Apolipoprotein E Genotype Association with Time to Stable Warfarin Dosing in African Americans

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Running head: Genotype and warfarin dose stabilization

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Abstract

Study Objective. To test the hypothesis that time to reach stable warfarin dosing is dependent on proteins involved in vitamin K_1 disposition.

Design. Retrospective cohort study.

Setting. Pharmacist-managed anti-thrombosis clinic

Patients. Ninety-two African Americans treated with warfarin.

Measurements and Main Results. A genetic sample was collected from each patient. Data from each anticoagulation clinic visit during the initial 6 months of warfarin therapy or until dose stabilization were collected retrospectively. Patients were genotyped for the apolipoprotein E (*APOE*) ε2, ε3, and ε4; NAD(P)H:quinone oxidoreductase (*NQO1*)*2; cytochrome P450 4F2 (*CYP4F2*) V433M; *CYP2C9**2, *3, *5, *8, and *11; and vitamin K epoxide reductase complex 1 (*VKORC1*) -1639G>A variants. The median time to reach stable warfarin dosing, defined as the dose that produced therapeutic anticoagulation for 3 consecutive clinic visits, was 83 days. Compared to patients reaching a stable warfarin dose within 83 days (n=46), those requiring longer for dose stabilization had a higher frequency of the *APOE* ε3/ε3 genotype (59% versus 37%, p=0.037). Sixty-one percent of patients with the ε3/ε3 genotype versus 40% with an ε2 or ε4 allele had a delay in achieving stable dosing. Neither the *CYP4F2* nor *NQO1* genotypes were associated with warfarin dose stabilization.

Conclusion. Our data ~~suggest~~ support the hypothesis that *APOE* genotype influences time to reach stable warfarin dosing in African Americans. Insight into genetic influences of warfarin dose stabilitzation could reveal novel methods to improve anticoagulation control during the warfarin initiation period.

Warfarin is commonly prescribed for the prevention of thromboembolism. However, warfarin is a challenging drug to manage, largely because of its narrow therapeutic index. Poor anticoagulation control increases the risks for thromboembolism and serious bleeding.[1, 2] These risks are greatest during the initial months of warfarin therapy.[3, 4] Thus, it is imperative to efficiently achieve therapeutic anticoagulation after warfarin initiation.

Warfarin exerts its anticoagulant effects by inhibiting vitamin K oxidoreductase (VKOR), thus preventing formation of vitamin K_1, as shown in figure 1. Vitamin K_1 is subsequently reduced to vitamin KH_2, a necessary cofactor for generation of functional clotting factors. It is well recognized that the genes for VKOR (*VKORC1*) and cytochrome P450 2C9 (*CYP2C9*) influence warfarin dose requirements.[5-12] Much less is known about genes influencing warfarin dose stabilization. While some studies support the *CYP2C9* gene as a contributor to dose stabilization, the data are inconsistent.[5, 8, 13, 14] Most studies show no association between *VKORC1* and time to attain stable dosing.[8, 14]

The apolipoprotein E (*APOE*), NAD(P)H:quinone oxidoreductase (*NQO1*), and *CYP4F2* genes are involved in vitamin K distribution and metabolism (Figure 1). As such, these genes could potentially influence response to vitamin K antagonism with warfarin. Specifically, APOE is involved in the hepatic uptake of vitamin K_1. Two *APOE* single nucleotide polymorphisms (SNPs), C130R (rs429358 T>C) and R176C (rs7412 C>T), result in 3 common alleles, designated as the ε2 (130C/176C), ε3 (130C/176R), and ε4 (130R/176R) alleles. Low, intermediate, and high clearance of vitamin K_1 from the plasma have been reported with the ε2, ε3, and ε4 alleles, respectively.[15] Increased hepatic uptake of vitamin K with the ε4 allele is proposed to increase vitamin K-dependent clotting factor activation.[16] The NQO1 enzyme reduces vitamin K_1 to vitamin KH_2. A common *NQO1* SNP, P187S (rs1800566 C>T) designated

as the *2 allele, has been associated with significant reductions in NQO1 activity.[17] In *in-vitro* studies, NQO1 activity is directly correlated with the enzyme's capacity to reduce vitamin K substrates and the concentration of the warfarin derivative dicoumarol necessary to inhibit NQO1 activity.[18] The CYP4F2 enzyme metabolizes vitamin K_1 to hydroxyvitamin K_1, thereby decreasing the amount of vitamin K_1 available for reduction to vitamin KH_2. A common *CYP4F2* SNP, V433M (rs2108622 C>T), decreases CYP4F2 concentration.[19] Thus, *CYP4F2* genotype influences the quantity of vitamin K_1 available for reduction to vitamin KH_2 by NQO1.

We hypothesized that through their effects on vitamin K availability, the *APOE*, *NQO1*, and *CYP4F2* genes influence warfarin dose stabilization. We sought to determine whether the *APOE*, *NQO1*, and *CYP4F2* genotypes are associated with time to achieve stable warfarin dosing. We focused our analysis on African Americans, a population largely underrepresented in warfarin pharmacogenomic studies, yet at particularly high risk for recurrent thromboembolism and adverse sequelae as a result of thromboembolism.[20, 21]

Methods

Study Population and Procedures

Patients were identified and enrolled from the pharmacist-managed Antithrombosis Clinic (ATC) at the University of Illinois Medical Center at Chicago (UIMCC). The ATC operates under a collaborative agreement protocol with oversight provided by a medical director. Approximately 450 warfarin-treated patients are managed in the ATC, the majority of whom are African American (60%) or Hispanic (25%). Clinic staffing is provided by 8 clinical pharmacists, with 2 to 3 pharmacists providing coverage at any given time. Clinic policies and procedures, including a protocol for warfarin dose changes based on international normalized

ratio (INR) values during both warfarin initiation and maintenance therapy, are approved by the UIMCC Pharmacy and Therapeutics committee and followed by each clinician. This assures consistency in following and adhering dosing guidelines for all ATC patients. Per these policies and procedures, stable patients are seen in clinic at least once every four weeks, whereas unstable patients or patient newly initiated on warfarin may be seen as often as once or twice a week. The INR is determined at each clinic visit through point-of-care testing using the ProTime® monitor (QAS, Orlando, FL). Patients with an INR >4.5 via point-of-care testing are sent to the medical center central laboratory for verification.

At the initial baseline clinic visit, all new patients receive intensive counseling on warfarin, including dietary counseling in regard to vitamin K intake, and literature on foods with high vitamin K content. Average vitamin K intake, in terms of servings of food with high vitamin K content, is assessed at the baseline visit, and patients are counseled to maintain a consistent intake of such foods during warfarin therapy. In addition to INR testing, a clinic pharmacist assesses the same basic criteria for each patient, regardless of INR results, at each clinic visit with documentation of such in the medical record. These criteria include adherence to warfarin, status of medical problems(s) warranting anticoagulation, and recent alterations in medications or intake of foods with high vitamin K content.

Inclusion criteria for the study were African American race, by self report, age ≥18 years, and warfarin initiation at the UIMCC. Patients with a history of liver dysfunction or serum transaminase levels greater than 3 times the upper limit of normal were excluded. All patients provided written informed consent for the collection of a buccal cell sample for genetic analysis and to allow retrospective review of their medical record, as previously described.[12] The date of warfarin initiation and data on the warfarin dose, INR, missed or extra warfarin doses, and

dietary changes at each anticoagulation clinic visit during the 6-month period following warfarin initiation or until stable dosing was achieved (whichever was longer) were collected from the medical record. The study was performed in accordance with the Declaration of Helsinki and approved by the institutional review board at the University of Illinois at Chicago.

Genotyping

Genomic DNA was isolated from buccal cells using a commercially available kit (PureGene,® Qiagen, Valencia, CA). The *APOE* C130R and R176C; *CYP2C9* R144C (*2), I359L (*3), and D360E (*5); and *VKORC1* rs9923231 (-1639G>A) genotypes were determined by PCR and pyrosequencing methods as previously described.[12] The *NQO1* P187S, *CYP4F2* V433M, and *CYP2C9* (R335W) *11 SNPs were determined by PCR and pyrosequencing. The *CYP2C9* R150H (*8) allele was determined by PCR and capillary sequencing. Primers are shown in Table 1. Each PCR reaction consisted of 25 μl of HotStarTaq™ Master Mix (Qiagen), primers (25 pmol), 15 μl of H_2O, and 20-100 ng of DNA. Thermocycling consisted of denaturation for 15 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds; annealing for 30 seconds at 62°C for *NQO1**2, 58°C for *CYP4F2* V433M, 61°C for *CYP2C9**8, and 60°C for *CYP2C9**11; and extension at 72°C for 45 seconds, with a final extension of 72°C for 10 minutes. All genotypes were assigned by investigators blinded to clinical data.

Statistical Analysis

Data were expressed as number (percentage), mean ± SD, or median (IQR). Hardy Weinberg Equilibrium assumption was tested by chi square analysis. Stable dosing was defined

as the same dose for 3 consecutive clinic visits spanning a period of ≥4 weeks for which the INR at each visit was within 0.1 units of the therapeutic range (e.g. 1.9 to 3.1 for a goal range of 2 to 3). The extended INR range was used because values within 0.1 units of the therapeutic range do not usually elicit a dose change. Time to stable warfarin dosing was defined as the time to the first of the 3 consecutive visits when the warfarin dose was stable. Adherence was expressed as the percent of visits with 100% patient-declared adherence to warfarin (no missed or extra warfarin doses) relative to the total number of clinic visits during the 6-month period following warfarin initiation. Genotype frequencies and other characteristics were compared between patients reaching a stable dose within the median timeframe for the study population and those requiring longer than the population median to achieve stable dosing by chi square analysis for categorical data and the Student's unpaired *t*-test or Mann Whitney *U* test for continuous data. In addition, a binary logistic regression analysis was used to estimate the *APOE* contributions to dose stabilization when accounting to the potential interaction with *VKORC1* and *CYP2C9* genotypes. Time to attain stable dosing was compared between genotypes using chi square analysis or the Fisher's exact test.

Results

A total of 97 unrelated African Americans were initially included; however, one patient had the *APOE* ε2/ε4 genotype and was subsequently excluded given the divergent effects of the ε2 and ε4 alleles on vitamin K clearance.[15] Four other patients moved, were lost to follow-up, or discontinued warfarin before reaching a stable warfarin dose. The mean age of the remaining 92 patients was 57 years (range 24 to 86 years). The majority were female (76%) and taking warfarin for secondary prevention of venous thromboembolism (55%).

The median time to reach stable dosing in the study population was 83 days (mean, 136 days; range, 7 to 541 days). ~~Forty-six (50%) patients required >83 days to achieve stable dosing.~~ Characteristics, including warfarin adherence rate, vitamin K intake, frequency of dietary changes, and *VKORC1* and *CYP2C9* genotypes, were similar between patients reaching and those not reaching a stable dose within 83 days (Table 2). The number of clinic visits required to reach stable dosing and percent of clinic visits with INRs >0.1 unit outside of the therapeutic range over the initial 6 months of therapy were significantly higher among those with a delay in reaching stable dosing.

None of the genotypes deviated from Hardy Weinberg equilibrium. Allele frequencies were 0.18 for *NQO1**2; 0.06, 0.72, and 0.22 for *APOE* ε2, ε3, and ε4, respectively; and 0.08 for *CYP4F2* 433M. Figure 2a shows the genotype distributions between those reaching and those not reaching a stable dose within 83 days. Individuals with a delay in achieving stable dosing had a higher frequency of the *APOE* ε3/ε3 genotype (p=0.037). Carriers of the ε2 or ε4 allele were similarly distributed between groups. There was no significant association between the *NQO1* or *CYP4F2* genotype and time to stable dosing. Logistic regression analysis showed that the association between *APOE* and time to stable dosing remained significant, regardless of the inclusion of *VKORC1* and *CYP2C9* genotypes in the model (odds ratio 2.6, 95% confidence interval 1.1 to 6.0; p=0.033). Next, we limited our analysis to those reporting alterations in vitamin K intake at any point during the initial 6 months of therapy (n=72). When doing so, the difference in *APOE* genotype distribution between those reaching and not reaching a stable dose within 83 days became more pronounced (Figure 2b).

In comparisons by genotype, significantly more *APOE* ε3 homozygotes required >83 days to achieve stable dosing than ε2 or ε4 allele carriers (61% versus 40%; p=0.037). As above,

the differences by genotype were more pronounced when limiting our analysis to those with dietary changes, with 68% of ε3 allele homozygotes versus 38% of ε2 or ε4 carriers reaching a stable dose after 83 days (p=0.013).

A previous warfarin pharmacogenetic study in African Americans used a definition for time to stable dosing that was similar to that in the current study, except that the difference between consecutive warfarin doses was allowed to vary up to 10%.[8] When analyzing our data according to this less stringent definition, the median time to achieve stable dosing was shortened to 67 days. The *APOE* ε3/ε3 genotype remained associated with a delay in dose stabilization. The ε3/ε3 genotype was especially overrepresented among those requiring longer than 90 days to achieve stable dosing compared to those reaching stable dosing in half that time (45 days; 65% versus 36%, p=0.020) .

Discussion

Alterations in vitamin K intake can affect anticoagulation response and influence warfarin dose stabilization. Previous investigators have observed significant inter-patient variability in anticoagulant response to alterations in dietary vitamin K.[22] We found that the *APOE* ε3/ε3 genotype was associated with a delay in achieving stable warfarin dosing in African Americans. Given that APOE mediates vitamin K uptake into the liver, we believe that the most likely explanation for our findings is that *APOE* genotype influences INR response to dietary vitamin K.[15] Specifically, our data suggest that alterations in vitamin K intake during the warfarin initiation phase have a greater impact on the INR in *APOE* ε3 allele homozygotes compared to those with other genotypes. Since vitamin K uptake is already increased in the presence of an ε4 allele and decreased in the presence of the ε2 allele, carriers of these allele may

have little further change in vitamin K uptake with alterations in vitamin K intake. On the other hand, those with the *APOE* ε3/ε3 genotype and "normal" uptake of vitamin K, could be more susceptible to changes in vitamin K intake. We believe that the more pronounced association between *APOE* genotype and time to stable dosing among those reporting dietary changes supports differential response to dietary alterations by APOE genotype.

Neither *CYP4F2* nor *NQO1* genotype was not associated with time to stable dosing in our study. There are *in-vitro* data to suggest that the NQO1 enzyme plays a smaller role in vitamin K recycling than previously thought, and this may account for our negative findings in regard to *NQO1* genotype.[23] Our negative findings in regard to the *CYP4F2* 433M allele may be reflective of its low frequency in African Americans. The *CYP4F2* 433M variant is more common in Caucasians, and its effects on warfarin stabilization in this population remains to be examined.

Although not a primary focus of our study, we also genotyped for *VKORC1* and *CYP2C9* alleles. Similar to previous findings,[8] neither genotype was associated with warfarin dose stabilization in our African American population. Numerous studies have demonstrated that *CYP2C9* and *VKORC1* influence warfarin dose requirements.[5-12] However, the ability of these genotypes to predict warfarin dose requirements declines over time.[24, 25] Whether the effect of *APOE* genotype on dose stability changes over time remains to be determined. However, it is conceivable that patients who experience large fluctuations in INR with dietary changes may become more conscientious about maintaining consistent vitamin K intake. This, in turn, could minimize genetic influences of INR response to diet over time. Other investigators have reported associations between *APOE* genotype and warfarin dose requirements. However, the data are inconsistent and, in some cases, conflicting.[16, 26-28] We found no association between *APOE* genotype and warfarin dose in a previous study of over 200 patients.[12]

The definition we used for stable dosing was more conservative than that used in previous studies and may account for the relatively long time until stable dosing was achieved. For example, other studies used the time to the first therapeutic INR rather than time to stable warfarin dose,[9, 14] based stable dosing on fewer than 3 INR values,[29] allowed the warfarin dose to vary somewhat rather than requiring the dose to remain unchanged,[8] allowed greater variability in INR values (e.g. 0.2 units outside of the target range),[30] or required very little time between INR measurements.[11, 31] We feel that our definition was appropriate since it is the same definition commonly used to define stable warfarin dosing during chronic therapy.[5, 7, 12, 32] In addition, using a less stringent definition that allowed warfarin doses to vary by up to 10% did not significantly influence our results.

One limitation of our study is that the retrospective method of data collection did not allow for more thorough assessment of vitamin K~~+~~ intake. In addition, we were unable to prospectively assess whether INR response to dietary changes by genotype. Thus, the mechanism underlying the associations we observed requires prospective confirmation. The influence of genotype on vitamin K supplementation to improve anticoagulation stability or use of vitamin K to treat over-anticoagulation would be of particular interest.[33] However, data such as ours are important to inform which candidate genes to prospectively study for their effects on vitamin K response. Another limitation is that our study population was limited to African Americans. While our study provides important pharmacogenomic data in African Americans, a population underrepresented in pharmacogenomic studies, it may not be applicable to other populations given racial differences in allele frequencies and vitamin K intake.

In summary, *APOE* genotype was associated with time to achieve stable warfarin dosing among African Americans. The mechanism underlying this association requires investigation,

but may relate to response to dietary changes. Given the retrospective nature of data collection, particularly in regard to assessment of vitamin K intake, our results should be considered hypothesis generating and require confirmation in a prospectively designed study. Insight into genetic influences of time to achieve stable dosing could ultimately reveal novel methods of improving anticoagulation control during the warfarin initiation period.

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Table 1. PCR and sequencing primers

Variant	**Primers (5’ to 3’)**
NQO1 P187S	PCR Forward: GCATTTCTGTGGCTTCCAAGTC
	PCR Reverse: Biotin-GTGTGCCCAATGCTATATGTCAG
	Sequencing: TGGCTTCCAAGTCTTAG
CYP4F2 V433M	PCR Forward: Biotin-GAGGGAGGTGATGTTGGATACT
	PCR Reverse: TTCTCTCCCACAGGCATTATC
	Sequencing: CCCATCACAACCCAG
*CYP2C9**8	PCR Forward and sequencing: CCTCCTAGTTTCGTTTCTCTTCC
	PCR Reverse: TGAGCTAACAACCAGGACTCA
*CYP2C9**11	PCR Forward: GAACGTGTGATTGGCAGAAAC
	PCR Reverse: Biotin-GCATCTGTGTAGGGCATGTG
	Sequencing: CGTGTGATTGGCAGAA'

Table 2. Characteristics of the study population

Characteristic	Stable dose ≤83 days (n=46)	Stable dose >83 days (n=46)	p value
Age (years)	55 ± 15	59 ± 14	0.255
Female sex, No (%)	32 (70)	38 (83)	0.143
BSA (m^2)	2.1 ± 0.2	2.0 ± 0.3	0.175
Indication for warfarin, No (%)			
Venous thromboembolism	26 (57)	25 (54)	0.834
Secondary stroke prevention	7 (15)	7 (15)	1.00
Peripheral vascular disease	4 (8.5)	6 (13)	0.503
Atrial fibrillation/ atrial flutter	4 (8.5)	5 (11)	0.743
Heart valve replacement	0 (0)	1 (2)	0.500
Other[a]	5 (11)	2 (4)	0.434
Stable warfarin dose (mg/d)[b]	5.7 (5.0-8.0)	6.6 (4.5-8.6)	0.412
Goal INR, No (%)			
2.5	44 (96)	43 (93)	1.00
3.0	2 (4)	3 (7)	
Heart failure, No (%)	5 (11)	5 (11)	1.00
Tobacco use, No (%)	9 (20)	5 (11)	0.246
Alcohol use, No (%)	11 (24)	6 (13)	0.179
Amiodarone use, No (%)	0	0	—
Phenytoin or carbamazepine use, No (%)	2 (4)	2 (4)	1.00

Number of clinic visits until stable	6 (5-8)	17 (13-27)	<0.001
Warfarin adherence rate[c] (%)	87 ± 13	87 ± 12	0.998
Intake of foods with high vitamin K content (servings/week)	1.5 (0.1-2.8)	1 (0.5-2.5)	0.970
Percent of visits with changes in vitamin K intake[c] (%)	13 (5-21)	12 (6-20)	0.835
Percent of visits with a nontherapeutic INR[c,d] (%)	37 (24-47)	50 (45-61)	<0.001
Genotype, No (%)			
VKORC1 -1639A allele	7 (15)	6 (13)	0.765
*CYP2C9**2, *3, *5, *8, or *11 allele	11 (24)	6 (13)	0.179

No (%), mean ± SD, or median (IQR)

[a]Left ventricular thrombus, atrial thrombus, sigmoid sinus thrombus, or cortical vein thrombosis

[b]Warfarin dose at the time of reaching stability

[c]Data for initial 6 months of therapy

[d]Nontherapeutic INR defined as an INR >0.1 units outside of the therapeutic range.

Figure 1. Proteins involved in vitamin K recycling and carboxylation of vitamin K-dependent clotting factors. NQO1, NAD(P)H:quinone oxidoreductase; CYP4F2, cytochrome P450 4F2; APOE, apolipoprotein E

Figure 2. Comparison of genotype frequencies between patients reaching a stable dose within 83 days and those not reaching a stable dose within 83 days of warfarin initiation. Figure 2A shows the data for all patients. Figure 2B is limited to patients reporting dietary alterations during the initial 6 months of warfarin therapy.

Figure 1

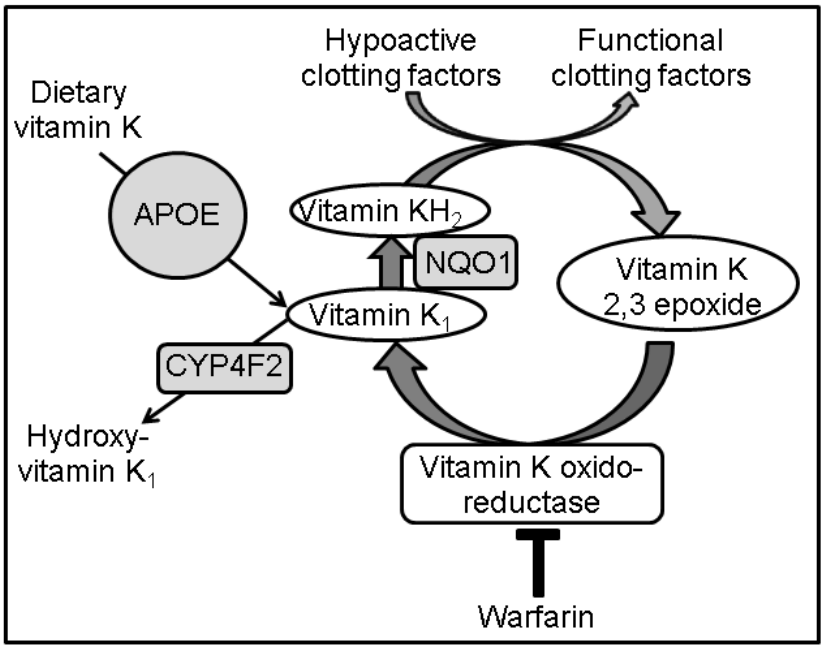
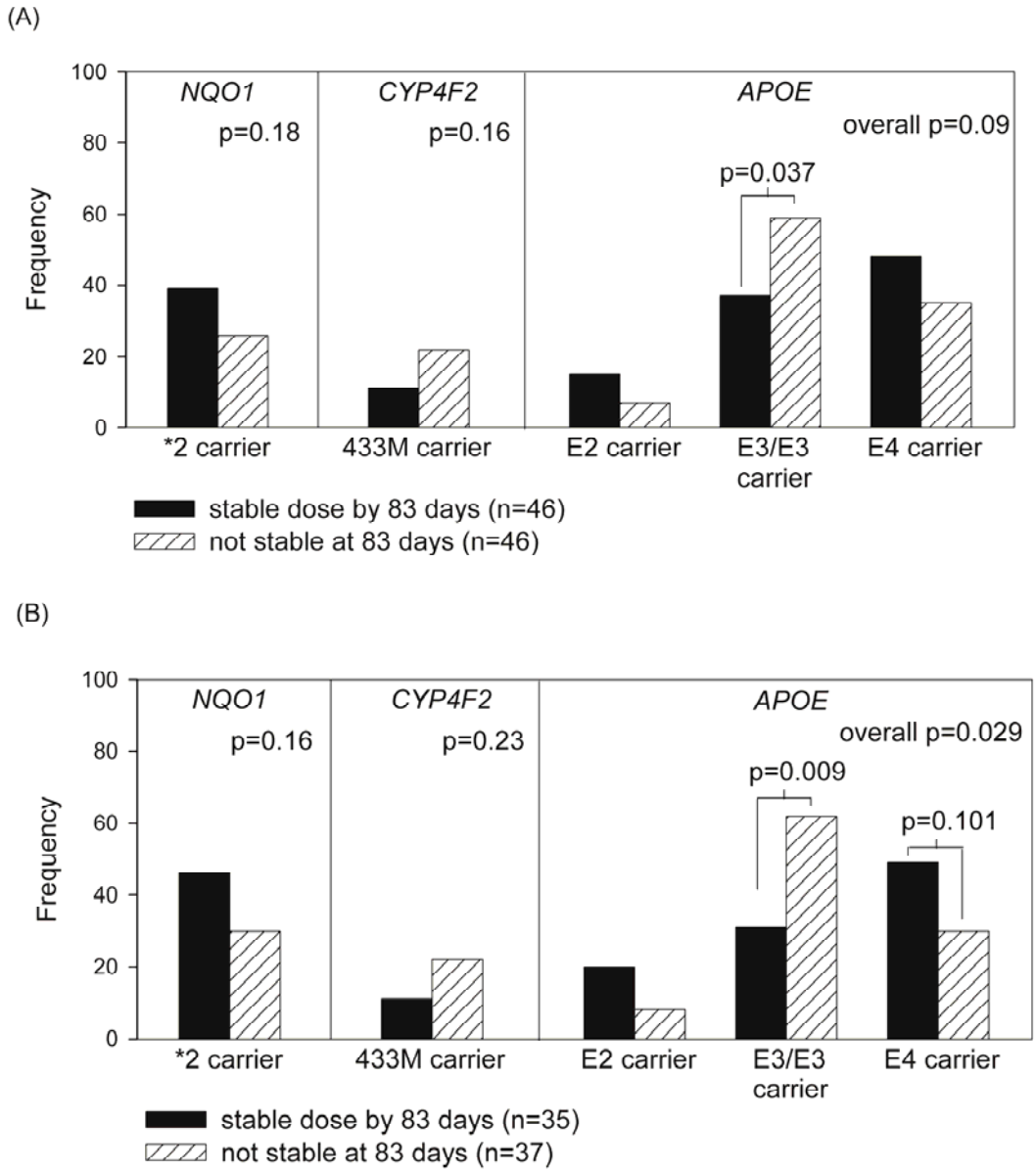

Hypoactive clotting factors
Functional clotting factors
Dietary vitamin K
APOE
Vitamin KH2
NQO1
Vitamin K 2,3 epoxide
Vitamin K1
CYP4F2
Hydroxy-vitamin K1
Vitamin K oxido-reductase
Warfarin

Figure 2


(A)
100
80
60
40
20
0
Frequency
NQO1
p=0.18
CYP4F2
p=0.16
APOE
overall p=0.09
p=0.037
*2 carrier
433M carrier
E2 carrier
E3/E3 carrier
E4 carrier
stable dose by 83 days (n=46)
not stable at 83 days (n=46)
(B)
100
80
60
40
20
0
Frequency
NQO1
p=0.16
CYP4F2
p=0.23
APOE
overall p=0.029
p=0.009
p=0.101
*2 carrier
433M carrier
E2 carrier
E3/E3 carrier
E4 carrier
stable dose by 83 days (n=35)
not stable at 83 days (n=37)