Genetic susceptibility to adverse drug effects

Common variants in the *ABCB1* and *NOS1AP* genes

Albert-Jan Aarnoudse

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Genetic Susceptibility to Adverse Drug Effects

Common variants in the *ABCB1* and *NOS1AP* genes

Genetische gevoeligheid voor bijwerkingen van geneesmiddelen

Veelvoorkomende varianten van de *ABCB1* en *NOS1AP* genen

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General Introduction

1
General Introduction

Differences in drug response are heritable. Ever since the introduction of drugs in medicine there have been problems with adverse reactions. In the absence of knowledge of the minimum effective dose, the occurrence of adverse events was even used to determine the appropriate dose of a drug. Doses were increased until adverse effects occurred. Nowadays, drugs are extensively tested in clinical studies to obtain information on the effective and safe dosage before they enter the market. Despite all these efforts not every drug is safe and beneficial for every patient. Different people may respond differently to the same drugs at the same dose in terms of efficacy of the drug, but also in the occurrence of adverse effects. For instance, as a rule of thumb, one third of people benefit from antidepressants, one third does not and one third does not take them or discontinues them (in part) because of adverse effects. Although some differences can be explained by differences in age (and the accompanying changes in metabolism), sex, organ function, concomitant therapy, drug-drug interactions and the nature of the disease, differences in reaction to drugs are often unpredictable. However, differences in efficacy and adverse effects do not occur random. It appears that differences are greater among members of a population than within the same person at different times (or between monozygotic twins). The existence of large population differences with small intra-patient variability suggests that inheritance is a determinant of drug response. It is estimated that genetics can account for 20-95% of variability in drug response.[1] Once a drug is administered, it is absorbed and distributed to its site of action where it interacts with targets (such as receptors or enzymes), undergoes metabolism and is then excreted. At each of these processes, genetic variation may influence the drug response.[2-4]

Single nucleotide polymorphisms (SNPs). In the initial sequencing of the human genome, more than 1.4 million single nucleotide polymorphisms (SNPs) were identified with over 60,000 of them in the coding region of genes.[5] Nowadays, it is estimated that 1 in every 300 nucleotides is polymorphic in a population and 1 in every 1200 base pairs differs between two randomly chosen subjects. In total, up to 12-15 million SNPs are present in the human

genome. Every SNP represents a common (>1%) variation in the DNA sequence in which one base-pair varies between individuals. Since allele frequencies of these SNPs may differ largely between populations it is not possible to determine which base-pair represents the 'original' code. Therefore, they are referred to as minor- and major alleles rather than wild type and mutant alleles.

Pharmacogenetics and pharmacogenomics. Two main strategies are currently being used to identify genes and gene variants that cause individual variations in drug response. Pharmacogenetics studies the variability in drug responses attributed to hereditary factors, such as genetic polymorphisms, in different populations. The concept of pharmacogenetics originated from the clinical observation that there were patients with very high or very low plasma or urinary drug concentrations leading to adverse drug reactions or lack of efficacy when given standard doses of drugs. These observations were followed by the recognition that the biochemical traits leading to this variation were inherited. Only later were the drug-metabolizing enzymes identified, and this discovery was followed by the identification of the genes that encoded the proteins and the DNA-sequence variation within the genes that were associated with the inherited trait.[3, 4, 6] Today there is a systematic search to identify functionally significant variation in DNA sequences in genes that influence the effects of various drugs. The search began with a focus on drug metabolizing enzymes,[2, 7] but has extended to membrane transporters that influence drug absorption, distribution and excretion.[3, 7, 8] The second strategy is pharmacogenomics, the whole genome application of pharmacogenetics. It encompasses a genome-wide search for genes and DNA sequence variations that are associated with drug response without restriction to known candidates.[2, 6] Although both approaches represent different tactics, they share technologies and the terms are commonly used interchangeably. With the completion of the Human Genome Project[9, 10] and the ongoing annotation of its data, the sequences of all genes that catalyze phase I and II drug metabolism are known. The same is true for drug transporters, drug receptors and other drug targets. As a result, the traditional phenotype-to-genotype pharmacogenetic research paradigm is reversing direction to create a complementary genotype-to-phenotype flow of information.[2] The recent development of large SNP databases,[11] genotyping arrays of great accuracy and genome wide coverage of common variation,[12] which allow genotyping of up to 1,000,000 SNPs in a person at once, together with analytical methods[13] has enabled unbiased surveys of the majority of common variation in the human genome. This greatly facilitates pharmacogenomics, allowing genome wide association studies (GWA) to search for new candidate variants which influence drug response and risk of adverse drug reactions.[14, 15]

***ABCB1*.** One of the products of classical pharmacogenetics is the discovery of the ATB-binding cassette family B member 1 (*ABCB1*) gene. *ABCB1* is a 200kb gene on chromosome 7p21, which encodes for the multi drug resistance 1 protein (MDR1, also known as P-glycoprotein) a multi-drug efflux transporter. It was first discovered in chemotherapy resistant tumor

cells where it was over expressed, but is also widely expressed in normal tissues such as the duodenum, kidneys, liver and the blood-brain barrier where it plays a role in the uptake, distribution and clearance of many drugs.[16-21] Since its first discovery, as many as 647 single nucleotide polymorphisms (SNPs) -with an established frequency- have been identified in the *ABCB1* gene.[22] Among the first SNPs to be identified was the C3435T polymorphism, which was found to be associated with decreased *ABCB1* expression and increased digoxin concentration.[23] Since then, many more studies on the association between *ABCB1* SNPs -mostly C1236T, G2677T/A and C3435T- and kinetics of digoxin,[24-29] a very old cardiovascular drug still in use today, as well as many other drugs[30-34] have been performed with varying results.[35-37]

NOS1AP. The nitric oxide synthase 1 adaptor protein (*NOS1AP*) gene can be seen as an exponent of both pharmacogenomics and classical pharmacogenetics. Common genetic variants of *NOS1AP* were recently discovered to be associated with QT-interval prolongation in a genome wide association study.[38] The electrocardiographic QT interval is a non-invasive measure of ventricular repolarization. Mendelian congenital long- and short QT syndromes (LQTS, SQTS) are both characterized by sudden cardiac death (SCD) from ventricular arrhythmias.[39] Moreover, non-syndromal long[40-43] and short[44] QT interval are associated with an increased risk of SCD in unselected populations. In addition, many drugs are known to prolong QT-interval duration,[45, 46] and medication-induced prolonged QT interval and ventricular arrhythmias have led to the withdrawal of many non-cardiac medications.[47, 48] *NOS1AP* was not previously known to play a role in cardiac repolarization and, so far, not much is known about the mechanism by which *NOS1AP* influences QT duration. Since QT-altering drugs may interact with genetic variants in QT-duration associated genes,[39, 49, 50] this makes *NOS1AP* an interesting candidate for pharmacogenetic studies with QT-altering drugs.

Aim and outline of this thesis. In this thesis we explore the effect of interaction of common variants in the *ABCB1* and *NOS1AP* genes with drugs on drug response and adverse effects of drugs in a number of epidemiological studies. We mainly focus on digoxin and on other cardiovascular drugs as drugs of interest. In chapter 2 we will quantify the risk of digoxin intoxication, to give a perspective of the possible impact of these interactions on drug safety. Chapter 3 will focus on variations in the *ABCB1* gene and their interaction with the commonly used drugs digoxin and mefloquine. In chapter 4 we study the effect of *NOS1AP* variants on QT-duration and their interaction with QT-altering drugs such as digoxin and cardiovascular drugs and with anti-diabetic medication. Chapter 5 describes the effect of the interaction of *ABCB1* and *NOS1AP* genotypes with digoxin on cardiac function as assessed with echocardiography. In the general discussion (Chapter 6) the main findings of this thesis are placed in the broader context of potential clinical implications of pharmacogenetics and we address methodological considerations. Finally, we discuss the direction and methods of future research in the field, especially the possibilities of genome wide association studies in pharmacogenomics.

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2

Quantifying the risk of adverse drug reactions to digoxin

2

Age- and gender specific incidence of hospitalization for digoxin intoxication

Abstract

Background. The safety of digoxin (digitalis) therapy has greatly improved over the past three decades, but recent incidence rates for digoxin intoxication-related hospitalization are not available. Recent literature suggests that women are at higher risk of digoxin toxicity.
Objective: To provide age- and gender-specific incidence rates for digoxin intoxication-related hospitalization and mortality during digoxin intoxication-related hospitalization in The Netherlands in the period 2001–2004.
Study design and methods. We conducted a nationwide population-based cohort-study of all hospital admissions in the years 2001–2004 using a national computerised hospital admission registry. All patients with acute admissions were included in the study (n = 2,987,580). From these admissions, we selected all hospitalizations that had digoxin intoxication coded as either a primary or secondary diagnosis. We obtained data on digoxin prescriptions from the Foundation for Pharmaceutical Statistics (Stichting Farmaceutische Kengetallen) pharmacy database, which extrapolates drug figures for The Netherlands from prescriptions dispensed by 90% of all community pharmacies. We computed age- and gender specific incidence rates of digoxin intoxication.
Results. Digoxin intoxication was identified in 0.04% (n = 1286) of acute admissions. The incidence rate for digoxin intoxication-related admission was 48.5 (95% CI 45.9;51.2) per 100 000 prescriptions, which corresponds to 1.94 admissions for intoxication per 1000 treatment-years. Women had a 1.4-fold higher risk of intoxication than men (95% CI 1.3;1.6). The age- and gender adjusted relative risk of mortality in patients with digoxin intoxication compared with those admitted for other reasons was 0.7 (95% CI 0.5;0.8).
Conclusion. This study shows that digoxin intoxication in patients receiving current therapy is presently infrequent and that women are at higher risk of digoxin intoxication than men.

Introduction

The cardiac glycoside digoxin has been successfully used in the treatment of heart disease for more than 200 years. Recently, digoxin was formally approved for ventricular response rate control in patients with atrial fibrillation and congestive heart failure, based on three clinical trials.[1-3] However, digoxin has been associated with toxicity problems since its introduction. In the past it was associated with a high frequency of intoxications with a high mortality. Studies on toxicity for the period 1969 to 1983 showed a frequency of intoxication in users that was as high as 11–30%.[4-6] One prospective study in 1971 reported a mortality of 41% in patients with definite digitalis intoxication[6] and in another study 47% of patients with digitalis intoxication experienced life threatening arrhythmias.[7]

Currently, there is widespread knowledge and awareness of digoxin pharmacokinetics and of drug interactions of digoxin, as well as a tendency towards a lower dosing regimen and more strict therapeutic drug monitoring than had been used in the past. These developments in therapy management have resulted in a major reduction of digoxin intoxication and an improved prognosis over the last three to four decades. Recent studies showed digoxin intoxication in 4.8% of inpatients admitted for heart failure who were using digoxin (0.8% definite),[8] 1.1% of randomly selected outpatients who were using digoxin,[9] and 2% and 1.2% of closely monitored outpatients who were using digoxin in trials.[1, 9] Furthermore, recent literature suggests that women are at an increased risk of digoxin toxicity.[10]

Current population estimates of the incidence rate of digoxin intoxication are lacking. In the present study, we investigated the incidence rate of digoxin intoxication in the general population and the mortality rate during admission. Specifically, we assessed whether women were at higher risk of digoxin intoxication than men.

Methods

Population. Data on hospital admissions were retrieved from a nationwide computer database for hospital discharge records (National Hospital Registration) in The Netherlands. This database contains basic patient characteristics, the dates of admission and discharge, the discharge diagnosis, additional diagnoses during or preceding admission, surgical procedures, the treating medical specialty and special codes indicating drug-related hospitalizations (E-codes), based on the International Classification of Diseases (9th edition), Clinical Modification coding system. All general and university hospitals in The Netherlands participate in the system. Characteristics of all hospitalizations are registered by medical doctors in hospital discharge letters and coded by professional code clerks. Reimbursement of hospital and specialist fees does not depend on the way an admission or disease is coded. For every admission, one discharge/main diagnosis (mandatory) and up to 9 secondary diagnoses (optional) are registered. All diagnoses are submitted in the same format, mostly electronically.

All patients with an acute (non-planned) admission to a Dutch academic or general hospital

in the period January 2001–December 2004 were included in the study (n= 2,987,580). A digoxin-related admission was defined as a hospitalization with the ICD-code 972.1 as the main diagnosis or one of the secondary diagnoses or the code E942.1 as a secondary diagnosis (usually diagnosis of the cause leading to main diagnosis).

Digoxin use. Data on the use of digoxin were obtained from the Stichting Farmaceutische Kengetallen (SFK)[Foundation for Pharmaceutical Statistics] database, which extrapolates drug figures for The Netherlands based on filled prescriptions from 90% of all community pharmacies. As community pharmacies deliver 90% of all outpatient prescriptions, this database covers more than 80% of such prescriptions in The Netherlands. Due to different anonymization procedures in the hospitals as well as in the SFK database, it was not possible to link individual prescription data to admission data. Prescription data were only available as the number of prescriptions per age group (eight fixed age groups) stratified by gender and by year. The range of the age categories was chosen on the basis of distribution of overall drug use over age and on the relevance of specific age groups (less drug use in age groups 20-55 results in wide age-ranges, special interest in children results in smaller age-ranges). This resulted in age groups with different age-ranges instead of more conventional age groups with equal age range. Since there were no data on drug regimens and digoxin dosages can vary greatly among patients, the data did not allow for the calculation of the incidence rate in terms of person-years of use.

Analyses. We calculated the proportion of acute admissions that were attributable to digoxin intoxication according to the coded discharge diagnoses. Since digoxin prescription rates vary across different patient groups, the proportion of admissions may not reflect the actual risk of digoxin intoxication. Therefore, we also calculated incidence rates of digoxin intoxication by dividing the number of admissions for digoxin intoxication by the number of filled digoxin prescriptions. Using similar methods, we calculated age- and gender-specific incidence rates.
Descriptive statistical methods were used for subgroup comparisons and consisted of Student's t-tests (unpaired) and Chi-squared tests. The relative mortality risk during admission for digoxin intoxication versus other reasons for admission was estimated by using age- and gender-adjusted logistic regression analysis. The association between the length of hospital stay and admission for digoxin intoxication was studied by using age- and gender-adjusted linear regression analysis. All analyses were carried out in SPSS 11.0 for Windows. The age adjusted relative risk (RR) of admission with digoxin intoxication per 100,000 prescriptions in females versus males, was computed using Mantel-Haenzel methods across the eight age strata.

Table 1 Characteristics of all acute admissions (N= 2,987,580)

Characteristic	
Mean age (years)	49.1
Age in years (no. of patients)	
0-10	380,827
11-20	128,653
21-40	641,91
41-54	383,175
55-64	349,304
65-69	203,664
70-74	242,968
>=75	657,079
Female sex (%)	53.5 %
Top 10 most frequent main diagnoses (% admissions)	
Chest pain	5.8
Myocardial infarction	3.0
Heart failure	2.7
Stroke	2.0
Pneumonia	1.9
Atrial fibrillation	1.9
Abdominal pain	1.7
Chronic obstructive pulmonary disease	1.4
Fetal distress	1.3
Acute appendicitis	1.3
Digoxin intoxication (% admissions)	0.04[a]
Mean duration of admission (days)	8.8
Died during admission,= (% [No] admissions)	4.9 (147,379)

a N= 1286, mean age 77.4 years

Results

Of all 9,729,819 admissions over the years 2001-2004, 30.7% (n= 2,987,580) were acute admissions. The baseline characteristics of the study population are shown in table 1. The mean age of acutely admitted patients was 49.1 years and 53.5% were female. The most frequent main diagnoses were of a cardiovascular nature including chest pain, myocardial infarction, heart failure and stroke (13.5% of all acute admissions). The mean duration of admission was 8.8 days and 4.9% of all acute admissions ended in death.

Digoxin intoxication was reported in 0.04% (n= 1286) of acute admissions. Patients with digoxin intoxication were significantly older (77.4 versus 49.1 years, $p< 0.001$) and more often female (68.0% versus 53.5%, $p<0.001$) than other acutely admitted patients. The mean

Table 2 Gender specific incidence rates for digoxin intoxication per 100,000 prescriptions, by age category (N= 1286)

	Males		Females	
Age	**Cases**	**Incidence Rate (95% CI)**	**Cases**	**Incidence Rate (95% CI)**
0-10	10	229.8 (123.6 – 427.1)	9	251.3 (114.9 – 477.0)
11-20	1	65.0 (1.7 – 362.3)	1	76.8 (1.9 – 427.6)
21-40	7	110.5 (44.4 – 227.7)	6	122.7 (45.0 – 267.1)
41-54	7	17.8 (7.2 – 36.7)	11	52.8 (29.3 – 95.4)
55-64	28	23.0 (15.9 – 33.3)	34	55.2 (39.4 – 77.2)
65-69	34	30.3 (21.7 – 42.4)	43	63.0 (46.7 – 85.0)
70-74	68	40.0 (31.6 – 50.8)	86	64.8 (52.5 – 80.0)
>=75	256	42.1 (37.2 – 47.6)	685	53.0 (49.2 – 57.1)
Total	411	38.6 (35.0 – 42.5)	875	55.2 (51.6 – 59.0)

duration of admissions for digoxin intoxication was 14.2 days. The age and gender adjusted difference in duration with other admissions was 2.1 days (95% CI: 1.3; 2.8). Admissions for digoxin intoxication ended in death more often than other acute admissions (7.7% [n= 99]of admissions for digoxin intoxication versus 4.9% of other acute admissions); however, patients who were admitted for digoxin intoxication were older than patients admitted for other causes. After adjustment for age and gender, the risk of death during admission appeared to be lower for patients admitted with digoxin intoxication than for those admitted for other reasons (RR 0.7, 95% CI: 0.5;0.8).

There were 2.6 million digoxin prescriptions during the study period, 1.6 million of which (61.5%) were prescribed to women. The number of digoxin prescriptions and intoxications was stable over the study period. The overall incidence rate of digoxin intoxication was 48.5 (45.9;51.2) per 100,000 digoxin prescriptions. Assuming an average prescription length of 3 months (maximum period dispensed per prescription for chronic use in The Netherlands) this corresponds to 1.94 intoxications per 1000 person years of digoxin use. The highest rates of intoxication were found in very young (0–10 years) users. After the age of 40 years, there was a slow increase in risk of intoxication with increasing age (table 2). Women were at a higher risk of digoxin intoxication than men, with 55.2 (95%CI 51.6;59.0) versus 38.6 (95%CI 35.0;42.5) intoxications per 100,000 prescriptions, resulting in an age adjusted relative risk of 1.4 (95%CI: 1.3;1.6) for women versus men.

Discussion

In this study, we show that the absolute number of cases of digoxin intoxication requiring hospitalization in The Netherlands between 2001-2004 was rather low (1286 in 4 years, around 2 per 1000 person years of use). This confirms the observation that current management of digoxin treatment has reduced the risk of digoxin toxicity dramatically. At the current time,

digoxin treatment appears to be rather safe, probably due to regimens that employ lower dosages than were used in the past and to increasing awareness of the risk factors for intoxication. This improved prognosis is confirmed by the fact that we found a lower risk of in-hospital death for patients admitted for digoxin intoxication compared with other acutely admitted patients. In the past, mortality from digoxin intoxications used to be as high as 41%.[6] By the 1980s, all cause mortality for patients with a discharge diagnosis of digoxin intoxication had already been reduced to 5%, and only 1.1% of intoxicated patients died as a direct result of the intoxication.[8] There have been several changes in the therapy of heart disease that explain this dramatic reduction in mortality. First, the dosage administered in most digoxin regimens are much lower than in the past, leading to less severe intoxication with fewer lethal arrhythmias. Second, the patient population receiving digoxin has changed. In the past, many patients received digoxin for the treatment of coronary heart disease, but currently the main indications for treatment are congestive heart failure and atrial fibrillation. Of the patients with digoxin intoxication in 1971, 74% also experienced coronary artery disease. In the 1980s, this was reduced to 35%. Old or recent myocardial infarction in intoxicated patients decreased from 60% to 20%.[6, 8] Third, the diagnosis of digoxin intoxication has changed over time, resulting in the recognition of less severe cases. In the past, the diagnosis was based on the patient history and ECGs. Therefore, only more severe cases of intoxication were identified. Presently, serum digoxin levels may support the diagnosis in patients with a less severe presentation.[11] Fourth, changes in the treatment of heart disease, especially the introduction of potassium-sparing diuretics and ACE-inhibitors, has drastically reduced the incidence of hypokalemia in patients treated with diuretics. In previous studies, hypokalemia often complicated digoxin intoxication giving rise to more serious arrhythmias.[7] Finally, patients with severe intoxication resulting in lethal arrhythmias might not reach the hospital alive. This might have resulted in the selection of cases of intoxication that had relatively good prognoses in this study.

In addition, we found that women were at increased risk of digoxin intoxication. This observation supports the concerns that were raised earlier in the gender-stratified analysis of the Digitalis Investigation Group (DIG) data about a higher risk of digoxin therapy complications in women. In a post-hoc analysis of the DIG trial, the group of women treated for heart failure with digoxin had a higher mortality rate than the placebo group and than men treated with digoxin. Women had slightly higher serum digoxin levels than men after 1 month of use, despite lower doses of digoxin per body mass index unit.[10] The only other study on the effect of gender on outcome of digoxin treatment (the SOLVD trial) showed no difference in survival between men and women.[12] However, the SOLVD trial included a much lower number of women than the DIG study, resulting in lower power to detect a difference. Furthermore, the outcome in both studies was death, and as we have shown that the current mortality from digoxin intoxication is low, this would make it more difficult to detect a difference in rates between men and women in those studies. However, studies on the subject of gender differences in digoxin intoxication remain scarce since gender-stratified results are not available for most digoxin trials and women are traditionally under-represented in trials. One hypothesis about the increased risk

of digoxin intoxication in women is the inhibition of P-glycoprotein by hormone replacement therapy (HRT), which reduces the excretion of digoxin in the renal tubes.[10, 13] However, HRT use in The Netherlands is relatively low, making this an unlikely explanation for our findings. An alternative hypothesis which seems more likely and also involves P-glycoprotein is the suggestion that women have lower P-glycoprotein expression,[14] resulting in higher uptake and lower excretion of digoxin. It is unlikely that the difference in the incidence of digoxin intoxication is explained by a difference in renal clearance, since renal function tends to deteriorate more with increasing age in men than in women.[15] Adjustment for renal function might therefore even further increase the RR of digoxin intoxication in women compared with men. On the other hand, it remains possible that there were differences in co-medication or co-morbidity between men and women that might account for at least part of the difference in incidence that was found. In the post-hoc analysis of the DIG trial, for instance, women used diuretics more often than men, which might lead to lower potassium levels and hence, higher susceptibility to digoxin toxicity.[10] However, we were not able to investigate the influence of these factors in the current study.

Although we used a nation-wide registry with complete coverage of all hospital admissions in The Netherlands, there may be some misclassification in our estimations. In patients with severe co-morbidity in addition to digoxin intoxication, the co-morbidity rather than digoxin intoxication may have been coded as the main diagnosis. Also, symptoms may not have been recognized as digoxin toxicity and instead have been coded as nausea or arrhythmia. In addition, digoxin intoxications causing death before reaching the hospital, and patients with only mild symptoms of toxicity who were not hospitalized but instead treated by a simple dose reduction, will have been missed. These inaccuracies may have led to a slight underestimation of the incidence rate. Furthermore, individual data on dose and duration of digoxin use were not available, and we were unable to adjust for co-morbidity or multiple hospitalizations per patient.

Conclusion. Despite its limitations we believe that this study provides an accurate quantification of digoxin intoxication-related admissions relative to digoxin prescriptions, showing that current digoxin treatment is rather safe. Added to the fact that digoxin therapy was proven to be effective[1] and its use has been recommended in heart failure treatment guidelines, digoxin might be used more often in heart failure treatment than it currently is. Furthermore, this study supports the previous finding that women are at higher risk of the negative consequences of digoxin treatment.

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3

Common variants in the *ABCB1* gene

3.1

Common *ABCB1* variants are associated with increased digoxin serum concentration

Abstract

Background and objective. Digoxin is a known substrate of ATP-Binding Cassette B1 (*ABCB1*/*MDR1*). However, the results of studies on the association between *ABCB1* polymorphisms and digoxin kinetics remain contradictory. Almost all studies were small and involved only single dose kinetics. The goal of this study was to establish *ABCB1* genotype effect on digoxin blood concentrations in a large cohort of chronic digoxin users in a general Dutch European population.

Methods. Digoxin users were identified in the Rotterdam Study, a prospective population-based cohort study of individuals aged 55 years and older. Digoxin blood levels were gathered from regional hospitals and laboratories. *ABCB1* SNPs C1236T, G2677T/A and C3435T were assessed on peripheral blood DNA using Taqman assays. We studied the association between the *ABCB1* genotypes and haplotypes and digoxin blood levels using linear regression models adjusting for potential confounders.

Results. Digoxin serum levels and DNA were available for 195 subjects (56.4% women, mean age 79.4 years). All three *ABCB1* variants were significantly associated with serum digoxin concentration (0.18 to 0.21 µg/l per additional T-allele). The association was even stronger for the 1236-2677-3435 TTT-haplotype allele (0.26 µg/l (95%CI 0.14;0.38)) but absent for other haplotypes (CGC-allele considered referent), suggesting an interaction of SNPs in a causal haplotype instead of individual SNP effects.

Conclusion. We found that the common *ABCB1* C1236T, G2677T and C3435T variants and the associated TTT-haplotype were associated with higher digoxin serum concentrations in a cohort of elderly European digoxin users in the general population.

Introduction

The ATP-Binding Cassette B1 (*ABCB1*) or Multidrug Resistance 1 (*MDR1*) gene is a 200kb gene on chromosome 7p21. It was first discovered in chemotherapy resistant tumor cells but *ABCB1* is also widely expressed in normal tissues such as the duodenum, kidneys, liver and the blood-brain barrier.[1-6] There, *ABCB1* is known to play an important role in the uptake, distribution and excretion of many drugs.[7] Since its first discovery, as many as 647 single nucleotide polymorphisms (SNPs) -with an established frequency- have been identified in the *ABCB1* gene.[8]

Among the first SNPs to be identified was the C3435T polymorphism, which was found to be associated with decreased *ABCB1* function, resulting in increased digoxin concentration.[9] Since then, many more studies on the association between *ABCB1* SNPs –mostly C1236T, G2677T/A and C3435T- and kinetics of digoxin and many other drugs have been performed with varying results. In a recent meta-analysis combining all studies of the C3435T SNP and digoxin , an association with serum digoxin kinetics could not be confirmed. However, all these studies consisted of small groups of healthy volunteers, the largest study encompassing 50 subjects. Furthermore, all but one of these studies concerned single dose kinetics. In the only multiple dose study, subjects received digoxin for no more than five consecutive days.[10]

The aim of our study was to investigate the association of the *ABCB1* SNPs C1236T, G2677T/A and C3435T and their haplotypes with digoxin serum concentrations in a large population based cohort of digoxin users.

Methods

Setting and study population. A cohort of digoxin users was identified from the Rotterdam Study, a prospective population-based cohort study of chronic diseases in the elderly. Objectives and methods of the Rotterdam Study have been described in detail elsewhere.[11] The medical ethics committee of the Erasmus Medical Center (Rotterdam, The Netherlands) approved the study and all participants provided signed, informed consent for participation, retrieval of medical records, use of blood and DNA for scientific purposes and publication of obtained results. Baseline examinations took place from March 1990 through July 1993. In addition to the follow-up examinations that take place approximately every four years, all drug prescriptions dispensed to participants by automated pharmacies are routinely stored in the database since January 1, 1991. DNA for genotyping was available for 6571 (82%) participants from the baseline visit.

For the present study, the study population comprised all subjects from the baseline cohort who were dispensed digoxin and who had a digoxin serum concentration assessment at any point during the study period. We excluded persons for whom DNA for genotyping was unavailable or information on dose was lacking. Follow-up started on January 1, 1991 or first digoxin prescription, whichever was latest, and lasted until the end of the study period on

Table 1 Primer sequences.

Assay	Primer/probe	Sequence
ABCB1 C1236T	Forward primer	TCTCACTCGTCCTGGTAGATCTTG
	Reverse primer	CACCGTCTGCCCACTCT
	VIC probe	TCAGGTTCAGGCCCTT
	FAM probe	TCAGGTTCAGACCCTT
ABCB1 G2677A	Forward primer	AATACTTTACTCTACTTAATTAATCAATCAT-ATTTAGTTTGACTCA
	Reverse primer	GTCTGGACAAGCACTGAAAGATAAGA
	VIC probe	TTCCCAGCACCTTC
	FAM probe	CTTCCCAGTACCTTC
ABCB1 G2677T	Forward primer	CTTAGAGCATAGTAAGCAGTAGGGAGT
	Reverse primer	GAAATGAAAATGTTGTCTGGACAAGCA
	VIC probe	TTCCCAGCACCTTC
	FAM probe	TTCCCAGAACCTTC
ABCB1 C3435T	Forward primer	ATGTATGTTGGCCTCCTTTGCT
	Reverse primer	GCCGGGTGGTGTCACA
	VIC probe	CCCTCACGATCTCTT
	FAM probe	CCCTCACAATCTCTT

The positions of the SNPs are underlined

August 30, 2005, digoxin serum concentration assessment, patient death or loss to follow-up, whichever came first.

Genotyping. All participants of the Rotterdam Study for whom DNA was available were genotyped for the *ABCB1* C1236T, G2677T/A and C3435T polymorphisms. Genotyping was done using Taqman allelic discrimination assays on the ABI Prism 7900 HT Sequence detection system (Applied Biosystems, Foster City, Ca., USA) on 1 ng of genomic DNA extracted from leukocytes, as previously reported.[12]. The primer and probe sequences, designed by Applied Biosystems (Assay-by-Design service) are listed in Table 1. For the tri-allelic variant G2677T/A , two separate assays were designed, one detecting G2677T and one detecting G2677A. Haplotypes were estimated using the estimation maximization algorithm and software as described in the statistical analyses section.

Outcome. All available serum digoxin concentrations of subjects from the study population were gathered from 3 hospital laboratories and 1 general practitioner's laboratory serving the area of the Rotterdam Study. To limit the effect of digoxin dose titration, only the first available digoxin serum concentration assessment of each subject was used. A potentially toxic digoxin concentration was defined as a serum concentration above the upper limit of the therapeutic range (i.e., above 2.00 µg/l).

Co-variables. Covariates were gathered at the baseline examination, and included height (in meters), weight (in kilograms) and serum creatinine. Lean body mass (LBM) was computed using Devines formula: LBM = (height (cm) -152) x 0.9 + (50 kg (men)) *or* (45.5 kg (women)). Body mass index (BMI) was calculated as: weight (kg) / height (m) squared. Renal clearance was estimated using Cockroft and Gault's formula. Information on daily digoxin dose was expressed as the number of defined daily dosages (DDD, 1 DDD is 0.25 mg) as gathered from the automated pharmacy records. Dose per unit LBM was calculated dividing the dose of digoxin (in DDD) by the LBM. In addition, we identified exposure to inhibitors or inducers of ABCB17 within 7 days of blood sampling.

Statistical analyses. Differences in baseline characteristics between men and women were tested by using a Chi-square test for binary variables and a t-test for normally distributed continuous variables. To ensure comparability of serum digoxin concentration assessments across the different laboratories we compared average digoxin concentrations by one-way ANOVA. Exact Hardy Weinberg equilibrium p-values were computed for bi-allelic SNPs and a simulated p-value for the tri-allelic SNP were computed using the Genetics 1.2.1 (G.Warns and F. Leisch) package for R 2.5.0 software (http://www.r-project.org/). Additionally, we compared genotype frequencies with those of the whole Rotterdam Study cohort. D' was calculated using the ldmax command from GOLD software.[13] All tests for differences in baseline characteristics and linear and logistic regression analyses for the individual SNPs were performed using SPSS for windows software, version 11.1 (Chicago, Illinois, USA). Expectation maximization and linear and logistic regression analyses for the haplotypes were performed with the HaploStats 1.3.0 package for R, using haplo.em and haplo.glm respectively.[14, 15] Inferred haplotypes with a frequency below 1.5% were pooled into one 'rare haplotype' group, since estimates become unreliable for rare haplotypes in HaploStats.
Both allelic and general genotype models were tested for the three SNPs and haplotypes, although the allelic model was considered primary. In haplotype analyses, the haplotype with C-G-C at positions 1236-2677-3435, respectively, was considered the referent, to which the other haplotypes were compared. Serum digoxin concentration was tested for association with genotype or haplotype as the sole predictor (crude) and adjusted for age, gender, daily digoxin dose, LBM and renal clearance (multivariate) in a linear regression model. Additionally, we accounted for the influence of digoxin dose by repeating the analyses with digoxin concentration/digoxin dose (L/D) ratio as the outcome. In the regression analyses, the 2677A variant carriers were excluded because the frequency of this variant (1.5%) was too low for a reliable effect estimation. To quantify the role of *ABCB1* variants in digoxin kinetics, the partial r^2 was calculated as a measure of the variability of serum digoxin concentrations explained by *ABCB1* variants, using the formula: partial $r = (T_k / \sqrt{(T_k^2 + (N - K - 1))}$, in which T_k is the T value for the variable of interest, N is the number of subjects and K is the number of variables in the model. Finally, two sensitivity analyses were carried out. Because for some of the laborato-

ries only part of the follow-up period was available, subjects who started digoxin before that date might have been titrated already to a lower daily dose by reference to earlier measurements.. Therefore, we restricted these analyses to subjects who had the start date of their first prescription after the date the laboratory started contributing data. Second, we excluded all subjects using inhibitors or inducers of *ABCB1*.[7]

To investigate an association between *ABCB1* variants and serum digoxin concentrations above 2.0 µg/l, an allelic model was tested using logistic regression analyses, both crude and adjusted for age, gender, LBM, digoxin dose and renal clearance. To keep the multivariate model stable despite the limited number of cases and some missing values in weight and renal function, LBM, digoxin dose and renal clearance were only kept in the model for toxic concentrations if they were tested significant as a risk factor in the overall fully adjusted digoxin concentration model at $p< 0.1$.

Since there are indications that high serum digoxin concentrations are more frequent in women[16, 17] and that *ABCB1* expression in women might be lower,[18] we performed an additional gender stratified analysis.

Results

Study subjects. During the study period, we identified 1359 (17% of total cohort) digoxin users in the Rotterdam Study. Digoxin serum concentrations, dose and DNA were available for 195 (14% of digoxin users) subjects. The average digoxin dose was 0.57 DDD and did not differ between men and women, but women received significantly higher doses of digoxin per unit LBM (DDD/LBM) (Table 2). We identified 50 subjects using *ABCB1* inhibitors: amiodarone 15-, propafenone 2-, verapamil 9-, atorvastatin 4-, dypiridamole 3-, spironolactone 18-, and

Table 2 Baseline characteristics of 195 genotyped digoxin users from the Rotterdam Study

Characteristic	Total	Males	Females	p-value
	N= 195	N= 85 (43.6%)	N= 110 (56.4%)	
Age in years	79.4 (7.0)	78.1 (6.1)	80.3 (7.6)	0.03
BMI kg/m^2	26.7 (3.6)	26.0 (3.1)	27.3 (3.9)	0.01
LBM	60.9 (10.1)	70.2 (5.8)	53.5 (3.9)	<0.001
Renal Clearance ml/min	61.8 (17.4)	67.5 (18.7)	57.5 (15.3)	0.001
Average digoxin dose (DDD)	0.57 (0.26)	0.57 (0.27)	0.56 (0.25)	0.81
Dose per unit LBM (x1000)[a]	9.6 (4.4)	8.3 (3.8)	10.6 (4.6)	<0.001
Serum digoxin concentration (µg/l)	1.04 (0.73)	1.01 (0.58)	1.07 (0.83)	0.62
Number of serum digoxin concentrations >2.00µg/l (%)	12 (6%)	6 (7%)	6 (5%)	0.64

Mean (standard deviation).

BMI: body mass index, LBM: lean body mass, DDD: defined daily dosage (1 DDD is 0.25mg for digoxin)

a Dose in DDD x 1000 / LBM

Table 3 Frequencies of *ABCB1* SNP and haplotype alleles in 195 genotyped digoxin users from the Rotterdam Study

SNP allele	Allele frequency	Nr of homozygotes
1236T	0.44	35
2677T	0.43	35
2677A	0.02	1
3435T	0.55	61
Haplotype allele 1236-2677-3435[a]		
C-G-C	0.40	38
T-T-T	0.40	33
C-G-T	0.13	9
T-T-C	0.02	5
T-G-C	0.02	3
C-T-T	0.01	5
C-A-C	0.01	5

a Determined using expectation maximization in Haplostats

paroxetine 3 users. Of these, 4 subjects used 2 inhibitors simultaneously. None of the participants used *ABCB1* inducers.

Genotyping results. Allele frequencies of the C1236T, G2677T/A and C3435T SNPs are shown in Table 3. Genotyping of the study population was successful in 96.9%, 95.9% and 94.9% respectively and all three SNPs were in Hardy-Weinberg equilibrium, with p-values of 0.88, 0.02 and 0.14, respectively. All three SNPs were in strong linkage disequilibrium, as previously reported[7, 19] with a D' of 0.93 between C1236T and G2677T/A, 0.87 between G2677T/A and C3435T, and 0.81 between C1236T and C3435T. Expectation maximization resulted in two major haplotype alleles, the 1236-2677-3435 C-G-C and T-T-T haplotypes, both at an allele frequency of 40% and a number of less frequent haplotypes (Table 3). Genotype distributions did not differ between the general population and digoxin users with or without a serum concentration assessment. Genotype and haplotype frequencies were consistent with other European populations.[19-22]

Serum digoxin concentrations. There was no significant difference in mean digoxin serum concentration or percentage of toxic concentrations between assessments from the four different laboratories. There was no association between genotype or haplotype and digoxin dose (results not shown). One woman, who was homozygous TT for all three SNPs, was found to have an extraordinary high serum digoxin concentration (7.6 µg/l). Even if the sample was taken correctly, and no (un)intentional overdose of digoxin was taken by the patient, such a single outlier might excessively influence the effect estimate for the association in such a relatively small group and introduce an erroneous association or mask a true association. Therefore, it

Table 4 Increase in digoxin serum concentration (µg/l) per variant allele copy

	Crude		Model 1[a]		Model 2[b]		Model 3[c]	
SNP allele	**β (95% CI)**	**r^2**	**β (95% CI)**	**r^2**	**β (95% CI)**	**r^2**	**β (95% CI)**	**r^2**
1236T	0.15 (0.04-0.26)	3.5%	0.15 (0.03-0.26)	3.5%	0.16 (0.06-0.26)	4.8%	0.18 (0.07-0.29)	7.5%
2677T[d]	0.15 (0.04-0.27)	3.8%	0.15 (0.04-0.27)	3.7%	0.17 (0.07-0.28)	5.4%	0.21 (0.10-0.32)	9.5%
3435T	0.12 (0.02-0.23)	2.6%	0.12 (0.01-0.23)	2.6%	0.13 (0.03-0.23)	3.8%	0.18 (0.07-0.29)	7.6%
Haplotype allele[e]								
CGC	Reference		Reference		Reference		Reference	
TTT	0.19 (0.07-0.32)	4.9%	0.19 (0.007-0.32)	4.9%	0.21 (0.09-0.32)	6.5%	0.26 (0.14-0.38)	11.5%
CGT	0.01 (-0.16-0.18)	0.0%	0.01 (-0.16-0.18)	0.0%	0.03 (-0.13-0.19)	0.1%	0.07 (-0.11-0.25)	0.4%
TTC	-0.16 (-0.60-0.28)	0.3%	-0.16 (-0.61-0.29)	0.3%	-0.05 (-0.46-0.36)	0.0%	0.01 (-0.40-0.42)	0.0%
TGC	0.13 (-0.29-0.55)	0.2%	0.13 (-0.29-0.55)	0.2%	0.17 (-0.22-0.56)	0.4%	0.04 (-0.37-0.45)	0.0%
CTT	0.08 (-0.49-0.58)	0.0%	0.08 (-0.43-0.59)	0.0%	0.12 (-0.35-0.59)	0.1%	0.16 (-0.29-0.61)	0.4%

β is the regression coefficient and reflects the increase in digoxin serum concentration (µg/l) per variant allele copy, r2 is partial r-squared, the percentage variability in digoxin serum concentration explained by the SNP or haplotype allele.

a Model 1: Adjusted for gender, age

b Model 2: Adjusted for gender, age, digoxin dose

c Model 3: Adjusted for gender, age, digoxin dose lean body mass, and renal clearance

d A-alleles (GA (n=4) and AA (n=1) genotypes, TA genotype was not found in this population) were excluded because of low numbers.

e All haplotype alleles with a frequency ≥1.5% were included in one model (haplotypes with frequency <1.5% were pooled in one 'rare haplotype' estimate)

Table 5 Risk of serum digoxin concentrations >2.00µg/l per genotype group and odds ratio per variant allele copy

				OR by allele		
SNP	**Cases/total (% within genotype group)**			**Crude**	**Model 1[a]**	**Model 2[b]**
C1236T	CC	CT	TT			
	2/59 (3.4%)	4/95 (4.2%)	5/35 (14.3%)	2.4 (1.0-6.1)	2.5 (1.0-6.4)	3.2 (1.2-8.7)
G2677T[c]	GG	GT	TT			
	1/55 (1.8%)	5/92 (5.4%)	4/35 (11.4%)	2.5 (1.0-6.6)	2.4 (0.9-6.4)	3.1 (1.1-8.4)
C3435T	CC	CT	TT			
	1/43 (2.3%)	4/81 (4.9%)	5/61 (8.2%)	1.9 (0.7-4.8)	1.8 (0.7-4.7)	2.1 (0.8-5.7)
Haplotype allele[d]						
CGC		NA		Reference	Reference	Reference
TTT				3.5 (1.2-10.5)	3.5 (1.2-10.6)	4.3 (1.4-13.4)
CGT				1.5 (0.3-8.0)	1.4 (0.3-7.8)	1.5 (0.2-9.5)

Odds ratios only for allelic models (OR per additional variant allele) due to low numbers of cases per genotype group

a Model 1: Adjusted for gender, age

b Model 2: Adjusted for gender, age, digoxin dose

c A-alleles (GA (n=4) and AA (n=1) genotypes, TA genotype was not found in this population) were excluded because of low numbers.

d Because of the limited number of cases, all haplotype alleles with a frequency ≥5% were included in one model (haplotypes with frequency <5% were pooled in one 'rare haplotype' estimate)

was excluded from further analyses. After adjustment for age and sex, an increase in digoxin with 1 DDD was associated with an increase in digoxin serum concentration of 0.93 µg/l (95% CI 0.63-1.23) with a partial r^2 estimate of 16.7%. The results of the genotypic model were consistent with an allelic model (results not shown). For each of the individual SNPs at positions 1236, 2677 or 3435, the serum digoxin concentration increased significantly per additional T-allele with the strongest association for the 1236-2677-3435 TTT haplotype (Table 4). The point estimates for haplotypes with a T variant at just one or two of the positions were around zero, but the numbers of these haplotypes were too low to obtain a statistically robust effect estimate (Table 4). Using L/D ratio as the outcome in stead of adjustment for digoxin dose in a linear regression model yielded similar results (data not shown). Stratification on sex showed similar results for men and women (results not shown). The partial r^2 estimate indicated that *ABCB1* variants explained up to 11.5% of the variability in serum digoxin concentration for the TTT haplotype (Table 4). The sensitivity analysis restricted to subjects who started taking digoxin after the date the laboratory they were referred to started contributing data (n=100) did not substantially change the effect estimates, neither did the analyses excluding subjects using *ABCB1* inhibitors (data not shown).

Although the number of subjects with a digoxin concentration above 2.0 µg/l (the upper level of the therapeutic range) was very low (n=12), a significant association between the T-variants

of the three SNPs and these potentially toxic digoxin concentrations was found. The association in the individual SNPs was strongest for the TTT haplotype allele: age sex and digoxin dose adjusted OR 4.3 (95% CI 1.4-13.4) per TTT allele (Table 5).

Discussion

To our knowledge, this is the first population based study on the consequences of *ABCB1* polymorphisms in a large population based group of European digoxin users. We demonstrated a statistically significant association between the common *ABCB1* variants C1236T, G2677T, C3435T and serum digoxin concentration, which was strongest for the associated TTT haplotype allele and explains an important part of the variability in digoxin concentrations as determined by the r^2 (up to 11.5% by the TTT haplotype). We observed no effect for the other haplotype alleles, including the CGT haplotype. This suggests interaction of the three SNPs in the TTT haplotype and is consistent with a recent study that demonstrated an effect of the silent C3435T SNP only in combination with one or two of the G2677T/A and C1236T SNPs. That study suggests an effect on protein folding through rare codon usage, resulting in changed substrate specificity.[23] Since, in our population, G2677T/A and C1236T best predicted the TTT haplotype, it may be sufficient to limit *ABCB1* genotype assessment for clinical purposes to these two SNPs. The majority of previous studies in Caucasians showed similar effects of *ABCB1* variants on digoxin kinetics (Table 6), but a meta-analysis could not confirm an effect for the C3435T SNP.[10] The main difference with our study is that most studies comprised only single dose kinetics and were underpowered to demonstrate small effects. Only one study was performed in actual digoxin using patients.[24] and showed no association of *ABCB1* variants with digoxin kinetics. The used dose was however almost twice that of the average daily dose in our study. At these higher dosages, the maximum transport capacity of *ABCB1* transport capacity might be saturated by the abundance of digoxin and subtle differences in *ABCB1* efflux-capacity may no longer contribute substantially to the variability in digoxin serum concentration. Studies in Asian populations and with other substrates are discussed in more detail elsewhere.[10, 25]

Although almost all observed serum digoxin concentrations fell within the generally accepted therapeutic range (0.5-2.0 µg/l), the observed differences in serum digoxin concentrations between genotype groups may still be large enough to have immediate clinical consequences. An increase of 0.20-0.25 µg/l per variant allele equals the effect of a 0.25DDD dose increase. Consequently, subjects with 2 variant alleles have an increase in serum concentration of 0.4-0.5 µg/l, equal to the effect of a dose increase of 0.5 DDD. Furthermore, even differences in serum digoxin concentrations within the accepted therapeutic range might influence the efficacy and safety of treatment.[26] Finally, this study suggests that T-variant carriers and TTT haplotype carriers are also at higher risk of digoxin concentrations that exceed the therapeutic range and are generally regarded as toxic. However, to calculate a reliable risk estimate a study with more cases of digoxin intoxications is necessary.

Table 6 Other studies on ABCB1 and digoxin kinetics in Caucasians

Study	Subjects	N	Dose	SNPs tested	Effect on digoxin concentration
Hoffmeyer et al (2000)[9] [a]	Healthy Caucasians	21 Single dose (+14 steady state)	1 mg (0.25 mg steady state)	C3435T	Higher for T-variants
				C1236T	No significant effect
				other SNPs	No significant effect
Gerloff et al (2002)[33]	Healthy Caucasians	50	1 mg	C3435T	No significant effect
Johne et al (2002)[34] [a]	Healthy Caucasians	24	2 days 2x0.25 mg, 3 days 1x0.25 mg	C3435T	Higher for T-variants
				G2677T	Higher for T-variants
				C1236T	No significant effect
				5 other SNPs	No significant effect
Verstuyft et al (2003)[35]	Healthy Caucasians, Africans, Asians	25, 6, 1	0.5 mg	C3435T	Higher for T-variants
				G2677T	Higher for T-variants (not significant)
Kurzawski et al (2007)[24] [a]	Heart failure patients	77	0.25 mg (or 0.5 mg methyldigoxin)	C3435T	No significant effect
				G2677T	No significant effect
Comets et al (2007)[36]	Healthy volunteers	32	0.5 mg	C3435T	Higher for T-variants
				G2677T	Higher for T-variants

a Steady state, all other studies were single dose

The main strength of our study compared to earlier studies on *ABCB1* SNPs and digoxin serum concentrations resides in the large number of digoxin users from a population based cohort. Selection bias is unlikely since SNPs were in HWE and the distribution of genotypes did not differ between the study population and the whole Rotterdam Study cohort or digoxin users without a serum concentration assessment. Furthermore, digoxin prescribing and serum concentration assessment were blinded for genotype. The influence of confounding, which was probably limited, was further minimized by applying a multivariate regression model including the most relevant determinants of digoxin serum concentration. One limitation is that the present study does not allow an estimation of the influence of *ABCB1* SNPs on treatment efficacy. Another limitation involves measurement inaccuracies. Digoxin concentration sampling under everyday circumstances is often, incorrectly, performed without regarding the time interval since last digoxin dose in clinical practice.[27] We could not verify the influence of such inaccuracy since the time of the last dose of digoxin and the time of drawing the blood sample had not been registered consistently. Any measurement errors (misclassification of the outcome) were most likely random and would have led to a conservative estimate of the actual effect. We limited the effect of dose titration based on serum concentrations by using only the first serum assessment of each subject. Furthermore, the sensitivity analyses, limited to subjects who had their first prescription of digoxin after the laboratory they were referred to started contributing data, showed similar effect estimates. Finally, this study was performed in a European population. The effect of *ABCB1* SNPs on serum digoxin concentrations may not automatically be valid for other populations since haplotype allele variation might differ and variation in expression of other transporter proteins might play a role. For instance, the 2677A variant is more frequent in certain Asian populations,[19, 28] but it was too rare in our study population to investigate its effect.

In conclusion, we found that the *ABCB1* C1236T, G2677T and C3435T variants were associated with higher digoxin serum concentrations in a cohort of elderly European digoxin users in the general population. The association was strongest for the 1236-2677-3435 TTT haplotype, suggesting an interaction of SNPs in a causal haplotype instead of individual SNP effects. The findings in this study might provide a starting point for strategies toward improving the efficacy and safety of digoxin therapy. As an efflux transporter, *ABCB1* influences pharmacokinetics of many drugs.[7] Therefore, these variants could have important implications for individual variation in drug concentrations and susceptibility to toxic effects.[29-32]

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3.2

Common *ABCB1* variants increase QTc shortening and risk of SCD in digoxin users

Abstract

Background and objective. Digoxin is one of the oldest cardiovascular drugs still in use today, but its use has always been associated with the risk of intoxication and life threatening arrhythmias. Digoxin is a known substrate of ATP-Binding Cassette B1 (*ABCB1*/*MDR1*). Therefore, we hypothesized that these *ABCB1* variants might also increase digoxin toxicity associated sudden cardiac death (SCD). In addition, to further evaluate the association with cardiac effects of digoxin, we studied the association of *ABCB1* variants with QTc interval duration in digoxin- and non-digoxin users.

Methods. We used the Rotterdam Study, a population-based, prospective cohort study of individuals ≥55 Years of age. *ABCB1* SNPs C1236T, G2677T/A and C3435T were assessed on peripheral blood DNA using Taqman assays. Hazard ratios (HRs) for time to SCD among *ABCB1* genotypes with and without digoxin were estimated by Cox proportional hazards modeling with time-dependent exposures. Heart rate corrected QT (QTc) interval duration was measured in prospectively collected ECGs and digoxin exposure was determined at the time of each ECG.

Results. There were 229 SCD cases, 40 of whom were exposed to digoxin at time of death. In digoxin users, homozygotes for 1236 or 2677 T-alleles and TTT-haplotype alleles had a two-fold increased risk of SCD (HR homozygous TTT: 2.36, 95%CI 1.02;5.45) in a recessive genetic model. No effect of *ABCB1* variants on SCD risk was observed within non-digoxin users. We included 13,694 ECGs from 5535 individuals (294 ECGs from 222 individuals on digoxin). In digoxin users, *ABCB1* T-allele or TTT-haplotype allele carriers increased QTc shortening compared to subjects homozygous for the reference allele.

Conclusion: *ABCB1* C1236T, G2677T/A and C3435T T-alleles and the associated TTT-haplotype allele increase the risk of SCD to digoxin and show increased QTc shortening to digoxin.

Introduction

Digoxin is one of the oldest cardiovascular drugs still in use today. Although digoxin therapy is relatively safe nowadays,[1] its use has often been associated in the past with considerable risks of intoxication, frequently resulting in of arrhythmias and death.[2-4] Toxic concentrations may occur at therapeutic doses. Moreover, toxic effects of digoxin can appear within the accepted therapeutic serum concentration range.[5] Digoxin is a known substrate of P-glycoprotein, a multi-drug efflux transporter encoded by the ATP-Binding Cassette B1 (*ABCB1*) also known as the Multidrug Resistance 1 (MDR1) gene, a 200kb gene on chromosome 7p21. It was first discovered in chemotherapy resistant tumor cells but is also widely expressed in normal tissues such as the duodenum, kidneys, liver and the blood-brain barrier where it plays a role in the uptake and clearance of many drugs.[6-11] P-glycoprotein is suggested to play a role in digoxin concentration management.

Many studies on single nucleotide polymorphisms (SNPs) of *ABCB1* and digoxin kinetics have been performed. However, most studies were small and the results were ambiguous.[12] In a recent study, we showed that common variants in *ABCB1* were associated with increased serum digoxin concentrations and risk of supra-therapeutic concentrations in a group of 195 chronic digoxin users from the Rotterdam Study.[13] Therefore, we hypothesized that common *ABCB1* variants might also be involved in digoxin toxicity associated fatal arrhythmia in the form of sudden cardiac death (SCD) to digoxin therapy. In addition, to further evaluate the association of *ABCB1* SNPs with cardiac effects of digoxin, we studied the association of *ABCB1* variants with QTc interval duration in users and non-users of digoxin.

Methods

Setting and study population. The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly. All inhabitants of Ommoord, a Rotterdam suburb, aged 55 years and older (10,278), were ascertained from the municipal register and invited to participate. Of them, 78% (7983, 58% female and 98% white) took part in the baseline examination from March 1990 through July 1993. Second and third examinations were conducted from September 1993 to August 1996 and from April 1997 to December 1999. Objectives and methods of the Rotterdam Study have been described in detail.[14, 15] The medical ethics committee of Erasmus Medical Center (Rotterdam, The Netherlands) approved the study and all participants provided signed informed consent for participation, including retrieval of medical records, use of blood and DNA for scientific purposes and publication of data.

Apart from extensive structured interview rounds, clinical characteristics including smoking, body mass index (BMI), hypertension, diabetes mellitus, heart failure and myocardial infarction are ascertained as previously described.[16-21] Active surveillance for incident diabetes mellitus, heart failure and myocardial infarction is conducted continuously between exams. DNA for genotyping is available for 6571 (82%) participants from the baseline visit. Exposure of

study participants to medications is gathered on a continuous basis as of January 1st, 1991 to the present through computerized pharmacy records of the pharmacies in the study area. Therefore, the start of the study period for both ECG measures and SCD was January 1st, 1991.
The study population comprised all participants from the first cohort for whom pharmacy data and genotype data were available. Subjects were followed form January 1st, 1991 until death, loss to follow-up or end of follow-up, whichever came first.

Genotyping. All participants of the Rotterdam Study for whom DNA was available were genotyped for the *ABCB1* C1236T, G2677T/A and C3435T polymorphisms. Genotyping was done using Taqman allelic discrimination assays as previously described.[13] Haplotypes were estimated using the estimation maximization algorithm and software as described in the statistical analyses section.

Adjudication of sudden cardiac death. All genotyped individuals who were alive at the start of the study period were included in the SCD analysis. The ascertainment of SCD cases in the Rotterdam Study has been described previously.[15, 21] SCD was defined operationally as: a witnessed natural death attributable to cardiac causes, heralded by abrupt loss of consciousness, within one hour of onset of acute symptoms, or an unwitnessed, unexpected death of someone seen in a stable medical condition <24 hours previously with no evidence of a non-cardiac cause.[22, 23]

Assessment of QTc interval and other ECG measurements. ECG measurements gathered during the study period were analyzed in all subjects with DNA for genotyping data available. The electrocardiographic phenotype studied was the heart rate corrected QT-interval (QTc) in msec, using Bazett's formula (QTc = QT / $\sqrt{RR}$).[24] As in previous studies of QTc in the Rotterdam Study[21] we used a 10-second resting 12-lead ECG (average of 8 to 10 beats), which was recorded on an ACTA electrocardiograph (ESAOTE, Florence, Italy) at a sampling frequency of 500 Hz and stored digitally. All ECGs were processed by the Modular ECG Analysis System (MEANS) to obtain ECG measurements.[25-27] MEANS determines the QT interval from the start of the QRS-complex until the end of the T-wave. MEANS also determines the presence of right (RBBB) or left bundle branch block (LBBB) and left ventricular hypertrophy (LVH). To study the association between NOS1AP variants and QTc duration, all eligible ECGs from subjects with DNA available were used. ECGs with RBBB or LBBB were excluded from the analyses. In addition, to minimize confounding by non-genetic influences on QT duration, all ECGs taken while the subject was on any QT prolonging drugs were excluded from the analyses. Drugs were considered as possibly QT prolonging if they appeared on any of lists 1-4 at www.qtdrugs.org.[28] We also excluded ECGs if subjects were on flupenthixol, levomepromazine, mefloquine, olanzapine or sertindole, which may also prolong QT interval. Finally, ECGs taken while digoxin dose >1 defined daily dosage (DDD) or with unknown digoxin dose were

excluded. Up to 4 QTc measurements per subject were recorded across the four examination cycles

Digoxin exposure. In order to assess exposure, we calculated the duration of dispensed digoxin prescriptions as the number of units dispensed divided by the number of units to be taken per day. Digoxin dose was expressed as the defined daily dose (DDD) as determined by the WHO (1 DDD is 0.25 mg of digoxin).
Subjects were considered as exposed to digoxin if the index date (date of death) of the case fell within a period of digoxin use. Similarly, in each remaining cohort participant, we assessed exposure to digoxin in this way on the same index date as the corresponding case of SCD.
For the analyses of QTc-interval duration, a study participant was considered to be exposed to digoxin if the ECG was taken on a date which fell within the duration of a dispensed digoxin prescription. Because up to 4 ECGs were take per individual, subjects could contribute ECGs to both the digoxin and the non-digoxin group.

Statistical analysis. Exact Hardy Weinberg equilibrium p-values for bi-allelic SNPs and a simulated p-value for the tri-allelic SNP (G2677T/A) were computed using the Genetics 1.2.1 (G.Warns and F. Leisch) package for R 2.5.0 software. Genetic linkage, as expressed by D' was calculated using the ldmax command from GOLD software.[29] Expectation maximization for the haplotypes was performed with the HaploStats 1.3.0 package for R 2.5.0, using haplo.em.[30, 31] Inferred haplotypes with a posterior probability < 0.95 were excluded from further analyses.
Hazard ratios for time to SCD from baseline were estimated using Cox proportional hazards models with time dependent variables for digoxin exposure. Based on the outcome from general genotype models, either allelic, dominant or recessive models were tested for the three polymorphisms and haplotypes. In addition to *ABCB1* genotype, known SCD risk factors including age, sex, and time dependent incident diabetes mellitus, heart failure and myocardial infarction were included as covariates. First, the effect of genotype on SCD risk was tested in strata of digoxin use and non-use. Additionally, we created an interaction dummy variable with six levels for the three genotypes with and without digoxin use in which non-digoxin users homozygous for the major allele were considered as referent. However, because of the vast impact of cardiovascular morbidity on SCD –which is much more frequent in digoxin users than in non-users- we considered the stratified analyses primary. In the stratified analyses we further adjusted for digoxin dose. The main variant haplotype (1236-2677-3435 T-T-T) was tested in Cox proportional hazards models similar to the individual SNPs using the 1236-2677-3435 C-G-C haplotype as the referent. We included the CGT haplotype as a separate covariate, and the rare variables as one pooled rare-haplotype covariate. To minimize bias by misclassification of SCD, we additionally performed a sub-analysis restricting the case definition to witnessed deaths only. All Cox proportional hazards analyses were performed using SPSS for Windows version 11.0 (Chicago, Illinois, USA).

Because the QTc in subsequent ECGs of the same subject are correlated, we used repeated measures analyses implemented in PROC MIXED (SAS 8.2, Cary, NC). To test for interaction of *ABCB1* genotypes with digoxin in QTc shortening, we used the same six level interaction dummies. Additionally, we compared the effect of digoxin on QTc duration in starters and stoppers of digoxin therapy between genotype groups (follow-up design). QTc was tested for association with genotype as the sole predictor (crude) and with adjustment for age, sex, heart failure, myocardial infarction and diabetes mellitus (multivariable). The main variant haplotype 1236-2677-3435 T-T-T was compared with the 1236-2677-3435 C-G-C haplotype as the referent. Other haplotypes were entered in the model similar to the SCD analyses.
Additionally, we studied the effect of digoxin on QTc interval within subjects in a follow-up design. For this, we determined the difference in QTc duration between two consecutive ECGs (one on digoxin and one off) of subjects starting or stopping digoxin therapy and applied descriptive statistics.
Differences in digoxin dose between ECGs from different genotype groups were compared using repeated measures analyses, because doses at the time of subsequent ECGs of the same subject are correlated. Digoxin dose of different genotype groups in SCD cases were compared using ANOVA.

Results

Study population and genotyping results. The study population comprised 6541 individuals with a mean follow-up time of 10.4 (SD 3.7) years. Of these, 871 subjects used digoxin at any time during the study period. SCD cases were older than the overall SCD sample and had more cardiovascular risk factors. A total of 13,694 eligible ECGs from 5535 individuals were available. Of these, 294 ECGs from 222 individuals were taken while the subject was using digoxin. Mean follow-up time was 11.4 (SD 3.7) years. Mean digoxin dose was 0.61 DDD (SD 0.27) and did not differ significantly between genotypes in the ECGs taken while using digoxin, ever digoxin users in the total SCD sample or SCD cases. Baseline characteristics are shown in Table 1.
Successful genotype calls were made in 96.7%, 95.5% and 95.0% for the C1236T, G2677T/A and C3435T SNPs. P-values for Hardy-Weinberg equilibrium were p= 0.52, p=0.0001 and p=0.20 respectively. The finding that the G2677T/A SNPs slightly deviates from Hardy-Weinberg equilibrium is explained by a relative excess of homozygotes for the rare A-allele. All three SNPs were in strong linkage disequilibrium, as previously reported[32, 33] with a D' of 0.94 between C1236T and G2677T/A, 0.84 between G2677T/A and C3435T, and 0.81 between C1236T and C3435T. Expectation maximization resulted in two major haplotype alleles, the 1236-2677-3435 C-G-C and T-T-T haplotypes, both at an allele frequency of 41%, a C-G-T haplotype with a frequency of 12% and a number of rare haplotypes. After exclusion of subjects with <0.95 posterior probability haplotype pairs, the mean posterior probability was 0.99. Genotype distributions did not differ between the general population and digoxin users. Geno-

Table 1 baseline characteristics of the study population

Characteristic		Total study population		SCD cases		QTc sample	
		Men	Women	Men	Women	Men	Women
Number		2655 (40.6%)	3886 (59.4%)	113 (49.3%)	116 (50.7%)	2259 (40.8%)	3276 (59.2%)
Age at baseline, years (SD)		68.1 (8.2)	70.3 (9.6)	71.4 (7.6)	74.3 (7.7)	67.5 (8.0)	69.6 (9.3)
Mean follow-up time (SD)		10.1 (3.8)	10.6 (3.7)	6.5 (3.7)	7.3 (3.8)	11.1 (3.8)	11.6 (3.6)
Current smoking		772 (29.1%)	679 (17.5%)	32 (28.3%)	15 (12.9%)	647 (28.6%)	593 (18.1%)
Past smoking		1629 (61.4%)	1032 (26.6%)	73 (64.6%)	37 (31.9%)	1391 (61.6%)	890 (27.2%)
Body Mass Index (SD)		25.7 (3.0)	26.7 (4.1)	25.3 (3.0)	27.3 (3.9)	25.7 (3.0)	26.7 (4.0)
Blood pressure (mmHg)							
Diastolic (SD)		74.6 (11.5)	73.1 (11.4)	74.4 (12.1)	77.0 (14.2)	74.8 (11.4)	73.1 (11.1)
Systolic (SD)		138.7 (21.7)	139.8 (22.6)	144.7 (24.4)	152.7 (27.8)	138.3 (21.5)	139.2 (22.3)
Hypertension		776 (29.2%)	1409 (36.3%)	52 (46.0%)	64 (55.2%)	542 (24.0%)	947 (28.9%)
Diabetes mellitus		279 (10.5%)	417 (10.7%)	14 (12.4%)	27 (23.3%)	229 (10.1%)	335 (10.2%)
Myocardial infarction		447 (16.8%)	318 (8.2%)	44 (38.9%)	19 (16.4%)	375 (16.6%)	266 (8.1%)
Heart failure		81 (3.1%)	127 (3.3%)	17 (15.0%)	7 (6.0%)	69 (3.1%)	108 (3.3%)
QTc, msec (SD)		NA	NA	NA	NA	421.6 (25.0)	431.6 (22.8)
Allele	1236T	43.3%	44.0%	40.5%	47.3%	43.4%	44.5%
frequency	2677T	42.7%	43.6%	39.4%	46.0%	42.8%	44.0%
	2677A	2.3%	2.2%	3.7%	1.8%	2.3%	2.1%
	3435T	53.1%	53.9%	48.1%	56.6%	53.4%	54.3%
Haplotype-	CGC	41.6%	41.0%	43.1%	38.2%	41.3%	40.5%
allele	TTT	40.3%	41.0%	35.8%	44.1%	40.5%	41.5%
frequency	CGT	11.7%	11.6%	12.3%	12.7%	11.9%	11.6%
	Rare	6.4%	6.4%	8.8%	5.0%	6.3%	6.4%

NA Not applicable since not all genotyped individuals have an eligible ECG available

Table 2 *ABCB1* Genotype effect on SCD risk, stratified on digoxin use

	Non-digoxin users				Digoxin users			
	***ABCB1* genotype (SCD cases)**				***ABCB1* genotype (SCD cases)**			
***ABCB1* SNP**	**HR (95% CI) by genotype[a]**				**HR (95% CI) by genotype[a]**			
C1236T	CC (65)	CT (80)	TT (38)	Recessive	CC (11)	CT (15)	TT (11)	Recessive
Crude	Reference	0.79 (0.57;1.09)	0.95 (0.64;1.42)	1.10 (0.77;1.57)	Reference	0.96 (0.44;2.10)	2.11 (0.91;4.87)	2.15 (1.06;4.37)
Full model[b]	Reference	0.81 (0.58;1.12)	0.97 (0.65;1.45)	1.10 (0.77;1.57)	Reference	0.94 (0.43;2.05)	2.03 (0.87;4.72)	2.11 (1.03;4.31)
Full model+dose[c]	NA.	NA	NA	NA	Reference	0.94 (0.43;2.05)	2.03 (0.87;4.73)	2.11 (1.03;4.31)
G2677T	GG (59)	GT (79)	TT (34)	Recessive	GG (11)	GT (15)	TT (11)	Recessive
Crude	Reference	0.83 (0.59;1.16)	0.90 (0.59;1.38)	1.01 (0.69;1.47)	Reference	0.93 (0.43;2.03)	2.09 (0.90;4.83)	2.18 (1.07;4.42)
Full model[b]	Reference	0.85 (0.61;1.19)	0.91 (0.60;1.39)	1.00 (0.69;1.46)	Reference	0.91 (0.42;1.98)	2.08 (0.90;4.84)	2.20 (1.08;4.50)
Full model+dose[c]	NA.	NA	NA	NA	Reference	0.91 (0.42;1.99)	2.08 (0.90;4.84)	2.20 (1.08;4.50)
C3435T	CC (43)	CT (82)	TT (52)	Recessive	CC (13)	CT (9)	TT (14)	Recessive
Crude	Reference	0.86 (0.60;1.25)	0.91 (0.61;1.36)	1.00 (0.73;1.39)	Reference	0.39 (0.17;0.90)	1.00 (0.47;2.13)	1.65 (0.85;3.23)
Full model[b]	Reference	0.90 (0.62;1.30)	0.90 (0.60;1.36)	0.97 (0.70;1.34)	Reference	0.38 (0.16;0.88)	0.98 (0.46;2.09)	1.64 (0.83;3.22)
Full model+dose[c]	NA.	NA	NA	NA	Reference	0.38 (0.16;0.88)	0.98 (0.46;2.09)	1.64 (0.83;3.21)
***ABCB1* haplotype**								
TTT vs CGC[d]	Ref. (66)	1 TTT (74)	TTT-TTT (29)	Recessive	Ref. (13)	1 TTT (13)	TTT-TTT (9)	Recessive
Crude	Reference	0.82 (0.58;1.16)	1.01 (0.63;1.62)	1.14 (0.74;1.74)	Reference	0.78 (0.36;1.69)	2.06 (0.82;5.18)	2.36 (1.03;5.41)
Full model[b]	Reference	0.85 (0.60;1.19)	1.00 (0.62;1.61)	1.11 (0.73;1.70)	Reference	0.74 (0.34;1.61)	2.00 (0.79;5.05)	2.36 (1.02;5.45)
Full model+dose[c]	NA..	NA	NA	NA	Reference	0.74 (0.34;1.61)	2.00 (0.79;5.05)	2.36 (1.02;5.45)

a Hazard ratio per genotype group stratified by digoxin use. Homozygous major allele groups are the referent.

b Full model: sex, time dependent age, td diabetes mellitus, td heart failure, td myocardial infarction

c (Users only) Full model 1 + digoxin dose at index date.

d Homozygous CGC-CGC subjects are the referent, non-TTT haplotype alleles are entered as dummy variables

Table 3 QTc difference in msec using a four-level *ABCB1* genotype-digoxin use interaction dummy variable in a dominant genetic model

	QTc increase msec (95% CI) by interaction dummy[b]			
	Dummies non-digoxin users		Dummies digoxin users	
	Genotype (N[a])		Genotype (N[a])	
C1236T	CC (1623)	CT + TT (3606)	CC (67)	CT + TT (148)
Crude	Reference	0.1 (-1.1;1.4)	-4.9 (-10.1;0.2)	-12.6 (-16.1;-9.0)
Full model[c]	Reference	0.2 (-1.0;1.4)	-10.7 (-15.8;-5.6)	-18.1 (-21.6;-14.6)
G2677T	GG (1497)	GT + GT (3445)	GG (60)	GT + GT (140)
Crude	Reference	0.1 (-1.2;1.4)	-4.1 (-9.5;1.4)	-13.1 (-16.7;-9.4)
Full model[c]	Reference	0.1 (-1.2;1.3)	-9.8 (-15.2;-4.4)	-18.9 (-22.5;-15.2)
C3435T	CC (1116)	CT + TT (4022)	CC (54)	CT + TT (155)
Crude	Reference	0.0 (-1.4;1.4)	-4.6 (-10.3;1.1)	-12.6 (-16.2;-9.1)
Full model[c]	Reference	0.2 (-1.2;1.6)	-10.1 (-15.7;-4.5)	-18.1 (-21.6;-14.6)
TTT vs CGC[e]	CGC-CGC (808)	TTT carriers (3206)	CGC-CGC (40)	TTT carriers (131)
Crude	Reference	-0.1 (-1.4;1.3)	-4.5 (-10.4;1.4)	-11.6 (-15.9;-7.3)
Full model[c]	Reference	0.0 (-1.3;1.3)	-10.2 (-16.0;-4.4)	-17.0 (-21.1;-12.8)

a Number of unique individuals

b QTc difference in msec. Homozygous major allele group without digoxin is the referent.

c Full model: adjusted for: age, sex, heart failure, myocardial infarction and diabetes mellitus at time of ECG

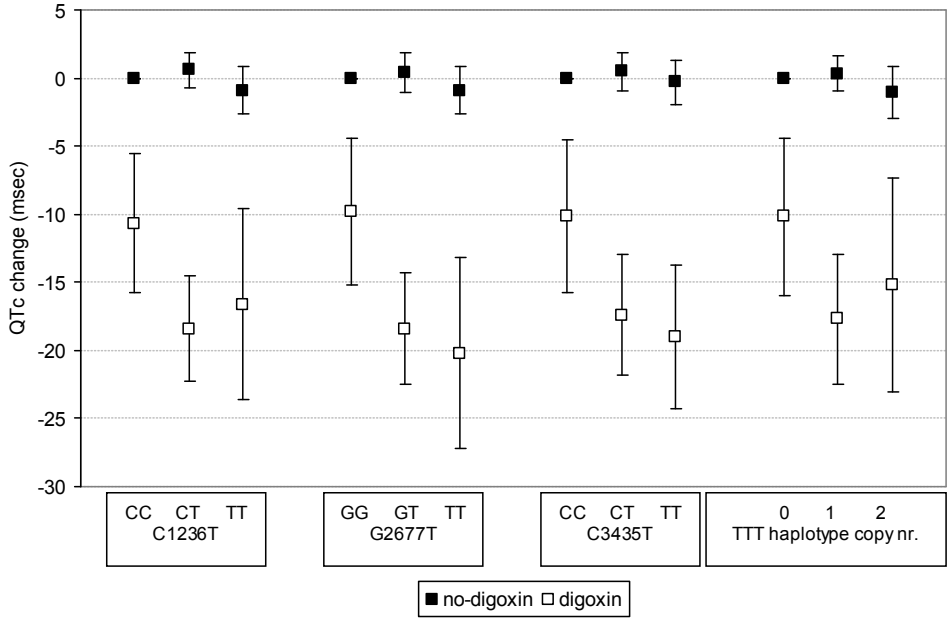
d Homozygous CGC-CGC subjects are the referent, non-TTT haplotype alleles are entered as dummy variables

type and haplotype frequencies were consistent with other European populations.[32, 34-36]

Sudden cardiac death. During the study period, we identified 229 SCD cases 121 of which were witnessed, and 40 of which were exposed to digoxin at time of death. Digoxin use resulted in a substantial SCD risk increase (HR 5.1 95%CI 3.7;7.2). However, there may be influence of confounding by the cardiac conditions (especially heart failure) that are the indication for digoxin. The hazard ratio decreased to 2.9 (95%CI 2.0;4.1) after adjustment for sex, smoking and time dependent age, diabetes, myocardial infarction and heart failure.

In digoxin users, both the C1236T and G2677T variants showed a two-fold increased risk of SCD according to a recessive model, with a HR of 2.11 (95%CI 1.03-4.31) and 2.20 (95%CI 1.08-4.50) respectively (Table 2). Unexpectedly, this effect was absent for the C3435T SNP, which results even suggested a protected effect for heterozygotes. However, digoxin users homozygous for the 1236-2677-3435 T-T-T haplotype allele also had an increased risk of SCD 2.36 (95%CI 1.02-5.45). We therefore believe that the observed protective effect for 3435CT digoxin users is a chance finding introduced by the small number of cases. Other haplotypes were too rare to assess their effect on SCD risk. In the stratum of non-digoxin users, no effect of *ABCB1* on SCD was observed. In the sensitivity analyses restricted to witnessed SCD cases, similar effects with slightly higher hazard ratios were found (recessive HR (95%CI) for C1236T 2.70 (1.06;6.90), G2677T 2.76 (1.09;6.97) and TTT haplotype 2.94 (0.91;9.49)).

Figure 1 QTc change by *ABCB1* genotype and digoxin use



QTc change in msec using six-level *ABCB1* genotype-digoxin interaction dummy variables. For each SNP, digoxin users homozygous for the major allele are the referent. For the TTT haplotype, digoxin users homozygous for the CGC haplotype are the referent. Black squares indicate non-digoxin users, open squares indicate non-digoxin users. Vertical bars indicate 95% confidence intervals.

QTc interval duration. In the total study population, ECGs taken while subjects were using digoxin showed a shorter QTc interval. The average QTc-interval in digoxin users was 9.5 (95%CI 6.9;12.1) msec shorter than in non-users. After adjustment for age, sex, heart failure, diabetes mellitus and myocardial infarction it was 15.7 (95%CI 13.0;18.3) msec shorter. Within the stratum of digoxin users, the effect of digoxin dose was 11.2 (95%CI 2.7;19.6) msec shortening per 0.5 DDD increase. T-variant carriers in the single SNP analyses and TTT haplotype allele carriers showed increased QTc shortening to digoxin. The effect appeared to be in accordance with a dominant genetic model (Figure 1 and Table 3).

We identified 80 persons starting on digoxin between ECGs, 24 persons discontinuing digoxin between ECGs and 11 subjects who started and later stopped digoxin therapy during the study period. On average, the QTc interval shortening to digoxin in starters and stoppers was larger among *ABCB1* T-allele and TTT-haplotype allele carriers in a in an allele-dose dependent way (1236 TT –24.9 msec versus CC 0.6 msec p-trend= 0.02, 2677 TT –25.5 msec versus GG –1.2 msec p-trend= 0.03, 3435 TT –22.0 versus CC –2.3 msec p-trend= 0.03 and TTT-TTT –22.0 msec versus –7.7 msec p-trend =0.17).

Discussion

In the present study, we found a more than two fold increased risk of SCD in digoxin users homozygous for the variant alleles of *ABCB1* C1236T and G2677T SNPs and the associated 1236-2677-3435 T-T-T haplotype allele. No increase of SCD risk was seen for digoxin users with the *ABCB1* C3435T variant nor in non- users of digoxin with *ABCB1* variant alleles. The interaction of *ABCB1* and digoxin was further confirmed by its influence on the QTc shortening properties of digoxin on ECG.

Digoxin is a known substrate of *ABCB1*.However, most studies on the effect of *ABCB1* SNPs on digoxin kinetics were small and the results were not unequivocal.[12]. As we previously demonstrated, *ABCB1* variants are associated with increased serum digoxin concentrations, as well as with an increased risk of concentrations exceeding the therapeutic range in chronic users in the Rotterdam Study.[13] In addition, some studies suggest that even higher digoxin serum concentrations that are still within the generally accepted therapeutic range may increase mortality.[5] *ABCB1* variant allele carriers might therefore be more susceptible to digoxin intoxication and to its pro-arrhythmogenic effects, explaining the observed interaction of *ABCB1* variants on SCD risk in digoxin users. The fact that we do not see a clear effect for the C3435T variant can be explained both by lack of power and our previous observation that (at least in our population) this variant showed the weakest association with digoxin serum concentration.[13] Also, because of its higher minor allele frequency, the C3435T variant occurs more often in haplotypes lacking the other two SNPs and a previous study shows that the C3435T variant only results in altered *ABCB1* function in combination with one of the other two variants.[37] Since we previously observed an additive effect of *ABCB1* T-alleles on digoxin concentration, it is striking that the effect of *ABCB1* on SCD appears to fit a recessive genetic model, whereas the effect on QTc follows a dominant pattern. A possible explanation is that different threshold digoxin concentrations may be needed for the different digoxin effects. Digoxin induced SCD may occur beyond a certain threshold concentration which is only reached in homozygous subjects resulting in an apparent recessive model. The effect of digoxin concentration on QTc shortening may already occur at a lower serum concentration and be limited to a maximum effect size resulting in an apparent dominant effect.

One major advantage in our study was the availability of up to four ECGs per subject at regular intervals during follow-up, resulting in more precise and unbiased long-term ECG measures. Furthermore, the use of digital ECG recordings all measured using the MEANS system likely reduced differential misinterpretation of the QTc interval. In addition, the intersection of the Rotterdam Study with detailed pharmacy exposure data allowed us not only to determine digoxin use at time of ECG or death, but also to exclude ECGs recorded in individuals on QT prolonging drugs, which could have attenuated the power to detect the association. Although no information on short QT syndrome cases was available, this is a rare condition and the number of relatives in the Rotterdam Study is low, the cohort is of older age and these syndromes are not known to be linked to *ABCB1* variants, which makes this an unlikely explana-

tion for our results. Another advantage of the Rotterdam Study is the prospective ascertainment of risk factors and the active surveillance for SCD events over a relatively long period of follow-up. Thus, extensive information surrounding SCD events was available, including the time between start of symptoms and death, enabling rigorous adjudication of SCD events.
The prospective design of the Rotterdam Study limits chances of selection or information bias and the multivariate models show that confounding is probably limited. However, although we stratified on digoxin use and so far *ABCB1* is not known to be associated with atrial fibrillation, cardiovascular disease or heart failure, we cannot fully exclude confounding by the underlying disease. However, the results from the ECG analyses make an effect of the underlying disease less likely as heart disease is associated with increased QTc duration while we observe more QTc shortening to digoxin in *ABCB1* variant carriers. One limitation of the study resides in the small number of exposed SCD cases. However, despite the limited power we still find a more than two-fold increase of SCD in subjects homozygous for the *ABCB1* variants in digoxin users. Another limitation lies in the variety of competing causes of abrupt death at increasing age, which may have led to misclassification of SCD events, especially in cases where death was unwitnessed. Since SCD coding was blinded to *ABCB1* genotype, this would likely have biased our study toward the null hypothesis that no effect exists. The stronger effect on SCD in analyses restricted to witnessed SCD lends some support to this hypothesis.

In conclusion, we found a two fold increased risk of SCD in digoxin users homozygous for the *ABCB1* C1236T, G2677T SNPs and the 1236-2677-3435 T-T-T haplotype allele. In addition, T-allele or TTT-haplotype allele variant carriers showed increased QTc shortening to digoxin. This is consistent with our previous finding that these common variants are associated with increased digoxin serum concentrations.[13] If this association can be reliably replicated, testing for *ABCB1* variants might further increase safe use of digoxin.

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3.3

ABCB1 (*MDR1*) gene polymorphisms are associated with neuropsychiatric adverse effects of mefloquine

Abstract

Background. Mefloquine, a drug used for treatment and prophylaxis of malaria, is known for its neuropsychiatric adverse effects. We hypothesized that neuropsychiatric adverse effects of mefloquine are associated with polymorphisms in the *MDR1*/*ABCB1* gene that encodes for the efflux pump P-glycoprotein.

Methods. The association between *MDR1* C1236T, G2677T and C3435T single nucleotide polymorphisms (SNPs) and the occurrence of neuropsychiatric adverse effects were examined in a prospective cohort study of 89 healthy white travellers taking mefloquine.

Results. Of the subjects, 27 (28%) reported neuropsychiatric adverse effects, women significantly more frequently than men. Allele frequencies of the C1236T, G2677T and C3435T polymorphisms were similar to those found in other white populations and there was no significant association between any of the individual polymorphisms and neuropsychiatric adverse effects. However, 1236TT, 2677TT and 3435TT women had a higher risk of neuropsychiatric adverse effects than the reference groups of women with heterozygous and homozygous CC or GG genotypes with odds ratios of 6.3 (95% CI 1.1;36.9), 10.5 (95% CI 1.1;100.6) and 5.4 (95% CI 1.1;30.0), respectively. The association for women homozygous for the 1236-2677-3435 TTT haplotype was even stronger (p= 0.004) than the effect of any of the individual polymorphisms. No associations with mefloquine blood levels were observed.

Conclusion. In this study, the *MDR1* 1236TT, 2677TT and 3435TT genotypes along with the 1236-2677-3435 TTT haplotype were associated with neuropsychiatric adverse effects of mefloquine in women. *MDR1* polymorphisms may play an important role in predicting the occurrence of neuropsychiatric adverse effects of mefloquine, particularly in female travellers.

Introduction

The quinolone derivative mefloquine is widely used in the treatment and prophylaxis of malaria in travellers to areas with chloroquine-resistant falciparum malaria. It has gained popularity because of its weekly dosing regimen. In the past decade, however, a number of case reports and studies on severe neuropsychiatric adverse effects of mefloquine have been reported.[1-3] Two main classes of neuropsychiatric adverse effects have been attributed to mefloquine: type I effects, consisting of mild neuropsychiatric events such as dizziness, dysphoria, lightheadedness and concentration problems occurring within 6 hours after intake and usually resolving quickly in the following days; and type II effects, including severe neuropsychiatric disorders such as acute psychosis with agitation or depression.[1, 3] Risk factors identified so far include history of seizures or psychiatric disorders, female gender and low body mass index (BMI).[4, 5] It has been suggested that the distribution volume or blood concentration of mefloquine plays a role, although no clear association between mefloquine blood level and neuropsychiatric adverse effects could be demonstrated.[5, 6]

P-glycoprotein (P-gp) is a product of the Multi Drug Resistance 1 gene/ATP-binding cassette B1 (*MDR1*/*ABCB1*), located on chromosome 7p21, and plays a role in the uptake and distribution of mefloquine. P-gp is an efflux pump for various toxins and drugs and is expressed in the intestine (regulating uptake into the systemic circulation) and the blood-brain barrier (affecting exposure to the brain).[7, 8] Mefloquine is a substrate for P-gp, but it is also an inhibitor of P-gp function.[9] A number of single nucleotide polymorphisms (SNPs) have been found to be associated with decreased P-gp expression and function,[7, 8, 10, 11] the most intensively studied SNPs being C1236T, G2677T/A and C3435T.

We examined the association between the *MDR1* SNPs C1236T, G2677T and C3435T and neuropsychiatric adverse effects in a subgroup of a cohort of mefloquine users previously studied to assess neuropsychiatric effects of mefloquine to determine whether *MDR1* genotype is associated with adverse effects.

Patients and methods

Study population. We performed a genetic re-analysis in a subgroup from a prospective cohort of mefloquine users previously studied to assess the neuropsychiatric effects of mefloquine.[2, 4] In brief, during a 1-year study period, we recruited subjects who received a prescription for mefloquine (baseline) for malaria prophylaxis at the Travel Clinic at the Institute for Tropical Diseases in the Harbour hospital, in the Rotterdam area in the Netherlands. Subjects with one or more contraindications for mefloquine or subjects who used mefloquine in the preceding two months or who had risk factors for concentration impairment (e.g. use of opioids, hypnotics or tranquilizers during the two weeks before testing or use of alcohol 4 hours before testing) were not included in the study. For the genetic analyses we further excluded subjects who refused to undergo blood sampling or who did not take 3 tablets of mefloquine

between baseline and follow-up. Subjects were followed from the time of prescription until the follow-up visit at 3-4 weeks (i.e. after 3 doses of mefloquine), but always before departure to the travel destination. All subjects received mefloquine in a dosage of 250 mg once a week. After consent was obtained to perform neuropsychiatric testing, report adverse events and provide blood, all personal identification was deleted from the data. Therefore, it was not possible to obtain further consent for this specific analysis, which was not foreseen at the time of sampling. The medical ethical committee gave permission for the genetic reanalysis of de de-identified data from the previously approved study.

Genotyping. Genomic DNA was isolated from whole EDTA blood by use of a total nucleic acid extraction kit (Roche, Basel, Switzerland) on a MagnaPure LC (Roche) according to the manufacturer's recommendations. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assays were performed by use of 5 ng of genomic DNA in a PCR volume of 25 µl containing 1x buffer (10 mmol/l Tris-HCl, pH 8.3; 1.5 mmol/l $MgCl_2$; 50 mmol/l KCl, and 0.001% [wt/vol] gelatin [Perkin-Elmer, Wellesley, Mass]), 0.2 mol/l each of the deoxynucleotide triphosphates (Roche), 1.25 U of AmpliTaq Gold (Perkin-Elmer), and 40 pmol of each forward and reverse primer. For the C1236T polymorphism, forward primer 5'-CCT GAC TCA CCA CAC CAA TG-3' and reverse primer 5'-TAT CCT GTG TCT GTG AAT TGC C-3' were used. PCR conditions were 7 min at 94^0C, followed by 35 cycles of 1 min at 94^0C, 1 min at 55^0C, and 1 min at 72^0C, and a final incubation of 7 min at 72^0C. The 370 bp PCR product was digested with HaeIII. For the G2677T polymorphism, forward primer 5'-TAG TTT GAC TCA CCT TCC CGG-3' and reverse primer 5'-GGC TAT AGG TTC CAG GCT TG-3' were used (the underlined and in bold nucleotide is a mismatch with the *MDR1* sequence, creating a restriction site in the PCR product). PCR conditions were 7 min at 94^0C, followed by 35 cycles of 1 min at 94^0C, 1 min at 57^0C, 1 min at 72^0C; and a final incubation of 7 min at 72^0C. The 218 bp PCR product was digested with BanI (New England Biolabs). For the C3435T polymorphism we used primers and PCR conditions and digestion as described previously.[12] All PCR digestion products were analyzed on agarose/Trisborate-EDTA gels with ethidium bromide.

Outcome assessment. The primary outcome was neuropsychiatric adverse effects using a broad definition that included type I complaints such as dizziness, nausea and sleeping problems.[1, 3] Subjects were asked to report the occurrence of adverse effects on the diary sheet used in previous studies.[2, 4] The diary sheet contained one question on adverse effects, with an open-ended follow-up question: 'Did you experience any adverse event(s) while using mefloquine? If yes what kind of adverse event(s)?' The answer to the question was only filled out if the subject had symptoms. Not returning the diary sheet or questionnaires was an exclusion criterion. Two independent physician-assessors classified reported adverse events as being neuropsychiatric or not. All classification was performed with blinding to genotype. We recorded neuropsychiatric side effects as present ('yes') or absent ('no') regardless of the number or severity of reported events.

In addition to the diary sheets, neuropsychiatric testing was performed at baseline and at the end of follow-up. We measured the intra-individual change in score of the Total Mood Disturbance (TMD), based on the validated Dutch shortened Profile Of Mood States (POMS).[13] The POMS is a standardised questionnaire, used to measure subjective mood. It contains 32 questions on 5 domains of feelings: tension, anger, depression, fatigue and vigor. The answers are graded on a 5-point scale ranging from 0 (not at all) to 4 (extremely). The TMD is an overall score, calculated by summing the raw scores and subtracting the score for vigor. The calculated TMD ranges from –20 to 108. An increase in TMD reflects an impaired mood state.

We measured the intra-individual change in hand-eye coordination, coding speed and sustained attention (Continuous Performance Test (CPT)), which are domains of the validated Neurobehavioral Evaluation System (NES). The NES is a series of computerized tests to provide quantitative neurobehavioural outcomes.[14, 15] A negative value in CPT, hand-eye coordination or coding speed indicates an increase in reaction time between the two measurements. Neuropsychiatric tests were not always performed at the same time of day and subjects were not restricted from smoking or drinking tea or coffee before testing.

Finally, we measured the serum concentration of mefloquine and its main carboxylic acid metabolite in blood samples collected at the end of follow-up. Fresh blood samples were centrifuged for 10 minutes (3000 rpm) within 30 minutes after collection. Serum was separated and stored at -20^{0}C until analysis. Concentrations were measured by a standardized HPLC method. The method used reversed-phase chromatography on a Xterra RP C18 column (5 µm; Waters, Milford, Mass). The mobile phase consisted of 40% acetonitrile in 50 mmol/l sodiumphosphate buffer pH 3.1. Quantification was accomplished with 2,8-bis(trifluoromethyl)-4-quinolinemethanol as the internal standard.[16] Calibration was done by calculating weighted linear regression from peak height ratios versus nominal concentration. Mefloquine and metabolite quality-control serum specimens were analyzed at concentrations of 50, 100, 200, 500, 1000 and 3000 ng/ml. The lower limits of quantification of mefloquine and metabolite were 50 ng/ml.

Covariates. All data on demographics, weight, height, previous use of mefloquine and long-term co-morbidity were gathered by use of a questionnaire at baseline. In addition, the time interval between last mefloquine dosing and follow-up visit was recorded. Body Mass Index (BMI) was calculated by dividing weight (in kg) by height (in squared meters).

Statistical analysis. Statistical analyses were carried out by use of SPSS for Windows, version 11 (SPSS, Chicago, Ill). We used standard descriptive statistics to describe population characteristics and outcome data. Comparisons between men and women were conducted by means of Student's t-tests or Chi square tests. Genotype frequency was tested against the Hardy-Weinberg equilibrium.

After crude odds ratios (ORs) for neuropsychiatric effects in the different genotype groups of

the SNPs were calculated, we decided on the genetic model (recessive, dominant or dose-effect) based on the results. Multivariate analysis was performed using a binary logistic regression model. Logistic regression analysis was chosen because time played no role in this study. Confounders were defined as covariates associated with neuropsychiatric effects at a p-value of 0.1 and changing the point estimate by 10% or more. Mefloquine concentration was tested as an intermediate by including it into the model as a covariate. In a second step, we identified effect modification by age, gender, BMI and previous mefloquine use by introducing interaction terms and stratified on these factors when statistically significant. Because the cohort size is limited and the outcome is frequent, in contrast to traditional case-control studies, ORs cannot be interpreted as relative risks.

Linear regression analysis was used to study the associations of the changes in scores on the neuropsychiatric tests and mefloquine concentrations with the genotypes, haplotypes and covariates. Time since last mefloquine dose was included in the model. Testing of confounders was similar to that for the logistic regression model. A trend test for an allele dose-effect relation of the SNP variants was obtained with the number of variant alleles entered as an ordered categorical variable.

Haplotypes were constructed using the program PHASE version 2.0.[17, 18] Linkage disequilibrium was computed using the Haploview software version 3.11.[19]

Results

Of the 151 subjects available,[4] 89 subjects (48 males and 41 females) with a mean age of 40 years, had blood samples available. The other 62 subjects refused to give a blood sample. All subjects included in the final cohort were white persons of European origin. General characteristics of the study population are presented in Table 1. Women had a significantly lower BMI compared with men (p= 0.02). Of all subjects, 37% had previously used mefloquine (Table 1). The concomitant use of drugs was higher in women than in men but this difference was mainly explained by 9 women who only used an oral contraceptive. One subject used a known P-gp inducer (acitretin, a synthetic retinoic acid analog) and one used a known P-gp inhibitor (atorvastatin). The prevalence of comorbidity was low (n=13) and included, among others, skin problems (n=2) and hypertension and other cardiovascular disease (n=5). The time interval since administration of the last mefloquine dose to follow-up ranged from 0 days (i.e., same day) to 7 days (i.e., just before fourth dose), with a median of 2 days.

Prevalence of SNPs. Frequencies of all three SNPs were consistent with other studies in Caucasian populations. For the C1236T the frequencies were CC 29.8%, CT 50.0% and TT 20.2%, for the G2677T they were GG 31.8%, GT 51.1%, TT 17.0% and for the C3435T polymorphism CC 22.6%, CT 51.2%, TT 26.2%.[7, 20, 21] Genotyping failed in 5 subjects(5.6%), 1 subject (1.1%) and 5 subjects (5.6%) respectively. All SNPs were in Hardy-Weinberg equilibrium with p= 0.8 for C3435T, p= 0.7 for G2677T and p= 0.9 for C1236T. The C1236T, G2677T

Table 1 General characteristics of the study population and comparisons between males and females

Characteristic	Males (n=48)	Females (n=41)
Age (years) mean, range	41.0 (21 – 68)	38.2 (18 – 59)
BMI (kg/m^2)[ab]	24.7 (19.0 - 33.0)	23.1 (18.3 - 31.3)
≤ 25	28 (59.6 %)	31 (75.6 %)
> 25	19 (40.4 %)	10 (24.4%)
Smokers	13 (27.1 %)	13 (31.7 %)
Concomitant drug	7 (14.6 %)	15 (36.6 %)
Previous mefloquine users	21 (43.8 %)	12 (29.3%)
Neuropsychiatric adverse events	9 (18.8 %)	16 (39.0 %)
Delta TMD[b]	-0.4 (-13 – 21)	5.3 (-16 – 48)
Delta hand-eye coordination (log RMSE)[b]	-0.19 (-0.64 – 1.04)	-0.23 (-0.69 – 0.55)
Mefloquine blood level (µg/ml)[b]	0.59 (0.31 - 1.14)	0.75 (0.37 - 1.51)

Statistically significant differences between males and females are printed in bold ($p \leq 0.05$), BMI: Body Mass Index, TMD: Total Mood Disturbance

a Numbers do not add up due to missing data on weight and height.

b Mean, range

and C3435T SNPs were found to be in linkage disequilibrium. D' for C3435T-G2677T was 0.87, for C3435T-C1236T and G2677T-C1236T it was 0.83 and 0.95 respectively. Construction of haplotypes, via estimation maximization, resulted in two frequent (1236-2677-3435 CGC and TTT) and four minor haplotypes; the other two theoretically possible haplotypes were not found in this population. Frequencies of the different haplotypes were: 1236-2677-3435 CGC 45.5%, TTT 38.6%, TTC 2.8%, CGT 9.1%, TGT 2.8% and CTT 1.1%.

Neuropsychiatric adverse effects. During the study period, 16 women (39.0%) and 9 men (18.8%) reported neuropsychiatric complaints. The most frequently reported adverse effects were insomnia or abnormal dreams (or both) (n= 10), fatigue (n= 8), headache (n= 8), dizziness (n= 5) and agitation, emotional instability or depression (n= 5). Women reported significantly more neuropsychiatric complaints then men (p = 0.03) and a higher change in TMD score (p =0.03) (Table 1).

SNPs and neuropsychiatric adverse effects. On the basis of the genotype-specific results we decided to use a recessive model for further analyses. There was no significant association between any of the SNPs and neuropsychiatric adverse effects in the combined analyses. In the gender-stratified analyses, women homozygous for the TT-variant of any of the three SNPs had a significantly higher risk of adverse effects from mefloquine than the reference groups (i.e., the combined groups of CT and CC [for C1236T and C3435T] or GT and GG [for G2677T]), with ORs of 6.3, 10.5 and 5.4 for C1236T, G2677T and C3435T, respectively (Table 2). In men, no significant association with these SNPs was seen. Although the interaction term for previous use was not significant, we stratified on previous use to explore potential

Table 2 Genotypes of individual SNPs and neuropsychiatric adverse events

	Whole cohort			Males			Females		
Genotype	**Cases**	**Total**	**OR (95% CI)**	**Cases**	**Total**	**OR (95% CI)**	**Cases**	**Total**	**OR (95% CI)**
C1236T									
CC/CT	17	67	Reference	7	36	Reference	10	31	Reference
TT	8	17	2.6 (0.9;7.9)	2	9	1.2 (0.2;7.0)	6	8	6.3 (1.1;36.9)
G2677T									
GG/GT	18	73	Reference	7	39	Reference	11	34	Reference
TT	7	15	2.7 (0.9;8.4)	2	9	1.3 (0.2;7.7)	5	6	10.5 (1.1;100.6)
C3435T									
CC/CT	16	62	Reference	7	32	Reference	9	30	Reference
TT	9	22	2.0 (0.7;5.5)	2	12	0.7 (0.1;4.0)	7	10	5.4 (1.1;30.0)

Numbers do not ad up to 89 due to failing of genotyping in some subjects

Table 3 Haplotypes and neuropsychiatric adverse events

	Whole cohort			Males			Females		
Haplotype	**Cases**	**Total**	**OR (95% CI)**	**Cases**	**Total**	**OR (95% CI)**	**Cases**	**Total**	**OR (95% CI)**
1236-2677-3435									
non TTT-TTT	18	76	Reference	7	41	Reference	11	35	Reference
TTT-TTT	7	12	4.5 (1.3;16.0)	2	7	1.9 (0.3;12.1)	5	5	∞ (p= 0.004)

Numbers do not ad up to 89 due to failing of genotyping in some subjects

confounding by contraindication. This revealed that the higher risks for adverse effects in the TT genotypes for the three SNPs was predominant in new users of mefloquine (results not shown). There was no effect modification by age or BMI. In multivariate analyses, no significant confounders were identified. Forcing age or BMI into the model resulted in only minor changes of the estimates. When mefloquine concentration was tested in the model as an intermediate this did not diminish the genotype effect.

Haplotypes and neuropsychiatric adverse effects. In the haplotype-based analyses, the homozygous 1236-2677-3435 TTT genotype showed a higher risk of neuropsychiatric adverse effects (OR 4.5, 95% CI 1.3;16.0) than was found in the reference group containing all other combinations of haplotypes (Table 3). This risk was higher than in any of the individual SNPs. Further stratification for gender resulted in a significantly higher risk for 1236-2677-3435 TTT-TTT women (p= 0.004; OR could not be calculated as all women homozygous for the TTT-variant had neuropsychiatric adverse effects). Again, we did not find a significant association in men. Being infinite, the OR for the TTT-TTT genotype in women was also higher than the OR for any of the individual SNPs.

SNPs and neuropsychiatric tests. We found no significant association between any of the neuropsychiatric tests and individual SNPs or haplotypes. However, increases in TMD values tended to be higher in subjects with TT-genotypes and those with homozygous TTT haplotypes than in the reference groups. This was most distinct for the C1236T and C3435T SNPs, with scores of 1.6 in the 1236CC/CT group, 6.0 in the 1236TT group (p= 0.18), 1.0 in the 3435CC/CT group and 5.1 in the 3435TT group (p= 0.17). On the hand eye coordination test, subjects with TT-genotypes and those with homozygous TTT haplotypes showed less improvement (i.e. less learning effect) than the reference groups. Again this effect was most obvious in the C1236T and C3435T SNPs (p= 0.16 and p= 0.09, respectively).
Forcing in age and BMI in the multivariate model did not significantly alter the results. No differences in coding speed and continuous performance were observed between the T-variants and other variants of the three SNPs.
Given the SDs of the tests and the frequencies of the SNPs and haplotypes, the smallest difference (irrespective of the direction of the difference) between groups that we were able to detect with 90% power was 9.7 for change in TMD (largest true difference, 4.4). For change in hand-eye-coordination it was 0.239sec (largest true difference, 0.122sec).

SNPs and mefloquine serum concentrations. Serum concentrations of mefloquine and its carboxylic metabolite were on average 0.67 µg/ml and 1.35 µg/ml. Women had significantly higher concentrations of the unchanged form (p=0.001). There was no association between serum concentrations of mefloquine or its metabolite and any of the investigated *MDR1* SNPs (Table 4). In addition we did not observe an association of serum levels with neuropsychiatric adverse effects.

Table 4 Mefloquine serum concentrations (µg/ml) by gender and genotype

	Males		Females	
Genotype	**µg/ml**	**t-test p-value**	**µg/ml**	**t-test p-value**
C1236T				
CC/CT	0.611	0.229	0.728	0.076
TT	0.518		0.889	
G2677T				
GG/GT	0.608	0.260	0.736	0.115
TT	0.523		0.894	
C3435T				
CC/CT	0.597	0.678	0.731	0.166
TT	0.568		0.846	
Haplotype				
non TTT-TTT	0.604	0.314	0.739	0.114
TTT-TTT	0.520		0.910	

Interpretation of mefloquine serum concentrations is complicated by differences in sampling time on the pharmacokinetic curve, resulting in non-steady state measurements

For the largest observed difference in mefloquine concentration, found in women homozygous for the TTT haplotype (0.171 µg/ml), the minimal difference (irrespective of the direction) to be identified with 90% power was 0.359 µg/ml.

Discussion

In this study, the C1236T, G2677T and C3435T *MDR1* polymorphisms were identified as risk factors for neuropsychiatric adverse effects in female mefloquine users. A haplotype-based analysis showed even more pronounced results.

Mefloquine is a substrate for the *MDR1* multidrug transporter and is expressed in the gut where it may play a role in the uptake of the drug, as well as in the liver where it is involved in the excretion of mefloquine in the bile.[8] Although our data on serum concentration should be cautiously interpreted because of differences in sampling time, we were not able to demonstrate an association of the three *MDR1* SNPs with mefloquine or metabolite concentrations and, as in literature reports, found no association between adverse effects of mefloquine and serum concentration.[5, 6]

Given that mefloquine, unlike its carboxylic acid metabolite, is able to cross the blood-brain barrier[22] and P-gp is expressed in the blood-brain barrier,[8] *MDR1* expression could modify the local mefloquine brain tissue concentration. Our finding that mefloquine serum concentration was not an intermediate in the association between genotype-haplotype and neuropsychiatric effects may further support this hypothesis. This might explain the association we found between the T-variants of the *MDR1* polymorphisms and the occurrence of neuropsychiatric

adverse effects of mefloquine: a lower expression of P-gp will result in lower mefloquine efflux from the brain, thus exposing it to higher tissue concentrations. Similar effects of *MDR1* polymorphisms have recently been shown for other drugs.[23, 24]

The effect of *MDR1* SNPs on neuropsychiatric functioning was almost exclusively found in women. We cannot exclude that this difference is explained by a greater awareness of disturbances in neuropsychiatric functioning by women. The fact that a greater vulnerability to neuropsychiatric effects in women than in men when taking mefloquine is genuine was demonstrated in a double-blind placebo-controlled trial comparing atovaquone plus chloroguanide (INN, proguanil) with mefloquine.[25] Possibly, a higher serum concentration (Table 1) of mefloquine in combination with a lower basal expression of *MDR1*[26] makes women more susceptible to the subtle effects of *MDR1* polymorphisms on its expression and functioning.

In line with several other studies, strong associations were found for the C3435T SNP,[7, 11, 23, 24, 27-29] although some other studies suggested an opposite or absent effect of this polymorphism.[10, 30-33] C3435T and the C1236T are synonymous SNPs, which do not result in an amino acid change in P-gp. A recent study showed that the C3435T T-variant affects mRNA stability,[34] which might result in lower P-gp expression. This might explain the association of the C3435T as a causal SNP for neuropsychiatric adverse effects. However, we found that the association of the 2677TT genotype with neuropsychiatric adverse effects was the strongest of the three individual SNPs, suggesting also a causal role for this amino acid changing (Ala893Ser) SNP. Furthermore, the association of the TTT-TTT haplotype with adverse effects was even stronger than with the 2677TT genotype alone. This could be a result of an additive effect of the G2677T and the C3435T SNPs. In addition, it cannot be excluded that other, unknown causal SNPs associated with the 1236-2677-3435 TTT haplotype might play a role in the association with neuropsychiatric adverse effects.

A possible limitation of the study is the limited number of subjects. Especially after stratification, the numbers in the different genotype groups became small which increases the risk of a false-positive result. The small number of subjects, in combination with large SDs, also resulted in low power, which might explain why no significant association with neuropsychiatric tests or mefloquine concentration was found. A second limitation may reside in the subjective reporting of our main endpoint. However, because the reporters were unaware of their genotype, this could not have biased our results. The fact that the objective neuropsychiatric tests were not very strongly correlated with the adverse effects might be because the tests are restricted to specific domains of brain function, whereas adverse effects are not. Furthermore, in our previous study using the larger cohort a significant association of adverse effects with TMD was seen.[2] In addition, results from the neuropsychiatric tests suggested impairment during mefloquine use and endorse the observed association with neuropsychiatric adverse effects. Because the *MDR1* C1236T, G2677T and C3435T polymorphisms are randomly distributed among the population at birth and are not known to be associated with the outcomes or covariates, no strong confounding effects or selection bias is expected to affect the association. However, the theoretical possibility of an association between *MDR1* variants and

neuropsychiatric events independent of mefloquine use cannot be entirely excluded in our study of mefloquine users. Finally, in different ethnic groups other SNPs may be linked with the 1236-2677-3435 haplotypes and frequencies of the different SNPs and haplotypes may differ. It is therefore unknown whether the results from this study can be generalized to other ethnic groups.

In conclusion, we found that the *MDR1* C1236T, G2677T and C3435T SNPs, along with the C1236T-G2677T-C3435T TTT haplotype, were associated with neuropsychiatric adverse effects to mefloquine in women. *MDR1* polymorphisms may therefore play an important role in predicting the occurrence of neuropsychiatric adverse effects of mefloquine, particularly in female subjects.

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4

Common variants in the *NOS1AP* gene

4.1

Common *NOS1AP* variants are associated with a prolonged QTc interval in the Rotterdam Study

Abstract

Background. QT prolongation is an important risk factor for sudden cardiac death (SCD). About 35% of QT interval variation is heritable. In a recent genome-wide association study, a common variant (rs10494366) in the Nitric Oxide Synthase 1 Adaptor Protein (*NOS1AP*) gene was found to be associated with QT-interval variation. We tested for association of two *NOS1AP* variants with QT duration and SCD.

Methods. The Rotterdam Study is a population based, prospective cohort study of individuals ≥ 55 years of age. The *NOS1AP* variants rs10494366 T>G and rs10918594 C>G were genotyped in 6571 individuals. Heart rate corrected QT interval (QTc) was determined with ECG analysis software on up to three digital ECGs per individual (total 11,108 ECGs from 5374 individuals). The association with QTc duration was estimated with repeated-measures analyses and the association with SCD was estimated by Cox proportional-hazards analyses.

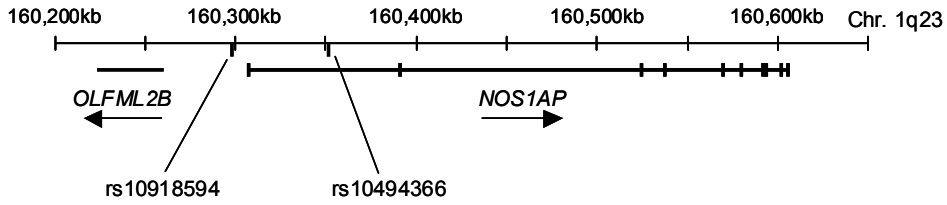
Results. The rs10494366 G-allele (36% frequency) was associated with a 3.8 msec (95%CI 3.0;4.6, $p= 7.8x10^{-20}$) increase in QTc-interval duration for each additional allele copy, and the rs10918594 G-allele (31% frequency) with 3.6 msec (95%CI 2.7;4.4, $p= 6.9x10^{-17}$) increase per additional allele copy. None of the inferred *NOS1AP* haplotypes showed a stronger effect than the individual SNPs. There were 233 sudden cardiac deaths over 11.9 years median follow-up. No significant association was observed with SCD risk.

Conclusions. Common variants in *NOS1AP* are strongly associated with QT-interval duration in an elderly population. Larger sample sizes are needed to confirm or exclude an effect on SCD risk.

Introduction

Sudden cardiac death (SCD) claims 300,000 lives annually in the US.[1] While certain high-risk groups have been identified,[2] most SCD occurs in individuals unrecognized to be at risk.[3]
Familial aggregation of SCD suggests a substantial contribution of genetic variation to SCD risk,[4-7] but Mendelian mutations identified to date individually explain little of the population burden of SCD.[8, 9] Until recently the search for sequence variants contributing to SCD risk has been restricted to candidate genes known for their role in arrhythmogenesis.[10] The recent development of large SNP databases,[11] genotyping arrays of great accuracy and genome wide coverage of common variation,[12] together with analytical methods,[13] has enabled unbiased surveys of most of the common variation in the human genome. Still, the relatively small size of existing SCD collections and etiologic heterogeneity limit the statistical power to detect causal variants; therefore, initial attention has focused on quantitative SCD risk factors in large cohorts.
The electrocardiographic QT interval is a non-invasive measure of ventricular repolarization. About 35% of the variation in QT interval duration in unselected community-based samples is heritable.[14, 15] Mendelian congenital long- and short QT syndromes (LQTS, SQTS) are both characterized by SCD from ventricular arrhythmias. Moreover, non-syndromal long QT interval[16-19] and short QT interval[20] impart increased risk of SCD in unselected populations. In addition, medication-induced prolonged QT interval and ventricular arrhythmias have led to the withdrawal of many non-cardiac medications,[21] making the QT interval an important phenotype to study.
Previously, we identified a locus on chromosome 3 with suggestive evidence of linkage to QT interval duration, but the genomic interval was large and the finding remains to be confirmed.[15] More recently, Arking et. al.[22] reported the finding from a genome-wide association study that a common variant (rs10494366, minor allele frequency 38%) in the *NOS1AP* gene was reproducible associated with QT interval variation in several large population samples. The *NOS1AP* gene, encoding the Nitric Oxide Synthase 1 Adaptor protein, has been found to regulate neuronal nitric oxide synthase (NOS1) activation[23] and to enhance Dexras1 activation by *NOS1* through a ternary complex.[24] *NOS1* knockout mice have been found to have altered cardiac contractility suggesting a role for *NOS1AP* in cardiac depolarization.[25-27] Furthermore, *NOS1AP* is capable of interaction with ion channels through its PDZ domain.[28-30] Nevertheless, the involvement of *NOS1AP* in myocardial repolarization was not known until the initial report of the association.
The impact of *NOS1AP* variants on QT-interval duration in older populations, in whom non-genetic factors might play a stronger role than heritable factors, is unknown.
The goal of the present study was to test for association of the *NOS1AP* variant with QT duration and to test for its association with SCD in the Rotterdam Study.

Figure 1 *NOS1AP* and location of rs10494366 and rs10918594



The ruler indicates the physical position on chromosome 1. Thick horizontal lines indicate genes in the region with *NOS1AP* exons as thick vertical lines, the arrows indicate direction of transcription. Thick vertical lines on the ruler indicate the positions of rs10918594 and rs10494366, approximately 55kb apart. The two SNPs were in linkage disequilibrium with an r^2 of 0.63 and D' of 0.89.

Methods

Study population. The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly. All inhabitants of Ommoord, a Rotterdam suburb, aged 55 years and older (10,278), were ascertained from the municipal register and invited to participate. Of them, 78% (7983, 58% female and 98% white) took part in the baseline examination from March 1990 through July 1993. Second and third examinations were conducted from September 1993 to August 1996 and from April 1997 to December 1999, respectively. Objectives and methods of the Rotterdam Study have been described in detail.[31] The medical ethics committee of Erasmus Medical Center (Rotterdam, The Netherlands) approved the study and all participants provided signed informed consent for participation, including retrieval of medical records, use of blood and DNA for scientific purposes, and publication of data. DNA for genotyping is available for 6571 (82%) participants from the baseline visit.
Clinical characteristics including smoking, body mass index (BMI), hypertension, diabetes mellitus, heart failure and myocardial infarction were ascertained as previously described.[19, 32-36] Active surveillance for incident diabetes mellitus, heart failure and myocardial infarction is conducted continuously between exams. In addition, exposure of study participants to medications has been gathered continuously from January 1st, 1991 to the present through computerized pharmacy records of the pharmacies in the study area.

Genotyping. All participants were genotyped for the *NOS1AP* SNP SNP rs10494366 T>G, previously shown to be associated with QT interval in 3 independent samples.[22] The correlated SNP rs10918594 C>G, which had evidence of association with QT interval in one of the original samples,[22] was also genotyped (Figure 1). Both were genotyped using Taqman assays C_1777074_10 and C_1777009_10 (Applied Biosystems, Foster City, Calif., USA) in 1 ng of genomic DNA extracted from leukocytes, as previously reported.[37]

Assessment of QTc interval and other ECG measurements. The electrocardiography (ECG) phenotype studied was the heart rate corrected QT-interval (QTc) in milliseconds, using Bazett's formula (QTc = QT / √RR).[38] As in previous studies of QTc in the Rotterdam Study[19] we used a 10-second resting 12-lead ECG (average of 8 to 10 beats), which was recorded on an ACTA electrocardiograph (ESAOTE, Florence, Italy) at a sampling frequency of 500 Hz and stored digitally. All ECGs were processed by the Modular ECG Analysis System (MEANS) to obtain ECG measurements.[39-41] MEANS determines the QT interval from the start of the QRS-complex until the end of the T-wave. MEANS also determines the presence of right- (RBBB) or left bundle-branch block (LBBB) and left ventricular hypertrophy. To study the association between *NOS1AP* variants and QTc duration, all eligible ECGs from subjects with DNA available were used. ECGs with RBBB or LBBB were excluded from the analyses. In addition, to minimize confounding by non-genetic influences on QT duration, all ECGs taken while the subject was on any QT altering drugs were excluded from analyses. Drugs were considered possibly QT prolonging if they appeared on any of lists 1 through 4 at www.qtdrugs.org.[42] We also excluded ECGs if subjects were on flupenthixol, levomepromazine, mefloquine, olanzapine or sertindole, which may prolong QT interval, or on digoxin, which shortens the QT interval. Up to 3 QTc measurements were recorded across the three examination cycles.
Finally, in additional analyses, the mean QTc interval per individual was divided into three gender-specific categories as previously described. For women, the cut points were ≤ 450 msec (normal), 451 to 470 msec (borderline) and >470 msec (prolonged); for men, the cut points were ≤ 430 msec (normal), 431 to 450 msec (borderline) and >450 msec (prolonged).[19, 43]

Adjudication of sudden cardiac death. For the SCD analyses, all genotyped subjects were included. The ascertainment of SCD cases in the Rotterdam Study has been described previously.[19] SCDs were defined operationally as a witnessed natural death attributable to cardiac causes, heralded by abrupt loss of consciousness, within one hour of onset of acute symptoms, or as an unwitnessed, unexpected death of someone seen in a stable medical condition <24 hours previously with no evidence of a non-cardiac cause.[44, 45]

Statistical analysis. Genotype frequencies were tested for Hardy-Weinberg equilibrium with a Chi-square test.
Because the QTc in subsequent ECGs of the same subject are correlated, we used repeated-measures analyses implemented in PROC MIXED (SAS 8.2, SAS Institute, Cary, NC). Both allelic and general genotype models were tested for the two polymorphisms, although the allelic model was considered primary because of the previously reported rs10494366-QT relationship.[22] Haplotypes were estimated using the estimation-maximization algorithm implemented in PHASE 2.0 (University of Washington, Seattle, USA),[46, 47] and only individuals with successful genotyping for both SNPs and a posterior probability of >0.95 for assigned haplotypes were included in haplotype analyses. In total we identified 2245 double heterozygotes, all of which were phased as heterozygous haplotype TC-GG because these are the

major haplotypes, with posterior probabilities in excess of 0.95. In haplotype analyses, the haplotype with major alleles for both SNPs was considered the reference, to which the three other haplotypes were compared individually. QTc was tested for association with genotype as the sole predictor (crude) and with adjustment for age and gender (multivariable). To compare the outcomes of haplotype analysis with individual SNP analysis, the latter analyses were also performed restricting the analysis to subjects in whom genotyping was successful for both SNPs. Finally a sensitivity analysis was carried out excluding ECGs with an abnormally prolonged QTc, using gender-specific cutoff points of >450 msec for men and >470msec for women. Jonckheere-Terpstra tests were used to test whether individuals carrying *NOS1AP* minor alleles had an increased frequency of borderline and abnormal mean QTc.
Hazard ratios for time to SCD from baseline were estimated using Cox proportional-hazards models. Again, both allelic and general genotype models were tested for the two polymorphisms. In addition to *NOS1AP* genotype, known SCD risk factors -including age, gender, BMI, smoking, hypertension, diabetes mellitus, heart failure and myocardial infarction at baseline and time dependent incident diabetes mellitus, heart failure and myocardial infarction- were included as predictors. To minimize misclassification of SCD, we additionally performed a sub-analysis restricting the case definition to witnessed deaths only. As we have previously shown, the risk of SCD for increasing QTc is stronger in the younger than in the older age group,[19] so we determined the hazard ratios for time to SCD separately in groups stratified by age above and below the median age at baseline. Finally, we performed a sensitivity analysis excluding subjects with a history of myocardial infarction at baseline from the analysis. All Cox proportional-hazards analyses were performed using SPSS for Windows version 11.0 (SPSS Inc, Chicago, Ill, USA).

Results

Study subjects. Baseline characteristics for the total study population, consisting of all genotyped subjects from the Rotterdam Study (n=6571), are summarized in Table 1. Within the study population, 12,967 ECGs were available from 6052 subjects across up to 3 examination cycles. After exclusion of ECGs with RBBB or LBBB (n= 640) and those performed in individuals taking QT prolonging or -shortening drugs (n= 1334) or both, a total number of 11,108 ECGs from 5374 subjects remained (on average, 2.1 ECGs per individual). The 5374 subjects included in the QTc analyses were 1.3 years younger at baseline, reflecting exclusions among older participants (Table 1). Women had an 8.9 msec longer age-adjusted QTc interval (431.4 msec vs. msec 422.5, p<0.0001) as has been previously shown,[38, 48] and were 2.2 years older than men (70.4 vs. 68.2 years at baseline, p<0.0001). The numbers of abnormally prolonged QTc in men and women of our study population were slightly higher than expected based on numbers from reference populations.[48, 49] However, our study population was on average already considerably older at baseline (69.5 years vs 53 years and 61 years respectively) and this mean further increased when follow-up ECGs were taken.

Table 1 Baseline characteristics of the study population

	Genotyped sample		QTc sample		SCD cases	
Characteristic	**Men**	**Women**	**Men**	**Women**	**Men**	**Women**
Number (%)	2666 (40.6%)	3905 (59.4%)	2191 (40.8%)	3183 (59.2%)	116 (49.8%)	117 (50.2%)
Age at baseline[a]	68.2 (8.2)	70.4 (9.6)	67.0 (7.7)	69.0 (9.1)	71.8 (7.8)	74.4 (7.7)
Follow-up time[a]	10.0 (3.8)	10.5 (3.7)	10.6 (3.4)	11.1 (3.2)	6.4 (3.8)	7.3 (3.8)
Smoking, number (%)						
Current	774 (29.0%)	680 (17.4%)	634 (28.9%)	582 (18.3%)	32 (27.6%)	15 (12.8%)
Past	1635 (61.3%)	1040 (26.6%)	1343 (61.3%)	872 (27.4%)	75 (64.7%)	38 (32.5%)
Body mass index (BMI)[a]	25.7 (3.0)	26.7 (4.1)	25.7 (3.0)	26.7 (4.0)	25.3 (3.0)	27.3 (3.9)
Blood pressure (mmHg)[a]						
Systolic	138.7 (21.7)	139.8 (22.6)	138.3 (21.5)	139.2 (22.2)	144.6 (24.2)	152.8 (27.7)
Diastolic	74.6 (11.5)	73.2 (11.4)	74.9 (11.3)	73.2 (11.1)	74.0 (12.5)	77.0 (14.1)
Hypertension	780 (29.3%)	1415 (36.2%)	621 (28.3%)	1102 (34.6%)	53 (45.7%)	65 (55.6%)
Diabetes mellitus	281 (10.5%)	422 (10.8%)	213 (9.7%)	309 (9.7%)	14 (12.1%)	27 (23.1%)
Myocardial infarction	447 (16.8%)	320 (8.2%)	345 (15.7%)	243 (7.6%)	44 (37.9%)	19 (16.2%)
Heart failure	81 (3.0%)	131 (3.4%)	34 (1.6%)	75 (2.4%)	17 (14.7%)	7 (6.0%)

Shown are characteristics of all individuals with DNA available for genotyping (Genotyped sample), of the subset of genotyped subjects with electrocardiograms without bundle branch block, or use of a QT-prolonging drug or digoxin (QTc sample) and of the SCD cases. The SCD source sample includes all genotyped subjects.

a Mean (standard deviation)

Table 2 Difference in QTc by *NOS1AP* genotype

	Genotypic model[a]				Allelic model[b]	
SNP and model	**Genotype**			**P-value**	**Per G-allele**	**P-value**
rs10494366 (36.4% MAF)	TT	TG	GG			
Number of subjects[c]	2100	2334	704		5138	
Crude	Reference	4.2 (3.0;5.5)	7.1 (5.3;8.9)	$2.2x10^{-17}$	3.7 (2.9;4.6)	$3.3x10^{-18}$
Age and gender adjusted	Reference	4.2 (3.0;5.4)	7.2 (5.5;9.0)	$5.9x10^{-19}$	3.8 (3.0;4.6)	$7.8x10^{-20}$
rs10918594 (31.4% MAF)	CC	CG	GG			
Number of subjects[c]	2456	2217	530		5203	
Crude	Reference	4.3 (3.1;5.5)	6.4 (4.4;8.3)	$1.7x10^{-15}$	3.6 (2.7;4.5)	$6.9x10^{-16}$
Age and gender adjusted	Reference	4.3 (3.2;5.5)	6.3 (4.4;8.2)	$1.5x10^{-16}$	3.6 (2.7;4.4)	$6.9x10^{-17}$

MAF = minor allele frequency, Values are difference from reference group (95% confidence interval) in msec.

a Linear regression model using dummies per genotype

b Linear regression model entering genotype as an ordinal variable

c Due to failures in genotyping for the individual SNPs, numbers do not add up to the total of 5374 individuals

Table 3 Number of individuals with normal, borderline and abnormal mean QTc per genotype group using gender specific cutpoints

	Number of individuals (% within genotype)			
Genotype	**Normal**	**Borderline**	**Abnormal**	**Test for trend**
rs10494366				P < 0.0001
TT	1679 (80.0%)	329 (15.7%)	92 (4.4%)	
TG	1715 (73.5%)	447 (19.2%)	172 (7.4%)	
GG	498 (70.7%)	144 (20.5%)	62 (8.8%)	
rs10918594				P < 0.0001
CC	1945 (79.2%)	390 (15.9%)	121 (4.9%)	
CG	1609 (72.6%)	448 (20.2%)	160(7.2%)	
GG	385 (72.6%)	96 (18.1%)	49 (9.2%)	

QTc interval divided into three gender specific categories. For women, the cutpoints were ≤450 msec (normal), 451 to 470 msec (borderline) and >470 msec (prolonged), and for men ≤430 msec (normal), 431 to 450 msec (borderline) and >450 msec (prolonged).[19, 43]

Genotyping. The G-allele (minor) frequency of rs10494366 T>G was 36.4% and of rs10918594 C>G was 31.4%. Successful genotype calls were made in 95.8% and 95.9% of subjects, respectively. Both SNPs were in Hardy-Weinberg equilibrium (p= 0.32 for rs10494366 and p= 0.89 for rs10918594). The two SNPs were in linkage disequilibrium with an r^2 of 0.63 and D' of 0.89. Upon phasing, we observed two common two-SNP haplotypes: TC (61.4%) and GG (29.1%), consisting of the two major and two minor alleles, respectively, and two remaining haplotypes containing one major and one minor allele each: GC (7.2%) and TG (2.3%). Genotype distributions did not differ between men and women and between quartiles of age at baseline.

***NOS1AP* polymorphisms and QTc.** Minor alleles of both *NOS1AP* SNPs were significantly associated with an increase in QTc duration. SNP rs10494366 T>G was associated with a 3.8 msec increase in multivariable-adjusted QTc interval for each additional G-allele, and SNP rs10918594 C>G was associated with a 3.6 msec increase per additional G-allele (Table 2). Additional adjustment for electrocardiographic left ventricular hypertrophy did not alter the results (data not shown). We observed no difference in effect of the SNPs between men and women. A sensitivity analysis excluding ECGs with an abnormally prolonged QTc (using gender-specific cut points) resulted in slightly lower estimates (2.9 and 2.7 msec for the allelic models); however the association of *NOS1AP* genotypes with QTc duration remained highly significant (all $p<10^{-11}$).

All three haplotypes containing one (GC and TG) or two (GG) minor alleles for the two SNPs were associated with increased QTc compared with the homozygous TC reference haplotype. The GG haplotype was associated with a 4.1msec longer multivariable-adjusted QTc per ad-

Table 4 Hazard ratio of all adjudicated sudden cardiac death and witnessed sudden cardiac death per *NOS1AP* genotype or allele

	All sudden cardiac death					
	Genotypic model[a]				Allelic model[b]	
SNP and model	**Genotype (number of SCD cases)**			**P-value**	**Per G-allele**	**P-value**
rs10494366	TT (n=90)	TG (n=95)	GG (n=36)			
Crude	Reference	0.97 (0.72;1.30)	1.26 (0.85;1.87)	0.41	1.08 (0.89;1.32)	0.42
Full model	Reference	0.99 (0.74;1.33)	1.27 (0.85;1.89)	0.44	1.09 (0.90;1.33)	0.37
rs10918594	CC (n=101)	CG (n=103)	GG (n=24)	P-value	Per G-allele	P-value
Crude	Reference	1.13 (0.85;1.50)	1.11 (0.70;1.76)	0.69	1.08 (0.88;1.32)	0.46
Full model	Reference	1.16 (0.88;1.54)	1.13 (0.71;1.80)	0.58	1.10 (0.90;1.34)	0.37
	Witnessed sudden cardiac death					
	Genotypic model[a]				Allelic model[b]	
SNP and model	**Genotype (number of SCD cases)**			**P-value**	**Per G-allele**	**P-value**
rs10494366	TT (n=47)	TG (n=43)	GG (n=26)			
Crude	Reference	0.82 (0.54;1.24)	1.66 (1.02;2.70)	0.02	1.20 (0.93;1.56)	0.17
Full model	Reference	0.84 (0.55;1.28)	1.68 (1.04;2.74)	0.02	1.22 (0.94;1.58)	0.14
rs10918594	CC (n=52)	CG (n=51)	GG (n=16)	P-value	Per G-allele	P-value
Crude	Reference	1.11 (0.75;1.64)	1.43 (0.80;2.54)	0.47	1.17 (0.89;1.53)	0.25
Full model	Reference	1.14 (0.77;1.68)	1.45 (0.81;2.59)	0.44	1.18 (0.91;1.55)	0.22

Cox proportional hazards model, HR (95% CI), n= number of SCD cases. Crude model: age and gender adjusted. Full model: including age, gender, BMI, smoking, hypertension, diabetes mellitus, heart failure and myocardial infarction

a Genotype specific HR

b HR entering genotype as an ordinal variable under an allelic model.

ditional GG haplotype copy ($p=2.0x10^{-18}$) using the TC haplotype as reference. The GC and TG haplotypes were associated with a 3.2 msec longer ($p= 7.0x10^{-4}$) and 4.1 msec longer (p= 0.01) multivariable-adjusted QT interval per additional copy, respectively. None of the haplotypes had a more significant effect than the individual SNPs.
Furthermore, rs10494366 and rs10918594 were associated with a larger proportion of borderline and prolonged QTc intervals using gender-specific cutpoints19 (test for trend both: $p < 0.0001$; Table 3).

***NOS1AP* polymorphisms and sudden cardiac death.** Within the study population (n=6571), we identified 233 sudden cardiac deaths, 121 of which were witnessed. Baseline characteristics of all adjudicated SCD cases are shown in Table 1. After adjustment for known risk factors, the *NOS1AP* polymorphisms rs10494366 T>G and rs10918594 C>G showed non-significant trends in the direction of increased hazard of SCD with hazard ratios per additional minor allele for time to SCD of 1.09 (0.90;1.33) and 1.10 (0.90;1.34), respectively. In the subset of 121 adjudicated SCD cases that were witnessed, a similar non-significant trend towards increased SCD risk was found (Table 4). Stratification for baseline age above and below the median showed no difference between age groups (data not shown). Finally, a sensitivity analysis excluding 767 subjects with a history of myocardial infarction at baseline did not result in a substantial change of the effect estimates or confidence intervals (data not shown).

Discussion

We observed strong replication in the Rotterdam Study, a large well-phenotyped cohort of European ancestry, of the finding from a prior genome-wide association study[22] that common *NOS1AP* variants are associated with increased age-, gender- and heart-rate-adjusted QT-interval duration. None of the haplotypes showed a more significant effect than the individual SNPs, which were not specifically selected to characterize haplotype variation at the locus. The two SNPs, which are 55kb apart, are not known to be functional, nor are they highly correlated with any known functional SNP. These results support the existence of a causal untyped SNP that is correlated with both rs10494366 and rs10918594.
The association with SCD was not statistically significant. Although we cannot fully exclude survival bias because of the older age of our study population, we did not find that the genotype distribution differed between different age groups at baseline, making this less likely. The modest QTc prolongation associated with *NOS1AP* variation, despite the strong effect of prolonged QTc on SCD risk suggests that a much larger study is needed to definitively confirm or rule out an increased risk of SCD by *NOS1AP* variants. At least 510 cases would be needed to detect an odds ratio of 1.2 per minor allele with 80% power. Even if no association with SCD is ultimately identified, the 7.2 msec increase in QTc interval in minor homozygotes compared with major homozygotes approximates the effect of medications which delay myocardial repolarization and increase liability to ventricular arrhythmias.

The mechanism by which common variation in *NOS1AP* affects QTc interval duration is unknown at present. However, the statistical evidence supporting the association with QTc interval of rs10494366 ($p<10^{-19}$) and rs10918594 ($p<10^{-16}$) in 5374 individuals confirms that this is a genuine association, consistent with evidence from four independent cohorts totaling >13,000 individuals of European ancestry. Our study examined the relationship of genetic variation, present at birth, in an elderly cohort in whom one might assume that genetic factors play a smaller role than in younger cohorts. However, these results demonstrate that genetic factors continue to play a role even at older age.

One major advantage in our study was the availability of up to three ECGs per subject at regular intervals during follow-up, resulting in more precise long-term ECG measures. Furthermore, the use of digital ECG recordings all measured with the MEANS system likely reduced systematic differences in assessment of the QTc interval. In addition, the intersection of the Rotterdam Study with detailed pharmacy exposure data allowed us to exclude ECGs recorded in individuals on QT prolonging or -shortening drugs, which could have attenuated the power to detect the association. Although no information on LQTS cases was available, the number of relatives in the Rotterdam Study is low and the sensitivity analysis excluding abnormally prolonged QTc further minimized influence of potential familial LQTS cases. Another advantage of the Rotterdam Study is the prospective ascertainment of risk factors and the active surveillance for SCD events over a relatively long period of follow-up. Thus, extensive information surrounding SCD events was available, including the time between start of symptoms and death, enabling rigorous adjudication of SCD events.

One limitation of the study resides in the variety of competing causes of abrupt death at increasing age, which may have led to misclassification, especially in cases where death was unwitnessed. Because SCD coding was blinded to *NOS1AP* genotype, this would likely have biased our study to detect no effect. This might explain our finding of a slightly increased, but still non-significant, hazard ratio when the analyses were restricted to witnessed sudden cardiac deaths. Our results and those of the prior study by Arking et al. were restricted to population samples of European ancestry. Further testing in samples of African and Asian ancestry is needed to establish the role of genetic variation at the *NOS1AP* locus in myocardial repolarization in these population groups. Moreover, substantial frequency differences are observed among European, African and Asian HapMap samples, which raises the possibility of natural selection in the region (www.hapmap.org accessed November 6, 2006). Attempts to validate the *NOS1AP* association in recently admixed populations, such as African Americans, will need to account for global and local chromosomal differences in ancestry because of the strong association with continental ancestry and the risk of population stratification.

In conclusion, we have strongly confirmed the association of *NOS1AP* variants with QT interval duration. With the limited number of SCD cases in our cohort, it was not possible to demonstrate that this association translates into an influence on risk of SCD, although the point estimates suggest that such a risk increase may truly exist. Additional larger studies will be required to determine whether *NOS1AP* genotype is associated with SCD.

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4.2

Common *NOS1AP* variants potentiate digoxin induced QT-shortening and risk of sudden cardiac death

Abstract

Background. Digoxin, widely used in the treatment of heart failure and atrial fibrillation, is associated with increased risk of life-threatening arrhythmias. Common variants of the *NOS1AP* gene were recently shown to be associated with QT-prolongation. We analyzed the combined effect of *NOS1AP* genotype and digoxin on QTc-interval duration and the risk of sudden cardiac death (SCD) to digoxin.

Methods. We studied the Rotterdam Study, a population-based, prospective cohort study of individuals ≥55 years of age. Heart rate corrected QT (QTc) interval duration was measured in prospectively collected ECGs. Digoxin exposure was determined at the time of each ECG. Hazard ratios (HRs) for time to SCD among *NOS1AP* genotypes with and without digoxin were estimated by Cox proportional hazards modeling with time-dependent exposures.

Results. We included 16,595 ECGs from 7771 individuals (315 ECGs from 243 individuals on digoxin). In digoxin users, *NOS1AP* minor alleles were associated with increased QTc shortening of 4.3 msec per additional allele compared to subjects homozygous for the reference allele. There were 229 SCD cases, 40 of whom were exposed to digoxin at time of death. In digoxin users, *NOS1AP* minor alleles were associated with an increased risk of SCD of 1.78 (95%CI 1.13;2.81) per additional allele, resulting in a 3-fold increased SCD risk for homozygous minor allele carriers. *NOS1AP* variants did not increase SCD risk in non-digoxin users.

Conclusions. Minor allele carriers of a common *NOS1AP* variant using digoxin have exaggerated QTc shortening and an increased risk of SCD.

Introduction

Digoxin (digitalis) has been used in the treatment of heart disease for over 200 years. More recently, digoxin was demonstrated to reduce the number of admissions for heart failure and to worsen heart failure on its withdrawal, without clear mortality advantage.[1-3] Digoxin is now widely used for treatment of atrial fibrillation and heart failure. Digoxin exerts its action, in part, by increasing intracellular Ca^{++} concentration ($[Ca^{++}]_i$), leading to higher contractile force and QT-shortening.[4] Digoxin has a narrow therapeutic window, and ever since its introduction, it has been associated with toxicity and, in the past, high risk of life-threatening arrhythmias and mortality.

Common variants of the nitric oxide synthase 1 adaptor protein (*NOS1AP*) gene were recently discovered to be associated with QT-interval prolongation in a genome wide association study.[5] We replicated this finding in the Rotterdam Study, a large population based cohort of persons 55 years and older.[6] *NOS1AP* was not previously known to play a role in cardiac repolarization. So far, not much is known about the mechanism by which *NOS1AP* influences QT duration. *NOS1AP* activates neuronal nitric oxide synthase (*NOS1*). *NOS1* deficiency increases $[Ca^{++}]_i$ and causes QT prolongation.[7-10]

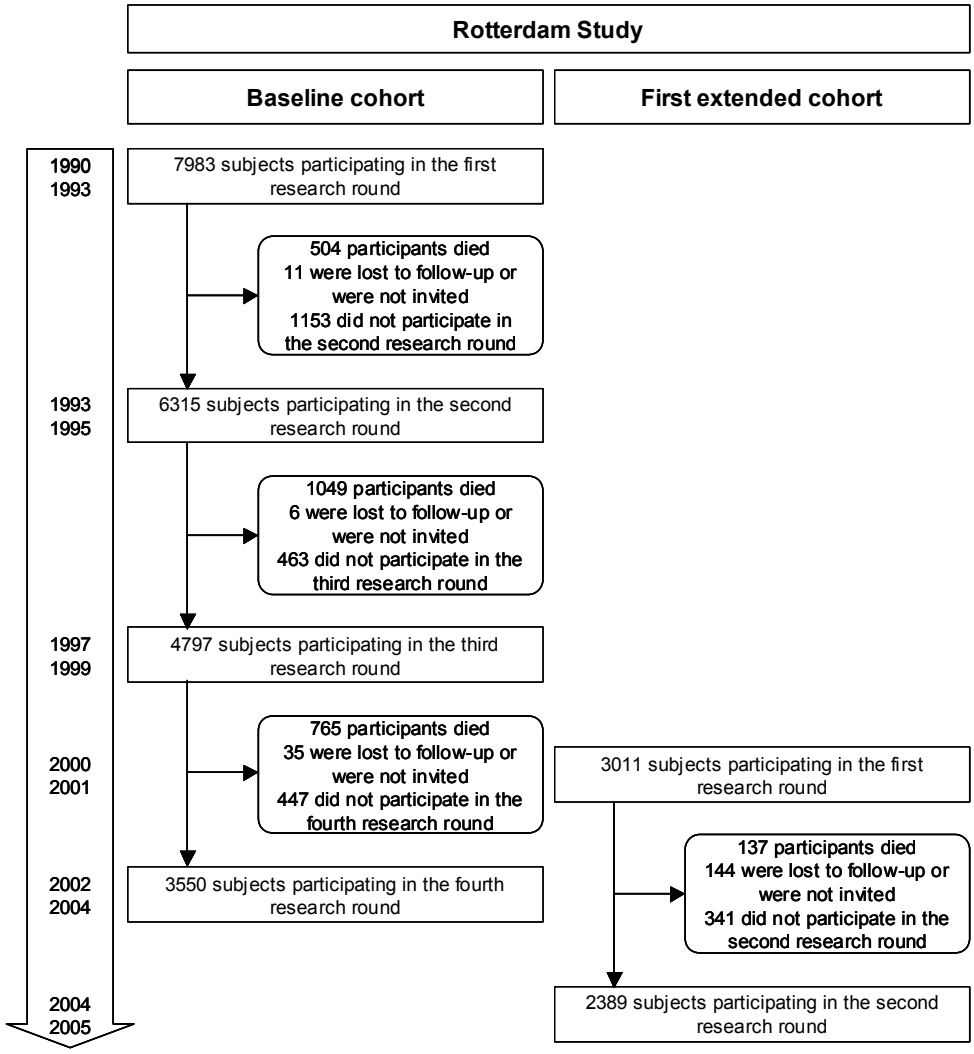
We hypothesized that subjects carrying the minor variants of two *NOS1AP* SNPs known to prolong the QT interval might be less sensitive to the QT shortening effects of digoxin. If so, they might also be less sensitive to its pro-arrhythmogenic effects.[11-13] In the present study, we tested for interaction of *NOS1AP* variants with digoxin use on QTc interval duration in a population based cohort study comprising both digoxin users and non-users. In addition, we studied whether these *NOS1AP* variants were associated with a reduced risk of sudden cardiac death (SCD) in digoxin users.

Methods

Study population. The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly. Starting from March 1990, 7983 inhabitants of Ommoord, a Rotterdam suburb, aged 55 years and older took part in the baseline examination. In 2000, a second cohort of 3011 inhabitants of Ommoord, aged 55 years and older at that time, was added. Follow-up examinations took place at 4 year intervals (Figure 1). Objectives and methods of the Rotterdam Study have been described in detail.[14, 15] ECGs are taken at every follow-up round. Clinical characteristics including smoking, body mass index (BMI), hypertension, diabetes mellitus, heart failure and myocardial infarction were ascertained as previously described.[16-21] Active surveillance for incident diabetes mellitus, heart failure and myocardial infarction is conducted continuously between follow-up examinations. In addition, exposure of study participants to medications has been gathered on a continuous basis since January 1st, 1991 through computerized pharmacy records from the study area covering >99% of prescriptions to the study population.

The medical ethics committee of the Erasmus Medical Center (Rotterdam, The Netherlands) approved the study. All participants provided signed informed consent for participation, including retrieval of medical records and use of blood and DNA for scientific purposes. Baseline visit DNA for genotyping is available for 6571 (82%) participants of the first cohort and 2607 (87%) participants of the second inception cohort. Our study population comprised all individuals from the Rotterdam Study for whom genotyping and pharmacy data were available.

Figure 1 Flowdiagram of the Rotterdam study



ECGs were taken at every research round.

Genotyping. All participants were genotyped for the *NOS1AP* SNP rs10494366 T>G and the correlated SNP rs10918594 C>G, which were both previously shown to be associated with QT interval duration,[5, 6] as previously described.[6, 22] Because SNP rs10494366 showed stronger evidence of association with QTc in the Rotterdam Study and both SNPs are in linkage disequilibrium,[5, 6] we considered it primary in the analyses.

Digoxin exposure. In order to assess exposure we calculated the duration of dispensed digoxin prescriptions as the number of units dispensed divided by the number of units to be taken per day. Digoxin dose was expressed as the defined daily dose (DDD) as determined by the WHO (1 DDD is 0.25mg of digoxin).
Subjects were classified as exposed to digoxin if the date of the ECG or the date of SCD (or the index date for the remainder of the cohort) fell within the duration of a dispensed digoxin prescription.

Assessment of QTc interval and other ECG measurements. All ECGs from subjects with DNA available taken between January 1st, 1991 (first date drug exposure available) and the end of the last follow-up round (March 1st, 2005) were checked for eligibility criteria. Heart rate corrected QT-interval (QTc) in msec, was calculated using Bazett's formula (QTc = QT / √RR).[23] ECG measures were determined using digital ECGs analyzed with the Modular ECG Analysis System (MEANS)[24-26] as in previous studies of QTc in the Rotterdam Study.[6, 21] ECGs with right or left bundle branch block were excluded from the analyses. In addition, all ECGs taken while the subject was on any QT-prolonging drugs were excluded from the analyses. The following drugs were considered to be QT-prolonging: drugs appearing on any of lists 1-4 at www.qtdrugs.org,[27] flupenthixol, levomepromazine, mefloquine, olanzapine or sertindole. Finally, ECGs taken while digoxin dose >1 DDD (to reduce influence of extreme dose effects) or with unknown digoxin dose were excluded. Up to 4 QTc measurements per subject were recorded across the four examination cycles.

Adjudication of sudden cardiac death. For the SCD analyses, we excluded subjects from the inception cohort since follow-up of this cohort was incomplete with respect to assessment of the causes of death. The end of the study period was January 1st, 2005, the last date for which follow-up has been completed for the baseline cohort. Since the inception cohort is a younger cohort with shorter follow-up, this did probably not result in loss of many SCD cases. The ascertainment of SCD cases in the Rotterdam Study has been described previously.[6, 21, 28, 29] SCD was defined as a witnessed natural death attributable to cardiac causes, heralded by abrupt loss of consciousness, within one hour of onset of acute symptoms, or an unwitnessed, unexpected death of someone seen in a stable medical condition <24 hours previously with no evidence of a non-cardiac cause.[28, 29]

Statistical analysis. Genotype frequencies were tested for Hardy-Weinberg equilibrium us-

ing a Chi-square test.

QTc interval was primarily studied, comparing all ECGs without digoxin exposure to those with digoxin exposure. Because measurements in ECGs of the same subject are correlated, we used repeated measures analyses with PROC MIXED (SAS 8.2, Cary, NC). To estimate the combined effect size of *NOS1AP* variants and digoxin use in one model, we created an interaction dummy variable with six levels (genotypes with and without digoxin) in which non-digoxin users homozygous for the major allele were considered as referent. Furthermore, multiplicative interaction terms (genotype (categorical) x digoxin use and genotype (additive) x digoxin use) were used to test for multiplicative interaction. Associations were tested in univariate models and in models adjusted for sex and age, heart failure, myocardial infarction and diabetes mellitus at the time of the ECG. The effect of genotype on QTc duration was also tested stratified on digoxin use to allow further adjustment for digoxin dose. Additionally, we studied the effect of digoxin on QTc interval within subjects in a follow-up design. For this, we determined the difference in QTc duration between two consecutive ECGs (one on digoxin and one off) of subjects starting or stopping digoxin therapy.

Hazard ratios for time to SCD from baseline were estimated using Cox proportional hazards models with time dependent digoxin exposure. Because of the strong impact of prevalent cardiovascular morbidity –which is much more frequent in digoxin users- *NOS1AP* genotype effect on SCD risk was tested stratified on digoxin use. First, the association was tested in univariate analyses and subsequently in multivariate models including known SCD risk factors as predictors: sex and smoking at baseline and time dependent age, diabetes mellitus, heart failure and myocardial infarction. In the stratum of digoxin users we further adjusted for digoxin dose. To test for interaction, we used multiplicative interaction terms similar to the QTc analyses. To exclude confounding by the underlying heart disease (confounding by indication), we also tested the effect of *NOS1AP* variants on SCD in participants with prevalent myocardial infarction or heart failure without digoxin use and in digoxin users without prevalent heart disease. To minimize bias by misclassification of SCD, we additionally performed a sensitivity analysis restricting the case definition to witnessed deaths only. All Cox proportional hazards analyses were performed using SPSS for Windows version 11.0 (Chicago, Illinois, USA).

Differences in digoxin dose between ECGs from different genotype groups were also compared using repeated measures analyses. Digoxin dose between different genotype groups in SCD cases were compared using ANOVA.

Results

Study population. For the QTc assesment, a total of 16,595 ECGs from 7771 individuals were included. Of these, 315 ECGs from 243 individuals were taken while the subject was using digoxin. Mean follow-up time was 9.2 (SD 4.6) years, slightly lower than in the SCD population due to inclusion of the second inception cohort which entered the Rotterdam Study later. The study population for SCD assessment comprised 6541 individuals with a mean

Table 1 Baseline characteristics

	QTc study population[a]		Total SCD study population[b]		SCD cases	
Characteristic	**Men**	**Women**	**Men**	**Women**	**Men**	**Women**
Number	3270 (42.1%)	4501 (57.9%)	2655 (40.6%)	3886 (59.4%)	113 (49.3%)	116 (50.7%)
Mean age at baseline, years (SD)	66.1 (7.7)	68.0 (9.1)	68.1 (8.2)	70.3 (9.6)	71.4 (7.6)	74.3 (7.7)
Mean follow-up time, years (SD)	8.9 (4.6)	9.5 (4.6)	10.1 (3.8)	10.6 (3.7)	6.5 (3.7)	7.3 (3.8)
Smoking status						
Current	834 (25.5%)	833 (18.5%)	772 (29.1%)	679 (17.5%)	32 (28.3%)	15 (12.9%)
Past	2012 (61.5%)	1367 (30.4%)	1629 (61.4%)	1032 (26.6%)	73 (64.6%)	37 (31.9%)
BMI	26.1 (3.1)	26.9 (4.2)	25.7 (3.0)	26.7 (4.1)	25.3 (3.0)	27.3 (3.9)
Hypertension	824 (25.2%)	1299 (28.9%)	776 (29.2%)	1409 (36.3%)	52 (46.0%)	64 (55.2%)
Blood pressure, mmHg (SD)						
Diastolic	76.8 (11.6)	74.4 (11.1)	74.6 (11.5)	73.1 (11.4)	74.4 (12.1)	77.0 (14.2)
Systolic	140.0 (21.4)	140.0 (22.1)	138.7 (21.7)	139.8 (22.6)	144.7 (24.4)	152.7 (27.8)
Diabetes mellitus	368 (11.3%)	453 (10.1%)	279 (10.5%)	417 (10.7%)	14 (12.4%)	27 (23.3%)
Myocardial infarction	461 (14.1%)	300 (6.7%)	447 (16.8%)	318 (8.2%)	44 (38.9%)	19 (16.4%)
Heart failure	98 (3.0)	132 (2.9%)	81 (3.1%)	127 (3.3%)	17 (15.0%)	7 (6.0%)
rs10494366 (MAF)	36.3%	36.6%	36.3%	36.5%	35.4%	38.9%
rs10918594 (MAF)	31.3%	31.7%	31.1%	31.5%	30.6%	35.6%
QTc	422.6 (24.5)	432.2 (22.2)	NA	NA	NA	NA

MAF = minor allele frequency, NA = Not applicable (since not all subjects in the SCD study population have an ECG available)

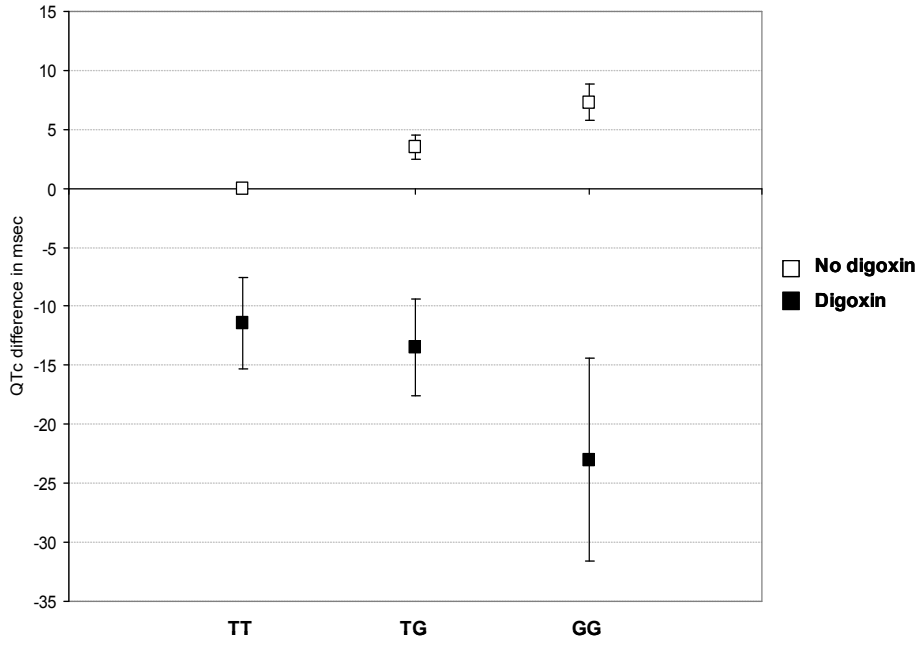
a All subjects with ECG measurements, those with multiple ECGs counted once

b Cases + controls

follow-up time of 10.4 (SD 3.7) years. Of these, 871 subjects used digoxin at any time during the study period. Baseline characteristics of the QTc and SCD study populations and of SCD cases are shown in Table 1.

Genotypes and digoxin exposure. Genotype assessment of rs10494366 T>G and rs10918594 C>G was successful in 96.4% and 97.6%, minor allele frequencies were 36.5% and 31.4% respectively. Both SNPs were in Hardy-Weinberg equilibrium (p= 0.53 for rs10494366 and p=0.78 for rs10918594). Genotype frequencies in digoxin users did not significantly differ from non-users (p= 0.93 and p= 0.71 respectively).
Mean digoxin dose was 0.61 DDD (SD 0.27) and did not significantly differ between genotype groups of ECGs taken while using digoxin, of those ever using digoxin in the total SCD sample or of SCD cases using digoxin on the index date.

Figure 2 QTc difference in msec using by *NOS1AP* genotype and digoxin use



QTc change in msec compared to persons with rs10494366 TT genotype without digoxin use (reference) adjusted for: age, sex, heart failure, myocardial infarction and diabetes mellitus at time of ECG (repeated measures analyses). Age, sex, heart failure, myocardial infarction and diabetes mellitus adjusted mean QTc for the reference group is 426msec. Vertical bars indicate 95% confidence intervals.

Table 2 *NOS1AP* genotype effect on QTc stratified on digoxin users

		QTc change in msec (95% CI) by genotype[b]		
Genotype	**N[a]**	**Crude**	**Adjusted[c]**	**Adjusted + dose[d]**
No digoxin				
Allelic model[e]	7342	3.6 (2.9;4.3)	3.6 (2.9;4.3)	NA
Genotypic model				
TT	2973	Reference	Reference	NA
TG	3379	3.4 (2.4;4.5)	3.5 (2.5;4.5)	NA
GG	990	7.3 (5.8;8.8)	7.3 (5.8;8.7)	NA
Digoxin				
Allelic model[e]	233	-3.4 (-10.2;3.3)	-4.5 (-11.1;2.2)	-4.3 (-10.8;2.3)
Genotypic model				
TT	107	Reference	Reference	Reference
TG	102	-1.7 (-11.1;7.6)	-1.5 (-10.8;7.8)	-1.8 (-11.0;7.3)
GG	24	-8.8 (-24.2;6.5)	-12.4 (-27.6;2.8)	-11.3 (-26.4;3.8)
P-value for multiplicative interaction				
Allelic model[e]		$8.8*10^{-4}$	$8.7*10^{-5}$	NA
Genotypic model		0.0033	$2.1*10^{-4}$	NA

Results for rs10494366 genotypes; NA= not applicable

a Number of unique individuals

b QTc change in msec with 95% confidence interval. Homozygous major allele group is the referent.

c Adjusted for known QT-prolonging factors: age, sex, heart failure, myocardial infarction and diabetes mellitus at time of ECG.

d (Users only) Additionally adjusted for digoxin dose at time of ECG

e Additive genetic model, QTc change per additional minor allele

QTc interval. In the total study population, ECGs taken while subjects were using digoxin showed QTc interval shortening of 9.5 (95%CI 6.9;12.1) msec compared to non-users. After adjustment for sex and time dependent age, heart failure, diabetes mellitus and myocardial infarction digoxin use was associated with 15.7 (95%CI 13.0;18.3) msec shortening. Within the stratum of digoxin users, the effect of digoxin dose was 11.1 (95%CI 3.2;18.9) msec shortening per 0.5 DDD increase. In non-digoxin users, QTc interval increased with approximately 3.5 msec per additional variant allele, similar to previous studies.[5, 6, 30]

Digoxin users with TT genotypes for rs10494366 had a 11.4 (95%CI 7.5;15.3) msec shorter QTc interval than TT subjects not using digoxin (reference group), whereas digoxin users homozygous for the G-allele showed 23.0 (95%CI 14.4;31.5) msec QTc shortening compared to the reference group (Figure 2). The interaction between digoxin exposure and *NOS1AP* genotypes in their effect on QTc interval exceeded multiplicative scale. Within the digoxin use stratum, the genotype effect on QTc interval was similar to that shown in Figure 2 and was not substantially changed by additional adjustment for digoxin dose (Table 2).

We identified 80 persons starting on digoxin between ECGs, 24 persons discontinuing digoxin

Table 3 *NOS1AP* Genotype effect on SCD risk, stratified on digoxin use at the index date

		HR (95% CI)[a]		
Genotype	**SCD cases**	**Crude**	**Adjusted[b]**	**Adjusted + dose[c]**
No digoxin				
Allelic model[d]	180	0.95 (0.77;1.18)	1.00(0.81;1.24)	NA
Genotypic model				
TT	80	Reference	Reference	NA
TG	73	0.81 (0.59;1.11)	0.86 (0.62;1.18)	NA
GG	27	1.01 (0.65;1.57)	1.11 (0.72;1.72)	NA
Digoxin				
Allelic model[d]	37	1.70 (1.09;2.66)	1.78 (1.13;2.81)	1.78 (1.13;2.81)
Genotypic model				
TT	10	Reference	Reference	Reference
TG	18	1.76 (0.81;3.83)	1.86 (0.85;4.10)	1.86 (0.85;4.11)
GG	9	2.89 (1.17;7.14)	3.17 (1.26;7.96)	3.16 (1.26;7.96)
P-value for multiplicative interaction				
Allelic model[d]		0.013	0.035	NA
Genotypic model		0.055	0.125	NA

Results for rs10494366 genotypes; NA = not applicable

a Hazard ratio and 95% confidence interval per genotype group, stratified by digoxin use. Homozygous major allele groups are the referent.

b Adjusted for known risk factors of SCD: sex, time dependent age, diabetes mellitus, heart failure, myocardial infarction, baseline smoking (past, present, never)

c (Users only) Additionally adjusted for digoxin dose at index date

d Additive genetic model , hazard ratio per additional minor allele

between ECGs and 11 subjects who started and later stopped digoxin therapy during the study period. On average, the QTc interval shortening within individuals (i.e. QTc interval without digoxin minus QTc interval with digoxin) was larger among *NOS1AP* minor allele carriers (35.2 msec shortening in GG vs 11.8 msec shortening in TT subjects, p-trend =0.37) but did not reach statistical significance.

Results of identical analyses for the partially correlated rs10918594 SNP were similar (results not shown).

Sudden cardiac death. During the study period, we identified 229 SCD cases 121 of which were witnessed, and 40 of which were exposed to digoxin at time of death. Digoxin use resulted in a substantial SCD risk increase (HR 5.1 95%CI 3.7;7.2). The HR decreased to 2.9 (95%CI 2.0;4.1) after adjustment for sex, smoking and time dependent age, diabetes, myocardial infarction and heart failure.

After stratification for digoxin use, we found no effect of *NOS1AP* genotypes on SCD risk in those not using digoxin (HR allelic model 1.00, 95%CI 0.81;1.24). However, in the stratum

of digoxin users we found an increased risk of SCD in minor allele carriers (HR 1.78 per additional minor allele, 95%CI 1.13;2.81). Multiplicative interaction significantly exceeded multiplicative scale (Table 3). The sensitivity analyses restricting the outcome to witnessed SCD cases only, resulted in a slightly higher point estimate of SCD risk for minor allele carriers using digoxin (HR fully adjusted allelic model 1.92, 95%CI 1.06-3.49). In participants with prior myocardial infarction or heart failure not using digoxin, no effect of *NOS1AP* genotype on SCD was observed (HR allelic model 1.01, 95%CI 0.68;1.50) and in digoxin users without previous myocardial infarction or heart failure, the effect estimate of *NOS1AP* was even larger (HR allelic model 2.01, 95%CI 0.84;4.85) making a strong effect of confounding by indication unlikely.
Results for the minor variant of rs10918594 were similar (results not shown).

Discussion

In the present study, we surprisingly found that digoxin users carrying the *NOS1AP* minor allele had significantly more pronounced QT shortening than major allele homozygous users, in contrast to the effect observed in non-users. Apparently, the QTc-shortening effect of digoxin is significantly worsened in those with the minor allele. Furthermore, minor allele carriers had an up to three-fold increased risk of SCD to digoxin. No genotype effect on SCD risk was observed in non-users of digoxin. Although the mechanism by which *NOS1AP* influences QT interval duration and interacts with digoxin is not known, it may involve calcium handling in the cardiomyocyte.[7-10] Digoxin is capable of increasing intracellular Na^+ by inhibition of the Na/K ATP-ase, resulting in an increased intracellular Ca^{++} concentration ($[Ca^{++}]_i$).[31, 32] In addition, the increased peak $[Ca^{++}]_i$ results in faster kinetics of $[Ca^{++}]_i$ decay,[4] thus leading to a faster repolarization and QT shortening. *NOS1AP* has been found to activate *NOS1*.[33] *NOS1* knockout cardiomyocytes have increased contractility through increased $[Ca^{++}]_i$ as well as a slower time to relaxation and a prolonged time-course of decay of the $[Ca^{++}]_i$ transient.[7-10, 34] Furthermore, *NOS1* seems to be able to interact with the L-type Ca^{++} channel,[35] which is known to be associated with both Short- and Long QT Syndrome and SCD.[36, 37] This suggests a model in which both digoxin and *NOS1AP* minor alleles increase $[Ca^{++}]_i$, resulting in excess $[Ca^{++}]_i$. Calcium overload can result in spontaneous cycles of Ca^{++} release and reuptake, resulting in after-depolarizations and increased risk of ventricular arrhythmias.[31] This would explain the increased risk of SCD in digoxin users with the variant *NOS1AP* alleles. If digoxin counteracted the slower decay of the $[Ca^{++}]_i$ transient caused by lower *NOS1* activity, the excess $[Ca^{++}]_i$ would also result in faster $[Ca^{++}]_i$ decline,[4] explaining the increased QTc-shortening in *NOS1AP* variants. In turn, short QT in itself, both as a congenital syndrome and in the general population, is a risk factor of SCD.[11-13, 36, 38]
A strength of our study was the extensive information surrounding SCD events, including the time between start of symptoms and death, enabling rigorous adjudication of SCD events. Furthermore, precise long-term ECG measures were available in up to four ECGs per subject.

The availability of detailed pharmacy exposure data allowed us to determine digoxin use at time of ECG or death, and to exclude ECGs recorded in individuals on QT prolonging drugs. The prospective design of the Rotterdam Study limits the risk of selection or information bias and the multivariate models show that confounding is limited. We cannot fully exclude confounding by (severity of) the underlying cardiovascular disease. However, this is unlikely to explain the results, since adjusting for concomitant heart disease slightly increased the effect estimates for *NOS1AP* minor alleles. Furthermore, in participants with prior myocardial infarction or heart failure not using digoxin no effect of *NOS1AP* minor alleles on SCD was observed, whereas a stronger genotype effect was observed in digoxin users without prevalent heart disease. Finally, the results from the ECG analyses show increased QTc-shortening to digoxin in *NOS1AP* minor allele carriers. Heart disease usually increases QTc duration, which further argues against an effect of the underlying disease.

One limitation of the study resides in the small number of SCD cases. However, despite the limited power we found a statistically significant, three-fold increased risk of SCD to digoxin in subjects homozygous for the *NOS1AP* minor allele. Another limitation lies in the variety of competing causes of abrupt death at increasing age, which may have led to misclassification of SCD events, especially in cases where death was unwitnessed. Since SCD coding was blinded to *NOS1AP* genotype, this would likely have biased our study toward the null hypothesis that no effect exists. The stronger effect on SCD in analyses restricted to witnessed SCD lends some support to this hypothesis.

In conclusion, we have found that common *NOS1AP* variants interact with digoxin, resulting in an increased QTc-shortening and an up to three-fold increased risk of SCD in digoxin users with *NOS1AP* minor alleles. These findings raise the possibility of testing for common *NOS1AP* variants with the goal of improving patient safety in digoxin therapy.

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4.3

Cardiovascular drugs, *NOS1AP* and QTc prolongation

Abstract

Aim. To study whether *NOS1AP* variant alleles rs10494366 T>G and rs 10918594 C>G modify the effect of cardiovascular drugs which may prolong the QTc interval and to study whether this combination was associated with a higher risk of sudden cardiac death.

Methods. This study was conducted as part of the prospective population-based Rotterdam Study, and included 16,603 ECGs from 7565 participants aged 55 years and older, after exclusion of ECGs from patients with left ventricular hypertrophy, left and right bundle branch block as well as carriers of pacemakers. The primary endpoint of the study was the length of the QTc interval of users with the minor alleles compared to non-users with the major alleles. The secondary endpoint was the risk of sudden cardiac death (SCD) of users with the minor alleles compared to non-users with the major alleles. Associations were examined by repeated measurement analyses and Cox proportional-hazards models, adjusted for age, gender, diabetes mellitus, hypertension, myocardial infarction, heart failure and use of other QTc prolonging drugs.

Results. Overall, 1061 participants developed QTc prolongation during follow-up. The G-allele (minor) frequency of rs10494366 T>G was 36.5% and of rs10918594 C>G was 31.4%. Users of triamterene and verapamil with the rs10494366 GG genotype showed significantly more QTc-prolongation than users with the TT haplotype. SNP rs10918594 C>G showed similar results. Although the numbers of SCD cases exposed to QT-prolonging drugs at time of death was very low, point estimates for amiodarone (15 exposed SCD cases) and verapamil (8 exposed cases) suggest that *NOS1AP* minor variant carriers might be at increased risk of SCD in users of these drugs.

Conclusions. We demonstrated that the minor alleles of both *NOS1AP* SNPs potentiated the QTc prolonging effect of several cardiovascular drugs, mainly in users of triamterene and verapamil. We also found indications that amiodarone, and verapamil in combination with *NOS1AP* minor alleles increase the risk of sudden cardiac death, which endorses the clinical importance of this gene-drug interaction.

Introduction

In the past decade, one of the most frequent causes of withdrawal or restriction of marketed drugs has been the prolongation of the heart-rate corrected QT (QTc) interval in combination with case-reports of sudden cardiac death. The QTc interval is the traditional measurement for assessing the duration of ventricular repolarization which may result in early after depolarizations (EAD) and re-entry, and thereby provoke Torsade de Pointes and fatal ventricular arrhythmias.[1-5] Previously, we have demonstrated that QTc prolongation is associated with an increased risk of sudden cardiac death.[6] An increasing number of drugs has been recognized to delay cardiac repolarization and to induce Torsade de Pointes.[4] The QTc interval is influenced by factors such as gender, age and use of certain drugs. However, it is mostly unknown which other underlying risk factors modify the risk of drug-induced QTc-prolongation. As the QT interval is a genetically quantitative trait with ~30% heritability,[7-9] it is likely that such genetic effect modifiers exist.
Recently, we reported the finding from a genome-wide association study that a common variant (rs10494366, minor allele frequency 38%) in the *NOS1AP* gene was associated with QT interval variation in several large population samples.[10,11] The *NOS1AP* gene encodes the nitric oxide synthase 1 activating protein.[10] The mechanism by which common variation in *NOS1AP* affects the QTc interval is presently unknown. *NOS1AP* is a regulator of neuronal nitric oxide synthase effected by forming a ternary complex with PSD95 (membrane-associated guanylate kinase)[11] and Dexras 1 (member of the Ras family of small monomeric G proteins).[12] *NOS1* has been shown to have a role in cardiac contractibility.[13, 14] It is hypothesized that nitric oxide signaling may be involved in cardiac repolarization.
We conducted a population-based prospective cohort study to investigate whether *NOS1AP* variant alleles rs10494366 T>G and rs10918594 C>G modify the effect of cardiovascular drugs which may prolong the QTc interval and to study whether this combination was associated with a higher risk of sudden cardiac death.

Methods

Setting and study design. The Rotterdam study is a prospective population-based cohort study, which started with a baseline visit between 1990 and 1993. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, the Netherlands, approved the study. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55 years and over, were invited to participate (n=10,275). Of them, 7983 (78%) gave their written informed consent and took part in the baseline examination. Objectives and methods of the Rotterdam Study have been described in detail elsewhere.[15] At baseline, all participants were visited at home for a standardized questionnaire, and 7151 were subsequently examined at the research center. Since the start of the study, follow-up visits took place in the period 1993 through 1996 for the second visit, in the period between 1997 through 1999 for the third visit and in the period between 2002 through 2004 for the fourth visit. Furthermore, in 2000 a second cohort (first extended cohort) was enrolled.[16] This included all inhabitants of Ommoord, at that time aged 55 years and over, who were invited to participate (n = 4504). Of them, 3011 (67%) entered the study and took part in the baseline examination. The second visit of the first extended cohort took place in the period between 2004 and 2005. In addition to follow-up

examinations, the total cohort is continuously being monitored for major morbidity and mortality through linkage of general practitioner and municipality records. Furthermore, all drug prescriptions dispensed to participants by automated pharmacies are routinely stored in the database since January 1, 1991.

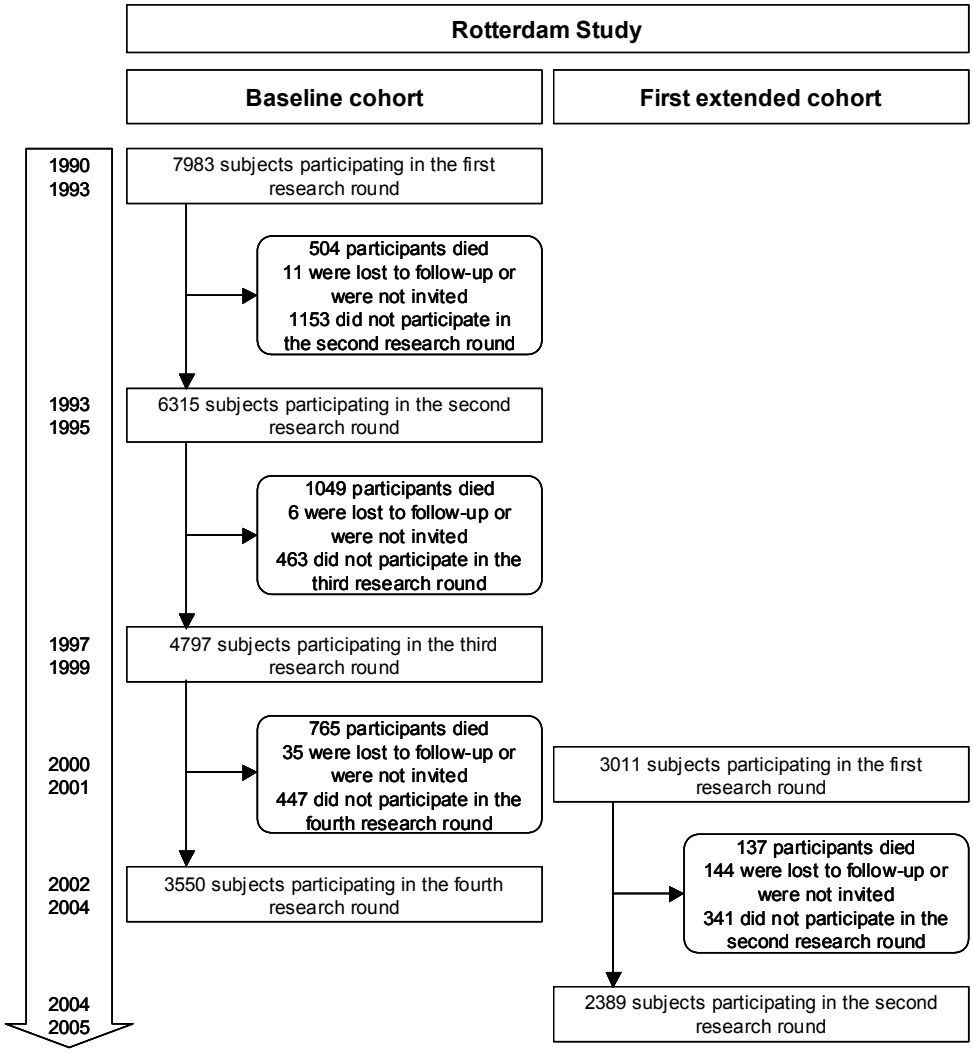
Study population. Two study populations were used, one for the QTc analysis and one for the sudden cardiac death analysis.

All cohort members of the Rotterdam Study and the first extended cohort, who had at least one ECG and was genotyped, were enrolled in the study population for the QTc analysis. Participants could contribute up to four ECGs to the analyses. Overall, 20,596 ECGs were available, 8586 in men and 12,010 in women. Digitally stored ECGs were available for 5397 participants at the time of the first visit (75% of 7151 participants visiting the research center), of 4798 participants at the time of the second visit (76% of 6315 participants visiting the research center), of 3818 participants at the time of the third visit (91% of 4215 participants visiting the research center), and of 3118 participants at the time of the fourth visit (99% of 3145 participants visiting the research center). There were 2273 ECGs available of the participants of the first extended cohort at the time of the first visit (84% of 2722 participants who visited the research center), and of 1190 at the time of the second visit (53% of 2249 participants who visited the research center). Missing ECGs were mainly due to temporary technical problems with ECG recording. Participants left the cohort mainly due to mortality while a minority was lost to follow-up (Figure 1). For the present study, the visit during which the first ECG was made was defined as baseline. ECGs of participants who used digoxin, which is a QTc shortening agent at the index date were excluded. In addition the ECGs were excluded of persons with a pacemaker, as well as of persons with evidence of left ventricular hypertrophy, left and right bundle branch block, since these conditions are associated with a prolonged QTc interval.[17, 18] Consequently, 6205 participants (3599 women and 2606 men) were included in the QTc interval study population.

For the sudden cardiac death study population, all cohort members of the Rotterdam Study who were genotyped, were enrolled. Since drug prescriptions dispensed to participants by automated pharmacies are routinely stored in the database since January 1, 1991, the follow-up lasted from January 1, 1991 until reaching one of the censoring dates (death or transferring out) or the end of the study period (January 1, 2006). Overall, 6214 participants (3705 women and 2509 men) were included in the sudden cardiac death study population..

ECG. The primary endpoint of the study was the length of the QTc interval in msec. A 12-lead resting ECG was recorded with an ACTA electrocardiograph (ESAOTE, Florence, Italy) at a sampling frequency of 500 Hz and stored digitally. All ECGs were processed by the Modular ECG Analysis System (MEANS) to obtain ECG measurements, the MEANS program has been evaluated extensively and has been validated.[19-22] MEANS determines common onsets and offsets for all 12 leads together on one representative averaged beat, with the use of template matching techniques.[20] The MEANS program determines the QT interval from the start of the QRS complex until the end of the T-wave. To adjust for heart rate, Bazett's formula ($QTc=QT/\sqrt{RR}$) was used.[23] European regulatory guidelines were used to categorize QTc prolongation into 3 categories. For men, the cut-off points were less than 430 ms (normal), 430-450 ms (borderline) and

Figure 1 Flowdiagram of the Rotterdam study



ECGs were taken at every research round.

more than 450 ms (prolonged), and for women less than 450 ms (normal), 450-470 ms (borderline), and more than 470 ms (prolonged).[24] Additionally, the MEANS program determines left ventricular hypertrophy and left and right bundle branch block.

Sudden cardiac death. The ascertainment of sudden cardiac death cases in the Rotterdam Study has been described previously.[6] In short, information on vital status is obtained from municipal health authorities in Rotterdam and general practitioners. In case of a fatal event, general practitioners filled in a questionnaire relating to the circumstances of death. Subsequently, research assistants gathered information

regarding these events at the general practitioners' offices. Two research physicians independently coded all reported events. In cases of disagreement, consensus was sought and finally, a cardiologist, whose judgment was considered decisive, reviewed all events. Sudden cardiac deaths were defined operationally as: 1) a witnessed natural death attributable to cardiac causes, heralded by abrupt loss of consciousness, within one hour of onset of acute symptoms, or 2) an unwitnessed, unexpected death of someone seen in a stable medical condition <24 hours previously with no evidence of a non-cardiac cause.[25, 26]

Medication. In this study, the exposure of interest included listed cardiovascular QTc-prolonging drugs, as specified in lists 1 through 4 from a commonly consulted internet based registry of QTc prolonging drugs from R.L.Woosley (www.qtdrugs.org/medical-pros/drug-lists.cfm); and in the list with QTc prolonging drugs from De Ponti et al. [27-30] The lists with QTc prolonging drugs are based on the medical literature and on the FDA database for reported adverse events. The QTc prolonging drugs from the internet based registry are classified into 4 categories, varying from drugs that are generally accepted by authorities to have a risk of causing Torsade de Pointes (list 1) to drugs that, in some reports, have a weak association with Torsade de Pointes and are unlikely to increase the risk when used in therapeutic dosages (list 4). In addition, De Ponti et al. have published a list of non-anti-arrhythmic drugs with pro-arrhythmogenic effects, based on a structured literature search including published (non-) clinical evidence and official warnings in the labelling.[27, 28]
In our analyses, we included the following listed cardiovascular QTc prolonging drugs: anti-arrhythmics: amiodarone, disopyramide, flecainide, quinidine, sotalol; and other cardiovascular drugs: diltiazem, indapamide, isradipine, ketanserin, losartan, nicardipine, triamterene and verapamil.
The index date in the QTc study population was the date of the ECG and in the sudden cardiac death study population the date of mortality. Cardiovascular drugs were considered to be currently used if the duration of the prescription covered the index date. The duration was the total number of units issued per prescription divided by the prescribed daily number of units.

Covariates. Diabetes mellitus, hypertension, myocardial infarction and heart failure are considered to be risk factors for QTc prolongation and presence of these conditions at each index date was included as a covariate.[31-34] Clinical measures were obtained during the visits at the Rotterdam Study research center. In 1990-1993 non-fasting blood samples were obtained, while in 1997-2000 blood samples were obtained after overnight fasting. Diabetes mellitus was defined as the use of blood glucose–lowering medication and/or a non-fasting serum glucose level of 11.1 mmol/l or higher and/or serum glucose levels ≥ 7 mmol/l (1997-2000).[35] Hypertension was defined as a systolic blood pressure ≥ 160 mm Hg and/or diastolic blood pressure ≥ 100 mm Hg and/or use of antihypertensive medication, encompassing grade 2 and grade 3 hypertension according to World Health Organization (WHO) criteria.[36] Myocardial infarction at baseline and during follow-up was assessed by hospital discharge diagnosis or in case a patient was not hospitalized, when signs and symptoms, analysis of the standard 12-lead electrocardiogram and cardiac enzyme data were diagnostic of a myocardial infarction.[37, 38] Heart failure at baseline and during follow-up were assessed by the presence of suggestive signs and symptoms and by the use of medication for the indication heart failure.[39, 40]

Table 1 Characteristics of the study population

	Study population QTc interval		Study population sudden cardiac death	
Characteristic	**Rotterdam study**	**First extended cohort**	**Rotterdam study**	**Sudden cardiac death**
Number of participants	5233	2332	6214	271
Gender (female)	3108 (59.4%)	1296 (55.6%)	3705 (59.6%)	145 (53.5%)
Mean age in years (SD)	68.5 (8.7)	64.4 (7.6)	69.4 (9.1)	73.0 (8.1)
Mean QTc interval (SD) (msec)	428.3 (24.1)	429.9 (22.3)		
ECGs with QTc prolongation	389 (7.4%)	170 (7.3%)		
NOS1AP rs10494366				
TT	2136 (40.8%)	930 (39.9%)	2534 (40.8%)	109 (40.2%)
TG	2380 (45.5%)	1094 (46.9%)	2837 (45.7%)	120 (44.3%)
GG	717 (13.7%)	308 (13.2%)	843 (13.6%)	42 (15.5%)
NOS1AP rs10918594				
CC	2442 (46.7%)	1091 (46.8%)	2910 (46.8%)	119 (43.9%)
CG	2258 (43.1%)	1006 (43.1%)	2687 (43.2%)	126 (46.5%
GG	533 (10.2%)	235 (10.1%)	617 (9.9%)	26 (9.6%

SD = standard deviation

Genotyping. All participants were genotyped for the *NOS1AP* SNP rs10494366 T>G which was previously shown to be associated with QT interval in 3 independent samples.[10] The partially correlated SNP rs10918594 C>G, which was associated with QT interval in one of the samples,[10] was also genotyped. Both were genotyped using Taqman assays C_1777074_10 and C_1777009_10 (Applied Biosystems, Foster City, Ca., USA) in 1 ng of genomic DNA extracted from leukocytes, as previously reported.[41] Haplotypes were estimated using the estimation-maximization algorithm implemented in PHASE 2.0 (University of Washington, Seattle, WA)[42, 43] and only individuals with successful genotyping for both SNPs were included in haplotype analyses.

Statistical analysis. Two types of analysis were conducted to assess the association between exposure to cardiovascular drug use, *NOS1AP* and QTc prolongation or sudden cardiac death.

The first analysis examined the association between exposure to cardiovascular drugs and the length of the QTc-interval. Since QTc measurements in subsequent ECGs in the same subject are correlated, the association was examined by means of linear regression repeated measures analyses implemented in PROC MIXED (SAS software, version 8.2). Analyses were adjusted for sex and the following time-depending covariates: age, diabetes mellitus, hypertension, myocardial infarction, heart failure and use of other listed QTc prolonging drugs.

Genotype frequencies were tested for Hardy-Weinberg equilibrium using a Chi-square test. Allelic genotype models were tested for the two polymorphisms rs10494366 T>G and rs10918594 C>G. In a separate allelic analysis, non-users with the major alleles were considered the referent, to which users with the other alleles were compared individually. For all above-mentioned cardiovascular drugs, we tested whether the 2 *NOS1AP* variant alleles modified the effect on a multiplicative scale.

In a second analysis, the association between exposure to cardiovascular drugs, the *NOS1AP* variant and the risk of sudden cardiac death was examined using Cox proportional hazards models. Non-users with the major alleles were considered the referent, to which users with the minor alleles were compared. The analyses were adjusted for sex and time-depending age. The analyses were performed using SPSS for Windows version 11.0 (Chicago, Illinois, USA).

Results

Study subjects. The baseline characteristics of all participants of the QTc study population after exclusion of left ventricular hypertrophy (538 ECGs), left and right bundle branch block (387 and 665 ECGs), use of digoxin (557 ECGs), participants with a pacemaker (53 ECGs) and with missing genotypes (2055 ECGs) are presented in Table 1. Overall 16,603 ECGs in 7,565 participants, remained for analysis.

The mean age of the study population at the first ECG in the Rotterdam Study and the first extended cohort was 67.1 years (Standard Deviation 8.2), women were significantly older than men. At baseline 574 participants had diabetes mellitus (9.8%), 127 participants had heart failure (2.2%), 1637 participants had hypertension (27.9%) and 509 participants have had a myocardial infarction (8.7%). At the time of an ECG, 319 participants used anti-arrhytmics and 676 participants used other cardiovascular drugs.

After exclusion of participants with missing genotypes (1685 participants) in the sudden cardiac death

Table 2 Cardiovascular drugs and QTc prolongation

Cardiovascular medication	Users	Prolongation QTc interval (95% CI)[a]
Anti-arrhytmics		
Amiodarone	62	19.1 (13.7 ; 24.4)
Disopyramide	17	31.1 (20.8 ; 41.5)
Flecainide	52	12.7 (6.9 ; 18.4)
Quinidine	7	34.4 (13.0 ; 55.7)
Sotalol	181	16.0 (12.9 ; 19.0)
Other cardiovascular		
Diltiazem	295	2.3 (-0.2 ; 4.8)
Indapamide	30	15.5 (7.7 ; 23.4)
Isradipine	44	1.1 (-5.7 ; 7.9)
Ketanserin	14	1.1 (-10.6 ; 12.8)
Losartan	146	4.3 (1.0 ; 7.6)
Nicardipine	6	-17.8 (-34.0 ; -1.7)
Triamterene	28	13.3 (5.3 ; 21.4)
Verapamil	113	6.7 (2.5 ; 10.9)

a Adjusted for age and sex, diabetes mellitus, hypertension, myocardial infarction, heart failure and use of other QTc prolonging drugs.

study population, 6214 participants remained for analysis (Table 1). Overall, 2503 participants died of all causes, of whom 271 participants experienced sudden cardiac death. At baseline, the mean age of the study population was 69.4 years (SD 9.1), 646 participants had diabetes mellitus (10.4%), 198 participants had heart failure (3.2%), 2077 participants had hypertension (33.4%), 727 participants had myocardial infarction (11.7%), 1383 participants currently smoked (22.3%), 2502 participants ever smoked (40.3%) and the mean body mass index was 26.3 kg/m^2 (SD 3.7). These variables did not significantly differ between participants with or without sudden cardiac death. The mean follow-up period was 10.4 years (SD 3.7).

Genotyping. The G-allele (minor) frequency of rs10494366 T>G was 36.5% and of rs10918594 C>G was 31.4%. Successful genotype calls were made in 96.3% and 97.3% of subjects, respectively. Both SNPs were in Hardy-Weinberg equilibrium (p= 0.53 for rs10494366 and p= 0.78 for rs10918594). The two SNPs were in linkage disequilibrium with an r2 of 0.63 and D' of 0.88 (95%CI 0.87 – 0.90). Upon phasing, we observed two common two-SNP haplotypes: TC (61.2%) and GG (29.2%), consisting of the two major and two minor alleles, respectively, and two minor haplotypes containing one major and one minor allele each: GC (7.2%) and TG (2.3%). Genotype distributions were similar for men and women and there were no age differences among genotypes.

Cardiovascular drugs and QTc. The mean QTc interval at study entry was significantly lower in males (421.8 msec.) than in females (431.5 msec.). 74.3% had normal QTc durations at baseline and 19.3% had a borderline QTc interval, using previously described gender specific cut-off points.
Overall, 559 participants developed QTc prolongation during follow-up (7.4%), with mean QTc levels of 473.9 milliseconds.
Current use of amiodarone (19.1 (95% CI 13.7 ; 24.4)), disopyramide (31.1 (20.8 ; 41.5)), flecainide (12.7 (6.9 ; 18.4)), quinidine (34.4 (13.0 ; 55.7)), sotalol (16.0 (12.9 ; 19.0)), indapamide (15.5 (7.7 ; 23.4)), losartan (4.3 (1.0 ; 7.6)), triamterene (13.3 (5.3 ; 21.4)) and verapamil (6.7 (2.5 ; 10.9)) was associated with a significant QTc interval prolongation, after adjustment for age, sex, diabetes mellitus, heart failure, hypertension, myocardial infarction and use of other listed QTc prolonging drugs (Table 2).

Cardiovascular drugs, *NOS1AP* variant alleles and QTc interval. Minor alleles of both *NOS1AP* SNPs in combination with several listed QTc prolonging drugs were associated with a significant increase of the QTc interval (Table 3). SNP rs10494366 T>G in combination with current use of triamterene and verapamil was associated with a significant QTc prolongation for the GG genotype compared to users with the TT genotype. Furthermore, although confidence intervals overlapped, point estimates indicated that a similar effect might exist for amiodarone, sotalol, diltiazem, indapamide, and perhaps isradipine and disopyramide. Point estimates suggest an opposite effect for losartan, i.e., less QTc prolongation for minor allele carriers. We observed no difference in effect of the SNPs between men and women.
SNP rs10494366 T>G modified the effect on QTc interval on a multiplicative scale in association with the following drugs: amiodarone, disopyramide, losartan, triamterene and verapamil.
Similar effects were observed for the rs10918594 C>G SNP.

Cardiovascular drugs, *NOS1AP* variant alleles and sudden cardiac death. The *NOS1AP* polymorphisms rs10494366 T>G and rs10918594 C>G were not associated with a significantly increased risk of sudden cardiac death. After adjustment for known risk factors, each additional minor allele was associated with hazard ratio for sudden cardiac death of 1.11 (0.91-1.34) and 1.09 (0.90-1.33), respectively.
The number of SCD cases exposed to QT-prolonging medication at the time of death was too low to make a proper assessment of the influence of *NOS1AP* variants on SCD risk in users of these drugs. However, although confidence intervals overlap, point estimates for users of amiodarone (15 exposed SCD cases, HR 21.30 vs 5.47) and verapamil (8 exposed cases, HR 3.97 and 2.50 vs 1.81) suggest that *NOS1AP* minor variant carriers might be at increased risk of SCD compared to major allele homozygotes (Table 4).

Discussion

We demonstrated, in this large prospective cohort study of an elderly population that the minor alleles of both *NOS1AP* SNPs significantly potentiated the QTc interval prolonging effect of current use of triamterene and verapamil. Furthermore, although confidence intervals overlapped, regression coefficients indicated that a similar effect might exist for amiodarone, sotalol, diltiazem, indapamide, and perhaps isradipine and disopyramide. Point estimates suggested an opposite effect for losartan. Furthermore, we

Table 3 Drugs, *NOS1AP* and QTc prolongation

	QTc prolongation (95% CI)					
	rs 10494366 TT[a]		rs 10494366 TG[a]		rs 10494366 GG[a]	
Cardiovascular medication	**Users**	**msec (95% CI)**	**Users**	**msec (95% CI)**	**Users**	**msec (95% CI)**
Anti-arrhytmics						
Amiodarone	26	27.3 (19.3 ; 35.3)	32	13.7 (5.7 ; 21.7)	4	44.3 (13.4 ; 75.2)
Disopyramide	9	17.1 (-13.6 ; 47.7)	8	52.9 (19.2 ; 86.5)	-	NA
Flecainide	20	17.0 (6.8 ; 27.1)	24	13.9 (5.3 ; 22.5)	8	17.7 (1.0 ; 34.5)
Quinidine	1	NA	6	NA	-	NA
Sotalol	71	13.9 (9.0 ; 18.7)	97	20.8 (16.6 ; 25.0)	13	26.8 (14.0 ; 39.5)
Other cardiovascular						
Diltiazem	120	4.8 (0.9 ; 8.7)	129	3.9 (-0.1 ; 7.8)	46	8.5 (2.4 ; 14.7)
Indapamide	14	15.6 (2.2 ; 29.0)	8	10.9 (-7.2 ; 28.9)	8	31.5 (11.8 ; 51.2)
Isradipine	22	-0.1 ; -11.2 ; 11.0)	17	2.0 (-10.6 ; 14.6)	5	20.2 (-0.1 ; 40.6)
Ketanserin	6	1.0 (-29.3 ; 31.4)	6	5.3 (-18.9 ; 29.4)	8	NA
Losartan	64	9.0 (3.9 ; 14.1)	70	5.6 (0.7 ; 10.4)	12	2.4 (-9.4 ; 14.1)
Nicardipine	3	NA	3	NA	3	NA
Triamterene	9	12.5 (-2.8 ; 27.8)	15	5.3 (-8.8 ; 19.4)	4	53.5 (30.3 ; 76.8)
Verapamil	47	3.0 (-3.6 ; 9.5)	48	9.5 (2.9 ; 16.1)	18	30.3 (18.0 ; 42.5)

QTc prolongation of participants with TT, TG or GG genotype using the drug compared to participants with the TT genotype not using the drug.

CI=Confidence Interval; QTc interval in msec. NA = not assessable, measures of association are not calculated for categories with less than 3 users.

a Adjusted for age and sex, diabetes mellitus, hypertension, myocardial infarction, heart failure and use of other QTc prolonging drugs.

Table 4 Cardiovascular drugs, *NOS1AP* and sudden cardiac death

	QTc prolongation (95% CI)					
	rs 10494366 TT[a]		rs 10494366 TG[a]		rs 10494366 GG[a]	
Cardiovascular medication	**Users**	**HR (95% CI)**	**Users**	**HR (95% CI)**	**Users**	**HR (95% CI)**
Anti-arrhytmics						
Amiodarone	6	5.47 (2.40 ; 12.48)		4.48 (1.82 ; 11.02)		21.30 (7.82 ; 58.06)
Disopyramide	-	NA	-	NA	-	NA
Flecainide	1	1.39 (0.19 ; 9.94)	1	1.10 (0.15 ; 7.86)	1	5.74 (0.80 ; 41.17)
Quinidine	-	NA	-	NA	-	NA
Sotalol	3	1.38 (0.44 ; 4.35)		0.38 (0.05 ; 2.71)	1	1.85 (0.26 ; 13.27)
Other cardiovascular						
Diltiazem	3	0.90 (0.29 ; 2.85)	3	0.91 (0.29 ; 2.86)		NA
Indapamide	-	NA	-	NA	-	NA
Isradipine	-	NA	-	NA	1	7.25 (1.01 ; 52.07)
Ketanserin	1	6.85 (0.96 ; 49.22)	-	NA	-	NA
Losartan	1	0.85 (0.12 ; 6.10)	1	0.91 (0.13 ; 6.53)	1	3.01 (0.42 ; 21.62)
Nicardipine	-	NA	-	NA	1	7.69 (1.01 ; 58.42)
Triamterene	-	NA	1	1.72 (0.24 ; 12.32)	-	NA
Verapamil	2	1.81 (0.45 ; 7.33)	5	3.97 (1.62 ; 9.73)	1	2.50 (0.35 ; 17.92)

Risk of sudden cardiac death of participants with TT, TG or GG genotype using the drug compared to participants with the TT genotype not using the drug. NA = not assessable; HR= Hazard Ratio; CI=Confidence Interval

a Adjusted for age and sex.

found indication that the *NOS1AP* minor alleles are associated with a significantly higher risk of sudden cardiac death in users of amiodarone, and verapamil. For some of the listed cardiovascular drugs we could not confirm a significant increase in the QTc interval or a higher risk of sudden cardiac death. This may have been due to limited power from small sample sizes. In general, the direction of change was as expected. The significant QTc interval shortening of nicardipine which we observed in our study population, has been described before in mice.[44]

Recently, one of us found that SNP rs10494366 T>G was associated with a 3.8 msec increase in multivariable-adjusted QTc interval for each additional G-allele, and SNP rs10918594 C>G was associated with a 3.6 msec increase per additional G-allele.[45] In participants with minor alleles of both *NOS1AP* SNPs the effect of the drugs on the QTc interval is reinforced. The effect we found exceeded an additive effect, while for several drugs the potentiation was even significant on a multiplicative scale.

Our study has several strengths. First, the fact that we had extensive information on potential confounders as well as 15 years of follow-up to obtain enough cases of sudden cardiac death. Second, the availability of genetic material, extensive information on potential confounders, and complete coverage of drug dispensing records allowed us to study the association between cardiovascular drugs, *NOS1AP* variant alleles and QTc prolongation. Since the Rotterdam Study is a prospective cohort study within a circumscribed population with little loss-to-follow-up, selection bias is unlikely. If exposure misclassification occurred it is probably non-differential. There is no information bias, since we used pharmacy data, which are registered prospectively and irrespective of disease status. One major advantage in our study was the availability of data on a large group of participants, including up to four ECGs per subject at regular intervals during follow-up, which allowed us to obtain more precise long-term ECG measures for each individual. Furthermore, the use of digital ECG recordings all measured using the MEANS system likely reduced intra- and interobserver variability in the assessment of the QTc interval. Confounding was minimized by adjusting for all known risk factors of QTc prolongation. An advantage of the Rotterdam Study is the prospective ascertainment of risk factors and the active surveillance for sudden cardiac death events over a relative long period of follow-up. Furthermore, we were able to take advantage of the fact that in most cases extensive information of the facts surrounding the event was available including, in many cases, the time between start of symptoms and death. This allowed rigorous adjudication of sudden cardiac death events.

Our study examined modification of the QTc prolonging effect of certain cardiovascular drugs by genetic variation. One might assume that genetic factors play a smaller role in our elderly cohort than in younger cohorts where long and short QT syndromes will add to mortality. This study confirms that the QTc interval is influenced by *NOS1AP* polymorphisms measured at a mean age at baseline of 67 years, increasing with follow-up. This serves as strong confirmation that genetic factors continue to play a role in an elderly population. While rare variants of strong genetic effects, such as are found in congenital Long QT Syndromes, are likely to be under negative selection as they reduce survival to reproduction, common variants of modest effects are more likely to escape negative selection as they contribute modestly and incrementally to a trait.[46] Common polymorphisms with a relatively small effect are capable of explaining a great degree of the population QTc interval variation. Also, the polymorphisms may modify the risk associated with QTc prolonging drugs.

In conclusion, we demonstrated that the minor alleles of both *NOS1AP* SNPs significantly potentiate the QTc prolonging effect of several cardiovascular drugs which were published on a list of QTc prolonging drugs, mainly in users of triamterene and verapamil. The fact that he minor alleles of *NOS1AP* are associated with a higher risk of sudden cardiac death in users of amiodarone, and verapamil, endorses the clinical importance of these gene-drug interactions.

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4.4

Common variation in the *NOS1AP* gene is associated with reduced glucose lowering effect and with increased mortality in users of sulfonylurea

Abstract

Objective. The single nucleotide polymorphism rs10494366 in the *NOS1AP* gene is associated with QTc prolongation, through an effect on the intracellular Ca^{2+} levels. As sulfonylurea stimulate insulin secretion by an increased influx of Ca^{2+}, we hypothesized that this polymorphism is associated with glucose lowering effect and mortality risk in sulfonylurea users.

Methods. Associations between the *NOS1AP* polymorphism, prescribed doses and mortality rates in sulfonylurea, metformin and insulin users were assessed in the Rotterdam Study, a population based cohort study of 7,983 elderly people.

Results. We identified 619 who were prescribed oral antidiabetic drugs during follow-up. In glibenclamide users carrying the TG genotype, the prescribed doses were higher compared to users carrying the TT genotype (0.38 DDD units, 95% CI 0.14; 0.63). Glibenclamide users with the TG or GG genotype had an increased mortality risk compared to glibenclamide users with the TT genotype (HR 2.80, 95% CI 1.09; 7.22). Tolbutamide users with the TG or GG genotype (HR 0.30, 95% CI 0.14; 0.63) and glimepiride users with the TG or GG genotype (HR 0.18, 95% CI 0.04; 0.74) had a decreased mortality risk compared to users with the TT genotype.

Conclusion. In subjects with the TG or GG genotype at rs10494366 in the *NOS1AP* gene, glibenclamide is less effective in reducing glucose levels and mortality rates were higher compared to glibenclamide users with the TT genotype. In tolbutamide and glimepiride users the TG and GG genotype were associated with a reduced mortality rate.

Introduction

Sulfonylurea drugs have been used extensively for decades in the treatment of type 2 diabetes. Since the publication of the University Group Diabetes Program trial in 1970, in which tolbutamide treatment was compared with other treatments and placebo, sulfonylurea have been associated with an increased risk of cardiovascular mortality.[1] However, there was criticism on this study in subsequent publications.[2-4] In 1998 the results of another trial with sulfonylurea were published. In this UK Prospective Diabetes Study trial, in which treatment with a sulfonylurea (chlorpropamide, glibenclamide or glipizide) was compared with insulin treatment and conventional policy with diet, no detrimental effects of sulfonylurea were seen.[5] Ever since, controversy remains as to whether sulfonylurea may increase the risk of cardiovascular death.

Sulfonylurea stimulate insulin secretion by the pancreatic β-cells.[6-8] The sulfonylurea receptor (SUR) is part of the ATP-sensitive K^+ (KATP) channel. Binding of the sulfonylurea to SUR causes inhibition of the KATP-channel, decreasing the K^+ efflux and depolarization of the cell membrane. This triggers the opening of voltage dependent Ca^{2+} channels, eliciting Ca^{2+} influx and a rise in intracellular Ca^{2+}. In the pancreatic β-cell, this rise stimulates the exocytosis of insulin-containing secretory granules.

Nitric oxide synthases (*NOS*) are the enzymes responsible for nitric oxide generation. Nitric oxide regulates cardiovascular homeostasis.[9] Recently, two nearby single nucleotide polymorphism (SNP), rs10494366 and rs10918594, in the gene encoding Nitric Oxide Synthase 1 Adaptor Protein (*NOS1AP*) have been associated with QTc-interval prolongation in electrocardiograms (ECGs).[10-12] *NOS1AP* is a regulator of neuronal *NOS* (*nNOS* encoded by *NOS1*), one of the isoforms of *NOS*. The *nNOS* enzyme is believed to regulate intracellular calcium levels.[9, 13] It is thought that *nNOS* inhibits the inward Ca^{2+} current through voltage dependent calcium channels, reducing the intracellular calcium concentrations. Thereby it suppresses β-adrenoreceptor stimulation of the heart. *nNOS* has also been associated with insulin release.[14, 15]

There are similarities between the effects of *nNOS* and sulfonylurea. Both *nNOS* and sulfonylurea influence the calcium influx through voltage dependent calcium channels. Moreover *nNOS* and sulfonylurea modulate the release of insulin by pancreatic β-cells. Both might be associated with cardiovascular mortality. In view of these similarities we hypothesized that genetic variation in the *NOS1AP* gene influences the glucose lowering effect of sulfonylurea and mortality risk in patients using sulfonylurea.

Methods

Setting. The data were obtained from the Rotterdam Study, a prospective population based closed cohort study in the suburb Ommoord in Rotterdam. All inhabitants who were 55 years of age or older and had lived in the district for at least one year, were invited between 1990 and 1993 to participate in the study. Of the 10,275 eligible persons, 7,983 participated and were followed since then. At baseline, trained interviewers administered a questionnaire during a home interview covering socioeconomic background and medical history, among other topics. During subsequent visits to the study center, laboratory assessments and clinical examinations were performed, including recording of ECGs. Follow-up examinations were carried out periodically (every 4 to 5 years). All participants of the Rotterdam Study gave written

informed consent. Ethical approval was obtained from the Medical Ethical Committee of the Erasmus Medical Center. The aim of the study was to investigate determinants of chronic and disabling cardiovascular, neurodegenerative, locomotor and ophthalmological diseases. The design of the Rotterdam Study has been described before.[16, 17] All mortality cases were identified, by obtaining at regular intervals the vital status of the participants from the municipal population registry. After notification of death, cause and circumstances were established by information from the general practitioner, letters, and in case of hospitalization, discharge reports from medical specialists were obtained. Two research physicians coded all events independently according to the International Classification of Diseases-10th edition.[18] In case of disagreement, consensus was sought. The seven pharmacies in Ommoord dispense the prescriptions of more than 99% of all participants. Information on all filled prescriptions from January 1st 1991 until January 1st 2005 was available and included the product name of the drug, the Anatomical Therapeutical Chemical (ATC) code, the amount dispensed, the prescribed dosage regimen and the date of dispensing.[19]

Cohort Definition. All participants of the Rotterdam Study, who received a prescription for an oral antidiabetic drug in the period between January 1st 1991 and January 1st 2005, were included in the study population at the time of the first prescription. These subjects were followed until death or end of the study period whichever came first.

Outcomes. Associations between the SNPs rs10494366 and rs10918594 in the *NOS1AP* gene, and prescribed doses of sulfonylurea, all-cause and cardiovascular mortality and first myocardial infarction were assessed. We used two types of study outcome, the change in prescribed dose of oral antidiabetic drugs compared to the first prescription and mortality while using antidiabetic drugs.

First, we analyzed differences between genotypes in prescribed doses in incident oral antidiabetic drug users. All subjects who received a first prescription for a sulfonylurea drug after July 1st, 1991 were included in this analysis and followed until the last prescription of that particular antidiabetic drug. July 1st, 1991 was chosen to ensure that we would have complete medication histories for at least half a year from January 1st, 1991. For every prescription of the oral antidiabetic drug the subject started on, the change in prescribed daily dose compared to the first prescription was calculated. As doses are titrated to avoid hypoglycemia and diabetes mellitus is a progressive disease, the prescribed doses of oral antidiabetic drugs usually increase over time. The prescribed daily dose is given as the number of defined daily dose (DDD) units, established by the World Health Organization, to make prescribed doses comparable between different drugs.[19] If subjects received more than one oral antidiabetic drug, the number of DDD units of the other drugs was added to the drug, which was prescribed first.

Second, we analyzed differences in all-cause mortality between genotypes within users of the same antidiabetic drug. We also analyzed differences in mortality in patients using metformin and insulin. In a subsequent analysis, we restricted to events that were coded as cardiovascular mortality. Similarly, we analyzed differences in the risk for a first (fatal and non-fatal) myocardial infarction.

Cofactors. The following characteristics were considered as potential determinants for affecting the change in prescribed daily dose of sulfonylurea after start: age, sex and calendar time. Determinants

potentially affecting the mortality rates were age, sex, QTc-interval at baseline, the cumulative prescribed dose of all oral antidiabetic drugs at the index date, the number of days the sulfonylurea of interest was prescribed until the index date and whether the subject used insulin at the index date. We also adjusted for current dihydropyridine calcium channel blocker use, because we recently found an association between genetic variation in *NOS1AP* and mortality in dihydropyridine calcium channel blocker users. The time of entrance in the Rotterdam Study was regarded as baseline and the results of physical examinations at the first visit were used in the analysis.

Genotyping. All participants were genotyped for the *NOS1AP* SNP rs10494366 T>G previously shown to be associated with QTc interval in five independent samples.[10-12] The correlated SNP rs10918594 C>G, which is in linkage disequilibrium, was also genotyped. Both were genotyped using Taqman assays C_1777074_10 and C1777009_10 (Applied Biosystems, Foster City, Ca., USA) in 1 ng of genomic DNA extracted from leukocytes, as previously reported.[11, 20]

Statistical analysis. A χ^2-test was used to test for deviation from Hardy-Weinberg equilibrium. We used unbalanced repeated measurements analysis to analyze the difference per genotype in the change in prescribed daily dose (in DDD units) in series of all consecutive prescriptions of oral antidiabetic drugs for the same subject compared to the prescribed daily dose of the first prescription. For these analyses, we used the PROC Mixed module of SAS (version 8.2). Cox proportional hazards analysis was used to analyze difference in mortality between genotypes in users of the same antidiabetic drug. For each antidiabetic drug, all subjects in the study population who died between July 1st,1991 and January 1st, 2005, while using that antidiabetic drug were identified as cases. The mortality date was taken as the index date. To each case we matched all persons in the cohort using that antidiabetic drug on the index date of the corresponding case. Subjects with missing values were excluded from the analyses. Cox proportional hazards analysis was also used for analyzing differences in first myocardial infarction between genotypes. These analyses were performed using SPSS software (version 11.0.1; SPSS, Chicago IL).

Results

In the Rotterdam Study, we identified 784 subjects who were prescribed oral antidiabetic drugs. One hundred thirty-four subjects were excluded because a blood sample was not available and 31 subjects were excluded because of failure to genotype successfully. Consequently, 619 subjects were available for the analysis (Table 1). We analyzed the associations between both SNPs rs10494366 and rs10918594 and the study outcomes. Since the associations with the SNP rs10494366 were stronger, only these results are presented. The minor allele frequency was 0.38 (G allele) and genotype distribution was in Hardy-Weinberg equilibrium (χ^2=1.94, p=0.38).

Four hundred fifty-two subjects received a first prescription for sulfonylurea between July 1st 1991 and January 1st 2005, and these patients were considered as incident users. There were no significant differences in starting dose among the genotypes. The average increase in prescribed daily dose for all consecutive prescriptions compared to the first prescriptions is given in Table 2. Among 74 patients using

Table 1 Characteristics of the study population by *NOS1AP* rs10494366 genotype

	rs10494366 genotype		
Characteristic	**TT**	**TG**	**GG**
Number	247	275	97
Gender, male	103 (41.7%)	118 (42.9%)	44 (45.4%)
Age (SD)	69.7 (8.3) years	69.1 (7.9) years	69.8 (8.5) years
Follow-up time (SD)	11.1 (3.3) years	11.0 (3.7) years	10.5 (4.0) years
Body mass index (SD)	28.0 (3.6) kg/m^2	28.2 (3.8) kg/m^2	28.6 (4.5) kg/m^2
Serum creatinine, µmol/l (SD)	85.0 (16.1) (n=198)	84.9 (17.0) (n=213)	84.3 (17.9) (n=70)
Drug use during follow up			
Glibenclamide	87 (35.2%)	109 (39.6%)	37 (38.1%)
Tolbutamide	137 (55.5%)	155 (56.4%)	55 (56.7%)
Gliclazide	43 (17.4%)	41 (14.9%)	10 (10.3%)
Glimepiride	56 (22.7%)	77 (28.0%)	23 (23.7%)
Metformin	141 (57.1%)	165 (60.0%)	55 (56.7%)
Insulin	49 (19.8%)	62 (22.5%)	19 (19.6%)

Figure 1 Change in prescribed daily dose (in DDD units) of glibenclamide plus co-prescribed oral antidiabetic drugs in patients starting with glibenclamide compared to the first prescription.

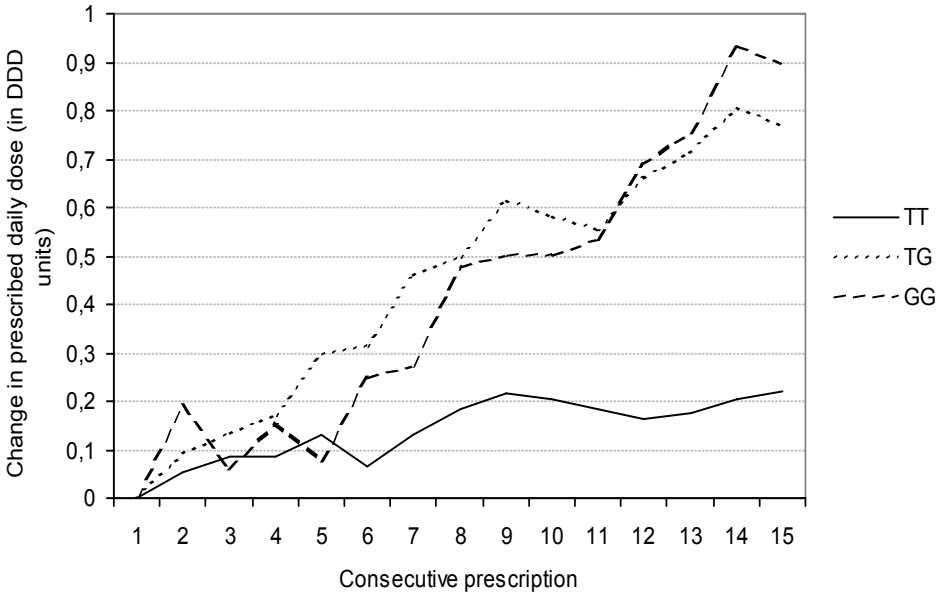

Table 2 Average change in prescribed dose of oral antidiabetic drugs (in DDD units) in incident sulfonylurea users by rs10494366 genotype.

Drug	TT		TG		GG	
	N		N	Change (95% CI)	N	Change (95% CI)
Glibenclamide	32	Reference	28	0.38 (0.14; 0.63)	14	0.11 (-0.32; 0.55)
Tolbutamide	115	Reference	116	-0.02 (-0.12; 0.08)	50	-0.05 (-0.19; 0.08)
Gliclazide	12	Reference	11	0.05 (-0.45; 0.55)	2	0.37 (-0.65; 1.39)
Glimepiride	24	Reference	41	0.16 (-0.22; 0.53)	7	0.26 (-0.43; 0.95)

Table 3 Association between the polymorphism rs10494366 in the *NOS1AP* gene and all-cause mortality in sulfonylurea users.

Drug and genotype	Cases[a]	Unadjusted HR	Adjusted HR (95% CI)[b]
Glibenclamide			
TT		Reference	Reference
TG	15	2.30	2.95 (1.02; 8.52)
GG	8	2.97	4.42 (1.23; 15.9)
Tolbutamide			
TT	21	Reference	Reference
TG	13	0.34	0.26 (0.11; 0.59)
GG	6	0.48	0.27 (0.09; 0.87)
Gliclazide			
TT	9	Reference	Reference
TG	3	0.41	0.32 (0.03; 3.27)
GG	0	-[c]	-[c]
Glimepiride			
TT	9	Reference	Reference
TG	7	0.50	0.15 (0.05; 0.75)
GG	2	-[c]	-[c]
Metformin			
TT	10	Reference	Reference
TG	12	0.86	0.82 (0.33; 2.06)
GG	7	1.45	1.65 (0.59; 4.55)
Insulin			
TT	16	Reference	Reference
TG	22	1.03	1.00 (0.48; 2.06)
GG	8	0.90	1.23(0.41; 3.68)

a Since patients can use more than one antidiabetic drug, these numbers do not add up to 142

b Adjusted for: age, sex, QTc time, prescribed doses oral antidiabetic drugs, duration of use, insulin use and dihydropyridine calcium channel blocker use

c Too few cases were available to calculate HR's

Table 4 Association between the polymorphism rs10494366 in the *NOS1AP* gene and cardiovascular mortality in sulfonylurea users.

	All-cause mortality		Cardiovascular mortality	
Drug and genotype	**N**	**HR (95% CI)[a]**	**N[b]**	**HR (95% CI)[a]**
Glibenclamide				
TT	6	Reference	4	Reference
TG or GG	23	2.80 (1.09; 7.22)	8	1.45 (0.34; 6.19)
Tolbutamide				
TT	21	Reference	11	Reference
TG or GG	19	0.30 (0.14; 0.63)	6	0.09 (0.02; 0.40)
Gliclazide				
TT	9	Reference	5	Reference
TG or GG	3	0.23 (0.02; 2.34)	0	-[c]
Glimepiride			3	
TT	9	Reference	3	Reference
TG or GG	9	0.18 (0.04; 0.74)		-[c]
Metformin				
TT	10	Reference	5	Reference
TG or GG	19	1.12 (0.50; 2.51)	7	1.10 (0.29; 4.23)
Insulin				
TT	16	Reference	7	Reference
TG or GG	30	1.03 (0.52; 2.01)	14	1.23 (0.43; 3.50)

a Adjusted for: age, sex, QTc time, prescribed doses oral antidiabetic drugs, duration of use, insulin use and dihydropyridine calcium channel blocker use

b Since patients can use more than one antidiabetic drug, these numbers do not add up to 63

c Too few cases were available to calculate HR's

glibenclamide, patients with the TG genotype received on average a daily-prescribed dose that was 0.38 DDD higher (95% CI 0.14; 0.63) than in patients with the TT genotype. The difference between patients with the GG genotype and the TT genotype was not significantly different (0.11 DDD, 95% CI –0.32; 0.55). The change in prescribed daily dose for consecutive prescriptions of glibenclamide is given in Figure 1. Patients with the GG genotype starting on glibenclamide were on average prescribed fewer prescriptions for glibenclamide than patients with the TG genotype (20.4 versus 27.4, p=0.04). For the other sulfonylurea no differences in prescribed doses were found.

In the cohort of 619 subjects, 156 subjects died during follow-up while using antidiabetic drugs. In 142 of the 156 cases we had complete follow-up information, including QTc-interval, and these subjects were used for the mortality analyses. In the group of glibenclamide users, both users with the TG genotype (Hazard Ratio (HR) 2.95, 95% CI 1.02; 8.52) and GG genotype (HR 4.42, 95% CI 1.23; 15.9) had a higher mortality rate than users with the TT genotype (Table 3). For tolbutamide (TG genotype HR 0.26, 95% CI 0.11; 0.59 and GG genotype HR 0.27, 95% CI 0.09; 0.27) and glimepiride (TG genotype HR 0.15, 95% CI 0.05; 0.75) the effects were the opposite. Subjects on these drugs with the TG and GG genotype

had lower mortality rates, although the numbers for the subjects with the GG genotype using glimepiride were too small to calculate hazard rates. In gliclazide users a non-significant protective effect for the TG genotype was found (95% CI 0.32, 95% CI 0.03; 3.27). No associations were found between all-cause mortality and *NOS1AP* genotype in metformin or insulin users.

In 63 of the 142 subjects who died during follow-up, the cause of death was categorized as cardiovascular. In Table 4 the associations between *NOS1AP* genotype and cardiovascular mortality are given. Since the hazard estimates for the all-cause mortality are suggestive of a dominant effect of the G-allele and the power is too low for separate analyses, we grouped patients with the TG and GG genotype. In glibenclamide users no differences were found in cardiovascular mortality between genotypes (HR 1.45, 95% CI 0.34; 6.19). With tolbutamide the decreased mortality in users with the TG or GG genotype seemed to be caused by a decrease in cardiovascular mortality. (HR 0.09, 95% CI 0.02; 0.40) For metformin and insulin, no differences in cardiovascular mortality were found.

Forty-nine cases of myocardial infarction were identified in subjects using antidiabetic drugs. The number of first myocardial infarctions in subjects using glibenclamide was too low to examine. In the group of subjects using tolbutamide, gliclazide or glimepiride, the hazard ratio for a myocardial infarction was 0.89 (95% CI 0.27; 2.97) for users with the TG or GG genotype compared to users with the TT genotype, albeit only 12 cases were identified. For subjects using insulin the hazard ratio was 1.34 (95% CI 0.48; 3.73).

Discussion

In this population based cohort study, the glucose lowering effect of glibenclamide seems to be less effective in users with the TG or GG genotype, because over time their daily dose is significantly higher than in users with the TT genotype. Moreover, for all sulfonylurea differences were found in mortality between patients with the TG or GG genotype and patients with the TT genotype. The effects of glibenclamide on mortality were opposite to the other sulfonylurea. In subjects using glibenclamide the TG and GG genotype were associated with an increased risk of mortality, while in subjects using tolbutamide and glimepiride this genotype was associated with a reduced risk of mortality. No differences were found in subjects using metformin or insulin.

Subjects with the TG genotype using glibenclamide were prescribed higher doses than subjects with the TT genotype. As prescribed doses are titrated according to glucose levels, it is likely that this is caused by a difference in glucose lowering effect. Subjects with the GG genotype, starting on glibenclamide, stopped sooner with using glibenclamide than subjects with the TG genotype. This may explain why no difference in the average prescribed dose was found for users with the GG genotype, although the changes in prescribed dose for users with the TG and GG genotype are similar in Figure 1.

Since the hazard rates for the TG and GG genotype are similar, we suggested a dominant effect of the G allele. If the underlying genetic effect operated through a recessive or additive effect, larger differences between the TG and GG would be expected. In the analyses of cardiovascular mortality we analyzed subjects with the TG or GG genotype as one group, because numbers were too low to analyze them separately. Only in subjects with the TG or GG genotype using tolbutamide, a decreased hazard rate for cardiovascular mortality was found.

The reduced all-cause mortality seen in subjects with the TG or GG genotype using tolbutamide and glimepiride may be caused by *NOS1AP* influencing the pharmacological pathway of sulfonylurea. In metformin and insulin users no differences were found, indicating that the differences are related to sulfonylurea use and not to the underlying disease. Also in gliclazide users a reduced mortality was seen, although not significant. Both *NOS1AP* and sulfonylurea regulate the Ca^{2+} influx by voltage dependent calcium channels. Sulfonylurea stimulate Ca^{2+} influx by blocking the KATP-channels, while the exact mechanism of *nNOS* is not known. In subjects with the TG or GG genotype using tolbutamide a reduced risk of cardiovascular mortality was seen. In subjects with the TG or GG genotype using gliclazide and glimepiride a reduction in cardiovascular mortality was the most likely explanation of the reduced all-cause mortality, although the differences were not significant. These effects on cardiovascular mortality in subjects using tolbutamide, gliclazide and glimepiride may be caused by the effect sulfonylurea have on the heart. More than one isoform of the SUR exist.[8, 21-23] The SUR1 isoform is found in the pancreas, the SUR2A isoform in the heart and skeletal muscle and the SUR2B isoform in vascular smooth muscles. The glucose lowering effect of sulfonylurea is accomplished by binding to the SUR1 receptor on the β-cell. Sulfonylurea also bind to other SUR isoforms. It is suggested that the affinity to the SUR2A isoform could be responsible for the effects on cardiovascular mortality.[21, 24-26] Under normal conditions the KATP-channels in the heart are closed. They open in response to metabolic stress such as ischemia, and the increasing total outward K^+ current shortens the action potential duration, decreases Ca^{2+} influx and contraction and conserves ATP. Binding to the SUR2A isoform by sulfonylurea may block this ATP conserving pathway and possibly influences survival of ischemic events.

The effects seen in subjects using glibenclamide were different from that seen in subjects using other sulfonylurea. Glibenclamide has a higher affinity for the SUR2A receptor than the other sulfonylurea.[8, 22, 24, 27-29] This difference in affinity by glibenclamide for the SUR2A receptor cannot explain all the results. Since SUR2A is only found on cardiac tissue, no differences would be expected in prescribed doses.

Glibenclamide is also an inhibitor of other channels than the KATP-channel.[30, 31] Studies have shown that beside the KATP-channel, other potassium channels are present in the β-cell, such as the Ca^{2+}-dependent K^+ channel.[32, 33] Blocking one or more of these channels by glibenclamide may be an alternative explanation for the results found in this study. A possible explanation for our results may be that there is a difference in effect on the Ca^{2+}-dependent K^+ channel between glibenclamide and other sulfonylurea. This explanation is supported by two observations. First, Ca^{2+}-dependent K^+ channel are also found in the pancreatic β-cell, influencing the firing of action potentials and possibly insulin release. Second, nitric oxide directly activates these Ca^{2+}-dependent K^+ channels, which could explain the role of *NOS1AP*.[34] Since we are not aware of studies assessing the influence of other sulfonylurea than glibenclamide on Ca^{2+}-dependent K^+ channels, we do not know whether differences in blocking these channels do attribute to the differences between glibenclamide and other sulfonylurea found in this study.

Although *nNOS* has previously been associated with insulin release we do not think that this association can explain the differences in prescribed doses and mortality risk in sulfonylurea users. The association with insulin release was too weak to explain the results and the associations were not found for metformin and insulin, suggesting that the association is related to sulfonylurea. As we adjusted for the QTc interval, also the QTc prolongating effect of *NOS1AP* is less likely to explain the observed results.

In population based studies, bias may affect the obtained results. We believe that bias in our study is minimal. Since diabetes mellitus is a progressive disease, co-prescription of other antidiabetic drugs and switching is common. Confounding by indication may have occurred if the risks at the start of a drug were different between genotypes, due to differences in the effect of previously prescribed drugs. This is for example the case if the genotype influences the rate of switching or co-prescription during previously prescribed drugs. However, if we adjusted for previously prescribed sulfonylurea, the results did not change. Therefore we do not think that confounding by indication did influence our results. Information bias is unlikely, since information was collected prospectively without prior knowledge of the study hypothesis. It is also unlikely that selection bias has occurred since we identified all patients with diabetes mellitus in a population based cohort study, and the absence of a blood sample and difficulties with genotyping were probably independent of the genotype.

Although there is always the possibility that the results are a chance finding, we think that this is probably not the case in our study. First, the analyses were not part of a genome wide association study. The SNP rs10494366 was associated with QTc prolongation in five independent populations before and we were testing whether this SNP affected prescribed doses and all-cause mortality in sulfonylurea users. Therefore, multiple testing did not bias our results. Second, significant associations with all-cause mortality were found for tolbutamide and glimepiride, while no significant associations were found for metformin and insulin. The point estimate for gliclazide was similar to the point estimate for tolbutamide and glimepiride, although not significant. Probably, this was due to lack of power in this group. For glibenclamide, we also found an association with all-cause mortality, although opposite to the effects of the other sulfonylurea. Differences in effect between glibenclamide and other sulfonylurea were seen before, although the differences were ascribed to differences in the affinity to the SUR2A receptor.

To conclude, the glucose lowering effect of glibenclamide in patients with the TG or GG genotype seems to be less effective. Moreover, genetic variation in the *NOS1AP* gene seems to predict the risk of mortality in patients using sulfonylurea. Although the exact mechanism has not been revealed, our results give a new insight into the pharmacological association between sulfonylurea use and cardiovascular mortality.

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5

Effect of *ABCB1* and *NOS1AP* genotypes on cardiac function

5

Effect of *ABCB1* and *NOS1AP* genotypes on cardiac function in digoxin users

Abstract

Background. Digoxin is one of the oldest cardiovascular drugs still in use today. It increases contractile force but delays relaxation of cardiomyocytes which may induce or aggravate diastolic dysfunction. Digoxin is a known substrate of *ABCB1* gene encoded P-glycoprotein and common *ABCB1* SNPs are associated with increased digoxin serum concentration. Also, we previously demonstrated that *NOS1AP* variants may interact with digoxin and influence cardiac function. Therefore, we hypothesized that *ABCB1* and *NOS1AP* SNPs might modify the effects of digoxin on systolic and diastolic cardiac function as assessed by echocardiography.

Methods. Digoxin users were identified in the Rotterdam Study, a prospective population-based cohort study of individuals aged 55 years and older. Participants who were not genotyped or with prevalent atrial fibrillation or flutter were excluded. Echocardiographic parameters were assessed using two-dimensional, M-mode and Doppler echocardiography. *ABCB1* and *NOS1AP* SNPs were assessed on DNA from peripheral blood lymphocytes using Taqman assays. We studied the association of *ABCB1* and *NOS1AP* genotypes and digoxin use with systolic and diastolic cardiac function. Analyses were performed stratified on prevalent heart failure or myocardial infarction.

Results. Echocardiograms and genotypes were available for 4827 participants, 59 of which used digoxin. We found some support for the hypothesis that common *ABCB1* variants potentiate the effects of digoxin on cardiac function. *NOS1AP* minor alleles seem to be associated with increased E/A ratio in digoxin users, with decreased systolic function in subjects with prevalent heart disease and with increased systolic function in those without. However, none of the associations was statistically significant.

Conclusion. The results point at a modulating effect of *ABCB1* and *NOS1AP* SNPs on the cardiac effects of digoxin, but the power was too low to reliably demonstrate or exclude any associations.

Introduction

Digoxin is one of the oldest cardiovascular drugs still in use today. It is widely used for treatment of atrial fibrillation and heart failure. Digoxin exerts its action, in part, by increasing intracellular Ca^{++} concentration ($[Ca^{++}]_i$), leading to higher contractile force of cardiomyocytes but to delayed relaxation which may induce or aggravate diastolic dysfunction.[1, 2]

Digoxin is a known substrate of P-glycoprotein, a multidrug efflux transporter, which is involved in the uptake, excretion and distribution of many drugs.[3, 4] P-glycoprotein is encoded by the ATP-Binding Cassette B1 (*ABCB1*) also known as the Multidrug Resistance 1 (MDR1) gene, a 200kb gene on chromosome 7p21. It was first discovered in chemotherapy resistant tumor cells but *ABCB1* is also widely expressed in normal tissues such as the duodenum, kidneys, liver and the heart.[5-10] The influence of *ABCB1* SNPs on digoxin kinetics has been subject of many studies. However, most studies were small and the results were not unequivocal.[11] In a recent study, we showed that common variants in *ABCB1* were associated with increased serum digoxin concentrations and risk of supra-therapeutic concentrations in a group of 195 chronic digoxin users from the Rotterdam Study.[12] These findings imply that *ABCB1* polymorphisms lead to increased serum digoxin concentrations and may thereby modulate the effects of digoxin on systolic and diastolic cardiac function.

Common variants of the nitric oxide synthase 1 adaptor protein (*NOS1AP*) gene were recently discovered to be associated with QT-interval prolongation in a genome wide association study.[13] We replicated this finding in the Rotterdam Study, a large population based cohort of persons 55 years and older.[14] So far, not much is known about the mechanism by which *NOS1AP* influences QT duration. *NOS1AP* activates neuronal nitric oxide synthase (*NOS1*). *NOS1* knockout cardiomyocytes have increased contractility through increased $[Ca^{++}]_i$ and a slower time to relaxation.[15-19] Furthermore, we recently demonstrated that *NOS1AP* variants and digoxin interact in QT-shortening and risk of sudden cardiac death to digoxin,[20] making a common pathway involving $[Ca^{++}]_i$ handling even more likely. Therefore, we hypothesized that these variants interact with digoxin possibly resulting in changed cardiac function.

We carried out a prospective cohort study within the Rotterdam Study, in order to analyze the influence of common *ABCB1* and *NOS1AP* variants on the effect of digoxin on systolic and diastolic cardiac function.

Methods

Study population. The study was performed within the framework of the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly.[21, 22] Baseline visits of the Rotterdam Study took place in 1990-1993. All inhabitants of a suburb of Rotterdam aged 55 years and over were invited and 7983 agreed to participate (response 78%). Follow-up visits took place approximately every four years. In 2000-2001, the cohort was extended with 3011 participants from the same suburb (response 67%), also aged 55 years and over. For the present study, data collection took place from 2002 to 2005. Within this period, the participants from the original cohort completed their fourth center visit (n=3550), and the participants of the extended cohort completed their

second center visit (n=2389). Of these, 3052 and 2235, respectively, underwent cardiac echocardiography. The large majority of missing echocardiograms was explained by incidental absence of echocardiographers.

Participants who were not genotyped for *ABCB1* (n=2431, *ABCB1* was not genotyped in the inception cohort) or *NOS1AP* (n=330), or those with atrial fibrillation or atrial flutter (n=137) at the time of echocardiography were excluded from the analysis. This resulted in a total of 2753 participants available for the *ABCB1* and 4827 for the *NOS1AP* analyses. The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians. The investigation is in accordance with the principles outlined in the declaration of Helsinki.

Assessment of risk factors and prevalent disease. Clinical characteristics including smoking, body mass index (BMI), hypertension, diabetes mellitus, heart failure and myocardial infarction were ascertained as previously described.[23-28] Active surveillance for incident diabetes mellitus, heart failure and myocardial infarction is conducted continuously between follow-up examinations. Presence of atrial fibrillation or atrial flutter at the time of echocardiography was assessed with electrocardiography by applying the Modular ECG Analysis System (MEANS), which has been extensively evaluated,[29, 30] and is characterized by a high sensitivity (96.6%) and a high specificity (99.5%) in coding arrhythmias.[31] For a small sample of participants, ECGs were not available at the time this report was written because of logistic problems and were approximated by using ECGs from the previous examination (year 2000-2001).

Genotyping. All participants from the baseline cohort of the Rotterdam Study for whom DNA was available were genotyped for the *ABCB1* C1236T, G2677T/A and C3435T polymorphisms. Genotyping was done using Taqman allelic discrimination assays as previously described.[12] Haplotypes were estimated using the estimation maximization algorithm and software as described in the statistical analyses section.

All participants from both cohorts for whom DNA was available were genotyped for the *NOS1AP* SNP rs10494366 T>G and the correlated SNP rs10918594 C>G, as previously described.[14, 32] Because SNP rs10494366 showed stronger evidence of association with QTc in the Rotterdam Study we considered it primary in the analyses.[14]

Digoxin exposure. Exposure of study participants to medications is gathered on a continuous basis since January 1st, 1991 through computerized pharmacy records from the study area covering >99% of prescriptions to the study population. In order to assess exposure to digoxin on the date of the echocardiography, we calculated the prescription duration of dispensed digoxin prescriptions as the number of units dispensed divided by the number of units to be taken per day. Digoxin dose was expressed as the defined daily dose (DDD) as determined by the WHO (1 DDD is 0.25mg of digoxin).

A study participant was considered to be exposed to digoxin if the echocardiography was taken on a date that fell within the duration of a dispensed digoxin prescription.

Echocardiography. Our primary endpoint was echocardiographic parameters of cardiac function. For each participant, an echocardiogram was obtained. The first 2188 echocardiograms were performed with a commercially available ultrasonography system (AU3 Partner, Esaote Biomedica, with a 3.5/2.5 MHz transducer). The following 3099 echocardiograms were performed with another commercially available system (Acuson Cypress, with a 3V2c transducer). A standardized protocol was used, including two-dimensional scanning in the parasternal long axis view, parasternal short axis view, apical view and subcostal view, M-mode scanning in the parasternal long axis view, and pulsed wave Doppler scanning in the apical four chamber view.[33] Echocardiograms were recorded onto VHS tape and assessed at the reading center, which was located at Erasmus Medical Center.

Several structural parameters were assessed.[34] Left ventricular end systolic dimension (LVES) and left ventricular end diastolic dimension (LVED), were measured in the parasternal long axis view using M-mode with two-dimensional guidance. With regard to systolic parameters, left ventricular ejection fraction was calculated as (end-diastolic volume minus end-systolic volume)/end-diastolic volume,[34] where the end-diastolic volume was $LVED^3$ and end-systolic volume was $LVES^3$, based on the cubed method. Fractional shortening at the endocardium was calculated as (LVED-LVES)/LVED*100%.[34] Global left ventricular systolic function was qualitatively assessed, without quantitative measurement, from the two-dimensional echocardiogram and classified as normal, fair, moderate or poor. Furthermore, diastolic parameters were measured.[35] Pulsed Doppler recordings of transmitral filling velocity were performed in the apical 4-chamber view, with the sample volume placed in the mitral valve orifice near the tips of the leaflets. Doppler peak E and peak A velocities were averaged over 3 cycles. E/A ratio was computed by dividing Doppler peak E velocity by Doppler peak A velocity. Early mitral valve velocity deceleration time was measured as the time between the peak E wave and the upper deceleration slope extrapolated to the zero baseline.

Echocardiograms were made and read by 4 trained echocardiographers blinded to the research question and exposure. To assess intra-reader and inter-reader agreement, 32 participants were examined in duplicate. For continuous variables, overall median percent intra-reader and inter-reader measurement variabilities were calculated as the absolute measurement difference divided by the average of the two measurements, multiplied by 100. Overall median intra-reader and inter-reader variabilities for left ventricular end-diastolic dimension were 3% and 4%, respectively. For left ventricular end-systolic dimension both variabilities were 6%, and for fractional shortening both were 8%. Overall median intra-reader and inter-reader variabilities for E/A ratio were 15% and 18%, respectively, and for deceleration time both were 16%. Finally, the percentage agreement for categorization of left ventricular systolic function as normal or other than normal was 82% within readers and 86% between readers.

Statistical analysis. We calculated means, medians and proportions of cardiovascular risk factors and echocardiographic characteristics in men and women. We categorized E/A ratio and deceleration time according to cut points used in previous reports. Normal diastolic function was defined as E/A ratio between 0.75 and 1.50 and deceleration time between 150 ms and 240 ms. Impaired relaxation was defined as E/A ratio < 0.75 and deceleration time > 240 ms. Restrictive diastolic dysfunction was defined as E/A ratio > 1.50 and deceleration time < 150 ms.[36, 37] Participants were required to have both Doppler criteria

consistent with impaired relaxation or restrictive dysfunction in order to be classified. Participants with one abnormal criterion were classified as indeterminate rather than as normal.

Exact Hardy Weinberg equilibrium p-values were computed for bi-allelic SNPs and a simulated p-value for the tri-allelic SNP were computed for both the total population and digoxin users, using the Genetics 1.2.1 (G.Warns and F. Leisch) package for R 2.5.0 software. Genetic linkage, as expressed by D' was calculated using the ldmax command from GOLD software.[38] Expectation maximization for the haplotypes was performed with the HaploStats 1.3.0 package for R 2.5.0, using haplo.em.[39, 40] Inferred haplotypes with a posterior probability < 0.95 were excluded from further analyses.

Linear regression was used to test the association of genotypes or haplotypes and digoxin use with systolic parameters (ejection fraction, fractional shortening) and diastolic parameters (mitral valve inflow peak E velocity, mitral valve inflow peak A velocity, E/A ratio and mitral valve inflow deceleration time) as dependent variables. To test for the effect of *ABCB1* and *NOS1AP* genotypes on digoxin induced changes in echocardiographic parameters, we created an interaction dummy variable with six levels for the three genotypes with and without digoxin use in which non-digoxin users homozygous for the major allele were considered as referent. Furthermore, to minimize the influence of differences in underlying cardiovascular disease -as present between digoxin users and non-digoxin users (confounding by indication)-, we performed the analyses stratified on prevalent heart failure or myocardial infarction. For the E/A ratio, the distribution of the residuals was skewed. After log-transformation the residuals were normally distributed with a constant variance. We adjusted for age, sex and type of ultrasonography system used. The low number of digoxin exposed subjects did not allow adjustment for additional risk factors or use of other drugs. All analyses were performed using SPSS 11.0 (Chicago, Illinois, USA).

Results

Study population and genotyping. Distribution of cardiovascular risk factors and drug use in *ABCB1* and *NOS1AP* study populations and in digoxin users is shown in Table 1. Echocardiographic characteristics for these groups are shown in Table 2.

Successful genotype calls for *ABCB1* C1236T, G2677T/A and C3435T were made in 97.1%, 95.5% and 95.5% respectively and for *NOS1AP* rs10494366 and rs10918594 in 96.9% and 97.8% respectively. Allele frequencies were: 1236T 44.2%, 2677T 43.9%, 2677A 2.2%, 3435T 54.0%, rs10494366 G 36.7% and rs10918594 G 31.5%. Hardy-Weinberg equilibrium p-values were: C1236T p= 0.39, G2677T/A p= 0.002, C3435T p= 0.21, rs10494366 p= 0.93 and rs10918594 p= 0.75; the finding that G2677T/A is out of Hardy-Weinberg equilibrium is explained by a relative excess of homozygotes for the rare A-allele and is probably a chance finding. We excluded *ABCB1* 2677A allele carriers from further analyses because this allele was too rare to gain reliable results. The three *ABCB1* SNPs are in strong linkage disequilibrium, as previously reported.[3, 41] D' was 0.94 between C1236T and G2677T/A, 0.84 between G2677T/A and C3435T, and 0.80 between C1236T and C3435T. D' between the two *NOS1AP* SNPs was 0.88. Expectation maximization resulted in two major haplotype alleles, the 1236-2677-3435 C-G-C and T-T-T haplotypes, both at an allele frequency of 40%, a C-G-T haplotype with a frequency of 12% and a number of rare haplotypes. After exclusion of subjects with <0.95 posterior probability haplotype pairs, the mean

Table 1 Population characteristics

Characteristic	*NOS1AP* genotyped	*ABCB1* genotyped	Digoxin users
Number	4827	2753	59
Women, %	57.0	58.2	55.9
Age at echo, years	72.1 (7.5)	75.4 (6.1)	80.3 (6.1)
Body mass index, kg/m2	27.6 (4.1)	27.4 (4.1)	26.0 (4.4)
Systolic blood pressure, mmHg	149.7 (21.2.)	152.9 (21.6)	145.6 (20.4)
Diastolic blood pressure, mmHg	79.8 (10.9)	79.4 (11.2)	70.9 (11.5)
Total cholesterol, mmol/l	5.6 (1.0)	5.6 (1.0)	5.3 (0.9)
HDL-cholesterol, mmol/l	1.5 (0.4)	1.4 (0.4)	1.3 (0.4)
Prevalent diabetes mellitus, %	14.0	15.2	28.8
Prevalent heart failure, %	5.2	7.0	52.5
Prevalent myocardial infarction, %	10.5	14.2	33.9
Smoking			
Never, %	29.7	29.7	33.9
Former, %	54.9	56.5	45.8
Current, %	15.4	13.9	20.3
Use of digoxin, %	1.2	1.8	100

The group of *NOS1AP* genotyped consists of all genotyped subjects from both the baseline and the extension cohort, the *ABCB1* group comprises only genotyped participants from the baseline cohort, the digoxin group consists of all genotyped digoxin users from both cohorts. Mean (SD) is shown for continuous variables and for proportions, percentages are shown.

posterior probability was 0.99. Genotype distributions did not statistically significantly differ between the general population and digoxin users. Genotype and haplotype frequencies were consistent with other European populations.[13, 41-45]

Digoxin. The effects of digoxin use on systolic and diastolic parameters is shown in Table 3. Digoxin was associated with a slightly increased E/A ratio and decreased systolic function. Also, mitral valve inflow deceleration time was increased in participants with prevalent heart failure or myocardial infarction but decreased in those without. However, none of these digoxin effects was statistically significant.

***ABCB1* and digoxin.** Although the number of digoxin was to low to obtain statistically robust estimates, the point estimates suggest a subtle effect of *ABCB1* TTT haplotype on cardiac function in digoxin users (Table 4). In the stratum of participants without prevalent heart disease, digoxin users carrying the TTT haplotype (heterozygotes plus 1 homozygous TTT) showed improved ejection fraction and fractional shortening compared to those not carrying this haplotype. However, the effect of TTT haplotype alleles in combination with digoxin seemed opposite in the prevalent heart disease stratum. Also, TTT-haplotype appeared to drive digoxin users more towards diastolic dysfunction. E/A ratio in digoxin users was lower (Table 3), and E/A ratio was lower in digoxin users with the TTT-haplotype in presence or absence of

Table 2 Echocardiographic characteristics

Echocardiographic parameter	*NOS1AP* genotyped	*ABCB1* genotyped	Digoxin users
Number	4827	2753	59
Structural parameters			
Left ventricular end systolic dimension, mm (median (IQR))	31.0 (28.0-35.0)	32.0 (28.0-35.0)	34.0 (28.0-43.0)
Left ventricular end diastolic dimension, mm (mean (SD))	51.2 (5.6)	50.6 (5.9)	53.1 (8.5)
Systolic parameters			
Ejection fraction, % (median (IQR))	78.0 (71.3-82.1)	73.7 (68.6-80.6)	71.1 (61.3-78.9)
Fractional shortening, % (mean (SD))	38.5 (7.1)	36.9 (7.5)	33.2 (10.1)
Left ventricular function, %[a]			
Normal	56.1	44.8	25.9
Fair	38.1	47.9	43.1
Moderate	4.1	5.1	19.0
Poor	1.7	2.3	12.1
Diastolic parameters			
Mitral valve inflow peak E, m/s (mean (SD))	0.65 (0.16)	0.64 (0.17)	0.68 (0.23)
Mitral valve inflow peak A, m/s (mean (SD))	0.77 (0.18)	0.78 (0.18)	0.75 (0.20)
E/A ratio (median (IQR))	0.83 (0.71-1.00)	0.80 (0.67-1.00)	0.75 ((0.65-1.00)
Mitral valve inflow deceleration time, msec (mean (SD))	215.3 (47.9)	219.3 (51.3)	223.5 (66.7)
Diastolic function, %[b]			
Normal	51.3	45.0	35.4
Impaired relaxation	12.2	15.1	14.6
Restrictive	0.5	0.5	2.1
Indeterminate	36.0	39.4	47.9

The group of *NOS1AP* genotyped consists of all genotyped subjects from both the baseline and the extension cohort, the *ABCB1* group comprises only genotyped participants from the baseline cohort, the digoxin group consists of all genotyped digoxin users from both cohorts. For normally distributed variables, mean (SD) is displayed. If a variable was not normally distributed, median (inter quartile range (IQR)) is shown.

a Qualitative assessment of ventricular systolic function

b Normal diastolic function: E/A ratio between 0.75 and 1.50 and deceleration time between 150 ms and 240 ms, impaired relaxation: E/A ratio < 0.75 and deceleration time > 240 ms, restrictive diastolic dysfunction: E/A ratio > 1.50 and deceleration time < 150 ms.

Table 3 Association between digoxin use and systolic or diastolic properties on echocardiography

Variable	B (95% CI)	
	No prevalent heart failure or myocardial infarction (22 users of digoxin)	Prevalent heart failure or myocardial infarction (37 users of digoxin)
Systolic function		
Ejection fraction (%)	-1.5 (-4.8;1.7)	-3.7 (-7.9;0.4)
Fractional shortening (%)	-0.2 (-2.8;2.5)	-2.7 (-5.6;0.3)
Diastolic function		
Mitral valve inflow peak E (m/s)	0.00 (-0.06;0.06)	0.06 (-0.01;0.13)
Mitral valve inflow peak A (m/s)	-0.10 (-0.17;-0.02)	-0.03 (-0.10;0.04)
Ln E/A ratio[a]	0.08 (-0.03;0.20)	0.06 (-0.06;0.18)
Mitral valve inflow deceleration time	-17.6 (-37.7;2.6)	11.6 (-10.0;33.3)

All *NOS1AP* genotyped subjects, adjusted for age, sex and ultrasonography system.

a Log-transformed because E/A ratio and residuals were not normally distributed, after log-transformation residuals were normally distributed.

prevalent heart disease (Table 4). Results were less equivocal for mitral valve inflow deceleration time, but digoxin users homozygous for the TTT haplotype seemed to have increased deceleration time, also driving towards impaired relaxation. For the individual SNPs we found similar effects (results not shown). In table 6, the results for an allelic model are displayed stratified on digoxin use and prevalent heart disease.

***NOS1AP* and digoxin.** The *NOS1AP* cohort was larger, with extra participants from the second inception cohort. Since this cohort was younger at the time of echocardiography, the extension resulted in only few extra digoxin users. Also, the *NOS1AP* variants were less frequent, resulting in little or no extra power compared to the *ABCB1* study population. Therefore, still no robust effect estimates could be obtained. Similar to our observations for the *ABCB1* TTT-haplotype, in the stratum of participants without prevalent heart disease, digoxin users carrying the rs10494366 minor allele haplotype showed improved ejection fraction and fractional shortening compared to those not carrying this haplotype. Again, the effect of the minor allele seemed opposite in the prevalent heart disease stratum (Table 5). In diastole, digoxin users carrying the minor allele seemed to have an increased E/A ratio. Deceleration time seemed increased in digoxin using minor allele carriers in the prevalent heart disease stratum, but the results might be opposite for the non-heart disease stratum. Similar results were observed for rs10918594 minor allele carriers.
We observed no effect of *NOS1AP* genotype on systolic or diastolic function in non-users of digoxin. In table 6, the results for an allelic model are displayed (where applicable) stratified on digoxin use and prevalent heart disease, but none of the results showed a significant association.

Table 4 Effect of digoxin use and *ABCB1* TTT haplotype on systolic and diastolic function

Echocardiographic variable	No prevalent heart failure or myocardial infarction		Prevalent heart failure or myocardial infarction	
	N	B (95% CI)	N	B (95% CI)
Ejection fraction (%)				
0 TTT no digoxin	652	Reference	129	Reference
1 TTT no digoxin	895	0.06 (-0.91;0.78)	194	-0.60 (-3.27;2.07)
2 TTT no digoxin	318	0.96 (-0.17;2.08)	68	-3.59 (-7.12;-0.06)
0 TTT digoxin	5	-11.70 (-19.08;-4.33)	15	-4.16 (-10.58;2.26)
1 TTT digoxin	9	3.13 (-2.38;8.63)	7	-2.95 (-12.1;6.20)
2 TTT digoxin	1	15.63 (not calculated)[b]	5	-11.62 (-22.31;-0.92)
Fractional shortening (%)				
0 TTT no digoxin	652	Reference	129	Reference
1 TTT no digoxin	895	-0.01 (-0.70;0.68)	194	-0.48 (-2.38;1.43)
2 TTT no digoxin	318	0.82 (-0.09;1.74)	68	-2.59 (-5.10;-0.08)
0 TTT digoxin	5	-6.40 (-12.39;-0.40)	15	-3.28 (-7.85;1.29)
1 TTT digoxin	9	2.39 (-2.09;6.86)	7	-2.63 (-9.14;3.88)
2 TTT digoxin	1	16.06 (not calculated)[b]	5	-7.58 (-15.19;0.03)
Ln E/A ratio[a]				
0 TTT no digoxin	676	Reference	137	Reference
1 TTT no digoxin	928	-0.01 (-0.03;0.02)	200	-0.04 (-0.11;0.04)
2 TTT no digoxin	325	-0.01 (-0.05;0.02)	76	0.01 (-0.09;0.10)
0 TTT digoxin	5	0.19 (-0.04;0.42)	14	-0.02 (-0.21;0.16)
1 TTT digoxin	6	0.21 (0.01;0.42)	8	0.19 (-0.05;0.43)
2 TTT digoxin	1	0.02 (not calculated)[b]	4	-0.37 (-0.70;-0.04)
Mitral valve inflow deceleration time				
0 TTT no digoxin	666	Reference	135	Reference
1 TTT no digoxin	914	-0.7 (-5.4;4.1)	202	3.8 (-9.8;17.4)
2 TTT no digoxin	318	6.1 (-0.2;12.3)	76	6.0 (-11.6;23.5)
0 TTT digoxin	5	-11.8 (-53.4;29.8)	15	2.9 (-30.4;36.1)
1 TTT digoxin	7	-41.2 (-76.3;-6.1)	7	-7.7 (-55.4;39.9)
2 TTT digoxin	1	7.9 (not calculated)[b]	4	39.0 (-22.8;100.9)

Numbers may differ between genotype and digoxin use groups due to missings in echography measures. All adjusted for age, sex and ultrasonography system.

a Log-transformed because E/A ratio and residuals were not normally distributed, after log-transformation residuals were normally distributed

b No confidence interval was calculated since n=1

Table 5 Effect of digoxin use and *NOS1AP* genotype on systolic and diastolic function

Echocardiographic variable	No prevalent heart failure or myocardial infarction		Prevalent heart failure or myocardial infarction	
	N	B (95% CI)	N	B (95% CI)
Ejection fraction (%)				
TT no digoxin	1518	Reference	218	Reference
TG no digoxin	1753	-0.02 (-0.49;0.52)	266	0.78 (-1.30;2.87)
GG no digoxin	521	0.07 (-0.66;0.80)	78	0.34 (-2.65;3.33)
TT digoxin	14	-4.55 (-8.49;-0.62)	16	-1.69 (-7.56;4.38)
TG digoxin	7	4.50 (-1.03;10.04)	16	-3.46 (-9.48;2.56)
GG digoxin	0	NA	2	-14.82 (-31.37;1.74)
Fractional shortening (%)				
TT no digoxin	1518	Reference	218	Reference
TG no digoxin	1753	-0.02 (-0.44;0.39)	266	0.39 (-1.11;1.89)
GG no digoxin	521	0.02 (-0.58;0.62)	78	0.30 (-1.85;2.44)
TT digoxin	14	-2.12 (-5.36;1.12)	16	1.50 (-5.86;2.86)
TG digoxin	7	3.71 (-0.86;8.27)	16	-2.45 (-6.77;1.88)
GG digoxin	0	NA	2	-9.76 (-21.66;2.13)
Ln E/A ratio[a]				
TT no digoxin	1543	Reference	225	Reference
TG no digoxin	1785	-0.01 (-0.03;0.01)	269	0.01 (-0.05;0.06)
GG no digoxin	532	-0.02 (-0.04;0.01)	80	0.00 (-0.08;0.09)
TT digoxin	14	-0.06 (-0.19;0.07)	11	-0.07 (-0.27;0.13)
TG digoxin	4	0.56 (0.33;0.81)	18	0.11 (-0.05;0.27)
GG digoxin	0	NA	2	0.37 (-0.09;0.83)
Mitral valve inflow deceleration time				
TT no digoxin	1539	Reference	224	Reference
TG no digoxin	1765	0.01 (-3.0;3.0)	274	-4.9 (-15.2;5.4)
GG no digoxin	524	3.4 (-1.0;7.7)	81	-2.1 (16.8;12.7)
TT digoxin	14	-9.5 (-33.0;14.0)	12	-10.2 (-44.7;24.3)
TG digoxin	5	-38.3 (-77.4;0.9)	18	-2.1 (-30.6;26.5)
GG digoxin	0	NA	2	221.5 (138.8;304.2)

NOS1AP results are shown for rs10494366, results for rs10918594 were similar.Numbers may differ between genotype and digoxin use groups due to missings in echography measures. All adjusted for age, sex and ultrasonography system. NA = not applicable

a Log-transformed because E/A ratio and residuals were not normally distributed, after log-transformation residuals were normally distributed

Table 6 Effect of *ABCB1* haplotype and *NOS1AP* genotype on systolic and diastolic function stratified on digoxin use, allelic model

Echocardiographic variable	No prevalent heart failure or myocardial infarction		Prevalent heart failure or myocardial infarction	
	B (95% CI)		B (95% CI)	
	No digoxin	Digoxin	No digoxin	Digoxin
Ejection fraction (%)				
Per *ABCB1* TTT-allele	0.4 (-0.2;0.9)	20.3 (4.5;36.0)	-1.6 (-3.3;0.1)	-2.8 (-9.0;3.3)
Per *NOS1AP* G-allele	0.1 (-0.3;0.4)	NA	0.3 (-1.1;1.7)	-3.5(-11.8;4.9)
Fractional shortening (%)				
Per *ABCB1* TTT-allele	0.3 (-0.1;0.8)	14.6 (5.6;23.6)	-1.1 (-2.4;0.1)	-1.7 (-5.9;2.5)
Per *NOS1AP* G-allele	0.0 (-0.2;0.3)	NA	0.2 (-0.8;1.2)	-2.3 (-8.0;3.3)
Ln E/A ratio[a]				
Per *ABCB1* TTT-allele	-0.01 (-0.02;0.01)	-0.17 (-0.85;0.51)	0.00 (-0.05;0.04)	-0.05 (-0.24;0.13)
Per *NOS1AP* G-allele	-0.01 (-0.02;0.00)	NA	0.01 (-0.03;0.05)	0.20 (-0.04;0.44)
Mitral valve inflow deceleration time				
Per *ABCB1* TTT-allele	2.4 (-0.7;5.4)	20.9 (-19.6;61.3)	3.1 (-5.6;11.8)	8.8 (-19.1;36.7)
Per *NOS1AP* G-allele	1.3 (-0.7;3.4)	NA	-2.1 (-9.1;5.0)	57.0 (13.7;100.3)

Estimates show the increase in the specific echocardiographic parameter per additional allele. All adjusted for age, sex and ultrasonography system. NA = not applicable. *NOS1AP* results are shown for rs10494366, results for rs10918594 were similar.

a Log-transformed because E/A ratio and residuals were not normally distributed, after log-transformation residuals were normally distributed

Discussion

Digoxin is known to improve systolic function and to increase the risk of diastolic dysfunction.[1, 2] It is also a known substrate for *ABCB1* encoded P-glycoprotein[3, 4] and we previously found evidence for interaction with *NOS1AP* variants.[20] In the present study, we found some indication that common variants in the *ABCB1* gene were associated with increased systolic function in digoxin users without prevalent heart disease, but wit decreased function in those with prevalent heart failure or myocardial infarction. Similar results were seen for *NOS1AP* minor allele carriers. Also, the *ABCB1* variants seemed to be associated with decreased diastolic function in digoxin users, driving their echocardiographic parameters towards impaired relaxation. In contrast with *ABCB1* variants, digoxin users with *NOS1AP* variants showed increased E/A ratios, but, similar to the observed effect in *ABCB1* 1236-2677-3435 TTT-allele carriers, increased deceleration time in subjects with prevalent heart disease and decreased deceleration time in those without. This might suggest a protective effect of the *NOS1AP* minor allele against impaired relaxation. However, the number of digoxin users with an echocardiogram was too low draw to solid conclusions and to reach statistical significance.

The suggested increased risk of diastolic failure through impaired relaxation in digoxin users with the *ABCB1* TTT-haplotype allele is in concordance with our previous finding that *ABCB1* TTT haplotype carriers have increased digoxin serum concentration.[12] Also, in another previous study using the population of the Rotterdam Study, we were able to demonstrate that *ABCB1* genotype was associated with increased QT-interval shortening to digoxin, further enforcing the potential of *ABCB1* TTT-haplotypes to potentiate digoxin effects.[46] The allele dose-effect of *ABCB1* TTT-haplotype on increased ejection fraction to digoxin in those without prevalent heart disease is also in line with these findings. These effects are expected as increased digoxin effects. The fact that we observed an opposite effect in those with prevalent myocardial infarction or heart failure is not, but might be partly explained by confounding by severity as it is likely that in those with prevalent heart failure, the more severe cases are treated with digoxin. This hypothesis is supported by the finding that digoxin use in the overall analyses in the group with prevalent heart disease was associated with decreased systolic function (Table 3). Furthermore, our results for participants with prevalent heart disease not using digoxin could suggest that *ABCB1* TTT-haplotype is associated with decreased systolic function in this group. If true, that might explain the observed discrepancy.

Since digoxin increases $[Ca^{++}]_i$ [1, 2] and *NOS1AP* minor alleles may do the same,[15-19] an increased systolic function, as observed in digoxin users without prevalent heart disease, and decreased diastolic function were expected. However, we observed decreased systolic function in minor allele carriers with prevalent heart disease using digoxin. Furthermore, E/A ratio was increased in digoxin with and without prevalent heart disease and the results for deceleration time were unequivocal. Too little is known about normal *NOS1AP* function to explain these findings. Also, numbers are too small to draw solid conclusions, increasing the possibility of a spurious association.

The main strength of the study lies in the population based prospective approach. Both echocardiography and medication prescription is blinded to genotype. Therefore, no selection bias is to be expected. Although confounding by indication, through the potential of an association between genotype and heart disease, cannot fully be excluded, the stratification on prevalent heart failure or myocardial infarction

should limit its influence. However, even such a stratification can not exclude confounding by severity of heart failure in those who receive digoxin. Moreover, the number of subjects who were exposed to digoxin at the time of the echocardiogram was very low, resulting in little power to detect an effect of genotype on the association of digoxin and systolic and diastolic function. The small sample size also increases the risk of finding a spurious association, since it limited the possibilities of adjusting for possible other cardiovascular risk factors. The fact that most findings for *ABCB1* genotypes are in line with our previous findings[12, 46] and with known mechanisms does, however, endorse the possibility of an effect of *ABCB1* SNPs on cardiac function in digoxin users.

In conclusion, in line with previous studies on *ABCB1* genotype and digoxin in the Rotterdam Study,[12, 46] we found some support that common *ABCB1* variants increase the effects of digoxin on systolic (increased) and diastolic (decreased) cardiac function. *NOS1AP* minor alleles seem to be associated with increased E/A ratio in digoxin users, with decreased systolic function in subjects with prevalent heart disease and with increased systolic function in those without. However, power was too low to reliably demonstrate or exclude any associations. Larger studies of echocardiograms -or other imaging techniques of the heart- in digoxin users will be needed to further clarify the exact role of these genes in the effects of digoxin on cardiac function.

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6

General discussion

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General discussion

Despite all research efforts on safety and efficacy preceding marketing authorization of drugs, not every drug is safe and beneficial for every patient. Different people do not always react in the same way to the same drugs at the same dose. Pharmacogenetic and pharmacogenomic research helps to identify genetic factors that may predict the chance of success and the risk of adverse outcomes of drug therapy. The objective of this thesis was to explore the effect of interaction of common variants in the *ABCB1* and *NOS1AP* genes with drugs on drug response and adverse effects. Most studies were conducted within the Rotterdam Study, a population-based cohort study among men and women aged 55 years and over.[1, 2] In this chapter, the main findings and methodological considerations are discussed. Furthermore, potential clinical implications and future directions for future research will be discussed.

Main findings

Risk of digoxin intoxication. One of the oldest drugs still in use today is the cardiac glycoside digoxin. It has been successfully used in the treatment of heart disease for more than 200 years. The use of an extract of Digitalis purpurea, commonly known in English as the Foxglove, containing cardiac glycosides for the treatment of heart conditions was first described by William Withering in 1785,[3] which is considered the beginning of modern therapeutics. However, since its first introduction digoxin has been associated with toxicity. Studies on toxicity over the period 1969 to 1983 showed a frequency in digitalis users of as high as 11 – 30%.[4-6] Digitalis intoxication was associated with high mortality and frequent occurrence of life-threatening arrhythmias.[6, 7] Improvements in therapy management and monitoring have resulted in a major reduction of the incidence of digoxin intoxication and an improved prognosis over the last decades. More recent studies showed digoxin intoxication in 4.8-0.9% of digoxin users.[8-12] However, the latest studies date from the late 1980s and no recent incidence figures of digoxin intoxication are available. Furthermore, recent literature suggests that women are at an increased risk of digoxin toxicity.[11, 13]

Using a national computerized hospital discharge registry and extrapolated drug figures, we performed a nationwide cohort-study of all hospital admissions in the years 2001-2004. In this study, we show that the relative number of digoxin intoxication requiring hospitalization is rather low in The Netherlands (around

2 per 1000 person years of use). Although there may have been some underrecognition of cases, these findings seem to confirm that current management of digoxin treatment has reduced the risk of digoxin toxicity dramatically. Furthermore, we found that women have a 40% higher risk of intoxication than men (95%CI: 30-60%)(**Chapter 2**). Our observations of a decreased incidence of digoxin intoxication and increased risk of intoxication for women were confirmed in a recent German study covering the same years.14

***ABCB1* variants and drug effects.** Digoxin serum concentration is an important factor in digoxin toxicity within one individual. However, toxic concentrations may occur at therapeutic doses and concentrations differ between persons receiving the same dose. Moreover, toxic effects of digoxin can appear within the accepted therapeutic serum concentration range.[15] Digoxin is a known substrate of P-glycoprotein, a multi-drug efflux transporter encoded by the ATP-Binding Cassette B1 (*ABCB1*). *ABCB1* is widely expressed in normal tissues such as the duodenum, kidneys, liver and the blood-brain barrier where it plays a role in the uptake and clearance of many drugs.[16-22] *ABCB1* is a large and polymorphic gene[23] and one of the first single nucleotide polymorphisms (SNPs) identified in the gene was found to be associated with digoxin concentration.[24] Since then, many studies on of *ABCB1* SNPs and digoxin kinetics have been performed, some showed an effect of *ABCB1* on digoxin kinetics,[24-29] whereas others did not.[30-33] However, most studies were small and they were performed in groups with different ethnic backgrounds, which may explain some of the differences by variation in SNP and haplotype allele frequencies between ethnic groups. For instance, the 2677A variant is more frequent in certain Asian populations but very rare in Caucasians.[34, 35]

In digoxin users from the Rotterdam Study with digoxin serum concentrations available, we found that the common *ABCB1* C1236T, G2677T and C3435T variants and the associated 1236-2677-3435 TTT-haplotype were associated with higher digoxin serum concentrations (**Chapter 3.1**). Moreover, we found that *ABCB1* variants explain an important part of the variability in digoxin concentrations in a model also containing age, sex, digoxin dose, lean body mass and renal clearance (up to 11.5% by the TTT haplotype as determined by the r^2). The increase in serum concentration in *ABCB1* TTT-haplotype allele carriers equaled the effect of a 0.25 DDD (1DDD= 0.25mg) dose increase. The majority of previous studies in Caucasians showed similar effects of *ABCB1* variants on digoxin kinetics, but a meta-analysis could not confirm an effect for the C3435T SNP.[36] The main difference with our study is that most studies comprised only single dose kinetics and were underpowered to demonstrate small effects. Only one study was performed in actual digoxin using patients.[33] and did not show an association of *ABCB1* variants with digoxin kinetics. However, the dose was almost twice that of the average daily dose in our study. At these higher dosages, the maximum efflux capacity of *ABCB1* might be saturated and other transport mechanisms may become more important.

In a study using all genotyped digoxin users and non-users of digoxin, we found a more than two fold increased risk of sudden cardiac death (SCD) in digoxin users homozygous for *ABCB1* variant alleles (**Chapter 3.2**). No increase of SCD risk was found in non-users of digoxin with *ABCB1* variant alleles. This is the first study to show an association between *ABCB1* variants and the risk of toxic effects of digoxin. The interaction of *ABCB1* and digoxin was further confirmed by its influence on the QTc short-

ening properties of digoxin on ECG. Apart from the influence on QTc-shortening to digoxin, we found echocardiographic indications that the *ABCB1* TTT-haplotype allele was associated with decreased diastolic function in digoxin users, driving their echocardiographic parameters towards impaired relaxation. The TTT-haplotype allele was also associated with an increased systolic function in digoxin users without prevalent heart disease, but with a decreased function in those with prevalent heart failure or myocardial infarction (**Chapter 5**). Although the latter associations were not statistically significant, probably due to low numbers, these observations (apart from the decreased systolic function in those with prevalent heart disease) are in line with the findings of increased digoxin concentrations in TTT-haplotype allele carriers and the known effects of digoxin on cardiac function.[37, 38] Unfortunately, the number of digoxin users with eligible echocardiograms available was too low to draw solid conclusions from this study.

The quinolone derivative mefloquine is widely used in the treatment and prophylaxis of malaria in travellers to areas with chloroquine resistant falciparum malaria but its use is associated with, sometimes severe, neuropsychiatric adverse effects.[39-41] Risk factors for adverse effects of mefloquine identified so far include history of seizures or psychiatric disorders, female gender and low body mass index (BMI).[42, 43] It has been suggested that the distribution volume or blood concentration of mefloquine plays a role, although no clear association between mefloquine blood level and neuropsychiatric adverse effects could be demonstrated.[43, 44] Mefloquine is able to cross the blood brain barrier[45] and *ABCB1* is not only expressed in the intestine and in the liver, where it may influence the uptake in the blood and excretion in the bile of mefloquine, but also in the blood-brain barrier.[22] Therefore, even at similar serum concentrations, *ABCB1* function may influence the risk of neuropsychiatric adverse effects by affecting the brain tissue concentration.
We studied a cohort of mefloquine users followed during the three weeks preceding their travel, as was previously studied for neuropsychiatric effects of mefloquine (**Chapter 3.3**).[40, 42] We found that *ABCB1* C1236T, G2677T and C3435T variants and the associated 1236-2677-3435 TTT haplotype were associated with an increased risk of neuropsychiatric adverse effects during the study period (relative risk (RR) for homozygous TTT subjects versus all other haplotype carriers 2.5, 95%CI 1.3-4.6). The effect was stronger in women (RR 3.2, 95%CI 2.0-5.2) in whom the frequency of adverse effects was much higher. In men, the effect was smaller and no longer statistically significant (RR 1.7, 95%CI 0.4-6.5). We found no association between *ABCB1* SNPs or haplotypes and serum concentration of mefloquine. Furthermore, consistent with literature,[43, 44] we found no association of mefloquine serum concentration and the occurrence of neuropsychiatric adverse effects. This suggests that the association of *ABCB1* variants with neuropsychiatric adverse effects of mefloquine is driven by differences in brain tissue concentration rather than in serum concentration. A lower expression of *ABCB1* will result in lower mefloquine efflux from the brain, thus exposing it to higher tissue concentrations. Similar effects of *ABCB1* polymorphisms have recently been shown for other drugs.[46, 47]

***NOS1AP* variants and drug effects.** Recently, common genetic variants of *NOS1AP* were discovered to be associated with QT-interval prolongation in a genome wide association study.[48] The *NOS1AP* gene, encoding the Nitric Oxide Synthase 1 Adaptor protein, was not previously known to be involved in myo-

cardial repolarization. Both syndromal[49] and non-syndromal[50-53] long- and short[54] QT-interval increase the risk of SCD, making QT-interval and QT-altering factors important to study. Many drugs are known to prolong QT-interval duration,[55, 56] and medication-induced prolonged QT interval and ventricular arrhythmias, which may result in SCD, have led to the withdrawal of many non-cardiac medications.[57, 58]

Since the impact of *NOS1AP* variants on QT interval duration in older populations, in whom non-genetic factors might play a stronger role than heritable factors, is unknown, we sought to replicate this finding in the Rotterdam Study, comprising participants of 55 years and older (**Chapter 4.1**). We also tested for association of *NOS1AP* variants with SCD. In the Rotterdam Study, we strongly confirmed the association of *NOS1AP* variants with a 3.8 (95%CI 3.0-4.6) msec increase in QT interval duration per additional minor variant allele. With the limited number of SCD cases in our cohort, it was not possible to demonstrate that this association translates into an influence on risk of SCD although the point estimates suggest that such a risk increase may truly exist. Additional larger studies will be required to determine whether *NOS1AP* genotype is associated with SCD.

Since QT-altering drugs may interact with genetic variants in QT-duration associated genes,[49, 59, 60] this makes *NOS1AP* an interesting candidate for pharmacogenetic studies with QT-altering drugs. One of these QT-altering drugs is digoxin. Digoxin exerts its action, in part, by increasing intracellular Ca^{++} concentration ($[Ca^{++}]_i$) through an increase of intracellular Na^+ by inhibition of the Na/K ATPase.[61, 62] This leads to higher contractile force and to QT-shortening.[38] Therefore, we hypothesized that subjects carrying the minor variants of two *NOS1AP* SNPs known to prolong the QT interval might be less sensitive to the QT shortening effects and pro-arrhythmogenic effects of digoxin.[54, 63, 64] In contrast with our expectations, in the Rotterdam Study, comprising both digoxin users and non-users of digoxin, we found that *NOS1AP* minor allele carriers had a significantly increased QT shortening on digoxin compared to major allele homozygotes, in contrast to the effect observed in non-digoxin users (**Chapter 4.2**). Participants homozygous for the *NOS1AP* major allele using digoxin had a 11.4 (95%CI 7.5;15.3) msec shorter QTc interval than major allele homozygotes not using digoxin (reference group), whereas digoxin users homozygous for the minor allele showed 23.0 (95%CI 14.4;31.5) msec QTc shortening compared to the reference group. Apparently, the QTc-shortening effect of digoxin is significantly worsened in those with a variant allele. Furthermore, minor alleles were associated with an increased risk of SCD to digoxin up to three-fold. Although the mechanism by which *NOS1AP* influences QT interval duration and interacts with digoxin is not known, it may involve calcium handling in the cardiomyocyte.[65-68] *NOS1AP* has been found to activate NOS1.[69] NOS1 knockout cardiomyocytes have increased contractility through increased $[Ca^{++}]_i$ and a slower decay of the $[Ca^{++}]_i$ transient.[65-68, 70] This slower decay of the $[Ca^{++}]_i$ transient is at contrast with normal $[Ca^{++}]_i$ kinetics,[38] but does explain the QT-prolonging effects of *NOS1AP* gene variants if these variants result in less activation of NOS1. Furthermore, NOS1 is able to interact with the L-type Ca^{++} channel,[71] which is known to be associated with both Short- and Long QT Syndrome and SCD.[72, 73] This suggests a model in which both digoxin and *NOS1AP* minor alleles lead to increased $[Ca^{++}]_i$, resulting in excess $[Ca^{++}]_i$. Cardiac calcium overload could in turn increase risk of ventricular arrhythmias.[61] This would explain the increased risk of SCD in digoxin users with the minor *NOS1AP* alleles. If either digoxin

or the excess $[Ca^{++}]_i$ counteracted the unexpected slower decay of the $[Ca^{++}]_i$ transient as found in *NOS1* knockout mice (and, as suggested by their QT-prolonging effect, in *NOS1AP* minor allele carriers), the excess $[Ca^{++}]_i$ would also result in faster $[Ca^{++}]_i$ decline38 explaining the increased QTc-shortening in users of digoxin carrying *NOS1AP* variants. In turn, short QT in itself, both as a congenital syndrome and in the general population, is a risk factor of SCD.[54, 63, 64, 72, 74]

In **Chapter 5**, we studied the effects of interaction of *NOS1AP* variants and digoxin on cardiac systolic and diastolic function. We found some indication that common variants in the *NOS1AP* gene are associated with an increased systolic function in digoxin users without prevalent heart disease, but with a decreased function in those with prevalent heart failure or myocardial infarction. Digoxin users with *NOS1AP* variants showed increased E/A ratios, but increased deceleration time in subjects with prevalent heart disease and decreased deceleration time in those without. This might suggest a protective effect of the *NOS1AP* minor allele against impaired relaxation. However, the number of digoxin users with an echocardiogram was too low to draw solid conclusions and to reach statistical significance.

Many cardiovascular drugs are known to prolong QT-interval duration.[55, 56] Since common *NOS1AP* variants are associated with QT-interval prolongation,[48, 75, 76] these QT-prolonging genetic variants and drugs might interact,[49, 59, 60] resulting in even further increased QT-prolongation and increased risk of SCD. In the Rotterdam Study, we studied QTc-interval duration of both users of QT-prolonging cardiovascular drugs and of non-users, to compare effects of QT-prolonging drugs in different *NOS1AP* genotype groups. Although numbers of drug-users per genotype group were low, we found that users of triamterene or verapamil homozygous for the minor variant showed statistically significantly more QTc prolongation than major allele homozygotes (**Chapter 4.3**). Furthermore, although confidence intervals overlapped, regression coefficients indicated that a similar effect might exist for amiodarone, sotalol, diltiazem, indapamide, and perhaps isradipine and disopyramide. The number of SCD cases exposed to QT-prolonging medication at the time of death was too low to make a proper assessment of the influence of *NOS1AP* variants on SCD risk in users of these drugs. However, point estimates for amiodarone (15 exposed SCD cases) and verapamil (8 exposed cases) suggest that *NOS1AP* minor variant carriers might be at increased risk of SCD in users of these drugs.

NOS1AP variants might also interfere with other drugs. *NOS1* is not only expressed in the heart where it may interact with the voltage dependent L-type Ca^{++} channel,[71] it also plays a role in insulin secretion.[77-79] The antidiabetic class of sulfonylureas stimulate insulin secretion by an increased influx of Ca^{++}.[80-82] As previously mentioned, *NOS1AP* variants may also influence Ca^{++} levels.[65-68, 70] Furthermore, sulfonylurea have since long been associated with increased risk of cardiovascular mortality, although there remains controversy on this matter.[83-87] Since both *NOS1* and sulfonylurea influence insulin secretion and both may be associated with increased cardiovascular mortality, we hypothesized that genetic variation in the *NOS1AP* gene influences the glucose lowering effect of sulfonylurea and mortality risk in patients using sulfonylurea. In the Rotterdam Study, we found that the glucose lowering effect of glibenclamide was less effective in patients carrying the *NOS1AP* minor allele (**Chapter 4.4**). Moreover, carriers of *NOS1AP* minor alleles using glibenclamide were at increased risk of mortality, whereas these genotypes were associ-

ated with reduced mortality in patients using tolbutamide, gliclazide or glimepiride. The reduced mortality risk for tolbutamide, gliclazide and glimepiride seemed to be explained by a reduction in cardiovascular mortality. The increased risk of death in glibenclamide users with the minor allele opposite to users of other sulfonylurea might be explained by a higher affinity of glibenclamide for the SUR2A receptor (sulfonylurea 2A),[82, 88-92] which is thought to be responsible for the effects of sulfonylurea on cardiovascular mortality.[89, 93-95]

Methodological considerations

The main limitation of genetic association studies, to which pharmacogenetics is no exception, is lack of replication. Many promising associations published later on appeared to be false positive findings. This is especially an issue for the two genes chosen in this thesis. Digoxin is known to be a substrate for *ABCB1*, but results of previous studies on *ABCB1* SNPs and digoxin kinetics were contradictory. The main problem may reside in the fact that most studies were very small.36 Another problem is the fact that most studies were performed in a setting with little clinical relevance. They comprised healthy volunteers receiving a single dose of digoxin. Only two studies were performed in a more steady state population,[26, 33] only one of which was performed in patients using the drug under everyday circumstances.[33] No studies on clinical effects of *ABCB1* SNPs in digoxin users have been performed so far. *NOS1AP* is such a new candidate that no studies on pharmacogenetic associations have been performed. Biological plausibility and clinical relevance of our findings have been discussed in the previous paragraphs. In the following sections we will discuss some methodological considerations that apply to the internal and external validity of the studies described in this thesis.

Setting. All studies described in this thesis were performed using population-based data sources. For the study on the incidence of digoxin intoxication in the Netherlands, we used the National Morbidity Registration (LMR, Landelijke Medische Registratie), a nationwide computer database containing all hospital discharge records using International Classification of Disease (ICD) coding. The major strength of this database is its nationwide coverage and the fact that coding of discharge diagnosis is in no way related to reimbursement of hospital or specialist. Its main limitation is its lack of linkage to medication data and other clinical information. This was in part solved using data on digoxin prescriptions from the Foundation for Pharmaceutical Statistics (SFK) pharmacy database, which extrapolates drug figures for the Netherlands from dispensed prescriptions from 90% of all community pharmacies.
For the study on the influence of *ABCB1* SNPs and haplotypes on the risk of adverse effects to mefloquine, we used a cohort of mefloquine users in the three weeks previous to their journey. Its main limitations reside in the relative small number of subjects and the relative short follow-up. The main strength is the fact that this design eliminates effects related to travelling itself.
All other studies were performed using data from the Rotterdam Study,[1, 2] a large population of older adults included in a follow-up program. Its strengths reside in the relatively long follow-up and extensive information available on various clinical characteristics of all participants gathered at every follow-up round. In addition, through linkage with general practitioner and municipal mortality records, the total cohort is

continuously monitored for major morbidity and mortality. Furthermore, clinical laboratory assessments were available (for a part of the period) from 3 hospital laboratories and 1 general practitioner's laboratory serving the area of the Rotterdam Study. Finally, virtually all participants fill their prescriptions in automated pharmacies linked to one computer network. Since January 1, 1991, data on all dispensed drugs, including the prescribed daily dose, are available in computerized format on a day-to-day basis. All information is gathered before, and irrespective of, the outcome under study. This limits the chance of selection bias and information bias.

Design. Although all studies described in this thesis were designed as prospective population-based cohort studies, they each have there specific problems. For the study on incidence of digoxin intoxication, we used two separate sources for the outcome (intoxication) and the exposure (digoxin use). Data from these two sources could only be studied in an ecological design, correlating group- (age-group and sex) and period (year) level. Also, no information of clinical relevant risk factors for digoxin intoxication was available. Therefore, only very crude incidence measures of intoxications per number of prescriptions could be obtained. The cohort of mefloquine users used in **Chapter 3.3** was not only limited by the relative small number of participants with genotype information available, the fact that it only included users of the drug did not allow assessment of the effect of *ABCB1* SNPs on the risk of neuropsychiatric complaints without the use of mefloquine. These problems did not occur in the Rotterdam Study, which contains individual drug exposure and a wide range of clinical characteristics for a large number of participants. However, the number of users of one specific drug, especially at the time of assessment of a specific clinical characteristic may still be small. Also, studies on associations of a drug with characteristics assessed at follow-up rounds (ECGs and echocardiograms) were assessed in a cross-sectional manner, assessing both exposure to the drug and outcome at that specific date. The most important shortcoming is the uncertainty of a temporal relationship. Since multiple ECGs per subject were available, some subjects both had ECG-measures available at moments that they used and moments that they did not use the drug. Although numbers for these analyses were very small, this allowed us to assess the effect of a drug within these persons, making a causal relationship more likely.

Availability of information on all dispensed drugs, including the number of dispensed units and prescribed daily dose in computerized format, since January 1, 1991 allowed us to calculate the duration of use per drug for each participant at each point in time. This results in a high level of information about medication use and fewer misclassification of exposure. In order to adjust for changing risks over time due to changes in medication use, we chose a time-dependent Cox-proportional hazards model in our analyses of drug use and mortality in the Rotterdam Study. Although compiling a data set with time-dependent exposure is laborious and may require much computing time in the analyses on standard PCs, it has several advantages.[96] This allowed us to assess the exposure status and to adjust for dosing regimen of both cases and the remainder of the cohort at each index date.

Bias and confounding. In observational studies, selection bias, information bias and/or confounding may jeopardize the validity. The population-based design of the studies probably limited the chances of

selection bias. However, the cohort of mefloquine users consists of travellers, who are probable healthier than the general population. On the other hand, this is no major jeopardy to external validity since this is also the target population for the drug. In the Rotterdam Study, there is a chance that non-participants to the study or to (part of) the examinations at the research center are sicker. However, the fact that genotypes of study populations from these two cohorts were in Hardy-Weinberg equilibrium make selection bias less likely.

Because all information in the Rotterdam Study is gathered prospectively, without knowledge of drug-use or genotype, the chance of information bias is unlikely. The same is true for the cohort of mefloquine users. Although misclassification of neuropsychiatric effects is not unlikely due to the self-reporting and the broad definition of the outcome, assessment of the outcome was performed before genotyping, making misclassification most likely random. In the digoxin intoxication incidence study, no drug use could be linked to the discharge diagnosis at patient level. Nevertheless, misclassification of digoxin intoxication is probably limited since intoxication has its own ICD code and can only occur in digoxin-users. It is, however, possible that we missed cases who did not require hospitalization or died before reaching the hospital. Also, in the case of severe co-morbidity digoxin intoxication might not be coded as one of the reasons of hospitalization whereas patients with elevated digoxin serum levels at hospitalization without clinical symptoms of digoxin intoxication may be coded as cases. Therefore, we might have slightly underestimated, or -less likely- overestimated, the true incidence of digoxin intoxication. It is, however, unlikely that this resulted in differential misclassification of potential cases for men and women.

Confounding is not very likely in our studies on *ABCB1* SNPs and digoxin, since there is no association between polymorphisms of this gene and use of digoxin. An association of *ABCB1* polymorphisms with neuropsychiatric complaints in persons not using mefloquine cannot be excluded since our study only comprised mefloquine users. Since *NOS1AP* SNPs are associated with QT-prolongation, they may also be associated with SCD or other cardiovascular conditions. Therefore, confounding by indication might be a problem in our studies on *NOS1AP* SNPs, drugs and risk of (cardiovascular or SCD) mortality if those with a certain genotype would more readily receive digoxin. In the study on the interaction of digoxin and *NOS1AP* we were able to exclude this by stratifying on digoxin use and sub-analyses limited to digoxin users without previous heart disease or to participants with heart disease not using digoxin. In the study on cardiovascular QT-prolonging drugs, however, numbers were too small for such analyses or even proper adjustment for known risk factors of SCD. The influence of confounding could not be assessed beyond age and sex in the study using LMR data because clinical information on co-variates was not available.

Clinical implications

The studies presented in this thesis clearly show the potential of pharmacogenetic studies. We were able to identify genetic high risk groups for unfavorable outcomes of drug therapy. Genotyping for *ABCB1* and subsequent dose adjustment of digoxin and perhaps also mefloquine might reduce the number of neuropsychiatric adverse effects to mefloquine and the number of digoxin intoxications and digoxin related SCD. Genotyping for *NOS1AP* might also reduce SCD to digoxin and potentially to other QT-prolonging

cardiovascular drugs. *NOS1AP* genotyping might also improve safety and efficacy of sulfonylurea treatment in diabetes mellitus.

Genotype information is already being used to improve drug safety, incorporating information on genotypic risk groups in the product information.[97] The benefits for all parties involved in drug therapy are obvious. The patient is not needlessly exposed to a drug or drug-dose that puts him/her at risk of serious adverse effects. The doctor knows which drug or dose should be avoided in a certain situation. The drug company avoids putting its drug at risk of being taken from the market because of serious adverse effects. However, there are also downsides to this. If a company and doctors know that a drug will not work for a certain (potentially large) group of patients, the drug can no longer be sold to all patients, making the market smaller and less economically profitable. Whether this would jeopardize drug development in the future, however, remains to be seen as most industries will probably be creative enough to overcome this hurdle. Another drawback would be that genotype information may not only inform on risk of adverse drug effects, but also on risk of future disease. This may raise ethical problems, especially if adequate therapy for that disease is not at hand.

Future directions

Currently, only a limited number of SNPs have been genotyped in the Rotterdam Study in the *ABCB1* and *NOS1AP* genes. Since there is still debate on the functionality of the three genotyped *ABCB1* SNPs and no evidence is at hand for a mechanism involved in the *NOS1AP* variants, there is enough interesting future work left.

In vitro and animal research may give more insight into the physiological role of *NOS1AP*. Studying this gene and protein in cultured myocardial cells or knockout mice could provide valuable clues to its role in both normal repolarization and the mechanisms involved in interaction with various drugs.

Also, the effects of both *NOS1AP* and *ABCB1* variants require further fine-mapping and potential identification of a causal SNP or haplotype. The availability of genotype information from the GWA arrays in the Rotterdam Study may allow this in the near future.

We realize that a number of studies in this thesis show results based on a very limited number of exposed individuals. This is especially the case for the (non-digoxin) cardiovascular drugs and sulfonylurea studies. These studies may therefore have to be replicated in other populations. As we were the first to report a number of gene variant-drug interactions (mefloquine with *ABCB1*, all drugs with *NOS1AP*), replication of these findings is needed in order to confirm them. One of the ways larger numbers for the various drugs could be obtained is by using genotype data from randomized clinical trials. Although these are considered the gold standard, they are very laborious and expensive. Alternatively, the phenomenon of 'Mendelian randomization' could be used to study these variants. One could think of a population-based cohort design with prospective follow-up of patients receiving a certain drug without interfering in normal therapy. The treating doctor will remain blinded to genotype to avoid bias. Since genotypes are distributed random in the population and genotype information is not related to receiving treatment, this will allow studying the outcome of patients receiving the drug in relation to their genotype. The idea of Mendelian randomization does, however, rely on a number of assumptions and therefore has its limitations.[98, 99] One

of the main problems is that the genotypes are not always randomly distributed over the study population. A certain genetic variant may, for instance, be related to the (severity of the) disease for which the drug is indicated and also to the outcome of interest. This might be the case for *NOS1AP* (which plays a role in cardiac function) and cardiovascular drugs in relation to SCD. These limitations of Mendelian randomization can often –but not always- be surmounted by restricting the analyses to incident users but should nevertheless be carefully considered in such studies.
Finally, the previously mentioned Genome Wide Association studies hold promising opportunities for pharmacogenomic and –genetic research. Allowing to search for new candidate variants which influence drug response and risk of adverse drug reactions.[100, 101] This could be done by taking advantage of the precise drug exposure data in the Rotterdam Study. If the number of users of the drug of interest is large enough within the Rotterdam Study, one could test for the association of genotypes with the outcome of drug therapy. Alternatively, the genotyping and drug use information from the Rotterdam Study can be used as a reference population against which one could test a series of cases with the outcome of interest. This might greatly increase the number of drug-exposed cases and therefore statistical power. Cases of (rare) adverse drug effects can be gathered through general practitioners or other databases. Subsequently, genotypes of these cases can be compared to those of 'normal' drug users or from the general population as available from the Rotterdam Study.

Main conclusions

Drug therapy has become safer over the last three to four decades. Nevertheless, almost 1300 hospital admissions because of digoxin intoxication in four years are still considerable. Identification of specific risk groups may even further improve the safe use of digoxin and other drugs.

The studies of *ABCB1* SNPs or haplotypes and both digoxin and mefloquine show that genotyping for *ABCB1* SNPs may contribute to safer use of these drugs. By prescribing lower doses to *ABCB1* TTT-haplotype allele carriers, the number of digoxin intoxications and SCD to digoxin, and, possibly, the number of neuropsychiatric adverse effects to mefloquine might be reduced. Given that many drugs are a substrate for *ABCB1*-derived P-glycoprotein,[22, 102] these findings might have implications for the safe use of many more drugs.

The studies on *NOS1AP* variants show that new pharmacogenomic techniques may not only be directly applied to research on susceptibility to (adverse) drug effects. Genome wide association studies looking for genetic variation that influences variation in physiological traits also yields new and unexpected candidates for 'classical' pharmacogenetic studies beyond known drug targets or drug-metabolizing enzymes. Our study of digoxin and *NOS1AP* variants is the first[49] to show interaction of a QT-altering drug with a QT-altering genetic variant in an epidemiological study comprising users of the drug. Not only do we demonstrate interaction at the level of QT-interval duration. We were also able to identify subjects with increased risk of SCD to digoxin. Apart from digoxin, we found indication for interaction of *NOS1AP* variants with a number of QT-prolonging cardiovascular drugs. Finally, *NOS1AP* is also an interesting candidate

for non-cardiovascular pharmacogenetics. In **Chapter 4.4** we show an association of *NOS1AP* variants with efficacy and risk of death to sulfonylurea. This endorses the chance that the findings considering the *NOS1AP* gene are of a physiologically fundamental character.

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7

Summary

7.1 Summary

Despite all research efforts on safety and efficacy preceding marketing authorization of drugs, not every drug is safe and beneficial for every patient. Different people do not always react in the same way to the same drugs at the same dose. Differences in efficacy and adverse effects do not occur at random. It is estimated that genetic variation can account for 20-95% of variability in drug response.
One of the products of classical research on pharmacogenetics is the discovery of the ATB-binding cassette family B member 1 (*ABCB1*) gene. It plays a role in the uptake, distribution and clearance of many drugs. Despite the many studies on *ABCB1* genotype and drug kinetics that have already been performed, the influence of common genetic variants of this gene on drug effects are still unclear. In addition, common genetic variants in the nitric oxide synthase 1 adaptor protein (*NOS1AP*) gene were recently discovered to be associated with QT-interval prolongation. QT-interval duration is associated with the risk of sudden cardiac death (SCD), making this gene an interesting candidate to study in relation to the risk of SCD and to its interaction with QT-altering drugs.
In this thesis we explore the effect of interaction of common variants in the *ABCB1* and *NOS1AP* genes with drugs on drug response and adverse effects of drugs in a number of epidemiological studies. We mainly focus on digoxin and on other cardiovascular drugs as drugs of interest because these drugs are widely used and are a common cause of adverse reactions. Most studies were conducted within the Rotterdam Study, a population-based cohort study. Additionally, we used the 'Landelijke Medische Registratie (LMR), a database with discharge information from all hospitalizations in the Netherlands, and a cohort of mefloquine users who were followed in the three weeks preceding their travel.

In **Chapter 2**, we studied the incidence of hospitalization for digoxin intoxication in the general population in the Netherlands. We showed that, although the safety of digoxin therapy has increased during the last few decades, the number of digoxin intoxication related hospitalizations is still considerable and that women are at 40% higher risk of digoxin intoxication than men.

In **Chapter 3** we studied the interaction of common genetic *ABCB1* variants with digoxin and mefloquine. In **Chapter 3.**1, we showed that among 195 users of digoxin in the Rotterdam Study, the *ABCB1* 1236-

2677-3435 TTT-haplotype allele is associated with higher digoxin serum concentrations and explains up to 11.5% of the variability in digoxin concentrations in a model also containing age, sex, digoxin dose, lean body mass and renal clearance. In **Chapter 3.2**, we studied the effect of the same *ABCB1* variants on digoxin induced SCD and QT-shortening. We found that the TTT-haplotype allele was associated with a two-fold increased risk of SCD in digoxin users. No effect of this haplotype on SCD was observed in non-users of digoxin. The increased QTc shortening to digoxin on ECG in TTT-haplotype allele carriers further confirmed the interaction of *ABCB1* and digoxin. The effect of *ABCB1* variants is not limited to digoxin. Mefloquine is widely used in the treatment and prophylaxis of malaria, but is notorious for its, sometimes severe, neuropsychiatric adverse effects. In **Chapter 3.3** we studied the effect of *ABCB1* genotypes and haplotypes on neuropsychiatric adverse effects of mefloquine. We found that the *ABCB1* 1236-2677-3435 TTT-haplotype allele increases the risk of these adverse events by 2.5 times. The effect was stronger in women (RR 3.2) than in men (RR 1.7) and, consistent with literature, seemed not to be mediated by the serum concentration of mefloquine, suggesting an effect at the level of brain-tissue concentration.

In **Chapter 4** we studied the effects of common genetic variants of the *NOS1AP* gene, both in the general population and in users of specific drugs. In **Chapter 4.1**, we replicated the finding from an earlier genome wide association study, that common variants in the *NOS1AP* gene are associated with a 3.5 msec increase in QTc interval per additional minor allele. We also found some indication that these variants might be associated with a slightly increased risk of SCD. Although the effect was in the expected direction, numbers were too low to statistically significantly demonstrate this association. In **Chapter 4.2**, we studied whether the QT-prolonging *NOS1AP* variants would counteract the QT-shortening effects of digoxin, and perhaps also its pro-arrhythmogenic effects. In contrast with our expectations, we found that digoxin users carrying the *NOS1AP* minor allele showed increased QT-shortening to digoxin. Furthermore, digoxin users carrying the minor allele showed increased risk of SCD with a HR of 1.8 per additional minor allele, resulting in a more than three fold increased risk of SCD in digoxin users homozygous for the minor allele compared to those homozygous for the major allele. Since QT-prolonging genetic variants might increase the risk of SCD to QT-prolonging drugs, we studied in **Chapter 4.3** whether *NOS1AP* variants interact with cardiovascular QT-prolonging drugs,. Although numbers of users of each specific drug per genotype group were low, we found that users of triamterene or verapamil homozygous for the *NOS1AP* minor variant showed statistically significantly more QTc prolongation than major allele homozygotes. Furthermore, regression coefficients indicated that a similar effect might exist for amiodarone, sotalol, diltiazem, indapamide, and perhaps isradipine and disopyramide. The number of drug exposed cases was too low to assess whether this increased QT-prolongation translates into an increased SCD risk. In **Chapter 4.4** we studied whether these *NOS1AP* variants affect the efficacy of the antidiabetic class of sulfonylureas, or the cardiovascular mortality to these drugs. Sulfonylureas stimulate insulin secretion by an increased influx of calcium. *NOS1*, which is activated by *NOS1AP*, may also influence cytoplasmatic calcium handling and also plays a role in insulin secretion. Furthermore, like *NOS1*, sulfonylureas are associated with cardiovascular mortality. We found that the glucose lowering effect of glibenclamide was less effective in patients carrying the *NOS1AP* minor allele. Moreover, carriers of *NOS1AP* minor alleles using glibenclamide were at increased risk of mortality, whereas these genotypes were associated with

reduced mortality in patients using tolbutamide, gliclazide or glimepiride. These differences may be explained by differences in affinity to the sulfonylurea 2A receptor.

In **Chapter 5**, we studied whether the observed interactions of *ABCB1* and *NOS1AP* genotypes with digoxin were also associated with differences in digoxin effect on cardiac systolic and diastolic function. In line with our previous findings in combination with the known effects of digoxin, we found that the *ABCB1* TTT-haplotype allele seemed to be associated with worsened diastolic function in digoxin users, driving their echocardiographic parameters towards impaired relaxation. The TTT-haplotype allele was also associated with an increased systolic function in digoxin users without prevalent heart disease, but with a decreased function in those with prevalent heart failure or myocardial infarction. Similar to our findings for *ABCB1*, we found some indication that common variants in the *NOS1AP* gene were associated with an increased systolic function in digoxin users without prevalent heart disease, but with a decreased function in those with prevalent heart failure or myocardial infarction. Results for *NOS1AP* and diastolic function were less clear. However, the number of digoxin users with an echocardiogram was too low to draw solid conclusions and to reach statistical significance.

In **Chapter 6**, the general discussion, the main findings form this thesis are placed in a broader perspective and methodological considerations with regard to these studies are described. Furthermore, the clinical implications of our findings with regard to improving drug safety by potential genetic testing are discussed. Finally, recommendations for future research on *ABCB1*, *NOS1AP* and pharmacogenetics and –genomics in general are discussed.

7.2 Samenvatting

Ondanks alle onderzoeken op het gebied van veiligheid en werkzaamheid voorafgaand aan het toelaten van geneesmiddelen op de markt, is niet ieder geneesmiddel veilig en werkzaam bij iedere patiënt. Verschillende mensen reageren niet altijd op dezelfde manier op hetzelfde geneesmiddel bij gelijke dosering. Verschillen in werkzaamheid en het optreden van bijwerkingen treden niet willekeurig op. Genetische variatie kan naar schatting 20-95% van de verschillen in reactie op geneesmiddelen verklaren.
Een van de resultaten van klassiek farmacogenetisch onderzoek is de ontdekking van het ATP-bindende casette familie B1 (*ABCB1*) gen. Dit gen speelt een rol in de opname, distributie en uitscheiding van vele geneesmiddelen. Ondanks het grote aantal studies naar *ABCB1* genotype en farmacokinetiek, is de invloed van veelvoorkomende genetische varianten binnen dit gen op geneesmiddeleneffecten nog steeds niet duidelijk. Daarnaast werd recent ontdekt dat veelvoorkomende genetische varianten van het stikstofoxide synthase 1 adapter eiwit (*NOS1AP*) gen geassocieerd zijn met verlenging van het QT-interval op het ECG. QT-interval duur is geassocieerd met een verhoogde kans op plotse hartdood. Dat maakt dit gen een interessante kandidaat om te bestuderen in relatie tot de kans op plotse hartdood en als effect modulator op QT-duur beïnvloedende geneesmiddelen.
In dit proefschrift onderzoeken we in een aantal epidemiologische studies hoe de interactie van veelvoorkomende varianten van de *ABCB1* en *NOS1AP* genen met geneesmiddelen de werkzaamheid en bijwerkingen van die geneesmiddelen beïnvloedt. We concentreren ons voornamelijk op het geneesmiddel digoxine en op andere cardiovasculaire geneesmiddelen, omdat deze geneesmiddelen veel worden gebruikt en vaak bijwerkingen veroorzaken. De meeste studies werden uitgevoerd binnen het Erasmus Rotterdam Gezondheids Onderzoek (ERGO), ook wel bekend als de Rotterdam Studie, een cohort dat een afspiegeling is van de algemene bevolking. Daarnaast gebruikten we de Landelijke Medische Registratie (LMR), een database met ontslaginformatie van alle ziekenhuisopnamen in Nederland, en een cohort van mefloquinegebuikers, die werden gevolgd in de drie weken voorafgaand aan hun reis.

In **Hoofdstuk 2** onderzochten we de incidentie van ziekenhuisopname vanwege digoxine intoxicatie in de algemene bevolking van Nederland. We toonden aan dat, hoewel het gebruik van digoxine veiliger is geworden gedurende de laatste decennia, het aantal ziekenhuisopnames vanwege digoxine intoxicatie nog

steeds aanzienlijk is en dat vrouwen een 40% hoger risico op digoxine intoxicatie hebben dan mannen.

In **Hoofdstuk 3** onderzochten we de interactie tussen veelvoorkomende varianten van het *ABCB1* gen met digoxine en mefloquine. In **Hoofdstuk 3.1** toonden we, onder 195 digoxine gebruikers uit de Rotterdam Studie aan, dat het *ABCB1* 1236-2677-3435 TTT-haplotype allel geassocieerd is met hogere digoxine spiegels in het bloed en tot 11.5% van de variatie in digoxine concentratie kan verklaren in een model dat ook leeftijd, geslacht, digoxine dosis, 'lean body mass' en nierfunctie bevat. In **Hoofdstuk 3.2** bestudeerden we het effect van dezelfde *ABCB1* varianten op digoxine gerelateerde plotse hartdood en QT-verkorting. We vonden dat het TTT-haplotype allel was geassocieerd met een twee maal zo hoge kans op plotse hartdood binnen digoxine gebruikers. Er werd geen effect van dit haplotype op plotse hartdood gezien bij mensen die geen digoxine gebruikten. Het toegenomen QT-verkortend effect van digoxine in TTT-haplotype allel dragers bevestigde de interactie tussen *ABCB1* varianten en digoxine. Het effect van *ABCB1* varianten is niet beperkt tot digoxine. Mefloquine wordt veel gebruikt in de behandeling- en ter voorkoming van malaria, maar is berucht vanwege, soms ernstige, neuropsychiatrische bijwerkingen. In **Hoofdstuk 3.3** onderzochten we het effect van *ABCB1* genotypes en haplotypes op het optreden van neuropsychiatrische bijwerkingern van mefloquine. We vonden dat het *ABCB1* 1236-2677-3435 TTT-haplotype allel het risico op dit soort bijwerkingen 2.5 maal verhoogde. Het effect was sterker in vrouwen (3.2 maal) dan in mannen (1.7 maal) en leek, in overeenstemming met de literatuur, niet gemedieerd te worden door de mefloquine serumconcentratie, hetgeen een effect op de concentratie in het hersenweefsel suggereert.

In **Hoofdstuk 4** onderzochten we de effecten van veelvoorkomende genetische varianten van het *NOS1AP* gen, zowel in de algemene bevolking als binnen gebruikers van bepaalde geneesmiddelen. In **Hoofdstuk 4.1** repliceerden we de bevinding uit een eerdere 'genome wide association study' dat veelvoorkomende varianten van het *NOS1AP* gen geassocieerd zijn met een QTc-interval verlenging van 3.5 msec per extra variant ('minor') allel. We vonden ook aanwijzingen dat deze varianten geassocieerd zijn met een licht verhoogde kans op plotse hartdood. Hoewel het effect in de verwachte richting was, waren de aantallen te klein om een associatie statistisch significant aan te tonen. In **Hoofdstuk 4.2** onderzochten we of QT-verlengende *NOS1AP* varianten het QT-verkortend effect van digoxine en mogelijk ook het pro-aritmogene effect ervan zouden tegengaan. In tegenstelling tot wat we verwachtten vonden we dat digoxine gebruikers met het variant *NOS1AP* allel een versterkte QT-verkorting hadden. Daarnaast hadden digoxine gebruikers met het variant *NOS1AP* allel een verhoogde kans op plotse hartdood van 1.8 maal per variant allel hadden, hetgeen resulteert in een meer dan drie keer verhoogde kans op plotse hartdood in digoxine gebruikers met twee variant allelen vergeleken met digoxine gebruikers met twee niet-variant ('major') allelen. Omdat QT-verlengende genetische varianten het risico op plotse hartdood door QT-verlengende geneesmiddelen zouden kunnen verhogen, onderzochten we in **Hoofdstuk 4.3** of *NOS1AP* varianten interactie vertonen met cardiovasculaire geneesmiddelen die het QT-interval verlengen. Hoewel de aantallen gebruikers per afzonderlijk geneesmiddel per genotype groep laag waren, vonden we dat gebruikers van triamtereen of verapamil met twee variant *NOS1AP* allelen statistisch significant meer QTc-verlenging vertoonden dan gebruikers met twee niet-variant allelen. Daarnaast sug-

gereerden de regressie coëfficienten dat er een vergelijkbaar effect zou kunnen bestaan voor amiodarone, sotalol, diltiazem, indapamide en mogelijk ook voor isradipine en disopyramide. Het aantal plotse hartdoden ten tijde van medicatie gebruik was te klein om vast te kunnen stellen of deze versterkte QT-verlenging ook resulteerde in een verhoogde kans op plotse hartdood. In **Hoofdstuk 4.4** onderzochten we of de *NOS1AP* varianten de effectiviteit van de antidiabetica uit de sulfonylurea geneesmiddelengroep de kans op cardiovasculaire mortaliteit bij gebruik van deze geneesmiddelen beïnvloedt. Sulfonylurea stimuleren insuline secretie door het verhogen van de calcium influx. *NOS1*, dat geactiveerd wordt door *NOS1AP*, zou ook de cytoplasmatische calcium huishouding kunnen beïnvloeden en speelt daarnaast een rol bij insuline secretie. Verder zijn sulfonylurea, net als *NOS1*, geassocieerd met cardiovasculaire mortaliteit. We vonden dat het glucose verlagende effect van glibenclamide minder sterk was in patiënten met het variant *NOS1AP* allel. Verder vertoonden de glibenclamide gebruikers die drager zijn van het variant *NOS1AP* allel een verhoogd sterfterisico, terwijl deze genotypes juist geassocieerd waren met verminderde sterfte in gebruikers van tolbutamide, gliclazide of glimepiride. Deze verschillen worden mogelijk verklaard door een verschil in affiniteit voor de sulfonylurea 2A receptor.

In **Hoofdstuk 5** onderzochten we of het *ABCB1* en *NOS1AP* genotype is geassocieerd met verschillen in digoxine-effect op cardiale systolische en diastolische functie. In overeenstemming met onze eerdere bevindingen en met de bekende effecten van digoxine, vonden we dat het *ABCB1* TTT-haplotype geassocieerd leek met een verslechterde diastolische functie in digoxine gebruikers, waarbij de echocardiografische parameters in de richting van verminderde relaxatie gedreven werden. Het TTT-haplotype allel was ook geassocieerd met een verbeterde systolische functie in digoxine gebruikers zonder prevalent hartfalen of myocard infarct, maar met een verslechterde systolische functie onder gebruikers met prevalent hartfalen of myocard infarct. Vergelijkbaar met onze bevindingen voor *ABCB1*, vonden we aanwijzingen dat veelvoorkomende varianten van het *NOS1AP* gen geassocieerd waren met verbeterde systolische functie bij digoxine gebruikers zonder prevalente hartziekte, maar met een verslechterde functie in gebruikers met een prevalente hartziekte. De resultaten voor *NOS1AP* en diastolische functie waren minder duidelijk. De aantallen digoxine gebruikers met een echocardiogram waren echter te laag om een duidelijke conclusie te trekken of om statistische significantie te bereiken.

In **Hoofdstuk 6**, de algemene discussie, worden de belangrijkste bevindingen uit dit proefschrift in een breder perspectief geplaatst en worden methodologische overwegingen met betrekking tot de verschillende studies beschreven. Daarnaast worden de klinische implicaties van onze bevindingen, met betrekking tot verbetering van de medicatieveiligheid door genetische testen, besproken. Ten slotte worden aanbevelingen gedaan voor verder onderzoek op het gebied van *ABCB1*, *NOS1AP* en farmacogenetica in het algemeen.

8
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About the author

Albert-Jan Aarnoudse was born on 8 February 1977, in Dordrecht, the Netherlands. In 1995 he graduated from the 'Scholengemeenschap Guido de Brès' and started medical school at the Erasmus University, Rotterdam, the Netherlands, from which he graduated in 2002. During his study he also attended a training in Molecular Medicine at the Erasmus University, including a full year of research at the department of Experimental Pathology under supervision of Dr. E.C. Zwarthoff during the year 2000, for which he obtained a Masters of Science degree in 2002 and received a Nijbakker-Morra award for excellent fundamental cancer research by students in 2001. After obtaining his medical degree, he worked for 17 months as a resident in Internal Medicine at the Albert Schweitzer hospital in Dordrecht (head Dr. A.C.M. van Vliet). In June 2004 he started the work described in this thesis at the Pharmacoepidemiology unit (head Prof.dr. B.H.Ch. Stricker) of the department of Epidemiology & Biostatistics (head Prof.dr. A. Hofman) of the Erasmus Medical Center in Rotterdam. During this period, he also worked at the Pharmacovigilance department of the Dutch Health Care Inspectorate in The Hague. In June 2006, he obtained a Master of Science in Clinical Epidemiology at the Netherlands Institute for Health Sciences (NIHES). He received an award for a part of this thesis as a second best abstract by a student, from the International Society for Pharmacoepidemiology in 2007. In May 2008, he will start his specialist training in Internal Medicine at the Albert-Schweitzer Hospital in Dordrecht (head Dr. E.F.H. van Bommel). He is married to Willemien Aanen. They have two children, a boy and a girl: Han and Anne-Linde.

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Manuscripts based on this thesis

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