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Evaluation of Cardiovascular Risk by Electrocardiographic Variables

Focus on heart rate and genetic variants of cardiac repolarization

ACADEMIC DISSERTATION
To be presented, with the permission of
the Board of the School of Medicine of the University of Tampere,
for public discussion in the Jarmo Visakorpi Auditorium
of the Arvo Building, Lääkärinkatu 1, Tampere,
on February 14th, 2014, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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Acta Universitatis Tamperensis 1895
ISBN 978-951-44-9334-8 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1377
ISBN 978-951-44-9335-5 (pdf)
ISSN 1456-954X
http://tampub.uta.fi

Suomen Yliopistopaino Oy – Juvenes Print
Tampere 2014

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four original publications, which are referred to in the text by their Roman numerals **I-IV**.

I Koskela J, Laiho J, Kähönen M, Rontu R, Lehtinen R, Viik J, Niemi M, Niemelä K, Kööbi T, Turjanmaa V, Pörsti I, Lehtimäki T, Nieminen T: Potassium channel KCNH2 K897T polymorphism and cardiac repolarization during exercise test. The Finnish Cardiovascular Study. Scandinavian Journal of Clinical and Laboratory Investigation 2008;68:31-38.

II Koskela J, Kähönen M, Fan M, Nieminen T, Lehtinen T, Viik J, Nikus K, Niemelä K, Kööbi T, Turjanmaa V, Pörsti I, Lehtimäki T: Effect of common KCNE1 and SCN5A ion channel gene variants on T-wave alternans, a marker of cardiac repolarization, during clinical exercise stress test: the Finnish Cardiovascular Study. Translational Research 2008;152:49-58.

III Koskela J, Kähönen M, Nieminen T, Lehtinen R, Viik J, Nikus K, Niemelä K, Kööbi T, Tobin MD, Samani N, Turjanmaa V, Pörsti I, Lehtimäki T: Allelic variant of NOS1AP effects on cardiac alternans of repolarization during exercise testing. Scandinavian Journal of Clinical & Laboratory Investigation. 2011;72:100-107.

IV Koskela J, Tahvanainen A, Haring A, Tikkakoski A, Ilveskoski E, Viitala J, Leskinen M, Lehtimäki T, Kähönen M, Kööbi T, Niemelä O, Mustonen J, Pörsti I: Association of resting heart rate with cardiovascular function: a cross-sectional study in 522 Finnish subjects. BMC Cardiovascular Disorders 2013;13:102.

2. LIST OF ABBREVIATIONS

AIx	Augmentation index
AIx@75	Augmentation index adjusted to heart rate 75 beats per minute
ANCOVA	Analysis of variances with covariates
ANOVA	Analysis of variances
BP	Blood pressure
BMI	Body mass index
bpm	Beats per minute
CHD	Coronary heart disease
95% CI	95 Percent confidence intervals
CI	Cardiac index
CO	Cardiac output
ECG	Electrocardiogram
FINCAVAS	Finnish Cardiovascular Study
HDL	High density lipoprotein
HR	Heart rate
$I_{Ca,L}$	Depolarizing L-type inward calcium current
I_{Kr}	Rapidly activating delayed outward rectified potassium current
I_{Ks}	Slowly activating delayed outward rectified potassium current
I_{Kur}	Ultrarapidly activating delayed outward rectified potassium current
I_{Na}	Inward sodium current
I_{to}	Transient outward potassium current
ICG_{WB}	Whole body impedance cardiography
KCNH2	Rapidly activating rectifying potassium channel α-subunit gene
KCNQ1	Slowly activating rectifying potassium channel α-subunit gene
KCNE1	Slowly activating rectifying potassium channel β-subunit gene
LCWI	Left cardiac work index
LDL	Low density lipoprotein
LQTS	Long QT syndrome

MAP	Mean arterial pressure
MinK	Slowly activating rectifying potassium channel β-subunit
nNOS	Neuronal nitric oxide synthase
NOS1AP	Nitric oxide synthase 1 adaptor protein gene
OR	Odds ratio
PP	Pulse pressure
PWV	Pulse wave velocity
QTc	Heart rate corrected QT interval
QTcBaz	Heart rate corrected QT interval by Bazzett's method
QTcFri	Heart rate corrected QT interval by Fridericia method
RANCOVA	Analysis of covariances for repeated measures
RANOVA	Analysis of variances for repeated measures
SCN5A	Inward sodium channel α-subunit gene
SI	Stroke index
SNP	Single nucleotide polymorphism
SV	Stroke volume
SVR	Systemic vascular resistance
SVRI	Systemic vascular resistance index
TWA	T wave alternans

3. ABSTRACT

Cardiovascular diseases are the major cause of death worldwide, and from these coronary heart disease is the most important cause of sudden death. The risk associated with cardiovascular disease can in many ways be estimated from electrocardiogram (ECG). The electrocardiographic variables prolonged QT interval, increased magnitude of T wave alternans (TWA) and higher heart rate (HR) are all associated with increased risk of unfavourable cardiac events.

Prolonged QT interval, and also increased TWA, can be a consequence of genetic variation in cardiac ion channels or other proteins affecting intracellular ion balance. The association of genetic variation with cardiac repolarization, measured as QT interval, has been widely studied, but most of the studies have focused on variables in ECG measured at rest, while systematic studies concerning these associations during exercise or recovery are largely missing. The genetic background of TWA has not been systematically studied.

Previous population based studies have repeatedly shown that higher resting HR is associated with less favourable prognosis in subjects with and without previous history of cardiovascular disease. Higher HR is also related with faster pulse wave velocity, an acknowledged marker of arterial stiffness. However, there is also a clear association of higher HR with decreased augmentation index, i.e. reduced central pressure wave reflection, which is considered to be haemodynamically beneficial.

The aim of the present thesis was to study the genetic and haemodynamic background of the ECG risk markers, QT interval, TWA, and HR at rest during physical challenge. The associations between genetic variants with cardiac repolarization during exercise testing were examined because the present knowledge in this context is limited, and also the reasons why higher HR is associated with less favourable prognosis are not well understood.

A total 2212 Finnish Cardiovascular Study (FINCAVAS) participants were genotyped using TaqMan assays, and their maximal TWA values and QT intervals were measured from continuous ECG recordings during clinical exercise test at rest,

exercise and recovery. The examined nucleotide polymorphisms were located in the cardiac ion channel genes KCNH2 (rs1805123), SCN5A (rs1805124), KCNE1 (rs727957 and rs1805127) and in nitric oxide synthase gene, NOS1AP (rs10494366), all of which have been previously found to be functionally relevant (**I-III**).

The DYNAMIC study subjects (n=522, 261 men, aged 20-72 years, without medication directly affecting HR) were examined in order to gain information about the association between higher HR and the less favourable prognosis observed in population studies. The relationship of resting HR with the principal haemodynamic variables peripheral and central blood pressure, stroke volume, cardiac output, systemic vascular resistance, and markers of left cardiac work, cardiac oxygen demand, arterial stiffness and central wave reflection were examined. The haemodynamic variables were noninvasively recorded in supine and upright position by the use of whole body impedance cardiography and radial applanation tonometry (**IV**).

In the FINCAVAS study the polymorphism rs1805123 of KCNH2 was associated with QT interval within women only at rest, but this polymorphism was not associated with TWA (**I**). From the studied polymorphisms the rs1805127 of KCNE1 was associated with TWA in the whole study population, but the relationship was most significant within women (**II**). The polymorphisms rs727957 and rs1805124 were not related with TWA (**II**). In addition, rs10494366 of NOS1AP was associated with TWA during exercise testing in a sex-specific manner (**III**).

In the DYNAMIC study population (**IV**) higher resting HR was significantly associated with lower stroke volume but also with higher cardiac output, reflecting increased cardiac work. Higher HR was also associated with increased arterial stiffness. Moreover, higher HR showed a relatively weak but significant association with elevated blood pressure. All relations remained remarkably similar during supine and upright positions.

In conclusion, this thesis studied the background of ECG risk markers. From the studied genetic variants the polymorphisms rs1805123, rs1805127 and rs727957 were associated with cardiac repolarization, measured as QT interval or TWA during clinical exercise testing. In addition, the basic and easily available ECG variable, HR, was associated with increased cardiac work and arterial stiffness, and to a lesser extent with elevated blood pressure, in both supine and upright position. Knowledge of these ECG-related characteristics may provide useful additional information for the risk stratification of cardiovascular diseases in the future.

4. TIIVISTELMÄ

Sydän- ja verisuonisairaudet on tärkein kuolinsyiden ryhmä maailmanlaajuisesti. Näistä sepelvaltimotauti on merkittävin syy äkkikuolemalle. Sydämen terveyttä voidaan arvioida sydänsähkökäyrästä (EKG) muuttujilla, joista pidentynyt QT-aika, lisääntynyt T-aallon vuorottelu (TWA) sekä nopea leposyke on monissa tutkimuksissa yhdistetty lisääntyneeseen sydäntapahtumien ja -sairauden riskiin.

QT-aika ja TWA edustavat sydänlihaksen sähköisessä toiminnassa repolarisaatiota ja sen muutoksia. Pidentynyt QT-aika on yhteydessä sydänsairauksiin sekä lisääntyneeseen kammioperäisten rytmihäiriöiden riskiin ja jopa kuolleisuuteen. Myös lisääntynyt TWA ilmenee monissa sydämen sairauksissa ja sen esiintyminen on yhdistetty henkeä uhkaaviin rytmihäiriöihin. Sekä pidentynyt QT-aika että lisääntynyt TWA voivat johtua sydämen ionikanavien geneettisestä muuntelusta. Myös muilla solunsisäiseen ionitasapainoon vaikuttavien proteiinien geneettisellä vaihtelulla on todettu olevan vaikutusta sydämen sähköiseen toimintaan. Geneettisen vaihtelun vaikutusta sydämen repolarisaatioon, QT-aikaan, on kuitenkin laajemmin tutkittu vain levossa, vaikka on oletettavaa, että muutokset voivat tulla esiin ja provosoitua rasituksessa tai rasituksesta palautumisessa. Geneettisen vaihtelun vaikutusta TWA:han ei ole systemaattisesti tutkittu.

Myös kohonneen leposykkeen yhteys huonoon ennusteeseen niin sydänsairailla kuin ennestään terveillä henkilöillä on tunnettu jo pitkään, mutta yhteyden syyt ovat pääosin selvittämättä. Lisäksi korkea leposyke on yhteydessä lisääntyneeseen valtimojäykkyyteen, jota voidaan mitata pulssiaallon etenemisnopeudella. Kuitenkin ristiriidassa tämän löydöksen kanssa on se, että kohonneella sykkeellä on todettu myös yhteys alhaisempaan pulssiaallon heijastumista kuvaavaan augmentaatioindeksiin, joka väestötutkimuksissa on yhdistetty suotuisampaan ennusteeseen sydän- ja verisuonitautiriskin osalta.

Tämän väitöskirjatutkimuksen tavoitteena oli selvittää EKG muuttujien, QT-ajan, sekä TWA:n ja leposykkeen, taustatekijöitä. Geneettisen vaihtelun vaikutusta sydämen repolarisaatiomarkkereihin tutkittiin kliinisen rasituskokeen aikana, koska tietoa

rasituksen aikaisista muutoksista on olemassa vain rajoitetusti. Lisäksi haluttiin selvittää leposykkeen ja huonon ennusteen välistä yhteyttä tutkimalla hemodynaamisia muuttujia: perifeerisiä ja sentraalisia verenpaineita, sydämen työtä ja ääreisvastusta, sekä valtimojäykkyyteen yhdistettyjä muuttujia, kuten pulssiaallon etenemisnopeutta sekä sentraalisia paineheijasteita levossa sekä yksinkertaisen toimintakokeen, passiivisen pystyyn nostamisen yhteydessä.

Sydämen repolarisaation genetiikkaa tutkittiin suomalaisessa rasituskoeaineistossa, jossa oli yhteensä 2212 tutkittavaa, joille tehtiin genotyypitys aikaisemmin repolarisaation liitettyjen polymorfismien suhteen. Tutkitut polymorfismit olivat sydämen ionikanavageeneissä, KCNE2 (rs1805123), KCNE1 (rs727957 ja rs1805127), SCN5A (rs1805124) sekä typpioksidisyntetaasin adaptoriproteiini geenissä, NOS1AP (rs10494366). Genotyyppien yhteyttä repolarisaatioon tutkittiin levossa, rasituksessa ja rasituksesta palautumisessa.

Leposykkeeseen liittyviä muita hemodynaamisia muuttujia tutkittiin kajoamattomalla mittauksella makuulla ja pystyasennossa koko kehon impedanssikardiografialla sekä perifeerisellä paineanturilla ranteesta. Mitattavia suureita olivat sentraaliset ja perifeeriset verenpaineet, iskutilavuus, minuuttitilavuus, perifeerinen vastus, pulssiaallon etenemisnopeus, sekä keskeisen verenkierron paineheijasteet kuten augmentaatioindeksi.

Tutkituista genotyypeistä QT-aikaan oli yhteydessä rs1805123 polymorfismi geenistä KCNH2, mutta vain naisilla lepomittauksen aikana, eikä yhteyttä TWA:han todettu. TWA:han olivat yhteydessä polymorfismit rs1805127 geenissä KCNE1 ja rs10494366 geenissä NOS1AP. Polymorfismin rs1805127 ja TWA:n yhteys oli selvempi naisilla kuin miehillä ja korostui rasituksessa ja palautumisvaiheessa verrattuna lepotilanteeseen. Myös polymorfismin rs10494366 yhteys TWA:han oli sukupuoleen sidottu, sillä miehillä genotyyppien välillä ei ollut tilastollista eroa.

Kohonnut leposyke oli tässä tutkimuksessa käänteisesti yhteydessä iskutilavuuteen, mutta suoraan verrannollinen minuuttitilavuuteen, mikä kertoo lisääntyneestä sydämen työstä. Tilastollisesti leposykkeen yhteys verenpaineisiin oli vähäinen, mutta yhteys valtimojäykkyyden mittariin, pulssiaallon etenemisnopeuteen, oli samankaltaista kuin aikaisemmissa väestötutkimuksissa. Lisäksi sentraaliset paineheijasteet olivat suotuisammat korkean leposykkeen vallitessa. Erot matalasykkeisten ja korkeasykkeisten välillä pysyivät hämmästyttävän samanlaisina makuu- ja pystyasennoissa.

EKG:stä mitattavia riskimarkkereita, QT ajan kestoa ja erityisesti T aallon vuorottelun määrää rasituskokeen aikana voidaan osaltaan selittää tavanomaisessa väestössä esiintyvillä erilaisilla genotyypeillä. Korkeaan leposykkeeseen liittyviä verenkiertoelimistön muutoksia ovat matalampi iskutilavuus ja korkeampi minuuttitilavuus ja kohonnut sydämen työ sekä kohonnut pulssiaallon etenemisnopeus. Tämä hemodynaaminen profiili säilyy levosta pystyyn samankaltaisena. Näiden EKG-markkereiden taustasyiden selvittäminen voi tulevaisuudessa auttaa yksilöllisempään riskinarvioon sydän- ja verisuonisairauksien suhteen.

5. INTRODUCTION

Cardiovascular diseases are the major cause of death worldwide from which coronary heart disease (CHD) is the most important cause of sudden cardiac death (Adabag *et al.* 2010). Existence of CHD and increased risk for malign ventricular arrhythmias can be estimated using electrocardiogram (ECG) during rest and clinical exercise test (Chou *et al.* 2011, John *et al.* 2012). The ECG variables prolonged QT interval, increased T wave alternans (TWA) and higher (HR) are associated with increased risk of unfavourable cardiac events and less favourable prognosis (Yi *et al.* 1998, Pham *et al.* 2003, Cook *et al.* 2006).

QT interval in ECG is representing the duration of cardiac repolarization. Prolonged QT interval is characteristic of QT syndromes, and is associated with increased risk of ventricular arrhythmias and mortality (Morita *et al.* 2008, John *et al.* 2012, Noseworthy *et al.* 2012). Increased QT interval duration can be consequence of genetic variation of cardiac ion channels or for example adverse effect of medications, and can be manifested not only at rest but also during exercise or recovery (Amin *et al.* 2010). TWA is another parameter characterizing ventricular repolarization and is indicating beat to beat alteration of T wave duration and shape (Narayan 2006). Increased TWA reflects the presence of heart disease and higher susceptibility of life-threating arrhythmias and sudden cardiac death (Narayan 2006). Moreover higher TWA during clinical exercise test is related with increased mortality (Nieminen *et al.* 2007). The mechanisms of repolarization alternans are not clear, but they may at least partly be explained by changes in intracellular calcium concentration (Pruvot *et al.* 2004). Thus, it seems probable that genetic variants of cardiac ion channels influencing repolarization duration have also associations with TWA.

Electrical activity of the heart is produced by ion currents through transmembrane ion channels, from which different sodium and potassium currents are mostly responsible for cardiac repolarization while calcium currents are related to excitation and the subsequent contraction of myocytes (Amin *et al.* 2010). Initiation and frequency of cardiac action potential is controlled by self-excitation of pacemaker cells

in sinoatrial node (Amin *et al.* 2010). Many polymorphisms of ion channel subunits are associated with medical conditions such as long QT syndrome, Brugada syndrome, atrial fibrillation as well as variation of QT interval in general populations (Amin *et al.* 2010). In addition, genetic variation of rapidly activating rectifying potassium channel α-subunit, KCNH2, has been associated with T wave morphology (Linna *et al.* 2006). There are also other genetic alterations than those detected in cardiac ion channels, which have been associated with cardiac repolarization. For example nitric oxide synthase 1 adaptor protein gene (NOS1AP) is associated with QT interval duration and mortality (Aarnoudse *et al.* 2007, Kao *et al.* 2009).

There are plenty of studies associating genetic variation with cardiac repolarization, but most of the studies have only focused on ECG variables measured at rest. However, the phenotype of a genetic variant may especially appear during exercise or the subsequent recovery, but systematic investigations concerning these associations is largely lacking (Takenaka *et al.* 2003, Amin *et al.* 2010).

Based on large epidemiological studies, higher resting HR is associated with less favourable prognosis in subjects with and without previous history of cardiovascular disease (Kannel *et al.* 1987, Gillman *et al.* 1993, Cooney *et al.* 2010). Higher HR is also related with faster pulse wave velocity (PWV), a widely accepted marker of arterial stiffness and increased cardiovascular risk (O'Rourke and Hashimoto 2007). But then there is also association of higher HR with decreased augmentation index (AIx) which is considered to be beneficial due to reduced central haemodynamic load (Wilkinson *et al.* 2000).

The aim of the present study was to investigate the genetic background of the electrocardiographic risk markers, QT interval, TWA and examine the haemodynamic associations of higher HR at rest. The associations between the above genetic variants with cardiac repolarization were studied during exercise testing, as the level of the present knowledge about these matters is scarce. In addition, to better understand the association between higher HR and compromised long-term prognosis, the relationship of resting HR with the principal haemodynamic variables peripheral and central blood pressures (BP), stroke volume (SV), cardiac output (CO), systemic vascular resistance (SVR), and markers of left cardiac work, cardiac oxygen demand, arterial stiffness and central wave reflection was investigated in supine and upright positions.

6. REVIEW OF THE LITERATURE

6.1 Electrical activity of heart muscle

6.1.1 Action potential of the heart

Cardiac function as a pump is based on electrical activity of heart muscle leading to contraction of myocardium, i.e. excitation-contraction coupling. Electrical activity is initiated from self-excitation of pacemaker cells in the sinoatrial node in right atrium. The excitation expands to other atrial cells and atrioventricular node from where it spreads to ventricular myocardium through Purkinje fibres (Nerbonne and Kass 2005). Contraction follows the electrical activity through the myocardium respectively (Nerbonne and Kass 2005). At the cellular level, electrical activity of the heart is produced by different ionic currents, which result in the generation of action potential in cardiomyocytes (Conrath and Opthof 2006). Changes in the magnitude of different ionic currents in separate myocardial cell types produce variation in action potential morphology and duration (Conrath and Opthof 2006). Cardiac action potential begins with rapid upstroke, i.e. depolarization, resulting from inward sodium current (I_{Na}, phase 0), which is followed by transient potassium outflow (I_{to}) and early repolarization (phase 1) (Amin *et al.* 2010). The plateau (phase 2) is a consequence of the balance between depolarizing inward L-type calcium current ($I_{Ca,L}$) and various delayed rectified outward potassium currents (ultra rapid, I_{Kur}; rapid, I_{Kr}; slow, I_{Ks}) and finally the dominance of outward potassium current leads to the repolarization (phase 3) when membrane voltage returns back to resting potential (phase 4) (Amin *et al.* 2010).

6.1.2 Cardiac ion channels

Transmembranic ion currents, and thus action potentials, are regulated by opening and closing the ion channels. The most important cardiac ion channels are voltage gated,

i.e. their function alters in response to changes in membrane voltage (Roden *et al.* 2002). The cardiac ion channels contain amino acid formatted subunits, the pore-forming α-subunit and accessory β-subunits which are encoded by different genes (Amin *et al.* 2010). The α-subunits of the sodium and calcium channels contain a chain of four homologous domains each including six transmembrane regions. The structures of α-subunits in the potassium channels are simpler and they contain a single domain including six or two transmembrane regions (Amin *et al.* 2010). At least 9 different ion channels are responsible for cardiac action potentials and each ion channel is encoded by a singular gene including different gene regions for α- and β-subunits (Amin *et al.* 2010). The most important cardiac ion channel genes and subunits and their relations with different ion currents are presented in Table 1. Heterogeneity of action potential waveforms in different regions of the heart results from variation in ion channel expression levels (Nerbonne and Kass 2005). Changes in ion channel function and expression can be influenced by exogenous factors, like drugs or ischemia, or variation of ion channel genes or their regulators (Roden *et al.* 2002). Polymorphisms and mutations of the ion channel genes have been shown to associate with inter-individual alteration of repolarization, as well as with certain medical conditions characterized by manifestation of arrhythmias and sudden cardiac death including long (LQTS) and short QT syndrome, Brugada syndrome and familial atrial fibrillation (Amin *et al.* 2010).

6.1.3 Genetic variants related with cardiac repolarization and prognosis

Background of genetic studies

Candidate gene studies and genome wide association studies are most commonly applied methods for investigating genetic basis of disease. Candidate gene studies focus on associations between genetic variation of a specific gene with phenotype (Lewis 2002). In contrast, genome wide association studies examine simultaneously many common variants in a large population to uncover whether any of the variants are more commonly represented in subjects with trait or disease than subjects without such a condition (Bush and Moore 2012).

An effective method for large-scale screening of known genetic variants or single nucleotide polymorhpisms (SNP) is the TaqMan assay, which is based on fluorescent labelled sequence-specific oligonucleotide probe that permits detection of SNPs during polymerase chain reaction (Livak 1999, Hui *et al.* 2008). In the TaqMan assay allelic discrimination is based on differently labelled fluorescent reporter dyes. During polymerase chain reaction the reporter dyes are released, producing an increase in fluorescence, which can be detected by the commercial ABI Prism Sequence Detection System (Livak 1999).

KCNH2 gene

The KCNH2 gene encodes cardiac potassium channel α-subunit, which produces the I_{Kr} current in phases 2 and 3 of action potential (Roden *et al.* 2002). This potassium channel is essentially responsible for repolarization duration and refractoriness in most of the cardiomyocytes (Tamargo *et al.* 2004). Mutations of KCNH2 gene have been linked with LQTS type 2 and variation of repolarization duration within general populations (Curran *et al.* 1995, Bezzina *et al.* 2003, Crotti *et al.* 2005, Gouas *et al.* 2005, Vandenberg *et al.* 2012).

The KCNH2 gene presents a SNP rs1805123 where the minor allele (G) results the change of lysine to threonine in position 897 in the pore region of the channel (Laitinen *et al.* 2000, Bezzina *et al.* 2003). The reported minor allele frequencies of SNP rs1805123 have been approximately 16% in general Finnish populations (Laitinen *et al.* 2000, Marjamaa *et al.* 2009). The SNP rs1805123 has been identified as a significant determinant of cardiac repolarization duration at rest, measured as QT interval in ECG, in several genome-wide association studies (Pietilä *et al.* 2002, Linna *et al.* 2006, Newton-Cheh *et al.* 2007, Marjamaa *et al.* 2009). However, in a large population based Cooperative Health Research in the Region Augsburg (KORA study) Akyol *et al.* came to the conclusion that SNPs rs1805123 was not associated with QT interval at rest, and a similar result was observed in a smaller study by Aydin *et al.*, but on the basis of these studies the relationship during exercise or during recovery could not be excluded (Aydin *et al.* 2005, Akyol *et al.* 2007). The SNP rs1805123 has also been related with changes in QT interval during clinical exercise testing in patients with type 1 LQTS caused by a mutation of the KCNQ1 gene, but there is no evidence

of possible exercise related associations within general populations (Paavonen *et al.* 2003, Akyol *et al.* 2007). The SNP rs1805123 minor allele carriers are associated with 8-fold risk of life-threatening ventricular arrhythmias after acute myocardial ischemia compared to controls (Crotti *et al.* 2012). In addition there is some evidence of the relation between SNP rs1805123 and sudden infant death (Nof *et al.* 2010).

KCNE1 gene

The ion channel gene KCNE1 encodes a β-subunit within a single transmembrane protein named minK, which together with an α-subunit coded by KCNQ1 gene produce the I_{Ks} current in the heart (Sanguinetti *et al.* 1996). The I_{Ks} activates slowly during depolarization and deactivates slowly during repolarization (Nerbonne and Kass 2005). Mutations in the KCNE1 gene results in loss of I_{Ks} current, which prolongs cardiac action potential duration particularly during sympathetic activation, and these mutations are associated with ventricular arrhythmogenesis characteristic of type 5 LQTS (Morita *et al.* 2008). Based on the present knowledge, there is a suspicion that minK β-subunit may also be linked with other potassium α-subunits, hence affecting other potassium currents during action potential (Nerbonne and Kass 2005).

MinK encoding the KCNE1 gene represents several polymorphisms, from which the SNP rs1805127 produces amino acid serine replacement by glycine at position 38 (Lai *et al.* 1994), and the SNP rs727957 results in the change of nucleotide G to A in non-coding intronic region of the KCNE1 gene (Pfeufer *et al.* 2005). The SNPs rs1805127 and rs727957 have been associated with cardiac repolarization duration at rest among healthy subjects and also within general population (Aydin *et al.* 2005, Friedlander *et al.* 2005, Pfeufer *et al.* 2005). In addition, the minor allele carriers of SNP rs1805127 have also been associated with elevated risk of atrial fibrillation in a Chinese population (odds ratio (OR) 1.66 for AG genotype, and OR 2.03 for GG genotype (minor allele homozygotes) (Yao *et al.* 2012)).

SCN5A gene

The SCN5A gene is encoding an α-subunit of cardiac sodium channel, and mutations in the gene can either increase I_{Na} current and produce delaying of repolarization when

altered sodium channels stay open during repolarization, or reduce I_{Na} current prolonging the conduction intervals (Amin *et al.* 2010). SCN5A gene mutations can cause inherited LQTS type 3, predisposing to life-threating ventricular arrhythmias (Wang *et al.* 1995). Furthermore, genetic variation of SCN5A is related with the Brugada syndrome, atrial fibrillation, inherited cardiomyopathy, sick sinus syndrome, and sudden cardiac death (Bezzina *et al.* 1999, Tan *et al.* 2007, Nguyen *et al.* 2008, Tsai *et al.* 2008, Albert *et al.* 2010).

SCN5A represents SNP rs1805124, a minor allele (G) of which results in replacement of amino acid histidine by arginine in position 558 affecting sodium channel function *in vitro* (Viswanathan *et al.* 2003). The SNP rs1805124 affects sodium channel properties via increasing I_{Na} current, and it has been associated with variation of repolarization duration at rest in general populations (Aydin *et al.* 2005, Gouas *et al.* 2005). The mean QT interval was 373 ms within the AA genotype group and 382 ms within the GG genotype group in study by Aydin *et al.*, and the minor allele was more frequent in the longest QT interval group (OR=1.52) in the study by Gouas *et al.* (Aydin *et al.* 2005, Gouas *et al.* 2005). The SNP rs1805124 minor allele increases also the risk of atrial fibrillation in the general population (OR=3.451 for minor allele carriers) (Chen *et al.* 2011).

NOS1AP gene

The nitric oxide synthase 1 adaptor protein, encoded by NOS1AP, regulates neuronal nitric oxide synthase (nNOS) activation and increases N-methyl-D-aspartic acid receptor –gated calcium influx (Jaffrey *et al.* 1998). Deficiency or inactivation of nNOS depresses cardiac excitation-contraction coupling via increased superoxide production and decreased calcium release in sarcoplasmic reticulum. Hence nNOS may contribute to cardiac disease, as based on findings in experimental studies (Barouch *et al.* 2002, Khan *et al.* 2004). Common variants of NOS1AP, including SNP rs10494366, have been associated with sudden cardiac death and prolonged QT interval at rest among different populations (Arking *et al.* 2006, Aarnoudse *et al.* 2007, Newton-Cheh *et al.* 2007, Post *et al.* 2007, Lehtinen *et al.* 2008, Eijgelsheim *et al.* 2009, Kao *et al.* 2009, Raitakari *et al.* 2009).

The SNP rs10494366 is leading to nucleotide change T to G in an intronic region of the NOS1AP, and this SNP has been related with the duration of QT interval for the first time in a genome wide association study by Arking *et al.* (Arking *et al.* 2006). They reported 36-39% minor allele frequency of the SNP rs10494366 in two different populations (KORA and Framingham study populations), and same size of minor allele frequencies has also been reported in other studies (Arking *et al.* 2006, Aarnoudse *et al.* 2007, Raitakari *et al.* 2009). In the study by Arking *et al.* the mean difference of the HR corrected QT interval (QTc) duration between the GG genotype group and the TT genotype group was 4.0-7.9 ms ($p \leq 0.004$), while within the Framingham study population the difference was not statistically significant within the male subgroup (Arking *et al.* 2006). On the basis of this study, the association of rs10494366 with QT interval is probably sex dependent (Arking *et al.* 2006). Moreover, SNP rs10494366 of NOS1AP has shown to enhance the risk of arrhythmias within patients with long QT syndrome (Crotti *et al.* 2009, Tomas *et al.* 2010). Cardiac ion channel genes and their relationships with ion currents and inherited disorders are summarized in Table 1.

Table 1. Cardiac ion channel genes and their relationships with ion currents and inherited disorders.

Current	Gene	Subunit type (name)	Related disorders	References
I_{Na}	SCN5A	α	LQTS 3, Brugada syndrome, cardiomyopathy, atrial fibrillation, sick sinus syndrome	(Wang *et al.* 1995) (Bezzina *et al.* 1999) (McNair *et al.* 2004) (Chen *et al.* 2011) (Tan *et al.* 2007)
	SCN1B	β1		
	SCN2B	β2		
	SCN3B	β3		
	SCN4B	β4	LQTS 10	(Medeiros-Domingo *et al.* 2007)
$I_{to, fast}$	KCND3	α		
	KCNE2	β (MiRP1)	Atrial fibrillation	(Yang *et al.* 2004)
	KCNE3	β (MiRP2)	Brugada syndrome	(Delpon *et al.* 2008)
$I_{to, slow}$	KCNA4	α		
	KCNB1	β1		
	KCNB2	β2		
	KCNB3	β3		
	KCNB4	β4		
$I_{Ca, L}$	CACNA1C	α	Timothy syndrome (LQTS 8)	(Splawski *et al.* 2004)
	CACNB2	β2		
$I_{Ca, T}$	CACNA1G	α		
	CACNA1H	α		
I_{Kur}	KCNA5	α	Atrial fibrillation	(Olson *et al.* 2006)
	KCNAB1	β1		
	KCNAB2	β2		
I_{Kr}	KCNH2	α	LQTS 2,	(Curran *et al.* 1995)
	KCNE2	β (MiRP1)	LQTS 6	(Abbott *et al.* 1999)
I_{Ks}	KCNQ1	α	LQTS 1, Atrial fibrillation, Jervell and Lange-Nielsen syndrome	(Wang *et al.* 1996) (Chen *et al.* 2003) (Tyson *et al.* 1997)
	KCNE1	β (minK)	LQTS 5, Jervell and Lange-Nielsen syndrome	(Splawski *et al.* 1997) (Tyson *et al.* 1997)
I_{K1}	KCNJ2	α	Andersen-Tawil syndrome (LQTS 7)	(Plaster *et al.* 2001)

Abbreviations: LQTS, long QT syndrome; $I_{Ca,L,}$Depolarizing L-type inward calcium current; $I_{Ca, T}$, Depolarizing T-type inward calcium current; $I_{Kr,}$ Rapidly activating delayed outward rectified potassium current; $I_{Ks,}$ Slowly activating delayed outward rectified potassium current; $I_{Kur,}$ Ultrarapidly activating delayed outward rectified potassium current; I_{K1}, Inward rectifying potassium current; $I_{Na,}$ Inward sodium current; $I_{to, fast,}$ Fast transient outward potassium current; $I_{to, slow,}$ Slow transient outward potassium current

6.2 Functional aspects of the heart and central wave reflection

6.2.1 Principal determinants of BP

The heart is an electromechanical pump where electrical activity causes contraction of muscle fibres in the particular order producing blood flow from the heart to arteries (Guyton and Hall 2006). Contraction duration and intensity as well as duration of relaxation phase are influenced by cardiac electrical activity (Guyton and Hall 2006). Plainly arterial BP is a product of prevailing blood flow and SVR. The role of heart in maintaining appropriate BP in association with vascular resistance will be discussed below.

HR and SV

Blood flow from heart means quantity of blood moving to aorta in a certain period of time and it is equal to CO (Guyton and Hall 2006). Most important factors determining CO are HR and SV. HR is controlled by sinoatrial node from where cardiac cycle, events between the heart beat to another, begins by self-excitation of the pacemaker cells (Guyton and Hall 2006). Sinoatrial node is innervated by sympathetic and parasympathetic branches of autonomous nervous system, which are responsible for extrinsic regulation of HR. HR is not only controlled by autonomous nervous system, but also by non-neuronal intrinsic cardiac factors, particularly during exercise (Mangoni and Nargeot 2008). Moreover, expression of different ion channels are involved with automaticity and conduction properties of sinoatrial node (Mangoni and Nargeot 2008). Thus, it is obvious that HR regulation is a complex mechanism, which is influenced by many extrinsic factors like temperature and extracellular ion balance. Normal resting HR has been appointed between 60 and 100 beats per minute (bpm), and in textbooks HR 75 bpm is informed to be normal average. There is no proper knowledge of the optimal HR, but probably it is approximately between 60 and 80 bpm (Fox *et al.* 2007).

SV is the amount of blood that is emptied to circulation from the left ventricle during systole, and that is approximately 70 ml at rest. SV is affected by venous return

by a mechanism called the Frank-Starling law, which is simplified as follows: the greater the ventricle is stretched during filling, the greater is the force of contraction (Guyton and Hall 2006). Force of contraction and thus magnitude of SV is controlled by balance of sympathetic and parasympathetic stimulation (Guyton and Hall 2006). Thus, CO depends not only on the body size, but also on the prevailing balance of HR, SV and SVR, which are all controlled by autonomic nervous system. Regulation of CO is mostly produced by venous return depending on total amount of local tissue flow.

SVR

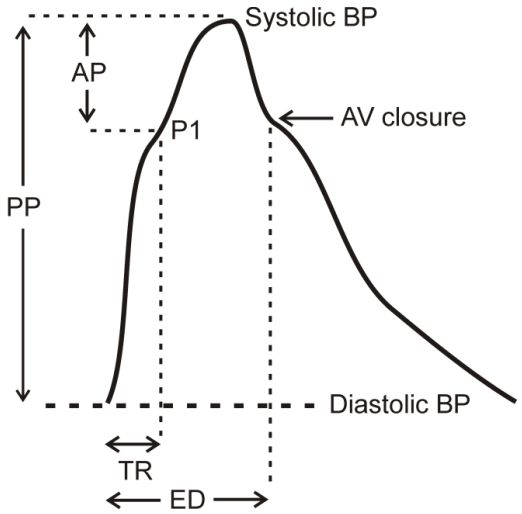
Local vascular resistance is maintaining appropriate blood flow in tissues during varying arterial pressures. SVR characterizes resistance to blood flow in the entire circulation, and is regulated by sympathetic vasoconstriction system, as well as by humoral and local factors (Guyton and Hall 2006). Arterial endothelium plays an important role in regulating short term vascular tone via releasing vasoactive substances such as nitric oxide and endothelin (Sudano *et al.* 2011, Tousoulis *et al.* 2012). Imbalance between vasoconstrictive and vasodilating substances, a condition often referred to as endothelial dysfunction, is related with increased cardiovascular morbidity (Wong *et al.* 2010, Sudano *et al.* 2011). Increased SVR is assumed to be an important determinant of hypertension via activation of sympathetic nervous system, renin-angiotensin-aldosterone system and local vasoconstrictive agents (Oparil *et al.* 2003). The long-term level of SVR is also reciprocally influencing CO when BP is maintained at a constant level.

6.2.2 Central wave reflection and arterial stiffness

Arterial pulse wave form consists of a forward pressure wave from the left ventricle to the aorta, and a backward wave that is reflected from the peripheral reflection sites (Figure 1). The variables that influence BP, arterial distensibility and arterial stiffness, can be determined from the arterial pulse wave form. The pulsatile component of arterial BP is characterized by pulse pressure (PP), which is equal to the difference between systolic and diastolic BP, and is influenced by arterial stiffness and SV (Hamilton *et al.* 2007). The following two parts can be distinguished from the systolic

pressure wave: i) the peak forward pressure wave from the root to the first inflection point (P1), and ii) the reflected pressure wave from the first inflection point to the peak of systolic pressure wave (i.e. augmentation pressure) (Chemla *et al.* 2008). Augmentation pressure depends on the distance of reflection sites of arteries from the aortic root and on arterial distensibility (O'Rourke and Pauca 2004). Arterial distensibility and arterial stiffness significantly influence the pulse transit time in the arterial tree and are hence strongly related with PWV (Hamilton *et al.* 2007). Further details of central wave reflection and arterial stiffness, measured as PWV, are given below.

Figure 1. Aortic pulse wave form.



Abbreviations: AP, augmentation pressure; AV, aortic valve; BP, blood pressure; ED, ejection duration; PP, pulse pressure; P1, first inflection point; TR, time to reflected wave

Central wave reflection

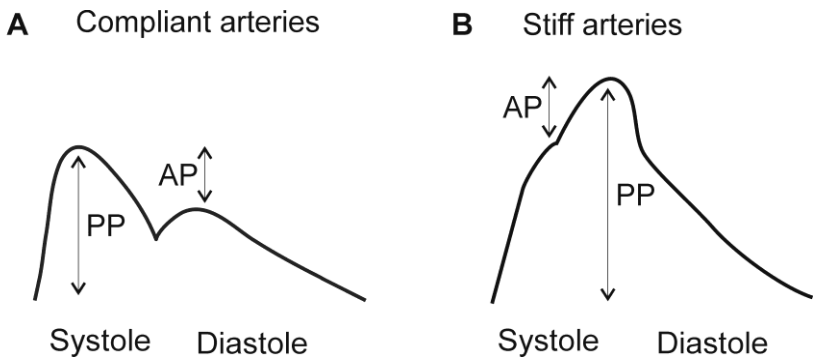
The amplitude and timing of pulse wave reflection in the aorta depend on the artery size, i.e. lumen area, distance of branching points, and arterial distensibility (O'Rourke and Pauca 2004, Laurent *et al.* 2006, Hamilton *et al.* 2007), and they can be evaluated from the central pulse wave form as shown in Figure 1. The variables in the central pressure wave form that represent wave reflection are augmentation pressure, AIx (determined by formula AP/PP, (Kelly *et al.* 1989)) and time to the reflected wave. Most of the wave reflection occurs at branches of arteries, where arterial resistance is increasing (Murgo *et al.* 1980, Latham *et al.* 1985, O'Rourke *et al.* 2001, Laurent *et al.*

2006). The pulse wave reflection sites are determined by subject's age, height, sex, and arterial stiffness (Smulyan *et al.* 1998, Gatzka *et al.* 2001, Laurent *et al.* 2006). Thus, within subjects with shorter height and thus closer reflection sites, or subjects with stiffened arteries and faster pulse transit time, the time to wave reflection is shorter. Subsequently, the reflected wave is shifted towards systole, augmentation pressure increases, and systolic BP is elevated. Actually, in compliant arteries the reflected wave may encounter the forwarded wave during diastole, whereupon the AIx will be negative (Figure 2).

AIx is the most commonly used index of central wave reflection in the literature. However, AIx is not only influenced by wave reflection and thus person's age, height, sex and arterial stiffness, but also by left ventricular outflow and HR (Wilkinson *et al.* 2000, Kingwell and Gatzka 2002, Wilkinson *et al.* 2002). The association of HR with AIx is presented in more detail below. The relationship of age with AIx is not linear and it is strongly affected by prevailing cardiovascular risk factors (Janner *et al.* 2010).

Higher central wave reflection, measured as AIx, has been related with increased cardiovascular risk (Weber *et al.* 2004, Stamatelopoulos *et al.* 2006, Sugawara *et al.* 2007), but negative results of this relationship have also been reported in a study with hypertensive women (Dart *et al.* 2006) and in a large population from the Framingham Heart Study (Mitchell *et al.* 2010). Higher AIx has also been linked with type 1 diabetes, hypercholesterolemia, acute ischemic stroke, and mortality within patients with end-stage renal failure (Wilkinson *et al.* 2000, London *et al.* 2001, Wilkinson *et al.* 2002, Tuttolomondo *et al.* 2010). AIx has also been shown to significantly associate with arterial stiffness, measured as PWV (Yasmin and Brown 1999).

Figure 2. Aortic pulse wave form in compliant (A) and stiff (B) arteries.



Abbreviations: AP, augmentation pressure; PP, pulse pressure

PWV

Pulse wave travelling in the arteries is largely affected by artery diameter and structure of arterial wall, which are varying between proximal and distal parts of the arterial tree. When arterial tree is narrowing and the compliance is reducing (i.e. stiffness is increasing), PWV increases approximately from 5 m/s in the ascending aorta to 8 m/s in the iliac arteries (Latham *et al.* 1985, Laurent *et al.* 2006). The measurement of PWV has been accepted as the gold standard for the assessment of arterial stiffness, and is most often measured from the distance and transit time of the two wave forms between the carotid and femoral arteries (Laurent *et al.* 2006). PWV is measured from aortic region, because changes in the aorta are mostly responsible for the noticeable effects of arterial stiffness (Boutouyrie *et al.* 2002). PWV can be measured by several different techniques, and there are many different devices on the market for the non-invasive assessment of carotid-femoral PWV (Laurent *et al.* 2006).

PWV is a marker of arterial stiffness and is affected by age and cardiovascular risk factors (Laurent *et al.* 2006, Hamilton *et al.* 2007). Aging induces changes in the arterial wall structure: fracturing and reducing of the elastin lamellae and increase in collagen fibres, and age is thus an important determinant of arterial stiffness (Benetos *et al.* 1993, O'Rourke and Hashimoto 2007). The effects of ageing on arterial stiffness have been demonstrated to be more pronounced within older than younger subjects, and hence the association of ageing with arterial stiffness is nonlinear (McEniery *et al.* 2005). Several risk factors have been shown to influence arterial wall and increase stiffness. Both high ambulatory and office blood pressures are related with increased arterial stiffness (O'Rourke 1990, Schillaci *et al.* 2011), and increased PWV has also been assumed to sometimes predict hypertension and other cardiovascular events (Gedikli *et al.* 2010, Mitchell *et al.* 2010). Also other medical conditions as CHD (Gatzka *et al.* 1998, Boutouyrie *et al.* 2002), carotid artery disease (Saba *et al.* 1993) and diabetes (Sipilä *et al.* 2007) are determinants of arterial stiffness. Recently high PWV was also associated with poor outcome after acute stroke (Gasecki *et al.* 2012). Moreover, cardiovascular risk factors such as hypercholesterolemia, metabolic syndrome and smoking influence arterial wall structure and therefore increase arterial stiffness (Kool *et al.* 1993, Wilkinson *et al.* 2002, Sipilä *et al.* 2007, Kim *et al.* 2012). In addition, arterial stiffness has even been related with increased mortality in end-stage renal disease (Blacher *et al.* 2003).

Association of HR with AIx and PWV

Higher HR decreases central augmentation pressure and thus AIx because of shortening of the duration of systole and subsequent shifting of the reflected wave towards diastole (Wilkinson *et al.* 2000). Higher HR has been shown to associate with decreased AIx, even though the relationship of HR with the time to wave reflection is rather weak (Gatzka *et al.* 2001). The correlation of HR with AIx has been studied in many different populations. AIx decreased 4-5%-units for every 10 bpm increase in HR during cardiac pacing of 22 patients, and correlation of the same magnitude was found in a large study on subjects with cardiovascular risk factors (Wilkinson *et al.* 2000, Williams and Lacy 2009). Due to the well-described influence of HR on AIx, the value is commonly adjusted for HR 75 bpm (AIx@75) in many reports. However, also a lesser decrease (2.5%-units) of AIx for every 10 bpm increase of HR has been reported within healthy subjects (Sugawara *et al.* 2007). Hence, the correlation of AIx with HR may not be quite the same in different populations, and the outcome depends on age, prevailing diseases and sex distributions.

HR has also been associated with arterial stiffness, measured as PWV, but contrary to the relationship with central wave reflection, this correlation is direct (Sa Cunha *et al.* 1997, Lantelme *et al.* 2002, Millasseau *et al.* 2005, Park *et al.* 2010). The association of HR with PWV has been supposed to be one of the links between higher HR and less favourable prognosis (Lantelme *et al.* 2002). Nonetheless, HR may be a significant determinant of PWV, since left ventricular ejection time was independently associated with PWV in healthy men, and the association of HR with PWV was also shown to be independent of the prevailing blood pressure (Lantelme *et al.* 2002, Nurnberger *et al.* 2003).

6.3 Non-invasive assessment of cardiac function, central wave reflection and PWV

Electrical activity of the heart can be evaluated noninvasively using electrocardiography, both at rest and during exercise. There are several different methods for the non-invasive evaluation of cardiac pump function, from which the most commonly applied method in clinical practice is echocardiography. In addition to

direct imaging using ultrasound, cardiac pump function can also be evaluated indirectly by the use of whole body impedance cardiography (ICG_{WB}). As an advantage, PWV can be simultaneously measured with cardiac pump function by the use of ICG_{WB} (Kööbi 1999, Kööbi *et al.* 2003). Non-invasive central pulse wave form can be derived from radial tonometry measurement by the use of a validated transfer function (Laurent *et al.* 2006). The methods for cardiac function assessment, central pulse wave analysis and PWV measurement are presented below.

6.3.1 ECG

ECG is a presentation of cardiac electrical activity recorded by electrodes placed on body surface. The ECG measures, in addition to electrical activity of the cardiac muscle, HR and rhythm, also present indirect information about blood flow to the heart muscle (Guyton and Hall 2006). Normal ECG is formed by P-wave, QRS-complex and T-wave, from which P-wave represents electrical activity of atriums, QRS-complex depolarization of ventricles, and finally T-wave is caused by repolarization of the ventricular myocytes (Guyton and Hall 2006, Kligfield *et al.* 2007). The QRS-complex consists of three separate waves Q, R and S-waves. Electrical activity of the heart can be evaluated from the shape, timing and amplitude of the different waves.

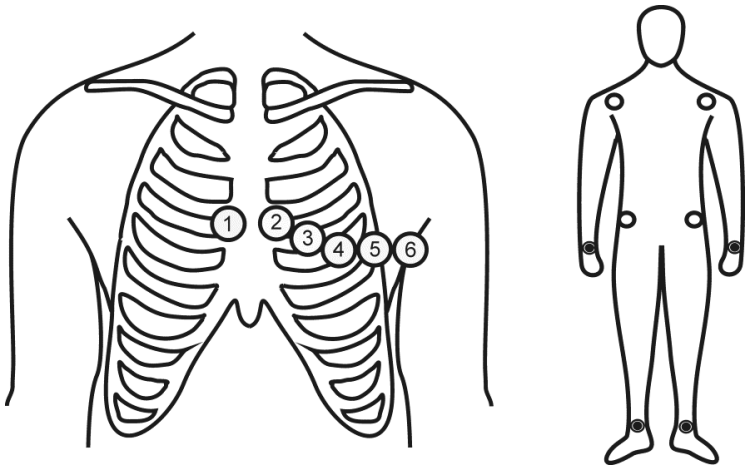
ECG recording at rest

Resting ECG is recorded for the evaluation of heart rhythm, HR, myocardial ischemia and conduction properties. The ECG is usually recorded with the standard 12-leads, where four electrodes are placed on wrists and ankles from which altogether 6 limb leads are derived (I, II, III, aVF, aVR, aVL). In addition, six electrodes are placed at specific locations on the surface of chest (chest leads V1-V6, Figure 3) (Kligfield *et al.* 2007). The 12-lead ECG enables several different views to inspect electrical activity of the heart.

The standard ECG recording is performed at rest in supine position to avoid the noise resulting from skeletal muscle activity. Different sources of errors like lead displacement, muscle tension, or poor contact of electrodes can cause misinterpretation of ECG. Hence the personnel recording the ECGs should be trained regularly for the

proper technique, as recommended by the American Heart Association (Kligfield *et al.* 2007).

Figure 3. Placement of chest electrodes during 12-lead electrocardiography recording and limb electrodes during standard recording (black circles) and during exercise testing recording (Mason-Likar system, white circles).



ECG during clinical exercise test

Clinical exercise test is performed for clarifying the cardiovascular responses to physical stress, which is generally induced by bicycle ergometer. The reasons for testing can be several like diagnosis of CHD or arrhythmias, evaluation of working capacity or drug therapy. During bicycle exercise test, the standard 12-lead ECG is recorded with the exception that the limb leads are placed on anterior iliac crest and upper outer arm according to the Mason-Likar system, Figure 3, (Mason and Likar 1966). This protocol reduces artefacts caused by limb movements during physical exercise. The different electrode placement may affect wave-forms in ECG, and the outcome is thus not fully comparable with the standard 12-lead ECG recording (Kligfield *et al.* 2007).

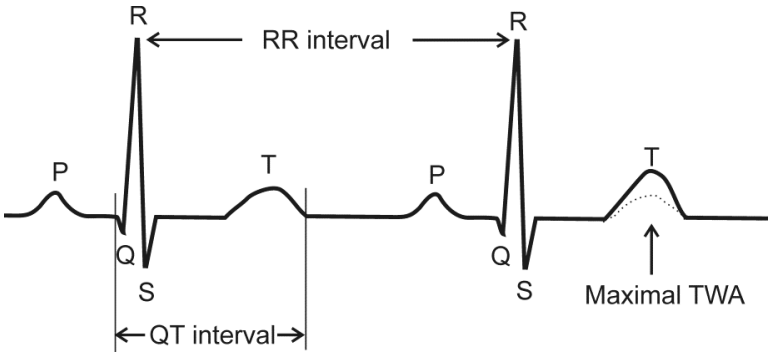
From the ECG recorded during exercise test the ECG parameters can be analysed automatically by appropriate software. The most important variables analysed are HR and rhythm, ST segment, QT interval, and also TWA.

Assessment of HR, QT interval and TWA from ECG

HR (i.e. ventricular rate) can be measured from the time difference of two consecutive R peaks in ECG (Figure 4). Because of beat to beat variability of HR, it is usually given as a mean of at least two separate RR-intervals in ECG (Guyton and Hall 2006). The duration of electrical activation of ventricles can be evaluated from QT interval (Figure 4), which mostly reflects the duration of repolarization and to lesser extent the duration of depolarization (Macfarlane *et al.* 2011). QT interval is strongly dependent on HR, i.e. QT interval is longer during lower HR, and thereby several algorithms have been developed for HR adjusting (Karjalainen *et al.* 1994, Luo *et al.* 2004). QT interval duration is also sex-dependent, and the QT interval tends to be longer within women. Hence the normal limits for QT interval are defined separately for men and women (Luo *et al.* 2004). The most commonly used HR correction methods for QT interval are Bazett's (QTcBaz) and Fridericia corrections (QTcFri), from which the first is performing better during lower HRs and the second during higher HRs (Luo *et al.* 2004). There are algorithms for automated QT interval analysis and HR corrections from resting and exercise test ECG (Kligfield *et al.* 2007).

TWA is an alternation of ventricular repolarization, and is defined as beat to beat variation of T-wave amplitude, shape or timing (Narayan 2006, Verrier *et al.* 2011) (Figure 4). TWA is considered to result from differences of repolarization timing between adjacent cardiomyocytes (Verrier *et al.* 2011). TWA analysing algorithms can also enable the measurement of nonvisible microvolt TWA. From the commercial TWA analysing methods, the time-domain Modified Moving Average (MMA) method enables TWA analysis during rest and exercise testing with standard ECG leads (Nearing and Verrier 2002, Nieminen *et al.* 2007, Verrier *et al.* 2011). The MMA method detects odd and even beats, and analyses the greatest difference of T wave shape between several consecutive odd and even beats (Verrier *et al.* 2011).

Figure 4. A typical electrocardiogram tracing from two consecutive cardiac cycles. Heart rate can be measured from the duration of RR interval and T wave alternans (TWA) from the change in two consecutive T waves (superimposed).



Electrocardiographic variables HR, QT interval, and TWA, and their association with prognosis

Over 25 years ago the relation of higher HR with increased all cause and cardiovascular mortality has been demonstrated for the first time in subjects with and without previous cardiovascular disease in epidemiological studies (Dyer *et al.* 1980, Kannel *et al.* 1987, Goldberg *et al.* 1996). In the studies by Kannel *et al.*and Dyer *et al.* higher HR was also a predictor of CHD (Dyer *et al.* 1980, Kannel *et al.* 1987). Later these relationships have been confirmed to be independent from other cardiovascular risk factors such as age, BP, BMI, diabetes, and smoking (Kovar *et al.* 2004, Diaz *et al.* 2005, Jouven *et al.* 2005, Cooney *et al.* 2010).

In some previous studies the associations of HR with mortality and morbidity have been weak or even lacking in women, and HR has therefore been thought to be only a weak predictor of prognosis in females (Kannel *et al.* 1987, Goldberg *et al.* 1996). However, Gillum *et al.* and Cooney *et al.* proved the relationship also within women in large epidemiological studies (Gillum *et al.* 1991, Cooney *et al.* 2010). Higher HR has also been shown to be a risk factor for adverse events in heart failure and hypertension (Bohm *et al.* 2010). Thus, higher HR has been identified as an independent risk factor for cardiovascular events in both sexes, but it is still not commonly utilised in cardiovascular risk assessment. In addition, HR has also been associated with other adverse cardiovascular findings like increased blood pressure and arterial stiffness (Sa Cunha *et al.* 1997, Wilkinson *et al.* 2002, Park *et al.* 2010, Fernandes *et al.* 2011).

However, the pathophysiology behind the relationship between higher HR and poor prognosis remains largely unresolved.

Prolonged QTc interval is related with increased all-cause and cardiovascular mortality and risk of sudden cardiac death in general populations of different ages (Karjalainen *et al.* 1997, Straus *et al.* 2006, Noseworthy *et al.* 2012). However, in the large epidemiological Framingham Study, QTc was not related with mortality when the Bazett correction method was utilised, but positive relationship was found in the same population when another HR correction method for QT interval was used (Goldberg *et al.* 1991, Noseworthy *et al.* 2012). The incidence of sudden cardiac death is also related with prolonged QTc interval during exercise testing within CHD patients, as QTc > 440 ms during peak exercise was more common within patients who would encounter sudden cardiac death (62%) vs. controls (15%) (Yi *et al.* 1998). In addition, prolonged QTc is also associated with more severe CHD and poor prognosis in patients with acute coronary syndrome (Gadaleta *et al.* 2003).

The association of QTc with mortality in CHD patients may be sex-related, since the association was more pronounced in men than women in a large coronary angiography population (n=19 252) (Williams *et al.* 2012). Prolonged QTc is found more often in cardiomyopathy patients than in the general population, and long QTc interval has also been related with the severity of cardiomyopathy (Johnson *et al.* 2011). In addition, inherited and drug-induced LQTS are related with mortality and ventricular arrhythmias in several studies (Morita *et al.* 2008). Prolonged QT interval increases the risk of life-threatening ventricular arrhythmias, which might be the explanation for these above findings.

Increased TWA is characteristic of different conditions of cardiac dysfunction like CHD, LQTS, congestive heart failure and cardiomyopathy, and higher magnitude of TWA at least moderately increases the risk of cardiac death and ventricular arrhythmias during these conditions (Narayan 2006, Chow *et al.* 2008, Gold *et al.* 2008, Slawnych *et al.* 2009, Calo *et al.* 2011, Gupta *et al.* 2012). In the aforementioned studies, different algorithms for TWA analyses were used, and the deviations in the methods might have influenced the results. Furthermore, increased magnitude of TWA is also associated with mortality in a low-risk clinical exercise test population, in which the relative risk for sudden cardiac death was 7.4, for cardiovascular mortality 6.0, and for all-cause mortality 3.3, in subjects with TWA $\geq$ 65 μV when compared with controls (Nieminen *et al.* 2007, Minkkinen *et al.* 2009). Of note, in the same

population TWA during exercise was a more powerful predictor for mortality than TWA during rest or recovery after exercise (Minkkinen *et al.* 2009). Finally, an increase in TWA may also precede ventricular fibrillation and ventricular tachycardia in implantable cardioverter-defibrillation patients when compared with the level of TWA during control measurements in the same patients (62.9 vs. 12.8 µV) (Swerdlow *et al.* 2011).

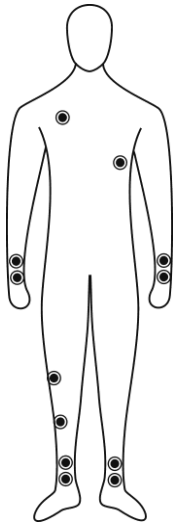
6.3.2 ICG_{WB}

The ICG_{WB} is a non-invasive method for CO measurement, and it has shown to be reliable when compared with other non-invasive CO measurement methods and also with the invasive thermodilution CO determination (Kööbi *et al.* 1997, Kööbi *et al.* 1997, Kööbi 1999, Cotter *et al.* 2004). During ICG_{WB} measurement high frequency (30 kHz) alternating current is applied to the extremities, and voltage is measured by other electrodes on the body surface. Because of the low resistance of blood, most of the current is passing through the main vessel tree in the body. The measurement of CO by ICG_{WB} is based on the changes in the conduction properties of the large vessels during wave pulsation within the cardiac cycle. It should be noted that ICG_{WB} also allows clinically reliable CO measurement during different body positions (Kööbi 1999).

PWV can also be determined by the use of the ICG_{WB} device. The device measures the decrease of the whole body impedance signal when the pressure wave is entering the aorta. Moreover, the decrease in distal impedance is measured from the knee joint level at region of the popliteal artery. The time difference of the whole body impedance signal and popliteal artery signal is measured (and the distance of distal electrode is measured from body surface), from which PWV can be calculated (Kööbi *et al.* 2003).

The voltage electrodes for ICG_{WB} recording are placed just proximally of both wrists and ankles, and another pair of electrodes (current electrodes) is placed about 5 centimetres distally of the voltage electrodes. In addition, two electrodes are placed on the thorax area responding to V5 channel in ECG, and electrodes for the recording of the distal impedance of popliteal artery are placed on knee-joint level and another electrode 20 centimetres distally from that (Figure 5).

Figure 5. Placement of whole-body impedance cardiography electrodes, including distal electrodes in the popliteal region for pulse wave velocity measurement.



6.3.3 Central pulse wave analysis by arterial applanation tonometry

Central pulse wave form can be determined noninvasively from carotid artery, or indirectly by peripheral applanation tonometry, so that the pressure sensor is placed on the artery and pulse wave form is measured (O'Rourke *et al.* 2001). Generally, a pen-like sensor is placed on the radial artery and 10 consecutive pulsations are recorded for pulse wave analysis. A technically more advanced method is the use of an automated tonometric sensor with a wrist band, which enables continuous pulse wave recording (Tahvanainen *et al.* 2009). For central pulse wave estimation, the peripheral pulse wave form is processed by the use of a generalized transfer function (Karamanoglu *et al.* 1993, Pauca *et al.* 2001). The transfer function has been validated against invasive aortic pulse wave measurements, as Pauca *et al.* (Pauca *et al.* 2001) studied 62 patients during cardiac surgery and found that the estimated central pulse wave measurements showed good agreement with direct invasive pressure measurements. In addition, a good correlation of the estimated pulse wave measurements with directly measured central pressure waves has been found during an exercise test, performed by cycling in supine position (Sharman *et al.* 2006). There are several different devices for pulse wave analyses on the market, from which the SphygmoCor system is most widely used. Peripheral tonometry provides information about central BP and central wave

reflection, given as augmentation pressure and AIx, as previously described in the section 6.2.2.

During peripheral applanation tonometry, BP is calibrated by the use of brachial BP measurements with a sphygmomanometer. However, the BP measurements by brachial cuff have not shown to be totally congruent with invasive BP measurements, and hence this matter is the greatest source of error during the pulse wave analysis (O'Rourke *et al.* 2001, Zuo *et al.* 2010). In some studies the SpygmoCor device may have underestimated the level of central BP (Zuo *et al.* 2010, Ding *et al.* 2011). In addition, calibration of radial BP is performed from brachial artery site, and it is commonly known that arterial pressure pulse increases when travelling towards periphery due to a phenomenon called amplification. A large epidemiological study has shown that the noninvasively measured amplification is reduced with increasing age, and it is also higher in men than women (Segers *et al.* 2009). Altogether, radial artery PP was approximately 8 mmHg higher when compared with the brachial artery PP (Segers *et al.* 2009). In addition, in a small invasive study, the difference between brachial and radial systolic BP was about 5 mmHg during cardiac catheterization ($p<0.002$) (Davies *et al.* 2010). Nevertheless, peripheral applanation tonometry is highly repeatable, even if applied by an inexperienced personnel, and it is a commonly used method for central pulse wave analysis (Crilly *et al.* 2007).

6.3.4 Tilt table test – a physical challenge

In addition to supine measurements of haemodynamics, marked changes in the function of the heart and vascular system can be produced by tilt table testing (Avolio and Parati 2011). The change in posture from supine to upright offers a simple physical challenge in laboratory circumstances, during which haemodynamic variables can be recorded for example by the use of sphygmomanometer, ICG_{WB}, ECG, or applanation tonometry. The haemodynamic changes arise from an increase in sympathetic activation and alteration of body fluid distribution during head-up tilt. The most widely applied clinical indication for tilt table testing is in the diagnostics of syncope, but a generally approved method or protocol for this is still lacking (Sheldon 2013).

7. Aims of the study

The aim of the present study was to identify the genetic and haemodynamic background of the electrocardiographic variables i.e., HR, QT interval, TWA, which can be used in risk stratification and evaluation of prognosis in cardiovascular diseases. The specific aims were as follows:

1. To evaluate whether the polymorphism rs1805123 of the KCNH2 gene influences QT interval and TWA during different phases of a physical exercise test (**I**).

2. To investigate the effects of three QT interval-related cardiac ion channel gene SNPs, rs1805127 and rs727957 in KCNE1, as well as rs1805124 in SCN5A, on TWA during clinical exercise test and 4-year mortality in a Finnish population (**II**).

3. To evaluate the association of SNP rs10494366 in NOS1AP with TWA during clinical exercise test and 4-year mortality in a Finnish population (**III**).

4. To examine the association of resting HR with the principal haemodynamic determinants of BP, in both supine and upright positions, in a cross-sectional study in a Finnish population without major diseases and medications that would have direct influences on cardiovascular function (**IV**).

8. SUBJECTS AND METHODS

8.1 Subjects and design of the studies I-III

The participant pool of studies **I-III** consisted of the patients who participated in the Finnish Cardiovascular Study (FINCAVAS), which is a follow-up study focusing on, among other issues, the genetic background of exercise stress test responses (Nieminen *et al.* 2006). The study subjects underwent clinical exercise stress tests at Tampere University Hospital between October 2001 and December 2004. All patients who participated in the study were recruited, but only those with successful exercise test data were included in analyses (n=2212). There were six different indications for the exercise stress test, while some patients had more than one indication. The indications were a suspected CHD (999 patients) or arrhythmias (473), evaluations of drug therapy (348) and working capacity (400), as well as ascertaining the patient's status prior to an invasive operation (294) or after an acute myocardial infarction (AMI, 179). Only patients with available genotype data after laboratory determinations were included in the final analyses, and in the study I 79 patients with temporarily ceased β-blocker medication 2-14 days before the test were excluded from the final analyses because of a possible rebound effect. A total of 1975, 2008, and 1963 participants were included in studies **I**, **II** and **II**, respectively.

Information of patients' medical history, lifestyle habits and cardiovascular risk factors were collected by a questionnaire. Also medication was recorded, and since β-blockers are altering cardiac repolarization duration, subjects were categorized as β-blocker users, non-users and subjects with β-blocker medication break. A temporary withdrawal of β-blocker medication was defined as 2–14 days because of a possible rebound effect. Digital angiographic data from 426 study patients was analysed in detail by a cardiologist blinded to all other study data. The existence of CHD was defined either as a previous myocardial infarction or more than 50% tapering in at least one major coronary artery in angiography. Blood samples were collected for genetic analyses.

8.2 Methods of the studies I-III

8.2.1 Clinical exercise test

The exercise tests with three phases of interest, rest before the test, during the exercise test, and recovery phase, were performed using a bicycle ergometer with electric brakes. The 12-lead ECG with standard Mason-Likar modification for the lead system (Mason and Likar 1966) was recorded at 500 Hz with CardioSoft exercise ECG system (Version 4.14, GE Healthcare, Freiburg, Germany). Completely automate analysis for the ECG signal was performed by MMA method released by GE Healthcare. During the test the systolic and diastolic blood pressures were measured with a brachial cuff every two minutes.

8.2.2 Measurement of QT interval and TWA

In study I, QT interval was measured at each test phase using the GE algorithm applied in Case Workstation software (GE Healthcare, Freiburg, Germany). The GE algorithm defines the earliest QRS onset and the latest T-wave offset in all used leads, thus providing the longest value for the QT interval. The QT interval was then corrected for HR by using Fridericia and Bazett's corrections. QT intervals were determined during rest before test, during maximal exercise, and during 1 minute and 3 minutes of recovery.

The algorithm for identification of TWA was based on the time-domain MMA analysis, which has been described earlier in detail by Nearing and Verrier (Nearing and Verrier 2002). The algorithm performs the measurement of TWA at an accuracy and discrimination of 1 μV. So, the TWA values were calculated continuously during the entire exercise test from rest to recovery. The TWA values during HR frequencies exceeding 125 bpm were considered unreliable, and were excluded. The highest magnitude of TWA during rest, exercise and recovery was taken into analysis.

8.2.3 DNA extraction and genotyping

DNA was obtained from peripheral blood leukocytes by using a commercially available kit and Qiagen BioRobot M48 Workstation under manufacturer's instructions (Qiagen Inc., Hilden, Germany). DNA genotyping was performed by employing the 5' nuclease assay and fluorogenic allele-specific TaqMan probes (Livak 1999) using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The samples were pipetted by TECAN Freedom EVO-100 automated robot (Tecan Group Ltd, Männedorf, Switzerland). The nucleotide sequences of the primers and probes used in the polymerase chain reactions were constructed from sequences published by the GenBank and Celera databases and synthesised by Applied Biosystems. The polymerase chain reactions, containing genomic DNA, 1 × Universal PCR Master Mix, 900 nM of each primer and 200 nM of each probe in a total volume of 5 μl, were performed in 384-well plates using the standard protocol. The final point fluorescence was measured and genotype calling carried out by the allelic discrimination analysis module after the polymerase chain reaction resulting in identification of the rs1805123 genotype of the KCNH2 gene, the rs1805147 and rs727957 genotypes of the KCNE1 gene, the rs1805124 genotype of the SCN5A gene, and rs10494366 of NOS1AP gene. Random duplicates were used as quality control.

8.2.4 Follow up of survival

Documents of deaths were received from the Causes of Death Register, maintained by Statistics Finland, in April 2007. The documents contained causes of death according to the International Classification of Diseases. The codes of the diagnoses and document texts were used to classify the deaths as all-cause, cardiovascular, and sudden cardiac death. Sudden death was defined as a death within 24 h after the onset of symptoms.

8.2.5 Statistical analyses of the studies I-III

To examine the possible differences of QTc intervals and TWA between the studied genotypes or allele carriers vs. non-carriers at the different exercise test phases analysis of variance for repeated measures (RANOVA) was applied. Age, β-blocker medication

status (yes/no in study **I** and yes/no/medication break in studies **II-III**), CHD (yes/no), and BMI were used as covariates. Because of different repolarization durations, male and female subjects were analysed within their own groups and against each other.

Distributions of categorical variables were compared between different genotype groups by χ^2-test and numerical demographic variables by Student's t-test. The unadjusted QTc intervals and TWA values for each genotype among men and women, respectively, were compared using analysis of variance (ANOVA) with LSD post-hoc test. All tests were performed for the QT intervals corrected with both Fridericia and Bazett's formulas in the study **I**. In addition, TWA was classified as normal and abnormal using 65 microvolts as cut-off point, since TWA is a significant predictor of death when using this cut-off point (Nieminen *et al.* 2007). Distributions of normal and abnormal TWA were compared between different genotype groups with χ^2-test.

Conformity of the genotype proportion to the Hardy-Weinberg equilibrium was determined for all genotypes studied. In the study **II** haplotypes of KCNE1 (SNP rs727957 and SNP rs1805127) were estimated from the 2 single-nucleotide polymorphisms using the PHASE 2.1.1 program (Stephens and Donnelly 2003) which uses a Bayesian statistical method for generating haplotypes and then lists the most probable haplotype pairs for each individual.

Survival of different genotype and haplotype carriers vs. non-carriers were examined by Cox regression analysis and reported by means of hazard ratios (HR) and 95 percent confidence intervals (95 % CI) in the studies **II** and **III**.

All analyses were performed by the SPSS 11.5 or 17.0 for Windows (SPSS Inc, Chicago, Illinois, USA).

8.3 Subjects and design of the study IV

Study **IV** subjects were involved in an on-going DYNAMIC study in which haemodynamics are noninvasively recorded from voluntary subjects. Study subjects were enrolled by announcements which were distributed at the University of Tampere, Tampere University Hospital, several occupational health care organizations, Varala Sports Institute, and two announcements were published in a local newspaper. The

subjects who contacted the research nurse were recruited in that order respectively. In October 2012 a total number of 830 subjects were recruited for the study.

In this study subjects with medical history of coronary artery disease, diabetes mellitus, cerebrovascular disease, valvular regurgitation or stenosis, long QT syndrome, chronic renal insufficiency, hemochromatosis or other considerable diseases were excluded. Also, subjects with regular medication influencing HR or BP were excluded, that is medication for hypertension, arrhythmias, and use of long-acting β2-sympathomimetics, α-adrenoceptor agonists, or the weight-reducing agent sibutramine. One person with recently initiated varenicline was also excluded. In total of 522 subjects (aged 20 to 72 years) with technically successful haemodynamic records were included in present study.

All subjects were submitted to physical examination performed by a physician, who also documented medical history, lifestyle habits, and cardiovascular risk factors by interview. Fourteen subjects were on lipid-lowering medication, and 81 of 261 women were on low-dose progesterone (intrauterine device) or combination of estrogen and progesterone therapy. All regular medications of the study population are presented in Table 2. Subjects who had quit smoking over a year before the investigation were defined as non-smokers.

Table 2. Regular medications of subjects in study IV.

	Resting heart rate tertiles			All
	1	2	3	
Medication	n=172	n=176	n=174	n=522
Allopurinol	1	0	1	2
Androgens	1	0	0	1
Antidepressants	14	5	10	29
Antiepileptic agents	2	1	2	5
Antihistamine	4	5	7	16
Anxiolytic agents	2	0	1	3
Bisphosphonate	1	0	0	1
Carbimazole	1	0	0	1
Corticosteroid, intranasal or inhaled	6	4	5	15
Dopamine agonists	0	0	1	1
Female hormones*	26	30	26	82
Glucosamine	2	4	0	6
Hypolipidemic agents (statins)	4	3	6	13
Isotretinoin	0	1	0	1
Melatonin	1	0	1	2
NSAID	2	1	1	4
Oxybutynin	0	0	1	1
Proton pump inhibitors	0	5	9	14
Salazosulphapyridine	0	1	0	1
Tamoxifen	0	1	0	1
Tamsulosin	1	1	0	2
Thyroxin	4	5	6	15
Warfarin	0	1	0	1

Abbreviations: n, number of subjects; NSAID, nonsteroidal anti-inflammatory drugs.

*Including peroral medications and intrauterine devices.

8.4 Methods of the study IV

8.4.1 Haemodynamic measurement

Haemodynamics were recorded in a quiet, temperature-controlled laboratory by a research nurse. The study subjects had refrained from smoking, caffeine-containing products and heavy meal for at least 4 h, and alcohol for at least 24 h prior to the measurement. Before the actual haemodynamic recording the subjects were resting supine for approximately 10 minutes, during which period the electrodes for ICG_{WB} were positioned on the body surface, a sensor for pulse wave analysis was fixed to the left wrist on the radial artery pulsation, and a brachial cuff for BP calibration was placed to the right upper arm. Then haemodynamic variables were continuously recorded in a beat-to-beat fashion for 5 minutes in supine position and 5 minutes during passive head-up tilt representing simple functional test.

8.4.2 ICG_{WB} recording

ICG_{WB} device (CircMonR, JR Medical Ltd, Tallinn, Estonia), was used to determine beat-to-beat HR, stroke index (SI, which is SV in proportion to body surface area, ml/m^2), cardiac index (CI, which is CO in proportion to body surface area, $l/min/m^2$), and PWV (m/s) (Kööbi *et al.* 1997, Kööbi 1999, Kööbi *et al.* 2003). In addition, left cardiac work index (LCWI, $kg*m/min/m^2$) was calculated by formula 0.0143*(MAP – PAOP)*CI, which has been derived from the equation published by Gorlin *et al.*(Gorlin *et al.* 1955) and where MAP is mean radial arterial pressure measured by tonometric sensor, PAOP is pulmonary artery occlusion pressure which was assumed to be normal (6 mmHg), and 0.0143 is the factor for the conversion of pressure from mmHg to cmH_2O, volume to density of blood (kg/L), and centimetre to metre. Systemic vascular resistance index (SVRI, which is SVR in proportion to body surface area, $dyn*s/cm5/m^2$) was calculated from the signal of the radial tonometric BP sensor and the CI measured by CircMonR device. Electrons for ICG_{WB} recording were placed on body surface according to instructions of CircMonR manual.

The measurement of the PWV is based on a decrease of impedance occurred by aortic diameter changes during pulse wave travelling. The CircMon software measures

the time difference between the decrease in impedance of the whole-body impedance signal and the signal of popliteal artery. From the time difference and the distance between the electrodes, the PWV can be determined. Because the ICG_{WB} slightly overestimates PWV when compared with Doppler ultrasound method, a validated equation was performed by CircMon software to calculate PWV corresponding to ultrasound method (PWV = (PWV-impedance *0.696) + 0.864) (Kööbi *et al.* 2003). The CO values measured with CircMonR are in good agreement with the values measured by the thermodilution method (Kööbi *et al.* 1997), and the repeatability and reproducibility of the measurement protocol have been shown to be reliable (Tahvanainen *et al.* 2009).

8.4.3 Pulse wave analysis

Radial BP and pulse wave form were continuously determined by the use of an automatic tonometric sensor (Colin BP-508T, Colin Medical Instruments Corp., USA), which was fixed on the radial artery pulsation with a wrist band. Radial BP signal was calibrated approximately every 2.5 minute by brachial BP measurement. Continuous aortic BP was derived with the SphygmoCor pulse wave monitoring system (Sphygmo-Cor PWMx, AtCor Medical, Australia) using the validated generalized transfer function (Chen *et al.* 1997). From the aortic pulse wave form, both AIx and AIx@75 were determined. In addition, the ratio of energy supply to energy demand of the heart was assessed using the Buckberg subendocardial viability ratio (Buckberg *et al.* 1972), which was also automatically derived from the aortic pulse waveform.

8.4.4 SV measurement by echocardiography

To evaluate the reliability of the SV estimation with ICG_{WB} during head-up tilt, echocardiography was performed to a subset of subjects (n=16). Three-dimensional echocardiography (Philips ie33 ultrasound system, Bothell, USA, with 1-5 MHz Matrix-array X5-1 transducer) was performed by cardiologist simultaneously with beat-to-beat ICG_{WB} recordings during head-up tilt. Mean SV from 7 consecutive heart beats (6 before and 1 after echocardiography) was analysed from ICG_{WB} recordings to cover approximately one respiratory cycle (approximately 5 seconds).

8.4.5 Laboratory analyses

Venous blood samples were drawn after 12 hours of fasting. Plasma sodium, potassium, glucose, creatinine, C reactive protein (CRP) , triglyceride, and total, high-density (HDL) and low-density lipoprotein (LDL) cholesterol concentrations were determined by Cobas Integra 700/800 (F. Hoffmann-LaRoche Ltd, Basel, Switzerland) or Cobas6000 module c501 (Roche Diagnostics, Basel, Switzerland) and blood cell count by ADVIA 120 or 2120 (Bayer Health Care,Tarrytown, NY, USA) devices. All analyses were done in accredited (FINANS, Finnish Accreditation Service nro. T043) Fimlab Laboratories Ltd. by using standardized methods and following international laboratory standards (EN ISO/IEC 17025, EN ISO 15189).

8.4.6 Statistical analyses of the study IV

The study population was divided into tertiles according to mean resting HR for the statistical analyses. Mean resting HR was determined by average of the last three minutes of the 5 minutes measurement period in the supine position as the recordings were most stable during this period. Studying the differences in the haemodynamic variables BP, SI, CI, LCWI, SVRI, AIx, and PWV within the HR tertiles the analysis of variance (ANOVA) was applied. RANOVA (test of within-subjects effects) was performed to study the haemodynamic responses to head-up tilt. For post hoc testing Tukey HSD test was performed for homogenous, and Tamhane's T2 test for nonhomogenous variables. In addition, sex, age, body mass index (BMI), smoking pack years, haematocrit, leukocyte count, CRP, creatinine, cystatin C, total cholesterol, triglycerides, HDL-cholesterol, fasting plasma glucose, and mean radial arterial pressure at rest were used as confounding factors in covariance analyses (ANCOVA and RANCOVA). Sidak post hoc test was used in adjusted analyses.

In addition resting HR was inspected as continuous variable. Associations between HR and other above mentioned haemodynamic variables were tested by linear regression analysis using BMI, age and sex as confounding factors.

For analysing the absolute changes in haemodynamic variables from supine to head-up tilt the mean value of the last three minutes of both positions were applied. Changes of haemodynamic variables from supine to upright position between the HR tertile groups were analysed by ANOVA and ANCOVA.

Correlations between echocardiographic and impedance cardiographic SV measurements were tested by Pearson's correlation and differences between methods were tested by Student's T-test.

Distributions of categorical variables among HR tertiles were tested by $\chi 2$ test and differences of numerical variables among HR tertiles were studied by ANOVA. Variable values are represented as means ± standard deviation if otherwise is not mentioned. Natural logarithms of CRP and triglyceride concentrations were used in analyses for normalizing their distribution. P-values < 0.050 were considered statistically significant. The analyses were performed with the SPSS Statistics 17.0 for Windows Software.

8.5 Ethical aspects

Both the clinical exercise test study (FINCAVAS) and the haemodynamic measurement study protocols (DYNAMIC study) were approved by the Ethics Committee of the Hospital District of Pirkanmaa, Finland, and all patients gave informed consent preceding study initiation, as stipulated in the Declaration of Helsinki.

9. RESULTS

9.1 Cardiac repolarization genetics, studies I-III

9.1.1 Population characteristics in the studies I-III

In total 2212 patients underwent clinical exercise testing, from whom genotyping was successfully carried out as follows: rs1805123 of KCNH2 from 1975 subjects, rs727957 of KCNE1 from 1962 subjects, rs1805127 of KCNE1 from 1904 subjects, rs1805124 of SCN5A from 1910 subjects, and rs10494366 of NOS1AP from 1963 subjects. All genotype distributions followed the Hardy-Weinberg equilibrium and they are presented in Table 3.

In the exercise test population (**I-III**), the number of male subjects was greater than the number of females, and also the background characteristics were different between sexes. As the duration of repolarization is known to be longer within women, the QTc and TWA variations were studied separately for both sexes. The population demographics during rest for each study, separately for men and women, are given in Table 4.

Table 3. Genotype distributions of the studied single nucleotide polymorphisms.

	Study I				Study II				Study III	
	KCNH2 rs1805123		KCNE1 rs727957		KCNE1 rs1805127		SCN5A rs1805124		NOS1AP rs10494366	
Genotype	n	%	n	%	n	%	n	%	n	%
AA	1412	71.5	1381	70.4	670	35.2	1203	63.5	865	44.1
Aa	513	26.0	540	27.5	916	48.1	604	31.5	862	43.9
aa	50	2.5	41	2.0	318	16.7	95	5.0	236	12.0

Table 4. Population characteristics (mean±SD) of the studies I-III at rest.

	Study I (n = 1975)		Study II (n= 2008)		Study III (n=1963)	
	Female	Male	Female	Male	Female	Male
	n = 716	n = 1259	n = 731	n = 1277	n = 718	n = 1245
Age (years)	57.5±13.2	56.8±13.0	57.6±13.2	56.9±13.1	57.7±13.0	56.9±13.0
Smoking (n / %)	110/15%†	420/33%	115/16%†	424/33%	110/15%†	408/33%
Body mass index (kg/m2)	27.3±4.9	27.5±4.2	27.3±4.7	27.5±4.3	27.3±4.8	27.5±4.2
Systolic blood pressure (mmHg)	139±23*	134±18	139±20*	135±18	139±20*	135±20
Diastolic blood pressure (mmHg)	79±10	80±10	79±10*	80±10	79±10	80±10
Heart rate (1/min)	65±11*	62±12	65±11*	62±12	65±11*	62±12
T wave alternans (μV)	20.0±12.5	20.4±13.0	19.9±12.4	20.3±13.0	19.9±12.3	20.4±13.2
Heart rate corrected QT interval (ms)	423±24	421±25	423±24	420±25	423±24	421±25
β-blocker medication (n / %)	371/52%†	812/65%	354/51%†	791/64%	352/49%†	772/62%
Coronary heart disease (n / %)	222/31%†	592/47%	225/31%†	588/46%	220/31%†	575/46%

Statistics: Statistically significant difference between sexes: *(T-test, $p<0.001$); †(χ^2 test, $p<0.001$). **Definitions:** Heart rate correction of QT intervals with the Fridericia formula.

Indications for the exercise test were suspicion of CHD (n=999, 45%), palpitation or sense of arrhythmia (n=473, 21%), evaluation of working capacity (n=400, 18%) and drug therapy (n=348, 16%), as well as evaluation of the patient's exercise performance preoperatively (n=294, 13%) or after an acute myocardial infarction (n=179, 8%). Some of the subjects had more than one indication.

9.1.2 Subject characteristics and repolarization

In the study I, QTc intervals were studied in addition to TWA responses. The QTc intervals differed significantly between sexes. The QTcFri interval tended to be longer within women than men at rest ($p=0.081$), while men presented with longer QTcFri at the maximal exercise and during the recovery phases ($p<0.001$).

Within patients with β-blocker medication QTcFri intervals were longer when compared with unmedicated patients at all recording phases ($p<0.001$). However, the finding was quite different with the other correction method. The QTcBaz intervals were shorter in patients on β-blocker medication when compared with QTcBaz of subjects without such medication during exercise test ($p<0.001$). QTcFri at rest did not differ between CHD and non-CHD patients ($p=0.273$), but QTcFri was longer within CHD patients during exercise and recovery ($p=0.023$ and $p=0.002$, respectively).

In the study I, sex did not affect TWA responses as significantly as it did with QT intervals. TWA did not significantly differ between sexes during rest (Table 5) or during exercise ($p>0.05$, for both). At recovery phase, the magnitude of TWA was significantly higher within men than women 28±19 vs. 25±14 ($p<0.001$). Sex differences in the magnitude of TWA were similar in the studies II and III, too. In addition, from confounding factors (presented as subgroups), age was related with TWA at all exercise test phases (rest, exercise and recovery, Table 5). The magnitude of TWA was higher with β-blocker medicated vs. non-medicated subjects during exercise and recovery but not at rest.

In the studies I-III age was not a determinant of TWA in any of the test phases when linear regression analysis was applied ($R^2 \leq 2.2\%$). However, TWA was higher within patients aged 60 years or older when compared with younger ones during the whole exercise test ($p \leq 0.003$ for all exercise test phases respectively). The use of β-adrenoceptor blockers did not affect TWA at rest, but TWA was higher in the presence

than absence of β-blocker medication during the exercise and the recovery phases ($p<0.001$, for both respectively).

In the study III, knowledge on the use of hormone replacement therapy was available from 589 (82.0%) women of which 24.1% were hormone users. The therapies included estrogen alone or a combination of estrogen and progesterone. Within women, estrogen medication had statistically a borderline influence on TWA responses during the exercise test (RANOVA, $p = 0.056$), so that TWA tended to be higher within hormone users during rest and exercise, but was lower during recovery compared to subjects without hormone therapy.

Table 5. T wave alternans (mean±SD) values according to the sexes, age group tertiles, and β-blocker medicated and non-medicated subjects during exercise testing.

	Rest (μV)	Exercise (μV)	Recovery (μV)
Sex			
Men	20±13	36±20	28±19
Women	20±12	36±21	25±14
p-value	0.427	0.861	<0.001
Age group			
Tertile 1: ≤ 53 years	19±12	33±17	25±21
Tertile 2: 54-63 years	20±13	37±25	25±14
Tertile 3: ≥ 60 years	22±13	38±21	31±16
p-value	<0.001*†	<0.001*‡	<0.001*‡
β-blocker medication			
No	20±13	33±19	25±18
Yes	20±13	38±21	29±18
p-value	0.475	<0.001	<0.001

Statistics: Age group comparison *p<0.001 1 vs. 2, †p<0.01 1 vs. 2 and ‡p<0.001 1 vs. 3 in Tukey's HSD post hoc testing.

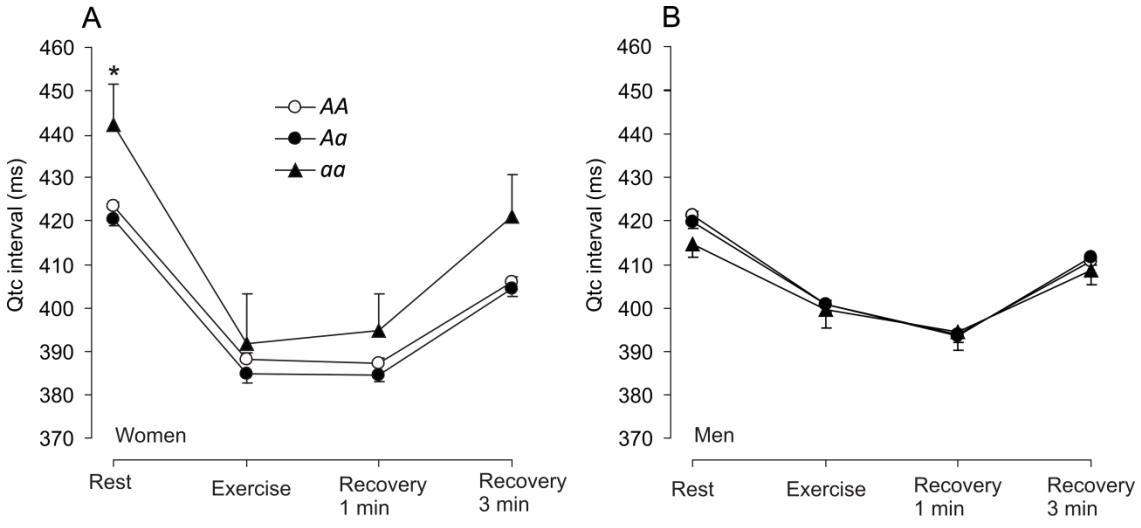
9.1.3 Association of SNPs with repolarization

SNP rs1805123 of KCNH2

In the study I, the QTcFri intervals did not differ between the three genotypes within the whole study population (p=0.470). Women with the minor *aa* genotype tended to have longer QTcFri intervals during the entire test, but the difference was statistically significant only during rest (p=0.011; LSD post hoc analysis: *aa* vs. *AA* p=0.011, *aa* vs. *Aa* p=0.004) (Figure 6A). The finding at rest remained significant after adjustment for β-blocker medication, CHD, BMI, and age (p=0.012). In men, no differences of QTc intervals (p=0.311) were observed between the genotypes (Figure 6B).

The results concerning QTc intervals did not significantly differ when the QTcBaz vs. QTcFri intervals were used. The present results are reported as Fridericia corrections, because this has been demonstrated to be more accurate in populations with high HR dispersion (Karjalainen *et al.* 1994, Luo *et al.* 2004).

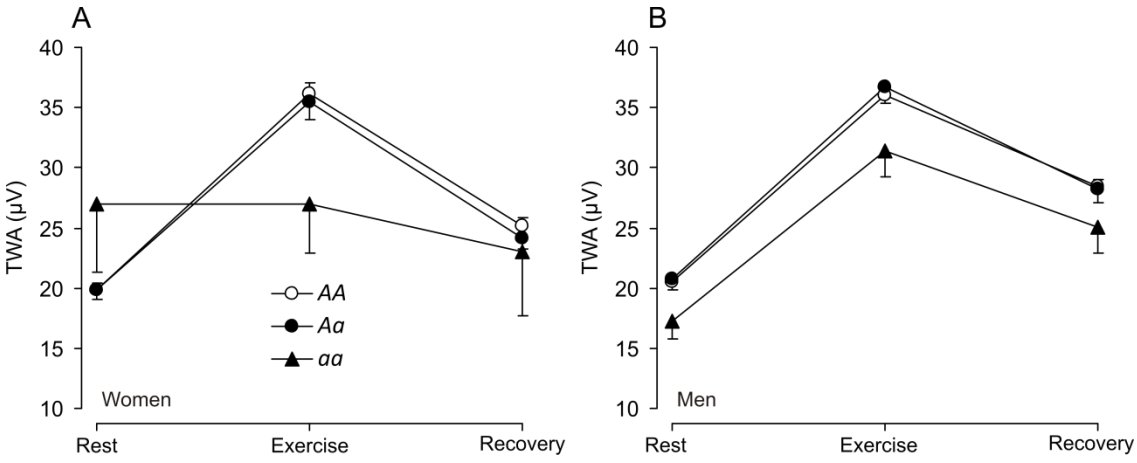
Figure 6. Fridericia corrected QTc intervals (mean±SEM) according to rs1805123 of KCNH2 genotypes within women (A) and men (B). *Statistically significant difference in QTc between *aa* and other genotypes (*aa* vs. *AA* p=0.011, *aa* vs. *Aa* p=0.004).



Within the whole population, TWA did not differ between the three genotypes during the exercise testing (p=0.20) or within women and men respectively (p=0.43 and p=0.20) (Figure 7). Within men, the minor *aa* genotype tended to present numerically smallest TWA during the entire exercise test, but the differences were not

statistically significant (p=0.07 *aa* vs. *AA*, and p=0.09 *aa* vs. *Aa*). The distributions of normal and abnormal TWA did not differ between the three genotype groups when analysed using the cut-off point 65 µV (p=0.54).

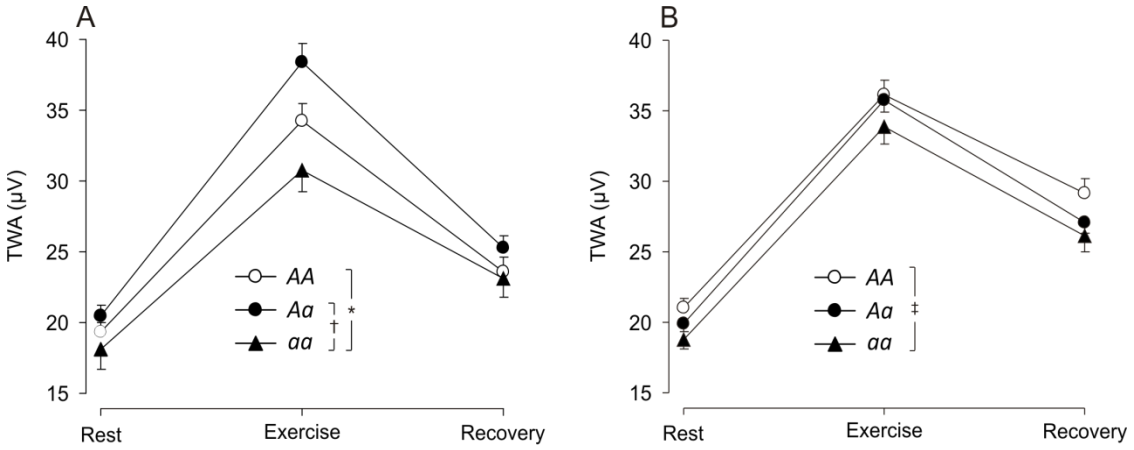
Figure 7. T wave alternans (mean±SEM) responses during exercise testing according to genotypes of rs1805123 in KCNH2 within women (A) and men (B).



SNPs rs1805127 and rs727957 of KCNE1

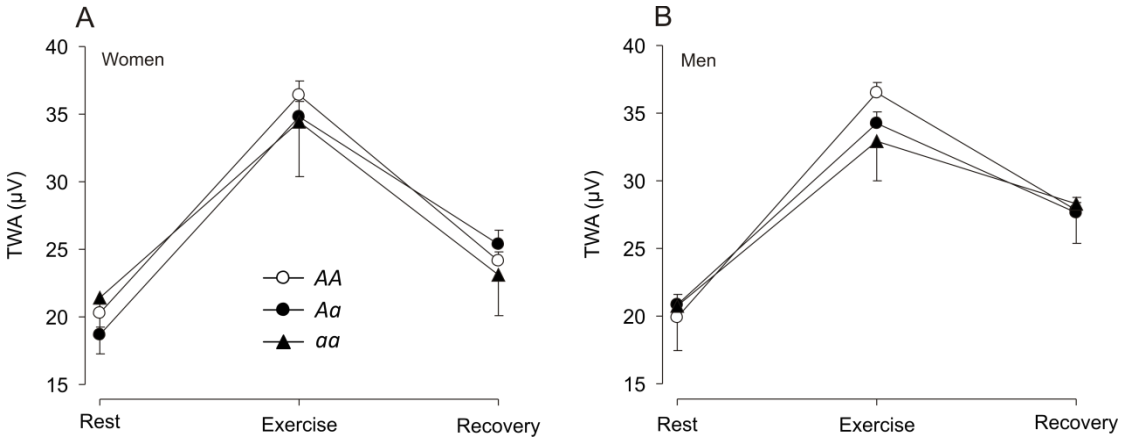
First the two polymorphisms were analysed separately. The subjects with *aa* genotype of rs1805127 showed significantly lower TWA during all phases of the exercise test when compared with other genotypes among all subjects (p≤0.01). The result remained significant after adjusting for potential confounding factors, i.e. age, CHD and β-blocker medication status (p=0.042). When analysing women and men separately, the *aa* genotype was still associated with the numerically lowest TWA during all phases of the exercise test in both sexes, but the finding was statistically significant only within women (p=0.004 for women, p=0.054 for men; Figure 8). The finding remained significant after adjustment for the above-mentioned covariates (p=0.004). QTcBaz did not differ between genotypes of rs1805127 within the entire population or among women and men separately (p≥0.05 for all).

Figure 8. Responses of T wave alternans (TWA, mean±SEM) to exercise testing according to KCNE1 rs1805127 genotypes within women (A) and men (B) separately. Statistically significant differences in post hoc test: *p=0.018, †p=0.003, ‡p=0.017.



The other studied KCNE1 polymorphism rs727957 alone was not associated with TWA, neither in the entire population nor separately within both sexes (p≥0.71 for all; Figure 9). Also the other repolarization marker, QTcBaz, was studied between the rs727957 genotypes, but no statistically significant differences were detected.

Figure 9. Responses of T wave alternans (TWA, mean±SEM) to exercise testing according to KCNE1 rs727957 genotypes within women (A) and men (B) separately.

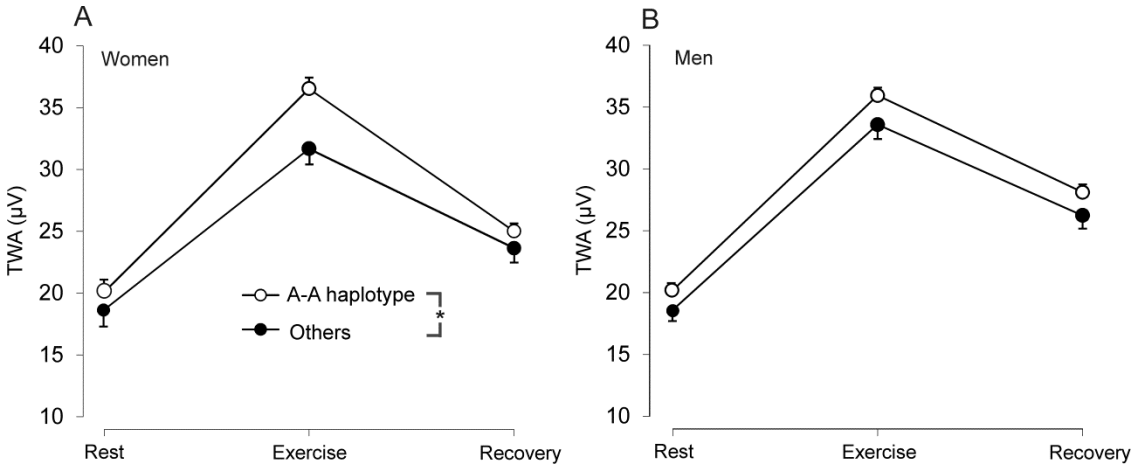


In addition, possible differences of repolarization between haplotypes of two polymorphisms were analysed. The association of the alleles in rs727957 and rs1805127 were confirmed by linkage disequilibrium analysis (D'=0.14; r2=0.005, p<0.001). Four haplotypes were detected within the polymorphisms rs727957 and rs1805127, respectively: *A-A* (54%), *A-a* (31%), *a-A* (6%) and *a-a* (10%). Among all

subjects, the most common *A-A* haplotype carriers presented with higher TWA than non-carriers during the entire exercise test (p=0.007), and the result remained significant after using age, β-blocker medication and CHD as covariates (p=0.015). Interestingly, in their medical history, 22% of the patients with the *A-A* haplotype, and 17% of those with other haplotypes, reported having arrhythmias (p<0.05). When comparing the *A-A* haplotype effect on TWA in women and men separately, the results were parallel but statistically significant only among women (p=0.031 for women, p=0.055 for men; Figure 10). There were no significant associations with TWA in analyses for other haplotypes.

Average resting QTcBaz duration for the *A-A* haplotype carriers was 457±33 ms, for *A-a* 457±33 ms, for *a-A* 459±33 ms and for *a-a* 456±33 ms, and these differences were not statistically significant. In corresponding statistical analyses for men and women separately, QTcBaz differences between haplotypes remained statistically insignificant.

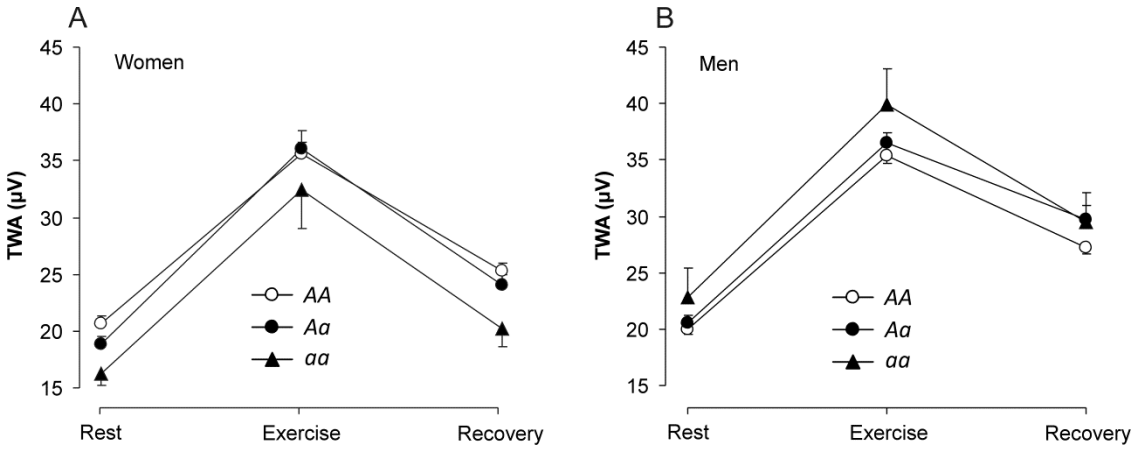
Figure 10. T wave alternans (TWA, mean±SEM) according to A-A haplotype versus other haplotypes of KCNE1 rs727957 and rs1805127 within women (A) and men (B). *Statistically significant difference, p=0.031.



SNP rs1805124 of SCN5A

Magnitude of TWA did not differ between the three genotypes of SCN5A rs1805124 in the entire population or in either sex (p>0.05 for all respectively, Figure 11).

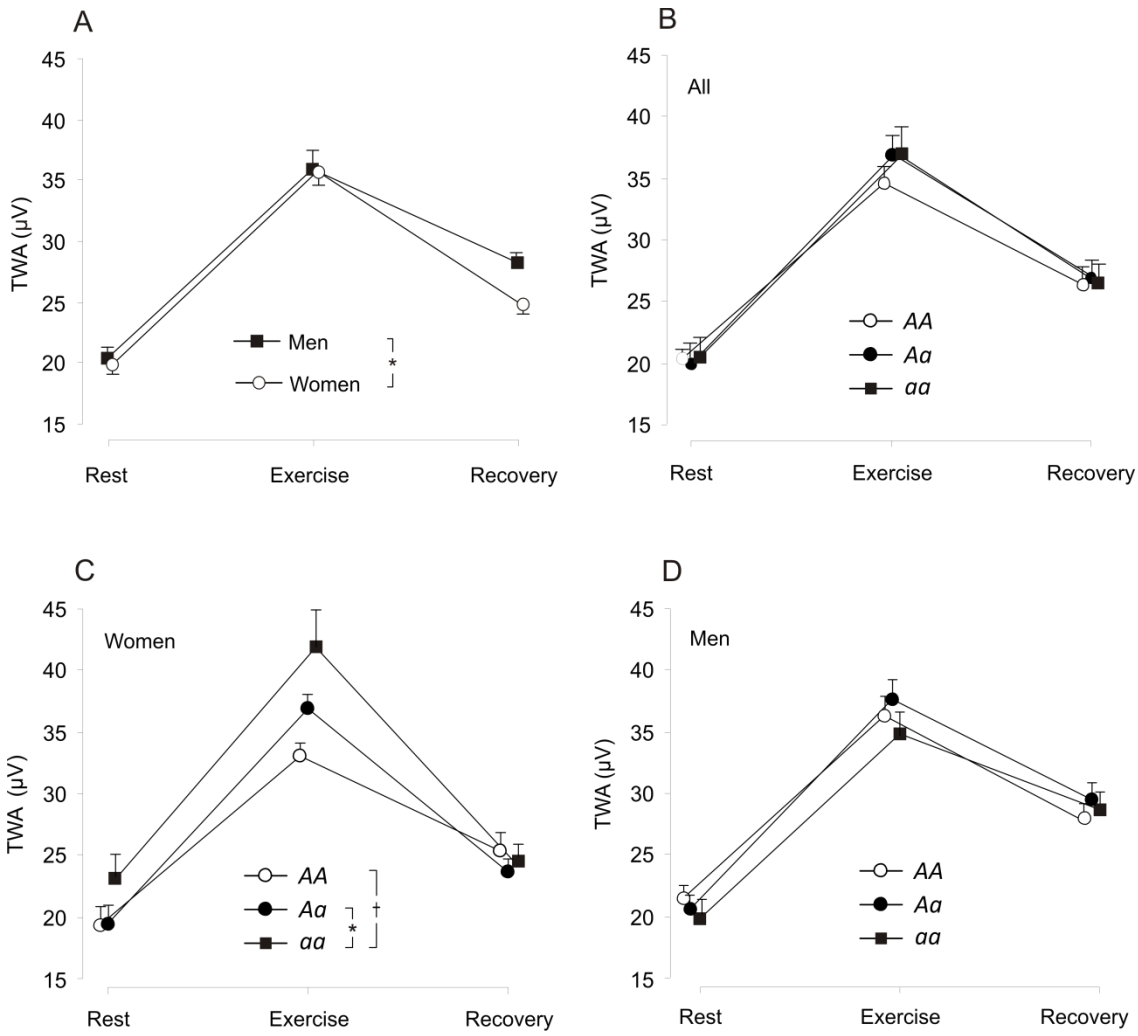
Figure 11. T wave alternans (TWA, mean±SEM) responses to exercise testing according to rs1805124 SCN5A genotypes within women (A) and men (B) separately.



SNP rs10494366 of NOS1AP

Within the entire population the rs10494366 polymorphism was not significantly associated with TWA variation during exercise testing (p=0.057, RANOVA, Figure 12B). Variation of TWA over the entire exercise test was significantly higher within men as compared to women (p=0.001, RANOVA, Figure 12A). Numerical TWA was significantly lower in women compared to men especially at the recovery phase (24.8±14.2 vs. 28.2±19.2, p<0.001, T test). Within women the rs10494366 of NOS1AP was associated with TWA during rest and exercise, while within men there was no such association (p<0.001 for women, Figure 12C, and p=0.16 for men, Figure 12D, RANOVA). Within women this finding remained significant after adjustment for age, CHD and β-blocker medication status (p=0.001, RANCOVA).

Figure 12. T wave alternans (TWA, mean±SEM) responses to exercise testing between women and men (A) and according to rs10494366 of NOS1AP genotypes within whole study population (B), within women (C) and men (D) separately. Statistically significant difference between genotypes in post hoc testing, *p=0.049, †p=0.050.



9.1.4 Association of SNPs with mortality

In the present study, the average follow-up was 47 months (from 0 to 66 months), during which 116 deaths were reported. From the studied SNPs, only the minor allele *a* carriers of KCNE1 rs1805127 had lower mortality compared to non-carriers (p=0.047, Table 6). However, other SNPs, the KCNE1 rs727957, SCN5A rs1805124, KCNH2 rs1805123 or NOS1AP rs10494366, did not relate with mortality in the Cox regression analysis (Table 6). Also the KCNE1 *A-A* haplotype was without an effect on mortality as compared with other haplotypes.

Table 6. Relation of minor allele *a* carriers versus non-carriers and haplotype *A-A* versus other haplotypes with mortality by Cox regression analysis.

Genotype/haplotype	Hazard ratio	95% CI
KCNH2 rs1805123	0.99	0.66 - 1.49
KCNE1 rs1805127*	0.68	0.46 - 0.99
KCNE1 rs727957	0.76	0.79 - 1.74
KCNE1 haplotype	1.19	0.72 - 1.96
SCN5A rs1805124	1.17	0.72 - 1.56
NOS1AP rs10494366	1.09	0.75 - 1.57

Statistics: *p<0.05. **Abbreviations:** 95% CI, 95% confidence interval

9.2 HR and haemodynamics, study IV

9.2.1 Population characteristics in study IV

Characteristics of the study population according to the HR tertiles are presented in the Table 7. The proportion of men to women was higher in the 1st tertile when compared with the 2nd and the 3rd tertile (p=0.019). Mean supine HR within entire population was 63±9 bpm, and within men 62±10 bpm and within women 64±9 bpm. BMI, white blood cell count, CRP, total cholesterol and triglycerides were highest within the 3rd tertile (p<0.050 for all). Probably due to sex distributions, creatinine was lowest within the 3rd HR tertile with higher proportion of women (p<0.005), but there were no statistically significant differences in cystatin C levels or estimated glomerular filtration rate between the tertiles. The other variables, age, alcohol usage, amount of smoking, haematocrit, sodium, potassium, HDL-cholesterol, LDL-cholesterol or glucose concentrations, did not differ between the groups (p>0.050, for all respectively). Based on the interview, the study subjects performed on average 3 physical exercise bouts per week that induced shortness of breath and sweating and lasted ≥ 30 min in all the tertiles (p=0.143).

Table 7. Characteristics of study population according to heart rate tertiles and characteristics of entire study population (mean ± standard deviation).

	Resting heart rate tertiles			All
Variables	**1**	**2**	**3**	
Sex (Males/Females) [#]	101/71	79/97	81/93	261/261
Age (years)	46±12	46±11	46±11	46 ± 12
Smoking (pack years)	2.3±7.5	1.8±5.7	3.5±10.1	2.5±8.0
Alcohol (Servings/week)	4±6	4±6	5±6	4±6
Body mass index (kg/m^2)	26.2±3.6	26.5±4.5	27.3±4.9*	26.7 ± 4.4
Waist circumference (cm)	92±12	92±14	94±15	92 ± 14
Leukocyte count (1*10^9/l)	5.4±1.1	5.9±1.6*	6.1±1.8*	5.8 ± 1.5
Haematocrit (%)	42±3	41±3	42±4	42 ± 3
C-reactive protein (mg/l)	1.2±1.2	1.6±2.6	2.2±4.2*	1.7 ± 3.0
Creatinine (µmol/l)	77±13	72±12*	71±13*	73 ± 13
Cystatin C (mg/l)	0.8±0.1	0.8±0.2	0.9±0.2	0.8±0.1
Sodium (mmol/l)	140±2	140±2	140±2	140±2
Potassium (mmol/l)	3.8±0.2	3.8±0.3	3.8±0.3	3.8±0.3
Total cholesterol (mmol/l)	5.2±1.1	5.0±0.9*	5.3±1.1†	5.1 ± 1.0
Triglycerides (mmol/l)	1.2±0.8	1.2±0.6	1.5±1.5*†	1.3±1.0
HDL cholesterol (mmol/l)	1.7±0.5	1.6±0.5	1.5±0.4	1.6 ± 0.5
LDL cholesterol (mmol/l)	3.0±1.0	2.9±0.9	3.1±1.0	3.0 ± 1.0
Fasting glucose (mmol/l)	5.4±0.6	5.4±0.5	5.5±0.6	5.4 ± 0.6
Estimated GFR (ml/min)	112±14	112±13	112±13	112±13

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; GFR, glomerular filtration rate. **Statistics:** *p<0.050 vs. tertile 1, †p<0.050 vs. tertile 2. #p<0.05, statistically significant difference in distributions between tertiles.

9.2.2 HR and BP

Linear association of HR and BP in supine position

In the whole study population supine HR showed relatively weak associations with peripheral or central BPs in linear regression analyses. Association of HR with radial systolic (R^2=0.021, p<0.001, Figure 13A) and diastolic BP (R^2=0.035, p<0.001, Figure 13B) was statistically significant, while that with radial pulse pressure was not (R^2=0.001, p=0.432). HR was not associated with aortic systolic BP (R^2=0.003, p=0.107, Figure 13C), but there was a minor association between higher HR and elevated aortic diastolic BP (R^2=0.044, p<0.001, Figure 13D). The results remained similar in multivariable regression model where sex, age and BMI were included as explanatory factors in addition to HR (Table 8).

Figure 13. Associations of supine heart rate with radial and aortic blood pressures.

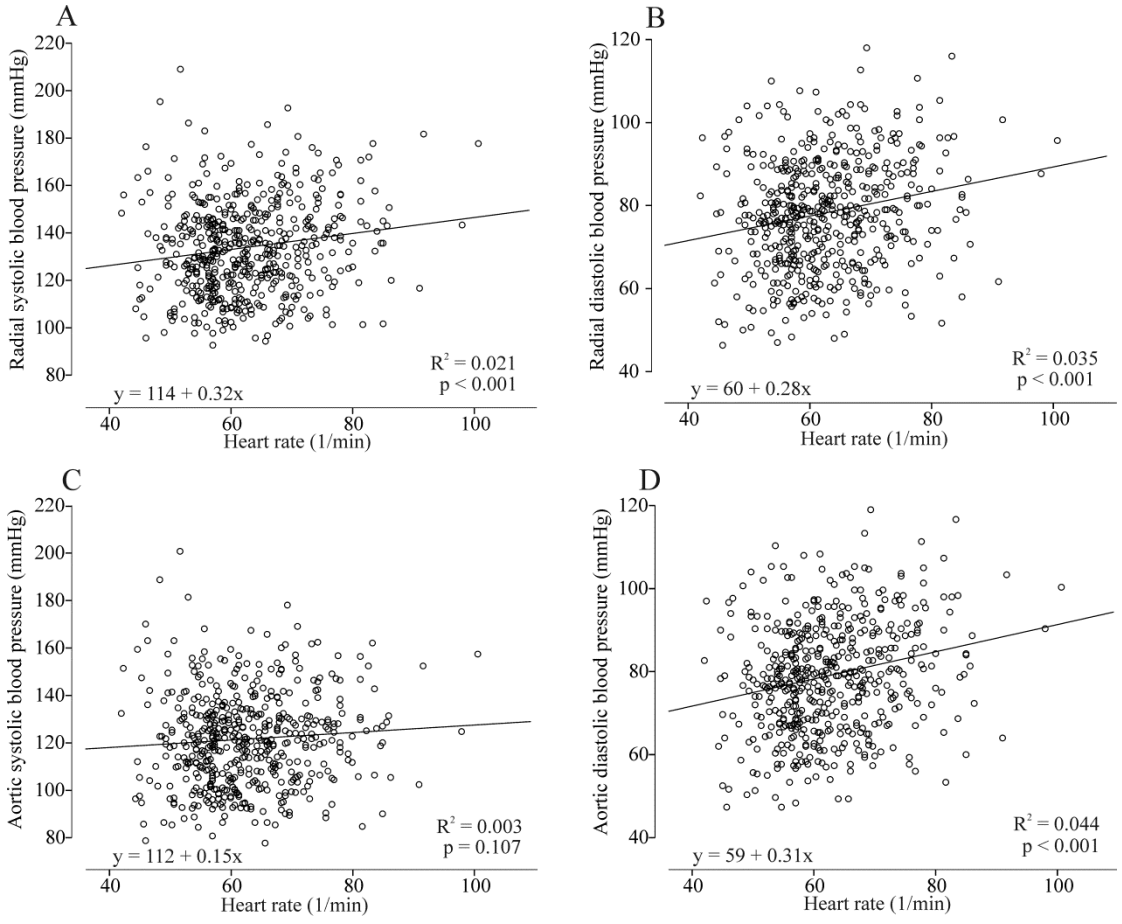


Table 8. Association of supine heart rate and confounding factors, age, body mass index and sex with radial and central blood pressures in stepwise multivariate linear regression analysis. Only statistically significant explanatory variables are presented.

Dependent and explanatory variables		**β**	**p-value**	**adjusted R^2**
Radial systolic BP (R^2 = 0.269)				
	Age	0.304	<0.001	0.144
	BMI	0.205	<0.001	0.065
	Sex	-0.225	<0.001	0.040
	HR	0.149	<0.001	0.020
Radial diastolic BP (R^2 = 0.261)				
	Age	0.325	<0.001	0.149
	Sex	-0.121	<0.001	0.050
	HR	0.188	<0.001	0.044
	BMI	0.161	<0.001	0.021
Central systolic BP (R^2 = 0.312)				
	Age	0.442	<0.001	0.259
	BMI	0.202	<0.001	0.043
	Sex	-0.104	<0.001	0.010
Central diastolic BP (R^2 = 0.266)				
	Age	0.323	<0.001	0.148
	BMI	0.161	<0.001	0.046
	HR	0.209	<0.001	0.032
	Sex	0.207	<0.001	0.040

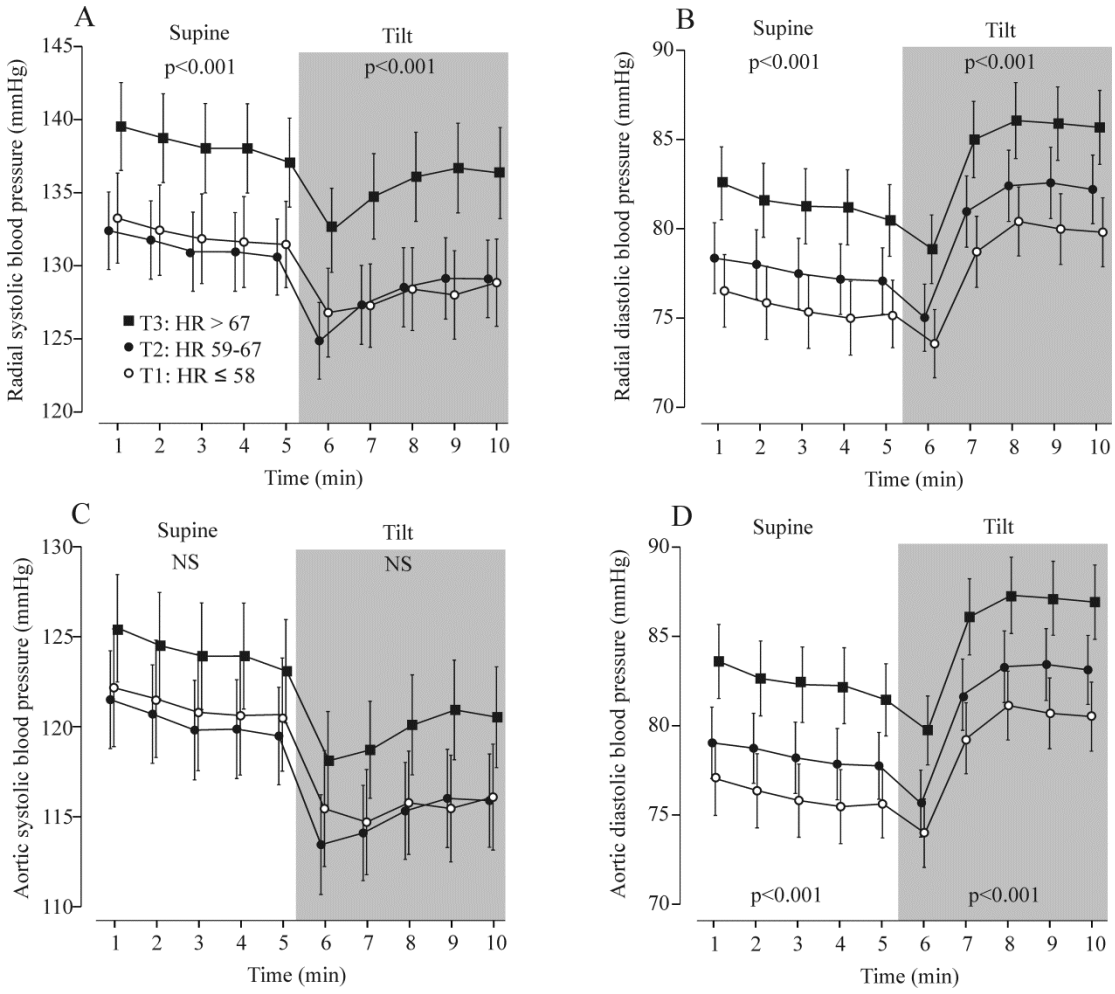
Abbreviations: BMI, body mass index; HR, heart rate.

Head-up tilt responses of BPs according to HR tertiles

Results of supine BPs remained similar as in regression analysis, when the associations were analysed according to HR tertile groups. Radial systolic and diastolic BPs were highest in the tertile 3 when compared with the other tertiles both in supine position (p=0.001 and p<0.001, for systolic and diastolic BP, respectively) and during head-up tilt (p<0.001, for both BPs, Figure 14). Differences in radial BPs between HR tertiles remained significant in supine position (p=0.004 for systolic BP, p<0.001 for diastolic BP) and during head-up tilt (p=0.003 for systolic BP, p=0.001 for diastolic BP) in adjusted analysis with above mentioned covariates, sex, age, BMI, smoking, hematocrit, white blood cell count, CRP, creatinine, cystatin C, total cholesterol, triglycerides, HDL-cholesterol and fasting plasma glucose.

Aortic systolic BP did not differ between tertiles in the supine (p=0.139) or upright positions (p=0.484). Aortic diastolic BPs increased according to HR tertiles both in supine position and during head-up tilt (p<0.001 for both positions, with and without adjustments).

Figure 14. Radial systolic (A) and diastolic (B) blood pressures and aortic systolic (C) and diastolic (D) blood pressures (mean ± 95% confidence interval) according to heart rate tertiles (T1-3) during 5 minutes supine position and 5 minutes head-up tilt.



9.2.3 Associations of HR with central wave reflection and arterial stiffness

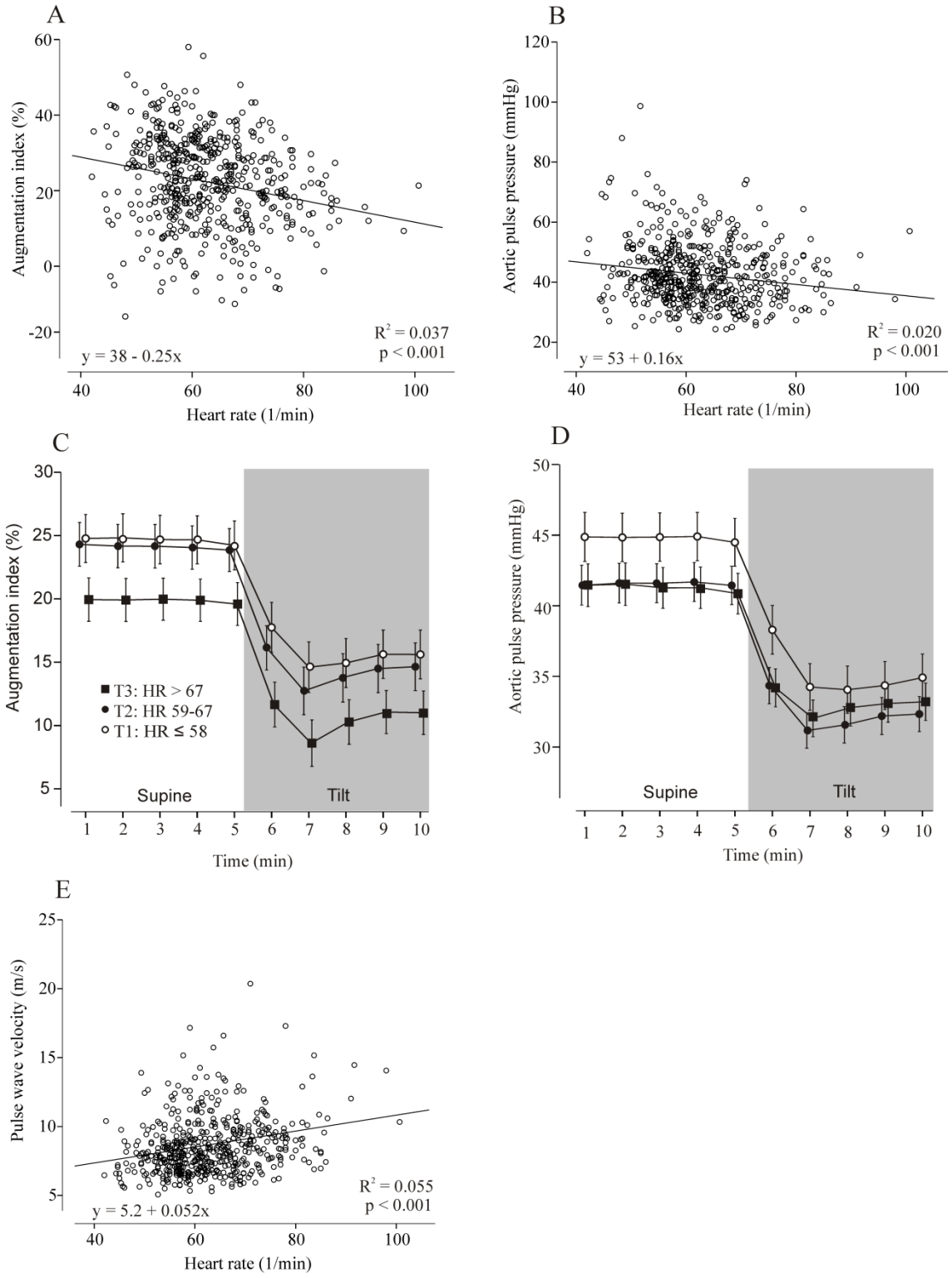
First, association of resting HR and indexes of central wave reflection were analysed as continuous variables. An expected negative association was found between resting HR and AIx (R^2=0.037, p<0.001, Figure 15A), so that AIx was reduced by 2.5%-units for

every 10 bpm elevation of HR. The association of HR with AIx@75 (as calculated by the SphygmoCor software) was also depicted: despite AIx was adjusted for HR, a minor direct relationship between HR and AIx@75 was observed (R^2=0.012, p=0.008). In addition, a small but statistically significant negative association was observed between higher HR and lower aortic PP (R^2=0.020, p<0.001, Figure 15B).

Then head-up tilt responses of central wave reflection were analysed according to HR tertiles. Supine AIx was lowest in the 3rd HR tertile (p<0.001, Figure 15C) and aortic pulse pressure was highest in the 1st HR tertile (p=0.001, Figure 15D), and the differences were similar also during the head-up tilt (p=0.001 for AIx, p=0.006 for aortic PP). The differences in AIx and aortic PP were statistically significant also after the adjustments (sex, age, BMI, smoking, haematocrit, white blood cell count, CRP, creatinine, cystatin C, total cholesterol, triglycerides, HDL-cholesterol, fasting plasma glucose and radial MAP) during both supine and upright position (p≤0.001).

Arterial stiffness was assessed by measuring supine PWV, because the measurement during head-up tilt has been shown to be less reliable. Mean PWV in the entire study population was 8.5±2.0 m/s. Higher HR was moderately correlated with increased arterial stiffness (R^2=0.055, p<0.001, Figure 15E) and in further, multivariable analysis HR was still statistically significant explanatory factor for PWV (R^2=0.055, p<0.001). In tertile group comparisons the outcome was similar, so that mean PWV was higher in the 2nd and 3rd tertiles when compared with the 1st tertile group (p<0.001, with and without adjustments).

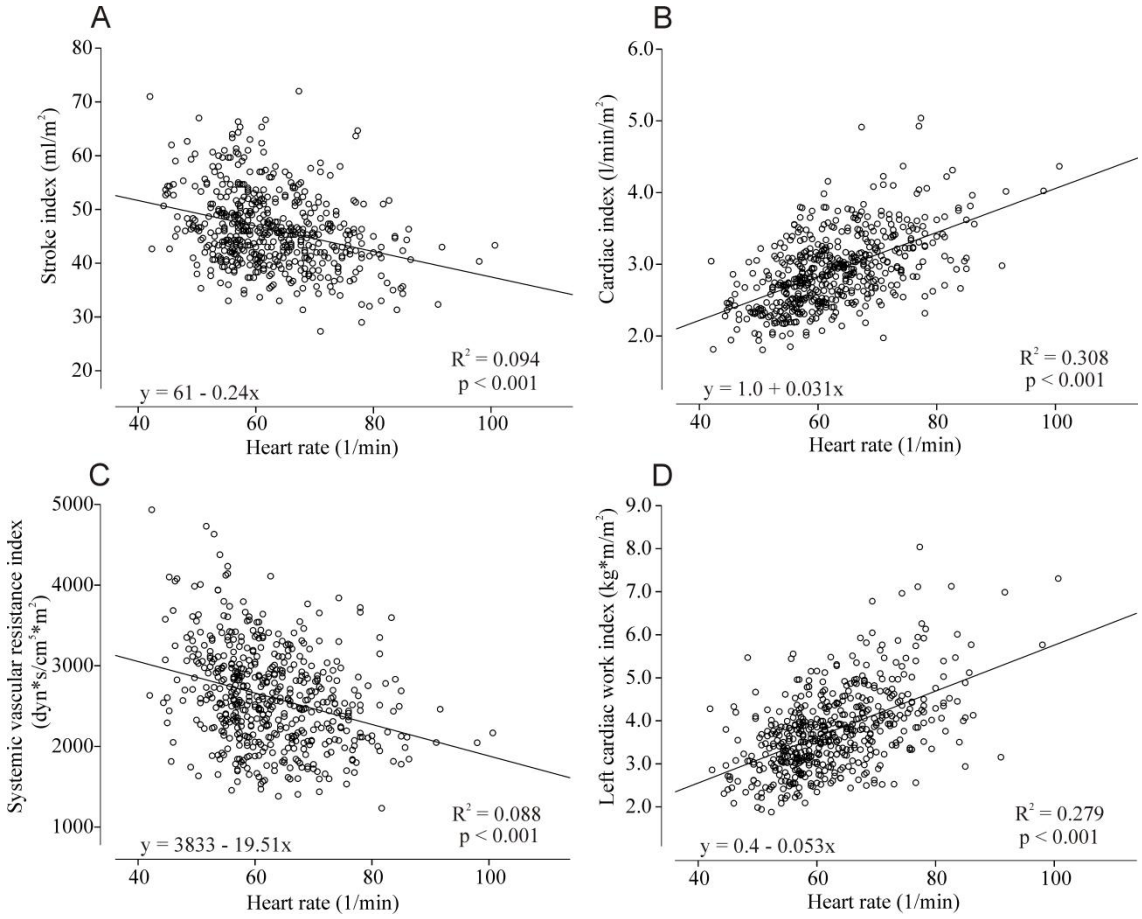
Figure 15. Linear associations of heart rate with indexes of central wave reflection, augmentation index (A), aortic pulse pressure (B), and pulse wave velocity (E). Augmentation index and aortic pulse pressure (mean ± 95% confidence interval) during five minutes in the supine position and during head-up tilt according to HR tertiles (C and D).



9.2.4 Relation of HR with SI, CI, SVRI and LCWI

HR was negatively correlated with SI (R^2=0.094, p<0.001, Figure 16A), and SVRI (R^2=0.088, p<0.001, Figure 16C), but higher HR was still strongly associated with increased CI (R^2=0.308, p<0.001, Figure 16B). Importantly, HR was a significant determinant of cardiac load, as estimated using LCWI, so that it explained 28% of the variability in LCWI (Figure 16D).

Figure 16. Relations of stroke index (A), cardiac index (B), systemic vascular resistance index (C) and left cardiac work index (D) with supine heart rate.



Differences in HR between the tertiles remained similar in supine position and during head-up tilt (p<0.001 for both), and the mean change in HR from supine to upright position was 12±7 bpm. Change of HR from supine to upright position was not statistically different between tertiles (18A). Supine SI was negatively associated with HR (p<0.001), and SI was smaller in the tertile 3 when compared with the tertiles 1 and

2 ($p \leq 0.001$ for both, with and without the adjustments; Figure 17A). SI decreased lesser in the tertile 3 when compared with the tertiles 1 and 2 in position change ($p \leq 0.001$ with and without the adjustments, Figure 18D). However, the difference in SI values between the tertiles was still significant during head-up tilt ($p=0.002$), although the differences between the groups were smaller than in supine values.

Despite negative association between SI and HR, CI was still strongly and directly related with higher HR also in adjusted analysis including MAP ($p<0.001$; Figure 17B). Differences in CI values between the tertile groups remained similar also in upright position ($p<0.001$, with and without adjustments) although change in CI from supine to upright position was statistically significantly smaller within 1st tertile versus 3rd tertile ($p<0.01$, Figure 18B).

Naturally, HR was related with left cardiac work both in supine and upright positions ($p<0.001$; Figure 17D). In further, adjusted analyses the finding remained similar ($p<0.001$ for both positions). SVRI was negatively related with HR in supine and upright positions ($p<0.001$ for both, without and with adjustments; Figure 17C). Change in SVRI from supine to up-right position was not statistically significant between HR tertiles (Figure 18C).

Figure 17. Mean (± 95% confidence interval) stroke index (A), cardiac index (B), systemic vascular resistance index (C) and left cardiac work index (D) at 5 minutes supine and 5 minutes up-right position according to HR tertiles.

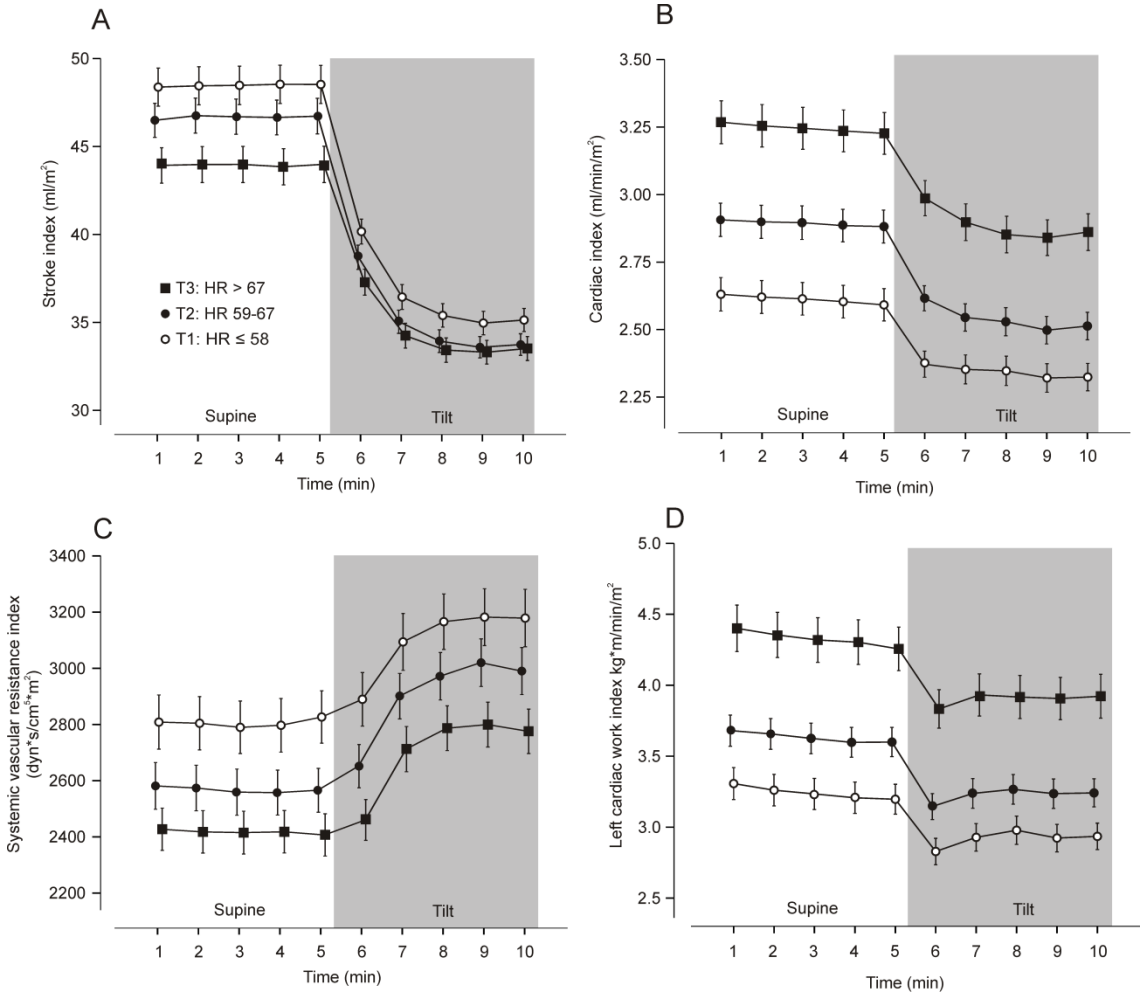
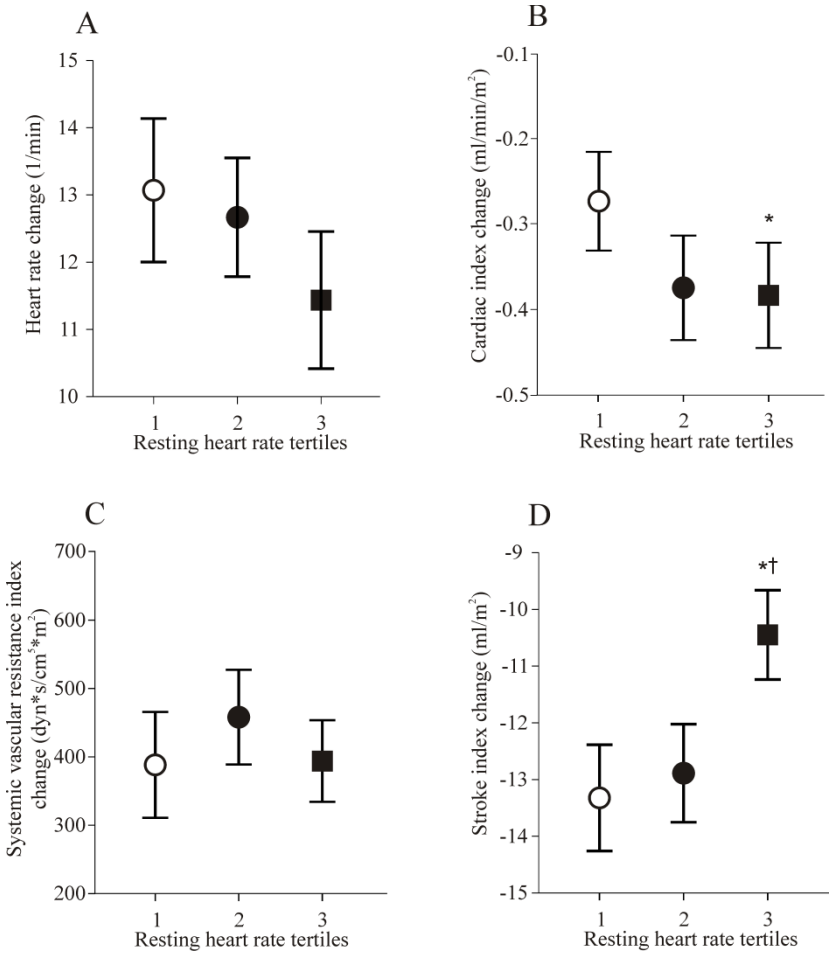


Figure 18. Mean (±95% confidence interval) change in heart rate (A), cardiac index (B), systemic vascular resistance index (C) and stroke index (D) from supine position to head-up tilt. *p<0.01 vs tertile 1, †p<0.001 vs tertile 2. Tertile 1: heart rate < 59/min , Tertile 2: heart rate 59-67/min, Tertile 3: heart rate > 67/min.



9.2.5 ICG_{WB} versus echocardiographic SV determination

For comparing two methods, SV was measured using ICG_{WB} and 3-dimensional echocardiography during head-up tilt from 16 subjects. Mean SV by ICG_{WB} was 72±19 ml and by ultrasound method 67±15 ml ($p>0.05$). Pearson's correlation between impedance cardiographic and echocardiographic SV measurement was remarkably good ($r=0.781$, $p<0.001$).

10. DISCUSSION

10.1 Cardiovascular risk stratification

Detection of increased cardiovascular risk is important in order to prevent mortality and morbidity. There are many acknowledged methods for cardiovascular risk assessment, but new methods are still needed, not the least because of continuously increasing healthcare costs. The conventional and generally known cardiovascular risk markers are age, male gender, obesity, smoking, increased cholesterol level, hypertension and diabetes. Various laboratory tests and technical devices have been applied for cardiovascular risk assessment (Hlatky *et al.* 2009). Furthermore, different risk assessment tools, like Framingham risk scores and SCORE (Conroy *et al.* 2003, D'Agostino *et al.* 2008, Parikh *et al.* 2008), have been built combining different risk factors by algorithm.

ECG offers an easily available and inexpensive tool for cardiac risk evaluation. Routinely ECG is measured at rest, but ECG recorded during exercise testing has been used as a diagnostic tool for CHD risk assessment at least in subjects with intermediate pre-test probability (Gibbons 2008). Prolonged QT interval, increased magnitude of TWA, and higher HR in ECG are associated with increased risk of unfavourable cardiac events (Yi *et al.* 1998, Pham *et al.* 2003, Cook *et al.* 2006).

In addition, genetic background is an important risk factor for CHD, and a great number of genetic variants has shown to associate with CHD (Swerdlow *et al.* 2012). The basic idea is that cardiovascular risk of an individual could be categorized according to genetic data, and subjects with higher cardiovascular risk could be treated more intensively or could be instructed to further medical examinations. The associations of various genotypes with cardiovascular risk factors, measured at rest, have been frequently determined, but a phenotype can also appear only during exercise or other physical challenge like change in posture during tilt table testing. These kind of functional associations have not been as extensively studied.

The combination of genetic information like polymorphisms and multifactorial risk scores could increase the sensitivity of risk stratification when compared with traditional risk factors alone. The area under the receiver operating characteristics (ROC) curve is the most frequently used method for risk assessment between healthy and diseased subjects (Cook 2007). However, more important question than the effect of a single risk factor on ROC curve is the capability of the risk factor identification to prevent disease or death (Cook 2007). Population differences have also effects on risk prediction, and thus a potential risk factor should be studied within various cohorts. Only thoroughly investigated risk factors are a worthwhile addition to prediction models. Hence, the pathophysiology of a potential cardiovascular risk factor, it's associations with other risk markers, and effects on morbidity and mortality should be carefully studied within different populations before it could be used in risk stratification.

10.2 Study populations

Studies I-III consisted of 2212 patients participating in the Finnish Cardiovascular Study (FINCAVAS). The FINCAVAS is a follow-up study investigating genetic associations with exercise stress test responses among patients who were coming to a diagnostic exercise test at the Tampere University Hospital. Reliable data of patients' survival was collected from the Causes of Death Register, maintained by Statistics Finland. The FINCAVAS study represents a unique outpatient population with detailed information of exercise test, during which continuously measured ECG was captured. In the FINCAVAS study, the proportion of CHD patients and use of medication differ from those of a normal population based sample. Furthermore, the most critical patients, in addition to patients with lower cardiovascular risk, are commonly referred to University hospitals, thus producing a potential selection bias.

The present population consisted of several different subgroups like CHD patients, patients with previous myocardial infarction, medicated patients, etc., thus leading to different risk profiles between subgroups. This was obvious in the present studies I-III, where men more often suffered from CHD than women (46% vs. 13%), and men were also more often treated with β-blockers than women (64% vs. 51%). Such differences may partially explain why the genetic variant KCNE1 rs1805127 was associated with

TWA within women but not men (**II**). The presence of CHD and usage of β-blocker medication were related with higher TWA (**II**). However, in a previous study there was no effect of β-blocker medication on TWA at rest (Zacks *et al.* 2007). The difference in TWA magnitudes between β-blocker medicated and non-medicated subjects in the present study is probably due to the large amount of CHD patients, which also were more often on β-blocker medication than the healthier controls.

The heterogeneity of the population was taken into account by using the most important factors affecting cardiac repolarization as covariates in statistical analyses, and comparing men and women separately (**I-III**). Moreover, in the present studies the mean age (57±13 years, **I-III**) was higher than in most of the previous SNP study populations but mortality was not (5.8% during average 4 years follow up, Studies II-III), suggesting the present population would be classified as having low-risk. It is difficult to generalize the findings to healthy populations, given the relative lack of examination test data in such populations. Regardless of different subgroups in the study, the minor allele frequencies of the studied SNPs (Table 2) were very similar to previous studies, where genetic associations with repolarization was investigated at rest (Aydin *et al.* 2005, Gouas *et al.* 2005, Pfeufer *et al.* 2005, Akyol *et al.* 2007).

The population of study IV was selected from on-going DYNAMIC study where haemodynamics are recorded noninvasively from voluntary subjects during supine and upright position. The subjects were recruited from several different organizations or societies and subjects were included in the order that they contacted the research nurse or personnel. From 830 persons recruited by November 2012, 522 subjects without medications directly affecting cardiovascular system were selected to the study (**IV**). It can be assumed that the subjects who were willing to participate in the haemodynamic measurements were also eminently interested in their health, and therefore selection bias cannot be totally avoided. However, this DYNAMIC cohort is representing very well the general Finnish population (Peltonen *et al.* 2008) according to their mean BMI (26.7 kg/m^2), proportion of smokers (18%), and total cholesterol level (5.5 mmol/l, Study **IV**). In this population the confounding factors like prevailing vascular diseases or medications were minimised by exclusion. Only subjects who were not receiving medications directly affecting the cardiovascular system were included in the present haemodynamic study (**IV**). Most of the previous haemodynamic studies can be criticized of consisting of subjects with various medications affecting blood pressure via different mechanisms.

The DYNAMIC study captures very original tilt table test data, including several continuously recorded haemodynamic variables with prognostic importance. The present study IV is a cross-over study without follow-up information on morbidity or mortality. Both DYNAMIC and FINCAVAS studies consist of exceptionally large populations with continuous haemodynamic and cardiac measurements during tilt and exercise testing.

10.3 Genotyping and cardiovascular recordings

10.3.1 Genotyping SNPs associated with cardiac repolarization

Various genetic polymorphisms have been related with cardiac repolarization in general populations (Bezzina *et al.* 2003, Aydin *et al.* 2005, Gouas *et al.* 2005, Pfeufer *et al.* 2005, Vandenberg *et al.* 2012). Many of these SNPs are coding cardiac ion channels or their non-coding regions affecting ion channel expression levels. In the present study five different SNPs were selected according to previous association studies. Most of the previous studies have been focused on QT interval measured at rest and repolarization genetics during exercise or recovery have only rarely been studied. However, increased magnitude of TWA has been shown to relate with cardiovascular mortality (Nieminen *et al.* 2007, Minkkinen *et al.* 2009, Leino *et al.* 2011), but TWA genetics have not previously been studied. The present study showed first time that *aa*-genotype of KCNE1 (rs1805127) and *a*-allele of NOS1AP (rs10494366) were associated with TWA and the association of NOS1AP (rs10494366) with TWA appeared mostly during exercise (**II-III**). In addition, the *aa*-genotype of KCNH2 (rs1805123) was associated with QTc during exercise but not with TWA (**I**). These results are supporting the view that genotypes are related with functional phenotypes rather than with static findings as in the case of resting ECG variables.

For genotype detection the 5' nuclease assay and fluorogenic allele-spesific TaqMan probes were used by automated ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The 5' nuclease assay is an accepted and suitable method for screening known SNPs from a large number of samples (Livak 1999). In the present studies (**I-III**) the genotype distributions followed the Hardy

Weinberg's equilibrium and distributions were similar to those reported previously supposing that genotyping was reliable.

10.3.2 Analysing TWA and QT interval during exercise testing

In studies I-III ECG was recorded continuously using the 12-lead Mason-Likar method by digital CardioSoft exercise system. The TWA analysis was performed using time-domain MMA method, which has been developed by Nearing and Verrier (Nearing and Verrier 2002) and commercialised by GE Healthcare Inc, Freiburg, Germany. The MMA method enables TWA measurement from routine ECG during rest and exercise (Nearing and Verrier 2002). However, during ambulatory ECG recording the MMA method probably overestimates the magnitude of TWA because of noise (Selvaraj and Chauhan 2009), but TWA analysis by MMA method has still been related with prognosis (Nieminen *et al.* 2007, Slawnych *et al.* 2009, Hoshida *et al.* 2013). Another main technique for TWA analysis, the fast Fourier transform method, has been well validated, but HR must be stabilised during the use of this method.

From continuously measured ECGs, the QT intervals were analysed by automated G.E. algorithm during exercise testing. In consequence of great HR variation during exercise testing, the method of QT correction is important in the present study. The Bazett's formula is the most popular correction method, but it works weakly especially in the HR range from 40 to 100 bpm (Karjalainen *et al.* 1994, Luo *et al.* 2004). In the present study, the Fridericia correction method was also used in the analysis, because the HR correlation of Fridericia QTc is clearly lower than that of Bazett's formula in all HR ranges (Luo *et al.* 2004). However, the relation of HR with QT interval is alternating considerably inter-individually suggesting that no perfect HR correction exists (Malik *et al.* 2002). Prolonged QT interval during rest has been related with mortality (Karjalainen *et al.* 1997, Straus *et al.* 2006, Noseworthy *et al.* 2012), while prolonged QT interval during exercise testing has been related with worse prognosis within CHD patients (Yi *et al.* 1998). Therefore, the QT interval was measured during rest, exercise and recovery in the present study.

10.3.3 Non-invasive haemodynamic measurements

In the present study haemodynamics were measured using ICG_{WB} and radial applanation tonometry. In the previous expert consensus of arterial stiffness measurements, the tonometric arterial pulse wave measurements have been recommended to be analysed from an average of 10 consecutive pulse waves (Laurent *et al.* 2006), but in the DYNAMIC study (**IV**) all measures were performed continuously for several minutes, which provides significantly more recording and thus the outcome can be argued to be more reliable. The ICG_{WB} is based on SV and HR measurements by identification of the changes in tissue conduction properties during cardiac cycle, and thus the other haemodynamic measures such as SVR and LCWI are derivatives of BP and CO. Of note, the CO measurement by ICG_{WB} has shown to be reliable against invasive thermodilution measurement in different body positions (Kööbi *et al.* 1997, Kööbi *et al.* 1997, Kööbi 1999, Cotter *et al.* 2004). In addition, in the present study SV measurement by ICG_{WB} in the upright position showed good correlation with SV measurement by 3D-echocardiograpy carried out by an experienced cardiologist (**IV**). Previously, the haemodynamic measurement method of DYNAMIC study has been shown to be repeatable and reproducible (Tahvanainen *et al.* 2009). The measurement of HR was very reliable, as it was based on the electrocardiographic method by ICG_{WB}. Resting HR was defined as mean of HR during three minutes of measurement, which is on average 180 consecutive cardiac cycles.

10.4 Main results of the study

10.4.1 Genotypes associating with cardiac repolarization

Both TWA and QT interval are markers of cardiac repolarization, and both higher magnitude of TWA and prolonged QT interval are related with morbidity (Karjalainen *et al.* 1997, Nieminen *et al.* 2007, Noseworthy *et al.* 2012). However, dynamic repolarization measures, as TWA, may provide more information about cardiac risks than static measures like QT interval (Exner 2009). In addition, according to prevailing knowledge the genetic background of TWA has not been previously studied.

In the study I SNP of KCNH2 (rs1805123) was related with QTcFri interval within women but not with TWA. The results of previous studies concerning resting QTc interval and KCNH2 (rs1805123) have not been congruent. In an earlier study with Finnish subjects the *a* allele rs1805123 was related with QTc prolongation (Pietilä *et al.* 2002), which is in line with present results. However, also contrary results have been reported where minor *a* allele carriers have been related with shorter QTc intervals (Bezzina *et al.* 2003, Pfeufer *et al.* 2005). Probably these differences are a consequence of population differences and low frequency of *aa* genotype in these populations.

Minor genotype *aa* in rs1805127 of other potassium channel gene KCNE1 was here related with lesser TWA during exercise testing also in the adjusted analysis (**II**). The effect of the minor genotype was more distinct within women compared with men, and during exercise than during rest or recovery. Also the haplotype result of two KCNE1 genotypes (rs727957 and rs1805127) strengthens the finding, because *A-A* haplotype seemed to associate with increased TWA (**II**). The effects of minor genotype in rs1805127 on cardiac repolarization have not been properly studied, because the sizes of the study populations have been small (Friedlander *et al.* 2005, Gouas *et al.* 2005). Within rs1805124 of SCN5A, the genotype with highest influence tended to be *aa,* but the effect was opposite between men and women, proposing that other factors than rs1805124 genotype are more significant in TWA regulation (Study II).

The NOS1AP rs10494366 minor allele *a* was related with higher magnitude of TWA during exercise within women (**III**). This is congruent with previous studies where NOS1AP rs10494366 minor allele *a* was associated with prolonged QTc interval within women (Tobin *et al.* 2008, Pfeufer *et al.* 2009). NOS1AP is functionally encoding protein, which controls nNOS activation and thus affects calcium influx to cells (Jaffrey *et al.* 1998), which might be the link between NOS1AP and TWA.

The exact mechanism causing TWA is not clear, but it has been linked to alterations of action potential duration caused by calcium cycling. Hence, putative explanations for the genotype findings of the present studies are i) genotypes are possibly changing structure or function of the proteins, which directly have effects on calcium cycling, as NOS1AP probably does, ii) the studied polymorphisms are in linkage disequilibrium with other more affective genes, or iii) SNPs are directly, via undetermined mechanisms, linked with TWA.

The effective genotypes studied here were related with cardiac repolarization sex-specifically, suggesting that sex hormones might influence TWA differences between men and women. Nonetheless, in the FINCAVAS study population, TWA responses during exercise testing were similar between female hormone users and non-users (**III**). According to previous knowledge, QT interval is longer within healthy women compared with men (Malik *et al.* 2002), but sex differences influencing TWA have not carefully been studied. In the present study, TWA at rest or at peak exercise did not differ between sexes, but men displayed higher magnitude of TWA at recovery compared to women (**III**). In addition, age, prevailing CHD, and β-blocker medication status were remarkably influencing TWA hence confounding the results, although these factors were included in the adjusted analyses in these investigations.

10.4.2 Genotypes and mortality

Previously, in the same FINCAVAS study population, TWA of at least 65μV during exercise testing has been related with mortality (Nieminen *et al.* 2007). Thus, in the present studies (**I-III**), the relations of five cardiac repolarization polymorphisms with mortality were studied. Only rs1805127 of KCNE1 tended to associate with mortality (**II**). The follow-up of the present study was rather short (mean 47 months). Moreover, subgroups of the most effective minor genotypes in all studied SNPs were quite small, and thus the power of detect the mortality was rather weak. Additional investigations with larger population sizes and longer follow-up times are needed to resolve this question.

10.4.3 HR and haemodynamic function

Functional haemodynamic characteristics in subjects with higher HR have not previously been studied, even though higher HR has been related with poor prognosis in various populations. The findings derived from this study show that higher HR is associated with some favourable phenotypes, namely lower SVR and diminished central wave reflection, but also with unfavourable characteristics, increased CO and cardiac work, elevated BP, and increased arterial stiffness (**IV**).

These findings were tested also during upright position, since the change in posture produces major haemodynamic alterations (Avolio and Parati 2011, Tikkakoski *et al.* 2013). Within subjects with highest resting HR the head-up tilt induced decrease in SV was smallest but changes in HR were similar regardless resting HR level. In addition, resting HR had an influence on changes in CO during head-up tilt, so that within the lowest HR tertile the upright decrease in CO was also lowest (**IV**). However, changes in haemodynamic determinants from supine to upright position remained remarkably similar between resting HR tertile groups, supposing that resting HR also provides substantial information about the upright haemodynamic profile.

Various mechanisms are suggested to relate higher HR with less favourable prognosis, for example elevated blood pressure, ventricular arrhythmias and increased arterial stiffness (Palatini and Julius 1997, Jouven *et al.* 2005, Fox *et al.* 2007, Verrier and Tan 2009, Julius *et al.* 2012). The present study showed relatively weak associations of resting HR with BP and increased arterial stiffness (**IV**). These findings are consistent with previous studies (Millasseau *et al.* 2005, Park *et al.* 2010, Fernandes *et al.* 2011). Previously Lantelme *et al.*(2002) showed that higher HR was related with increasing PWV during cardiac pacing without any effect on BP, and thus HR might be a confounding factor during PWV assessment. However, in the present study the association of HR with PWV remained significant in adjusted analyses including BP. In addition to increased cardiac output, HR was also related with increased left cardiac work, and thus higher oxygen demand, during tilt testing independently from BP level (**IV**). An experimental study previously showed that increasing HR produces higher oxygen demand for excitation-contraction coupling even though the work of heart was maintained stable (Tanaka *et al.* 1990). So maybe HR lowering by drugs like β-blockers and ivabradine, and the following decrease in cardiac work, plays an important role in the treatment of certain groups of patients.

An increase in AIx, a measure of central wave reflection, has been related with cardiovascular disease (Weber *et al.* 2004). However, higher HR is associated with lower central wave reflection according to the present and previous studies (Wilkinson *et al.* 2000, Williams and Lacy 2009). This might arise from i) timing of reflected wave during shorter duration of diastole (Wilkinson *et al.* 2000) or ii) moving of the reflection point more peripherally as a result from a decrease in SVR during higher HR (Wilkinson *et al.* 2000).

10.5 Future aspects

Previously higher resting HR has been related with adverse cardiovascular events and the present study IV showed that higher resting HR is related with increased cardiac work, higher BP, and faster PWV. Higher TWA has also been linked with cardiovascular mortality during exercise testing and the genetic background of TWA variation was studied here (**I-III**). In addition, the potassium channel gene, KCNE1, was related with both cardiac repolarization and mortality (**II**). Previously, within the FINCAVAS population, Leino *et al.* (2009) showed that high TWA during exercise or recovery phase in exercise testing together with low HR recovery increased the risk of cardiovascular mortality up to eight-fold. In the present study different functional cardiovascular risk factors and their genetic background were studied. Cardiovascular response, for example to tilt or exercise testing, could provide more detailed information of individual's risk profile. Probably functional tests should also be assessed for genetic investigations in risk stratification.

Only five SNPs of four genes, which previously were associated with cardiac repolarization, were studied here. Large genome wide association studies with larger populations are needed for understanding the functional cardiovascular responses, since dozens of genes have complex influences on the regulation of cardiac repolarization and cardiac cycle.

Different cardiovascular phenotypes, as shown here, higher resting HR with lower SVR and increased cardiac work, or higher magnitude of TWA during exercise testing, could be related with known, harmful genotypes. This genotype-phenotype data could offer plenty of information of cardiovascular risk profiles and could help in future clinical decisions: who is in the need of more aggressive treatment or additional clinical investigations, and with whom can these be avoided?

11. SUMMARY AND CONCLUSIONS

On the basis of this study, together with previous knowledge, genetic variation has an effect on the cardiac repolarization variables, QT interval and TWA, and increased cardiac work load and arterial stiffness are associated with higher HR. In addition, functional measurements during clinical exercise test and orthostatic physical challenge provide additional information of these associations when compared with conventional measurements only performed at rest. More studies in different populations are warranted to clarify whether these haemodynamic and genetic characteristics represent true cardiac risk factors and to provide further details of the mechanisms behind these relationships.

The principal findings and conclusions of the study are as follows:

1. The polymorphism rs1805123 in KCNH2 gene does not have obvious effect on QTc interval or TWA during different phases of physical exercise testing. The minor genotype of this SNP tended to influence on QTc responses during exercise test within women, but greater study populations with larger group for the minor genotype are needed for additional evidence (**I**).

2. The rs1805127 of KCNE1 potassium channel gene may have an influence on cardiac repolarization responses, measured as TWA, during clinical exercise test particularly in women. The same effective polymorphism tended to associate with mortality during a rather short follow-up period. Additional investigations are necessary for further evaluation whether this SNP characterises a true cardiovascular risk marker. The rs727957 of KCNE1, as well as rs1805124 of SCN5A were not associated with TWA during exercise test or with mortality (**II**).

3. The common variant, rs10494366 of NOS1AP, has an influence on TWA responses during clinical exercise test in a Finnish population, especially within women. Although this SNP was not associated with mortality within an average follow-up period of 47 months, these results suggest that NOS1AP has clinical significance in the regulation of cardiac repolarization (**III**).

4. Within a population devoid of medications with direct influences on cardiovascular function and without major diseases, the haemodynamic characteristics of subjects with higher resting HR were increased CO and lower SVRI during supine and upright position. In addition, higher resting HR was related with elevated BP and arterial stiffness. These findings may be the explanation for the association of higher HR and less favourable prognosis in population studies (**IV**).

12. ACKNOWLEDGEMENTS

This study was carried out at the School of Medicine, University of Tampere; Department of Internal Medicine, Tampere University Hospital; and Fimlab Laboratories, Pirkanmaa Hospital District. In addition, research was performed in Pirkanmaa Hospital District's Medical Imaging Center and Hospital Pharmacy.

My sincerest gratitude goes to my supervisor Professor Ilkka Pörsti for introducing me to clinical research. I have been impressed of his intelligence and enthusiasm towards science. From Ilkka I have learned genuine scientific thinking and, curiously enough, English writing. Without his support this never could be possible.

I wish to express my sincere gratitude to my other supervisor Professor Terho Lehtimäki for giving me the opportunity to carry out this doctoral thesis. Under his guidance I have had a chance to develop as a researcher during these years.

I greatly appreciate the valuable comments and suggestions I have received from Professor Jukka Mustonen, Professor Mika Kähönen, Professor Onni Niemelä, Docent Tiit Kööbi, and Docent Erkki Ilveskoski.

I am extremely grateful to Docent Tuomo Nieminen for his advice with statistical and technical issues in studies I, II and III. He was always ready to help when I asked for it.

I thank Docent Panu Taskinen and Docent Olavi Ukkola for their valuable comments and advice, and for their work in reviewing the thesis.

I am grateful to our dynamic research group for their inspirational attitude. My warmest thanks go to my friend and colleague Anna Tahvanainen, MD, PhD, for valuable encouragement and continuous support during this project; and to Antti Tikkakoski, MD, for being always ready to help with SPSS and data processing. I wish

to thank Jani Viitala, MSc, for developing the data management system; Antti Haring, MD, and Miia Leskinen, MD, for their contribution with data collection. In addition I thank Arttu Eräranta, MSc, Elina Hautaniemi, MSc, Pauliina Kangas, MD, and Matias Wilenius, MD, for their assistance and all the refreshing discussions we shared during research.

My sincere thanks are due to all other co-authors: Professor Martin Tobin, Professor Nilesh Samani, Professor Väinö Turjanmaa, Docent Kari Niemelä, Kjell Nikus, MD, PhD, Rami Lehtinen, PhD, Jari Viik, PhD, Mari Niemi, MD, PhD, Riikka Rontu, PhD, Meng Fan, MD, PhD, and Jarno Laiho, MD.

I express my sincere gratitude to the research nurses Paula Erkkilä, Reeta Kulmala and Pirjo Järventausta for their priceless work for DYNAMIC and FINCAVAS studies. I am grateful to the staff of Medical Imaging Center and Hospital Pharmacy for collecting the exercise test data.

I am grateful to my family: my mother Raija and my deceased father Osmo for all their love and support, my admirable older sisters Tiina and Hanna and my brave younger sister Sanni for broadening my view on life. Thanks go also to my dear friends Tanja, Hamppa, Minna, Elina, and others, for always staying by my side.

Finally, I am most indebted to my husband Anssi for his considerable love and support. Last, I wish to thank our brilliant sons Touko and Väinö for the happiness and joy they bring to our everyday life.

This work has been financially supported by Pirkanmaa Fund of Finnish Cultural Foundation, Competitive State Research Financing of the Expert Responsibility Area of Tampere University Hospital, Finnish Medical Foundation, Finnish Foundation for Cardiovascular Research, Aarne Koskelo Foundation, Orion-Farmos Research Foundation, Emil Aaltonen Foundation, Tampere Tuberculosis Foundation, Sigrid Jusélius Foundation, and Paavo Nurmi Foundation.

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14. ORIGINAL PUBLICATIONS

The Scandinavian Journal of Clinical & Laboratory Investigation,
Vol. 68, No. 1, February 2008, 31–38

informa
healthcare

ORIGINAL ARTICLE

Potassium channel KCNH2 K897T polymorphism and cardiac repolarization during exercise test: The Finnish Cardiovascular Study

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Objective. Cardiac repolarization is regulated, in part, by the KCNH2 gene, which encodes a rapidly activating component of the delayed rectifier potassium channel. The gene expresses a functional single nucleotide polymorphism, K897T, which changes the biophysical properties of the channel. The objective of this study was to evaluate whether this polymorphism influences two indices of repolarization – the QT interval and T-wave alternans (TWA) – during different phases of a physical exercise test. ***Material and methods***. The cohort consisted of 1,975 patients undergoing an exercise test during which on-line electrocardiographic data were registered. Information on coronary risk factors and medication was recorded. The 2690A > C nucleotide variation in the KCNH2 gene corresponding to the K897T amino acid change was analysed after polymerase chain reaction with allele-specific TaqMan probes. ***Results***. Among all subjects, the QTc intervals did not differ between the three genotype groups ($p \geq 0.31$, RANOVA). Women with the CC genotype tended to have longer QT intervals during the exercise test, but the difference was statistically significant only at rest ($p = 0.011$, ANOVA). This difference was also detected when the analysis was adjusted for several factors influencing the QT interval. No statistically significant effects of the K897T polymorphism on TWA were observed among all subjects ($p = 0.16$, RANOVA), nor in men and women separately. ***Conclusions***. The K897T polymorphism of the KCNH2 gene may not be a major genetic determinant for the TWA, but the influence of the CC genotype on QT interval deserves further research among women.

Keywords: Arrhythmia; beta-adrenergic blockers; electrocardiography; ion channels; polymorphism

Introduction

The long-QT syndrome (LQTS) is an inherited or acquired ventricular repolarization disorder that manifests as a prolonged QT interval in electrocardiographic (ECG) recording [1,2]. The syndrome may manifest as polymorphic ventricular tachycardias described as torsade de pointes which cause syncope, seizures and sudden death [1]. The onset of symptoms in patients with LQTS is usually related to physical stress [3]. LQTS type 2 (LQT2), however, has been demonstrated to cause cardiac events during rest/sleep or emotional stress, although arrhythmias during physical exercise are also possible [4]. LQT2 is an example of an inherited aetiology with mutations in the KCNH2 gene that encodes the α-subunits of the rapidly activating component of the delayed rectifier potassium channel (I_{Kr}) [5]. This channel has an important part to play in ventricular repolarization and cardiomyocyte action potential duration and refractoriness [6].

The KCNH2 gene presents a single nucleotide polymorphism (SNP) at locus 2690, where adenine is replaced by cytosine (2690A > C) [7]. This SNP causes basic lysine to be replaced by a neutral threonine at amino acid position 897 (K897T) in the pore region of an I_{Kr}, leading to a change in the biophysical properties of the channel [7,8]. The I_{Kr} channel is formed by α-subunits coded by KCNH2 which co-assemble to form heterotetrameric channels that are probably regulated by a β-subunit such as MiRP1, coded by KCNE2, or minK, coded by KCNE1 [9,10]. Mutations in these β-subunit coding genes have been described previously, but their clinical significance is yet to be determined [11].

Previous publications have reported contradictory information on the influence of the K897T amino acid polymorphism on the electrophysiological properties of the I_{Kr} channel and QT interval [7,8,12–17]. Although the symptoms of LQT2 can appear during physical exercise [4], previous studies have

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(Received 6 March 2007; accepted 4 June 2007)

ISSN 0036-5513 print/ISSN 1502-7686 online

DOI: 10.1080/00365510701496488
http://www.informaworld.com

mainly focused on QT intervals measured at rest, and there is little detailed information available on the influence of this polymorphism on QT intervals during exercise [13,18].

T-wave alternans (TWA) is another marker of cardiac repolarization. It is defined as beat-to-beat alternation in the amplitude of the T-wave in ECG, and it has been shown to predict ventricular arrhythmias [19]. TWA is often manifested in LQTS, and is even one of the diagnostic criteria of LQTS [20]. However, the effects of ion channel gene or other gene mutations on TWA have not been reported.

The present study cohort consists of patients undergoing a clinical exercise stress test, thus reflecting the actual population being treated by physicians. The aim of the present study was to analyse the effects of the KCNH2 gene K897T polymorphism of the potassium channel I_{Kr} on the two indices of repolarization – QT intervals and TWA –during different phases of a physical exercise test.

Methods

Participants

The Finnish Cardiovascular Study (FINCAVAS) is an ongoing follow-up study focusing on, among other issues, the genetic background of exercise stress test responses [21]. The participant pool comprises the patients who underwent exercise stress tests at Tampere University Hospital between October 2001 and December 2004. All the patients who were willing to participate in the study were recruited (n=2,210). Those with unsuccessful storage of exercise test data and no available genotype data were excluded from the present analysis. Moreover, we excluded patients who had ceased to take β-blocker medication for 2 to 14 days before their exercise stress, due to a possible rebound effect (89 patients). Therefore, 1,975 participants (1,259 M and 716 F) were included in the analysis. The demographics are given in Tables I and II. Indications for the exercise stress test were: a diagnosis of coronary heart disease (CHD, 880 patients) or arrhythmias (430); evaluations of drug therapy (310) and working capacity (357); the patient's status prior to an invasive operation (263) or after an acute myocardial infarction (AMI, 161). Some patients had more than one indication. The study protocol was approved by the Ethics Committee of the Hospital District of Pirkanmaa, Finland, and all patients gave informed consent prior to study initiation, as stipulated in the Declaration of Helsinki.

Study design

Data on demographics, cardiovascular risk factors, lifestyles, medications and medical history were gathered using a computer-based questionnaire after the patients signed the informed consent form and before the exercise stress test. Blood samples were drawn for deoxyribonucleic acid (DNA) analyses after the exercise test.

Digital angiographic data on 426 study patients were analysed in detail by a cardiologist blinded to all other study data.

Exercise test protocol

Prior to the exercise stress test, the subject lay down in the supine position for 10 min, and the resting ECG was recorded digitally. The maximal exercise test was performed using a bicycle ergometer with electric brakes. Blood pressure was measured with a brachial cuff every 2 min.

During the exercise test, a 12-lead ECG Mason-Likar modification [22] was measured continuously, and the following four phases were chosen for the analysis: immediately before (rest, phase 1) and at the end of the test (maximal exercise, phase 2), in addition to 1 and 3 min after the test (recovery, phases 3 and 4). Continuous ECG was digitally recorded at 500 Hz with the CardioSoft exercise ECG system (version 4.14; G.E. Healthcare, Freiburg, Germany). QT interval was measured at each test phase using the G.E. algorithm implemented in Case Workstation software (G.E. Healthcare). The G.E. algorithm defines the earliest QRS onset and the latest T-wave offset in all used leads, thus providing the longest value for the QT interval. The QT interval was then corrected (QTc) for HR using Fridericia and Bazett's corrections [23]. The TWA values were analysed by the G.E. Healthcare modified moving average (MMA) method [24]. The maximum TWA value at HR $<$125 beats per minute at three exercise test phases (rest, maximal exercise and recovery) was taken into account in the analysis. TWA values at higher HR were excluded because these measurements may be inaccurate [25].

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leucocytes using a commercially available kit and the Qiagen BioRobot M48 Workstation in accordance with the manufacturer's instructions (Qiagen Inc., Hilden, Germany). KCNH2 (rs1805123) was genotyped by employing the 5′ nuclease assay and fluorogenic allele-specific TaqMan MGB probes [26] using the ABI Prism 7900HT Sequence Detection

System (Applied Biosystems, Foster City, Calif., USA). The nucleotide sequences of primers and probes used in the polymerase chain reaction (PCR) were deduced from published sequences deposited in the GenBank and Celera databases and synthesized in conjugation with Applied Biosystems. PCR reaction containing genomic DNA, 1 × Universal PCR Master Mix, 900 nM of each primer and 200 nM of each probe was performed in 384-well plates using the standard protocol in a total volume of 5.0 μL. End-point fluorescence was measured and genotype calling carried out by the allelic discrimination analysis module after PCR, resulting in clear identification of the 2690A > C genotype of the KCNH2 gene. Negative and positive controls and random duplicates were used as quality control.

Statistical analysis

Since gender is known to be a significant determinant of repolarization [15,27], men and women were compared within their own groups as well as against each other. Age is also one of the factors influencing the QT interval [15,27,28] and, as a continuous variable, was therefore used as a covariate in the analyses. β-adrenoceptor blocker has been described as altering QT interval and QT dispersion [29–32], and therefore the subjects with β-blocker medication were included as their own subgroup and compared to those with no β-blocker therapy. β-blocker medication was also used as a covariate. In addition, analyses were adjusted for CHD (yes/no), as the ischaemic changes during the exercise stress test can alter the QTc interval [26,33] and TWA [34] values. The CHD group was formed by combining subjects with a previous myocardial infarction and subjects with ⩾50 % stenosis in at least one of the coronary arteries. Furthermore, body mass index (BMI), a considerable modulator of physical characteristics, was selected as a covariate.

The QTc intervals and degree of TWA at the different phases of the exercise stress test were compared between genders, between CHD patients and those who did not suffer from CHD, as well as between β-blocker users and non-users with Student's *t*-test for independent samples. Correlation between age and QTc intervals was tested with linear regression analysis using the covariates described above.

To examine the possible differences in QTc intervals and TWA at the different phases described above between KCNH2 genotypes, analysis of variance for repeated measures (RANOVA) was used. Age, β-blocker medication, CHD and BMI were taken as covariates. Male and female subjects were also compared within their own groups. Categorical variables (CHD, previous AMI, β-blocker medication, hypertension) were compared for the different genotype groups with the chi-square test. The unadjusted QTc intervals and TWA values for each genotype among men and women, respectively, were compared using analysis of variance (ANOVA) with the LSD post-hoc test. All tests were calculated for the QT intervals corrected with both Fridericia and Bazett's formulas. In addition, TWA was classified as normal and abnormal using 65 microvolts as cut-off point, since TWA is a significant predictor of death when using this cut-off point (unpublished observation). Distributions of normal and abnormal TWA were compared between different genotype groups with the chi-square test.

All statistical analyses were performed with the SPSS release 11.5 for Windows (SPSS Inc., Chicago, Ill., USA). A $p < 0.05$ was considered statistically significant.

Results

Participant characteristics

Subject characteristics are summarized in Table I. The number of patients for each gene KCNH2 genotype is given in Table II; genotype distribution between the genders was heterogeneous ($p=0.03$, chi-square test). The genotype distributions followed the Hardy-Weinberg equilibrium, and the allele frequencies (A 0.845 and C 0.155) were similar to those in another

Table I. Population demographics.

	Women (n=716)				Men (n=1,259)			
Variable	Mean	SD	Min	Max	Mean	SD	Min	Max
Age (years)	57	13	15	85	56	13	17	84
Height (cm)	162	6	144	182	176*	6	136	204
Weight (kg)	71	12	39	126	85*	14	45	146
Body mass index (kg/m^2)	27	5	16	47	27	4	17	48

* Statistically significant difference between the genders ($p < 0.001$, Student's *t*-test).

Table II. Population demographics.

Variable	Women	Men	All	%
Genotype AA	496	916*	1,412	71.5
Genotype AC	207	306*	513	26.0
Genotype CC	13	37*	50	2.5
CHD	222	592†	814	41.2
Previous MI	86	386†	472	24.0
HT	282	499	781	39.7
LVH	24	68†	92	4.4
Cardiomyopathy	9	27	36	1.7
β-blocker therapy	372	812†	1,184	59.9
Smoking	110	420†	503	26.8

CHD, coronary heart disease; MI, myocardial infarction; HT, hypertension; LVH, left ventricular hypertrophy. * and †, statistically significant difference between genders (*$p=0.034$, †$p<0.05$, chi-square test).

study with a Finnish population [7]. Approximately 60 % of the patients ($n=1,184$) were on β-blocker medication (Table II). The proportion of patients receiving β-blocker therapy and of smokers was higher in men than in women ($p<0.001$, chi-square test). Men presented with CHD more frequently than women ($p<0.001$, chi-square test), and were more likely to have suffered a previous myocardial infarction ($p<0.001$, chi-square test). The prevalence of hypertension did not differ between the genders ($p=0.49$, chi-square test). These demographic attributes were not associated with the three genotypes.

Fridericia-corrected QT intervals, TWA, diastolic (DBP) and systolic blood pressures (SBP) and HR at rest by gender are presented in Table III. The HR and SBP where higher in women than in men, and the differences were statistically significant ($p<0.001$, 95 % confidence interval (CI) -3.6 to -1.4, $p<0.001$ 95 % CI -5.8 to -2.4).

QTc interval and covariates without genotype considerations

The Fridericia-corrected QT interval tended to be longer in women than in men at rest ($p=0.081$, t-test), while men presented with longer QTc at maximal exercise and during the recovery phases ($p<0.001$, t-test). In linear regression analysis, age had a very minor effect on QTc ($R^2 \leqslant 4.2$ %). The patients treated with β-blockers had longer Fridericia-corrected QT intervals than those without medication at all recording phases ($p<0.001$, t-test). However, the QT intervals corrected with Bazett's formula were shorter in patients on β-blocker medication at phases 1, 2 and 3 ($p<0.001$, RANOVA).

Genotype and QTc interval

Among all subjects, the Fridericia-corrected QT intervals did not differ between the three genotypes ($p=0.47$, main effect in RANOVA). Women with the CC genotype tended to have longer QTc intervals during the entire test, but the difference was statistically significant only at phase 1 ($p=0.011$, ANOVA, LSD post hoc analysis, CC versus AA $p=0.011$, CC versus AC $p=0.004$) (Figure 1A). The finding at rest remained significant after adjustment for β-blocker medication, CHD, BMI and age ($p=0.012$, ANCOVA). In men, no interactions ($p=0.31$, RANOVA) were observed between genotype and QTc intervals (Figure 1B).

The results concerning QTc intervals did not differ significantly when Bazett's versus Fridericia-corrected QT intervals were used. The present results are reported as Fridericia corrections, because this has been demonstrated to be more accurate in populations with high HR dispersion [23,35].

TWA

Variation in TWA did not differ between men and women, except during the recovery phase of the exercise test ($p<0.001$, t-test). Age was not a determinant of TWA in any of the test phases when examined using linear regression analysis ($R^2 \leqslant 2.2$ %). The use of β-adrenoceptor blockers did not affect TWA at rest, but during both the

Table III. Fridericia-corrected QT interval, T-wave alternans (TWA), blood pressure and heart rate in women and men at rest.

	Women ($n=716$)				Men ($n=1,259$)			
Variable	Mean	SD	Min	Max	Mean	SD	Min	Max
QTc interval (ms)	423	24	344	519	421	25	345	546
TWA (μV)	20	12	0	154	20	13	0	140
SBP (mmHg)	143	23	88	220	134*	18	82	218
DBP (mmHg)	79	10	46	112	80	10	54	130
Heart rate (beats/min)	65	11	34	118	62*	12	35	127

SBP and DBP, systolic and diastolic blood pressure, respectively. *Statistically significant difference between genders ($p<0.001$, Student's t-test).

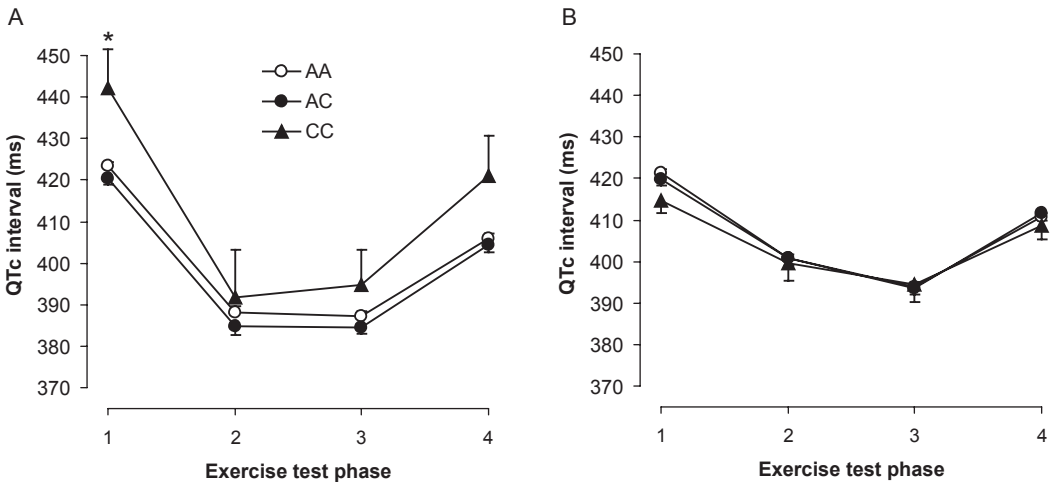

Figure 1. The Fridericia method corrected QT interval (QTc) in (A) women and (B) men at rest (phase 1), at maximal exercise (phase 2) and after 1 min (phase 3) and 3 min (phase 4) recovery by the K897T genotypes. Mean ± SEM. *Statistically significant difference between genotypes (ANOVA, $p=0.011$).

exercise and the recovery phase TWA was higher in the presence than it was in the absence of β-blocker medication ($p<0.001$, t-test).

TWA did not differ in the entire population between the three genotypes during the exercise test ($p=0.20$, main effect in RANOVA; for women $p=0.43$, for men $p=0.20$) (Figure 2A). Within men, the CC genotype presented the numerically smallest TWA during the entire exercise test, but the differences were not statistically significant (LSD post hoc analysis $p=0.07$ CC versus AA, and $p=0.09$ CC versus AC) (Figure 2B). The distributions of normal versus abnormal TWA did not differ between the three genotype groups when analysed using the cut-off point 65 μV ($p=0.54$, chi-square test).

Discussion

We studied the effect of functional KCNH2 gene 2690A > C polymorphism – which leads to an amino acid replacement (K897T) in the pore region of a rapidly activating component of the delayed rectifier potassium channel [8,12,13,15] – on cardiac repolarization in a Finnish exercise stress test population. While most of the previous results concerning the effects of various genotypes on the length of QT intervals have been derived at rest, we assessed the effect of this genotype on QT intervals during different phases of physical exercise. We considered measuring QTc during the exercise test as appropriate, because changes in body position and physical exercise amplify abnormal repolarization in LQT2

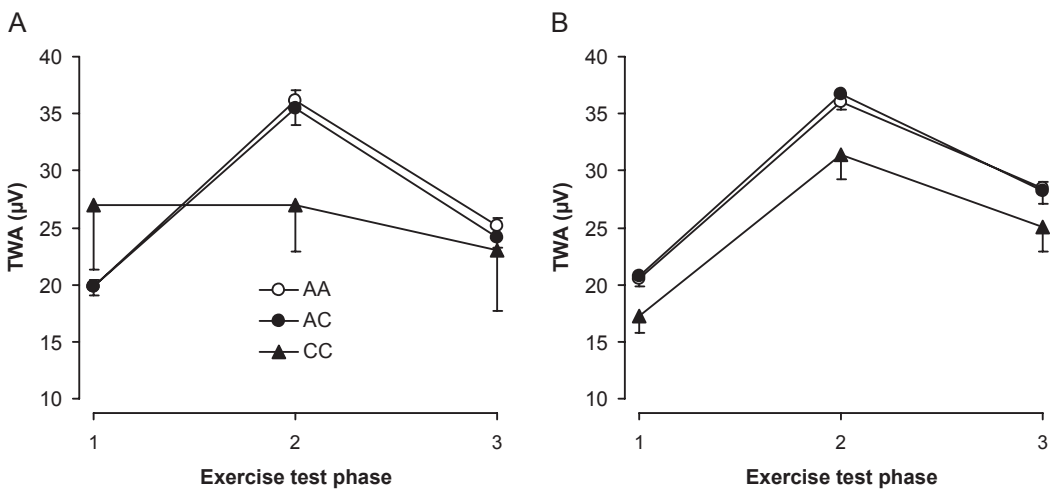

Figure 2. TWA values during different exercise test phases, rest (phase 1), exercise (phase 2) and recovery (phase 3), in (A) women and (B) men by the K897T genotypes. Mean ± SEM. Differences between genotypes were not statistically significant (RANOVA, $p=0.64$ for women; $p=0.20$ for men).

patients [18]. In addition, prolongation of the QTc interval during exercise differentiates patients at high risk of sudden cardiac death from those at low risk [36]. Besides the QTc interval duration, we also investigated the effects of this well-known mutation on TWA, another predictor of cardiac arrhythmias. The study cohort presented here is larger than those used previously [15], and the population consisted of actual patients who were examined with a clinical exercise test, with no other inclusion criteria.

HR variation influences the QT interval, thus making the method of QT correction important in this study. However, no perfect heart rate correction method yet exists [37]. Even though Bazett's formula is the most popular correction method, it works poorly, particularly in the HR range from 40 to 100 beats per minute [23,35]. In the present study, the Fridericia correction method was also used in the analysis, because the HR correlation of Fridericia QTc is clearly lower than that of Bazett's formula in all HR ranges [23].

The results of earlier studies on KCNH2 K897T polymorphism are not concordant regarding the QTc interval. One study with Finnish middle-aged women demonstrated that genotypes with the C allele were associated with prolonged QT intervals at rest [15]. In that study, AC and CC were combined due to low frequency of CC genotypes. In another study with a larger study cohort, the results were quite the opposite: women with the CC genotype had shorter QTc intervals at rest than those with AC or AA [8]. The authors of a large population-based KORA study concluded that Caucasians of both sexes demonstrated shorter QTc intervals in C allele carriers [14]. Our results on the effect of the CC genotype among women ($n=13$) coincide well with the earlier study consisting solely of Finns [15]. These different results may arise from population differences. However, the present finding needs to be tested in trials with larger CC groups.

Within all patients, resting SBP and HR were significantly higher in women than in men, which could be due to differences in β-blocker medication between genders. Our results present either a prolonged or decreased QTc interval in subjects medicated with β-blockers, depending on the method of QT interval correction for HR. Previous results have shown decreased intervals or no effect at all with Bazett's correction [29–32], which is in line with our results. Women tended to have longer QTc than men at rest, which is in line with earlier studies [28]. However, men had longer QTc intervals during peak exercise and recovery. Prevailing cardiovascular diseases may be the explanation for the longer QTc in men than in women in these two phases, since men are more likely to suffer from heart diseases prolonging repolarization: CHD, left ventricular hypertrophy and cardiomyopathy (Table II). Another possibility is that the QTc dynamics during exercise differ in men and women. However, there have been few studies in this area.

TWA is another ECG variable reflecting abnormalities in cardiac repolarization, and both TWA and prolongation of QT interval are predictive of cardiac arrythmias [1,19]. The effects of K897T polymorphism on TWA have not been examined previously. In our study, this polymorphism influenced TWA neither during exercise nor at rest.

TWA was higher during exercise and recovery in those with than in those without β-blocker treatment. In contrast, β-blockers did not influence TWA in an earlier study with CHD patients [38]. Our study included both CHD patients, who were more often β-blocker medicated, and healthy individuals, who did not have such medication. This is probably the explanation for the differences in TWA values between the β-blocker medicated and non-medicated groups.

Although we examined quite a large study cohort, the low frequency of the CC homozygotes limits the statistical power of the present study. Furthermore, more than 100 mutations have been described in the KCNH2 gene [6], and only one of them was studied in this article. Possible gene–gene interactions and influences of various haplotypes on cardiac repolarization cannot therefore be excluded [4]. Moreover, the heterogeneity of the study population may be considered a study limitation. Further studies are therefore warranted to expand our understanding of the clinical significance of genetic variation on cardiac repolarization.

In conclusion, the K897T polymorphism of the KCNH2 gene does not appear to have a marked influence on cardiac repolarization in a clinical exercise stress test population, but the effect of the CC genotype in women needs to be reassessed in larger populations.

Acknowledgements

We thank Ms. Marita Koli and Nina Peltonen for their skilful technical assistance, and the staff in the Department of Clinical Physiology for collecting the exercise test data. Grants: the Emil Aaltonen Foundation, the Finnish Foundation of Cardiovascular Research, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Tuberculosis Foundation of Tampere and Competitive research funding of the Pirkanmaa Hospital District. J.K. and J.L. contributed equally to this work.

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ORIGINAL ARTICLES

Effect of common KCNE1 and SCN5A ion channel gene variants on T-wave alternans, a marker of cardiac repolarization, during clinical exercise stress test: the Finnish Cardiovascular Study

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T-wave alternans (TWA) in electrocardiography (ECG) is a marker of cardiac repolarization, the molecular regulation of which is incompletely understood. High TWA and prolonged QT intervals are both associated with ventricular arrhythmias and sudden death. Therefore, we tested the hypothesis of whether the same mutations that influence the QT interval also affect TWA variation. We examined the effect of 3 ion channel gene single nucleotide polymorphisms (SNPs), rs1805127, rs727957 KCNE1, and rs1805124 SCN5A, on TWA during a clinical exercise test. A total of 2008 subjects from the Finnish Cardiovascular Study underwent an exercise test with online ECG recording. TWA was measured by using the time-domain, modified moving average method. Maximum values at rest, during maximal exercise, and during recovery were used as outcome measures in statistical analysis. Moreover, 4-year survival data were collected and ion channel SNPs were determined. TWA was lowest in subjects with the TT genotype of rs1805127 during all phases of the exercise test (RANOVA main effect for genotype, $P = 0.018$). The result remained significant after adjustment for age, existing coronary heart disease, and β-blocker medication status (RANCOVA, $P = 0.035$). Of the polymorphisms studied, only rs1805127 had a significant association with mortality ($P = 0.047$). The most common G-C haplotype, formed by rs727957 and rs1805127, was associated with TWA (RANOVA, $P = 0.007$) but not with mortality. The rs1805124 polymorphism was not associated with TWA. The common KCNE1 gene variant rs1805127 is associated with TWA during an exercise test in a Finnish population, which provides additional evidence that KCNE1 genetics may influence cardiac repolarization and cardiovascular mortality. (Translational Research 2008;152:49–58)

Abbreviations: AMI = acute myocardial infarction; ANOVA = analysis of variance; CHD = coronary heart disease; CI = confidence interval; ECG = electrocardiography; FINCAVAS = Finnish Cardiovascular Study; HR = hazard ratio; LQTS = long QT syndrome; LSD = least significant difference; MMA = modified moving average; PCR = polymerase chain reaction; RANCOVA = analysis of variance for repeated measures with covariates; RANOVA = analysis of variance for repeated measures; SCD = sudden cardiac death; SEM = standard error of the mean; SNP = single nucleotide polymorphism; TWA = T-wave alternans.

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Supported by the Emil Aaltonen Foundation (to T.L.), the Finnish Foundation of Cardiovascular Research, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Tuberculosis Foundation of Tampere, and the Medical Research Fund of Tampere University Hospital.

Submitted for publication December 13, 2007; revision submitted June 18, 2008; accepted for publication June 20, 2008.

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1931-5244/$ – see front matter

doi:10.1016/j.trsl.2008.06.003

AT A GLANCE COMMENTARY

Background

Electrocardiographic T-wave alternans (TWA) and QT interval are markers of cardiac repolarization. High TWA and prolonged QT interval are associated with ventricular arrhythmias and sudden cardiac death. Molecular regulation of TWA is incompletely understood. Therefore, we examined the effect of 3 QT interval-related, cardiac, ion channel gene, single nucleotide polymorphisms, rs1805127, rs727957 KCNE1, and rs1805124 SCN5A, on TWA during clinical exercise test.

Translational Significance

The results of this study prove that common gene variant (rs1805127) of KCNE1 are associated with TWA during the exercise test. This finding provides additional evidence that cardiac ion channel genetics may influence cardiac repolarization.

Electrocardiographic T-wave alternans (TWA) represents beat-to-beat alternation in the shape, amplitude, or timing of the T wave.[1,2] Both TWA and another measure of cardiac repolarization, the QT interval, have been used as diagnostic tools in LQTS.[3,4] High TWA is also associated with both ventricular arrhythmias[2,5] and sudden cardiac death (SCD).[2,6,7] Several prospective studies have demonstrated the relationship between TWA and an increased risk of ventricular arrhythmias in patients with prior myocardial infarction,[1,8] implantable cardioverter-defibrillator,[9,10] and congestive heart failure.[11,12] TWA has also been linked with increased risk of arrhythmias in patients with long QT syndrome (LQTS),[13] hypertrophic cardiomyopathy,[14] and cardiac autonomic dysfunction.[15] TWA has even been suggested to distinguish between patients likely and not likely to benefit from implantable cardioverter-defibrillator therapy.[10]

Cardiac repolarization, which is measured as the QT interval, is a highly genetically influenced quantitative trait with heritability estimates varying between 25% and 52%. Interestingly, some potassium ion channel mutations previously associated with different classes of LQTS also cause phenotypic variation in the general population.[16-19]

The major potassium currents that contribute to action potential duration are delayed rectifiers (I_{Kr} and I_{Ks}), whereas the inward rectifier (I_{K1}) participates in the terminal phase of repolarization.[20] The ion channel gene KCNE1 encodes a single transmembrane domain protein, which coassembles with a 6-transmembrane domain protein coded by KCNQ1 to form the α- and β-subunits of the potassium channel that conducts the slow component (I_{Ks}) of the delayed rectifier current.[21] Reduced I_{Ks}, which may accompany mutations within KCNE1, is implicated in the prolongation of cardiac action potential and associated ventricular arrhythmogenesis characteristic of type 5 LQT.[22,23] The KCNE1 gene presents several mutations out of which the rs1805127 and rs727957 polymorphisms as well as the KCNE1 gene haplotypes have been associated with the QT interval among the healthy population in previous studies.[16,17,19,24] The KCNE1 single nucleotide polymorphism (SNP) rs1805127 (112A>G) causes amino acid replacement of the amino acid serine by glycine at position 38 (S38G),[25] whereas the other SNP, rs727957, causes a noncoding G>A nucleotide change in the intronic gene region.[17] Furthermore, mutations in the gene SCN5A, which code the α-subunit of the voltage-gated cardiac sodium channel (I_{Na}), can cause LQTS.[26] The cardiac sodium channel SCN5A gene presents a functional SNP rs1805124 at locus 1673 where adenine is replaced by guanine (1673A>G), which results in the replacement of the amino acid histidine by arginine at position 558 (H558R).[27] This mutation can also influence cardiac repolarization, which is measured as a resting QT interval, in the general population.[16,18]

The detailed ionic mechanism of TWA appearance is still unclear, but changes in cellular and extracellular levels of potassium, sodium, and calcium ions are speculated to influence the beat-to-beat alteration of the T wave.[1] Therefore, the selected ion channel genes may be implicated in the regulation of TWA in a way that can eventually translate into inter-individual differences in the risk for ventricular arrhythmias[2,5] and SCD.[2,6,7] Only 1 study has evaluated the importance of genetic variation in the regulation of TWA.[28] Moreover, TWA has been recently shown to predict mortality in the exercise test population of the current study.[7] In this context, it is relevant to test the hypothesis of whether the same cardiac ion channel mutations, which have been previously shown to influence QT interval,[16,17,27] could also affect the variation of another trait that reflects cardiac repolarization (ie, TWA in patients who underwent a clinical exercise test FINCAVAS).[29] Our unpublished results suggest that TWA measured at any phase of the exercise test seems to have prognostic significance in the FINCAVAS study population. Therefore, we analyzed the effects of the KCNE1 gene polymorphisms rs1805127 and rs727957 (and their haplotypes), and the SCN5A gene rs1805124 polymorphism, on TWA at rest, during exercise, and during recovery, in addition to investigating their subsequent impact on 4-year mortality.

Table I. Population demographics (mean ± SEM) at rest

	Female (n = 760)	Male (n = 1319)
Age (years)	57.6 ± 0.5	56.9 ± 0.4
Body mass index (kg/m^2)	27.3 ± 0.2	27.5 ± 0.1
Diastolic blood pressure (mm Hg)*	79.1 ± 0.3	79.7 ± 0.3
Systolic blood pressure (mm Hg)*	138.6 ± 0.72	134.5 ± 0.5
Heart rate (beats per min)*	65.0 ± 0.4	62.1 ± 0.3
T-wave alternans (μV)	20.0 ± 0.5	20.4 ± 0.4

*Note: t-test, *$P < 0.001$, difference between genders.*

METHODS

Study cohort and design. The Finnish Cardiovascular Study (FINCAVAS) is an ongoing follow-up study that focuses on the genetic background of exercise stress test responses and coronary heart disease (CHD). This population consisted of 2212 patients who underwent an exercise stress test at the Tampere University Hospital between October 2001 and October 2004. Patients with a technically successful test and genotype information were included (2008 patients, 731 females, and 1277 males, aged 57.0 ± 13.1 years). The population demographics are given in Table I. The indications for the exercise stress test were a suspicion or evaluation of CHD (n = 898 patients), arrhythmias (n = 443), working capacity (n = 369), drug therapy (n = 308), and status preoperatively (n = 267) or after an acute myocardial infarction (AMI; n = 162). Some patients had more than 1 indication. The patients' medical history, lifestyle information, and cardiovascular risk factors were recorded. Blood samples were collected for DNA isolation and genetic analyses. Information concerning the patients' survival was collected from the Finnish cause of death register in April 2007. The Ethical Committee of the Hospital District of Pirkanmaa has approved the study protocol, and patients gave informed consent as stipulated in the Declaration of Helsinki.

Exercise test. An exercise stress test was performed with a bicycle ergometer with electric brakes. Standard 12-lead electrocardiography (ECG) using Mason-Likar modification for the lead system[30] was recorded at 500 Hz with the CardioSoft exercise ECG system (Version 4.14; GE Healthcare, Freiburg, Germany) at rest before the test, during the exercise test, and during the recovery phase. The ECG signal was analyzed by the released version of the GE Healthcare modified moving average (MMA) method. During the test, systolic and diastolic arterial pressures were measured with a brachial cuff every 2 min.

Measurement of TWA. The algorithm used in the identification and quantification of TWA in FINCAVAS is based on the time-domain MMA analysis and has been described earlier in detail.[31] In brief, the algorithm separates odd and even beats. Average morphologies of both the odd and even beats are calculated separately and updated continuously by a weighting factor of 1/8 or 1/32 of the difference between the ongoing average and the new incoming beat. The update is calculated for every incoming beat and results in continual moving averages of the odd and even beats. The TWA values were calculated continuously during the entire exercise test from rest to recovery using all the standard leads. The maximum TWA value at a heart rate less than 125 beats per min was taken into analysis; TWA values at a higher heart rate were considered unreliable and were excluded.

DNA extraction and genotyping. Genomic DNA was extracted from peripheral blood leukocytes using a commercially available kit and Qiagen BioRobot M48 Workstation (Qiagen Inc., Hilden, Germany). DNA samples were genotyped by the 5' nuclease assay and fluorogenic allele-specific TaqMan MGB probes[32] using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif). The nucleotide sequences of primers and probes used in the polymerase chain reaction (PCR) were deduced from published sequences deposited in the GenBank and Celera databases and were synthesized in conjugation with Applied Biosystems. PCR reaction that contains genomic DNA, 1 × Universal PCR Master Mix, 900 nM of each primer, and 200 nM of each probe was performed in 384-well plates using the standard protocol in a total volume of 5 μL. End-point fluorescence was measured and genotype calling was carried out by the allelic discrimination analysis module after PCR, which results in clear identification of the rs1805147 and rs727957 genotypes of the KCNE1 gene and the rs1805124 genotype of the SCN5A gene. Negative and positive controls as well as random duplicates were used as a quality control.

Statistical analysis. The degrees of TWA at the different phases of the exercise stress test were compared between the genders, between CHD patients, and between those who did not suffer from CHD as well as between β-blocker users and nonusers with a Student t-test for independent samples. The effect of age group and β-blocker medication (yes/no/pause) on TWA was also tested using univariate analysis of variance (ANOVA) with least significant difference (LSD) post hoc test.

To examine the possible differences in TWA between the genotypes, we used analysis of variance for repeated measures (RANOVA) with LSD post hoc test. Genotypes were compared also as recessive and dominant models between allele carrier and noncarrier groups. Age, β-blocker medication (yes/no/pause), and CHD (yes/no), which are known modulators of cardiac repolarization,[33-37] were used as covariates in RANOVA (RANCOVA). Temporary withdrawal of β-blocker medication was defined as 2–14 days because of a possible rebound effect. The CHD group was formed by combining subjects with a previous myocardial infarction and subjects with at least 50% narrowing in at least 1 major coronary artery in coronary angiography. Categorical variables (CHD, previous AMI, and β-blocker medication) were compared for the different genotype groups with a χ^2 test. Males and females were also compared within their own groups. TWA values for each genotype among men and women, respectively, were compared using ANOVA. Left ventricular hypertrophy

was defined from resting ECG with the Sokolow-Lyon equation.[38]

In addition, TWA assessed by time-domain MMA methodology was classified as normal and abnormal using a previously published cutoff point (65 μV) to predict total and cardiovascular mortality in the FINCAVAS study population.[7] Distributions of normal and abnormal TWA were compared between different genotype groups with the χ^2 test. The survival of different genotype and haplotype carriers versus noncarriers was estimated with the aid of Cox regression analysis and was reported by means of hazard ratios (HR) and 95% confidence intervals (CI).

A P less than 0.05 was considered statistically significant. All statistical analyses were performed with the SPSS 11.5 for Windows Software (SPSS Inc., Chicago, Ill).

Haplotype analysis. The χ^2 test was used to compare the frequencies of the polymorphisms between the study groups. Conformity of the genotype proportion to the Hardy-Weinberg equilibrium was examined in the whole population. Linkage disequilibrium analysis between polymorphisms was carried out with the free software LDA.[39] Haplotypes were estimated from the 2 single-nucleotide polymorphisms using the PHASE 2.1.1 program,[40] which uses a Bayesian statistical method for reconstructing haplotypes from population genotype data and lists the most likely pairs of haplotypes for each individual. The results are shown in the order KCNE1 SNP rs727957 and SNP rs1805127 (eg, haplotypes are G-C, G-T, T-C, and T-T, respectively).

RESULTS

Study cohort characteristics. The genotype distributions in the studied gene SNPs are shown in Table II. All 3 genotype distributions followed the Hardy-Weinberg equilibrium, which supports the integrity of our genetic data. No statistically significant differences were observed in the KCNE1 rs727957 and SCN5A rs1805124 genotype distributions between the genders (χ^2 test,P $\geq$ 0.79 for both), but the prevalence of the KCNE1 rs1805127 TT genotype was greater among males than females (χ^2 test, P = 0.03).

Among all patients, 1145 (57.0%) were on β-blocker medication on the exercise test day, whereas 88 (4.4%) were on medication break. The number of patients with CHD was 849 (40.8%). When compared with females, males suffered more often from cardiovascular diseases such as CHD (χ^2 test, $P < 0.001$), left ventricular hypertrophy (χ^2 test, $P = 0.020$), and previous AMI (χ^2 test, $P < 0.001$).

In this study population, 443 patients(22%) had a suspicion of arrhythmia as the indication for the exercise test, and 304 (69%) of them had also reported subjective symptoms of arrhythmias. Among the patients with the arrhythmia indication, 192 patients (43%) also had other reasons for the exercise test, such as a diagnosis of CHD or an evaluation of working capacity, whereas 97 patients (22%) already had a previous diagnosis of CHD. No patients examined because of suspected arrhythmia were found to have LQTS.

Table II. Ion channel genotype distributions between genders (%)

	Female (%)	Male (%)	All (%)
SCN5A rs1805124			
TT	63.2	63.6	63.5
CT	31.5	31.6	31.6
CC	5.3	4.8	5.0
Total (n)	699	1211	1910
KCNE1 rs1805127*			
CC	36.5	34.5	35.2
CT	49.8	47.1	48.1
TT	13.7	18.4	16.7
Total (n)	693	1211	1904
KCNE1 rs727957			
GG	69.5	71.0	70.4
GT	28.4	27.0	27.5
TT	2.1	2.0	2.0
Total (n)	718	1244	1962

Note: χ^2 test, *P = 0.03 difference between distributions of genders.

The relation of TWA with potential covariates. TWA was higher in patients aged over 60 years when compared with younger age groups at all 3 exercise test phases (ANOVA, $P \leq 0.003$ for all) (Table III). β-blocker medication was also a significant modulator of TWA during the exercise test and recovery, but not at rest (Table III). TWA was higher in the β-blocker-treated group when compared with patients without medication and those taking a break in medication, both at maximal exercise and during the recovery phase (ANOVA, P $<$ 0.001 for both).

During the entire exercise test, females had numerically lower TWA than males, but the finding was statistically significant only at the recovery phase (t-test, $P = 0.03$) (Table III). Existing CHD increased TWA significantly during the recovery phase (t-test, $P = 0.049$), whereas at rest and during exercise, TWA was almost equal between the patients with and without CHD.

The relation of variation in TWA with cardiac ion channel gene polymorphisms. Variations in either the SCN5A or KCNE1 gene were not significantly associated with the above-mentioned potential confounding factors modulating TWA, such as age, use of β-blocker medication, or the occurrence of CHD or AMI, except for the minor gender diffrence in KCNE1 rs1805127 distribution. These polymorphisms were also not significantly associated with BMI, heart rate, or blood pressure values (data not shown). Among all subjects, TWA

Table III. T-wave alternans (mean ± SEM) according to the age groups, use of β-blocker medication, and gender at different exercise test phases

	Rest	Exercise	Recovery
Age group (years)			
0–39	19.1 ± 0.88	31.7 ± 1.28	22.4 ± 1.34
40–59	19.3 ± 0.41	34.5 ± 0.60	25.5 ± 0.61
60+	21.3 ± 0.45	38.3 ± 0.74	29.6 ± 0.54
P-value*	0.003‡	<0.001‡	<0.001§
β-blocker medication			
No	20.4 ± 0.49	33.1 ± 0.68	24.9 ± 0.65
Yes	20.0 ± 0.37	38.1 ± 0.62	28.6 ± 0.52
Pause (2–14 days)	21.1 ± 1.34	31.5 ± 1.56	24.3 ± 1.44
P-value*	0.76	<0.001‖	<0.001‖
Gender			
Female	20.0 ± 0.46	36.2 ± 0.87	25.9 ± 1.10
Male	20.3 ± 0.37	36.2 ± 0.61	28.2 ± 0.54
P-value†	0.47	0.99	0.034

Notes: *ANOVA and †*t*-test. Statistically significant difference in LSD post hoc analysis.
‡$P \leq 0.03$ between the oldest age group and other groups.
§$P \leq 0.023$ between all age groups.
‖$P < 0.001$ between β-blocker medication users and nonusers.

was significantly lower in those with the TT (rs1805127, that is, 38G/G) genotype during all phases of the exercise test when compared with subjects with other genotypes (RANOVA main effect for genotype, P = 0.018; LSD post hoc analysis, P = 0.013 for TT vs CC and P = 0.006 for TT vs CT). The result remained significant after adjusting for potential confounding factors such as age, CHD, and β-blocker medication status (RANCOVA, P = 0.042).

When analyzing females and males separately, the TT genotype was associated with the numerically lowest TWA during all phases of the exercise test in both genders, but the finding was statistically significant only in females (RANOVA main effect for genotype, $P = 0.004$ for females, $P = 0.054$ for males) (Fig 1). In females the finding remained significant after adjustment for the above-mentioned covariates (RANCOVA, $P = 0.004$). No statistically significant gender by genotype (for rs1805127) interactions were observed in relation to TWA over the whole exercise test (RANOVA interaction, $P = 0.36$).

The KCNE1 rs727957 polymorphism was not associated with TWA, neither in the entire population nor in either gender (both RANOVA and RANCOVA main effect for genotype, $P \geq 0.71$ for all, Fig 2). When comparing the T allele carriers with noncarriers, the differences in TWA values were not statistically significant (RANOVA main effect for genotype group, $P = 0.12$).

TWA values did not differ among the 3 genotypes of SCN5A rs1805124 in the entire population or in either gender (RANOVA for main effect for genotype, $P =$ NS for all, Fig 3). However, in LSD post hoc analysis, the CC tended to differ from the TT genotype in both genders ($P \leq 0.050$ for both). Within males, the C allele carriers had significantly higher TWA ($P < 0.05$) than subjects with the TT genotype, but when adjusting for all potential covariates, the finding had only borderline significance (RANCOVA for main effect for genotype group, $P = 0.05$).

The relation of abnormal variation in TWA with ion channel polymorphisms and haplotypes. In all, 178 patients (8.9%) had TWA of 65 μV or more, which was a cutoff value classified to constitute a significantly greater mortality risk according to our previous study from the same population.[7] In any of the investigated polymorphisms or their forming haplotypes, the distributions of normal and abnormal TWA did not differ among the 3 genotype or haplotype groups when compared using the cutoff point of 65 μV (χ^2 test, $P \geq 0.14$ for all).

The relation of TWA and QTc with KCNE1 gene haplotypes. Linkage disequilibrium analysis showed that the alleles of 2 polymorphisms (rs727957 and rs1805127) were associated (LDA program, $D' = 0.14$; $r^2 = 0.005$, $P < 0.001$, χ^2 test). Based on the genotype data for KCNE1 rs727957 and rs1805127, 4 haplotypes were detected within this sample: G-C (54%), G-T (31%), T-C (6%), and T-T (10%).

Among all subjects, the most common G-C haplotype carriers presented with higher TWA than noncarriers during the entire exercise test (RANOVA main effect for haplotype, $P = 0.007$), and the result remained significant after using age, β-blocker medication, and CHD as covariates (RANCOVA main effect for haplotype, $P = 0.015$). Interestingly, in medical history, 22% of patients with the G-C haplotype and 17% of those with other haplotypes reported having arrhythmias (χ^2 test, $P < 0.05$ for difference).

When comparing the G-C haplotype effect on TWA in males and females separately, the results were parallel but statistically significant only among females (RANOVA main effect for haplotype, $P = 0.031$ for females, $P = 0.055$ for males; RANCOVA, $P = 0.038$ for females, $P = 0.13$ for males). In corresponding statistical analyses for other haplotypes, no significant associations with TWA were observed.

The average QTc duration (heart rate correction carried out with Bazett's model) for the G-C haplotype carriers was 457 ± 33 ms, for G-T 457 ± 33 ms, for

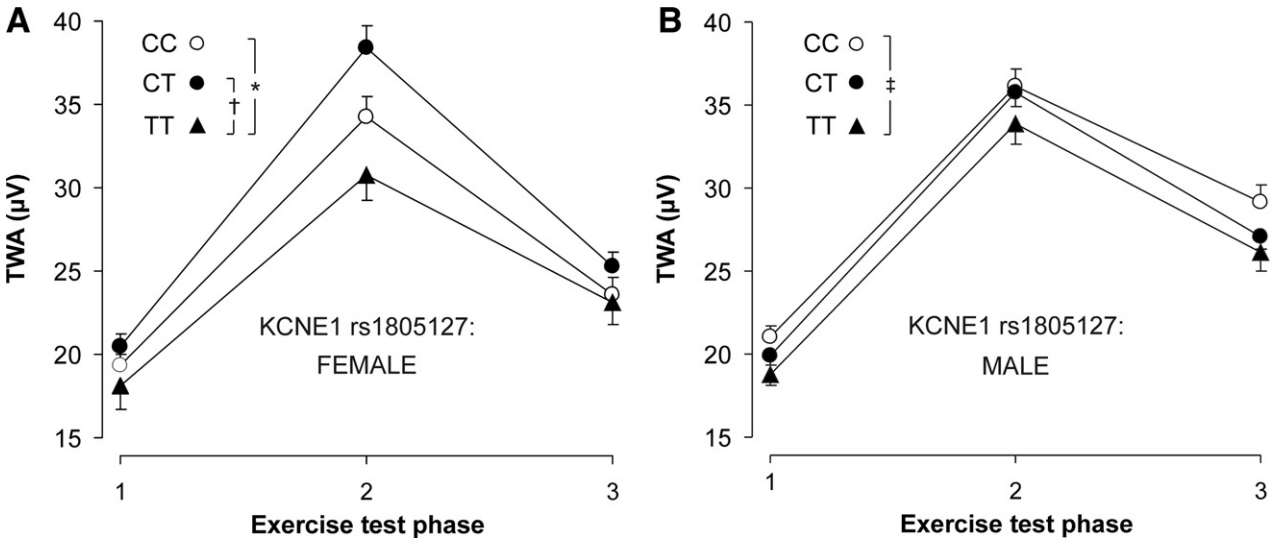

Fig 1. TWA values during different exercise test phases by KCNE1 rs1805127 genotype: 1 = rest, 2 = exercise, and 3 = recovery. Statistically significant difference between the genotypes in females (**A**), RANOVA $P = 0.004$, LSD post hoc test $^{*}P = 0.018$ and $^{\dagger}P = 0.003$; no major differences between the genotypes in males (**B**), RANOVA $P = 0.054$; LSD post hoc test $^{\ddagger}P = 0.017$. Mean ± S.E.M.

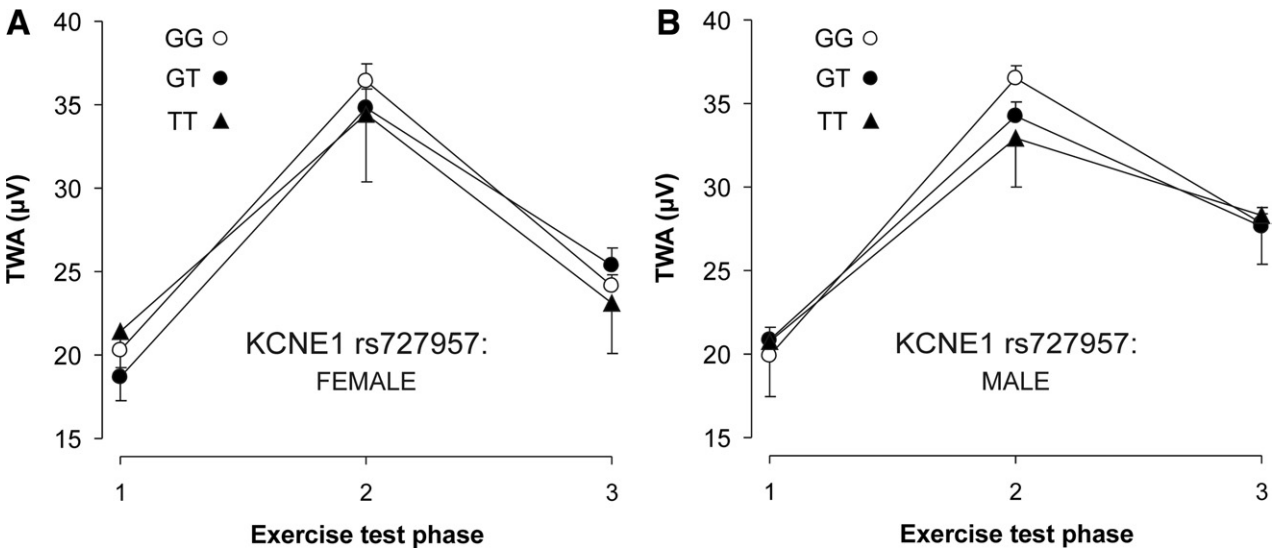

Fig 2. TWA values during different exercise test phases by KCNE1 rs727957 genotype: 1 = rest, 2 = exercise, and 3 = recovery. No statistically significant differences were observed among the 3 genotypes in females (**A**) or males (**B**). Mean ± SEM.

T-C 459 ± 33 ms, and for T-T 456 ± 33 ms. The mean QTc duration of G-C haplotype carriers did not differ from the QTc duration of other haplotype carriers (*t*-test, $P > 0.53$ for all comparisons). The other demographics or phenotypic variables, such as BMI, resting systolic or diastolic blood pressure levels, or heart rate, of all subjects with the G-C haplotype did not differ significantly from that of other haplotype carriers. In corresponding statistical analyses for men and women, these differences also remained statistically insignificant.

The association of polymorphisms with mortality. During an average follow-up of 47 months (from 0 to 66 months), 116 deaths were reported. From the genotypes studied, the minor allele T carriers of KCNE1 rs1805127 tended to have lower mortality compared with noncarriers (HR 0.68, 95% CI 0.46–0.99, P = 0.047). The mortality of the minor allele carriers of

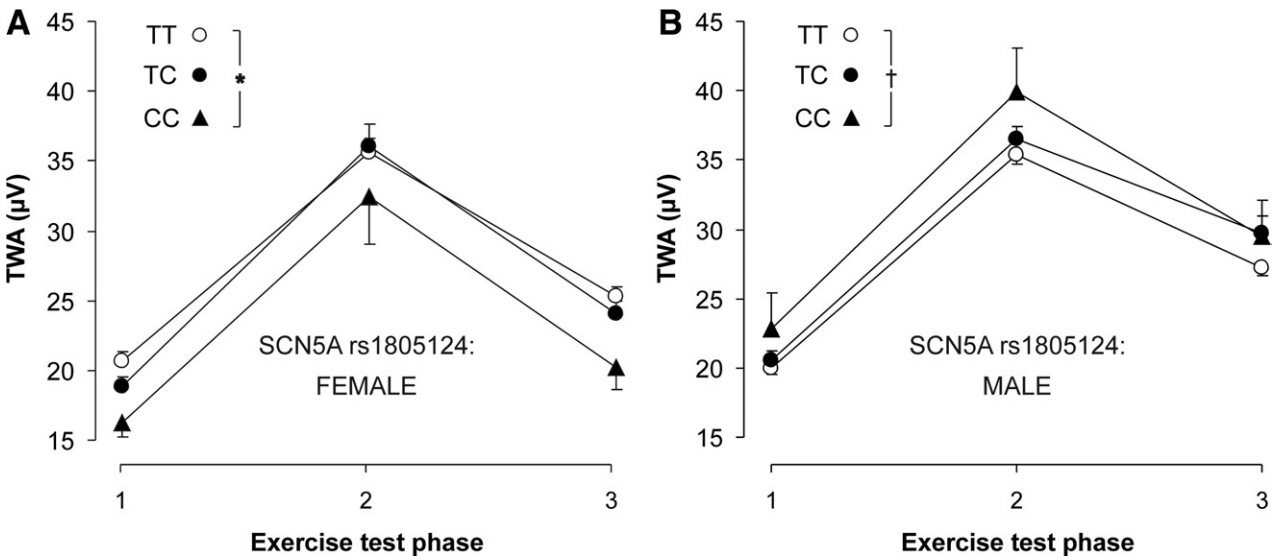

Fig 3. TWA values during different exercise test phases by SCN5A rs1805124 genotype: 1 = rest, 2 = exercise, and 3 = recovery. No statistically significant differences between the genotypes in RANOVA, $P = 0.11$ for females (**A**), $P = 0.054$ for males (**B**); LSD post hoc test $*P = 0.049$ (**A**), $\dagger P = 0.050$ (**B**). Mean ± SEM.

KCNE1 rs727957 and SCN5A rs1805124 did not differ from noncarriers in the Cox regression analysis (HR 1.17, 95% CI 0.79–1.74, and 0.76, 95% CI 0.72–1.56, respectively). The KCNE1 G-C haplotype was also without an effect on mortality (HR 1.19, 95% CI 0.72–1.96) as compared with noncarriers.

DISCUSSION

We recently showed in the FINCAVAS study that a high magnitude of TWA predicts all-cause and SCD mortality especially above the cutoff point of 65 μV.[7] This observation forms the basis for the rationale to study the possible genetic determinants of the variation in TWA in our study population. As cardiac potassium and sodium channel mutations are determinants for 1 marker of cardiac repolarization, namely the QT interval, genetic polymorphisms known to affect this interval also served as natural candidate genes for the study of the variation in TWA in the same Finnish exercise test population.

One major finding is that the SNP rs1805127 of KCNE1 affects TWA during a clinical exercise test. Subjects with the TT genotype presented lower TWA than the other genotypes, even when age, β-blocker medication status, and current CHD were taken into consideration as covariates. The haplotype analyses of the same gene corroborated our finding, because the most common G-C haplotype (ie, the G allele in rs727957 and the C allele in rs1805127) of the KCNE1 gene seemed to be associated with increased TWA. No previous results associate these SNPs with TWA, whereas evidence of a positive association exists between QT interval and the rs1805127 polymorphism in a relatively small study population.[24] In the study by Friedlander et al,[24] the CT genotype group was associated with longer QTc when compared with the CC group among males. However, the population size of this previous study was too small to evaluate the effects of the minor genotype TT,[24] which was the most important genotype that affected TWA in our study. In this regard, the data on the KCNE1 gene are inconclusive, because no previous studies have found any association between the SNP rs1805127 and cardiac repolarization at rest, as measured by QT interval.[16,41] In addition, Gouas et al[19] found an association with the KCNE1 haplotype (consisted of rs1805127 and rs2236609) and the QT interval, but again the study population was small (n = 200). It is important to note that these previous results are not fully comparable with ours, because the QT interval and TWA are different indicators of cardiac repolarization. In addition, the previous results have been derived from the resting state, although SCD often occurs during physical exercise.

Previous studies show a positive association of rs1805124 with cardiac repolarization as measured by QT interval.[16,18] However, in our study, the rs1805124 in SCN5A did not affect TWA, even though we cannot exclude the possibility that other variants or their forming haplotypes in this gene may also be important. Furthermore, no previous information exists about the sex-specific influence of ion channel genes in relation

to TWA. It is known that action potential duration, as measured by the QT interval, is prolonged in healthy women as compared with men[42] and that QT shortens in men after puberty.[33] In the current study, the effects of KCNE1 rs1805127 on TWA tended to be different between the genders, and the association between rs1805127 and TWA was more obvious in females than males (Fig 1). According to our knowledge, the gender differences of TWA have not been as well studied as the gender differences of the QT interval.

The genetic determinants of QT or cardiac repolarization may represent risk factors for arrhythmias or SCD.[43-45] In the current study, 116 deaths were detected during a mean follow-up of 47 months. Of the SNPs studied, only the rs1805127 had an association with both mortality and TWA.

Outward rectifier potassium channels, which open after the cells are depolarized, generate 2 different types of current. One is a brief transient current that causes rapid repolarization (I_{Kr}), whereas the other causes more sustained delayed rectifier currents (I_{Ks}). As mutations in the KCNE1 gene that encodes the slow outward rectifier potassium channel cause lengthening of the QT interval, it is not surprising that the polymorphism of the same gene, which is described in the current study, also affects TWA, another marker of repolarization. The pathophysiology of TWA has been linked to altered excitation−contraction coupling and calcium cycling[46]; however, modulation of cardiac potassium currents can affect action potential duration alteration in experimental studies.[47,48] Therefore, the detailed mechanism of TWA appearance is still unclear and the possible mechanisms behind these findings remain to be solved in future studies. It is conceivable that changes in the structure of potassium channel proteins affect the regulation of ionic currents, which influence cardiac action potential duration and repolarization. Another explanation might be that the studied KCNE1 polymorphisms are in linkage disequilibrium with some unknown but more important gene (or SNP) involved in regulating TWA and, thus, cardiac repolarization. Another possibility is that KCNE1 polymorphism (rs1805127) is linked directly with some yet unknown mechanisms associated with TWA.

Two main techniques have been introduced for measuring TWA. In this study, we employed the MMA approach[31] because the patients performed a normal routine exercise stress test in which heart rate was not stabilized to meet the data-stationary requirements of the fast Fourier transform analysis.[49]

In addition to genotype effects, we also examined the influences of CHD, β-blocker medication, and age on TWA. As proposed, β-blocker medication status affected TWA, but only during the exercise and recovery phases and, in our sample, not at rest like in most previous studies.[50,51] It is of note that in 1 large study with CHD patients, no differences in TWA were observed between β-blocker medicated patients and those without such medication.[52] Our study included a considerable number of CHD patients who were also more often treated with β-blockers than the healthy individuals. This observation is the probable explanation for the differences in TWA values between the β-blocker medicated and nonmedicated groups. In this population, age also had a significant effect on TWA, in that the oldest group (age ≥ 60 years) presented with the highest magnitude of TWA, which could be a consequence of higher cardiac morbidity within the oldest study group.

The current study includes several limitations. First, the FINCAVAS study cohort represents a typical Finnish outpatient hospital population, and thus, the proportion of CHD patients and use of medication differs from those of a normal population-based sample. The lack of original exclusion criteria has made the study population heterogeneous, which was taken into account by using the most important disparities as covariates in our multinomial statistical analyses. Like in most association studies, the results cannot be directly generalized to other populations.[53] Moreover, in this study population, the groups with minor alleles were too small for final survival analyses, which made the results concerning mortality tentative. In addition, several other SNPs in the KCNE1 and the SCN5A gene not studied here may be more functional and therefore more important.[16-19,24,27] From the SNPs studied, only rs1805124 in SCN5A has been tested functionally.[27]

In summary, the polymorphism rs1805127 of the cardiac potassium channel gene KCNE1 may be a genetic factor that influences TWA during a clinical exercise test. Interestingly, the same allelic variation also tended to be associated with mortality, and our results together with previous observations imply that additional investigations are warranted to assess whether this genetic marker represents a risk factor for arrhythmias and sudden cardiac death at the population level. However, the rs727957 polymorphism of KCNE1 and the rs1805124 polymorphism of SCN5A are not major determinants of TWA or mortality.

The authors thank Ms. Marita Koli and Ms. Nina Peltonen for their skillful technical assistance and the staff of the Department of Clinical Physiology for collecting the exercise test data.

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Scandinavian Journal of Clinical & Laboratory Investigation, 2011; 00: 1–8

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ORIGINAL ARTICLE

Allelic variant of *NOS1AP* effects on cardiac alternans of repolarization during exercise testing

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Abstract

Introduction. A repolarization abnormality manifested as T-wave alternans (TWA) in electrocardiogram (ECG) predicts cardiovascular mortality. A common variant in the *NOS1AP* gene is associated with mortality and QT interval duration, possibly in a gender-specific manner, but data is lacking on potential association with TWA. This study tested association between rs10494366 in *NOS1AP* and both TWA and 4-year mortality. ***Material and methods.*** A total of 1963 Finnish Cardiovascular Study participants (36.6% female, 57.1 ± 13.0 years) were genotyped and their maximal TWA values were measured from continuous ECG recordings during clinical exercise test at rest, exercise and recovery. ***Results.*** We observed a significant gender-specific effect of NOS1AP genotype on TWA. In all subjects, there was no statistically significant difference between the three genotypes (TT, TG, GG) in the responses of TWA over the entire exercise test (time-by-genotype interaction $p = 0.057$). In women, after adjustment for age, coronary heart disease and β-blocker medication status, changes of TWA over different phases of exercise test were significantly associated with NOS1AP genotype (time-by-genotype interaction $p = 0.001$). In men, *NOS1AP* rs10494366 was not associated with TWA. During follow-up (mean 47 months), 113 patients died. *NOS1AP* rs10494366 was not a statistically significant predictor of mortality. ***Conclusion.*** The NOSIAP variant rs10494366 influences TWA and TWA response during clinical exercise test in females. Gender-specific effects have also been previously reported for the influence of the variant on QT interval. If replicated, these findings should prompt studies to further elucidate the mechanisms underlying the gender differences in *NOS1AP* effects on repolarization.

Key Words: *Cardiac electrophysiology, exercise test, mortality, polymorphism*

Introduction

T-wave alternans (TWA) in the electrocardiogram (ECG) is an important index of variability in cardiac repolarization that measures beat-to-beat alternation in T-wave shape, amplitude, and timing [1]. There are several approaches to characterizing TWA but two [2], the fast Fourier Transform spectral method [3–5] and the modified moving average (MMA) method [6–8] have been most extensively utilized. Intrinsic flexibility and demonstrated capacity of the MMA method to measure TWA accurately under dynamic conditions including changing heart rates, myocardial ischemia, exercise, and behavioural stress [6–9] enables identification of individuals whose risk is elevated, but who are otherwise not identified by contemporary routine ECG tests [2].

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(Received 21 June 2011; accepted 7 October 2011)

ISSN 0036-5513 print/ISSN 1502-7686 online © 2011 Informa Healthcare
DOI: 10.3109/00365513.2011.638725

Increased magnitude of TWA predicts cardiac arrhythmias [1,5], cardiovascular death [10] as well as sudden cardiac death in many cardiac patient populations [11], and even in a low-risk clinical exercise test population [7,12]. Furthermore, the prognostic power of TWA measured during exercise is preferable to TWA defined before or after exercise [13]. There is also uncertainty about predictive accuracy of TWA on mortality and ventricular tachyarrhythmias especially TWA measured by the spectral method [14,15].

Recently, several different variants in the *NOS1AP* gene have been associated with variation in the heart rate corrected QT (QTc) interval, possibly in a sex-specific manner [16–19]. There is also evidence of the single nucleotide polymorphism (SNP) rs10494366 of NOS1AP increasing risk of sudden cardiac death in patients with long QT syndrome [20]. The association between QTc duration and the SNP rs10494366 has been widely replicated [16–18] but effect on TWA has not been investigated. Therefore, our hypothesis was based on the analysis of the relationship of this strongly QTc-associated SNP of *NOS1AP*, rs10494366, circumventing some of the multiple-testing issues inherent in less-focused genetic association studies. Here, we report an association analysis of this variant with TWA measured at rest, during exercise and during recovery in the Finnish Cardiovascular Study (FINCAVAS). Previously, we have shown that high TWA measured by MMA method is a risk marker for mortality in this population [7]. Therefore, we also investigated the association of this *NOSIAP* variant with mortality in these subjects.

Methods

Study cohort and design

The FINCAVAS is an ongoing follow-up study focusing on the genetic background of exercise stress test responses. The present study population was collected between October 2001 and October 2004 and it consisted of 2212 patients who were coming for an exercise test at the Tampere University Hospital. Only technically successful tests and patients with acquired genotype information were included (1963 patients, 718 females and 1245 males). The population demographics are given in Table I. There were several different indications for the exercise stress test and some patients had more than one indication. The indications were a suspicion of coronary heart disease (CHD) ($n = 887$ patients), palpitation or sense of arrhythmia ($n = 423$), evaluation of working capacity ($n = 357$) and drug therapy ($n = 303$), as well as ascertaining the patient's exercise performance preoperatively ($n = 268$) or after an acute myocardial infarction ($n = 152$). Data of patients' medical history, lifestyle and cardiovascular risk factors were collected by a computerized questionnaire. Temporary withdrawal of β-blocker medication was defined as 2–14 days, because of a possible rebound effect. Blood samples were collected for genetic analyses. In addition, digital angiographic data from 419 study patients was analysed in detail by a cardiologist blinded to all other study data. The presence of CHD was defined as follows: subjects who had either a previous myocardial infarction or showed more than 50% narrowing in at least one major coronary artery in coronary angiography.

The Ethical Committee of the Hospital District of Pirkanmaa has approved the study protocol and patients gave informed consent as stipulated in the Declaration of Helsinki.

Exercise test

The exercise tests were performed by bicycle ergometer with electric brakes. Standard 12-lead ECG using Mason-Likar modification for the lead system [21] was recorded at 500 Hz with CardioSoft exercise ECG system (Version 4.14, GE Healthcare, Freiburg, Germany) at rest before the test, during the exercise test, and recovery phase. The ECG signal was analysed fully automatically by the released version of the GE Healthcare MMA method. During the test the systolic and diastolic arterial pressures were measured with a brachial cuff every 2 minutes.

Measurement of TWA

Identification and quantification of TWA in FINCAVAS was based on the time-domain MMA analysis, and the algorithm we used has been described

Table I. Population demographics. The values are (mean ± SD) at rest.

Variable	Female ($n = 747$)	Male ($n = 1290$)	All ($N = 2037$)
Age (years)	57.7 ± 13.0	56.9 ± 13.0	57.1 ± 13.0
Body mass index (kg/m^2)	27.3 ± 4.8	27.5 ± 4.2	27.5 ± 4.4
Systolic blood pressure (mmHg)*	138.7 ± 19.8	134.6 ± 18.1	136.0 ± 18.9
Diastolic blood pressure (mmHg)	79.1 ± 9.6	79.8 ± 9.8	79.5 ± 9.7
Heart rate (beats/min) *	65.0 ± 11.5	62.2 ± 11.9	63.2 ± 11.8
T-wave alternans (μV)	19.9 ± 12.3	20.4 ± 13.2	20.2 ± 12.8

Statistics: *$p < 0.001$, *t*-test comparison between sexes.

earlier in detail [22]. We have used the same method in our previous TWA analyses [23]. Briefly, the TWA values were calculated continuously during the entire exercise test from rest to recovery. The maximum TWA value at heart rate < 125 beats per minute was taken into analysis. TWA values at higher heart rate were considered unreliable and were therefore excluded. The highest magnitude of TWA during rest, exercise and recovery was taken into analysis. The TWA algorithm performs the measurement of T wave variation at an accuracy and resolution of 1 μV.

Follow-up

Death certificates were received from the Causes of Death Register, maintained by Statistics Finland, in April 2007; this source has been shown to be reliable [24]. The certificates contained causes of death using the tenth revision of the International Classification of Diseases. The diagnosis codes and certificate texts were used to classify the deaths as all-cause, cardiovascular, and sudden cardiac death (SCD; defined as a death within 24 h after the onset of symptoms).

DNA extraction and genotyping

DNA was extracted from peripheral blood leukocytes using a commercially available kit and Qiagen BioRobot M48 Workstation according to the manufacturer's instructions (Qiagen Inc., Hilden, Germany). DNA samples were genotyped for *NOS1AP* SNP rs10494366 by employing the 5' nuclease assay and fluorogenic allele-specific TaqMan MGB probes [25] using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as we previously reported [26]. Random duplicates were used as a quality control.

Statistical analysis

To examine the possible genotype and sex differences in the responses of TWA over the different phases of the clinical exercise test we used analysis of variance for repeated measures (RANOVA) with the Least Significant Difference (LSD) test in post hoc analyses. In addition to main effects we calculated time-by-sex and time-by-sex-by-genotype interactions. Age, β-blocker medication (yes/no/temporarily withdrawn) and CHD (yes/no), all important modulators of cardiac repolarization [27–30], were used as covariates in analysis of covariance for repeated measures (RANCOVA) and Cox regression. Cox regression models were used to calculate genotype specific hazard ratios (HR) and their 95% confidence intervals (CI) for all cause and cardiovascular mortality as well as SCD. The proportional hazards assumption was confirmed to be valid using log-log plots and assessment of weighted residuals. As there were significant sex- and genotype-related differences (RANOVA both time-by-sex interaction and time-by-sex-by-genotype interaction were significant $p = 0.001$ in relation to TWA responses) we also performed the TWA response analysis separately in men and women.

Genotype frequencies were tested for Hardy-Weinberg equilibrium with a 2 degree-of-freedom χ^2 test. Resting TWA values and other numerical haemodynamic variables are presented as means ($\pm$ SD) and the sex groups were compared using Student's *t*-test for independent samples. The proportion of CHD patients and β-blocker medication users between sexes, as well as distributions of high and low mortality risk (defined using a previously published cut-off point (65 μV) for TWA), were compared between three genotype groups with a 2 degree-of-freedom χ^2 test. In the FINCAVAS study population, more than 3, 6 and 7 times relative risk for total, cardiovascular and SCD mortality, respectively, was proposed for the cut-off point [7].

A p-value < 0.05 was considered statistically significant. The statistical analyses were performed with the SPSS 17.0 for Windows Software (SPSS Inc, Chicago, Illinois) and power calculations were made with free Power and Sample Size Calculation program (PS). Power calculations in our data showed that we had 90% power at an alpha of 0.05 to detect a 4 μV difference in mean TWA between TT and TG subjects in women. A minimal TWA value 4 μV was chosen in power calculations because the TWA algorithm performs the measurement of this variation at an accuracy and resolution of 1 μV.

Results

Population characteristics

The main cohort demographics are shown in Table I. In the whole study population, rs10494366 had a minor allele (G) frequency of 34.7%, comparable to previously reported frequencies [17]. The genotype distribution did not deviate from the Hardy-Weinberg equilibrium.

Table II shows the distribution of *NOS1AP* rs10494366 genotype, CHD and β-blocker medication status separately in males and females. Genotype frequencies were similar between sexes ($p = 0.77$, χ^2 test). In the total cohort the prevalence of CHD was 40.5% ($n = 795$) with a higher prevalence in males (46.2%) than females (30.6%, $p < 0.001$ for difference) (Table II). Most of the CHD patients were on β-blocker medication (73.1%), while in 3.0% this medication was temporarily withdrawn. From all subjects 1124 (57.3%) patients were on β-blocker medication during the exercise test, while

Table II. Prevalence of nitric oxide synthase regulator gene *NOS1AP* (rs10494366) genotypes, coronary heart disease and β-blocker medication in males and females.

Variable	Female *n* (%)	Male *n* (%)	*p*-value* (χ^2 test)
Genotype TT	329 (44.0)	579 (44.9)	0.932
Genotype TG	330 (44.2)	560 (43.4)	
Genotype GG	88 (11.8)	151 (11.7)	
Coronary heart disease	232 (31.1)	602 (46.7)	<0.001
β-blocker medication	407 (54.5)	847 (65.7)	<0.001

Statistics: *χ^2 test comparison between sexes.

the medication was discontinued for 2–14 days before the test in 85 (4.3%) patients. Data of hormone replacement therapy was available for 589 (82.0%) women, of which 142 subjects (24.1%) used hormones, either estrogen alone or a combination of estrogen and progesterone. In women estrogen medication had no statistically significant influence on TWA responses during the exercise test (RANOVA, $p = 0.056$ for time-by-hormones interaction).

During an average 4-year follow-up time (range 0–66 months) 113 (5.7%) deaths were documented, of which 55 were cardiovascular deaths and 31 were classified as SCD.

Effect of NOS1AP rs10494366 polymorphism and gender on TWA variation

In all subjects the rs10494366 polymorphism was not significantly associated with TWA variation during the entire exercise test ($p = 0.057$, RANOVA time-by-genotype interaction, Figure 1A). Women had significantly greater variation of TWA over the entire exercise test as compared to men ($p = 0.001$, RANOVA time-by-sex interaction, Figure 2). Post-hoc testing showed that numerical TWA was significantly lower in women compared to men especially at the recovery phase (24.8 ± 14.2 vs. 28.2 ± 19.2, $p < 0.001$, *t*-test). In females the *NOS1AP* SNP rs10494366 was associated with TWA during rest and exercise (with higher maximum TWA in those with GG genotypes), while in males there was no such association ($p < 0.001$ for females, Figure 1B, and $p = 0.16$ for males, Figure 1C, RANOVA time-by-genotype interaction). Within females this finding remained significant after adjustment for age, CHD and β-blocker medication status ($p = 0.001$, RANCOVA time-by-genotype interaction).

When we dichotomized TWA into high ($\geq$ 65 μV) and low ($<$ 65 μV) mortality risk groups [7], the GG genotype was significantly associated with high mortality risk compared to TT or TG genotypes in females ($p = 0.021$, χ^2 test). In a similar analysis for men *NOS1AP* genotypes were not associated with high- or low-risk groups.

NOS1AP and mortality

During mean follow-up of 47 months, 113 of the original patients died but the total mortality was not related to *NOS1AP* rs10494366 in unadjusted Cox regression analysis (HR = 1 for TT, HR = 1.00 for TG, CI 0.67–1.48 and HR = 1.24 for GG, CI 0.70–2.18). None of the three genotypes of *NOS1AP* rs10494366 were associated with all cause (Table III) or cardiovascular mortality or SCD when age, β-blocker medication and CHD were taken into consideration. Of the covariates used, age and sex were related to mortality (Table III).

Discussion

Within a short period after its identification, the *NOS1AP* locus rs10494366 in chromosome 1q23.3 has proven to have a robust association with QTc duration in a wide range of populations [16,17,31]. Here we show, for the first time, that the G allele of rs10494366, which is associated with longer QTc duration, is also associated with higher values of another clinically important marker of abnormal repolarization, cardiac repolarization alternans (TWA), in women assessed during a clinically indicated exercise test. The results remained significant after adjustment for several important factors having influence on cardiac repolarization including age, CHD and β-blocker medication.

Functionally, the *NOS1AP* gene encodes the nitric oxide synthase 1 adaptor protein, which regulates neuronal nitric oxide synthase (nNOS) activation and increases N-methyl-D-aspartic acid (NMDA) receptor-gated calcium influx [32]. TWA may result from the complementary mechanism of temporal dispersion of repolarization, or alternans of action potential shape or action potential duration [1,33]. Alternation of cytosolic calcium concentration [34] may underlie action potential duration alternans [35]. Interestingly, binding of *NOS1AP* results in the reduction of NMDA receptor-PSD95-nNOS complexes, leading to a decreased access to the NMDA receptor-gated calcium influx [32] suggesting that cytosolic calcium might be mediating the effect of this locus on cardiac repolarization alternans. Further functional studies and replication of these findings are required to assess whether allelic variation of *NOS1AP* is involved in the regulation of NMDA receptor-gated calcium influx [32] but this could provide a potential mechanism by which the locus affects cardiac repolarization alternans, i.e. TWA.

We found both sex-by-time and sex-by-time-by-*NOS1AP* genotype differences in relation to TWA responses over different phases (rest, exercise, recovery) of a clinically indicated exercise test. These sex differences were attenuated after exercise, suggesting that nNOS signalling may be especially important in

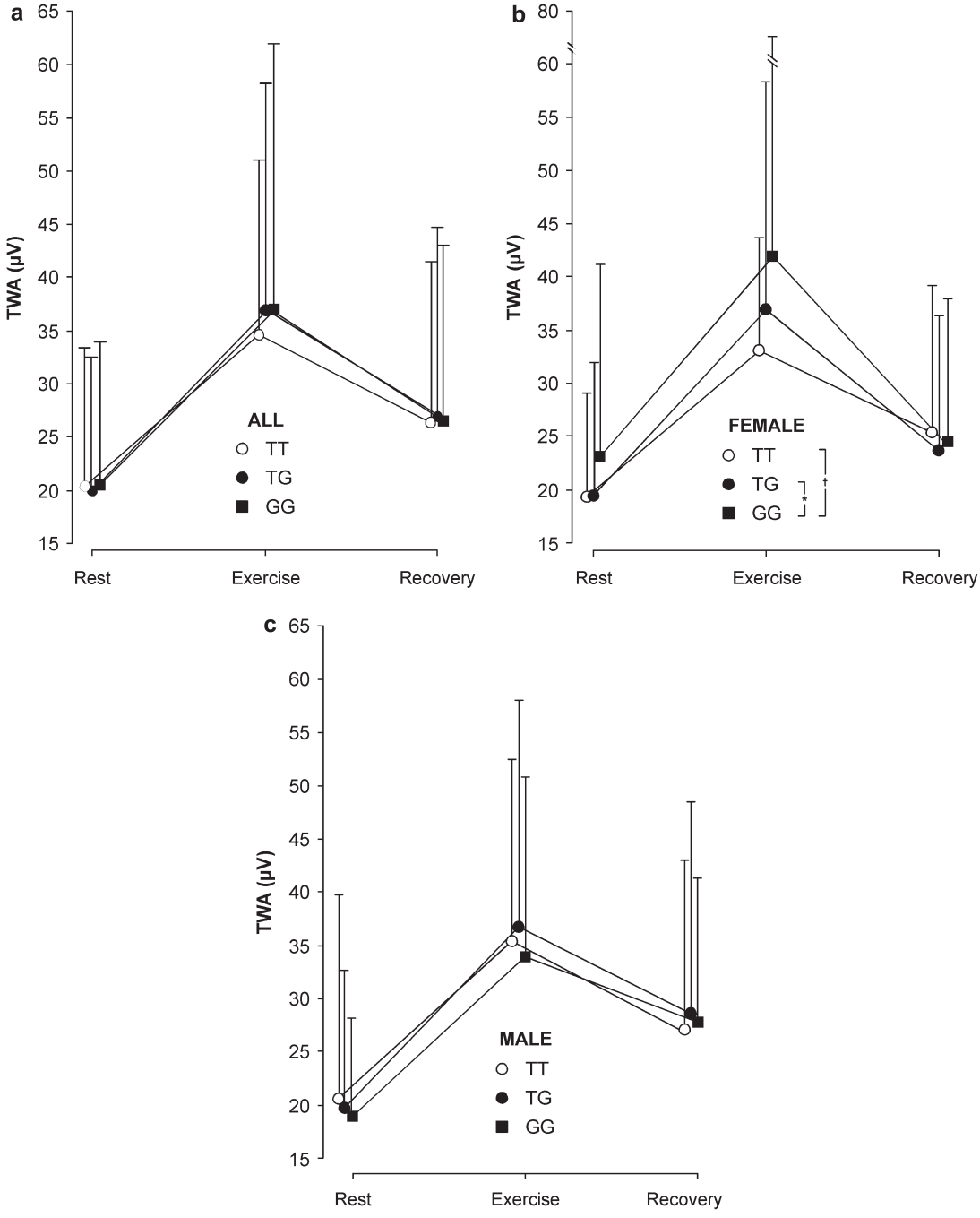

Figure 1. T-wave alternans (TWA) responses during different exercise test phases by the NOS1AP rs10494366 genotypes (Mean ± SD). In all subjects (A) no statistically significant differences of TWA responses between genotypes are present. Statistically significant difference between the genotypes in females (B), RANOVA time-by-genotype interaction, $p < 0.001$; Least Significant Difference post hoc test $*p = 0.007$ and $^{\dagger}p = 0.03$, is shown. In males no such differences of TWA responses are presented (C).

regulating TWA under physical stress. Sex differences in QT prolongation at puberty [30] together with greater susceptibility of women than men to drug-induced QT prolongation [36] suggest that sex hormones influence cardiac repolarization. However, we did not find any support for estrogens mediating the associations observed in women, since the TWA responses over the exercise test were similar in female hormone users and non-users. In addition, testosterone levels have been implicated in QT duration in humans [37]. In animal models testosterone shortens cardiac repolarization and administration of NG-nitro-L-arginine methyl ester, which suppresses the activities of all isoforms of NOS [38] and reverses testosterone effects [37]. The nNOS appears to have a role in intracardiac vagal stimulation [39], but has also other effects on cardiac physiology which are incompletely understood [40], and alternative pathways could explain the associations we observed in females and with TWA after exercise.

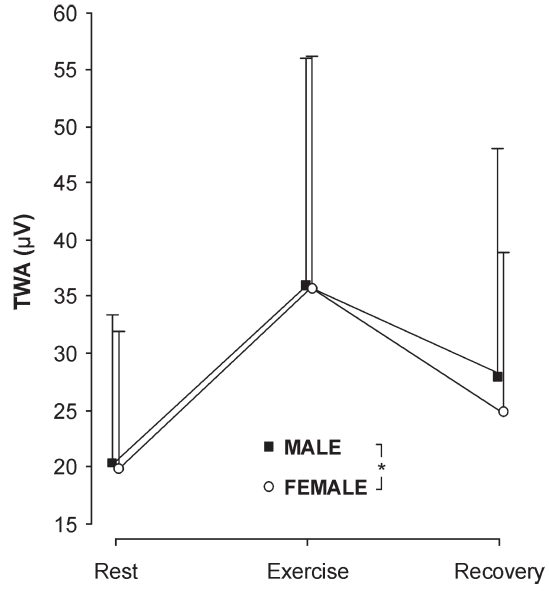

Figure 2. T-wave alternans (TWA) responses during the exercise test by gender (Mean ± SD). A higher magnitude of TWA changes during the entire exercise test was observed in women compared to men ($^*p < 0.001$ for time-by-sex interaction in RANOVA).

Previous studies have also suggested a gender difference in the effect of the *NOS1AP* variant on QT interval. Notably, a large genome-wide association study showed more pronounced effect of *NOS1AP* on QT interval in women than men [41]. In a previous study of GRAPHIC and FINCAVAS study populations we showed similar sex-specific effects of SNP rs10494366 on cardiac repolarization [18]. However, the previous studies tested the effect of the SNP of *NOS1AP* on cardiac repolarization, measured as QT interval at rest and thus the results of sex differences are not completely comparable with our findings.

We showed previously that TWA at higher levels than 65 μV [7] and also TWA as a continuous variable [13] predict cardiovascular and all-cause mortality in the FINCAVAS population undergoing clinical exercise test. Therefore, it is of interest to investigate whether the *NOS1AP* variant impacted on mortality. However, we found no association between *NOS1AP* rs10494366 genotype and all-cause or cardiovascular mortality or SCD over the period of follow-up (average 47 months). Aarnoudse et al. came to the same conclusion when studying the association of *NOS1AP* genetics with SCD with an average follow-up of more than 10 years [16]. However, given the relatively low power of our study to detect effects on mortality, especially given the sex-specific effects we observed for the association of the variant with TWA, larger studies or longer follow-up time are needed to clarify the possible association of *NOS1AP* polymorphisms on mortality.

Table III. Effects of *NOS1AP* (rs10494366) genotype, sex, CHD, β-blocker medication status and age on all-cause mortality during average follow-up of 47 months.

Variable	Hazard ratio*	95% CI	*p*-value
Genotype TT	1	—	—
Genotype TG	0.99	0.66–1.49	0.951
Genotype GG	1.21	0.66–2.20	0.538
Age (years)	1.06	1.04–1.08	<0.001
Sex (male/female)	2.26	1.40–3.65	<0.001
Coronary heart disease (yes/no)	1.04	0.69–1.56	0.849
β-blocker medication (yes/no)	0.55	0.33–0.92	0.022

Statistics: *Cox regression analysis; CI, confidence interval.

The present population was rather heterogeneous, including patients with CHD and several subjects also used medications affecting cardiac repolarization. However, factors affecting cardiac repolarization were taken into consideration in the statistical analyses. In our study the mean age was higher than in most of the previous study populations [17,31] and the genetic factors may play a more minor role in the regulation of cardiac repolarization than extrinsic factors. At this stage it is also difficult to generalize our findings to healthy populations, given the relative lack of data on this relatively unique clinical examination test in such populations. Even so, our results support the previous finding that SNP rs10494366 in *NOSIAP* is associated with cardiac repolarization.

In conclusion, a common variant, rs10494366 of *NOS1AP*, is associated with TWA responses during a clinical exercise test, in a sex-specific manner. However, this allelic variation was not associated with mortality in our study. Our results support previous findings and provide supplemental insight into the mechanisms through which NOS1AP regulates cardiac repolarization. Further studies will be required to confirm gender-specific effects and elucidate their mechanisms.

Acknowledgements

The authors wish to thank the staff of the Department of Clinical Physiology for collecting the exercise test data. This work was supported by the Medical Research Fund of Tampere University Hospital (grant 9M048), the Finnish Cultural Foundation, the Finnish Foundation for Cardiovascular Research (T.L., I.P.), the Tampere Tuberculosis Foundation, the Emil Aaltonen Foundation, and MRC Clinician Scientist Fellowship (G0501942 to M.D.T.). N.J.S. holds a chair funded by the British Heart Foundation.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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BMC Cardiovascular Disorders

RESEARCH ARTICLE Open Access

Association of resting heart rate with cardiovascular function: a cross-sectional study in 522 Finnish subjects

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Abstract

Background: High resting heart rate (HR) is associated with increased cardiovascular risk in general populations, possibly due to elevated blood pressure (BP) or sympathetic over-activity. We studied the association of resting HR with cardiovascular function, and examined whether the hemodynamics remained similar during passive head-up tilt.

Methods: Hemodynamics were recorded using whole-body impedance cardiography and continuous radial pulse wave analysis in 522 subjects (age 20–72 years, 261 males) without medication influencing HR or BP, or diagnosed diabetes, coronary artery, renal, peripheral arterial, or cerebrovascular disease. Correlations were calculated, and results analysed according to resting HR tertiles.

Results: Higher resting HR was associated with elevated systolic and diastolic BP, lower stroke volume but higher cardiac output and work, and lower systemic vascular resistance, both supine and upright ($p < 0.05$ for all). Subjects with higher HR also showed lower supine and upright aortic pulse pressure and augmentation index, and increased resting pulse wave velocity ($p < 0.001$). Upright stroke volume decreased less in subjects with highest resting HR ($p < 0.05$), and cardiac output decreased less in subjects with lowest resting HR ($p < 0.009$), but clear hemodynamic differences between the tertiles persisted both supine and upright.

Conclusions: Supine and upright hemodynamic profile associated with higher resting HR is characterized by higher cardiac output and lower systemic vascular resistance. Higher resting HR was associated with reduced central wave reflection, in spite of elevated BP and arterial stiffness. The increased cardiac workload, higher BP and arterial stiffness, may explain why higher HR is associated with less favourable prognosis in populations.

Trial registration: ClinicalTrials.gov, NCT01742702

Keywords: Arterial stiffness, Cardiac output, Heart rate, Head-up tilt, Systemic vascular resistance

Background

Measurement of heart rate (HR) is an easily available cardiovascular phenotype in clinical practice. Increased resting HR is associated with higher cardiovascular mortality and morbidity in general populations [1-5], even when other cardiac risk factors are taken into consideration [2,4,5]. Higher HR is also a risk factor for elevated blood pressure (BP) in both children and adolescents [6]. Moreover, many studies have reported that HR is associated with atherosclerosis and elevated risk of adverse cardiovascular events [1,7-9]. However, the results concerning possible benefits of pharmacological HR lowering are inconsistent [7,10-14].

The mechanisms linking elevated HR with cardiovascular pathology and pathophysiology are not well understood. The association has been assumed to be a consequence of sympathetic over-activity [15,16]. In addition, elevated HR and reduced HR variability have

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been shown to accelerate atherosclerotic process in coronary arteries through local hemodynamic changes [17,18]. Higher resting HR has also been associated with increased pulse wave velocity (PWV), i.e. increased arterial stiffness [19-22]. High PWV is a marker of cardiovascular aging and an acknowledged independent risk factor for cardiovascular morbidity [23].

On the other hand, there is a significant negative association between HR and central BP and central wave reflection [24]. Augmentation index (AIx) is commonly measured to evaluate central wave reflection, and higher AIx can result from increased arterial stiffness, older age, short stature, and lower HR [24]. Increased AIx and higher central wave reflection have also been associated with increased cardiovascular risk [25,26]. Although β-blocking agents seem to increase AIx, this class of drugs appears to have a beneficial influence on prognosis in subjects with coronary artery disease, at least after myocardial infarction [14,24,27]. In addition to the anti-arrhythmic properties of β-blockers, this may be attributed to improved oxygen delivery due to increased time for coronary flow during prolonged diastole [24,27].

Taken together, even though higher HR at rest is associated with lower central wave reflection which is considered to be beneficial, it is associated with less favourable prognosis in observational studies [2,4,5,25,26]. In order to understand the adverse influence of increased HR on prognosis we should identify the underlying hemodynamic differences. Previously, simultaneous analysis of central and peripheral BP, vascular resistance, cardiac function, arterial stiffness and central wave reflection during standard physical challenge has only seldom been performed. The aim of this study was to examine the association of HR with principal hemodynamic variables and their functional responses during head-up tilt in a cross-sectional study including 522 subjects without medications directly influencing HR or BP.

Methods

Study population

All study subjects participated in an on-going study, in which hemodynamics are noninvasively recorded from voluntary subjects (DYNAMIC-study; Clinical Trials registration number NCT01742702). The ethics committee of the Tampere University Hospital approved the study protocol and patients gave an informed consent, as stipulated in the Declaration of Helsinki. An announcement for the recruitment of subjects was distributed at the University of Tampere, Tampere University Hospital, several occupational health care organizations, Varala Sports Institute, and two announcements were published in a local newspaper. The subjects who responded were successively recruited in the order that they contacted the research nurse. In November 2012 a total number of 830 subjects had been recruited to the study.

In present investigation, those subjects with a history of coronary artery disease, diabetes mellitus, peripheral arterial or cerebrovascular disease, valvular regurgitation or stenosis, long QT syndrome, chronic renal insufficiency, hemochromatosis, or medication for hypertension were excluded. Also, subjects with regular medication influencing HR or BP were excluded, i.e. subjects using anti-arrhythmic agents, long-acting β_2-sympathomimetics, α-adrenoceptor agonists, varenicline, or the weight-reducing agent sibutramine.

Altogether 522 subjects (261 males, aged 20 to 72 years) with technically successful hemodynamic recordings were included in the present study. The majority of subjects were without concurrent diseases or medications (for medication details please see Additional file 1: Table S1). In total 80 of 261 women were on low-dose progesterone (intrauterine device) or combination of oestrogen and progesterone therapy (contraception or hormone replacement therapy). Subjects with the following medical conditions with established and stable drug treatment were included in the study: depression (n = 29), allergies or asthma (n = 26), dyspepsia (n = 15), hypothyroidism (n = 15), hyperlipidaemia (n = 14), musculoskeletal problems (n = 10), and epilepsy (n = 5). All subjects with thyroid problems were euthyroid clinically and on the basis of laboratory tests. Moreover, mean HR was not statistically different between any medicated versus corresponding unmedicated subgroups of subjects.

All subjects underwent a physical examination performed by a physician, who also documented medical history, lifestyle habits, and cardiovascular risk factors by interview. The amount of smoking was calculated in pack years, and the use of alcohol was evaluated as average consumption of standard drinks (corresponding to 12 grams of absolute alcohol) per week. Physical exercise frequency was interviewed, and was expressed as the number of bouts of physical activity per week that lasted for at least 30 min each time and caused sweating or shortness of breath.

Laboratory analyses

Venous blood samples were drawn after ~12 hours of fasting. Plasma sodium, potassium, glucose, creatinine, cystatin C, C-reactive protein (CRP), and total, high-density (HDL) and low-density lipoprotein (LDL) cholesterol concentrations were determined using Cobas Integra 700/800 (F. Hoffmann-LaRoche Ltd, Basel, Switzerland), or Cobas 6000, module c501 (Roche Diagnostics, Basel, Switzerland), and white blood cell count and haematocrit using ADVIA 120 or 2120 analyzers (Bayer Health Care, Tarrytown, NY, USA). Glomerular filtration rate was

estimated using the Rule formula [28], since the measured creatinine values were within the normal range.

Hemodynamic measurements

Hemodynamics recordings were carried out in a quiet, temperature-controlled laboratory by a research nurse. The subjects were instructed to refrain from caffeine-containing products, smoking, and heavy meals for at least 4 h, and from alcohol for at least 24 h prior to the investigation. Before the actual measurement the subjects were resting supine for approximately 10 min, during which period electrodes for impedance cardiography were placed on the body surface, a tonometric sensor for pulse wave analysis was fixed to the left wrist on the radial pulsation, and a brachial cuff for BP calibration was placed to the right upper arm. Then hemodynamic variables were continuously captured in a beat-to-beat fashion for 5 min in supine position and for 5 min during passive head-up tilt to 60 degrees. Mean values of each measured minute of the experiment were calculated and used in statistical analyses.

Whole-body impedance cardiography

A whole-body impedance cardiography device (CircMon^R, JR Medical Ltd, Tallinn, Estonia), which records the changes in body electrical impedance during cardiac cycles, was used to determine beat-to-beat HR, stroke index (stroke volume in proportion to body surface area, ml/m^2), cardiac index (cardiac output/body surface area, l/min/m^2), and PWV (m/s) [29-31]. Left cardiac work index (kg*m/min/m^2) was calculated by formula 0.0143*(MAP–PAOP)* cardiac index, which has been derived from the equation published by Gorlin et al. [32]. MAP is mean radial arterial pressure measured by tonometric sensor, PAOP is pulmonary artery occlusion pressure which is assumed to be normal (default 6 mmHg), and 0.0143 is the factor for the conversion of pressure from mmHg to cmH_2O, volume to density of blood (kg/L), and centimetre to metre. Systemic vascular resistance index (systemic vascular resistance/body surface area, dyn*s/cm^5/m^2) was calculated from the signal of the tonometric BP sensor and cardiac index measured by CircMon^R.

To calculate the PWV, the CircMon software measures the time difference between the onset of the decrease in impedance in the whole-body impedance signal and the popliteal artery signal. From the time difference and the distance between the electrodes, PWV can be determined. As the whole-body impedance cardiography slightly overestimates PWV when compared with Doppler ultrasound method, a validated equation was utilized to calculate values that correspond to the ultrasound method (PWV = ($PWV_{impedance}$*0.696) + 0.864) [30]. PWV was determined only in the supine position because of less accurate timing of left ventricular ejection during head-up tilt [30]. A detailed description of the method and electrode configuration has been previously reported [31]. PWV was also recorded after the head-up tilt in all subjects, and the average difference between the mean PWV before and after the head-up tilt was 0.024 ± 0.388 m/s (mean ± standard deviation), showing the good repeatability of the method (repeatability index R 98%, Bland-Altman repeatability index 0.8) [33]. The cardiac output values measured with CircMon^R are in good agreement with the values measured by the thermodilution method [31], and the repeatability and reproducibility of the measurements (including PWV recordings) have been shown to be good [34,35].

Pulse wave analysis

Radial BP and pulse wave form were continuously determined by the use of an automatic tonometric sensor (Colin BP-508 T, Colin Medical Instruments Corp., USA), which was fixed on the radial pulse with a wrist band. The extended left arm was lying on a stable bracket at the level of the heart, whether supine or upright. Radial BP signal was calibrated by brachial BP measurement at the onset of the recording. Continuous aortic BP was derived with the SphygmoCor monitoring system (SphygmoCor PWMx, AtCor Medical, Australia) using the previously validated generalized transfer function [36]. From the aortic pulse wave form AIx (augmented pressure/pulse pressure*100,%) was determined.

Stroke volume determination with cardiac ultrasound

To evaluate the accuracy of stroke volume determination with impedance cardiography during head-up tilt, echocardiography was performed by a cardiologist (author E.I.) to a subset of subjects (n = 16) during an extra visit. The 3D echocardiography (Philips ie33 ultrasound system, Bothell, USA; 1-5 MHz Matrix-array X5-1 transducer) was performed simultaneously with beat-to-beat impedance cardiography recordings during head-up tilt to 60 degrees. Mean stroke volume from 7 consecutive heart beats (6 before and 1 after echocardiography) was calculated from impedance cardiography recordings to cover approximately one respiratory cycle (~6 seconds).

Statistical analyses

For the statistical analyses, the study population was divided into tertiles according to mean resting HR, determined as an average HR of the last 3 min during the 5-min measurement period in supine position. Analysis of variances for repeated measures was applied to study the differences in the hemodynamic variables BP, stroke index, cardiac index, left cardiac work index, systemic vascular resistance index, AIx, and PWV between the HR tertile groups during rest and head-up tilt. For post hoc

testing Tukey HSD test was performed for homogenous, and Tamhane's T2 test for nonhomogeneous variables. In adjusted analysis of variances for repeated measures, the variables sex, age, body mass index, smoking in pack years, haematocrit, leukocyte count, CRP, creatinine, cystatin C, total cholesterol, triglycerides, HDL cholesterol, fasting plasma glucose, and mean radial arterial pressure at rest were used as covariates.

Pearson's correlation coefficients were calculated, as appropriate, and possible differences in stroke volume determined using impedance cardiography and 3D echocardiography were tested using Student's T-test. Distributions of categorical variables among resting HR tertiles were tested using χ^2 test, and differences of numerical variables among HR tertiles were studied using analysis of variances. Variable values are given as means and 95% confidence intervals (CI). Natural logarithms of CRP and triglyceride concentrations were used in analyses to normalize their distributions. P-values <0.05 were considered statistically significant. The analyses were performed using SPSS Statistics 17.0 for Windows software (SPSS Inc., Chicago, Ill., USA).

Results

Study population

The characteristics of the study population according to the HR tertiles at rest, with average values of 54, 62 and 75 beats/min in the 1st, 2nd and 3rd tertile, respectively, are shown in Table 1. Mean resting HR among men was 62 (CI: 61 to 64), and among women 64 (CI: 63 to 65) beats/min ($p = 0.028$). The proportion of men was higher in tertile 1 (with lowest HR) when compared with tertiles 2 and 3 ($p = 0.019$). Age, use of alcohol, amount of smoking, hematocrit, and plasma concentrations of sodium, potassium, HDL cholesterol, LDL cholesterol, and glucose did not differ between the HR tertiles ($p > 0.05$ for all). Body mass index, white blood cell count, C-reactive protein, plasma total cholesterol and triglycerides were highest within tertile 3 ($p < 0.05$ for all). Although plasma creatinine was lowest in tertile 3 ($p < 0.005$), there were no significant differences in cystatin-C concentrations or estimated glomerular filtration rate between the groups. The self-reported amount of physical exercise bouts per week was 3.4 in tertile 1 (CI: 3.1 to 3.7), 3.0 in tertile 2 (CI: 2.7 to 3.3), and 3.1 in

Table 1 Characteristics of the study population

	Resting heart rate tertiles		
	1	2	3
	n = 172	n = 176	n = 174
Resting heart rate (1/min)	54 (53–54)	62 (62–63)*	75 (73–75)*†
Age (years)	46 (44–48)	46 (44–47)	46 (44–47)
Sex (M/F)	101/71‡	79/97	81/93
Body mass index (kg/m^2)	26.2 (25.7–26.7)	26.5 (25.8–27.1)	27.3 (26.6–28.1)*
Waist circumference (cm)	92 (90–93)	92 (90–94)	94 (92–96)
Smoking (pack years)	2.3 (1.2–3.4)	1.8 (0.9–2.6)	3.5 (2.0–5.0)
Alcohol (drinks/week)	4 (3–5)	4 (3–5)	5 (4–6)
Leukocyte count (1*10^9/l)	5.4 (5.2–5.6)	5.9 (5.6–6.1)*	6.1 (5.8–6.4)*
Haematocrit (%)	42 (42–43)	41 (41–42)	42 (41–43)
C-reactive protein (mg/l)	1.2 (1.0–1.4)	1.6 (1.2–2.0)	2.2 (1.6–2.9)*
Creatinine (μmol/l)	77 (75–79)	72 (70–74)*	71 (70–73)*
Estimated GFR (ml/min/1.73 m^2)	112 (109–114)	111 (110–113)	112 (110–114)
Cystatin C (mg/l)	0.83 (0.81–0.85)	0.82 (0.80–0.84)	0.86 (0.83–0.88)
Sodium (mmol/l)	140 (140–141)	140 (140–141)	140 (140–140)
Potassium (mmol/l)	3.8 (3.8–3.9)	3.8 (3.8–3.9)	3.8 (3.7–3.8)
Fasting plasma			
Total cholesterol (mmol/l)	5.2 (5.0–5.4)	5.0 (4.8–5.1)*	5.2 (5.1–5.4)†
Triglycerides (mmol/l)	1.1 (1.0–1.2)	1.2 (1.1–1.3)	1.3 (1.2–1.5)*†
HDL cholesterol (mmol/l)	1.7 (1.6–1.7)	1.6 (1.5–1.6)	1.5 (1.5–1.6)
LDL cholesterol (mmol/l)	3.0 (2.9–3.2)	2.9 (2.7–3.0)	3.1 (3.0–3.3)
Glucose (mmol/l)	5.4 (5.3–5.5)	5.4 (5.3–5.4)	5.5 (5.4–5.6)

Values are means and 95% confidence intervals; *$p < 0.05$ vs. tertile 1, †$p < 0.05$ vs. tertile 2, analysis of variance with Tukey HSD post hoc test; ‡$p < 0.05$ for distributions between the tertiles, χ^2 test; GFR = glomerular filtration rate using the Rule formula [28].

tertile 3(CI: 2.7 to 3.5), and the differences between the tertiles were not statistically significant (p = 0.143).

BP in the HR tertiles

In the entire study population HR at rest showed a moderate association with resting radial systolic BP (r = 0.14, p < 0.001) and diastolic BP (r = 0.19, p < 0.001). In tertile 3 radial systolic and diastolic BPs were higher than in other tertiles, in both supine position and during head-up tilt (Figure 1). The differences in radial BPs between HR tertile 3 versus other tertiles in supine and upright positions remained significant in adjusted analyses including the covariates given in the Statistical analyses section of Methods.

Aortic systolic BP did not differ between the tertiles in supine (p = 0.139) or upright positions (p = 0.452). However, supine aortic diastolic BP was higher in tertile 3 than tertiles 1 and 2 (p ≤ 0.033, unadjusted and adjusted comparisons). Upright aortic diastolic BP was higher in tertile 3 than tertile 1 (p < 0.001).

Stroke volume, cardiac work, and systemic vascular resistance among HR tertiles

All HR tertiles showed a corresponding upright chronotropic effect (11.5-13.1 beats/min, p > 0.05), and the clear differences in HR between the tertiles persisted during the head-up tilt (Figure 2A). The mean supine to upright increase in HR in the whole population was 12.4 (CI: 11.8 to 12.9) beats/min.

Supine stroke index showed a significant negative correlation with HR (r = −0.31, p < 0.001), and in tertile 3 supine stroke index was lower than in other tertiles (Figure 2B, p < 0.001 also after adjustments). In response to head-up tilt, the decrease in stroke index was smaller in the 3rd tertile (10.4 ml/m^2, CI: 9.7 to 11.2) , than in the 1st (13.3 ml/m^2, CI: 12.4 to 14.2), and 2nd tertiles (12.9 ml/m^2, CI: 12.0 to 13.7) (unadjusted and adjusted p < 0.001). Upright stroke index was lower in tertiles 2 and 3 than in tertile 1 (p = 0.002).

In spite of the above negative correlation between stroke index and HR, supine cardiac index and also left cardiac work index were highest in tertile 3, and were also higher in tertile 2 than tertile 1 (Figures 2C and 2D, p < 0.001 also in adjusted analyses). The upright decrease in cardiac index was slightly higher in the 2nd (−0.38 ml/min/m^2, CI: -0.31 to -0.44), and 3rd tertiles (−0.38 ml/min/m^2, CI: -0.32 to −0.44), than in the 1st tertile (-0.28 ml/min/m^2, CI: -0.22 to −0.33) (p < 0.034), but in adjusted analyses the difference remained significant only between the 1st and 3rd tertiles (p < 0.009). Importantly, the clear differences in cardiac index and left cardiac work index between the HR tertiles persisted in the upright position (p < 0.001 in unadjusted and adjusted analyses).

Systemic vascular resistance index was lowest in tertile 3, and also lower in tertile 2 than tertile 1, and the differences remained significant in the upright position (Figure 2E, p < 0.001 also after adjustments).

HR, central wave reflection, and arterial stiffness

In the whole population, an expected negative association was found between resting HR and AIx (r = −0.19, p < 0.001), so that AIx was mathematically reduced by 2.5%-units for every 10 beats/min increase in HR.

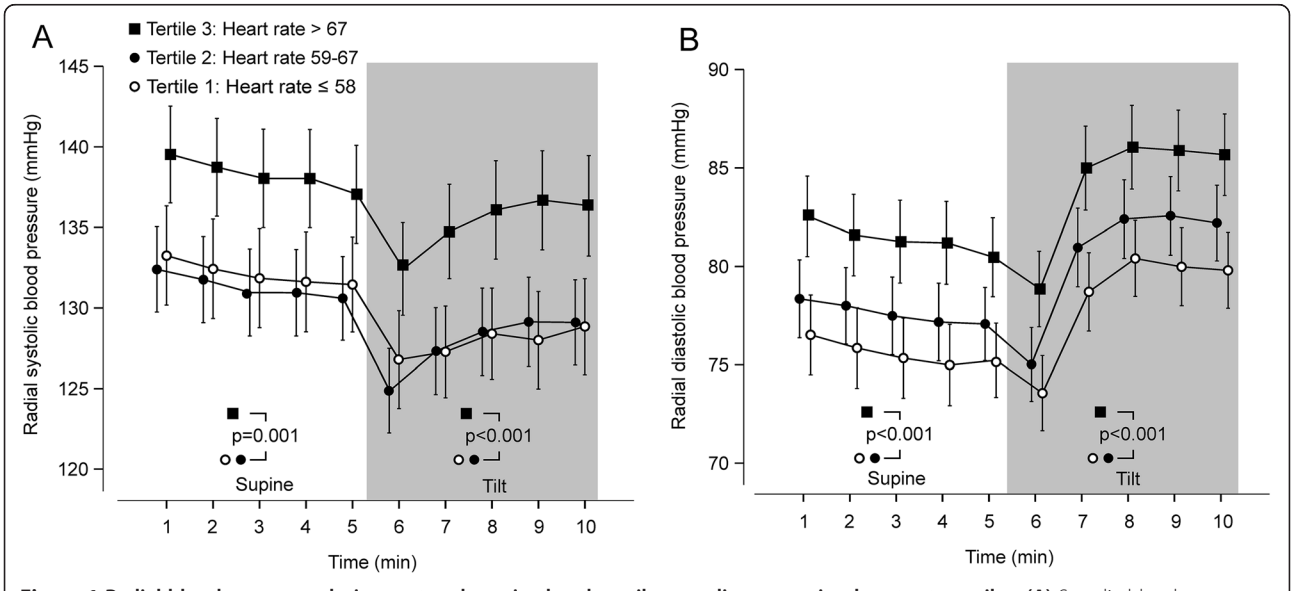

Figure 1 Radial blood pressures during rest and passive head-up tilt according to resting heart rate tertiles. (A) Systolic blood pressure, **(B)** diastolic blood pressure; mean (95% confidence intervals), p values denote significant differences between the tertiles depicted with different symbols.

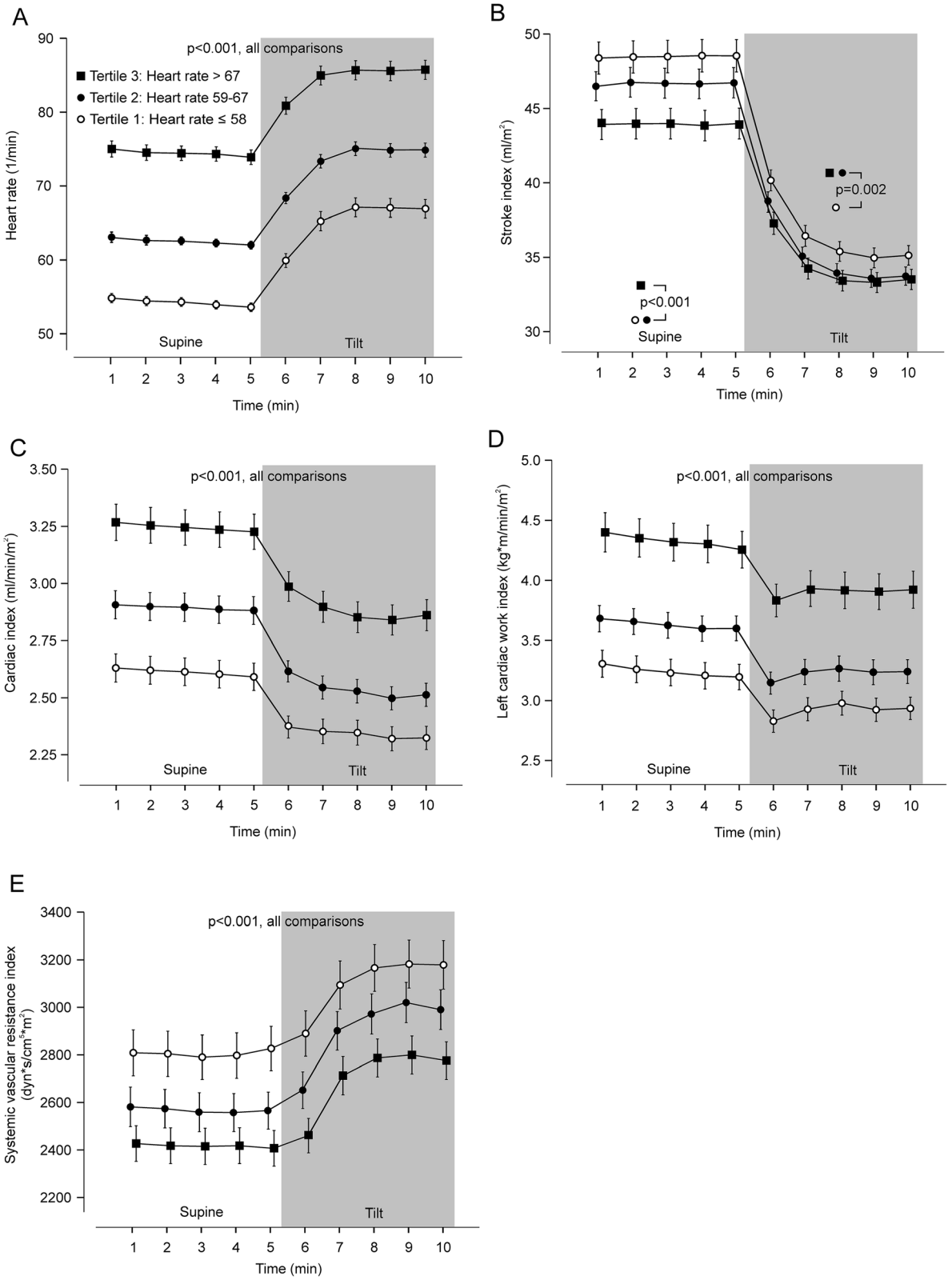

Figure 2 Principal hemodynamic variables during rest and passive head-up tilt according to resting heart rate tertiles. (A) Heart rate, **(B)** stroke index, **(C)** cardiac index, **(D)** left cardiac work index, **(E)** systemic vascular resistance index; mean (95% confidence intervals).

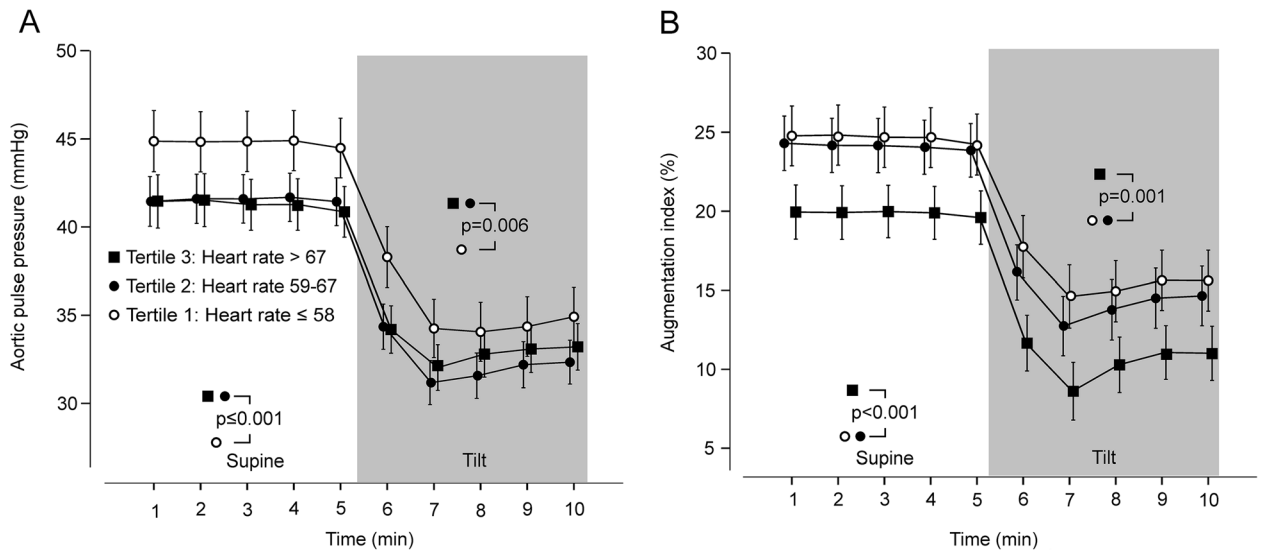

Figure 3 Central pulse pressure and wave reflection during rest and passive head-up tilt according to resting heart rate tertiles. **(A)** Aortic pulse pressure, **(B)** augmentation index; mean (95% confidence intervals).

Supine aortic pulse pressure was highest in the 1st HR tertile (Figure 3A), while supine AIx was lowest in the 3rd HR tertile (Figure 3B), and corresponding differences were also observed during the head-up tilt. The differences in aortic pulse pressure and AIx remained significant in adjusted analyses ($p \leq 0.001$ for all, supine and upright). As the subject's height may influence AIx, we performed an additional analysis so that body mass index was replaced by height and weight in the adjustments, but the outcome of the analysis did not change.

Arterial stiffness was evaluated by measuring resting PWV, the mean value of which in the whole study population was 8.5 m/s (CI: 8.3 to 8.7). HR was notably correlated with PWV ($r = 0.23$, $p < 0.001$). PWV was significantly higher in tertiles 2 and 3 than in tertile 1 (Figure 4; $p < 0.001$ also in adjusted analysis).

Determination of stroke volume using impedance cardiography and echocardiography

Stroke volume during the head-up tilt was determined by means of impedance cardiography and cardiac 3D ultrasound in 16 subjects. Mean stroke volume by impedance cardiography was 72 ml (CI: 61 to 82) and by echocardiography 67 ml (CI: 59 to 75) ($p > 0.05$, Figure 5A). The correlation between the impedance cardiography and echocardiography recordings was good (Figure 5B).

Discussion

To our knowledge, the association of resting HR with cardiovascular response to head-up tilt has not been studied previously. Here we demonstrated that higher resting HR was associated with reduced central wave reflection and lower vascular resistance, but in spite of these beneficial characteristics, cardiac output and work were increased in tertiles with higher resting HR, both supine and upright. Moreover, BP was moderately increased in the 3rd HR tertile, while arterial stiffness was increased in both 2nd and 3rd HR tertiles when compared with the lowest HR tertile. Altogether, the present

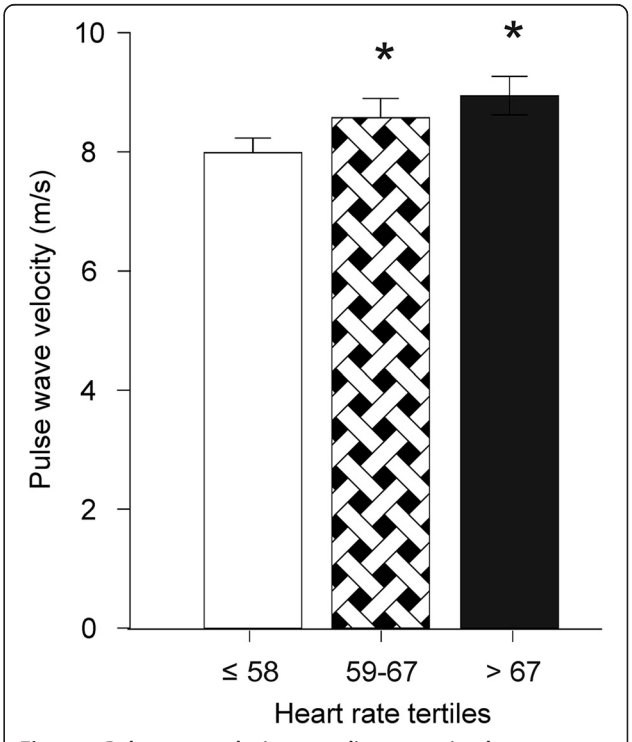

Figure 4 Pulse wave velocity according to resting heart rate tertiles; mean (95% confidence intervals), *p < 0.001 vs. tertile 1.

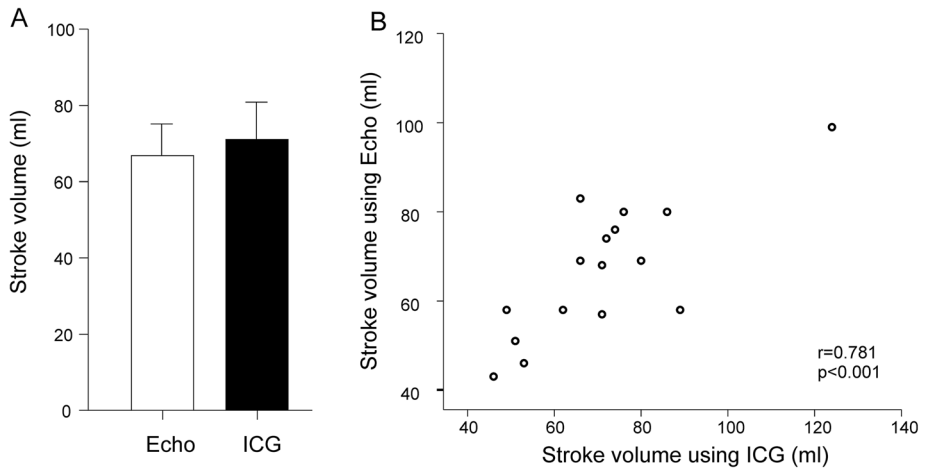

Figure 5 Evaluation of stroke volume in 16 subjects during passive head-up tilt using echocardiography (Echo) and impedance cardiography (ICG). (A) Comparison of stroke volume measurements mean (95% confidence intervals). **(B)** Scatterplot depicting the measurements in each individual.

findings support the view that lower resting HR represents a more beneficial hemodynamic profile.

Since the change in posture induces significant hemodynamic changes in blood volume distribution, vascular resistance, and autonomic nervous function, head-up tilt can be regarded as a test addressing cardiovascular reactivity [37,38]. The head-up tilt -induced decrease in stroke volume was slightly lesser in subjects with highest resting HR, while the decrease in cardiac output was somewhat lower in the subjects with lowest resting HR, despite the changes in HR between the tertiles did not differ. However, the clear differences in cardiac output and vascular resistance between the HR tertiles persisted in the upright position, and the observed differences between the hemodynamic profiles in the HR tertiles were surprisingly similar both supine and upright. This suggests that resting HR provides significant information about upright hemodynamics, so that higher resting HR indicates a more hyperdynamic upright hemodynamic profile.

The increased cardiovascular risk related to higher resting HR has been attributed to genetic factors, impaired myocardial oxygen delivery, increased BP, arrhythmias, sympathetic over-activity, and increased arterial stiffness [1-5,7,15,16], but the mechanisms are not completely understood. Elevated BP is considered one of the most potential mechanisms associating higher HR with cardiovascular risk [6,39]. In the present study, despite lower stroke volume and vascular resistance, radial BP was highest in the 3rd tertile with the highest resting HR, in line with previous results [39]. However, as the difference in central and peripheral BP between the HR tertiles was rather small, other factors in addition to elevated BP may play a more important pathophysiological role in the cardiovascular risk associated with elevated HR. Moreover, increased HR may merely be a marker, but not a cause, for higher risk of cardiovascular end-points.

A change in heart rate is a major factor by which the cardiovascular system adjusts cardiac output [40], and in the absence of a heart disease, higher HR is commonly assumed to indicate higher cardiac output. Although cardiac output cannot be reliably predicted from HR alone [40], the present results suggest that higher HR is associated with increased cardiac output. The known determinants of cardiac output are the interaction of i) cardiac function, which is determined by heart rate, contractility, afterload and preload; and ii) return function, which is determined by vascular volume, venous compliance, blood draining from the venous compliant regions, and right atrial pressure (for a review, see [40]).

We found that higher HR was related with increased left cardiac work, and thus higher cardiac oxygen demand. In patients with coronary artery disease, reducing HR is an acknowledged treatment modality, which reduces myocardial oxygen consumption and improves subendocardial blood flow [14,24,27]. In an experimental study in dogs, increasing HR was found to increase cardiac oxygen demand even when the external work performed by the heart was kept constant, and this effect was attributed to the greater oxygen requirement for excitation-contraction coupling during higher HR [41]. Moreover, although the benefits of HR lowering in the treatment of hypertension have recently been questioned [10-13], pharmacological reduction of HR and the subsequent decrease in cardiac workload by the use of drugs like β-adrenoceptor blockers might still benefit distinct subgroups of patients [15,16].

Higher resting HR is thought to reflect enhanced sympathetic tone, and this may predispose to cardiac arrhythmias and hypertension [6,42,43]. In the heart increased sympathetic tone has both chronotropic and inotropic effects, while in the resistance arteries higher sympathetic tone induces vasoconstriction and thus elevates BP [44]. Counteracting these effects, parasympathetic tone plays an important role in the regulation of HR and cardiac output, and it also influences vascular resistance via secondary mechanisms [45]. In this study, we observed an inverse relation between higher HR and systemic vascular resistance and stroke index, and these findings seem to contradict with the concept of sympathetic over-activity as the cause of higher HR. On the other hand, higher HR was associated with higher BP, which suggests that vascular resistance was not sufficiently reduced to compensate for the increased cardiac output resulting from higher HR. Therefore, lower resistance in peripheral arteries cannot exclude the possibility of increased sympathetic tone in subjects with higher HR. A thorough analysis of autonomic tone would require the recording of HR variability, baroreceptor sensitivity, or direct muscle sympathetic nerve activity, and such analyses make an interesting topic for further investigations.

We found an inverse relation with HR and AIx and central pulse pressure, in line with previous reports [13,24,46]. Increased AIx, which is an indicator of central wave reflection, has been related to elevated cardiovascular risk [25,26]. When higher HR leads to shorter duration of systole, this shifts the reflected wave towards diastole, and the reduction in AIx during higher HR can thus be regarded as a beneficial hemodynamic change [24]. The present results suggest that the relationship between HR and AIx could also arise from the inverse association of HR with systemic vascular resistance: the reflection point of the forward arterial pressure wave is shifted more peripherally during lower systemic vascular resistance index, and this prolongs the time to wave reflection shifting it towards diastole [24].

Our results showed a small but significant relationship between higher HR and increased PWV, a marker of arterial stiffness [23], in agreement with previous studies [19,22,47,48]. However, HR itself may be an important confounder during PWV assessment. Higher HR exerted a significant increasing influence on PWV in 22 elderly subjects during cardiac pacing, in the absence of changes in BP [19]. In a study with 102 young, healthy males, left ventricular ejection time was an important determinant of PWV both under resting conditions and during adrenergic stimulation: shorter ventricular ejection time was associated with higher PWV [49]. Therefore, the associations between HR and PWV must be interpreted with caution. Although higher arterial stiffness increases pulse pressure [50], we found that aortic pulse pressure was lower in the highest HR tertile when compared with the lowest HR tertile, in spite of higher PWV in the former group. This can be explained by the lower augmentation index during higher heart rate, i.e. lower summation of the reflected pressure wave to the systolic volume wave. In addition, pulse pressure is significantly determined by stroke volume [50], and stroke index was inversely correlated ($r = -0.31$) with heart rate. Thus, lower aortic pulse pressure in the highest versus lowest heart rate tertile was probably a consequence of reduced augmentation index and lower stroke volume in the former group.

The majority of the present subjects were devoid of medications or prevalent diseases, while those with a diagnosed disorder were in a stable condition and on a constant medication without direct cardiovascular influences. HR was slightly higher in women versus men (64 vs. 62 beats/min), while there was no relation between HR and age. Previously, resting HR has been reported to be 2–7 beats/min higher in female than male subjects, while the effect of age on resting HR has been minor [1,39,51]. Importantly, age and sex were included as confounding variables in the statistical analyses. On the basis of a recent epidemiological FINRISKI survey, the present study population with a mean body mass index of 26.7, total cholesterol level of 5.1 mmol/l, and 18% proportion of smokers, represented well the prevalent Finnish population [52]. The applied methods were non-invasive, safe, and easy to perform, and the accuracy of pulse wave analysis and impedance cardiography has been tested against invasive methods [30,31,53]. To strengthen the results, we performed a small validation study that showed a good correlation between impedance cardiography and echocardiography -derived stroke volume.

When measuring the human hemodynamics noninvasively, many of the variables are calculations or derivatives, and most of the calculations include HR in the procedure. The formulas used here have been found to be reliable [30-32,54], but HR is actually a major determinant in most of the calculated cardiac and vascular hemodynamic variables. Furthermore, some of the background characteristics were strongly correlated with each other, like the associations of body mass index with age, sex, and fasting glucose. These points cause a multicollinearity problem, which cannot be completely controlled for by statistical methods, but has to be taken into consideration when evaluating the results of this study.

Conclusions

We found that the hemodynamic profile associated with higher resting HR was characterized by higher cardiac output, higher cardiac workload, and lower systemic

vascular resistance. The hemodynamic features of the distinct HR tertile groups persisted during the head-up tilt. Higher resting HR was also associated with lower augmentation index and aortic pulse pressure, in spite of elevated BP and arterial stiffness. Thus, possible factors associating higher resting HR with less favourable prognosis in the population studies are higher cardiac workload, elevated BP, and increased arterial stiffness.

Additional file

Additional file 1: Regular medications used by the study population.

Abbreviations

AIx: Augmentation index; BP: Blood pressure; CI: Confidence interval; CRP: C-reactive protein; HDL: High-density lipoprotein; HR: Heart rate; LDL: Low-density lipoprotein; PWV: Pulse wave velocity.

Competing interests

The authors have no conflicts of interest to disclose.

Authors' contributions

JK, AT, IP, JM designed and conducted the study. JK, AT, IP, AJT analysed and interpreted the data, and drafted the first version of the manuscript. JK, AT, AJT, AH, ML, IP, EI, ON and JV performed experiments. JM, AJT, EI, ON, TL, TK and MK gave critical intellectual input and contributed to drafting revised versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Reeta Kulmala, RN, Paula Erkkilä, RN, Marika Päällysaho, RN, Pirjo Järventausta, RN, and Mirja Ikonen, RN, for invaluable technical assistance. The study was supported by Competitive State Research Financing of the Expert Responsibility Area of Tampere University Hospital, Finnish Cultural Foundation, Finnish Foundation for Cardiovascular Research, Sigrid Jusélius Foundation, Tampere Tuberculosis Foundation, Paavo Nurmi Foundation, Aarne Koskelo Foundation, Finnish Medical Foundation, and Orion-Farmos Research Foundation.

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Received: 30 August 2013 Accepted: 5 November 2013
Published: 15 November 2013

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doi:10.1186/1471-2261-13-102
Cite this article as: Koskela *et al.*: Association of resting heart rate with cardiovascular function: a cross-sectional study in 522 Finnish subjects. *BMC Cardiovascular Disorders* 2013 **13**:102.