

ARTICLE

Impact of *GGCX*, *STX1B* and *FPGS* Polymorphisms on Warfarin Dose Requirements in European-Americans and Egyptians

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Genotype-based algorithms that include *VKORC1* and *CYP2C9* genotypes are less predictive of warfarin dose variability in Africans as opposed to Europeans. Polymorphisms in *GGCX*, *FPGS*, or *STX1B* are associated with warfarin dose requirements in African-Americans. We sought to determine if they influenced warfarin dose in European-Americans, and another African population, specifically Egyptians. We genotyped 529 adults ($n = 325$ European-Americans, 204 Egyptians) on a stable warfarin dose for *GGCX* rs12714145 and rs10654848, *FPGS* rs7856096, and *STX1B* rs4889606. Rs12714145, rs10654848, and rs7856096 were not associated with warfarin dose, whereas *STX1B* rs4889606 was a significant determinant in univariate analysis ($P < 0.0001$) in both cohorts. However, *STX1B* rs4889606 was in high linkage disequilibrium with *VKORC1*-1639 G>A, and was no longer significant after including *VKORC1*-1639 G>A in the regression model. Based on these data, the polymorphisms do not appear to influence, in a clinically important way, warfarin dose requirements in European-Americans and Egyptians.

Clin Transl Sci (2016) 9, 36–42; doi:10.1111/cts.12385; published online on 11 January 2016.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THIS TOPIC?

✓ The percent of interindividual variability in the warfarin maintenance dose explained by current genetic-based algorithms varies by ethnicity, emphasizing the need to examine genetic associations across different ethnic groups.

WHAT QUESTION DID THE STUDY ADDRESS?

✓ Previous studies have shown that *GGCX*, *STX1B*, or *FPGS* influence warfarin dose in African-Americans. In this study we sought to interrogate these genes for their association with warfarin dose requirements in European-Americans and Egyptians, a population from the African continent.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ Results from univariate analysis indicate that only the single nucleotide polymorphism (SNP) in *STX1B* (rs4889606) was significantly associated with warfarin dose. However, after accounting for *VKORC1* rs9923231 SNP, the effect of *STX1B* rs4889606 was no longer significant. This finding can be explained by high linkage disequilibrium between two SNPs in both populations.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ Based on these data, there is no reason to consider inclusion of *GGCX*, *FPGS*, or *STX1B* genotypes into warfarin pharmacogenetic dosing algorithms for European-Americans and Egyptians.

Despite the advent of new oral anticoagulants with a more predictable dose–response profile, fewer drug–drug interactions, and no requirement for frequent monitoring, warfarin remains the mainstay of anticoagulation therapy for the treatment and prevention of thromboembolism. Since its approval in 1954, warfarin dosing has presented significant challenges clinically. Optimal warfarin dosing mandates that an international normalized ratio (INR) in the range of 2 to 3 be achieved for the majority of indications for anticoagulation. Accordingly, regular and vigilant monitoring of the INR is warranted, particularly in the early phases of

warfarin initiation since values outside of the target range may have detrimental health consequences, i.e., an INR less than 2 is associated with an increased risk of thrombosis,^{1,2} whereas an INR above 3 carries a heightened risk for bleeding complications including intracranial hemorrhage.^{3,4} Of note, there is considerable interpatient variability in the warfarin dose that produces therapeutic anticoagulation. As an illustration, the stable warfarin dose could be as low as 0.5 mg per day for some individuals, whereas for others the dose needed for therapeutic anticoagulation could exceed 10 mg/day.⁵ This has spurred the formulation of several

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Received 2 November 2015; accepted 17 December 2015; published online on 11 January 2016. doi:10.1111/cts.12385

pharmacogenetic-based algorithms^{6–9} that set the framework for a personalized rather than an empiric approach to dosing warfarin. These algorithms incorporate single nucleotide polymorphisms (SNPs) in *VKORC1* and *CYP2C9* genes, which have been shown to regulate the pharmacodynamics and pharmacokinetics of warfarin, respectively. *VKORC1* codes for the target protein of warfarin,^{10–13} vitamin K epoxide reductase complex 1, and *CYP2C9* encodes the principal cytochrome P450 (CYP2C9), responsible for metabolism of the more potent S-warfarin enantiomer.^{14–16} Together with clinical factors (e.g., age, body surface area, smoking, and amiodarone use), the *VKORC1* rs9923231 (-1639 G>A) and *CYP2C9**2 (rs1799853) and *CYP2C9**3 (rs1057910) variants account for ~50% of the variation in the warfarin daily dose among patients of European ancestry.^{17,18} However, these variants explain less of the dose variability in African-Americans and Egyptians,¹⁹ also a population residing on the African continent. Conversely, other variants demonstrate a significant association with warfarin dose requirements in African-Americans, namely, the rs7856096 SNP in the gene coding for folate polyglutamate synthase (*FPGS*) and rs10654848 in *GGCX*, which codes for the vitamin K-dependent enzyme, gamma glutamyl carboxylase. These variants have not been sufficiently interrogated for their association with warfarin in other racial groups. On the other hand, there are genetic markers that have been found to be highly associated with reduced therapeutic warfarin doses in African-Americans^{17,20–23} only, such as the African-specific *CYP2C9* variants (*CYP2C9**5, *CYP2C9**6, *CYP2C9**8, and *CYP2C9**11), and rs12777283 in *CYP2C9*, which was identified in a recent genome-wide association study (GWAS).^{24–26} Furthermore, *STX1B* rs4889606, which is 90 kb downstream of the *VKORC1* gene, has been associated with *VKORC1* expression,^{27,28} and recent data suggest that the effect of this SNP on warfarin dose requirements in African-Americans is independent of rs9923231 in the *VKORC1* gene.²⁹ Uncovering genetic factors that provide contributions to warfarin response beyond the *CYP2C9* and *VKORC1* genotypes could potentially improve the accuracy of pharmacogenomics dosing algorithms in predicting warfarin maintenance dose.

We sought to determine the association between the *GGCX*, *FPGS*, or *STX1B* genotypes and warfarin dose requirements in European-Americans and Egyptians. While these genes have been previously associated with warfarin dose in other populations, their inclusion in dosing algorithms is hampered by the paucity of data across different racial groups, thereby precluding the generalizability of results.

METHODS

Patient selection and intervention

The study design and patient selection are described elsewhere.^{30,31} In brief, our patient cohort included a total of 529 patients (325 European-Americans and 204 Egyptians) who were taking a stable warfarin maintenance dose for the prevention of recurrent venous thromboembolism (VTE) or stroke due to atrial fibrillation. Per protocol, a stable maintenance dose was defined as the dose (not varying by more than 10% between visits) that produced an INR within the target therapeutic range (± 0.2) for each patient at three con-

secutive visits. The study protocol was approved by the University of Florida Review Board (for the European-American cohort) and the Research Ethics Committee at the Faculty of Medicine, Ain Shams University in Cairo (for the Egyptian cohort). Each patient provided written informed consent for use of genetic material and clinical information for evaluating the genetic determinants of warfarin dose variability.

DNA isolation and genotyping

Genomic DNA was isolated either from buccal cells obtained from mouth wash samples (European cohort) or leukocytes in peripheral blood samples (Egyptian cohort) using the manufacturers' guidelines.³²

Genotyping for *GGCX* C>T, rs12714145; *STX1B* A>G, rs4889606; *FPGS* A>G, rs7856096; *VKORC1* -1639 G>A (rs9923231); *CYP2C9**2 430 C>T (rs1799853); and *CYP2C9**3 1075 A>C (rs1057910) was performed by polymerase chain reaction (PCR) and pyrosequencing³³ according to the manufacturer's recommendations (Qiagen, Valencia, CA). The PCR and sequencing primers for PCR and pyrosequencing reactions are shown in **Supplementary Table S1**. The microsatellite (CAA) tandem repeats in intron 6 of *GGCX* gene (rs10654848) were genotyped by fragment analysis.²⁷

Haplotype analysis

Since the *STX1B* haplotype block in the Egyptian population had not been identified or characterized in any previous study, the genotyping results obtained for rs9923231 (in *VKORC1*) and rs4889606 (in *STX1B*) were uploaded into Haploview software (v. 4.2) to calculate the D' and r^2 linkage disequilibrium values, and also to construct the haplotype block. The Gabriel *et al.* block method was used to define the confidence interval.³⁴

Statistical analysis

The chi-square test with one degree of freedom was used to test for deviation from Hardy–Weinberg equilibrium (HWE) for each genotype. The nonparametric Mann–Whitney or Kruskal–Wallis statistical test was used to compare the median weekly maintenance warfarin dose between genotypes. P values less than 0.012, adjusted for multiple comparisons (0.05/4), were considered statistically significant. A stepwise linear regression model was developed to determine whether each of the tested SNPs in the *GGCX*, *FPGS*, and *STX1B* genes remained associated with warfarin dose requirements after accounting for other genotypes and clinical factors (e.g., age, body surface area (BSA), smoking status, SNPs in *VKORC1* and *CYP2C9*). Under the additive model, the *STX1B*, *GGCX*, and *FPGS* genotypes were coded as 0 (homozygous wildtype), 1 (heterozygous), and 2 (homozygous variant). For *CYP2C9*, a composite score was created, with a score of 0 for those without a variant allele and 1 or 2 for those with 1 or 2 variant alleles (*2 or *3), respectively. All statistical analyses were performed on SAS (v. 9.3, SAS Institute, Cary, NC).

RESULTS

Baseline characteristics of both cohorts (European-Americans and Egyptians) were previously reported.^{30,31}

Table 1 Genotype percentages of *FPGS* rs7856096, *GGCX* rs12714145, *STX1B* rs4889606, and *GGCX* CAA microsatellite in the European-American and Egyptian cohorts

Genotype	European-Americans (n)	Egyptians (n)
<i>FPGS</i> rs7856096		
AA	95.7 (291)	97 (155)
AG	4.3 (13)	3 (5)
GG	0	0
<i>GGCX</i> rs12714145		
CC	32.2 (97)	42 (55)
CT	47.8 (144)	42.7 (56)
TT	20 (60)	15.3 (20)
<i>STX1B</i> rs4889606		
AA	40 (125)	22.2 (32)
AG	51 (160)	56.2 (81)
GG	9 (30)	21.6 (31)
<i>GGCX</i> (CAA) rs10654848		
8/10	0.3 (1)	0.4 (1)
10/10	24.8 (74)	28.2 (57)
10–11/11	11.4 (34)	14.3 (29)
10–12/12	3.3 (10)	7.6 (16)
10–13/13	34.8 (104)	26.2 (53)
10–14/14	24.1 (72)	20.6 (42)
10–16/15	1.3 (4)	2.7 (6)

In brief, the mean age of the participants was 69 (± 11) years in the European-American cohort and 47.4 (± 14.7) years in the Egyptian cohort; 12.6% of the study participants in the European-American cohort and 55.1% in the Egyptian cohort were females. The main indications for initiating warfarin in the European-American and Egyptian cohorts were atrial fibrillation and mitral valve replacement, respectively. The genotypes for the tested SNPs in *FPGS*, *GGCX*, and *STX1B* are shown in **Table 1**. None of the participants in either cohort was homozygous for the rs7856096 G allele in *FPGS*. The genotype frequencies for rs7856096, rs12714145, and rs4889606 did not depart from HWE. For the *GGCX* CAA microsatellite (rs10654848), the 10 CAA tandem repeat was the most prevalent allele; 24.8% of European-Americans and 28.2% of Egyptians harbored the *GGCX* 10/10 microsatellite genotype (**Table 1**). Only one study participant in each cohort had 16 CAA tandem repeats, previously associated with high warfarin dose in African-Americans.³⁵

Analysis of warfarin dose requirement by gene

Only samples with genotype calls for each of the tested SNPs were included in the final analysis. Neither the *FPGS* rs7856096 nor *GGCX* rs12714145 genotype was associated with median weekly warfarin dose in either ethnic group (**Table 2**); however, there was a significant association between the *STX1B* genotype and warfarin dose on univariate analysis. Each *STX1B* variant G allele was associated with about a 10 mg reduction in the weekly warfarin dose in the European-American cohort (**Table 2**, $P < 0.0001$), and about a 7 mg reduction in the Egyptian cohort (**Table 2**, $P = 0.0001$). However, the association with the *STX1B* allele was no longer significant in either group when the *VKORC1* -1639 G>A was added to the regression model (**Table 3**). Haplotype

Table 2 Comparison of median warfarin dose (mg/week) by genotype for *GGCX*, *STX1B* and *FPGS* in the European and Egyptian cohorts

Genotype	Median warfarin dose (mg/week) by genotype (IQR)			
	European-Americans	P value	Egyptians	P value
<i>GGCX</i> rs12714145				
CC	34.5 (25.5–40)	0.46	31.5 (24.5–38.5)	0.24
CT	35 (27.5–45)		38.5 (28–49)	
TT	35 (25–43.75)		35 (24.5–47.2)	
<i>STX1B</i> rs4889606				
AA	42.5 (35–52.5)	<0.0001	42 (29.7–59.5)	0.0001
AG	32.2 (25–40)		35 (28–42)	
GG	21.5 (17.5–27.5)		24.5 (17.5–35)	
<i>FPGS</i> rs7856096				
AA	35 (25–42.5)	0.4	33.2 (24.5–42)	0.86
AG	28 (25–40)		33.2 (22.7–45.5)	
GG				

IQR: interquartile range.

Table 3 Multiple regression stepwise analysis for evaluating effect of *STX1B* on weekly warfarin dose variability

Variable	European-Americans			Egyptians		
	β	S.E.	P value	β	S.E.	P value
<i>STX1B</i>	-2.4	2.4	0.3	-1.4	3.3	0.6
<i>VKORC1</i>	-9.6	2.3	<0.0001	-9.1	3.6	0.008
<i>CYP2C9</i>	-8.2	1.1	<0.0001	-7.4	2.1	0.0005
Age (years)	-0.2	0.1	0.0003	-0.3	0.1	0.0015
Smoking	8.0	2.0	<0.0001	14.0	4.7	0.0026
BSA (m ²)	15.6	2.6	<0.0001	0.9	7.2	0.8

analysis of the *STX1B* block (**Figure 1**) indicated high linkage disequilibrium (LD) between the *STX1B* and *VKORC1* -1639 G>A SNPs in European-Americans ($D' = 0.91$, $r^2 = 0.83$) and Egyptians ($D' = 0.88$, $r^2 = 0.71$).

For the *GGCX* microsatellite (rs1064848), weekly warfarin dose was compared between the following genotype groups: 8–10/10 repeats, 10–11/11 repeats, 10–12/12 repeats, 10–13/13 repeats, 10–14/14 repeats, and 10–16/15 repeats.³⁵ As depicted in **Figure 2a**, there was no significant difference in the weekly warfarin dose requirement between these groups in European-Americans ($P = 0.87$). On the other hand, the difference in the warfarin dose requirement tended to be higher in Egyptians, with 15 or more repeats compared with those with fewer than 15 repeats, but the difference did not reach statistical significance (**Figure 2b**, $P = 0.16$). Even when the comparison was restricted to the extreme *GGCX* CAA tandem repeat genotypes, i.e., 8–10/10 vs. 10–16/15, the difference in the weekly warfarin dose was not statistically significant in either population (**Figure 2c,2d**). Only one Egyptian and one European-American carried the 16 CAA repeat allele; their weekly maintenance warfarin doses were 70 mg and 40 mg, respectively. Both also had the *VKORC1* -1639 GA genotype associated with intermediate dose requirements.

DISCUSSION

There are significant differences across race/ancestry groups in the portion of warfarin dose variability explained by known

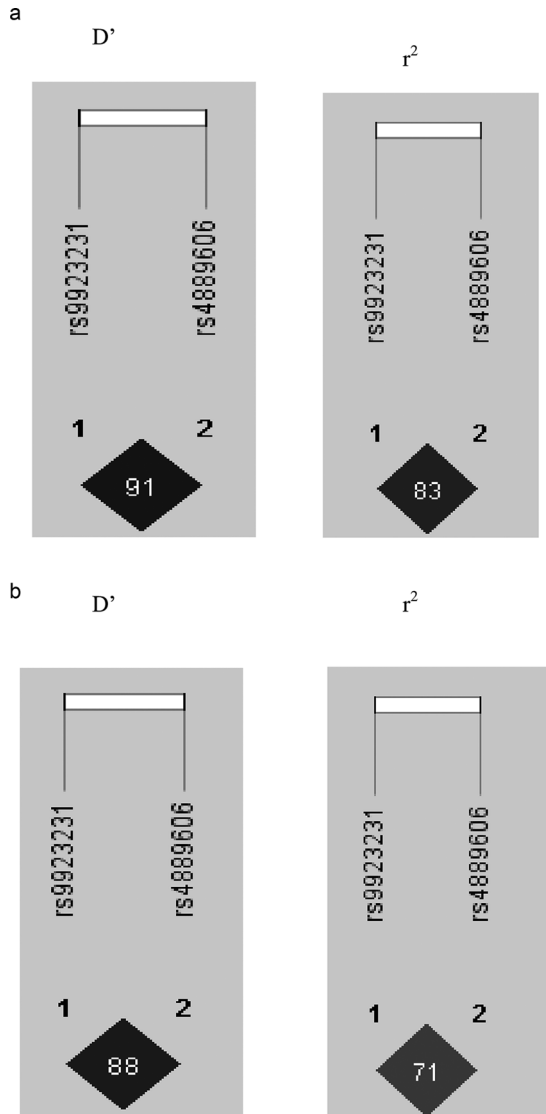


Figure 1 Linkage disequilibrium (LD) plots showing D' and r^2 values for the European-American population (a) and the Egyptian population (b).

genetic and nongenetic factors, and thus warfarin pharmacogenetic dosing algorithms perform variably across different groups. The most widely cited algorithms^{6,7} include only the *VKORC1* and *CYP2C9**2 and *3 genotypes. It has been shown that inclusion of additional genotypes improves dosing accuracy in African-Americans.^{23,24} However, whether genotypes associated with warfarin dose in African-Americans, such as *FPGS* and *GGCX*, are also important in other populations is not well known.

In this study we evaluated variants in the *GGCX*, *FPGS* and *STX1B* genes to determine whether they contribute to the interindividual variability in the warfarin dose requirement in either the European-American or Egyptian population. We found no significant association between the *GGCX* and *FPGS* variant and warfarin maintenance dose, and the association with the *STX1B* genotype was no longer observed

on regression analysis including *VKORC1* -1639G>A. A previous study²⁸ reported an association between the *STX1B* variant and warfarin dose in a small cohort of mostly European-American patients. Although the authors of that study proposed that the association might be secondary to linkage between the *STX1B* gene and *VKORC1* expression, they did not provide LD values (D' and r^2) to demonstrate this. While we replicated the results reported by this group in European-Americans and extended the association to Egyptians, we also ruled out an independent effect of the *STX1B* genotype on warfarin response in these cohorts. The Egyptian population, by virtue of the geographic location of Egypt, carries a portion of West African ancestry.³⁶ Nevertheless, informative markers were not available to confirm this for our population. In defining the *STX1B* haplotype structure and LD patterns, we found that the *STX1B* haplotype block in Egyptians resembled to a great extent the *STX1B* haplotype block of individuals of European ancestry, indicating that at least in this gene region, Egyptians closely resembled European-Americans. Similarly, the observed minor allele frequency of *FPGS* rs7856096 among Egyptians was comparable to that in European-Americans rather than the frequencies previously reported in Africans. Such findings indicate that the Egyptians are a highly admixed population where the allele frequencies of SNPs vary from being either close to Europeans or close to Africans.³¹ Hence, results from genetic association studies involving Africans cannot be extrapolated to other populations within the African continent.

The contribution of rs12714145 (C>T) in intron 2 of *GGCX* to warfarin dose variability was previously assessed in European-Americans, with contrasting results, where only one³⁷ of three studies demonstrated that homozygous carriers of the T variant required higher warfarin maintenance doses compared to the other genotypes. Specifically, in 201 Caucasian patients, rs12714145 was shown to account for about 3% of the overall variability in dose. Our results in both European-Americans and Egyptians are consistent with the negative findings by Rieder *et al.* and King *et al.*,^{38,39} suggesting that *GGCX* rs12714145 (C>T) is not an important determinant of the warfarin maintenance dose.

As for the microsatellite tandem (CAA) repeats in intron 6 of *GGCX* (rs10654848), there is some evidence showing an association with warfarin dose requirements. Among Japanese patients, higher warfarin doses were needed to achieve a therapeutic INR in patients with at least 13 CAA repeats compared with those with fewer repeats. In an African-American population, the presence of at least 16 repeats was found to be higher than previously reported and associated with higher warfarin dose requirements.²⁷ The 16 CAA repeat was observed in only one patient in a previous study in European-Americans. Similarly, we found that only one European-American and one Egyptian patient carried 16 CAA repeats, and none carried more than 16 repeats, which translated into an allele frequency of 0.3% and 0.1% in Egyptians and European-Americans, respectively, compared with 5.6% reported in African-Americans.³⁵ Interestingly however, both patients with a 16 CAA repeat in our study required doses higher than typically needed to attain therapeutic anticoagulation, despite also having the

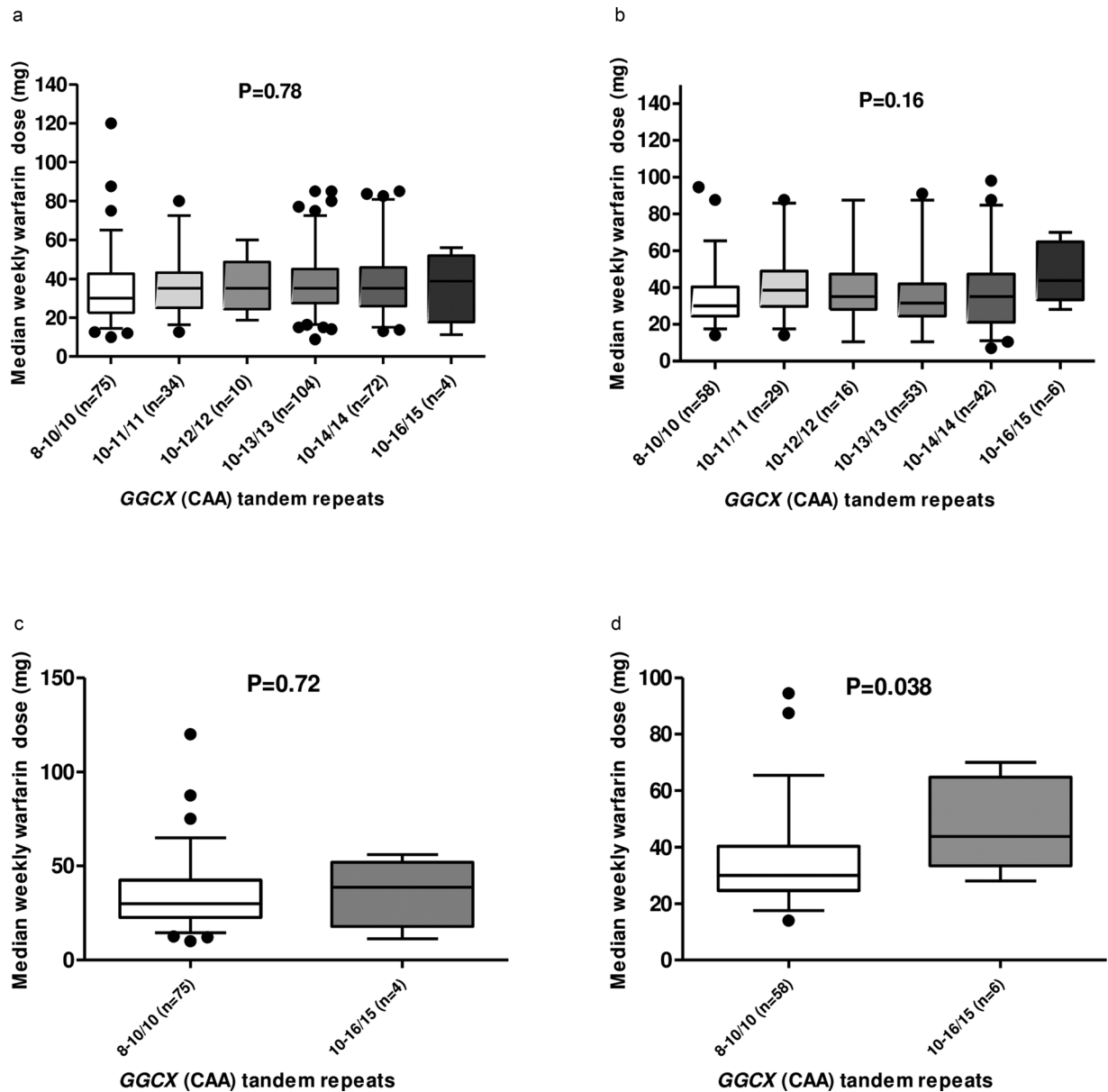


Figure 2 Differences in weekly warfarin maintenance dose based on number of *GGCX* microsatellites. Comparison of median weekly warfarin dose by number of *GGCX* CAA tandem repeats in the European-American cohort (a). The bottom and top of the box represent the 25th and 75th percentiles, respectively, and the band in the middle represents the median (50th percentile). The lower whisker represents 5th percentile of the data and the upper whisker represents 95th percentile of the data. Comparison of median weekly warfarin dose by number of *GGCX* CAA tandem repeats in the Egyptian cohort (b). The bottom and top of the box represent the 25th and 75th percentiles, respectively, and the band in the middle represents the median (50th percentile). The lower whisker represents 5th percentile of the data and the upper whisker represents 95th percentile of the data. Comparison of median weekly warfarin dose between the extreme CAA tandem repeat genotypes (8–10/10 vs. 10–16/15) in the European-American cohort (c). The bottom and top of the box represent the 25th and 75th percentiles, respectively, and the band in the middle represents the median (50th percentile). The lower whisker represents 5th percentile of the data and the upper whisker represents 95th percentile of the data. Comparison of median weekly warfarin dose between the extreme CAA tandem repeat genotypes (8–10/10 vs. 10–16/15) in the Egyptian cohort (d). The bottom and top of the box represent the 25th and 75th percentiles, respectively, and the band in the middle represents the median (50th percentile). The lower whisker represents 5th percentile of the data and the upper whisker represents 95th percentile of the data.

heterozygous *VKORC1* genotype, which typically leads to a requirement for lower than average dose. In the light of this extremely low frequency of the *GGCX* 16 CAA tandem repeats in both cohorts, we opted to group the 15 and 16 CAA tandem repeat genotypes together to ascertain their

impact on warfarin dosing. In both cohorts, our results did not reveal a statistically significant difference in the weekly warfarin maintenance dose between carriers of at least 15 CAA tandem repeats and those with 10 or less. Nonetheless, larger studies are warranted to validate the contribution of

15 and more CAA tandem repeats to the weekly warfarin dose. Unlike previous studies,^{19,40} the presence of 13 and 14 CAA tandem repeats in *GGCX* did not translate into higher warfarin dose requirements in either cohort.

In a recent whole-exome sequencing study, Daneshjoui et al.⁴¹ identified a novel association between warfarin dose requirements in an African-American population and rs7856096 (A>G) in the *FPGS* gene, which encodes for the mitochondrial enzyme involved in folate homeostasis. They found that the G variant allele was associated with a reduction in warfarin dose by 0.83 mg/day or 5.81 mg/week. Our study was the first study to examine the association between *FPGS* rs7826096 and warfarin dose in a non African-American population. In contrast to previous findings in African-Americans, we did not observe a correlation between *FPGS* genotype and warfarin maintenance dose in either European-Americans or Egyptians. However, the prevalence of this risk allele in these cohorts was low (2% in European-American and 5% in Egyptians) compared with that reported in African-Americans (23%), and none of the participants in our study was homozygous³⁶ for the variant allele, which could account for our negative association.

Given the multiple gene approach that we undertook in this study, it is important to point out that we did not investigate other polymorphisms such as SNPs in *CYP4F2* since they were evaluated elsewhere, and explained a small portion of the warfarin dose variability in European-Americans,⁴² whereas in Egyptians the contribution was insignificant.²³

In summary, this study shows that *GGCX* rs12714145, *GGCX* rs10654848, *FPGS* rs7856096, and *STX1B* rs4889606 are not significant determinants of the weekly warfarin dose requirements in either European-Americans or Egyptians.

Acknowledgments. This work was supported in part by National Institutes of Health grant GM074492.

Conflicts Of Interest/Disclosure. The authors declared no conflict of interest.

Author Contributions. I.S.H. wrote the article; M.H.S., S.I.K., L.H.C, R.M.C.-D., and J.A.J. designed the research; I.S.H, M.H.S., S.M.L., F.O., and L.W. performed the research; I.S.H., M.H.S., S.M.L., and L.W. analyzed data; T.Y.L. contributed new reagents/analytical tools.

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Supplementary information accompanies this paper on the *Clinical and Translational Science* website.
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