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Genetic determinants of warfarin maintenance dose and time in therapeutic treatment range: a RE-LY genomics substudy

Aims: We investigated associations between genetic variation in candidate genes and on a genome-wide scale with warfarin maintenance dose, time in therapeutic range (TTR), and risk of major bleeding. **Materials & methods:** In total, 982 warfarin-treated patients from the RE-LY trial were studied. **Results:** After adjusting for SNPs in *VKORC1* and *CYP2C9*, SNPs in *DDHD1* (rs17126068) and *NEDD4* (rs2288344) were associated with dose. Adding these SNPs and *CYP4F2* (rs2108622) to a base model increased R^2 by 2.9%. An SNP in *ASPH* (rs4379440) was associated with TTR (-6.8% per minor allele). *VKORC1* was associated with time less than INR 2.0. *VKORC1* and *CYP2C9* were associated with time more than INR 3.0, but not with major bleeding. **Conclusions:** We identified two novel genes associated with warfarin maintenance dose and one gene associated with TTR. These genes need to be replicated in an independent cohort.

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Introduction

Warfarin is a commonly prescribed anti-coagulant for prevention of stroke in patients with atrial fibrillation (AF). Compared to placebo, warfarin reduces the risk of stroke in AF by approximately 64%; however, the treatment also has some shortcomings, mainly high inter-individual variation in dose needed to reach therapeutic effect, a narrow therapeutic range and increased risk of bleeding [1,2]. The anticoagulant effect of warfarin is measured by the international normalized ratio (INR). During the initiation phase of warfarin treatment, the dose is individualized through monitoring of the INR value and dose changes in order to reach and maintain a therapeutic range of anticoagulation, which commonly is INR 2.0–3.0 in AF [3]. There is a close relationship between INR and risk of bleeding where the risk increases at INR

above 4 and rises sharply at INR above 5 [4]. Among other risk factors for bleeds during warfarin treatment are age, renal function, concomitant antithrombotic medication, concomitant diseases and unstable INR [4,5].

Genetic variability related to the anti-coagulant response to warfarin, and possibly related to the risk of bleeding, has been intensely investigated over the last years [2,6–10]. The two genes *VKORC1* and *CYP2C9* have been identified as the most common sources of genetic variation affecting warfarin dose requirements along with *CYP4F2* [11,12]. In combination, variants in the two genes *VKORC1* and *CYP2C9* explain around 20–40% of the variance in dose needed to reach INR 2.0–3.0. The large genetic effects on warfarin dose requirements have enabled the development of pharmacogenetic dose prediction models and today

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prediction models exist for maintenance dose, loading dose and dose revisions [13]. Pharmacogenetic prediction models for warfarin dose have been compared with standard care or a clinical algorithm in randomized clinical trials with varying results [14,15].

Patients in the warfarin arm of the genomic substudy of the RE-LY clinical trial are included in this genome-wide association study (GWAS). We hypothesized that genetic markers other than the known *CYP2C9*, *VKORC1* and *CYP4F2* might affect warfarin maintenance dose and that there might exist genetic variants affecting time in therapeutic range (TTR). We also investigated if any of the additional variables renal function, smoking, *CYP4F2* or in this study identified genetic markers might improve performance of future prediction models for warfarin dosing. Furthermore, we investigated the possible clinical associations of the previously known variants rs9923231 from *VKORC1*, *CYP2C9**2 and *3 and rs2108622 from *CYP4F2* with TTR, time below INR 2.0, time above INR 3.0 or risk of major bleeding.

Materials & methods

RE-LY was a randomized clinical trial comparing two doses of dabigatran (110 or 150 mg twice daily) with warfarin for stroke prevention in 18,113 patients with documented AF and at least one additional risk factor for stroke. The primary efficacy end point of the RE-LY trial was stroke or systemic embolism and the primary safety outcome was major bleeding during a mean follow-up of 2 years. The study design and results have been described previously [16,17]. In the genomic substudy of the RE-LY trial, 3076 patients consented to provide blood samples for DNA analyses. The current study focuses on 982 warfarin-treated patients with genetic samples.

Eligibility for the trial required documented AF and at least one of the following additional risk factors: first, history of previous stroke, transient ischemic attack or systemic embolism; second, ejection fraction less than 0.40; third, symptomatic heart failure, New York Heart Association class 2 or higher in the last 6 months; fourth, age at least 75 years or, fifth, age at least 65 years and any of diabetes mellitus, hypertension or coronary artery disease. Among the exclusion criteria were severe heart valve disorder, recent stroke, increased risk of hemorrhage, creatinine clearance less than 30 ml/min or active liver disease. Patients on vitamin K antagonist (VKA) treatment at the time of randomization stopped their treatment on the day of randomization and began treatment at INR below 3.0 if assigned to warfarin. The local investigator was responsible for warfarin dose adjustments required to reach and maintain an

INR of 2.0–3.0. After the initial dose titration phase, patients randomized to warfarin underwent INR testing at least once a month and measures were adopted to maximize the TTR 2.0–3.0 [17]. A dose-adjustment algorithm including an initiation nomogram and an action table that proposed dose adjustments was provided, but its use was not mandatory according to the study protocol [17].

Study end points

Warfarin maintenance dose was calculated as the mean of all doses during stable anticoagulation periods. A stable anticoagulation period was defined as a period of at least three measures of INR within 2.0–3.0. In patients where no stable anticoagulation periods were observed, the maintenance dose was calculated as the mean of all doses associated with an INR between 2.0 and 3.0. TTR 2.0–3.0, time below INR 2.0 and time above INR 3.0 for the first 3 months (TTR 3M) and TTR during the whole treatment period (TTR Total) were calculated using linear interpolation according to the Rosendaal method [18]. TTR 3M was calculated using all values regardless of treatment initiation or interruption and TTR Total was calculated excluding the first 7 days of treatment and treatment interruptions. Major bleeding was defined as a reduction in hemoglobin concentration by at least 20 g/l, transfusion of at least two units of blood or symptomatic bleeding in a crucial area or organ [16]. Creatinine clearance (CrCL [ml/min]) was calculated according to the Cockcroft–Gault formula [16].

Genotyping

The 982 warfarin patients with DNA samples were genotyped using the Illumina Human610-quad DNA analysis BeadChip, which has 620,901 markers. Data management and quality control (QC) procedures were performed using PLINK [19]. In short, SNPs were excluded if the call rate was less than 98% or minor allele frequency (MAF) was less than 1%. The 3405 SNPs that deviated from the Hardy–Weinberg equilibrium ($p < 10^{-6}$) were flagged, but not excluded from analyses. Patients were excluded if their total call rate was less than 98%, if self-reported sex did not match genetic sex or if the genetic relatedness to another patient was high (π_{hat} from PLINK > 0.2). Reported genetic coordinates were based on the NCBI human genome build 36.

Statistical analysis of genome-wide data

In general, all genome-wide statistical analyses of all outcomes were performed assuming an additive genetic model, in other words, the genotypes for each SNP were coded 0, 1 and 2 and handled as continuous

variables. To account for the amount of multiplicity, a p-value threshold of genome-wide significance was set at $0.05/554,725 = 9 \times 10^{-8}$ according to the Bonferroni method. To account for possible population stratification, all genome-wide analyses were adjusted for the first four genetic principal components [20]. Furthermore, genome-wide significant findings were validated in the large cluster 1, shown in the plot of the first two principal components in **Supplementary Figure 1**, for consistency of regression coefficient (beta) estimates. The clusters were identified using k-means clustering (the Hartigan and Wong method with $k = 6$) on the four first genetic principal components.

GWAS of warfarin maintenance dose was performed using linear regression on the square root of warfarin dose (transformed to obtain normality). The analyses were adjusted by the nongenetic clinical variables from the International Warfarin Pharmacogenetics Consortium (IWPC) model and the full set of IWPC variables [7]. The full IWPC pharmacogenetic prediction model for warfarin maintenance dose includes the following variables: age, height, weight, race, enzyme inducer status (treatment with carbamazepine, phenytoin or rifampicin), amiodarone, *VKORC1* rs9923231 and *CYP2C9**2/*3.

TTR during the first 3 months and total TTR were analyzed using linear regression. Due to the low consistency of factors affecting TTR in the literature [21,22], selection of variables for adjustments were driven by the data. This was done as follows for each outcome. First, univariate effects on the outcomes were estimated. Variables with univariate $p < 0.10$ were analyzed in a multiple model and selected for inclusion for adjustment in the GWAS analyses if $p < 0.10$ in the multiple model. Variables with previously known clinical effect could be added to the final adjustment variables although $p \geq 0.10$ based on judgment of beta estimates and precision. Assumptions of linear effect of continuous variables were inspected and where nonlinear effects were present, appropriate transformation was made. The variables eligible for inclusion as covariates were: age, weight (kg), height (cm), BMI (kg/m^2), gender, systolic and diastolic blood pressure at baseline (mmHg), CrCl (ml/min), type of AF, CHADS₂ score [23], previous stroke, previous diabetes mellitus, previous hypertension, history of VKA use, VKA use class at entry, baseline INR, treatment with aspirin, angiotensin-II receptor antagonists, ACE-inhibitors, clopidogrel, amiodarone, statins, proton pump inhibitors, H₂-blockers, P-glycoprotein (P-gp) inhibitors and intake of any liver enzyme inducer (carbamazepine, phenytoin or rifampicin).

All statistical analyses on a genome-wide scale were performed using GenABEL [24].

Table 1. Baseline characteristics and outcomes.

Characteristics	Outcomes
Total number of patients	956
Age (years)	72 (68–77)
Gender (male)	632 (66%)
Height (cm)	173 (166–178)
Weight (kg)	85 (75–96)
BMI (kg/m^2)	28.4 (25.7–32.2)
Systolic blood pressure (mmHg)	133 (120–146)
Diastolic blood pressure (mmHg)	80 (70–85)
CrCl (ml/min)	73 (58–90)
Smoker: yes	70 (7%)
History of VKA use	772 (81%)
VKA naive at baseline [†]	387 (40%)
Oral anticoagulation at baseline	726 (76%)
Previous stroke	93 (10%)
Diabetes mellitus	189 (20%)
Hypertension	729 (76%)
Baseline INR	1.8 (1.2–2.5)
Medications at baseline	
Aspirin	289 (30%)
Angiotensin-II receptor antagonists	230 (24%)
ACE inhibitors	448 (47%)
Clopidogrel	45 (5%)
Amiodarone	82 (9%)
Statins	465 (49%)
Proton pump inhibitors	123 (13%)
H ₂ blockers	39 (4%)
P-gp inhibitors	200 (21%)
Inducer status [‡]	12 (1%)
CHADS₂ score	
0	34 (4%)
1	329 (34%)
2	327 (34%)
3	168 (18%)
4	74 (8%)
5	22 (2%)
6	2 (0%)
Type of AF	
Paroxysmal	321 (24%)

Continuous variables are presented as median (interquartile range) and categorical variables are presented as n (%).

[†]Duration of previous VKA treatment ≤ 2 months.

[‡]Taking any of carbamazepine, phenytoin or rifampicin [7].

AF: Atrial fibrillation; CrCl: Creatinine clearance; INR: International normalized ratio; TTR: Time in therapeutic range (2–3); VKA: Vitamin K antagonist.

Table 1. Baseline characteristics and outcomes (cont.).	
Characteristics	Outcomes
Permanent	344 (36%)
Persistent	291 (30%)
Ethnicity	
Asian	19 (2%)
African	7 (1%)
Other	100 (10%)
White	830 (87%)
Warfarin maintenance dose (mg/week)	29.6 (21.9–37.6)
TTR	
First 3 months (%)	61.7 (42.1–81.6)
Total (%)	72.2 (61.4–81.2)
Stroke or systemic embolism	24 (2.5%)
Major bleeding	53 (5.5%)
Continuous variables are presented as median (interquartile range) and categorical variables are presented as n (%).	
*Duration of previous VKA treatment ≤2 months.	
*Taking any of carbamazepine, phenytoin or rifampicin [7].	
AF: Atrial fibrillation; CrCl: Creatinine clearance; INR: International normalized ratio;	
TTR: Time in therapeutic range (2–3); VKA: Vitamin K antagonist.	

Imputation using reference panels

To enrich for SNPs in regions with genome-wide hits, variants were imputed one megabase upstream and one megabase downstream of each hit using public

available reference panels. Impute v2.2.2 was used for the imputation [25,26] and SHAPEIT was used for prephasing the RE-LY data [27]. The 1000 Genomes Phase I integrated variant set (February 2012) was used as reference set for the imputations [28].

Statistical analysis of phenotype & candidate gene data on maintenance dose

Univariate and multiple analyses of candidate variables evaluated for predictive ability of warfarin dose were performed using linear regression on the square root transformed dose. R² values were reported on the original scale of the variable by retransforming the predicted values and calculating the squared correlation between the predicted and observed values. To reduce the effects of overfitting, the added R² to the pharmacogenetic base model that included all the IWPC covariates, was evaluated using cross validation with 10,000 resamples of data; 70% of patients as training data set and 30% of patients as validation data set. The median and the 2.5 and 97.5 percentiles of the resulting retransformed R² distribution were presented as point estimate and 95% confidence interval. As R² values can be quite abstract, we also calculated a set of more clinically intuitive measures. These included the percentage of patients predicted within 20% of the observed dose in total and in subclasses of patients with observed dose ≤21 mg/week, >21 to <49 mg/week and ≥49 mg/week as well as the number needed to treat

Figure 1. The analysis of warfarin maintenance dose. (A) Adjusted for clinical factors and the first four genetic principal components. (B) Adjusted for clinical factors, genetic factors (*VKORC1* rs9923231 and *CYP2C9**2/*3) and the first four genetic principal components. In (A & B), the red dashed line indicates the threshold for genome-wide significance.

Table 2. Genome-wide hits.

Outcome	SNP	Chr	Pos	Gene	N	MAF	Beta (95% CI)	p-value	HWE p-value
Warfarin dose	rs10871454	16	30955580	<i>STX4</i>	951	37.9	0.82 (-0.91 to -0.72)	4.40×10^{-64}	0.19
Warfarin dose	rs9923231	16	31015190	<i>VKORC1</i>	951	38.0	-0.82 (-0.91 to -0.72)	7.26×10^{-64}	0.19
Warfarin dose	rs4917639	10	96715525	<i>CYP2C9</i>	951	19.4	-0.72 (-0.84 to -0.60)	8.47×10^{-32}	0.76
Warfarin dose	rs1057910 (*3)	10	96731043	<i>CYP2C9</i>	951	6.6	-0.93 (-1.11 to -0.74)	1.77×10^{-22}	0.30
Warfarin dose	rs1799853 (*2)	10	96692037	<i>CYP2C9</i>	951	12.4	-0.48 (-0.62 to -0.34)	4.98×10^{-11}	0.77
Warfarin dose (adjusted [†])	rs2288344	15	53909639	<i>NEDD4</i>	951	30.2	0.20 (0.13–0.28)	3.96×10^{-8}	0.76
Warfarin dose (adjusted [†])	rs17126068	14	52569647	<i>DDHD1</i>	951	1.4	-0.80 (-1.09 to -0.52)	4.14×10^{-8}	1.00
TTR in total	rs4379440	8	62628368	<i>ASPH</i>	938	7.6	-6.82 (-9.27 to -4.37)	5.00×10^{-8}	0.25
TTR in total	rs17791091 [‡]	8	62626151	<i>ASPH</i>	938	6.9	-7.84 (-10.45 to -5.23)	4.13×10^{-9}	0.80

All genome-wide significant results were validated in cluster 1, shown in [Supplementary Figure 1](#).

[†]In addition to the clinical factors, this analysis was adjusted for rs9923231 of *VKORC1* and *2/*3 of *CYP2C9*.

[‡]Imputed SNP.

HWE: Hardy–Weinberg equilibrium test; TTR: Time in therapeutic range (2–3).

(NNT) to prevent one patient from being predicted outside 20% of the actual dose [7,29]. Furthermore, to graphically evaluate additional factors, we plotted the difference in the absolute residual of a base model to a new model including an additional covariate. In these graphs, the reported values of difference are on the original scale of the variable. A negative value indicates that the new

prediction is worse and a positive value indicates that the new prediction is better.

Statistical analysis of candidate gene data on TTR & major bleeding

Univariate effects of *VKORC1*, *CYP2C9* and *CYP4F2* on TTR outcomes were evaluated using the non-

Figure 2. The analysis of TTR in total adjusted for clinical factors and the first four principal components. The red dashed line indicates the threshold for genome-wide significance.

Table 3. Univariate and multiple effects for potential candidates for inclusion in future prediction models of warfarin dose.						
Variable	Univariate			Multiple [†]		
	R ²	p-value	Beta (95% CI)	Added R ² (95% CI) to the base model [‡]	p-value	beta (95% CI)
Base model including all IWPC variables	–	–	–	51.6% (44.5–58.3) [§]	–	–
CrCl (ml/min)	9.6%	6.30 × 10 ^{–23}	0.013 (0.011–0.016)	0.4% (–1.5–1.1)	6.51 × 10 ^{–4}	0.005 (0.002–0.008)
Smoking (yes)	0.0%	8.57 × 10 ^{–1}	–0.025 (–0.300–0.250)	0.0% (–0.7–0.1)	2.79 × 10 ^{–1}	–0.102 (–0.288–0.083)
CYP4F2 rs2108622 (G>A)	0.8%	1.96 × 10 ^{–3}	0.159 (0.046–0.272)	1.0% (–0.9–2.0)	3.64 × 10 ^{–6}	0.178 (0.103–0.253)
DDHD1 rs17126068 (A>G)	1.2%	2.26 × 10 ^{–4}	–0.827 (–1.264 to –0.389)	1.1% (–0.6–2.7)	7.03 × 10 ^{–8}	–0.809 (–1.100 to –0.517)
NEDD4 rs2288344 (A>C)	1.1%	5.53 × 10 ^{–3}	0.172 (0.062–0.283)	1.3% (–0.9–2.5)	4.20 × 10 ^{–7}	0.191 (0.117–0.264)
R ² values are reported on the original scale of the variable. [†] After adding the variable to the base model including the variables in the IWPC model: age, weight, height, amiodarone, inducer status (any of carbamazepine, phenytoin and rifampicin), ethnicity, VKORC1 rs9923231 and CYP2C9*2/*3. [‡] Calculated as the median and 95% CI (2.5 percentile; 97.5 percentile) of the resulting R ² distribution created by 10,000 cross validations (70% of data as training and 30% as validation). [§] Total R ² of the base model. CrCl: Creatinine clearance; IWPC: International Warfarin Pharmacogenetics Consortium.						

parametric Kruskal-Wallis test (due to the skewed distribution of time below INR 2.0 and time above INR 3.0). Major bleeding was evaluated using Fisher’s exact test and hazard ratios were estimated using Cox-regression. In addition to the analysis of single variants of *VKORC1* and *CYP2C9*, a responder category was created according to Mega *et al.* [30] as following:

- Normal responders: *VKORC1* G/G and (*CYP2C9**1/*2 or *1/*2);
- Sensitive responders: *VKORC1* G/G and (*CYP2C9**1/*3 or *2/*2 or *2/*3) or *VKORC1* A/G and (*CYP2C9**1/*2 or *1/*3 or *2/*2) or *VKORC1* A/A and (*CYP2C9**1/*1 or *1/*2);
- Highly sensitive responders: *VKORC1* G/G and (*CYP2C9**3/*3) or *VKORC1* A/G and (*CYP2C9**2/*3 or *3/*3) or *VKORC1* A/A and (*CYP2C9**1/*3 or *2/*2 or *2/*3 or *3/*3).

In the analyses of phenotype and candidate gene data, the significance level was set at 0.05. All statistical analyses were done using R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics & outcomes

Baseline characteristics and outcomes are given in Table 1. All subsequent analyses are reported based on

the 956 patients and 554,725 markers that passed QC in the warfarin treatment arm. These patients had a median follow-up of 816 days. 81% had a history of VKA use and 40% were warfarin naive at baseline (duration of previous VKA treatment ≤2 months). The maintenance dose of warfarin could be calculated in 951 patients and the median value was 29.6 mg/week. 38 (4%) of the 951 patients did not achieve any stable anticoagulation period, hence the mean of all doses achieving INR 2.0–3.0 was used for these patients. Overall median TTR was 72.2% (mean: 70.1%), whereas the median TTR in the first 3 months was 61.7% (mean: 61.0%). Fifty-three major bleeding events were reported corresponding to an incidence rate of 2.6% per patient year.

GWAS in relation to warfarin maintenance dose

A Manhattan plot of the GWAS results for warfarin maintenance dose, adjusted for clinical factors and principal components, is shown in Figure 1A. As expected, there were two major peaks in the areas around *VKORC1* and *CYP2C9* [12]. The top hit SNPs were in a region of high LD around *VKORC1* on chromosome 16 and the SNP with the highest signal was in *STX4* (rs10871454, p = 4.40 × 10^{–64}) (Table 2 & Supplementary Table 1). However, this SNP is in high LD (r² = 0.994) with the third most significant SNP, rs9923231, which is the *VKORC1* SNP commonly used in prediction models

for warfarin maintenance dose [6,7,10]. The top hit SNP in the *CYP2C9* region (rs9332220, $p = 5.64 \times 10^{-32}$) is in complete LD ($r^2 = 1.00$) with the SNP rs4917639 that is known to tag the *2 and *3 variants in *CYP2C9* (Table 2 & Supplementary Table 2) [2,10].

Figure 1B shows the results of the analysis of warfarin maintenance dose when, in addition to the first model, also adjusting for *VKORC1* (rs9923231) and *CYP2C9*(*2/*3). Two SNPs reach genome-wide significance (Table 2 & Supplementary Table 2), one rare SNP (MAF: 1.4%) in the intergenic region close to *DDHD1* on chromosome 14 (rs17126068, $p = 4.14 \times 10^{-8}$) as well as one more common variant (MAF: 30.2%) located in the intron of *NEDD4* on chromosome 15 (rs2288344, $p = 3.96 \times 10^{-8}$). The *DDHD1* SNP was associated with a lowering of dose per minor allele (beta estimate: -0.80 mg/week on square root scale); however, no homozygotes for the minor allele were present. On the contrary, the *NEDD4* SNP was associated with an increase of 0.20 mg/week (on square root scale) per minor allele. Descriptive statistics per genotype of the two SNPs are given in Supplementary Table 3. Imputation around each top hit in the regions *VKORC1*, *CYP2C9*, *DDHD1* and *NEDD4* revealed no additional findings.

Subgroup analyses were performed for the two variants from *NEDD4* and *DDHD1* in the largest cluster (based on PCA) to rule out the risk of confounding by population stratification. The results were similar and are presented in Supplementary Table 4.

GWAS in relation to TTR

Concerning TTR during the first 3 months, the results were adjusted for the following covariates that were significant in our study: diastolic blood pressure at baseline, previous stroke, baseline INR and the first four principal components. The results concerning total TTR were adjusted for the following factors: age, age squared (to account for nonlinear effect of age), height (cm), AF type, CHADS₂ score, previous stroke, VKA use class at entry, baseline INR, P-gp inhibitors under treatment and the first four principal components. The analysis of TTR in the first 3 months revealed no genome-wide significant signals. However, for total TTR there was a genome-wide significant hit in *ASPH* on chromosome 8 (Figure 2 & Table 2), namely rs4379440 ($p = 5.00 \times 10^{-8}$). The SNP has a MAF of 7.6% and the effect on TTR was -6.8% (95% CI: -9.3 to -4.4) per minor allele. Imputation in the region around rs4379440 revealed an even stronger signal

Table 4. Estimations of clinical usefulness of potential candidates for future prediction models of warfarin dose.

Variable	R ² (%)	Predicted ideal [†] dose (n total = 951); n (%)	NNT [‡] compared with clinical model or base model	Predicted ideal [†] dose for observed dose category, n (%)		
				≤21 mg/week (n = 215); n (%)	>21 to <49 mg/week (n = 648); n (%)	≥49 mg/week (n = 88); n (%)
Clinical model including all nongenetic IWPC variables	13.3	404 (42.5)	–	11 (5.1)	389 (60.0)	4 (4.5)
Add <i>CYP2C9</i> *2/*3	26.2	425 (44.7)	45	34 (15.8)	387 (59.7)	4 (4.5)
Add <i>VKORC1</i> rs9923231	39.4	500 (52.6)	10	70 (32.6)	417 (64.4)	13 (14.8)
Add <i>VKORC1</i> and <i>CYP2C9</i>	53.2	542 (57.0)	7	89 (41.4)	430 (66.4)	23 (26.1)
Base model including all IWPC variables	53.2	542 (57.0)	–	89 (41.4)	430 (66.4)	23 (26.1)
Add CrCl (ml/min)	53.2	541 (56.9)	-951	89 (41.4)	428 (66.0)	24 (27.3)
Add smoking	52.9	540 (56.8)	-476	89 (41.4)	429 (66.2)	23 (26.1)
Add <i>CYP4F2</i> rs2108622 (G>A)	53.8	545 (57.3)	317	89 (41.4)	431 (66.5)	25 (28.4)
Add <i>DDHD1</i> rs17126068 (A>G)	54.1	544 (57.2)	476	88 (40.9)	432 (66.7)	24 (27.3)
Add <i>NEDD4</i> rs2288344 (A>C)	54.2	549 (57.7)	136	87 (40.5)	436 (67.3)	26 (29.5)
Add <i>CYP4F2</i> , <i>DDHD1</i> and <i>NEDD4</i> to base model [§]	56.1	551 (57.9)	106	97 (45.1)	425 (65.6)	29 (33.0)

R² values are reported on the original scale of the variable.

[†]Ideal dose was defined as prediction within 20% of the observed maintenance dose [7].

[‡]Number needed to treat (NNT) was calculated as 1/(proportion predicted ideal for comparison model – proportion predicted ideal for base model). A negative value indicates that the comparison model does worse than the base model.

[§]The increase in R² when adding *CYP4F2*, *DDHD1* and *NEDD4* to the base model including all IWPC variables is 56.1% - 53.2% = 2.9%.

CrCl: Creatinine clearance; IWPC: International Warfarin Pharmacogenetics Consortium.

Figure 3. Added value of *CYP4F2* rs2108622, *DDHD1* rs17126068 and *NEDD4* rs2288344 to a base prediction model including all the variables from the IWPC algorithm [7]: age, height, weight, amiodarone, inducer use, ethnicity, *CYP2C9**2/*3 and *VKORC1* rs9923231. Each dot illustrates a patient's difference in ABS residual between the base model and the new model. The red line is a loess smoother showing the trend in the data. ABS: Absolute.

from an imputed SNP, rs17791091 ($p = 4.13 \times 10^{-9}$). It has an MAF of 6.9% and the effect on TTR was -7.8% (95% CI: -10.5 to -5.2) per minor allele. This SNP is positioned in the intron region of *ASPH* close to the typed rs4379440 SNP, which also is located in the intron, the LD (r^2) between them is 0.936.

Subgroup analysis was performed for rs4379440 in the largest cluster (based on PCA) to rule out the risk of confounding by population stratification. The effects on total TTR were similar and are presented in [Supplementary Table 4](#).

Evaluation of additional variables for dose prediction models

We evaluated a list of phenotypes and genotypes that could be candidates to be included in future prediction models for warfarin dosing. The variables evaluated were CrCl, smoking and *CYP4F2* rs2108622 with the addition of the novel two SNPs found in this GWAS:

DDHD1 rs17126068 and *NEDD4* rs2288344. The univariate and adjusted effects on dose are shown in [Table 3](#). CrCl showed a large univariate effect with an R^2 of 9.6% ($\beta = 0.013$; $p = 6.30 \times 10^{-23}$), but much of this effect was due to the correlation of CrCl with age ($r = -0.60$) and weight ($r = 0.69$). When adding CrCl to a base model including the variables used in the IWPC model [7], the added R^2 was 0.4% but CrCl was still statistically significant ($\beta = 0.005$; $p = 6.51 \times 10^{-4}$). As an example, the lowering of dose for patients moving from the median CrCl in this study of 73 to 30 ml/min is -1.3 mg/week (-13.1%) for patients with a 10 mg/week dose, -2.3 mg/week (-7.7%) for patients with a 30-mg/week dose and -3.0 mg/week (-6.0%) for patients with a 50-mg/week dose. Smoking showed no signs of being important for determination of warfarin dose (adjusted $p = 0.279$). All three SNPs evaluated (*CYP4F2* rs2108622, *DDHD1* rs17126068 and *NEDD4* rs2288344) had statistically significant

effects and showed univariate R^2 values of approximately 1% and added roughly the same amount to the base model.

The clinical value of adding the variables to a base model including the variables used in the IWPC model are shown in Table 4 [7]. The NNT to prevent one patient from being predicted outside 20% of the actual dose was negative for CrCl and smoking (-951 and -476, respectively) indicating that these factors would not improve dosing accuracy. The genetic factors *CYP4F2* rs2108622, *DDHD1* rs17126068 and *NEDD4* rs2288344 presented with positive NNT values of 317, 476 and 136, respectively. All variables with positive NNT (i.e., the genetic factors) were used in a combined model resulting in a NNT of 106. An illustration of the performance of this model compared with the base model is shown in Figure 3. For comparison, adding *CYP2C9**2/*3 and *VKORC1* rs9923231 to a clinical model including all nongenetic variables

from the IWPC model was also calculated (Table 4). Adding *VKORC1* rs9923231 and *CYP2C9**2/*3 to the clinical model gives a NNT of 7, an illustration of the performance of this model compared with the clinical model is shown in Figure 4.

Effects of *VKORC1*, *CYP2C9* & *CYP4F2* on time in or out-of-range & major bleeding

VKORC1 rs9923231, *CYP2C9**2, *3 and the *2/*3 composite, *CYP4F2* and the responder categories according to Mega *et al.* [30] were evaluated against time below INR 2.0, time above INR 3.0 and TTR 2.0–3.0 for the first 3 months and for the whole treatment period. The same variables were also analyzed against major bleeding events. During the first 3 months, *VKORC1* rs9923231 was associated with time below INR 2.0 (median per genotype A/A = 7.5%, A/G = 15.1%, G/G = 17.2%, Kruskal–Wallis $p = 6.35 \times 10^{-4}$) as well as time above INR 3.0 (median

Figure 4. Added value of *CYP2C9**2/*3 and *VKORC1* rs9923231 to a base prediction model including the clinical variables from the IWPC algorithm [7]: age, height, weight, amiodarone, inducer use and ethnicity. Each dot illustrates a patient's difference in residual between the base model and the new model. The red line is a loess smoother showing the trend in the data.

ABS: Absolute.

per genotype, A/A: 13.0%, A/G: 8.1%, G/G: 5.2%; Kruskal–Wallis $p = 1.11 \times 10^{-2}$). *CYP2C9*2/*3* was associated with time above INR 3.0 (Kruskal–Wallis $p = 9.21 \times 10^{-4}$). The *CYP2C9*2/*3* effect was due to the effect of *CYP2C9*3* (median per genotype, A/A: 6.5%, A/C: 13.4%, C/C: 21.3%; Kruskal–Wallis $p = 5.65 \times 10^{-4}$), whereas *CYP2C9*2* had no significant

effect on time above target range (Kruskal–Wallis $p = 2.81 \times 10^{-1}$). Furthermore, during the first 3 months, the responder categories were associated with time above INR 3.0 (median per category: normal responder 3.4%, sensitive responder 12.6% and highly sensitive responder 13.3%, Kruskal–Wallis $p = 4.35 \times 10^{-5}$) and time below INR 2.0 (median per category, nor-

mal responder: 17.0%, sensitive responder: 11.9% and highly sensitive responder: 12.3%; Kruskal–Wallis $p = 1.30 \times 10^{-2}$). No other time in or out-of-range outcomes were statistically significant for either TTR during the first 3 months or in total. However, trends toward what would be expected for *CYP2C9**3 could be seen for all time in or out-of-range outcomes (Figure 5). There was no significant difference between the numbers of major bleeding events for the genotypes of the analyzed markers (Table 5).

Discussion

We performed a genome-wide association study on 956 warfarin-treated patients from the RE-LY study. The major findings were two novel genes affecting warfarin maintenance dose as well as one novel gene affecting TTR.

In concordance with previous GWAS of warfarin maintenance dose on patients of mainly European descent, we found major peaks around *CYP2C9* and *VKORC1* [12,31]. After adjustment of *CYP2C9* (*2 and *3), *VKORC1* (rs9923231) and clinical covariates, we found two novel genes affecting warfarin maintenance dose. The findings were located in or close to *DDHDI* (rs17126068) on chromosome 14 and *NEDD4* (rs2288344) on chromosome 15. *DDHDI* is a phospholipase that hydrolyzes phosphatidic acid. *NEDD4* is an E3 ubiquitin-protein ligase and a receptor-potentiating factor.

The GWAS analyses of warfarin maintenance dose were followed up by investigating the effect of adding the new novel markers as well as other known variables that affect warfarin maintenance dose (CrCl, smoking and *CYP4F2* rs2108622) to a warfarin dose predic-

Table 5. Major bleeding events per genotype of *VKORC1* rs9923231, *CYP2C9**2, *3, the *2/*3 composite and *CYP4F2* rs2108622.

SNP	Genotype	Major bleeding		p-value [†]	Hazard ratio (95% CI) vs reference
		Yes (n = 53)	No (n = 903)		
<i>VKORC1</i> rs9923231	A/A	10 (19%)	138 (15%)	0.75	1.25 (0.59–2.66)
	A/G	22 (42%)	409 (45%)		0.94 (0.52–1.71)
	G/G	21 (40%)	356 (39%)		Reference
<i>CYP2C9</i> *2/*3	*1/*1	30 (57%)	595 (66%)	0.51	Reference
	*1/*2	15 (28%)	182 (20%)		1.61 (0.86–2.99)
	*1/*3	7 (13%)	94 (10%)		1.42 (0.62–3.24)
	*2/*2	1 (2%)	12 (1%)		1.51 (0.21–11.04)
	*2/*3	0 (0%)	14 (2%)		Too few observations
	*3/*3	0 (0%)	6 (1%)		Too few observations
<i>CYP2C9</i> *2	G/G	37 (70%)	695 (77%)	0.35	Reference
	G/A	15 (28%)	196 (22%)		1.43 (0.20–10.43)
	A/A	1 (2%)	12 (1%)		1.41 (0.78–2.58)
<i>CYP2C9</i> *3	A/A	46 (87%)	789 (87%)	0.88	Reference
	A/C	7 (13%)	108 (12%)		1.07 (0.48–2.38)
	C/C	0 (0%)	6 (1%)		Too few observations
<i>CYP4F2</i> rs2108622	G/G	31 (58%)	449 (50%)	0.30	Reference
	G/A	17 (32%)	385 (43%)		0.63 (0.35–1.14)
	A/A	5 (9%)	69 (8%)		1.04 (0.41–2.68)
Responder according to Mega et al. [‡]	Normal responder	32 (60%)	561 (62%)	0.91	Reference
	Sensitive responder	20 (38%)	322 (36%)		1.08 (0.62–1.90)
	Highly sensitive responder	1 (2%)	20 (2%)		0.90 (0.12–6.61)

[†]Fisher's exact test.

[‡]According to Mega et al. [30], Normal responders: *VKORC1* G/G and (*CYP2C9**1/*2 or *1/*2); Sensitive responders: *VKORC1* G/G and (*CYP2C9**1/*3 or *2/*2 or *2/*3) or *VKORC1* A/G and (*CYP2C9**1/*2 or *1/*3 or *2/*2) or *VKORC1* A/A and (*CYP2C9**1/*1 or *1/*2); Highly sensitive responders: *VKORC1* G/G and (*CYP2C9**3/*3) or *VKORC1* A/G and (*CYP2C9**2/*3 or *3/*3) or *VKORC1* A/A and (*CYP2C9**1/*3 or *2/*2 or *2/*3 or *3/*3).

tion model including the covariates used in the IWPC prediction model [7]. The genetic variables *CYP4F2* (rs2108622), *DDHD1* (rs17126068) and *NEDD4* (rs2288344) showed promising results with an added R^2 of approximately 1% each or approximately 3% combined. However, given that adding the three genetic factors gave a NNT of 106 while doubling the number of SNPs that have to be genotyped, one can hypothesize that prospective genotyping of these additional SNPs is not cost effective in clinical practice. Although there might exist rare variants affecting warfarin sensitivity or resistance, this study indicates that for patients of European descent, the published clinical trials utilizing pharmacogenetic driven warfarin dosing were performed using appropriate genetic variants of *VKORC1* and *CYP2C9* [14,15].

Interestingly, our study shows no effect of smoking on warfarin maintenance dose. Smoking is thought to potentially increase warfarin metabolism, and to increase warfarin maintenance doses by approximately 10% compared with nonsmokers; however, there is also conflicting evidence showing no effect of smoking [32].

To our knowledge, this is the first study reporting GWAS results on TTR. For TTR during the whole treatment period, a genome-wide significant signal was found in *ASPH* (rs4379440) on chromosome 8. *ASPH* is thought to play an important role in calcium homeostasis. The gene is expressed from two promoters and undergoes extensive alternative splicing. The longest isoforms (a and f) include a C-terminal domain that hydroxylates aspartic acid or asparagine residues of some proteins, including protein C, coagulation factors VII, IX, and X, and the complement factors C1R and C1S [33].

When analyzing the effect of the established warfarin-related genes *VKORC1* rs9923231, *CYP2C9**2/*3 and *CYP4F2* rs2108622 on time in and out-of-TTR, rs9923231 was associated with both time below INR 2.0 and time above INR 3.0 during the first 3 months. Furthermore, *CYP2C9**3 was associated with time above INR 3.0 during the first 3 months. The responder categories according to Mega *et al.* [30], which combines *VKORC1* and *CYP2C9*, also had effect on INR above 3.0 and below 2.0 during the first 3 months. These results are in line with previous results on TTR measures showing that *VKORC1* does have modest effect on the stability of anticoagulation in patients on warfarin, whereas the largest effect (the lowest TTR) is seen in patients homozygous for the *CYP2C9**3 allele [10,34]. We saw no statistically significant effects on TTR during the whole treatment period. This could be due to the effect of genetic factors affecting dose which in turn affects TTR are expected to diminish over time as patient dosing is data driven and adjusted according to

the INR value. Thus, TTR in the long term is expected to be more dependent on the skill of the person doing the dosing or if algorithm based dosing is used, as was encouraged in the RE-LY trial [35].

Our study did not show an effect of *VKORC1* or *CYP2C9* on the hard outcome major bleeding. The ENGAGE AF-TIMI 48 trial with 4833 warfarin-treated patients showed an increased risk of bleeding during the first 3 months in warfarin-sensitive patients [30]. Patients were classified as sensitive if they carried the following combinations of variants: *CYP2C9**1/*3, *2/*2, *2/*3 or *3/*3 with any *VKORC1* genotype, in addition *1/*2 with *VKORC1* A/G or *VKORC1* A/A and *1/*1 with *VKORC1* A/A. A conclusion from the ENGAGE trial was that non-vitamin K oral anticoagulants could be reserved for individuals classified as sensitive responders and therefore more likely to experience early warfarin bleeding [36]. We used the same definition of responder as in the ENGAGE AF-TIMI 48 trial but did not see any increased risk of major bleeding in the sensitive categories.

There are limitations to this study. First of all, only 40% of patients were warfarin naive at baseline and 76% of the patients were on oral anticoagulants at baseline, which affects TTR measurements. Although we could see genetic effects on TTR in the first 3 months, these results are probably underestimated due to the number of patients on oral anticoagulants at baseline. Second, a majority of the patients are white (87%) with a low percentage of Asians (2%) and Africans (1%) why the results are not generalizable to these ethnic groups. Third, no patients with severe renal impairment (CrCl <30 ml/min) were included in the RE-LY study creatinine clearance <30 ml/min was an exclusion criterion. Fourth, multiple outcomes were studied that increase the chance of spurious findings and the novel findings against warfarin dose were found after adjusting for the previously known variants in *VKORC1* and *CYP2C9*. Fifth, the genome-wide hits found in this study, have not yet been replicated in an independent cohort. And last, even though the study was fairly large with 982 warfarin-treated patients, it was underpowered with respect to the outcome major bleeding.

Conclusion

We conducted a GWAS analyzing warfarin maintenance dose and TTR in the warfarin-treated patients from the RE-LY genomics study. For both outcomes, we identified novel genome-wide significant findings. The main finding in the current study was the identification of two novel SNPs having an effect on warfarin maintenance dose. However, these SNPs provide limited incremental

information for prediction of a patient's dose.

Future perspective

This study confirms the importance of variants within *CYP2C9* and *VKORC1* for the prediction of warfarin dose in patients of European descent. By adding variants from *CYP4F2*, *DDHD1* and *NEDD4* to a pharmacogenetic dose model including *CYP2C9* and *VKORC1*, the variance in stable warfarin dose explained increased by ~3%. Since warfarin dosing is closely monitored by INR with subsequent dose changes if the INR is out of range, dose prediction based on *CYP2C9* and *VKORC1* is probably good enough in Europeans. However, in the future patients may have their genome sequenced and the results available in their medical records. In that case, even genetic variants with a limited effect on dose could be used to influence prescribing decisions.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/pgs-2016-0061

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Executive summary

Objective

- Warfarin treatment has some shortcomings, mainly high inter-individual variation in dose needed to reach therapeutic effect, a narrow therapeutic range and increased risk of bleeding. We hypothesized that genetic markers other than the known *CYP2C9*, *VKORC1* and *CYP4F2*, might affect warfarin maintenance dose and that there might exist genetic variants affecting time in therapeutic treatment range (TTR).

Materials & methods

- Patients in the warfarin arm of the genomic substudy of the RE-LY clinical trial are included in this genome-wide association study (GWAS).
- Outcomes analyzed on a GWAS scale were: warfarin maintenance dose, TTR within 3 months and TTR in total.
- TTR, as well as time below INR 2.0 and time above INR 3.0, was evaluated within 3 months and in total were analyzed for the genetic factors rs9923231 of *VKORC1*, *2/*3 of *CYP2C9*, rs2108622 of *CYP4F2* and sensitivity groups based on *VKORC1* and *CYP2C9*. The same genetic variables were also analyzed versus major bleeding.

Results

- Novel genome-wide significant SNPs affecting warfarin dose were found in *DDHD1* (rs17126068) and *NEDD4* (rs2288344). Adding the new SNPs to a model including *VKORC1* (rs9923231), *CYP2C9* (*2/*3) and clinical factors increased R^2 by 2.9%.
- A SNP in *ASPH* (rs4379440) on chromosome 8 was associated with TTR in total (-6.8% per minor allele).
- During the first 3 months, *VKORC1* (rs9923231) was associated with time below INR 2.0 ($p = 6.35 \times 10^{-4}$) and time above INR 3.0 ($p = 1.11 \times 10^{-2}$). *CYP2C9* (*2/*3) was associated with time above INR 3.0 ($p = 9.21 \times 10^{-4}$). Combining *VKORC1* and *CYP2C9* into sensitivity groups affected the same TTR measures as the individual variants. No other time in or out-of-range outcomes were statistically significant for either TTR during the first 3 months or in total.
- Major bleeding was not significantly associated with either *VKORC1*, *CYP2C9**2/*3 or *CYP4F2* genotypes as well as sensitivity groups defined by *VKORC1* and *CYP2C9*.

Conclusion

- We identified two novel genes, *DDHD1* (rs17126068) and *NEDD4* (rs2288344), associated with warfarin maintenance dose and one gene, *ASPH* (rs4379440), associated with TTR.
- The incremental information provided by these SNPs for prediction of a patient's dose is probably limited.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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