominant and recessive genetic models, with best P values retained. Stage II analysis (600 women) added the next 200 women from each extreme of QTc_RAS and was performed as above. Stage III samples

(3,366 subjects comprising both men and women) and the replication sample F3 (2,646 subjects comprising both men and women) were analyzed using standard ANOVA under additive, dominant and recessive genetic models. P values are adjusted for the testing of multiple genetic models by permutation tests. Model-free analyses were performed using ANOVA with 2 degrees of freedom. Analyses were performed separately for males and females, as well as for the combined sample. To estimate the variance explained, we calculated R2 using linear regression. Regression analyses were performed using SPSS ver13.0. The FHS analyses of SNPs at the seven loci identified in stage II involved testing the association of genotypes at these loci with adjusted QT interval duration in 1,805 unrelated FHS participants, using linear regression testing dominant, additive and recessive models (SAS v 8.1). Nominal P values for the best genetic model are reported for the replication samples.
Power calculation: Power was estimated by Monte Carlo simulation. Phenotypes (P) for 2,000 subjects were randomly generated from a normal distribution. To simulate a genetic effect (d) under an additive model, genotypes were generated under Hardy-Weinberg equilibrium, and phenotypes were simulated. with the following means: AA = P + (d / 2), Aa = P; aa = P – (d / 2). These subjects were ranked by phenotype and ANOVA was performed on the top and bottom 100 subjects (stage I analysis). If $P < 10^{-4}$ was obtained, the next 200 ranked subjects at the top and bottom were added and ANOVA performed (stage II). If $P < 0.005$ was obtained, an additional 2,000 subjects were simulated as above, and ANOVA was performed on the 3,400 subjects not tested in stages I and II (stage III). A positive result was assigned if $P < 0.005$, which was empirically derived and corresponds to a genome-wide type I error of 0.05. Power to replicate findings in the F3 population (nominal $P < 0.05$) was determined by simulating 2,700 subjects as described above, using the genetic effect observed in the S4 population.

2.2.3. Results and Discussion

We initially identified three loci based on sex-pooled analyses of stage III samples with nominal significance ($P < 0.05$): these loci correspond to rs10494366 at NOS1AP (P < 10–7), rs1559578 at QTc_5.3 ($P < 0.004$) and rs7341478 at CACNA2D1 ($P < 0.024$). NOS1AP (CAPON) is the C-terminal PDZ domain ligand to neuronal nitric oxide synthase (nNOS, encoded by the NOS1 gene) and affects

NMDA receptor–gated calcium influx. It has not been previously suspected to have a role in cardiac repolarization. CACNA2D1 encodes an L-type voltage dependent calcium channel regulatory subunit expressed in the heart. The third locus, QTc_5.3, does not correspond to a known gene, but rather to a GeneScan31 prediction, so its potential biological relationship to the QT interval is unknown. None of the four remaining loci in stage III was significant in the overall sample, but KCNK1 (P ~ 0.005) showed significant effects in females only. KCNK1 (TWIK1) is the weakly inward-rectifying potassium channel subfamily K member 1 and may be involved in the control of background potassium membrane conductance. The gene is transcribed in many tissues but is particularly highly expressed in the brain and heart. However, given the number of hypotheses tested (seven SNPs, sex-pooled and sex-specific), only the NOS1AP SNP achieves genome-wide significance after correcting for multiple testing. This finding was not dependent on the genetic model, with a similar result obtained from model-free analysis ($P < 10^{-5}$).

				AA		Aa		Aa			
Gene or locus	SNP	Gender	MAF	*n*	QTc_RAS	*n*	QTc_RAS	*n*	QTc_RAS	*P*	P_{adj}
Genome-wide association											
NOS1AP	rs10494366	Both	0.36	1,542	–2.2 (0.4)	1,771	1.3 (0.4)	491	2.7 (0.8)	**$<10^{-10}$**	**$<10^{-7}$**
		Male		758	–1.6 (0.6)	867	1.5 (0.6)	257	2.9 (1.2)	**0.0001**	**0.0001**
		Female		784	–2.8 (0.6)	904	1.2 (0.6)	234	2.5 (1.0)	**$<10^{-7}$**	**0.021**
QTc_5.3	rs1559578	Both	0.36	1,542	0.9 (0.4)	1,775	–0.1 (0.4)	504	–2.4 (0.8)	**0.001**	**0.004**
		Male		762	1.4 (0.7)	899	0.1 (0.6)	243	–2.6 (1.2)	**0.007**	**0.007**
		Female		780	0.3 (0.6)	876	–0.3 (0.6)	261	–2.3 (1.0)	0.067	0.47
FGFR2	rs6585682	Both	0.46	1,102	0.3 (0.5)	1,820	0.1 (0.4)	793	–0.2 (0.7)	0.78	0.93
		Male		553	0.1 (0.8)	898	0.1 (0.6)	385	1.8 (1.0)	0.20	0.20
		Female		549	0.6 (0.6)	922	0.1 (0.5)	408	–2.0 (0.9)	**0.03**	0.46
QTc_14.1	rs7146384	Both	0.33	1,702	0.3 (0.4)	1,603	0.5 (0.4)	416	–0.4 (0.8)	0.21	0.52
		Male		838	–0.2 (0.6)	788	0.9 (0.7)	213	0.1 (1.2)	0.69	0.69
		Female		864	0.8 (0.6)	815	0.1 (0.6)	203	–0.9 (1.1)	0.20	0.052
Candidate genes											
KCNK1	rs2282428	Both	0.34	1,652	–0.5 (0.4)	1,753	0.4 (0.4)	423	0.6 (0.8)	0.20	0.41
		Male		838	0.7 (0.6)	847	0.3 (0.6)	211	–0.6 (1.0)	0.56	0.56
		Female		814	–1.7 (0.6)	906	0.6 (0.5)	212	1.9 (1.1)	**0.002**	**0.005**
ITPR1	rs3804999	Both	0.28	1,994	0.6 (0.4)	1,523	–0.6 (0.5)	307	0.0 (0.9)	0.12	0.42
		Male		1,003	0.5 (0.6)	758	0.3 (0.7)	145	0.1 (1.3)	0.91	0.91
		Female		991	0.6 (0.5)	765	–1.5 (0.6)	162	–0.1 (1.3)	**0.03**	0.32
CACNA2D1	rs7341478	Both	0.27	2,027	–0.6 (0.3)	1,412	0.3 (0.5)	283	2.7 (1.0)	**0.009**	**0.024**

Table 6. Genetic effects of QT interval-associated polymorphisms in 3,996 individuals from the KORA S4 sample

Gene or locus	SNP	Gender	MAF	AA		Aa		Aa		P
				n	QTc_RAS	n	QTc_RAS	n	QTc_RAS	
Genome-wide association										
NOS1AP	rs10494366	Both	0.36	1,085	–2.6 (0.5)	1,170	0.7 (0.6)	345	5.3 (1.1)	**$<10^{-11}$**
		Male		535	–2.6 (0.8)	562	0.2 (0.8)	171	6.9 (1.5)	**$<10^{-7}$**
		Female		550	–2.6 (0.7)	608	1.1 (0.8)	174	3.8 (1.5)	**$<10^{-5}$**
QTc_5.3	rs1559578	Both	0.38	989	–0.3 (0.6)	1,161	0.2 (0.6)	376	–0.6 (1.0)	0.82
		Male		476	–0.3 (0.9)	570	0.0 (0.8)	180	–0.4 (1.5)	0.98
		Female		513	–0.3 (0.8)	591	0.4 (0.8)	196	–0.7 (1.3)	0.83
FGFR2	rs6585682	Both	0.46	777	–0.8 (0.7)	1,264	0.6 (0.5)	558	–0.5 (0.7)	0.37
		Male		379	–1.7 (0.9)	617	1.1 (0.8)	273	–0.4 (1.1)	0.12
		Female		398	0.1 (0.9)	647	0.1 (0.7)	285	–0.6 (1.0)	0.84
QTc_14.1	rs7146384	Both	0.33	1,172	–0.4 (0.5)	1,135	0.5 (0.6)	286	–1.0 (1.1)	0.65
		Male		581	–0.8 (0.8)	547	1.0 (0.8)	136	–2.1 (1.5)	0.38
		Female		291	0.0 (0.7)	588	0.0 (0.8)	150	0.1 (1.6)	1.00
Candidate genes										
KCNK1	rs2282428	Both	0.35	1,091	0.6 (0.6)	1,179	–0.6 (0.5)	330	–0.4 (1.0)	0.26
		Male		527	0.8 (0.8)	576	–0.8 (0.8)	166	–0.5 (1.5)	0.29
		Female		564	0.4 (0.8)	603	–0.3 (0.7)	164	–0.3 (1.3)	0.73
ITPR1	rs3804999	Both	0.29	1,321	–0.1 (0.5)	1,076	–0.2 (0.6)	211	0.8 (1.4)	0.72
		Male		647	0.6 (0.7)	507	–1.1 (0.9)	118	0.9 (2.0)	0.42
		Female		674	–0.7 (0.7)	569	0.5 (0.8)	93	0.8 (2.1)	0.37
CACNA2D1	rs7341478	Both	0.28	1,336	0.7 (0.5)	1,045	–1.1 (0.6)	214	–0.5 (1.3)	**0.040**
		Male		670	1.1 (0.8)	509	–1.8 (0.8)	87	0.4 (2.0)	**0.035**
		Female		666	0.4 (0.7)	536	–0.4 (0.8)	127	–1.1 (1.7)	0.54

QTc_RAS values are displayed as the difference (s.e.m.) from the population mean (405.0 ms). *P* values are reported for the best genetic model adjusted by permutation analysis for the multiple genetic models tested. MAF: minor allele frequency in the sex-pooled analysis. Boldface indicates statistically significant results.

Table 7. Genetics effect of stage III SNPs with QT interval in 2,646 individuals from the KORA F3 replication sample

Gene or locus	SNP	Gender	MAF	AA		Aa		aa		P
				n	ΔQT	n	ΔQT	n	ΔQT	
Genome-wide association										
NOS1AP	rs10494366	Both	0.39	663	–1.3 (0.6)	864	0.1 (0.6)	257	2.7 (1.0)	**0.004**
		Male		309	–1.5 (0.9)	440	1.0 (0.8)	119	–0.4 (1.5)	0.17
		Female		354	–1.1 (0.9)	424	–0.8 (0.8)	138	5.4 (1.4)	**0.0001**
QTc_5.3	rs1559578	Both	0.36	740	–0.2 (0.6)	794	0.5 (0.6)	229	–0.7 (1.1)	0.84
		Male		349	0.5 (0.9)	380	0.5 (0.8)	120	–2.2 (1.5)	0.27
		Female		391	–0.9 (0.9)	414	0.6 (0.8)	109	–0.9 (1.6)	0.43
FGFR2	rs6585682	Both	0.47	509	–1.5 (0.7)	870	0.7 (0.6)	390	–0.2 (0.9)	0.09
		Male		257	–1.9 (1.0)	425	1.3 (0.8)	182	–0.2 (1.2)	0.07
		Female		252	–1.0 (1.1)	445	0.2 (0.8)	208	–0.2 (1.2)	0.80
QTc_14.1	rs7146384	Both	0.31	830	0.2 (0.6)	724	0.1 (0.6)	214	–1.1 (1.1)	0.68
		Male		414	0.1 (0.8)	348	0.2 (0.9)	92	–0.5 (1.7)	0.98
		Female		416	0.2 (0.8)	376	0.1 (0.9)	122	–1.5 (1.5)	0.67
Candidate genes										
KCNK1	rs2282428	Both	0.33	188	–0.1 (0.6)	807	0.2 (0.6)	188	–0.5 (1.2)	0.97
		Male		370	0.6 (0.8)	405	–0.8 (0.8)	92	0.7 (1.7)	0.69
		Female		421	–0.7 (0.8)	402	1.1 (0.8)	96	–1.7 (1.7)	0.60
ITPR1	rs3804999	Both	0.28	906	–0.6 (0.6)	691	0.4 (0.6)	142	–0.1 (1.4)	0.60
		Male		442	–0.6 (0.8)	339	1.1 (0.9)	69	–1.3 (2.0)	0.60
		Female		464	–0.6 (0.8)	352	–0.3 (0.9)	73	1.0 (2.0)	0.87
CACNA2D1	rs7341478	Both	0.25	993	–0.1 (0.5)	659	–0.1 (0.7)	118	2.0 (1.5)	0.45
		Male		487	–0.3 (0.7)	311	–0.3 (0.9)	58	4.9 (2.1)	0.31
		Female		506	0.0 (0.8)	348	0.0 (0.9)	60	–0.9 (2.2)	0.97

Table 8. Genetics effect of stage III SNPs with QT interval in 1,805 individuals from the FHS replication sample.

After stahe III we genotyped SNPs from the seven loci of stage III in 1,805 participants from the Framingham Heart Study (FHS) (Table 8) and 3996 participants KORA S4 (Table 6) and participants 2646 KORA F3 Study (Table 7).

In these two completely independent samples we could only positively replicate SNP rs10494366 SNP from CAPON. All the other loci thererfore have to be considered as nonreplications. rs10494366 acted on QT intervall in an additive allelic model explaining overall 1,5% of the variation of the corrected QT interval. This finding is the first description of the detection of a common genome variant associated with a proarrhythmogenic trait in humans.

2.3. Validation of significant SNPs from the Genome-wide Association Study in The DESIR Study from France (Paper III)

From the genome-wide association study (Chapter 5.3) SNP rs10494366 near the NOS1AP (CAPON) gene has been identified as a quantitative trait locus for QT interval. The most strongly associated SNP marker rs10494366 explained some 1.5% of corrected QT interval variance in general population samples from Germany and the U.S. We intended to replicate this finding in the DESIR Study from France.

2.3.1. Study Population

The French D.E.S.I.R. study includes n=4115 individuals from France, a general population sample of men and women aged 30 to 65 years who had been recruited between 1994 and 1996 and followed up 9 years later. QT intervals were measured in lead 11 on a Cardionic machine using Cardionic software. Correction factors were determined separately for each gender, as dictated by our sampling strategy, and the resulting QT interval, corrected for heart rate (R), age (A) and sex (S).

2.3.2. Genotyping and Association Study

DNA was extracted from EDTA anticoagulated blood by phenol extraction. The SNP variant rs10494366 was determined by Kbiosciences (Hoddesdon, UK) using its own form of competitive PCR system (KASPar). Hardy-Weinberg-equilibrium (HWE) p-values were calculated. The SNP was tested for association with QT interval by fitting linear regression models to either uncorrected QT and covariates or QT after correction for covariates.

2.3.3. Results and Discussion

In the study sample of n=2,392 individuals QT interval had a mean value of 388,0 ms and a standard deviation of ± 34,9 ms. A multivariate linear regression model we used to demonstrate a significant association between rs10494366 and QT interval. The effect on QT interval was +1.52 ms for TG - heterozygotes and +4.67 ms for GG - homozygotes. The association explained 1% of trait variance in a multivariate linear regression model ($p=1.1*10^{-3}$). Effect size was not significantly different from the effect observed in the two German and the one US American samples reported in the initial study. In our study subjects carrying a minor allele had significantly longer QT intervals.

2.4. Replication of the KCNE Gene Variant G38S in the KORA Population (Paper IV).

A recent family based study found an association between QT interval and the common variant of the KCNE1 (MinK) gene potassium channel subunit. The G38S polymorphism explained 2.2% of QTc variability (p = 0.003) from family based association study (Friedlander et al., 2005).

2.4.1. Study Population

We used the KORA S4 population as in the previous chapters. We studied 3966 individuals from the KORA S4 study.

2.4.2. Genotyping and Association Study

DNA was extracted from EDTA anticoagulated blood using a salting out procedure. The G38S variant of the KCNE1 gene was determined using PCR, primer extension, and MALDI-TOF mass spectrometry in a 384-well format (Sequenom, San Diego, USA) as previously described. Hardy–Weinberg equilibrium (HWE) P-values were calculated using the STATA statistical software package. SNPs were tested for association to QT, QTc-Bazett, and QTc-RAS as the dependent variables by applying

two-tailed one-degree-of-freedom linear regression test (1df) and two-tailed two-degree-of-freedom ANOVA analysis (2df).

2.4.3. Results and Discussion

The G38S variant of the KCNE1 coding sequence was genotyped with a call rate of 98.7%. Genotypes showed no significant deviation from HWE (P = 0.08). The minor allele frequency (MAF = 36%) was well in common with those in other Caucasian samples. Genotyping results revealed no significant association between G38S and the uncorrected or corrected QT interval. For the uncorrected QT interval, we found all P-values to be 0.16. For QT corrected according to Bazett's formula, P-value was 0.64 and for QTc-RAS, P-value was 0.16 (Table 9, Table 10).

Table 9. Power Calculation

	Effect size estimator (δ)	Significance level (α)	GG38	GS38	SS38
Entire sample (n)			1591	1770	555
Power to discriminate	0.2 SD	0.001		0.994	0.936
		0.05		>0.999	0.998
	1.0 SD	0.001		>0.999	>0.999
		0.05		>0.999	>0.999
Men only (n)			772	872	293
	0.2 SD	0.001		0.776	0.545
Power to discriminate		0.05		0.982	0.926
	1.0 SD	0.001		>0.999	>0.999
		0.05		>0.999	>0.999

Power calculation to detect an effect of the G38S variant in the study population. Power was calculated separately for each allelic step (GG to GS and GS to SS) in two-tailed T-tests (1df) with the detected genotype frequencies from the study population. An effect size (δ) in the published magnitude of 21.7 ms QTc equals about 1 SD, an effect size of 0.2 SD corresponds to a change of 3.4 ms in QTc.

Table10. Association results.

	GG38	GS38	SS38	P (1df)	P (2df)
Total (n)	1591	1770	555	–	–
QT	408.1 ± 28.0	407.2 ± 28.2	409.7 ± 27.5	0.5797	0.1655
QTc-Bazett	423.1 ± 22.0	422.6 ± 21.2	423.5 ± 21.4	0.9263	0.6493
QTc-RAS	417.7 ± 17.5	417.2 ± 17.0	418.7 ± 16.8	0.5498	0.1650
Men (n)	772	872	293	–	–
QT	407.7 ± 30.3	406.8 ± 29.4	407.7 ± 28.3	0.8393	0.8187
QTc-Bazett	419.9 ± 22.8	419.2 ± 21.9	421.7 ± 23.0	0.5006	0.2460
QTc-RAS	418.1 ± 18.6	417.3 ± 17.7	419.2 ± 17.5	0.6904	0.2995
Women (n)	819	898	262	–	–
QT	408.5 ± 25.6	407.5 ± 27.0	412.0 ± 26.4	0.2636	0.0548
QTc-Bazett	426.0 ± 20.7	426.0 ± 20.0	425.6 ± 19.3	0.7747	0.9444
QTc-RAS	417.3 ± 16.4	417.0 ± 16.4	418.2 ± 15.9	0.6842	0.5589

3. CONCLUSION

The QT interval is a quantitative trait with significant genetic components. The influence of rare exonic mutations on QT has long been recognized and the molecular genetics of monogenic long-QT syndrome has been elucidated in the last decade. Likewise the influence of common genetic variation has also been evident by the notion of the heritability of QT in the general population. In dispite of this evidence available in favour of genetic factors involved. Only few common gene variants have been discovered until now.

In the candidate gene study (paper I) we could not only replicate the only previusly identified common QT modifying variant KCNH2-K897T, but could discover a novel one, rs727957 in intron 1 of KCNQ1. An important finding of this work was that, although not coding, rs727957 influences repolarization to a similar extent than the common nonsynonymous variant KCNH2-K897T. In the meantime the effect of this intronic variant has been replicated in a different population (Gouas et al., 2007).

The successful candidate gene study served as a proof of principle study to conduct a genome-wide association (GWAS) study for QT interval. The genome wide study identified association between a common variant in the NOS1AP gene promoter (rs10494366) and QT interval in German population as well as in American adults with European ancestry. The NOS1AP variant explains about 1,5% of the variation of the corrected QT interval and is the strongest single QT modifying genetic variant identified up to date (Paper II). The association could be replicated in the French DESIR study population (Paper III).

Another nonsynonymous variant KCNE1-G38S has been identified in an earlier study from the candidate gene approach to be associated with QTc (Friedlander et al., 2006). This polymorphism could not be replicated in a German population. The possible interpretation of there conflicty of results is the influence of different genetic backgrounds on the phenotype of QT interval. The G38S variant may be a causal variant only on certain genetic background or may be not associated to QT interval at all (Paper IV).

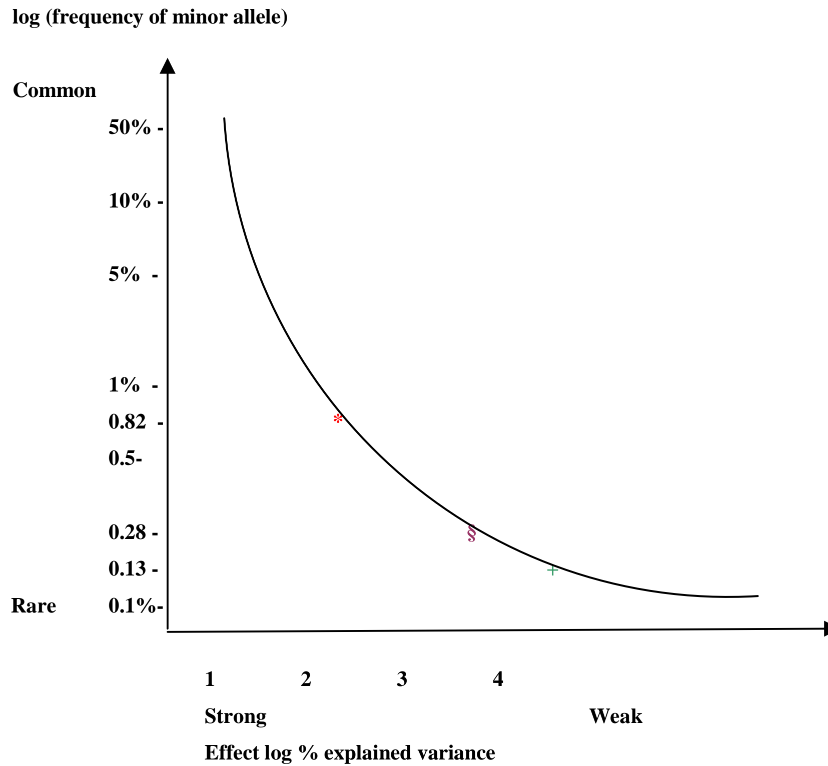

Figure6. The explained variant distributions of the overall frequency of the minor alleles.

*** KCNH2 K897T,;+ KCNQ1 rs757092; § NOS1AP rs10494366**

The work presented papers confirms the concept that cardiac repolarization as measured with the QT interval is a complex genetic trait with a significant heritable component. High resolution SNP mapping in candidate genes or in genome-wide association studies can successfully be used to identify common variants for such a traits in known and in novel genes and common variants explained stronger effects (Fig6).GWA provides a valuable new tool for elucidating the genetic basis of the common causes of diseases.

4. References

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PUBLISHED PAPERS

PAPER I

Common Variants in Myocardial Ion Channel Genes Modify the QT Interval in the General Population

Results From the KORA Study

Arne Pfeufer, Shapour Jalilzadeh, Siegfried Perz, Jakob C. Mueller, Martin Hinterseer, Thomas Illig, Mahmut Akyol, Cornelia Huth, Andreas Schöpfer-Wendels, Bernhard Kuch, Gerhard Steinbeck, Rolf Holle, Michael Näbauer, H.-Erich Wichmann, Thomas Meitinger, Stefan Kääb

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Pathological alteration of myocardial ventricular repolarization is a leading cause of ventricular tachycardia and fibrillation.[1] It is also suspected to contribute to sudden cardiac death in the context of myocardial hypertrophy or heart failure as well as in drug-induced arrhythmias.[2]

The cardiac repolarization process is known to be strongly dependent on various parameters, among them heart rate,[3] age,[4] sex,[5,6] plasma levels of electrolytes,[7] and medications,[8] as well as inherited and acquired pathological conditions.[9] The QT interval measured in the surface ECG is the most accessible noninvasive marker of repolarization. After correction for heart rate, its strongest covariate, it is usually referred to as the corrected QT or QTc interval.

Apart from monogenic long QT syndrome (LQT), heritability studies have suggested that genetic factors are also involved in the control of cardiac repolarization at the population level. The heritability of the QTc interval has been estimated between 25% and 52% in three sibpair and in one family-based study.[10–13] In a nonparametric linkage analysis, the authors of one of the above studies could demonstrate a significant linkage of the QTc interval to the *KCNQ1* (LQT1) and the *ANK2* (LQT4) gene loci.[12]

Several authors have investigated nonsynonymous SNPs in candidate genes for their effect on repolarization. The K897T variant in exon 11 of the *KCNH2* gene encoding the α-subunit of the voltage-gated myocardial IKr channel (LQT2) was examined in a study of 226 males and 187 females of Finnish descent. Only in females the 897T-allele had a prolonging influence on the maximum QTc interval measured over all 12 leads, but not in lead V2.[14] In 39 LQT

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TABLE 1. Population Characteristics

	Screening Sample	Confirmation Sample	*P*
Survey population, No.	880	3358	
Exclusion criteria			
Atrial fibrillation	8	30	0.97
Pacer/ICD implant	8	36	0.67
Pregnancy	2	15	0.36
Randomly excluded to meet requirements of 384-well genotyping	173	0	
Study sample, No.	689	3277	
Male, %	342 (49.6%)	1617 (49.3%)	0.8887
Age, y	57.7±12.3	47.4±13.4	<0.00005
Age range, y	25–74	25–74	
RR, ms	925.8±147.2	942.6±151.1	0.0076
QT, ms	409.3±28.4	407.6±28.0	0.1513
QT range, ms	330.0–538.0	322.0–550.0	
QTc_RAS, ms	418.9±18.5	417.3±16.9	0.0278
QTc_RAS range, ms	322.8–525.6	345.8–541.8	

In the screening sample, the age groups above 55 years were overrepresented, reflecting the age distribution of typical cardiovascular patient populations in an attempt to make the sample also suitable for matched case control designs. Differences in age, RR, and QTc_RAS are due to this overrepresentation.

patients with the KCNQ1-G589D mutation, the QT interval during exercise was prolonged in those with at least one KCNH2–897T-allele.[15] The authors of this and another functional study[16] noted that the IKr-897T channel exhibited a decreased current density.

In a study of 1316 Europeans, the 897T-allele shortened the QTc interval at rest in both males and females.[17] The effect appeared to be recessive with a shortening of QTc by −10.0 ms in 897TT-homozygotes and was stronger in females than in males. The IKr-897T channel showed a decrease in steady state activation potential predicting a shortening of action potential duration due to an increase in IKr current.

Myocardial repolarization is a fine-tuned process dependent on the delicate coordination of low strength ionic currents at the end of the action potential.[18] Gene variants conferring only subtle differences to gene regulation or function, such as intronic or promoter variants, may well influence the repolarization process similar to nonsynonymous variants. We tested the hypothesis that frequent gene variants in the long QT syndrome potassium channel genes *KCNQ1*, *KCNH2*, *KCNE1*, and *KCNE2* cause phenotypic variation of myocardial repolarization in the general population and conducted a systematic and high-density linkage disequilibrium (LD)–based SNP association study with a resolution similar to the current HapMap effort[19] in search of novel quantitative trait loci (QTL) of the QT interval.

Materials and Methods

Individuals

Between 1999 and 2001, we conducted an epidemiological survey of the general population living in or near the city of Augsburg, Southern Germany (KORA S4). This was the fourth in a series of population-based surveys originating from our participation in the WHO MONICA project. The study population consisted of residents of German nationality born between July 1, 1925 and June 30, 1975 identified through the registration office. A sample of 6640 subjects was drawn with 10 strata of equal size according to gender and age. After a pilot study of 100 individuals, 4261 individuals (66.8%) agreed to participate in the survey, which were ethnic Germans with very few exceptions (>99.5%). During 2002 and 2003, we reinvestigated a subsurvey of 880 persons specifically for cardiovascular diseases. From that subsurvey, 689 individuals were studied to screen for positive genetic associations (screening sample), whereas 3277 different individuals from the total survey were used to confirm positive findings (confirmation sample). A detailed description of samples and the list of exclusion criteria are given in Table 1. Blood samples were drawn after informed consent had been obtained. All studies involving humans were performed according to the declarations of Helsinki and Somerset West and were approved by the local medical ethics committee.

ECG Phenotyping

In the initial survey, we recorded 12-lead resting electrocardiograms (ECGs) using a digital recording system (Bioset 9000, Hörmann Medizinelektronik). QT intervals were determined using the Hannover ECG analysis software (HES-Version 3.22-12) by computerized analysis of an averaged cycle computed from all cycles of the 10-second recording after exclusion of ectopic beats. The QT interval determined by this algorithm represents the earliest begin of depolarization until the latest deflection of repolarization between any two leads. In an international validation study, the HES software was among the best performing digital ECG systems.[20] Reproducibility of HES QT-measurements over short- and long-term time intervals has been investigated.[21]

Covariate Analysis and Phenotype Correction

We adjusted QT for known covariates by a correction formula. Traditional formulas like Bazett's[3] correct only for heart rate in a nonlinear fashion. A linear correction formula for QT has been derived from Framingham Heart Study data.[22] We based correction of QT on a multivariate linear regression model including covariates heart rate (RR interval), sex, and age. Correction factors were determined separately for each sex; the QT interval corrected for

rate-, age-, and sex was called QTc_RAS. With the correction factors derived from the total sample of 3966 individuals, the formulas for QTc_RAS were determined for males:

$$(1)\quad QTc_RAS = QT - [0.152 \times (RR - 1000\text{ ms})] - [0.318\text{ ms/year} \times (\text{age} - 60\text{ years})]$$

and for females:

$$(2)\quad QTc_RAS = QT - [0.154 \times (RR - 1000\text{ ms})]] - [0.207\text{ ms/year} \times (\text{age} - 60\text{ years})] - 4.58\text{ ms},$$

where RR denotes RR interval in milliseconds.

Genotyping, Determination of Haplotype Blocks, and Haplotypes

We investigated genes encoding the α- and β-subunits of the myocardial delayed rectifier potassium channels IKs (IKs-α: *KCNQ1*; IKs-β: *KCNE1*) and IKr (IKr-α: *KCNH2*; IKr–β: *KCNE2*). A total of 270 SNPs distributed in and around these genes were chosen from the public dbSNP database, databases on monogenic long QT-syndrome genes,[23–25] and diagnostic LQT-patient resequencing. SNPs in exons or intron/exon boundaries were chosen without exception (19 SNPs). Outside those regions SNPs were selected on the criterion of equidistant spacing of ≈1 SNP every 5 kb (251 SNPs). Information about local patterns of LD from HapMap or other sources was not available at the time of SNP selection.

DNA was extracted from EDTA anticoagulated blood using a salting out procedure.[26] SNP genotypes were determined using PCR, primer extension, and MALDI-TOF mass spectrometry in a 384-well format (Sequenom). LD measures (D′, r^2) and haplotypes were determined with Haploview software.[27] Haplotype block boundaries were defined based on the confidence interval of the D′ measure as described in Gabriel et al.[28] Haplotype-phenotype association analysis based on sliding window haplotypes was performed using the haplotype trend regression test as described in.[29]

Of the 270 SNP assays, 33 were not functional, with call rates below 0.8, and 36 were monomorphic. And 174 SNPs had call rates ≥0.8, minor allele frequencies ≥0.02, and Hardy-Weinberg-equilibrium (HWE) *P* values ≥0.01. The low cut-off value for HWE was accepted because of the relatively large number of SNPs genotyped in the project.

Genotype Phenotype Association Analysis

SNPs were tested for association by linear regression analysis using QTc_RAS as the dependent variable. Significance levels were determined for both the one-degree (1df) and the two-degree of freedom (2df) test. In the 1df test, the independent variable was derived by transforming SNP's genotypes (AA, Aa, aa) to a relational scale by counting the number of minor alleles (0, 1, 2) assuming a strictly codominant model with identical trait increases between genotypes. This test has a relatively higher power to detect weak effects and was our primary test used during screening and confirmation. In the 2df test, a SNP was decomposed into two variables representing the two genotypic changes and both were included into a bivariate regression. This test accounts for dominance and recessivity by allowing the trait increase of each genotypic change to take an individual value. It was used to specifically quantify each genotype's effect and significance level in the total sample. The average trait increase per allele was calculated as the mean of both genotypic changes weighted by the genotype frequencies and the variance attributable to a SNP was calculated as the adjusted r^2 value from the bivariate regression analysis.

To determine the independence of effects, we performed multivariate linear regression analysis, incorporating the genotypic changes of several SNPs into one model. To determine combined effects, we counted the number of significant genotypic changes in each person to give a QT-prolongation score and performed ANOVA analysis using the score as the independent variable. To investigate if associated SNP-markers had also been identified in a categorical trait analysis, we analyzed groups of individuals with extreme QTc_RAS values in both and individual sexes in a case control–like design using the Cochran-Armitage test for trend.

Association Study Design and Adjustment for Multiple Testing

We designed a two-step association procedure using a small screening and a larger confirmation sample in an attempt to minimize the false-positive error rate. We genotyped the screening sample for all designed SNP assays. Without adjusting for multiple testing, we genotyped all SNPs significantly associated with QTc_RAS ($P<0.05$ in the 1df test) and nonredundant to each other (pairwise $r^2<0.6$) in the confirmation sample. To adjust for multiple testing in this step, we calculated an adjusted table-wide significance level using 1000 rounds of permutation. As the question if adjustment for multiple testing is necessary in two-step designs is not resolved, we used both the unadjusted and the adjusted significance levels in the discussion of confirmation results. In haplotype blocks of confirmed SNPs, we investigated additional nonredundant markers even if in the screening sample they had not been significantly associated with QTc_RAS.

To determine gender-specific differences of SNP-phenotype associations, we performed sex-specific regression analysis in the total sample. Sample sizes of males (n=1959) and females (n=2007) were similar and therefore comparable for effect strength. To investigate if SNPs with confirmed association to QTc_RAS had also been identified by a categorical trait analysis, we analyzed groups of individuals with extreme QTc_RAS values in both and individual sexes against each other using the Cochran-Armitage test for trend.

Results

Association Analysis of Individual SNPs

In the total sample of 3966 individuals, QTc_RAS corrected to a 60-year-old male with a heart rate of 60 bpm had a mean value of 417.6 ms and a SD of ±17.2 ms.

Of the 174 successfully genotyped SNPs, the average call rate was 0.953 and the average minor allele frequencies were 0.258 (mean) and 0.251 (median). Haplotype blocks are shown in Figure 1 and described in Table 2. In the screening sample, 34 of these SNPs showed association to QTc_RAS in the 1df-test, 18 of these being also significant in the 2df test. We genotyped 13 nonredundant SNPs in the confirmation sample (Table 3a; supplemental Table I, available online at http://circres.ahajournals.org) plus one additional SNP that tags another frequent haplotype in the block of an associated marker. Association was confirmed for four SNPs if the unadjusted significance level of 0.05 was used and for three SNPs if the adjusted table-wide significance level of 0.0041 was applied.

We detected a previously undescribed QTL in intron 1 of the *KCNQ1* gene. Although the gene shows remarkably little LD, intron 1 contains a large haplotype block of ≈50-kb size and high LD (both D′ ≥0.94 and r^2≥0.79 for 6 of 7 markers) (Figure 1). It contains two major haplotypes with frequencies of 0.570 and 0.379 that can be tagged by rs757092. This SNP showed association in both subsamples (Table 3a), the rare G-allele being associated to a QTc_RAS prolongation of +1.7 ms in heterozygotes and +3.3 ms in homozygotes (Table 3b; 0.38% of variance; P=0.0002).

In the *KCNH2* gene, we confirmed the previously published effect of SNP KCNH2-K897T (rs1805123) on the QT interval. The rare 897T-allele was associated with a shortening of QTc_RAS of −1.9 ms in heterozygotes and −3.5 ms in homozygotes (0.36% of variance; P=0.0006). The effect was stronger in females. The K897T variant resides on a large haplotype block extending over 60 kb from exon 3 to 30 kb

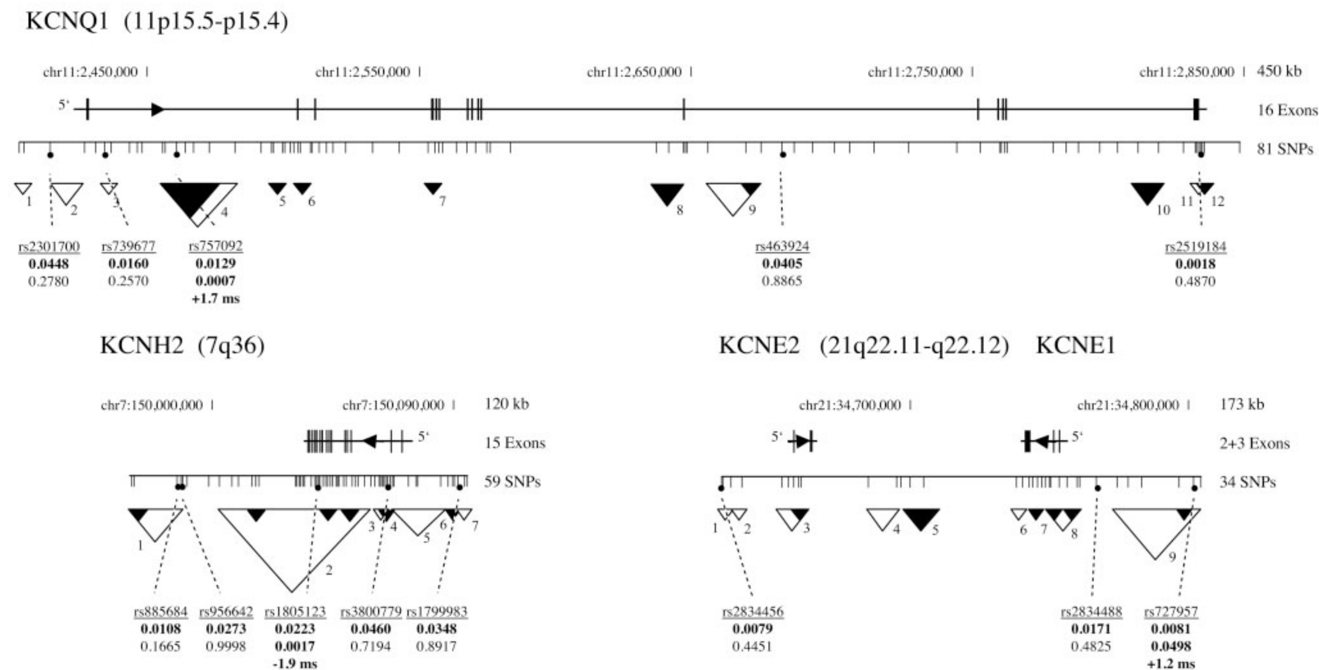

Figure 1. Genomic structure, LD-structure, and genotyped SNPs in the investigated gene regions. 174 SNPs genotyped in the screening sample are denoted as (|), 13 SNPs genotyped in the confirmation sample are marked by ●. LD-structure in the regions is marked in form of D′-based haplotype block boundaries[28] (△) and in form of neighboring SNPs exceeding r^2-values of 0.5 (▲). For SNPs genotyped in both samples, *P* values for association with QTc_RAS in the screening (top) and in the confirmation sample (bottom) and for associated SNPs the effect of one minor allele on QTc_RAS in the entire sample are given.

3′ of the gene (KCNH2-block 2 in Figure 1), in which four haplotypes with allele frequencies above 0.05 exist (Table 4) among which KCNH2-K897T tags haplotype h2 (Hf=0.205). Typing the confirmation sample with a SNP tagging haplotype h3 (Hf=0.195) revealed a second effect. The rare A-allele of SNP rs3815459 was associated with a prolongation of QTc_RAS of +1.5 ms in heterozygotes and +4.5 ms in homozygotes (0.35% of variance; P=0.0004).

TABLE 2. Investigated Gene Regions

Gene	*KCNQ1*	*KCNH2*	*KCNE1*	*KCNE2*
Genomic region	11p15.5-p15.4	7q36	21q22.11-q22.12	
Genotyped region				
Start	2 406 312	149 970 666	34 630 685	
End	2 856 274	150 090 991	34 803 884	
Length of genotyped region	450 kb	120 kb	173 kb	
Length of gene	404 kb	33 kb	13 kb	7 kb
Exons in gene model	16	15	3	2
SNP assays setup	131	90	49	
Succesfully genotyped SNPs for association in the screening sample	81	59	34	
Genomic density of succesfully genotyped SNP assays	1 SNP/5.6 kb	SNP/2.0 kb	1 SNP/5.1 kb	
Average call rate of successfully genotyped SNPs	95.4%	94.4%	96.6%	
SNPs significantly associated with QTc_RAS in the screening sample	11	16	7	
SNPs genotyped in the confirmation sample	5	5 (6)*	3	
SNPs significantly associated with QTc_RAS in the confirmation sample	1	1 (2)*	1**	
No. of haplotype blocks in the entire genotyped region	12	7	9	
No. of haplotype blocks between Start and Stop codon of the gene	11	4	3	1
No. of SNP-markers not in LD blocks in the entire genotyped region	45	4	6	

KCNE1 and *KCNE2* genes are adjacent on Chr. 21 and treated as one region. Start of each gene is given as the position of the first known start of mRNA transcription. All positions are given from human genome assembly hg16. *In the *KCNH2* gene, one of five SNPs was significantly associated to QTc_RAS in the two-step design. One additional associated SNP was identified by tagging the third haplotype in the associated block. **This SNP upstream of the *KCNE1* gene only showed significant association in the confirmation step when no adjustment for multiple testing was performed.

TABLE 3A. Association Results

Gene Region	SNP-Marker	Alleles (A/a)	Minor Allele Effect on QT	Screening Sample (n=689)							Confirmation Sample (n=3277)						
				(AA)		(Aa)		(aa)			(AA)		(Aa)		(aa)		
				n	QTc_RAS	n	QTc_RAS	n	QTc_RAS	*P* (1df)	n	QTc_RAS	n	QTc_RAS	n	QTc_RAS	*P* (1df)
KCNQ1	rs2301700	G/A		530	418.0	110	420.9	8	428.2	0.0448	2590	417.5	600	416.9	35	415.0	0.2780
KCNQ1	rs739677	A/G		368	417.5	244	421.0	47	422.0	0.0160	1707	417.8	1230	416.7	213	417.6	0.2570
KCNQ1	rs757092	A/G	↑	284	417.5	305	418.8	100	423.3	0.0129	1216	416.1	1541	417.9	445	418.9	0.0007
KCNQ1	rs463924	C/T		310	417.6	294	419.7	68	422.2	0.0405	1557	417.4	1347	417.3	292	417.4	0.8865
KCNQ1	rs2519184	G/A		535	419.9	114	414.6	6	408.7	0.0018	2220	417.2	460	417.1	29	423.2	0.4870
KCNH2	rs885684	T/G		303	420.7	302	417.9	82	415.6	0.0108	1261	417.5	1515	417.6	421	415.7	0.1665
KCNH2	rs956642	A/G		258	416.9	322	419.7	106	421.0	0.0273	1111	417.6	1413	417.2	507	417.8	0.9998
KCNH2	rs1805123	A/C	↓	389	420.7	245	416.4	31	419.2	0.0223	1665	418.0	1032	416.7	187	414.3	0.0017
KCNH2	rs3800779	G/T		272	417.4	317	419.1	82	422.0	0.0460	1109	416.9	1193	417.7	327	416.7	0.7194
KCNH2	rs1799983	G/T		320	420.7	290	418.3	50	415.9	0.0348	1467	417.3	1330	417.4	355	417.4	0.8917
KCNE1	rs2834456	A/G		207	416.3	354	419.7	122	421.6	0.0079	966	417.2	1554	417.0	611	418.0	0.4451
KCNE1	rs2834488	T/C		598	419.5	76	414.0	2	414.9	0.0171	2783	417.3	329	417.6	15	422.0	0.4825
KCNE1	rs727957	G/T	↑	447	417.7	199	420.2	26	427.2	0.0081	2046	416.9	948	417.6	106	420.1	0.0498

Results of 13 SNPs in both samples in the two-step approach. Significance levels were determined by the 1df test as described in the Materials and Methods section. All SNPs are located in introns or in flanking genomic regions of the genes except for SNP rs1805123 (KCNH2-K897T). For significance levels of the 2df test, see supplemental Table I.

In the *KCNE1* gene region, SNP rs727957 showed a positive association to QTc_RAS in both the screening ($P=0.0081$) and the confirmation sample ($P=0.0498$), but did not exceed the adjusted significance level. In the total sample, the rare T-allele of the marker was associated with a prolongation of QTc_RAS of +1.0 ms in heterozygotes and +4.5 ms in homozygotes (0.23% of variance $P=0.0051$).

Among the 174 investigated common SNPs were two further nonsynonymous gene variants, KCNH2-R1047L [Af(min)=0.024], for which functional data indicate no allele differences,[16,17] and KCNE1-S38G [Af(min)=0.355], neither of which were associated to QTc_RAS. To clarify the importance of rare nonsynonymous SNPs, we additionally genotyped KCNE2-T8A [Af(min)=0.0073], which had previously been described associated to drug-induced long QT syndrome[30] and KCNQ1-G643S [Af(min)=0.00072], for which only one heterozygote was observed. Also, these showed no significant effect (supplemental Table III).

TABLE 3B. Association Results

Gene Region	SNP-Marker	Alleles (A/a)	Gender	Minor Allele Effect on QT	Total Sample (n=3966)											
					(AA)		(Aa)			(aa)						
					n	QTc_RAS	n	Δ QTc_RAS	*P* (Aa)	n	Δ QTc_RAS	p(aa)	Explained Variance	Average Δ QTc_RAS Per Allele	*P* (1df)	*P* (2df)
KCNQ1	rs757092	A/G	Both	↑	1500	416.4	1846	+1.7 ms	0.005	545	+3.3 ms	0.001	0.38%	+1.7 ms	<0.00005	0.0002
KCNQ1	rs757092	A/G	Male	↑	747	416.8	899	+1.3 ms	0.143	275	+3.4 ms	0.009	0.26%	+1.5 ms	0.0087	0.0296
KCNQ1	rs757092	A/G	Female	↑	753	416.0	947	+2.0 ms	0.011	270	+3.3 ms	0.004	0.44%	+1.9 ms	0.0012	0.0046
KCNH2	rs1805123	A/C	Both	↓	2054	418.5	1277	−1.9 ms	0.002	218	−3.5 ms	0.004	0.36%	−1.9 ms	0.0001	0.0006
KCNH2	rs1805123	A/C	Male	↓	1027	418.6	601	−1.2 ms	0.181	104	−3.8 ms	0.043	0.18%	−1.4 ms	0.0288	0.0791
KCNH2	rs1805123	A/C	Female	↓	1027	418.5	676	−2.5 ms	0.002	114	−3.2 ms	0.046	0.52%	−2.3 ms	0.0010	0.0031
KCNH2	rs3815459	G/A	Both	↑	2469	416.9	1251	+1.5 ms	0.011	178	+4.5 ms	0.001	0.35%	+1.7 ms	0.0001	0.0004
KCNH2	rs3815459	G/A	Male	↑	1226	417.0	612	+2.1 ms	0.022	92	+4.3 ms	0.028	0.35%	+2.1 ms	0.0031	0.0127
KCNH2	rs3815459	G/A	Female	↑	1243	416.8	639	+1.0 ms	0.198	86	+4.7 ms	0.010	0.28%	+1.3 ms	0.0134	0.0230
KCNE1	rs727957	G/T	Both	↑	2493	417.0	1147	+1.0 ms	0.085	132	+4.5 ms	0.003	0.23%	+1.2 ms	0.0030	0.0051
KCNE1	rs727957	G/T	Male	↑	1239	417.0	555	+1.5 ms	0.100	66	+6.4 ms	0.005	0.41%	+1.5 ms	0.0044	0.0080
KCNE1	rs727957	G/T	Female	↑	1254	417.1	592	+0.6 ms	0.444	66	+2.6 ms	0.202	<0.01%	+0.7 ms	0.1946	0.3682

Effect of the associated SNPs in the total sample and stratified for gender as determined by linear regression over genotypes. Significance levels are given separately for each genotypic change and for the entire effect of the SNP with both the one and two degrees of freedom tests.

TABLE 4. Haplotypes in LD-Block 2 of the *KCNH2* Gene

	rs2968864	rs2968863	rs2907948	rs2968853	rs1547958	rs3815459	rs1805123 (K897T)	rs1137617 (Y652Y)	rs1805121 (L564L)	rs1805120 (F513F)	rs740952 (I489I)	rs3807376	rs3778874	rs4725386	haplotype frequency
haplotype h1	A	G	C	A	G	G	A	T	A	C	C	A	C	G	0.344
haplotype h2	G	A	T	G	A	G	C	C	A	C	C	A	C	A	0.205
haplotype h3	A	G	C	G	G	A	A	C	G	T	T	G	T	G	0.195
haplotype h4	A	G	C	G	G	G	A	C	G	C	C	A	C	G	0.096

Four major haplotypes with frequencies above 0.05 exist in that block of 60 kb size. Haplotype h2 is tagged by SNPs KCNH2-K897T, rs2968864, rs2968863, rs2907948, rs1547958, and rs4725386. Haplotype h3 is tagged by SNPs rs3815459, rs1805120 (KCNH2-F513F), rs740952 (KCNH2-I489I), rs3807376, and rs3778874.

Combined and Categorical Association Analysis

The comparison of multivariate linear regression analysis of both QTc_RAS and QT including covariates demonstrated highly similar significance levels from both methods and independence of SNPs' effects enabling combined association analysis (Table 5; supplemental Table II). Analysis of the intragenic KCNH2 variants K897T and rs3815459 against all other haplotypes of that block revealed an increase of QTc_RAS mean values among the six genotype groups from 415.0 to 421.1 ms (0.52% of variance; $P=0.0002$; Table 6a; Figure 2b). The combined intergenic analysis of the KCNH2-K897T and the KCNQ1-rs757092 variants showed an increase of QTc_RAS mean values among the nine genotype groups from 412.9 to 421.2 ms (0.74% of variance; $P<0.00005$; Table 6b; Figure 2c).

Five of six genotypic changes of the three confirmed SNPs KCNQ1-rs757092, KCNH2-rs1805123, and rs3815459 were independently significant ($P<0.05$; Table 5). Individuals harboring the maximum possible number of five QT-prolonging alleles had on average a 10.5 ms longer QTc_RAS than individuals that had no QT-prolonging allele (0.95% of variance; $P<0.00005$) (Table 6c). When the genotypic change KCNE1-rs727957(aa) was included, a +14.3 ms increase was observed (1.13% of variance; $P<0.00005$)

Categorical analysis of individuals with extreme QTc_RAS values for all SNPs genotyped in the total sample detected significant effects in 2 of the 4 associated SNPs in 200 individuals from the extremes and in 3 of the 4 associated SNPs in 600 individuals from the extremes (supplemental Table IV). After adjustment for multiple testing, categorical analysis results were only significant for KCNQ1-rs707592 in the analysis of 600 individuals.

Discussion

Covariate Correction of QT

The linear correction factors for heart rate we determined were well in agreement with published ones.[22] The comparison between a formula correction and a multivariate linear regression model of QT for detecting SNP association (Table 5; supplemental Table II) supports the view that none of the two methods is superior.

Effects of Individual SNPs

The *KCNQ1* gene locus had previously been shown to influence the QT interval in a quantitative trait linkage study.[12] We could map this QTL to a 50-kb haplotype block in intron 1 in which only two major haplotypes existed. Of all identified effects, this was the most significant and most robust against testing in both genders. The 50-kb block does not contain any known or predicted exonic or regulatory sequences. Its high LD precludes further fine mapping in our population. The causal variant and its functional nature thus remain elusive at this point. Several association studies have demonstrated the nonsynonymous KCNH2-K897T variant to be significantly associated with repolarization, but results were conflicting. Our data show that in Caucasians of both sexes, the 897T-allele (block 2, h2) shortens the QT interval. The conflicting results may indicate that in other ethnic groups, the LD-relationship of the K897T variant may vary or that the smaller previous association studies were affected by increased type 1 error rates. We have identified another QT-modifying haplotype (h3) in the same block. In Caucasians, the presence of a common nonsynonymous SNP on h3 is unlikely, given the large number of individuals others and we have sequenced to detect mutations in the *KCNH2* gene. An effect of the two synonymous SNPs I489I and F513F

TABLE 5. Multivariate Linear Regression Models of QTc_RAS

	Total sample (n=3327)				Males (n=1620)				Females (n=1707)			
	Δ QTc_RAS (Aa)	Δ QTc_RAS (aa)	*P* (Aa)	*P* (aa)	Δ QTc_RAS (Aa)	Δ QTc_RAS (aa)	*P* (Aa)	*P* (aa)	Δ QTc_RAS (Aa)	Δ QTc_RAS (aa)	*P* (Aa)	*P* (aa)
KCNQ1-rs757092	+1.9ms	+3.0ms	0.002	0.001	+1.1ms	+2.7ms	0.261	0.053	+2.7ms	+3.4ms	0.001	0.006
KCNH2-rs1805123	−1.8ms	−2.9ms	0.005	0.023	−1.3ms	−2.6ms	0.190	0.175	−2.4ms	−3.3ms	0.005	0.055
KCNH2-rs3815459	+1.4ms	+2.9ms	0.035	0.054	+2.1ms	+2.9ms	0.038	0.189	+0.7ms	+2.9ms	0.397	0.159
KCNE1-rs727957	+1.2ms	+4.2ms	0.068	0.011	+1.6ms	+6.3ms	0.095	0.011	+0.7ms	+2.0ms	0.406	0.359

In the multivariate linear regression analysis of QTc_RAS in the total sample, *P* values represent the additive significance contributed by each genotypic change. Six out of eight genotypic changes were significantly associated; five of them were from SNPs confirmed by our two-step design. None of the other SNPs genotyped in the total sample contributed significantly.

TABLE 6A. Analysis of Combined Effects of SNPs

	KCNH2-rs3815459		
KCNH2-K897T (rs1805123)	GG	GA	AA
AA (897KK)	417.7±17.4	419.2±16.8	421.1±19.0
	1072 (hn/hn)	797 (h3/hn)	158 (h3/h3)
AC (897KT)	416.4±16.9	417.0±18.5	—
	937 (h2/hn)	322 (h3/h2)	
CC (897TT)	415.0±16.7	—	—
	212 (h2/h2)		

Combined intragenic effect of the KCNH2-K897T (h2) and rs3815459 (h3) ($D' > 0.99$, $r^2 = 0.08$) in haplotype block 2 of the *KCNH2* gene (Figure 1). hn denotes all haplotypes other than h2 and h3 (Table 4). For QTc_RAS mean, standard deviation and the no. of genotype carriers are given.

TABLE 6B. Analysis of Combined Effects of SNPs

	KCNQ1-rs757092		
KCNH2-K897T (rs1805123)	AA	AG	GG
AA (897KK)	417.3±16.9	418.9±17.0	421.2±18.7
	793	944	297
AC (897KT)	415.4±17.0	417.6±17.2	416.5±18.5
	489	612	161
CC (897TT)	412.9±13.1	416.3±17.6	417.1±21.5
	85	104	27

Combined intergenic effect of two SNPs KCNH2-K897T and rs757092 (KCNQ1-intron 1) on QTc_RAS. SNPs are not in LD ($D' < 0.01$, $r^2 < 0.01$). Rare allele KCNH2–897T shortens QTc_RAS, whereas the rare allele of rs757092 (G) prolongs QTc_RAS.

TABLE 6C. Analysis of Combined Effects of SNPs

QT-Prolongation Score	QTc_RAS±SD	From Total Sample (n=3966), n
0	412.7±13.4	79
1	415.5±16.9	462
2	416.6±16.9	1021
3	418.3±17.8	1132
4	419.3±16.9	641
5	423.2±19.4	135

Effect of the five genotypic changes significant in the multivariate regression analysis from the three confirmed SNPs KCNQ1-rs757092 (Aa, aa), KCNH2-K897T (Aa, aa), and KCNH2-rs3815459 (Aa) was determined by a QT-prolongation score ($P < 0.00005$). For each score-class the average QTc_RAS, standard deviation and the no. of individuals are given.

might be causal as for both amino acids less common codons are present on h3 [I489I: ATC (0.48) > ATT (0.35); F513F: TTC (0.55) >TTT (0.45)].[31] In humans, codon usage has been shown to correlate with expression breadth, which covaries with expression levels[32] but convincing evidence for codon usage effects in humans and other higher organisms has not been demonstrated.

SNP KCNE1-rs727957 did not fulfill all our significance criteria but showed evidence for association in the combined analysis ($P = 0.0051$). It is located in the 5′ region 50 kb upstream, its haplotype block ending 20 kb upstream of the *KCNE1* gene. We argue that an independent replication of this SNP's effect should be conducted before it is considered a significant QTL.

Sliding window haplotype analysis did not reveal additional associations or improve significance levels (data not shown). For the effect in KCNQ1-intron 1 this result is intuitive, as most of the associated haplotype block's diversity can be captured by typing only a single SNP. Using the information from the International HapMap Project, currently providing data at an average coverage of 1 SNP per 3.8 kb,[19] will aid the capturing of relevant haplotype diversity in future studies.

Combined Effects at Several SNP Loci

We have observed two kinds of combined QTL effects on the QT interval. SNPs in high LD tagging different haplotypes in one block had opposite additive effects on QTc_RAS as seen in the *KCNH2* gene. Similarly, SNPs in complete linkage equilibrium also exerted additive effects as seen between the *KCNQ1* and *KCNH2* genes. The fact that these three gene variants, although together only explaining 0.95% of trait variance, were associated to a monotonous rise in average QTc_RAS of up to 10.5 ms, supports their concerted mode of action irrespective whether they are in LD or not.

Gender Effects

Previous publications of the KCNH2-K897T variant's association to the QT interval had noted its more pronounced effect in females. We have confirmed and extended this finding, as especially marker KCNE1-rs727957 and to a lesser extent also KCNQ1-rs757092 and KCNH2-rs3815459 showed gender-dependent association. This underscores the importance of considering gender as a potent confounder variable when designing complex trait genotype-phenotype association studies.

Categorical Analysis of the QT Interval

A categorical analysis in the confirmation step using only 200 individuals with extreme QTc_RAS values would only have confirmed KCNQ1-rs757092 ($P = 0.002$, OR=1.92) and KCNH2-K897T ($P = 0.004$, OR=0.44) (supplemental Table IV). Notably, these effects were the most significant ones from the quantitative association analysis. Using a larger

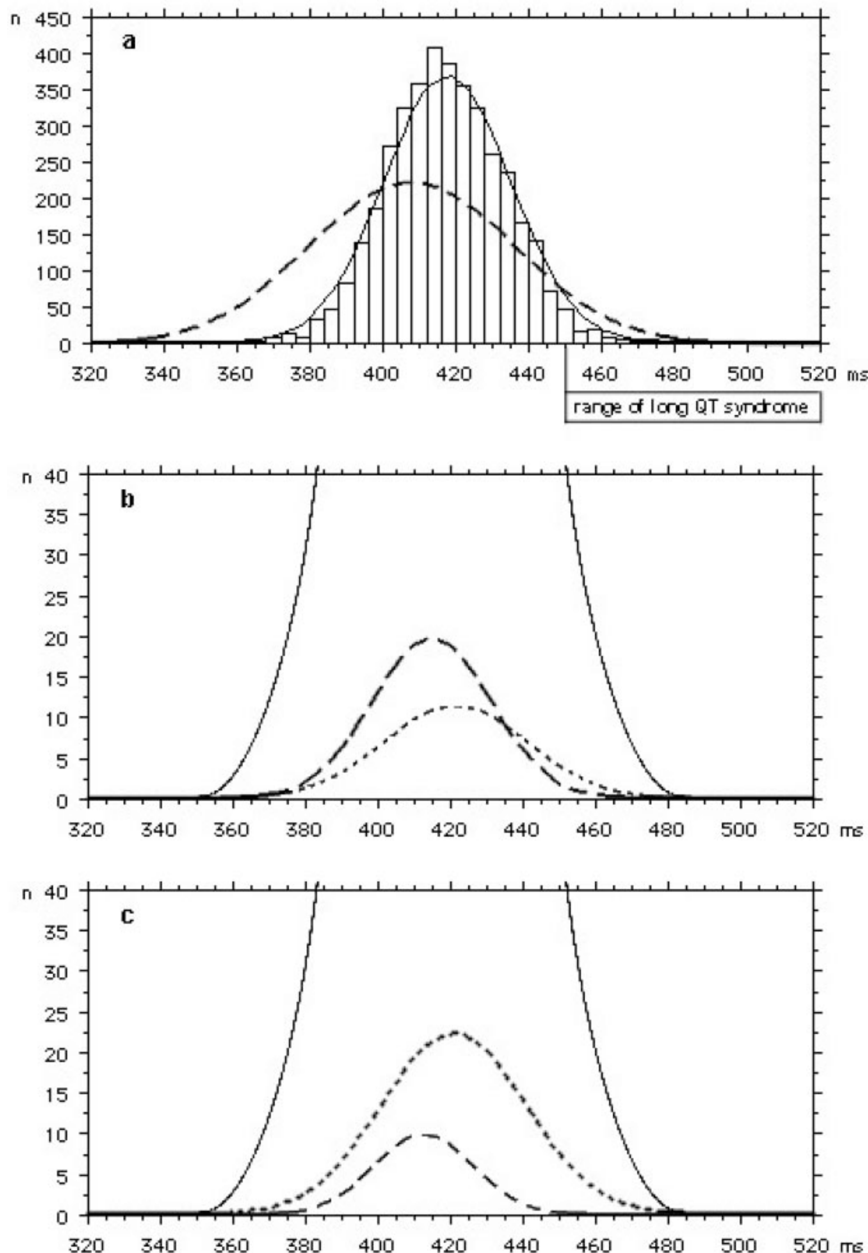

Figure 2. Normal distribution of QT and QTc_RAS. a, Distribution of QT (dashed line) and QTc_RAS (solid line, with bars) in the total sample (n=3966). QTc values longer than 450 ms are typically suggestive of long QT syndrome. Mean of QTc_RAS is shifted to higher values compared with QT due to correction to a 60-year-old male. b, Combined effect of the two SNPs KCNH2-K897T and rs3815459 on QTc_RAS in the total sample (see Table 6a). Individuals with 897TT/GG genotype (n=212, dashed) have the shortest, and individuals with 897KK/AA genotype (n=158, dotted) have the longest QTc_RAS. c, Combined effect on QTc_RAS of the two SNPs KCNH2-K897T and rs757092 (KCNQ1-intron 1) (see Table 6b). Individuals with 897TT/AA genotype (n=85, dashed) have the shortest, and individuals with 897KK/GG genotype (n=297, dotted) have the longest QTc_RAS.

sample (n=600) also KCNH2-rs3815459 (P=0.008, OR=1.47) would have been confirmed. Categorical confirmation analysis thus can be considered in similar projects if a focus on the strongest effects at significantly reduced cost is desirable.

Implications for Future Investigations

Although the overall heritability of the QT interval is high, all gene variants identified in this study are only minor quantitative trait loci each explaining less than 1% of trait variance. This finding is in common with the view that important physiological mechanisms are unlikely to tolerate large genetic variance at a single locus. The authors of an early heritability study on electrocardiographic traits already noted that these reflected critical biologic functions, which evolved to an evolutionary optimum and the attainment of this optimum would necessarily tend to eliminate interindividual differences.[33]

We show that in a large sample of thoroughly phenotyped individuals even minor QTLs can be detected. The population-representative recruiting of individuals from one geographic area with limited recent immigration was helpful to this aim, as complex population genealogies can confound association signals. In two of the known monogenic long QT disease genes *KCNQ1* and *KCNH2*, the common disease or in this case common phenotypes/common variants hypothesis holds true. The confirmation of this hypothesis for cardiac rhythm phenotypes appears a prerequisite to investigate whether common gene variants also influence cardiac patients' predisposition toward arrhythmias.

Common intronic gene variants may influence repolarization to a similar extent as common nonsynonymous exonic variants. Future fine mapping studies of complex and quan-

titative trait loci should avoid to focus on exonic effects, but apply SNP coverage based on LD.

Besides studies of monogenic arrhythmogenic diseases and functional studies of recombinant cardiac ion channels, the genome-wide investigation of heritable surface ECG signatures may provide a valuable third route toward the identification of novel genes involved in cardiac electrophysiology that up to now went undetected by other methods.

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PAPER II

A common genetic variant in the *NOS1* regulator *NOS1AP* modulates cardiac repolarization

Dan E Arking[1,2,16], Arne Pfeufer[3,4,16], Wendy Post[2,5], W H Linda Kao[5], Christopher Newton-Cheh[6,7,8], Morna Ikeda[1], Kristen West[1], Carl Kashuk[1], Mahmut Akyol[3,4], Siegfried Perz[9], Shapour Jalilzadeh[3,4], Thomas Illig[10], Christian Gieger[10], Chao-Yu Guo[6,11], Martin G Larson[6,11], H Erich Wichmann[10,12], Eduardo Marbán[2], Christopher J O'Donnell[6,7,8], Joel N Hirschhorn[7,13,14], Stefan Kääb[15], Peter M Spooner[2], Thomas Meitinger[3,4] & Aravinda Chakravarti[1]

Extremes of the electrocardiographic QT interval, a measure of cardiac repolarization, are associated with increased cardiovascular mortality. We identified a common genetic variant influencing this quantitative trait through a genome-wide association study on 200 subjects at the extremes of a population-based QT interval distribution of 3,966 subjects from the KORA cohort in Germany, with follow-up screening of selected markers in the remainder of the cohort. We validated statistically significant findings in two independent samples of 2,646 subjects from Germany and 1,805 subjects from the US Framingham Heart Study. This genome-wide study identified *NOS1AP* (*CAPON*), a regulator of neuronal nitric oxide synthase, as a new target that modulates cardiac repolarization. Approximately 60% of subjects of European ancestry carry at least one minor allele of the *NOS1AP* genetic variant, which explains up to 1.5% of QT interval variation.

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The electrocardiographic (ECG) QT interval, a measure of cardiac repolarization, is a genetically influenced quantitative trait with ~30% heritability[1–3]. The QT interval has considerable medical relevance, as both high and low values are associated with increased risk of cardiovascular morbidity and mortality[4–10]. Moreover, extremely long or short QT intervals occur in a heterogeneous collection of mendelian disorders (long-QT syndrome (LQTS) and short-QT syndrome (SQTS)) and are usually due to rare, highly penetrant mutations in ion channel genes and are associated with increased risk of sudden cardiac death (SCD)[11].

Despite major advances in understanding the etiology of cardiovascular disease and concomitant reduction in cardiovascular disease–related mortality, the incidence of SCD has remained largely unchanged. Familial clustering of SCD has been observed, but the vast majority of subjects who are at risk for SCD do not have mutations in the known genes for LQTS or SQTS. To date, common variants in these genes have been associated with disease in only a few studies in selected populations[12,13]. Thus, the major genetic mechanisms by which an altered QT interval and other arrhythmogenic conditions may contribute to SCD risk remain incompletely characterized. To identify previously unknown genetic pathways that may modulate SCD risk, we examined the QT interval directly, as opposed to the SCD phenotype. Several reasons prompted this choice: (i) the QT interval is a genetically modulated intermediate trait for SCD with modest heritability; thus, genes affecting the QT interval have been implicated in the pathogenesis of SCD; (ii) the QT interval is a quantitative trait that can be accurately and reliably measured in large samples from standard ECG recordings[14]; (iii) quantitative rather than qualitative traits are more powerful for genetic analysis and (iv) large populations of uniformly ascertained individuals with SCD are presently unavailable. Notably, the QT interval has been examined in large numbers of healthy volunteers so that findings can be replicated in population-based settings.

To maximize our chance of identifying QT interval genetic factors, we focused on discovering alleles with a large genetic effect or with a genetic effect that could be amplified by study design. Some alleles of large effect have been identified for categorical traits[15–18]. For a quantitative trait such as the QT interval, we examined the extremes

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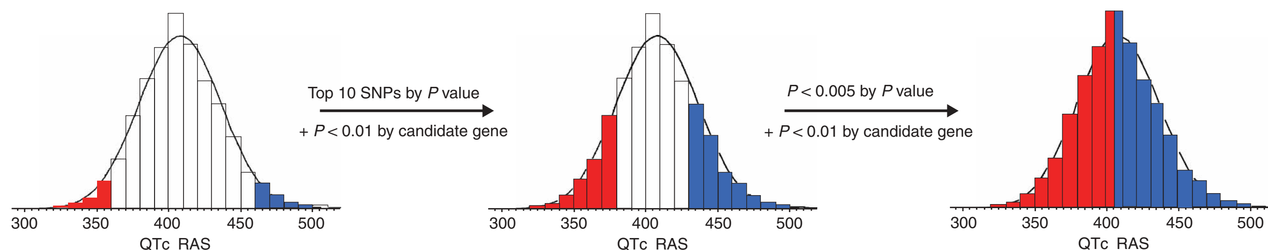

Figure 1 Genome-wide association study of the QT interval. In stage I, genome-wide genotyping was performed on 100 females from each extreme of QTc_RAS. Two analysis approaches were taken: (i) a genome-wide scan (above the arrows) and (ii) a candidate gene scan (below the arrows), with significance criteria indicated for the following stage. In stage II, an additional 200 females from each extreme were genotyped, and the combined stage I and II samples were analyzed. In stage III, all samples not typed in stages I and II were genotyped, including both males and females, and both combined and stratified analyses were performed.

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of the distribution, as these can be expected to be enriched for the alternate alleles at many quantitative trait loci (QTLs), thereby creating a large detectable effect by design and maximizing the statistical power for detecting such alleles[19,20]. These models assume that the alleles underlying QTLs are biallelic, common, additive and of small effect but cannot exclude the existence of numerous, rare variants of large effect at the extremes as well. Currently, the impact of rare variants can be comprehensively tested only by DNA sequencing of known candidate genes, as recently shown for low high-density lipoprotein (HDL) cholesterol[21]. However, common variants can now be efficiently tested by a genome-wide association study, with the added advantage that new pathways can be uncovered.

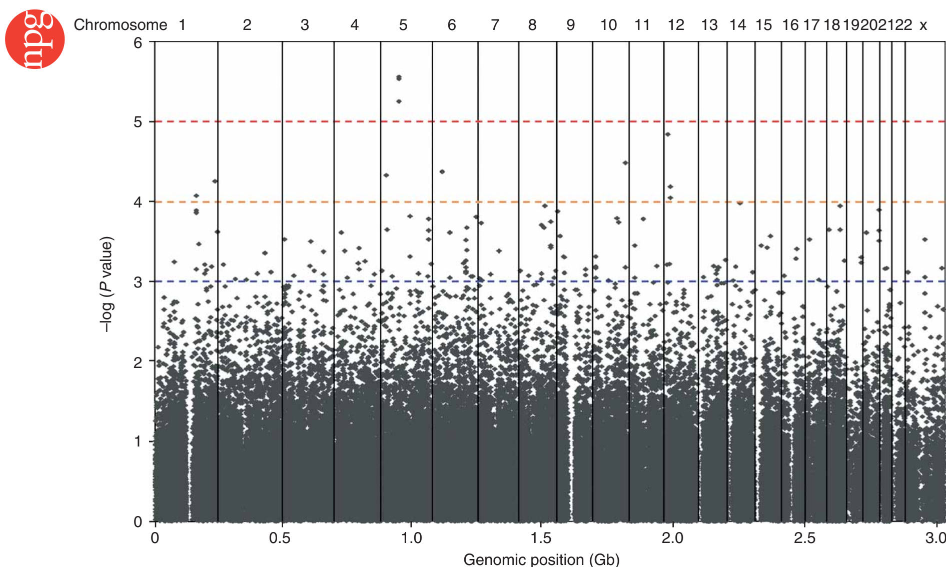

Figure 2 Genome-wide significance of QTc_RAS. The analysis compared 100 females from each extreme of QTc_RAS (stage I). The *x*-axis is genomic position, and the *y*-axis is the negative base-10 logarithm of the *P* value. None of the SNPs showed genome-wide significance, but the top ten positives had $P < 10^{-4}$.

A multistage design for genome-wide association

Our genetic experiment had three key features. First, we studied subjects from a population-based survey of volunteers aged 25–75 years from Germany ($n = 3{,}966$ from the KORA S4 survey[22]). Second, we performed the genome-wide analysis only on a subset of the 2,001 women from this survey. This strategy was designed to avoid the heterogeneity due to sex in the QT interval[22,23] and because women have a lower prevalence of cardiovascular disease, which could confound QT interval measurement. Third, using a three-stage study design, we attempted to minimize false positive findings yet maximize power and efficiency by examining samples with phenotypic means of decreasing deviations from the population average but with increasing sample size, for SNPs significantly associated in the previous stage (**Fig. 1**). Multiple-stage designs have been shown to be both powerful and cost-efficient in such settings[24]. Indeed, simulation studies show that in our specific design, the statistical power to identify a variant that explains 5% of QT interval variation exceeds 40% and 87% for alleles with frequency of 20% and 40%, respectively; for a variant that explains 2% of QT interval variation, the corresponding power is 7% and 22%, respectively, assuming that we are typing either the functional variant or a marker in complete linkage disequilibrium (LD). The power estimates are for each QTL that might exist, and, thus, under the assumption of multiple contributing variants, the power to identify any QTLs of such magnitude is roughly proportional to the number of QTLs. Fourth, to reduce false-positive reporting, we performed replication studies in two population-based samples of European ancestry.

In stage I, we selected 100 women from each extreme of the QT interval distribution in the KORA S4 cohort, corrected for the covariates known to influence QT interval: heart rate, age and sex (termed 'QTc_RAS').

This selection corresponds to QT intervals below the 7.5 percentile (385.7 ± 7.7 ms) or above the 92.5 percentile (444.8 ± 3.6 ms). These samples were genotyped using Affymetrix Centurion arrays containing probes for ~115,000 SNPs, with an average heterozygosity of 0.30 and with average spacing of 23.6 kb.

Each of the 88,500 SNPs that passed quality criteria and was polymorphic (see Methods) was tested individually for association with QTc_RAS using a truncated measure test[25,26], under the recessive, dominant and additive genetic models, with the most significant value retained. No single SNP in stage I reached genome-wide significance at the $\alpha = 5.6 \times 10^{-7}$ level, based on permutation testing. Nevertheless, for follow-up in stage II, we selected the best SNP from each of the ten most significant loci, all of which showed nominal P values $<10^{-4}$ (**Fig. 2**; **Supplementary Table 1** online). Approximately nine false positives were expected by chance, so further follow-up was critical. Statistical tests of population stratification confirmed that the observed allele frequency differences between the samples at the extremes were not from inherent population substructure ($F_{ST} = 0.0009 \pm 0.0759$ across the genome).

Based on our current understanding of the biology of cardiac repolarization, we also selected *a priori* 45 candidate genes that have been implicated in SQTS or LQTS or cardiac cellular electrophysiology or that are homologous to the selected genes (**Supplementary Table 2** online) and that each had at least one SNP represented on the array within 10 kb of its 5′ or 3′ UTR. We used a less stringent significance threshold to choose candidate gene SNPs for follow-up in stage II ($P < 0.01$), as their prior probability of involvement was higher than that for anonymous markers.

Each SNP selected for follow-up was supplemented with a partially correlated 5′ and 3′ flanking SNP (see Methods). As we were unlikely to have identified the causal SNPs in stage I, the addition of flanking SNPs in the neighborhood of a positive signal could identify a SNP more strongly associated with QTc_RAS and thus more highly correlated with a causal variant.

In stage II, we included an additional 400 females having QTc_RAS below the 15th ($n = 200$) or above the 85th percentile ($n = 200$). All 600 women who had their QTc_RAS means ~2 s.d. apart and a mean trait difference of 45.5 ms were genotyped for SNPs that passed stage I criteria and flanking SNPs. The second stage should lead to fewer false positives, because under the null hypothesis of no association, adding samples will decrease significance and should lead to greater power, as we used a sample size three times larger than the initial screening set while still maintaining a sample enriched with subjects from either tail of the phenotypic distribution. In stage III, we genotyped anonymous SNPs significant at $P < 0.005$ and candidate gene SNPs with $P < 0.01$ in stage II in the remaining 3,366 subjects of both genders. Importantly, we performed significance tests separately on the men and women specific to stage III (that is, excluding the 600 females analyzed in stages I and II), allowing stage III to serve as a validation study for stages I and II. This replication approach has comparable power to the joint analysis proposed by others[27], given the smaller sample size of the first two stages compared with the third stage and the small proportion of markers chosen for follow-up in the third stage.

Genome-wide analysis identifies *NOS1AP*

Twelve of 57 SNPs (rs2282428 was in common among the anonymous and candidate gene SNPs) were significant in stage II, representing seven SNPs from four anonymous loci studied at $P < 0.005$ (**Supplementary Table 3** online) and five SNPs from four candidate gene loci at $P < 0.01$ (**Supplementary Table 4** online). Two SNPs,

Table 1 Genetic effects of QT interval–associated polymorphisms in 3,966 individuals from the KORA S4 sample

				AA		Aa		aa			
Gene or locus	SNP	Gender	MAF	n	QTc_RAS	n	QTc_RAS	n	QTc_RAS	P	P_{adj}
Genome-wide association											
NOS1AP	rs10494366	Both	0.36	1,542	–2.2 (0.4)	1,771	1.3 (0.4)	491	2.7 (0.8)	**$<10^{-10}$**	**$<10^{-7}$**
		Male		758	–1.6 (0.6)	867	1.5 (0.6)	257	2.9 (1.2)	**0.0001**	**0.0001**
		Female		784	–2.8 (0.6)	904	1.2 (0.6)	234	2.5 (1.0)	**$<10^{-7}$**	**0.021**
QTc_5.3	rs1559578	Both	0.36	1,542	0.9 (0.4)	1,775	–0.1 (0.4)	504	–2.4 (0.8)	**0.001**	**0.004**
		Male		762	1.4 (0.7)	899	0.1 (0.6)	243	–2.6 (1.2)	**0.007**	**0.007**
		Female		780	0.3 (0.6)	876	–0.3 (0.6)	261	–2.3 (1.0)	0.067	0.47
FGFR2	rs6585682	Both	0.46	1,102	0.3 (0.5)	1,820	0.1 (0.4)	793	–0.2 (0.7)	0.78	0.93
		Male		553	0.1 (0.8)	898	0.1 (0.6)	385	1.8 (1.0)	0.20	0.20
		Female		549	0.6 (0.6)	922	0.1 (0.5)	408	–2.0 (0.9)	**0.03**	0.46
QTc_14.1	rs7146384	Both	0.33	1,702	0.3 (0.4)	1,603	0.5 (0.4)	416	–0.4 (0.8)	0.21	0.52
		Male		838	–0.2 (0.6)	788	0.9 (0.7)	213	0.1 (1.2)	0.69	0.69
		Female		864	0.8 (0.6)	815	0.1 (0.6)	203	–0.9 (1.1)	0.20	0.052
Candidate genes											
KCNK1	rs2282428	Both	0.34	1,652	–0.5 (0.4)	1,753	0.4 (0.4)	423	0.6 (0.8)	0.20	0.41
		Male		838	0.7 (0.6)	847	0.3 (0.6)	211	–0.6 (1.0)	0.56	0.56
		Female		814	–1.7 (0.6)	906	0.6 (0.5)	212	1.9 (1.1)	**0.002**	**0.005**
ITPR1	rs3804999	Both	0.28	1,994	0.6 (0.4)	1,523	–0.6 (0.5)	307	0.0 (0.9)	0.12	0.42
		Male		1,003	0.5 (0.6)	758	0.3 (0.7)	145	0.1 (1.3)	0.91	0.91
		Female		991	0.6 (0.5)	765	–1.5 (0.6)	162	–0.1 (1.3)	**0.03**	0.32
CACNA2D1	rs7341478	Both	0.27	2,027	–0.6 (0.3)	1,412	0.3 (0.5)	283	2.7 (1.0)	**0.009**	**0.024**
		Male		977	–0.5 (0.6)	715	1.3 (0.7)	154	2.4 (1.3)	**0.037**	**0.037**
		Female		1,050	–0.6 (0.5)	697	–0.6 (0.6)	129	3.0 (1.4)	**0.034**	0.72

QTc_RAS values are displayed as the difference (s.e.m.) from the population mean (417.6 ms). P values are reported for the best genetic model adjusted by permutation analysis for the multiple genetic models tested. P indicates significance for all 3,966 samples. P_{adj} indicates significance in the 3,366 stage III samples only (excluding the women from the extremes of the QT interval). MAF: minor allele frequency in the sex-pooled analysis. Boldface indicates statistically significant results.

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Table 2 Genetic effects of stage III SNPs with QT interval in 2,646 individuals from the KORA F3 replication sample

				AA		Aa		aa		
Gene or locus	SNP	Gender	MAF	*n*	QTc_RAS	*n*	QTc_RAS	*n*	QTc_RAS	*P*
Genome-wide association										
NOS1AP	rs10494366	Both	0.36	1,085	–2.6 (0.5)	1,170	0.7 (0.6)	345	5.3 (1.1)	**$<10^{-11}$**
		Male		535	–2.6 (0.8)	562	0.2 (0.8)	171	6.9 (1.5)	**$<10^{-7}$**
		Female		550	–2.6 (0.7)	608	1.1 (0.8)	174	3.8 (1.5)	**$<10^{-5}$**
QTc_5.3	rs1559578	Both	0.38	989	–0.3 (0.6)	1,161	0.2 (0.6)	376	–0.6 (1.0)	0.82
		Male		476	–0.3 (0.9)	570	0.0 (0.8)	180	–0.4 (1.5)	0.98
		Female		513	–0.3 (0.8)	591	0.4 (0.8)	196	–0.7 (1.3)	0.83
FGFR2	rs6585682	Both	0.46	777	–0.8 (0.7)	1,264	0.6 (0.5)	558	–0.5 (0.7)	0.37
		Male		379	–1.7 (0.9)	617	1.1 (0.8)	273	–0.4 (1.1)	0.12
		Female		398	0.1 (0.9)	647	0.1 (0.7)	285	–0.6 (1.0)	0.84
QTc_14.1	rs7146384	Both	0.33	1,172	–0.4 (0.5)	1,135	0.5 (0.6)	286	–1.0 (1.1)	0.65
		Male		581	–0.8 (0.8)	547	1.0 (0.8)	136	–2.1 (1.5)	0.38
		Female		291	0.0 (0.7)	588	0.0 (0.8)	150	0.1 (1.6)	1.00
Candidate genes										
KCNK1	rs2282428	Both	0.35	1,091	0.6 (0.6)	1,179	–0.6 (0.5)	330	–0.4 (1.0)	0.26
		Male		527	0.8 (0.8)	576	–0.8 (0.8)	166	–0.5 (1.5)	0.29
		Female		564	0.4 (0.8)	603	–0.3 (0.7)	164	–0.3 (1.3)	0.73
ITPR1	rs3804999	Both	0.29	1,321	–0.1 (0.5)	1,076	–0.2 (0.6)	211	0.8 (1.4)	0.72
		Male		647	0.6 (0.7)	507	–1.1 (0.9)	118	0.9 (2.0)	0.42
		Female		674	–0.7 (0.7)	569	0.5 (0.8)	93	0.8 (2.1)	0.37
CACNA2D1	rs7341478	Both	0.28	1,336	0.7 (0.5)	1,045	–1.1 (0.6)	214	–0.5 (1.3)	**0.040**
		Male		670	1.1 (0.8)	509	–1.8 (0.8)	87	0.4 (2.0)	**0.035**
		Female		666	0.4 (0.7)	536	–0.4 (0.8)	127	–1.1 (1.7)	0.54

QTc_RAS values are displayed as the difference (s.e.m.) from the population mean (405.0 ms). *P* values are reported for the best genetic model adjusted by permutation analysis for the multiple genetic models tested. MAF: minor allele frequency in the sex-pooled analysis. Boldface indicates statistically significant results.

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rs945713 (*NOS1AP*) and rs7341478 (*CACNA2D1*), showed increased significance in the stage II analysis compared with stage I. Notably, rs10494366, the 5′ flanking SNP of rs945713, achieved genome-wide significance ($P < 2.57 \times 10^{-8}$) with a *P* value three orders of magnitude lower than that for rs945713, the sentinel SNP on the Centurion array, supporting our rationale for incorporating flanking SNPs in stage II (**Supplementary Table 3**). From each of the eight loci (four anonymous and four candidate genes), we selected the most significant SNP from stage II for validation in stage III (**Supplementary Tables 3** and **4**); seven of these SNPs, all in Hardy-Weinberg equilibrium, were successfully genotyped in the remainder of the KORA S4 sample.

We identified three loci based on sex-pooled analyses of stage III samples with nominal significance ($P < 0.05$; **Table 1**): these loci correspond to rs10494366 at *NOS1AP* ($P < 10^{-7}$), rs1559578 at *QTc_5.3* ($P < 0.004$) and rs7341478 at *CACNA2D1* ($P < 0.024$). *NOS1AP* (*CAPON*) is the C-terminal PDZ domain ligand to neuronal nitric oxide synthase (nNOS, encoded by the *NOS1* gene)[28] and affects NMDA receptor–gated calcium influx. It has not been previously suspected to have a role in cardiac repolarization. However, using RT-PCR, we found *NOS1AP* expression in human left ventricular heart tissue (data not shown). *CACNA2D1* encodes an L-type voltage-dependent calcium channel regulatory subunit expressed in the heart[29,30]. The third locus, *QTc_5.3*, does not correspond to a known gene, but rather to a GeneScan[31] prediction, so its potential biological relationship to the QT interval is unknown. None of the four remaining loci in stage III was significant in the overall sample, but *KCNK1* ($P \sim 0.005$) showed significant effects in females only. *KCNK1* (TWIK1) is the weakly inward-rectifying potassium channel subfamily K member 1 and may be involved in the control of background potassium membrane conductance. The gene is transcribed in many tissues but is particularly highly expressed in the brain and heart[32]. However, given the number of hypotheses tested (seven SNPs, sex-pooled and sex-specific), only the *NOS1AP* SNP achieves genome-wide significance after correcting for multiple testing. This finding was not dependent on the genetic model, with a similar result obtained from model-free analysis ($P < 10^{-5}$).

Validation in the KORA F3 cohort

We genotyped a separate sample of 2,646 subjects from the KORA F3 for the seven SNPs from stage III (**Table 2**). Power estimation by permutation testing revealed that for the non–sex specific effects, we had >95% power to replicate a true finding from stage III and >85% power to replicate the sex-specific effect seen in KCNK1. rs10494366 in *NOS1AP* was highly significant in the replication cohort even after adjusting for the number of SNPs tested ($P < 10^{-10}$), but no SNP from the other six loci was significant. The nominal significance observed for rs7341478 in CACNA2D1 was actually for an effect in the direction opposite from that observed in the S4 samples, suggesting a false positive association. Nevertheless, these results clearly demonstrate that the multistage genome-wide approach was able to unequivocally identify at least one common variant of a gene previously unrecognized as being associated with cardiac repolarization.

The average genetic effect (δ), measured as the difference in means of QTc_RAS between the two homozygotes, is 4.9 ms for *NOS1AP* in the total S4 sample and 7.9 ms in the F3 sample, with *NOS1AP* accounting for 1.2% and 1.9% of the variance, respectively. The minor allele frequency was the same in both populations (36%), and genetic effects were observed in both males and females.

Table 3 Genetic effects of stage III SNPs with QT interval in 1,805 individuals from the FHS replication sample

Gene or locus	SNP	Gender	MAF	AA *n*	AA ΔQT	Aa *n*	Aa ΔQT	aa *n*	aa ΔQT	*P*
Genome-wide association										
NOS1AP	rs10494366	Both	0.39	663	–1.3 (0.6)	864	0.1 (0.6)	257	2.7 (1.0)	**0.004**
		Male		309	–1.5 (0.9)	440	1.0 (0.8)	119	–0.4 (1.5)	0.17
		Female		354	–1.1 (0.9)	424	–0.8 (0.8)	138	5.4 (1.4)	**0.0001**
QTc_5.3	rs1559578	Both	0.36	740	–0.2 (0.6)	794	0.5 (0.6)	229	–0.7 (1.1)	0.84
		Male		349	0.5 (0.9)	380	0.5 (0.8)	120	–2.2 (1.5)	0.27
		Female		391	–0.9 (0.9)	414	0.6 (0.8)	109	–0.9 (1.6)	0.43
FGFR2	rs6585682	Both	0.47	509	–1.5 (0.7)	870	0.7 (0.6)	390	–0.2 (0.9)	0.09
		Male		257	–1.9 (1.0)	425	1.3 (0.8)	182	–0.2 (1.2)	0.07
		Female		252	–1.0 (1.1)	445	0.2 (0.8)	208	–0.2 (1.2)	0.80
QTc_14.1	rs7146384	Both	0.31	830	0.2 (0.6)	724	0.1 (0.6)	214	–1.1 (1.1)	0.68
		Male		414	0.1 (0.8)	348	0.2 (0.9)	92	–0.5 (1.7)	0.98
		Female		416	0.2 (0.8)	376	0.1 (0.9)	122	–1.5 (1.5)	0.67
Candidate genes										
KCNK1	rs2282428	Both	0.33	188	–0.1 (0.6)	807	0.2 (0.6)	188	–0.5 (1.2)	0.97
		Male		370	0.6 (0.8)	405	–0.8 (0.8)	92	0.7 (1.7)	0.69
		Female		421	–0.7 (0.8)	402	1.1 (0.8)	96	–1.7 (1.7)	0.60
ITPR1	rs3804999	Both	0.28	906	–0.6 (0.6)	691	0.4 (0.6)	142	–0.1 (1.4)	0.60
		Male		442	–0.6 (0.8)	339	1.1 (0.9)	69	–1.3 (2.0)	0.60
		Female		464	–0.6 (0.8)	352	–0.3 (0.9)	73	1.0 (2.0)	0.87
CACNA2D1	rs7341478	Both	0.25	993	–0.1 (0.5)	659	–0.1 (0.7)	118	2.0 (1.5)	0.45
		Male		487	–0.3 (0.7)	311	–0.3 (0.9)	58	4.9 (2.1)	0.31
		Female		506	0.0 (0.8)	348	0.0 (0.9)	60	–0.9 (2.2)	0.97

Adjusted QT values are shown as the difference (s.e.m.) from the sex-pooled or sex-specific mean QT adjusted to age = mean, RR = 1,000 ms (sex-pooled, 394.5 ms, s.d. 16.7; men, 388.0 ms, s.d. 16.3; women, 402.3 ms, s.d. 16.9). Nominal *P* values for the best genetic model have been Bonferroni-adjusted for the models tested. MAF: minor allele frequency in the sex-pooled analysis. Boldface indicates statistically significant results.

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Validation in the Framingham Heart Study

We genotyped SNPs from the seven loci of stage III in 1,805 participants from the Framingham Heart Study (FHS), a population-based sample of predominantly European ancestry (**Table 3**). We confirmed the association of rs10494366 in *NOS1AP* with QT interval in the sex-pooled analysis (the prespecified primary test of replication as supported by the S4 and F3 results) with $P = 0.004$. The average genetic effect for QT interval adjusted for heart rate (RR interval), age and sex was 4.0 ms. In secondary analyses, the effect of rs10494366 on QT interval in FHS was stronger in women ($\delta = 6.5$ ms), with a more modest effect in the same direction in men ($\delta = 1.1$ ms). However, because findings in the KORA S4 and F3 samples did not show a clear difference by sex, the apparent differences in effect between men and women observed in the FHS sample may reflect chance statistical fluctuations, although a small influence of gender in the effect of the *NOS1AP* SNP cannot be excluded.

Fine mapping of the common variant at *NOS1AP*

To identify the underlying functional variant(s), we undertook fine-scale association mapping of the *NOS1AP* locus by genotyping 13 SNPs in the region in the 600 stage II samples; the *P* values for each SNP by genomic position are plotted in **Figure 3**. We found the strongest association in the 5′ upstream region of the *NOS1AP* gene, at rs4657139, although the entire region between the SNPs rs10494366 and rs2880058 (~120 kb) shows strong LD and, consequently, strong association with QT interval. Similar results were observed for a subset of SNPs typed in the entire KORA S4 cohort, including the sentinel SNP from the Affymetrix array (**Supplementary Table 5** online). The rapid drop in association between QT interval and SNPs further upstream (toward *OLFML2B*) strongly suggests that a functional variant in the *NOS1AP* gene is mediating the observed variation in QT interval. This observation is supported by reference genotyping in the HapMap CEU sample, which demonstrates strong correlation of SNPs in the *NOS1AP* gene with a significant drop in association 3′ of *OLFML2B*. To identify the mutational site, we sequenced all exons and, in addition, 13 noncoding conserved sequences presumed to have regulatory function (see Methods) localized to this 120-kb region, in ten subjects of each rs4657139 homozygous genotype. We did not find any missense mutations in the exons, but we identified three SNPs associated with QT interval (rs12096347, rs4656349, rs11579080) in the noncoding conserved sequences (**Fig. 3**), and functional analysis of these SNPs is a high priority. Thus, there is a high likelihood that the *NOS1AP* functional allele is in noncoding DNA. This observation is consistent with the increasing evidence that regulatory SNPs have a significant role in complex inheritance[15].

DISCUSSION

Previous attempts to dissect genetic contributions to the QT interval have focused on monogenic LQTS disease genes[22] or on family-based linkage studies of the quantitative trait[1,3]. The latter approach is well suited to uncovering rare alleles with large to moderate effects. Instead, we have used a genome-wide association study to uncover common polymorphisms of small effect that are capable of explaining a greater degree of the population QT interval variation.

We have identified *NOS1AP* as a gene that is significantly associated with QT interval variation in a general population of ~4,000 German adults and have replicated this finding in a second sample of ~2,700 adults from the same population and in a third sample of ~1,800

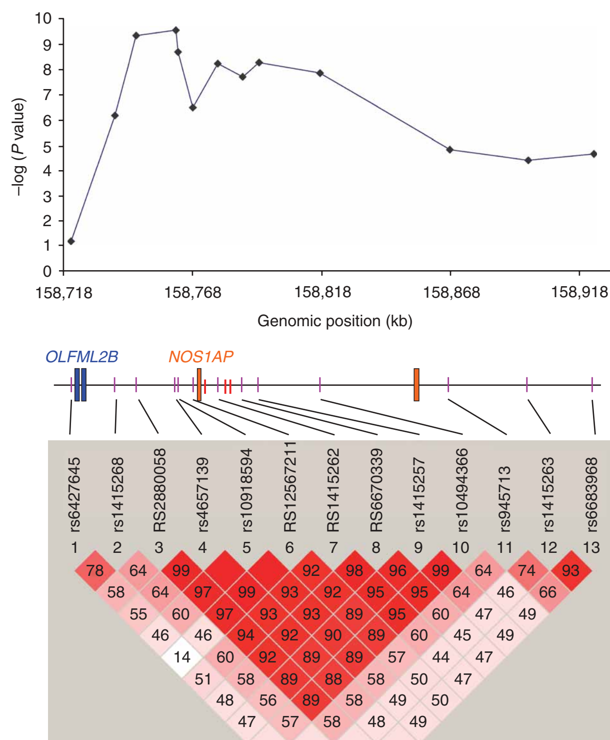

Figure 3 Fine mapping of the *NOS1AP* gene. The lower panel shows pairwise LD between SNPs at *NOS1AP*. The value within each diamond represents the pairwise correlation between SNPs (measured as D') defined by the top left and the top right sides of the diamond. Diamonds without a number correspond to $D' = 1$. Shading represents the magnitude and significance of pairwise LD, with a red-to-white gradient reflecting higher to lower LD values. *NOS1AP* exons 1 and 2 are shown in orange. The upper panel shows significance for each SNP, with genomic position on the *x*-axis and the negative base-10 logarithm of the *P* value on the *y*-axis, indicating that the most likely location of the underlying functional variant is in the 5′ region of *NOS1AP*. SNPs detected by sequencing conserved regions in individuals from the QT interval extremes are shown in red.

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American adults of European ancestry. Differences in effect size and statistical significance across the three populations could reflect methodological differences in measurement (see Methods) or statistical fluctuation around a common effect. SNPs at the other six loci were not confirmed in the replication samples.

These results emphasize that genome-wide association studies, which are not limited by our current understanding of cardiac repolarization biology, can be used to identify common variants that show previously unanticipated genetic associations. Indeed, the involvement of the *NOS1AP* gene in QT interval variation was unsuspected, yet it explains 1.5% of the variance in our combined sample of ∼6,600 German adults and 0.6% in the FHS sample. Although the variant identified explains a small fraction of the total variation in QT duration, the identification of a gene not previously known to be implicated in myocardial repolarization opens up a completely new area for QT interval biology and brings to attention drug targets that could be of benefit to SCD patients. *NOS1AP* is a regulator of neuronal nitric oxide synthase effected by forming a ternary complex with PSD95 (membrane-associated guanylate kinase[28]) and Dexras1 (member of the Ras family of small monomeric G proteins[33]). Notably, *NOS1* has been shown recently to have a role in cardiac contractility[34]. Consequently, nitric oxide signaling may be an important effector of cardiac repolarization and seems to have a role in balancing nitric oxide and superoxide production[35]. In addition, several cardiac and neuronal ion channel genes contain PDZ domains capable of binding NOS1AP[36–38]. Through such binding, direct modulation of channel activity may occur, or NOS1AP may displace other PDZ-binding regulators of channel expression or function.

Keeping in mind that the ultimate goal is a comprehensive genome-wide scan, it is important to ask how much of the genome our study has missed. The Phase I HapMap data suggests that an efficient set of 100,000 SNPs with minor allele frequency >5% would cover ∼70% of the genome for subjects of Northern European origin with an $r^2 \geq 0.8$ (ref. 39). The marker set we used has a smaller number of SNPs, and they were not chosen with particular regard to their linkage disequilibrium patterns to neighboring SNPs; thus, we believe that we have covered closer to 50% of the genome. Our scan also did not interrogate a number of gene loci known to be involved in regulating cardiac electrogenesis: for example, the depolarizing sodium channel SCN5A (**Supplementary Table 2**). Nevertheless, a few conclusions are warranted: (i) we did not find a major QTL (> 5% explained variance); (ii) the *NOS1AP* SNP explains a larger percentage of the variance than previous findings for the QT interval based on a candidate gene approach[22] and (iii) given that the heritability of QT interval is ∼30% (refs. 1–3), this would suggest that many more genes with small effects are likely to be involved. If this effect size holds true for other complex phenotypes, it would suggest that the vast majority of studies are substantially underpowered, and sample sizes will need to be much larger than those currently studied.

METHODS

Study population. The KORA S3 and S4 surveys are representative samples from the general population living in or near Augsburg, Germany and were conducted between 1994 and 2004. Consequent to informed consent, each of the surveys sampled subjects from ten strata according to sex (equal ratio) and age (range 25–75 years) with a minimum stratum size of >400 subjects. KORA procedures and samples have been previously described extensively[22,40]. Briefly, for sample S4, 4,261 probands were studied between 1999 and 2001, and for sample S3, 4,856 subjects were studied between 1994 and 1995. In 2003 and 2004, 2,974 participants from S3 returned for follow-up (KORA F3). For this analysis, we excluded subjects with atrial fibrillation, pacemaker implant and/or pregnancy. We included 3,966 subjects from S4 for the three-stage genome-wide study, and 2,646 subjects from F3 were used for an independent replication. All studies involving humans were performed according to the declarations of Helsinki and Somerset West and were approved by the local medical ethics committees in Germany and the US.

The FHS, the second replication sample, is a prospective epidemiologic study established in 1948 to evaluate potential risk factors for coronary heart disease. In 1971, 5,124 subjects were entered into the Framingham Offspring Study, including children or spouses of the children of the original cohort. We measured the QT interval in electrocardiograms (ECG) from offspring participants of the Framingham Heart Study examined between 1971 and 1975 who were free of atrial fibrillation or QT-influencing medication and survived to provide DNA collected during 1995–1998. This community-based cohort is predominantly of European ancestry (mean age 36.9 years; 51.4% women).

QT interval measurement. QT interval in all KORA samples was measured in ms from 10-s, 12-lead digitally recorded resting ECGs (S4: Bioset 9000, Hörmann Medizinelektronik; F3: Mortara Portrait, Mortara) as previously described[22]. In S4, QT intervals were determined by computerized analysis of an averaged cycle computed from all recorded cycles after exclusion of ectopic beats using the Hannover ECG analysis software (v 3.22-12). The QT interval determined by this algorithm represents the earliest start of depolarization until the latest deflection of repolarization between any two leads. QT measurements over short- and long-term time intervals have been investigated and shown to

be highly reproducible[14]. In F3, QT intervals were determined by the proprietary algorithm implemented in the ECG system[41]. Absolute measurement values for QT are known to depend strongly on the individual algorithm used, which explains the significant differences in mean QT interval measurements between KORA S4 and F3. In contrast, the relative differences in QT interval between subjects (the measurement relevant to QTL studies) have been shown to be well preserved across ECG measurement platforms[42]. The raw QT interval measured in the ECG has several significant covariates that need to be normalized to perform genetic studies; we used a multivariate linear regression model including heart rate (RR interval), sex and age. Correction factors were determined separately for each gender, as dictated by our sampling strategy, and the resulting QT interval, corrected for heart rate (R), age (A) and sex (S) was termed QTc_RAS. The correction formulas were as follows:

S4 males:

$$\mathrm{QTc_RAS} = \mathrm{QT} - (0.152 \times (\mathrm{RR} - 1{,}000\ \mathrm{ms})) - (0.318\ \mathrm{ms/year} \times (\mathrm{age} - 60\ \mathrm{years}))$$

S4 females:

$$\mathrm{QTc_RAS} = \mathrm{QT} - (0.154 \times (\mathrm{RR} - 1{,}000\ \mathrm{ms})) - (0.207\ \mathrm{ms/year} \times (\mathrm{age} - 60\ \mathrm{years})) - 4.58\ \mathrm{ms}$$

F3 males:

$$\mathrm{QTc_RAS} = \mathrm{QT} - (0.139 \times (\mathrm{RR} - 1{,}000\ \mathrm{ms})) - (0.465\ \mathrm{ms/year} \times (\mathrm{age} - 60\ \mathrm{years}))$$

F3 females:

$$\mathrm{QTc_RAS} = \mathrm{QT} - (0.145 \times (\mathrm{RR} - 1{,}000\ \mathrm{ms})) - (0.210\ \mathrm{ms/year} \times (\mathrm{age} - 60\ \mathrm{years})) - 6.49\ \mathrm{ms}$$

where RR denotes RR interval in ms.

QT intervals in the FHS sample were measured using digital calipers in leads II, V2 and V5 from digitized electrocardiograms, as previously published[3]. A single cycle from each lead was regressed for each sex separately on age and RR interval in linear models. QT residuals were standardized to mean 0 and s.d. 1 and averaged across the three leads. These average residuals represent the age, sex and RR-adjusted QT phenotype studied and have a demonstrated heritability of 35% in the FHS sample[3]. Although minor differences between the QT trait definition exist, adjustment for age, sex and RR interval were comparable, and the association of a variant with *NOS1AP* using either QT trait definition attests to the robustness of the finding and the applicability of the results to QT intervals measured using multiple methods.

Genome-wide assays and SNP genotyping. Stage I genome-wide analysis was performed using Affymetrix oligonucleotide arrays containing 115,571 SNPs, which were hybridized with genomic DNA as described[43]. Genotypes were determined using the software tool GDAS3.0, with a setting of 0.05 for both homozygous and heterozygous genotype calls. Fourteen (7%) arrays with <85% overall genotyping call rates (across all SNPs) as well as 9,616 (8.3%) SNPs with overall genotyping call rates of <85% (across samples) were removed from the data set because their accuracy was 99.5%, as determined by extensive internal validation of repeat samples. We also removed 17,367 SNPs with minor allele frequency (MAF) <2.5%, as they would have no power under any study design. These procedures left us with 186/200 subjects (93.0%) and 88,548/115,571 SNPs (76.6%) for analysis. Additional genotyping was performed in S4, F3 and FHS using either TaqMan Assays on Demand or Assays by Design (Applied Biosystems) or primer extension MALDI-TOF genotyping technology (Autoflex HT, Sequenom), according to the manufacturers' protocols. Sequenom primer sequences used are available in **Supplementary Table 6** online.

Testing for population stratification. All SNPs were tested for Hardy-Weinberg equilibrium using methods previously described[44]. F_{ST} for each SNP, with no missing data in the stage I analysis (11,431 SNPs), was calculated as the complement of the observed to expected heterozygosity.

SNP selection for stage II and III genotyping. SNPs representing the ten most significant loci from the genome-wide screen and $P < 0.01$ from the candidate genes were selected for follow-up in stage II. For loci with multiple SNPs in high linkage disequilibrium (LD) ($r^2 > 0.4$), only the most significant SNP was selected. Flanking SNPs were chosen from the International HapMap project[39]. LD was measured (r^2) for each HapMap SNP (genotyped in a sample of 60 independent subjects of Northern European origin) within 500 kb of the target SNP, and one was chosen on each side of the target SNP with an r^2 value between 0.4 and 0.8 and MAF ≥ 0.2. In those cases where there was no flanking SNP within the r^2 limits, the closest SNP with MAF ≥ 0.2 was chosen. The SNPs showing $P < 0.005$ and $P < 0.01$ from the stage II genome-wide and candidate gene analyses, respectively, were selected for stage III genotyping.

Statistical analyses of genetic effects. Stage I analysis (200 women), including 100 from each extreme of QTc_RAS, was performed using a truncated measure analysis of variance (ANOVA)[25,26] under additive, dominant and recessive genetic models, with best P values retained. Stage II analysis (600 women) added the next 200 women from each extreme of QTc_RAS and was performed as above. Stage III samples (3,366 subjects comprising both men and women) and the replication sample F3 (2,646 subjects comprising both men and women) were analyzed using standard ANOVA under additive, dominant and recessive genetic models. P values for the best fit model are reported; however, P values are adjusted for the testing of multiple genetic models by permutation tests. Model-free analyses were performed using ANOVA with 2 degrees of freedom. Analyses were performed separately for males and females, as well as for the combined sample. To estimate the variance explained, we calculated R^2 using linear regression. Regression analyses were performed using SPSS ver13.0.

The FHS analyses of SNPs at the seven loci identified in stage II involved testing the association of genotypes at these loci with adjusted QT interval duration in 1,805 unrelated FHS participants, using linear regression testing dominant, additive and recessive models (SAS v 8.1). Nominal P values for the best genetic model are reported for the replication samples.

Identification and sequencing of conserved noncoding regions. Conserved noncoding regions were identified using the phastCons[45] track from the University of California, Santa Cruz genome browser with a threshold of lod $\geq$ 25. Automated dideoxy sequencing was performed on an ABI3100 with the BigDye Terminator Sequencing Kit according to the manufacturer's protocol (Applied Biosystems). Primer sequences are available in **Supplementary Table 7** online.

Power calculations. Power was estimated by Monte Carlo simulation. Phenotypes (P) for 2,000 subjects were randomly generated from a normal distribution. To simulate a genetic effect (δ) under an additive model, genotypes were generated under Hardy-Weinberg equilibrium, and phenotypes were simulated with the following means: AA = P + (δ / 2), Aa = P; aa = P – (δ / 2). These subjects were ranked by phenotype and ANOVA was performed on the top and bottom 100 subjects (stage I analysis). If $P < 10^{-4}$ was obtained, the next 200 ranked subjects at the top and bottom were added and ANOVA performed (stage II). If $P < 0.005$ was obtained, an additional 2,000 subjects were simulated as above, and ANOVA was performed on the 3,400 subjects not tested in stages I and II (stage III). A positive result was assigned if $P < 0.005$, which was empirically derived and corresponds to a genome-wide type I error of 0.05. Power to replicate findings in the F3 population (nominal $P < 0.05$) was determined by simulating 2,700 subjects as described above, using the genetic effect observed in the S4 population.

URLs. The International HapMap project[39]: http://www.HapMap.org; University of California, Santa Cruz genome browser: http://genome.ucsc.edu/index.html?org=Human. See http://www.broad.mit.edu/mpg/haploview/ for further details on mapping in **Figure 3**.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHORS' CONTRIBUTIONS

The KORA GWA study was designed by D.E.A., A.P., W.P., W.H.L.K., H.E.W., E.M., S.K., P.M.S., T.M. and A.C. Phenotype assessment was performed by A.P., W.P., S.P., C.G. and S.K. T.I. and H.E.W. were responsible for the management of KORA data and biological samples. Affymetrix genotyping was conducted by K.W., M.I. and D.E.A. Sequenom genotyping and analysis was performed by M.A., S.J. and A.P.; T.M. and A.C. supervised the resequencing and all marker typing. Statistical analyses were performed by D.E.A., A.P., W.H.L.K. and C.K. under the supervision of A.C. The Framingham replication study was designed and carried out by C.N.-C. under the supervision of J.N.H. and C.J.O.; the statistical analyses were conducted by C.-Y.G. under the supervision of M.G.L.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

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PAPER III

Replication of Association in the NOS1AP region to general population QT Interval in the D.E.S.I.R. study

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The QT interval is a noninvasive measure of the cardiac repolarization process at the end of systole. Disturbances of repolarization, often revealing themselves by QT prolongation, have been shown to increase the risk of ventricular tachycardia and sudden cardiac death.

QT is a complex genetic trait with a heritability estimate above 30%. In a recent genomewide association study a quantitative trait locus for QT interval has been identified in a region of the NOS1AP (CAPON) gene. The most strongly associated SNP marker rs10494366 explained some 1.5% of heart-rate, age and gender-corrected QT interval (QTc_RAS) variance in general population samples from Germany and the U.S..

We intended to replicate this association in n=4125 individuals from the French D.E.S.I.R. study, a general population sample of men and women aged 30 to 65 years who had been recruited between 1994 and 1996 and followed up 9 years later. After exclusion of 10 individuals affected by atrial fibrillation (AF) we positively replicated the effect of the QTL. QTc RAS was prolonged +2.4±0.6 ms in TG-heterozygotes and +5.5±0.9 ms in GG–homozygotes when compared to TT-homozygotes ($p=8.3*10^{-10}$). The association explained 1.0% of QTcRAS trait variance in a multivariate linear regression model. Testing for heterogeneity revealed that the effect size was not significantly different from those observed in the two German and the one US American samples reported in the initial study (Q-Test, p=0.38).

We conclude that the effect of NOS1AP-QTL on cardiac repolarization process under a "common trait – common variant" paradigm was confirmed in another caucasian sample. Future investigation will be necessary to reveal the causal mechanism behind this association and to investigate whether beyond QT, interval genomic variance in this locus also influences the risk of cardiac arrhythmias and sudden cardiac death.

Replication of Association in the NOS1AP region to general population QT Interval in the D.E.S.I.R. study

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1. Abstract

The QT interval is a noninvasive measure of the cardiac repolarization process at the end of systole. Disturbances of repolarization, often revealing themselves by QT prolongation, have been shown to increase the risk of ventricular tachycardia and sudden cardiac death.

QT is a complex genetic trait with a heritability estimate above 30%. In a recent genomewide association study a quantitative trait locus for QT interval has been identified in a region of the NOS1AP (CAPON) gene. The most strongly associated SNP marker rs10494366 explained some 1.5% of heart-rate, age and gender-corrected QT interval (QTc_RAS) variance in general population samples from Germany and the U.S..

We intended to replicate this association in n=4125 individuals from the French D.E.S.I.R. study, a general population sample of men and women aged 30 to 65 years who had been recruited between 1994 and 1996 and followed up 9 years later. After exclusion of 10 individuals affected by atrial fibrillation (AF) we positively replicated the effect of the QTL. QTc RAS was prolonged +2.4±0.6 ms in TG-heterozygotes and +5.5±0.9 ms in GG–homozygotes when compared to TT-homozygotes ($p=8.3*10^{-10}$). The association explained 1.0% of QTcRAS trait variance in a multivariate linear regression model. Testing for heterogeneity revealed that the effect size was not significantly different from those observed in the two German and the one US American samples reported in the initial study (Q-Test, p=0.38).

We conclude that the effect of NOS1AP-QTL on cardiac repolarization process under a “common trait – common variant” paradigm was confirmed in another caucasian sample. Future investigation will be necessary to reveal the causal mechanism behind this association and to investigate whether beyond QT, interval genomic variance in this locus also influences the risk of cardiac arrhythmias and sudden cardiac death.

2. Introduction

Genomic approaches to identify common SCD predisposing variants have recently received increased attention (i). Especially the direct approach of examining the heart-rate corrected QT interval as a quantitative endophenotype trait in large population-based samples has much appeal. Recently in a genome-wide association scan in the KORA cohort in Germany a previously unrecognized association between QT interval and variants near the neuronal Nitric Oxide Synthase activating protein (NOS1AP or CAPON) were identified (ii). The most strongly associated variant was rs10494366 with a minor allele frequency 36% acting under a recessive model. The finding was replicated in a second German cohort and in a sample from the U.S. Framingham cohort. The proportion of explained variance in QT interval length was around 1.5% and larger than all previous findings based on candidate gene approaches (iii,iv).

3. Methods

3.1. Subjects

The D.E.S.I.R (Data from an Epidemiological Study on the Insulin Resistance syndrome) cohort has investigated 5212 Caucasian men and women aged 30-64 years. Participants were recruited in 1994-1996 from volunteers who agreed to be followed for 9 years and to have a clinical examination every three years. This study involved ten health examination centres from the western and the central parts of France. All individuals gave informed consent to the clinical and genetic study, which was approved by the CCPPRB (Comité Consultatif de Protection des Personnes pour la Recherche Biomédicale) of Bicêtre Hospital.

3.2. ECG recording and QT correction

From 4125 randomly selected individuals out of the entire sample a 12-lead resting ECG recording was available. The ECG recording and analysis procedure in DESIR has been previously reported in detail (v, vi). Briefly QT and RR intervals were measured in lead II (ms) and digitally analyzed by the Cardionics® software.

QT interval was corrected for heart-rate, age gender and gender by multivariate linear regression to give QTc_RAS as described in (4).

3.3. DNA extraction and genotyping

DNA was extracted from EDTA anticoagulated blood by phenol extraction The SNP variant rs10494366 was determined by Kbiosciences (Hoddesdon, UK) using its own form of competitive PCR system (KASPar).. Hardy-Weinberg-equilibrium (HWE) p-values were calculated using the STATA 8.0 statistical software package.

3.4. Statistical association analysis

The SNP was tested for association with QT interval by fitting linear regression models to QTc_RAS. Both the allelic and the genotypic tests were performed. In the genotypic test the three genotypes of the SNP were decomposed into two variables representing the two genotypic changes and accounting for two degrees of freedom in the model. This test can account for dominance effects by allowing the trait increase of each genotypic change to take an individual value and can independently quantify each genotype's effect and significance level in the sample. The variance attributable to the SNP was calculated as the adjusted r^2 value from each regression model.

3.5. Heterogeneity testing.

Heterogeneity testing was performed by the Q-Test in order to test if effect size was significantly different from the effects observed in the two German and the one US American samples reported in the initial study.

4. Results

4.1. Population characteristics

The demographic and clinical characteristics of the n=5212 DESIR population have been reported more fully elsewhere (vii). For the original subjects recruited in 1994 with available ECG data, (n= 4125, Stratum 1) we excluded 10 due to atrial fibrillation (Stratum 2). Of the remaining ones n=3901 had useable genotype data of rs10494366 (callrate 94.8%). For further stratified analysis we excluded another n=178 due to diabetes or bundle branch block (QRS interval > 120 ms., Stratum 3). In no stratum did the genotypes show significant deviation from Hardy-Weinberg-Equilibrium (Table 1). The minor allele frequency was well in common with those in the initial publication and in other Caucasian samples.

4.2. Genotype phenotype association

In the Stratum 1 study sample with ECG (n=4125 individuals) QTc_RAS interval had a mean value of 381.3 ms and a standard deviation of ±17.8 ms (Table 1). All allelic and genotypic linear regression models of QTc_RAS in all three strata revealed a significant association between rs10494366 and QTc_RAS interval (Table 2). In the n=4125 (Stratum 1) genotypic regression model the effect on QTc_RAS was +2.32 ms for TG-heterozygotes and +5.53 ms for GG-homozygotes. The association explained 1,0% of QTc_RAS trait variance.

4.3. Exclusion criteria and Heterogeneity testing

Exclusion of individuals with AF, pacer pregnancy (Stratum 2) and additionally of bundle branch block and diabetes (Stratum 3) both led to increases in the strength of the association (Table 2). The individuals in whom the individual exclusion criteria were present were too small in number to perform meaningful association analysis. Nevertheless we noted that in the n=93 diabetics not only was the association insignificanz (p=0.77) but also was the effect size estimate negative (-0.78 ± 2.72 ms) suggesting a significant interaction between rs10494366 and diabetes.

Heterogeneity testing by the Q-Test was performed in Stratum 2 as its exclusion criteria have also been employed in the initial pubication of the association. Effect size estimate of rs10494366 on QTc_RAS interval in the allelic test was not significantly different from those observed in the two German and the one US American samples in the initial study (Table 3) (p=0.38).

5. Discussion

We could confirm the previously published strong effect of the SNP variant rs10494366 in the promoter of the NOS1AP gene on QT interval. The effect size estimate is betweem +2.5 and +3.0 ms per allele depending on the exclusion criteria employed. The effect of rs10494366 on QT-interval is stronger than that of the previously detected nonsynonymous K897T variant in the KCNH2 gene (4).

The excluded substrata were to small to perform association analysis but showed indication for interaction effects on this association. Meaningful analysis of such interaction effects will have to await larger samples and metaanalysis.

The identification of the causal genetic variant in the NOS1AP locus will have to await further analysis of the locus employing genomic and functionla genomic analysis methods.

Our finding underscores the use of large sample sizes and Meta-analysis for future studies of QTc interval and sudden cardiac death in order to obtain statistically significant and reproducible associations as - despite the relatively high heritability of the QTc- interval - the contribution of individual variants is recognized to be rather low.

Nevertheless the QTc interval is a valuable endophenotype to investigate the predisposition to complex arrhythmias but can nevertheless replace disease phenotypes such as VT/VF or sudden cardiac death in future investigations. We are now starting to anticipate the substantial size of study samples carrying these phenotypes that will have to be recruited in order to obtain reliable and reproducible genetic associations for complex arrhythmias.

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7. Conflict of interest

none declared.

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PAPER IV

The common non-synonymous variant G38S of the *KCNE1*-(minK)-gene is not associated to QT interval in Central European Caucasians: results from the KORA study

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KEYWORDS
Cardiac repolarization;
Genetic association study;
Single nucleotide polymorphism (SNP);
Genetic epidemiology

Aims The QT interval in the general population is a complex trait with 30–50% heritability. QT prolongation is associated with an increased risk of sudden death. A recent family-based study found an association between QT interval and the common non-synonymous Glycin 38 Serine variant (G38S, rs1805127) of the *KCNE1* gene coding for the minK-potassium channel subunit. We intended to replicate this finding in a large population sample of central European Caucasian ancestry as part of our ongoing search for genetic variants predisposing to arrhythmias.

Methods and results We studied 3966 unrelated individuals from the KORA S4 population-based study without atrial fibrillation, pacemaker implant, or pregnancy. Individuals were genotyped by MALDI-TOF mass spectrometry. We did not detect any significant association between the genotypes of the G38S variant and the QT interval in the entire population or in any gender.

Conclusion Unlike the common Lysine 897 Threonine variant of *KCNH2* (K897T, rs1805123) the G38S variant of *KCNE1* does not appear to have a strong modifying effect on QT interval. However, we cannot rule out an effect of G38S on QT in other ethnic groups, under exercise or medications or on the risk for arrhythmias and sudden death.

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Conclusion Unlike the common Lysine 897 Threonine variant of *KCNH2* (K897T, rs1805123) the G38S variant of *KCNE1* does not appear to have a strong modifying effect on QT interval. However, we cannot rule out an effect of G38S on QT in other ethnic groups, under exercise or medications or on the risk for arrhythmias and sudden death.

Introduction

The analysis of genetic variants modifying cardiac electrophysiological traits has met increased attention in recent years.[1] Beyond cellular electrophysiology of ion channels and other candidate proteins[2] and molecular cloning of disease genes in families affected by mendelian arrhythmogenic diseases,[3] it is believed to provide a third and independent route to the identification of genes and gene products involved in cardiac electrophysiology.[4] One of the prerequisites of this approach is the use of population samples that are large, free of underlying population stratification, have been carefully phenotyped, and have biosamples available. In addition, these studies are motivated to enable tests that can identify individuals predisposed to arrhythmias useful in primary and secondary prevention.

One trait finding particular attention of researchers is the QT interval. This is mostly because of its relatively high heritability,[5,6] accurate measurability,[7] and its associated predisposition to sudden cardiac death.[8] Several previous association studies have analysed whether non-synonymous SNPs in cardiac ion channel candidate genes modify the QT interval. One of these SNPs is the Lysine 897 Threonine variant of the *KCNH2* gene (K897T, rs1805123).[9] The rarer T897 allele was reproducibly found to be associated with a shortened QT interval following an additive model of allelic effects.[4]

A recent study of 441 men and women sampled within families reported association of another common

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non-synonymous SNP in another cardiac ion channel gene, the Glycine 38 Serine variant of the *KCNE1* gene (G38S, rs1805127).[10] The *KCNE1* gene encodes the MinK protein, which forms the beta-subunit of the cardiac IKs channel. The allele frequency of the minor S38 allele has been reported to be between 16.4 and 48.5% in different ethnic groups.[11] In the study, however, G38S had an exceptionally low minor allele frequency of 3.3%. It was found to be associated to QT interval only in men, in whom it accounted for 2.2% of QT variance in a multivariate linear regression model ($P < 10e - 4$). Male probands with heterozygous GS38 genotype had on an average 21.7 ms longer QT intervals compared with GG38 homozygotes, equaling to a difference of almost one standard deviation (SD) ($\sigma = 23.7$ ms). In our study, we intended to replicate the previous result in 3966 probands from the KORA S4 survey, a large population-based sample of Central European Caucasian origin.

Methods

Individuals

Between 1999 and 2001, we conducted an epidemiological survey of the general population living in or near the city of Augsburg, Southern Germany (KORA S4). This was the fourth in a series of population-based surveys originating from our participation in the WHO MONICA project. The study population consisted of unrelated residents of German nationality born between 1 July 1925 and 30 June 1975 identified through the registration office. A sample of 6640 subjects was drawn with 10 strata of equal size according to gender and age. Following a pilot study of 100 individuals, 4261 individuals (66.8%) agreed to participate in the survey, who were ethnic Germans with very few exceptions (>99.5%). From 4115 probands, a positive consent, a DNA sample as well as an electrocardiogram (ECG) recording were available. After the application of exclusion criteria, atrial fibrillation, pacemaker or defibrillator implant, or ongoing pregnancy, 3966 individuals were used for association analysis. A detailed description of probands and phenotypic measurements is given in *Table 1*. The same study population has been previously used to screen for associations in ion channel candidate genes[4] and in a genome-wide approach.[12] Blood samples were drawn after informed consent had been obtained. All studies involving humans were performed according to the declarations of Helsinki and Somerset West and were approved by the local medical Ethics Committee.

Table 1 General characteristics of the study sample

Population sample drawn (n)	6640
Exclusion criteria for survey KORA S4 (n)	
Dead	51
No contact possible, moved away	344
No sufficient command of German language	37
Too sick to participate	199
No time to participate	338
Unwilling to participate	1410
Survey population of KORA S4 (n)	4261
Exclusion criteria for this study (n)	
Atrial fibrillation	38
Pacer/ICD implant	44
Pregnancy	17
No DNA sample or no ECG available	196
Sample of this study (n)	3966
Male n (%)	1957 (49.4%)
Age (years)	49.1 ± 13.8
Age range (years)	25–74
RR (ms)	939.9 ± 150.5
RR range (ms)	512–1644
QT (ms)	407.9 ± 28.1
QT range (ms)	322.0–550.0
QTc-Bazett (ms)	423.0 ± 21.6
QTc-Bazett range (ms)	329.5–545.9
QTc-RAS (ms)	417.6 ± 17.2
QTc-RAS range (ms)	322.8–541.8

Applicable data are presented as mean ± standard deviation. Values refer to the respective units given in paretheses.

ECG recording

In the S4 survey, 12-lead resting ECGs were recorded using a digital recording system (Bioset 9000, Hörmann Medizinelektronik, Germany). QT intervals were determined with the Hannover ECG analysis software (HES-Version 3.22).[13] Computerized analysis of an averaged cycle was performed from all cycles of the 10 s recording after exclusion of ectopic beats as previously described. The QT interval determined by this algorithm represents the earliest beginning of depolarization until the latest deflection of repolarization between any two leads. In an international validation study, the HES-software was among the best performing digital ECG systems.[14] Reproducibility of HES QT measurements over short- and long-term time intervals has been investigated.[7]

QT interval correction

For the purpose of this replication study, we analysed the raw QT interval as well as the corrected QT interval according to Bazett's formula,[4] which corrects QT only for heart rate in a nonlinear fashion. In addition, we used a linear correction formula for QT as has been suggested from Framingham Heart Study data[15] with the correction parameters derived from a multivariate linear regression model including the covariates heart rate (RR interval), sex, and age within KORA S4 as previously described.[4] Correction factors were determined separately for each sex. The QT interval corrected for rate-, age-, and sex was determined for men:

$$QT_c\text{-RAS} = QT - [0.152 \times (RR - 1000\,\text{ms})] - [0.318\,\text{ms/year} \times (\text{age} - 60\,\text{years})]$$

and for women:

$$QT_c\text{-RAS} = QT - [0.154 \times (RR - 1000\,\text{ms})] - [0.207\,\text{ms/year} \times (\text{age} - 60\,\text{years})] - 4.58\,\text{ms}$$

where RR denotes RR interval in milliseconds.

DNA extraction and genotyping

DNA was extracted from EDTA anticoagulated blood using a salting out procedure.[16] The G38S variant of the *KCNE1* gene was determined using PCR, primer extension, and MALDI-TOF mass spectrometry in a 384-well format (Sequenom, San Diego, USA) as previously described.[4] Hardy–Weinberg equilibrium (HWE) *P*-values were calculated using the STATA statistical software package.[17]

Genotype phenotype association analysis

SNPs were tested for association to QT, QTc-Bazett, and QTc-RAS as the dependent variables by applying two-tailed one-degree-of-freedom linear regression test (1df) and two-tailed two-degree-of-freedom ANOVA analysis (2df). The 1df test has a relatively higher power to detect weak effects, whereas the 2df test accounts for dominance and recessivity by allowing the trait increase of each genotypic change to take an individual value.

To determine gender specific differences of SNP-phenotype associations, we performed sex-specific regression analysis in the total sample. Sample sizes of males ($n = 1959$) and females ($n = 2007$) were similar and therefore comparable for effect size. Although this study was intended to replicate a previous significant finding, in light of the grossly different allele frequency we did not use one-tailed but two-tailed statistics. All reported significance levels have not been adjusted for multiple testing.

We designed our study using $n = 3966$ individuals to be able to detect the effect of the GS38 heterozygous genotype described to prolong QT interval by about one standard deviation at the published Caucasian allele frequency of 30% with near certainty. In fact, our study had >99% power in the entire sample and >98% in men to detect with $\alpha < 0.05$, an effect prolonging QT by only 0.2 standard deviations at the given allele frequencies (*Table 2*).

Results

In the total sample of 3966 individuals, QT interval had a mean value of 407.9 ms and a standard deviation of ±28.1 ms, QT corrected according to Bazett's formula had a mean value of 423.0 ms and a standard deviation of ±21.6 ms, and linearly corrected QTc-RAS had a mean value of 417.6 ms and a standard deviation of ±17.2 ms when corrected to a 60-year-old man with a heart rate of 60 b.p.m. (*Table 1*).

The G38S variant of the *KCNE1* coding sequence was genotyped with a call rate of 98.7%. Genotypes showed no significant deviation from HWE ($P = 0.08$). The minor allele frequency (MAF = 36%) was well in common with those in other Caucasian samples (*Table 3*).

Genotyping results revealed no significant association between G38S and the uncorrected or corrected QT interval. For the uncorrected QT interval, we found all *P*-values to be >0.16. For QT corrected according to Bazett's formula, *P*-values were >0.64 and for QTc-RAS >0.16 (*Table 3*). The associations between QT and G38S stratified by gender and age were also not significant (all $P > 0.05$). Applying the same model as in the initial publication, the QTc-Bazett difference between GG38 homozygous and GS38 heterozygous men was −0.7 ms ($P = 0.53$).

Discussion

We could not confirm the previously published strong effect of the non-synonymous G38S variant of the *KCNE1* gene in men or any other effect of this variant on QT interval.

Table 2 Power calculation

	Effect size estimator (δ)	Significance level (α)	GG38		GS38		SS38
Entire sample (*n*)			1591		1770		555
Power to discriminate	0.2 SD	0.001		0.994		0.936	
		0.05		>0.999		0.998	
	1.0 SD	0.001		>0.999		>0.999	
		0.05		>0.999		>0.999	
Men only (*n*)			772		872		293
	0.2 SD	0.001		0.776		0.545	
Power to discriminate		0.05		0.982		0.926	
	1.0 SD	0.001		>0.999		>0.999	
		0.05		>0.999		>0.999	

Power calculation to detect an effect of the G38S variant in the study population. Power was calculated separately for each allelic step (GG to GS and GS to SS) in two-tailed T-tests (1df) with the detected genotype frequencies from the study population. An effect size (δ) in the published magnitude of 21.7 ms QTc equals about 1 SD, an effect size of 0.2 SD corresponds to a change of 3.4 ms in QTc.

Table 3 Association results

	GG38	GS38	SS38	*P* (1df)	*P* (2df)
Total (*n*)	1591	1770	555	–	–
QT	408.1 ± 28.0	407.2 ± 28.2	409.7 ± 27.5	0.5797	0.1655
QTc-Bazett	423.1 ± 22.0	422.6 ± 21.2	423.5 ± 21.4	0.9263	0.6493
QTc-RAS	417.7 ± 17.5	417.2 ± 17.0	418.7 ± 16.8	0.5498	0.1650
Men (*n*)	772	872	293	–	–
QT	407.7 ± 30.3	406.8 ± 29.4	407.7 ± 28.3	0.8393	0.8187
QTc-Bazett	419.9 ± 22.8	419.2 ± 21.9	421.7 ± 23.0	0.5006	0.2460
QTc-RAS	418.1 ± 18.6	417.3 ± 17.7	419.2 ± 17.5	0.6904	0.2995
Women (*n*)	819	898	262	–	–
QT	408.5 ± 25.6	407.5 ± 27.0	412.0 ± 26.4	0.2636	0.0548
QTc-Bazett	426.0 ± 20.7	426.0 ± 20.0	425.6 ± 19.3	0.7747	0.9444
QTc-RAS	417.3 ± 16.4	417.0 ± 16.4	418.2 ± 15.9	0.6842	0.5589

Association of uncorrected and corrected QT interval measurements to the G38S genotypes in the entire sample and stratified by gender. Significance levels are given from two-tailed tests. A total of 3916 of 3966 individuals were successfully genotyped corresponding to a call rate of 98.7%. The genotype frequencies were in HWE ($P = 0.080$).

Our non-replication is in line with a previous investigation that also could not find any evidence of association between G38S and QT interval length[18] but was less powered to do so.

Power simulations showed that we would have been adequately powered to detect an effect if it existed in the range of 1 SD as in the initial publication as well as down to the range of 0.2 SD, which equals the magnitude of the effect of K897T in *KCNH2*.

It appears unlikely that the non-replication can be explained by a lack of precision of the QT interval measurement or another unsuitability of the samples as we have successfully replicated the association of the *KCNH2* K897T SNP to QT interval in the same sample in a previous publication.[4] In addition, a novel QTL for QT interval in the promoter of the *NOS1AP (CAPON)* gene could be identified in this sample and reproducibly confirmed in others.[12] The population-representative recruitment of individuals from one geographic area with limited recent immigration increases the homogeneity of the sample and thus increases the power to detect true positives and likewise reduces the probability of false positives due to population stratification.

Non-synonymous variants are generally considered to be likely causal variants themselves and not just markers associated by linkage disequilibrium to causal variants in their vicinity. Therefore, also in individuals from other ethnic groups we would expect no association between this variant and the QT interval. However, we cannot rule out the possibility that G38S may be a causal variant only on certain genetic backgrounds or that it may be in linkage disequilibrium to neighbouring causal variants only in some ethnic groups. Independent replication studies of similar size in individuals of such groups will be the only way to resolve the issue whether this negative association result is dependent on ethnicity or is universally valid.

Similarly, the non-replication does not rule out the possibility that the two alleles of G38S may still exert subtle differences on the repolarization process. It makes it likely that such an effect does not exist on repolarization at rest, but an effect limited to exercise, intake of medications, or other conditions may well exist. We likewise cannot rule out a modification of the risk of arrhythmias or sudden cardiac death by G38S via more complex repolarization- or non-repolarization-driven effects.

Effects of other variants within the *KCNE1* gene may also be present. Two other QT-modifying variants have been previously described, the Intron 2 variant IVS2−128 G $>$ A[19] and the promoter variant rs727957.[4] Both of them await independent replication.

This finding necessitates the use of large sample sizes for future studies of QT interval and sudden cardiac death in order to obtain statistically significant and reproducible associations as—despite the relatively high heritability of the QT interval—the contribution of individual variants is recognized to be rather low.

The QT interval is a valuable endophenotype to investigate the predisposition to complex arrhythmias but can nevertheless replace disease phenotypes such as VT/VF or sudden cardiac death in future investigations. We are now starting to anticipate the substantial size of study samples carrying these phenotypes that will have to be recruited in future to obtain reliable and reproducible genetic associations for complex arrhythmias.

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Clinical vignette

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Failed Amplatzer Septal Occluder device implantation due to an embryonic septal remnant

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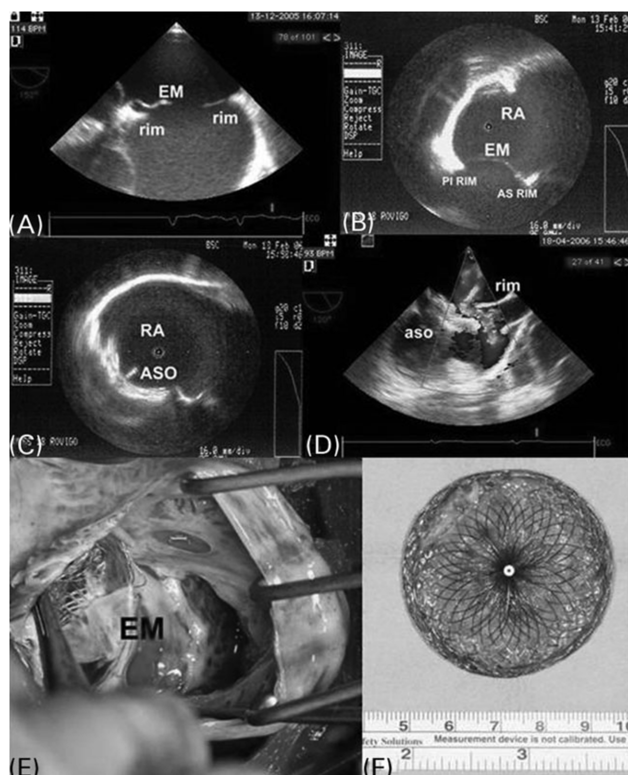
Embryonic remnants of incomplete septation may complicate occlusion device implantation in secundum atrial septal defects (sASD) even if stiff devices such as the Amplatzer Occluder are used.

A 35-year-old woman was referred to our center for evaluation of a sASD. Transesophageal echocardiography revealed a haemodinamically relevant sASD with a mean diameter of 22 mm, a virtually absent anterosuperior rim (aortic rim) and remaining floppy rims, and an apparently soft membrane at the inferior aspect of the interatrial septum which was concluded to be a remnant of incomplete septation (EM) (Panel A). Because the patient initially refused the surgical option, a percutaneous closure was attempted.



By intracardiac echocardiography (UltraICE, Boston Scientific Corp.), the diameters of the sASD at the aortic valve and four-chamber planes were 29 and 32 mm, respectively. A soft membrane (EM) was visible at the aortic valve plane but was felt to be too soft to influence device opening (Panel B; AS, anterosuperior rim; EM, embryonic membrane; PI, postero-inferior rim; RA, right atrium). To test the stiffness of the rims, a sizing balloon was passed across the ASD and inflated till an indentation was evident thus obtaining a sizing balloon ASD with a diameter of 28 mm. After calculating a mean diameter from the sizing balloon and the ICE measurement, the operators opted for a 32 mm Amplatzer ASD Occluder. The device was successfully implanted (Panel C; RA, right atrium; ASO, Amplatzer Septal Occluder) with excellent pre-discharge transthoracic echocardiography. At 1-month transesophageal echocardiography follow-up, the device appeared to be perfectly stable but deviated from the true rim with a moderate high flow shunt (Panel D; ASO, Amplatzer Septal Occluder). It was postulated that once the embryonal septal remnant had been caught by the device, it became stiffer and anchored to the true septum primum rim. It was therefore decided to remove the device surgically. Direct surgical inspection confirmed the suspicion (Panel E; EM, embryonic membrane). The partially endothelized device (Panel F) was removed and the defect repaired with a patch. Embryonic remnants of interatrial septum formation, such as the one presented above, are quite rare but should be carefully evaluated for potential technical constraints to percutaneous closure. Once this structure has been caught up by the two retention disks of the device, it may become quite stiff and can deviate the disks from the true rim.

See online supplementary material for a colour version of the figure available at *European Heart Journal* online.

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