A *NOS1AP* gene variant is associated with a paradoxical increase of the QT-interval shortening effect of digoxin

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Abstract:

Digoxin is characterized by a small therapeutic window and a QT-interval shortening effect. Moreover, it has been shown that the genetic variants of the nitric oxide synthase-1 adaptor protein (*NOS1AP*) gene are associated with QT-interval prolongation. We investigated whether the rs10494366 variant of the *NOS1AP* gene decreases the QT-interval shortening effect of digoxin in patients using this drug. We included 10,057 individuals from the prospective population-based cohort of the Rotterdam Study during a median of 12.2 (interquartile range (IQR) 6.7-18.1) years of follow-up. At study entry, the mean age was 64 years and almost 59% of participants were women. A total of 23,179 ECGs were longitudinally recorded, of which 334 ECGs were from 249 individuals on digoxin therapy. The linear mixed model analysis was used to estimate the effect of the rs10494366 variant on the association between digoxin use and QT-interval duration, adjusted for age, sex, RR interval, diabetes, heart failure, and history of myocardial infarction. In non-users of digoxin, the GG genotype was associated with a significant 6.5 ms [95% confidence interval (CI) 5.5; 7.5] longer QT-interval duration than the TT variant. In current digoxin users, however, the GG variant was associated with a significantly -23.9 [95%CI -29.5; -18.5] ms shorter mean QT-interval duration than in those with the TT variant with -15.9 [95%CI -18.7; -13.1]. This reduction was strongest in the high digoxin dose category [≥ 0.250 mg/day] with the GG genotype group, with -40.8 [95%CI -52.5; -29.2] ms changes compared to non-users. Our study suggests that the minor homozygous GG-genotype group of the *NOS1AP* gene rs10494366 variant is associated with a paradoxical increase of the QT-interval shortening effect of digoxin in a population of European ancestry.

Keywords: Digoxin, QT-interval, Genetic Variants, *NOS1AP* gene

Introduction

Digoxin is a cardiac glycoside, prescribed to control ventricular rate in chronic atrial fibrillation (AF) and patients with mild to moderate heart failure (HF). Still, the latter indication has become less common(1). Although the use of digoxin is declining, up to one-third of the AF patients are still treated with this medication worldwide(2). In the most recent European guidelines, digoxin therapy is recommended for rate control in AF patients with left ventricular ejection fraction (LVEF) < 40% as a class I indication(3). The only large randomized trial of digoxin in heart failure provides some evidence for the effect of digoxin on reduced admissions for heart failure(4), although without a clear mortality advantage along with observational studies(5, 6). Digoxin's primary mechanism of action is thought to result from increased intracellular Ca^{2+} concentration due to inhibition of the sodium-potassium ATPase, with resultant increased ventricular contraction, followed by a shortened ventricular refractory period with QT-interval shortening on electrocardiogram (ECG)(1, 7).

QT-interval duration reflecting myocardial repolarization time on the ECG explains approximately 35% of the heritability of QT variation in the general population. This suggests that a considerable proportion of its variation is related to genetic factors(8). Some factors modifying electrophysiology of the heart are considered to be associated with an increased risk of sudden cardiac death (SCD). QT-interval prolongation is associated with medication-induced arrhythmias and SCD(9, 10), which makes it an important intermediate phenotype to study. In recent years, Genome-wide association studies (GWAS) have discovered that genetic variants at the locus including the nitric oxide synthase-1 adaptor protein (*NOS1AP*) gene, located on chromosome 1, are associated with QT-interval prolongation(11, 12). This gene encodes a cytosolic ligand of neuronal NOS, and some of its genetic variants are associated with SCD(13, 14). Common variants of rs10918594 and rs10494366 significantly potentiate the QT-interval prolonging effect of verapamil(15).

The association of the *NOS1AP* variants and QT interval has been studied and confirmed in the large population-based cohort of the Rotterdam Study (RS), where the minor G allele of the rs10494366 variant was associated with a longer mean QT-interval duration(16, 17). The rs10494366 variant is a common genetic variant with a minor allele frequency (MAF) of more than 5% in the general population. The rs10494366 is also associated with an increased incidence of cardiac events and SCD in the rare Mendelian disorder of long QT syndrome (LQTS)(18, 19). According to a recent meta-analysis, the minor allele of the rs10494366 variant at the *NOS1AP* locus is associated with an increased QT-interval duration and potentially plays a role in SCD in people of European descent(20). Therefore, we considered investigating this variant in our study.

Because the minor allele of the rs10494366 variant is associated with QT-prolongation while digoxin causes QT-shortening, we hypothesized that the variant allele would compensate for that effect. We included participants from the large population-based cohort of the RS during a long

follow-up period. We had available data of repeatedly measured ECGs, enabling us to perform longitudinal analyses. Therefore, we studied whether the minor allele of rs10494366 was associated with a decreased QT-shortening effect of digoxin use.

Methods

Study population and setting

This longitudinal prospective study was performed within the large, population-based cohort of the RS. The RS started in 1990 in the district of Ommoord, in the city of Rotterdam, The Netherlands. The original study population consisted of 7,983 residents aged ≥55 years old at the time of enrollment(21). In 2000, the cohort population was increased by the addition of 3,011 eligible participants. A further extension started in 2006 in which 3,932 participants aged ≥45 were included, with a total of 14,926 subjects by the end of 2008. The recruitment of new subjects is an ongoing process, and the follow-up examinations are performed every four years. The objectives and methods of RS have been described in detail(22). The RS has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare, and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The RS entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalog number NTR6831. All participants who provided written informed consent for participation and sharing their medical records and samples for scientific purposes, were enrolled. A total of 10,057 participants with available medication use data, genotyping data, and repeated ECG measurements from the three RS cohorts were included in this study analysis. **Figure 1** shows the flowchart of the study population. Subjects were followed from baseline until their death or the end of the study period on January 1st, 2014.

Exposure definition

Digoxin dispensing data, including prescribed daily dose, product name, Anatomical Therapeutic Chemical (ATC)-code, dispensing date, and the amount prescribed was obtained from all pharmacies in the study district, which use one collaborative computer system. According to the World Health Organization (WHO), the defined daily dose (DDD) of digoxin [ATC code: C01AA05] is equivalent to 0.250 mg(23). Each subject was considered as currently exposed to digoxin if the ECG recording date fell within the duration of a dispensed digoxin prescription that was calculated by dividing the number of tablets in the prescription by the daily prescribed number of units. We categorized current digoxin use into three daily dose categories of 1 (digoxin dose< 0.125 mg), 2 (0.125 ≤ digoxin dose< 0.250 mg) and 3 (digoxin dose≥ 0.250 mg).

Genotyping

DNA samples from almost 12,000 participants of the three RS cohorts were genotyped using Illumina 550 K (single + duo format) and Illumina Human 610 K (Quattro format) arrays. The RS GWAS dataset was imputed with 1000 Genomes (version Iv3 and IIIv5) reference datasets(22). All participants were genotyped for the *NOS1AP* single nucleotide polymorphism (SNP) rs10494366 T>G and the correlated SNP rs10918594 C>G, which were previously shown to be associated with QT-interval duration(12, 17). Because SNP rs10494366 showed stronger association with QT intervals in the Rotterdam Study and both SNPs are in linkage disequilibrium (with an r^2 of 0.63)(12, 17), we considered it primary in the analyses. We extracted data on the SNP rs10494366. Imputation quality for this SNP was high (>0.99).

Outcome definition

An ACTA electrocardiograph (ESAOTE, Florence, Italy) at a sampling frequency of 500 Hz was used to record the standard 12-lead ECGs. ECG measurements, including QT, QRS, and RR-interval durations, were obtained by digital processing using a modular ECG analysis system (MEANS)(24, 25). The MEANS program determines the QT interval from the start of the QRS complex until the T wave end. The ECGs with left or right bundle branch block (BBB) and also those recorded while the subject was on any medication associated with QT-prolongation and Torsade de Pointes (TdP) risk, provided by CredibleMeds.org,(26) were excluded. A maximum of five QT-interval measurements were recorded for each subject during the examination cycles, in an average of every four years.

Covariates

The information on covariates including age, sex, type 2 diabetes mellitus (T2D), heart failure (HF), and myocardial infarction (MI) was gathered at baseline. Diagnosis of T2D at baseline was based on fasting blood glucose (>7.0 mmol/L), non-fasting blood glucose >11.0 mmol/L values, or glucose-lowering medication use. HF was diagnosed in patients with typical symptoms according to the European society of cardiology (ESC)-guidelines and objective evidence of cardiac dysfunction(27). A medical specialist confirmed definite MI cases in the RS at baseline through an interview, available medical records, and objective indicative ECGs(27).

Statistical analysis

Descriptive statistics were performed by reporting mean (standard deviation (SD)) or median (interquartile range (IQR)) for continuous variables and numbers (percentage) for categorical variables. The Hardy-Weinberg equilibrium *P*-value was calculated for genotype frequencies using the chi-square test. Primarily, we assessed the association between digoxin use and QT-interval duration in the total population. Then, we tested the association of the rs10494366 variant and QT interval among digoxin non-users.

The mean QT-interval duration changes in the patients with digoxin use as exposure at the time of measurement were compared to those of non-users, stratified by genotype and according to an allele-effect model. We also studied the effect of rs10494366 genotype groups on the association between the mean QT-interval duration and digoxin daily dose categories of 1 [dose< 0.125 mg (low dosage)], 2 [0.125 mg ≤ dose< 0.250 mg (moderate dosage)], and 3 [dose≥ 0.250 mg (high dosage)]. Since subjects had more than one recorded ECG, correlated in the same person, we used a repeated-measures analysis with a linear mixed model adjusted for age, sex, RR interval (crude model) and additionally adjusted for prevalent T2D, HF, and MI (adjusted model). We assessed the model assumptions by examining the normality and homoscedasticity assumptions of residuals in the longitudinal data.

A two-sided *P* value <0.05 was considered statistically significant. An expectation-maximization (EM) single imputation method was used to impute missing values. Data were analyzed using IBM SPSS statistics for windows, version 25 (IBM Corp., Armonk, N.Y., USA). Plots were created using SAS statistics for windows, version 9.4 (SAS Institute Inc, Cary, NC, USA).

Results:

Baseline characteristics of the study populations (n=10,057) are shown in **Table 1**. A total of 23,179 ECGs were recorded. The baseline mean age was 63.9 (SD 9.3) years, and almost 59% of participants were female. In total, 925 patients used digoxin at any time during the median follow-up period of 12.2 (IQR 6.7-18.1) years. Among them, there were 249 individuals whose ECGs were recorded while the subjects were on digoxin therapy. We considered this group as current users of digoxin in the model analyses. The minor allele (G) frequency (MAF) for the rs10494366 SNP was 35.8%, and the genotype distribution was in Hardy-Weinberg equilibrium (χ^2*=0.08, P*-value=0.77).

Digoxin current use, QT-interval change and genetic influence: A total of 334 ECGs were recorded in 249 current digoxin users. Current digoxin use was associated with a shorter QT interval -18.4 [95%CI -20.3; -16.4] milliseconds (ms) when compared to non-users in the total population regardless of the *NOS1AP* genotypes.

The analysis results for the *NOS1AP* gene rs10494366 variant effects on mean QT-interval differences among digoxin non-users are shown in **Table 2.** The mean QT-interval duration in the reference TT genotype group is 400.7 ms. The adjusted model revealed that subjects with a homozygous GG genotype and heterozygous TG genotype had longer mean QT-interval duration than subjects with a homozygous TT genotype, respectively 6.5 [95%CI 5.5; 7.5] ms and 3.4 [95%CI 2.7; 4.0] ms (**Figure 2**).

We investigated the mean QT-interval differences among digoxin users and compared them to non-users in the population stratified on rs10494366 genotypes. The adjusted model showed that digoxin users with GG genotype had a -23.9 [95%CI -29.5; -18.5] ms shorter mean QT-interval than non-users with GG genotype. Digoxin users with TG and TT genotypes had respectively –18.2 [95%CI -21.2; -15.2] ms and -15.9 [95%CI -18.7; -13.1] ms differences in the mean QT interval compared to non-users with the same genotypes. These findings are shown in **Figure 3**.

Homozygous minor allele carriers (GG) with high digoxin dose usage category 3 [≥ 0.250 mg/day] demonstrated the shortest mean QT-interval compared to non-users, among other dosage categories. As shown in **Table 3**, the analysis of the high-digoxin dose category 3 associations in the adjusted model revealed that the mean QT-interval was -21.6 [95%CI -26.7; -16.5] ms in TT, -23.2 [95%CI -28.8; -17.6] ms in TG and -40.8 [95%CI -52.5; -29.2] ms in GG genotypic group shorter than digoxin non-users with the same genotypes. The mean QT-interval differences compared in categorical prescribed digoxin daily doses among genotypic groups are shown in **Figure 4**.

Discussion

To the best of our knowledge, this is the first study to investigate the association of digoxin use and QT-interval duration within the genotypic groups of the *NOS1AP* gene rs10494366 variant. Although we expected to find that the QT-interval prolonging effect of the *NOS1AP* gene rs10494366 GG genotype would attenuate the well-known QT-shortening effect of digoxin, the opposite was observed. Digoxin users with the homozygous minor allele GG genotype of the *NOS1AP* gene rs10494366 variant had a significantly shorter QT-interval duration than the GG genotype digoxin non-users. Higher doses of digoxin were associated with a shorter QT-interval, especially in minor homozygous genotypes.

Digoxin inhibits the Na^+/K^+-ATPase pump and causes an increased intracellular Na^+ concentration. This inhibition promotes Na^+/Ca^{2+} exchange, which results in increased intracellular Ca^{2+} influx, available to contractile proteins(28). The higher peak of intracellular free Ca^{2+} concentration ($[Ca]_i$) is associated with faster $[Ca]_i$ decline(29), leading to faster repolarization and QT-interval shortening.

The exact mechanism by which this *NOS1AP* gene variant causes differences in QT-interval duration in digoxin users is unknown. However, it could involve calcium handling in cardiomyocytes(30). NOS1AP protein, also known as the carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase (CAPON), regulates the activation of neuronal nitric oxide synthase (NOS1) enzyme. NOS1 expresses in cardiomyocytes and plays a role in regulating intracellular Ca^{2+} level and contractibility by NOS1-derived nitric oxide (NO) (30). Chang et al. demonstrated that overexpression of the *NOS1AP* gene product, CAPON, in ventricular

myocytes of guinea pigs (GP) activates NOS1-derived NO signaling pathway, which results in the abbreviation of the action potential duration (APD) mediated by a diminished L-type Ca^{2+} current ($I_{Ca,\ L}$) and an enhanced delayed rectifier K^+ current (I_{Kr}), (31) which brings about accelerated cardiac repolarization. In murine ventricular myocytes, NOS1 disruption enhanced basal contraction and the inotropic response to β-adrenergic stimulation(32). Its location in the cardiac sarcoplasmic reticulum suggests that NOS1 is involved in the regulation of intracellular calcium fluxes(33). Furthermore, it was shown that the minor alleles in some of the *NOS1AP* gene variants are associated with NOS1 loss of function(34). NOS1 inhibition (in GP and human pluripotent stem cell-derived cardiomyocytes) and NOS1AP downregulation (in pluripotent stem cell-derived cardiomyocytes) increased $I_{Ca,\ L}$ density, and prolonged APD, resulted by a prolongation in transient $[Ca]_i$ decay in patients affected by long QT syndrome (LQTS)(34). These findings suggest that the expression level of the *NOS1AP* gene influences the cellular electrical properties, contributing to the imbalance between inward and outward currents that can alter cardiac repolarization. It has been demonstrated that the rs10494366 variant is a predictor of prolonged repolarization in acute coronary syndrome survivors and is associated with SCD(35).

Based on the above findings, it seems possible that the combination of the *NOS1AP* variant minor allele and digoxin intake increase $[Ca]_i$. Their simultaneous activity on the cardiomyocyte leads to perturbed intracellular Ca^{2+} signaling and excess intracellular $[Ca]_i$. Calcium overload induces spontaneous cycles of calcium release from the sarcoplasmic reticulum and its reuptake, promoting delayed afterdepolarizations and early afterdepolarizations, eventually causing severe ventricular arrhythmias and increased risk of sudden death(36, 37). On the other hand, high $[Ca]_i$ due to digoxin use could counteract with slower decay of $[Ca]_i$ resulting from lower NOS1 activity and this excess Ca^{2+} concentration could lead to faster $[Ca]_i$ decline(29), explaining the shorter QT-interval in *NOS1AP* variant homozygous minor allele carriers.

We observed that carrying a minor G allele is associated with greater QT-interval shortening effects of current digoxin use. This was in contrast to the observed association in the digoxin non-user group in which minor allele carriers had a longer QT-interval, consistent with prior reports(16, 17). Moreover, higher doses of digoxin use were associated with a shorter mean QT-interval in the GG genotype group.

This study has some strengths and limitations. Our study's main strength was the availability of up to five recorded ECGs per subject at regular intervals during a relatively long follow-up period, which enabled us to obtain more precise ECG measures along with digoxin use. Additionally, the precise and detailed pharmacy data available in the RS allowed us to determine digoxin use at the time of ECG and exclude participants on QT-prolonging medications. The limitations of our study should also be acknowledged. It is worth noting that the frequency of the rs10494366 minor G allele is 36% in people of European descent. In contrast, other populations including, American, African, East Asian, and South Asians, have higher frequencies of the G allele, varying from 43% to 68% according to the 1000 Genomes reference(38). Hence, it can be hypothesized that the revealed associations of this variant and digoxin use in patients with

European ancestry in the present study cannot be generalized to other populations unconditionally. Consequently, further studies in other populations with different ancestral backgrounds are needed. Furthermore, whether we have selected the functional variant and rs10494366 is probably in linkage disequilibrium with other causal SNPs is uncertain. Future studies on other common variants of the *NOS1AP* gene may help refine the findings in our study.

To conclude, we observe a significant genetic association of minor homozygous genotype of the rs10494366 variant with a greater QT-shortening effect of digoxin use. Since the rs10494366 variant alleles are common in the general population, replication of our findings could improve our understanding of digoxin's effects and facilitate more safe medical usage.

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Conflict of interest

The authors have no conflicts of interest relevant to this article to disclose.

References:

1. Ehle M, Patel C, Giugliano RP. Digoxin: clinical highlights: a review of digoxin and its use in contemporary medicine. Crit Pathw Cardiol. 2011;10(2):93-8.
2. Lopes RD, Rordorf R, De Ferrari GM, Leonardi S, Thomas L, Wojdyla DM, et al. Digoxin and Mortality in Patients With Atrial Fibrillation. J Am Coll Cardiol. 2018;71(10):1063-74.
3. Hindricks G, Potpara T, Dagres N, Arbelo E, Bax JJ, Blomström-Lundqvist C, et al. 2020 ESC Guidelines for the diagnosis and management of atrial fibrillation developed in collaboration with the European Association for Cardio-Thoracic Surgery (EACTS) The Task Force for the diagnosis and management of atrial fibrillation of the European Society of Cardiology (ESC) Developed with the special contribution of the European Heart Rhythm Association (EHRA) of the ESC. Eur Heart J. 2021;42(5):373-498.
4. Digitalis Investigation G. The effect of digoxin on mortality and morbidity in patients with heart failure. N Engl J Med. 1997;336(8):525-33.
5. Ahmed A, Rich MW, Fleg JL, Zile MR, Young JB, Kitzman DW, et al. Effects of digoxin on morbidity and mortality in diastolic heart failure: the ancillary digitalis investigation group trial. Circulation. 2006;114(5):397-403.
6. Aguirre Davila L, Weber K, Bavendiek U, Bauersachs J, Wittes J, Yusuf S, et al. Digoxin-mortality: randomized vs. observational comparison in the DIG trial. Eur Heart J. 2019;40(40):3336-41.

7. Gheorghiade M, Van Veldhuisen DJ, Colucci WS. Contemporary use of digoxin in the management of cardiovascular disorders. Circulation. 2006;113(21):2556-64.
8. Newton-Cheh C, Larson MG, Corey DC, Benjamin EJ, Herbert AG, Levy D, et al. QT interval is a heritable quantitative trait with evidence of linkage to chromosome 3 in a genome-wide linkage analysis: The Framingham Heart Study. Heart Rhythm. 2005;2(3):277-84.
9. Straus SMJM, Sturkenboom MCJM, Bleumink GlS, Dieleman JP, Lei Jvd, Graeff PAd, et al. Non-cardiac QTc-prolonging drugs and the risk of sudden cardiac death. Eur Heart J. 2005;26(19):2007-12.
10. Straus SMJM, Kors JA, De Bruin ML, van der Hooft CS, Hofman A, Heeringa J, et al. Prolonged QTc interval and risk of sudden cardiac death in a population of older adults. J Am Coll Cardiol. 2006;47(2):362-7.
11. Newton-Cheh C, Eijgelsheim M, Rice KM, de Bakker PI, Yin X, Estrada K, et al. Common variants at ten loci influence QT interval duration in the QTGEN Study. Nat Genet. 2009;41(4):399-406.
12. Arking DE, Pfeufer A, Post W, Kao WH, Newton-Cheh C, Ikeda M, et al. A common genetic variant in the NOS1 regulator NOS1AP modulates cardiac repolarization. Nat Genet. 2006;38(6):644-51.
13. Kao WH, Arking DE, Post W, Rea TD, Sotoodehnia N, Prineas RJ, et al. Genetic variations in nitric oxide synthase 1 adaptor protein are associated with sudden cardiac death in US white community-based populations. Circulation. 2009;119(7):940-51.
14. Eijgelsheim M, Newton-Cheh C, Aarnoudse AL, van Noord C, Witteman JC, Hofman A, et al. Genetic variation in NOS1AP is associated with sudden cardiac death: evidence from the Rotterdam Study. Hum Mol Genet. 2009;18(21):4213-8.
15. van Noord C, Aarnoudse A-JLHJ, Eijgelsheim M, Sturkenboom MCJM, Straus SMJM, Hofman A, et al. Calcium channel blockers, NOS1AP, and heart-rate-corrected QT prolongation. Pharmacogenet Genomics. 2009;19(4).
16. Eijgelsheim M, Aarnoudse AL, Rivadeneira F, Kors JA, Witteman JC, Hofman A, et al. Identification of a common variant at the NOS1AP locus strongly associated to QT-interval duration. Hum Mol Genet. 2009;18(2):347-57.
17. Aarnoudse AJ, Newton-Cheh C, de Bakker PI, Straus SM, Kors JA, Hofman A, et al. Common NOS1AP variants are associated with a prolonged QTc interval in the Rotterdam Study. Circulation. 2007;116(1):10-6.
18. Tomás M, Napolitano C, De Giuli L, Bloise R, Subirana I, Malovini A, et al. Polymorphisms in the NOS1AP gene modulate QT interval duration and risk of arrhythmias in the long QT syndrome. J Am Coll Cardiol. 2010;55(24):2745-52.
19. Earle N, Yeo Han D, Pilbrow A, Crawford J, Smith W, Shelling AN, et al. Single nucleotide polymorphisms in arrhythmia genes modify the risk of cardiac events and sudden death in long QT syndrome. Heart Rhythm. 2014;11(1):76-82.
20. Zang X, Li S, Zhao Y, Chen K, Wang X, Song W, et al. Systematic Meta-Analysis of the Association Between a Common NOS1AP Genetic Polymorphism, the QTc Interval, and Sudden Death. Int Heart J. 2019;60(5):1083-90.
21. Hofman A, Grobbee DE, De Jong P, Van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. Eur J Epidemiol. 1991;7(4):403-22.
22. Ikram MA, Brusselle G, Ghanbari M, Goedegebure A, Ikram MK, Kavousi M, et al. Objectives, design and main findings until 2020 from the Rotterdam Study. Eur J Epidemiol. 2020;35(5):483-517.
23. World Health Organization. ATC/DDD Index. 2020. http://wwwwhoccno/atc_ddd_index.
24. Van Bemmel JH, Kors JA, Van Herpen G. Methodology of the modular ECG analysis system MEANS. Methods Inf Med. 1990;29(04):346-53.
25. Willems JL, Abreu-Lima C, Arnaud P, van Bemmel JH, Brohet C, Degani R, et al. The diagnostic performance of computer programs for the interpretation of electrocardiograms. N Engl J Med. 1991;325(25):1767-73.

26. Woosley RL, Black K, Heise CW, Romero K. CredibleMeds. org: what does it offer? Trends Cardiovasc Med. 2018;28(2):94-9.
27. Leening MJG, Kavousi M, Heeringa J, van Rooij FJA, Verkroost-van Heemst J, Deckers JW, et al. Methods of data collection and definitions of cardiac outcomes in the Rotterdam Study. Eur J Epidemiol. 2012;27(3):173-85.
28. Smith TW, Antman EM, Friedman PL, Blatt CM, Marsh JD. Part II Digitalis glycosides: Mechanisms and manifestations of toxicity. Prog Cardiovasc Dis. 1984;26(6):495-540.
29. Bers DM, Berlin JR. Kinetics of [Ca] i decline in cardiac myocytes depend on peak [Ca] i. American Journal of Physiology-Cell Physiology. 1995;268(1):C271-C7.
30. Sears CE, Bryant SM, Ashley EA, Lygate CA, Rakovic S, Wallis HL, et al. Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. Circ Res. 2003;92(5):e52-e9.
31. Chang KC, Barth AS, Sasano T, Kizana E, Kashiwakura Y, Zhang Y, et al. CAPON modulates cardiac repolarization via neuronal nitric oxide synthase signaling in the heart. Proc Natl Acad Sci U S A. 2008;105(11):4477-82.
32. Ashley EA, Sears CE, Bryant SM, Watkins HC, Casadei B. Cardiac nitric oxide synthase 1 regulates basal and β-adrenergic contractility in murine ventricular myocytes. Circulation. 2002;105(25):3011-6.
33. Xu KY, Huso DL, Dawson TM, Bredt DS, Becker LC. Nitric oxide synthase in cardiac sarcoplasmic reticulum. Proceedings of the National Academy of Sciences. 1999;96(2):657-62.
34. Ronchi C, Bernardi J, Mura M, Stefanello M, Badone B, Rocchetti M, et al. NOS1AP polymorphisms reduce NOS1 activity and interact with prolonged repolarization in arrhythmogenesis. Cardiovasc Res. 2021;117(2):472-83.
35. Earle NJ, Poppe KK, Pilbrow AP, Cameron VA, Troughton RW, Skinner JR, et al. Genetic markers of repolarization and arrhythmic events after acute coronary syndromes. Am Heart J. 2015;169(4):579-86 e3.
36. Souza DS, Menezes-Filho JER, Santos-Miranda A, Jesus ICG, Silva Neto JA, Guatimosim S, et al. Calcium overload-induced arrhythmia is suppressed by farnesol in rat heart. Eur J Pharmacol. 2019;859:172488.
37. Hauptman PJ, Kelly RA. Digitalis. Circulation. 1999;99(9):1265-70.
38. Auton A, Abecasis GR, Altshuler DM, Durbin RM, Abecasis GR, Bentley DR, et al. A global reference for human genetic variation. Nature. 2015;526(7571):68-74.

Figure titles and legends

Figure 1. Flow chart of the study population selection

RS: Rotterdam study; ECG: electrocardiogram; BBB: bundle branch block

Figure 2. Mean QT-interval differences among *NOS1AP* rs10494366 genotypic groups in digoxin non-users

The mean QT-interval duration in the reference group is 400.7 milliseconds (ms)
Model adjusted for age, sex, RR-interval, prevalent diabetes mellitus, heart failure, and myocardial infarction
Vertical lines represent a 95% confidence interval

Figure 3. Mean QT-interval differences in digoxin users compared to non-users stratified on *NOS1AP* rs10494366 genotypic groups

Model adjusted for age, sex, RR-interval, prevalent diabetes mellitus, heart failure, and myocardial infarction
Vertical lines represent a 95% confidence interval
ms, millisecond

Figure 4. Mean QT-interval differences comparing digoxin dose categories in *NOS1AP* rs10494366 genotyping groups

Digoxin daily dose categories: **0**: non-users, **1**: dose< 0.125 mg (low dosage), **2**: 0.125 mg ≤ dose< 0.250 mg (moderate dosage), **3**: dose≥ 0.250 mg(high dosage)
Model adjusted for age, sex, RR-interval, prevalent diabetes mellitus, heart failure, and myocardial infarction
Vertical lines represent 95% confidence interval
ms, millisecond

Table 1. Baseline characteristics of the study populations

Characteristic	Total (10,057)	Digoxin current users (249)	Digoxin non-users (9,808)	p_value
Age (years), mean (SD)	63.9 (9.3)	74.3 (9.4)	63.6 (9.2)	**<0.001**
Female, N (%)	5903 (58.7)	144 (57.8)	5759 (58.7)	0.78
BMI (kg/m2), median (IQR)	26.4 (24.2-28.9)	25.9 (23.5-28.3)	26.4 (24.2-28.9)	**0.02**
Ever Smokers, N (%) (Current or past)	5664 (56.3)	107 (42.9)	5557 (56.6)	0.24
Hypertension, N (%)	5289 (52.6)	125 (50.2)	5164 (52.7)	0.44
Type 2 Diabetes, N (%)	971 (9.7)	29 (11.6)	942 (9.6)	**0.001**
Heart Failure, N (%)	194 (1.9)	34 (13.7)	160 (1.6)	**<0.001**
Myocardial Infarction, N (%)	387 (3.8)	20 (8)	367 (3.7)	**<0.001**
Glucose level (mmol/L), median (IQR)	5.5 (5.1-6.0)	5.9(5.4-6.6)	5.5 (5.1-6.0)	**<0.001**
HDL cholesterol (mmol/L), median (IQR)	1.4 (1.1-1.6)	1.2 (0.9-1.5)	1.4 (1.1-1.6)	**<0.001**
Total cholesterol (mmol/L), median (IQR)	5.7 (5.1-6.4)	5.3 (4.6-6.1)	5.7 (5.1-6.4)	**0.001**
RR Interval (ms), mean (SD)	875.2 (142.8)	813.2 (175.0)	876.7 (141.6)	**<0.001**

BMI, body mass index; HDL, high-density lipoprotein; IQR, interquartile range; SD, standard deviation

Table 2. *NOS1AP* gene rs10494366 variant effects on mean QT-interval changes in digoxin non-users

Mean QT-interval change in ms (95% CI)			
Genotype	**Number of ECGs**	**Crude model[a]**	**Adjusted model [b]**
TT	9314	Reference	Reference
TG	10498	3.3 (2.7; 4.0)	3.4 (2.7; 4.0)
GG	3033	6.4 (5.4; 7.4)	6.5 (5.5; 7.5)

a Crude model adjusted for age, sex, RR-interval

b Adjusted model additionally adjusted for prevalent diabetes mellitus, heart failure, and myocardial infarction

ECG, electrocardiogram; ms, millisecond; CI, confidence interval

Table 3. *NOS1AP* gene rs10494366 variant effects on mean QT-interval differences per digoxin dose categories compared to non-user group

		Mean QT-interval difference in ms (95% CI)			
Digoxin Daily Dose Categories[a]		**0 (Non-users)**	**1 (Low dose)**	**2 (Moderate dose)**	**3 (High dose)**
	Number of ECGs	22845	49	188	97
Crude model[b]					
TT	148	Reference	-6.3 (-12.6; 0.1)	-16.4 (-20.0; -12.8)	-22.2 (-27.3; -17.1)
TG	143	Reference	-14.1 (-20.9; -7.3)	-17.2 (-21.0; -13.3)	-23.4 (-28.9; -17.8)
GG	43	Reference	-20.4 (-36.1; -4.6)	-20.7 (-27.2; -14.2)	-41.4 (-53.1; -29.8)
Adjusted model[c]					
TT	148	Reference	-5.9 (-12.4; 0.4)	-15.9 (-19.5; -12.3)	-21.6 (-26.7; -16.5)
TG	143	Reference	-14.2 (-21.0; -7.4)	-17.0 (-20.9; -13.2)	-23.2 (-28.8; -17.6)
GG	43	Reference	-18.3 (-33.9; -2.5)	-19.6 (-26.1; -13.0)	-40.8 (-52.5; -29.2)

[a] Digoxin daily dose categories: **0**: non-users, **1**: dose< 0.125 mg (low dosage), **2**: 0.125 mg ≤ dose< 0.250 mg (moderate dosage), **3**: dose≥ 0.250 mg(high dosage)

[b] Crude model adjusted for age, sex, RR-interval

[c] Adjusted model, additionally adjusted for prevalent diabetes mellitus, heart failure, and myocardial infarction

ECG, electrocardiogram; ms, millisecond; CI, confidence interval