The Effect of Gene-Obesity and Gene-Care Setting Interactions on Risk of Major Bleeding Events in Warfarin Users

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Abstract

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A commonly prescribed anticoagulant, Warfarin (Coumadin®), reduces thromboembolic (blood clotting) event rates in patients with atrial fibrillation, stroke, joint replacement, and other conditions that present increased clotting risk. Unfortunately, pharmacogenetic-guided dosing algorithms have not yielded the predicted effect clinicians and researchers anticipated compared to using the clinically guided dosing methods. Further explanations for effects modifying the genetic effects present on bleeding risk in warfarin patients are warranted. We analyzed a case-control study to investigate gene-environment interaction effects on risk of serious bleeding in warfarin users in a community setting. No interaction was found between *CYP2C9/VKORC1* and obesity. A statistically significant interaction was found between *CYP4F2* x obesity, however, the p-value of 0.049 would not hold its statistical significance to Bonferroni correction for multiple comparisons. No statistically significant interaction was found between *CYP2C9*/*VKORC1* x care setting or between *CYP4F2* x care setting. These findings need to be validated in a larger population to inform future clinical guidelines in order for warfarin users to receive the optimal benefit.

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DEDICATION

To my parents.

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1. INTRODUCTION

1.1 Warfarin

A commonly prescribed anticoagulant, Warfarin (Coumadin®), reduces thromboembolic (blood clotting) event rates in patients with atrial fibrillation, stroke, joint replacement, and other conditions that present increased clotting risk (Deitelzweig, 2013; Roth et al, 2014). However, risk of bleeding still poses serious harm across populations, thus it is critical that the anticoagulation level be monitored using the international normalized ratio (INR), a standardized measure of how long it takes the blood to clot, to maximize clinical benefit and minimize clinical harm (Bussey, Bussey, Bussey-Smith, & Frei, 2013; Han, Ren, & Wang, 2013; Kamali & Wynne, 2010; Pirmohamed et al., 2013). Wide variability in dosing requirements for patients taking warfarin has motivated the majority of pharmacogenetics research investigating the safety of warfarin management during initiation of treatment (Deitelzweig, 2013; Furie, 2013; Han, Ren, & Wang, 2013; Higashi, 2002; Kamali & Wynne, 2010; Kimmel et al., 2013; Limdi, Wiener, Goldstein, Acton, & Beasley, 2009). The risk of bleeding during the first month of therapy is 10 times higher than the risk after the first year of therapy (Moyer et al., 2009), and the risk of bleeding is greatest in the first three months (Baker et al., 2004), hence the substantial research focused on outcomes during therapy initiation, which also provides adequate power to detect associations.

In 2009, the International Warfarin Pharmacogenetics Consortium released a pharmacogenetic algorithm to estimate more closely the appropriate initial stable therapeutic dose of warfarin compared to doses calculated from a clinical algorithm or a fixed-dose method, hoping to reduce the number of warfarin-related major bleeding events (IWPC, 2009). The dosing algorithm includes clinical characteristics (age, height, weight, and race),

pharmacogenetic information (*VKORC1* genotype and *CYP2C9* genotype), and whether the patient is taking an enzyme inducer (i.e. rifampin, phenytoin, and carbamazepine) and/or an amiodarone (an antiarrhythmic drug). Their study was limited by not having sufficient data to include additional potentially important factors such as, vitamin K intake, smoking status, genetic factors (e.g. *CYP4F2, ApoE,* etc.), or environmental factors, all of which could strengthen the dosing prediction and have an effect on bleeding risk (IWPC, 2009).

Unfortunately, the pharmacogenetic-guided dosing algorithms have not yielded the predicted effect clinicians and researchers anticipated compared to using the clinically guided dosing methods during the first four weeks of therapy (Kimmel et al., 2013). No significant differences were found between the genotype-guided group and the clinically guided group, with a mean percentage of time spent in the therapeutic range (INR, 2-3), 45.2% and 45.4%, respectively. In the Limdi et al study, the overall median time it took the patient to attain the therapeutic range (INR, 2-3) using these dosing techniques was 9 days (Inter-quartile range-IQR: 4.2-26.4), though the median time varied significantly among patients with *VKORC1* variant only (median 6.0, IQR 3.4-19.2), with variants in both *CYP2C9* and *VKORC1* (median 5.0, IQR 2.5-11.2), with *CYP2C9* variant (median 12.7, IQR 4.3-26.3) and with no *CYP2C9* and *VKORC1* variants (median 12.3, IQR 5.7-31.7) (Limdi et al., 2009). Higher variation in the therapeutic range may influence over-anticoagulation, which could increase the risk of hemorrhagic complications.

1.2 Pharmacogenetics

1.2a CYP2C9

Researchers have identified three genes that influence the variability in warfarin therapeutic dose requirement, and could potentially be associated with major bleeding risk

(Caldwell et al., 2008; Higashi, 2002; Kamali & Wynne, 2010; Limdi et al., 2009). Cytochrome P450 2C9 (*CYP2C9)* is primarily responsible for the metabolism of the warfarin S-enantiomer into its inactive metabolites, and common genetic variants decrease enzymatic activity (Limdi et al., 2009; Meckley, Wittkowsky, Rieder, Rettie, & Veenstra, 2008; Wells et al., 2010). As a result, the *CYP2C9*2* and **3* alleles (collectively defined as **1/ *2, *1/ *3, *2/ *2, *2/ *3,* and **3/ *3* as *CYP2C9* genotypes)*,* are known to require altered warfarin dose requirements, have been associated with higher risk of serious bleeding events, and require a significantly longer time to reach stabilized dose (Higashi, 2002; Meckley et al., 2008). Limdi et al found that *CYP2C9* variants confer a significantly increased bleeding risk since patients remain in the therapeutic target range only 40-60% of the time, even after stabilization of therapy (Limdi et al., 2009). In a warfarin pharmacogenetics review, Limdi and Veenstra found a major bleeding risk associated with variant *CYP2C9* genotype with a risk ratio of 3.9 (95% Confidence Interval (CI): 1.3-12.1) during initiation and risk ratio of 2.4 (95% CI: 1.2-4.9) (Limdi & Veenstra, 2008). Another study showed that patients with a variant genotype in *CYP2C9* have a significantly increased risk of serious bleeding event (hazard ratio: 2.39; 95% CI: 1.18-4.86) (Higashi, 2002). Tomek et al found a significant association with *CYP2C9* variants and increased risk of major bleeding events during initiation and the maintenance phase with the adjusted hazard ratio (HR) for major bleeding event risk for patients with any *CYP2C9* variant allele to be 1.962 [95% CI: 1.08–3.56, p = 0.027] (Tomek et al., 2013).

1.2b VKORC1

Second, the vitamin K epoxide reductase complex 1 (*VKORC1*) gene encodes the warfarin target enzyme, vitamin K epoxide reductase (Rieder et al., 2005), and warfarin inhibits vitamin K-dependent coagulation factors (Kabagambe, Beasley, & Limdi, 2013). Together, *CYP2C9* and

VKORC1 variants account for approximately 50% of the genetic effect on the inter-individual variability related to dosing requirements (Wells et al., 2010). Tomek et al found a non-significant trend in *VKORC1* variants and increased risk of major bleeding events during initiation and the maintenance phase (Tomek et al., 2013). The adjusted hazard ratio for major bleeding event risk for patients with the *VKORC1* AA haplotype was 1.841 (95% CI 0.97–3.48, p = 0.06), while for three variant allele carriers of both genes (*CYP2C9* and *VKORC1*), HR was 4.34 (95% CI 1.95–9.65, p < 0.001). In a race-adjusted multivariable analysis, Limdi et al found over-anticoagulation was more frequent (P < 0.0001) among patients with the *VKORC1* variant compared to patients without the variant and this could be associated with an increased bleeding risk (Limdi et al., 2009).

1.2c CYP4F2

Third, additional evidence suggests an association of Cytochrome P450 4F2 (*CYP4F2*3,* rs2108622*)* and variability in warfarin dose (Caldwell et al., 2008; Liang et al., 2012). Evidence indicates that *CYP4F2* variants likely influence Vitamin K metabolism (Kamali & Wynne, 2010), affecting warfarin dose requirement and contributing 2%-7% of the variability due to genetic effects (Caldwell et al., 2008; Cen et al., 2010).

In a recent study, Roth et al found that variants in *CYP2C9* (**2, *3)* and *VKORC1* (1173) did not result in statistical significance, but did show a trend toward increased major bleeding risk in their predominately long-term users of warfarin, and they showed the variants in both genes have a potentially weaker association during initiation than those previously reported (Roth et al., 2014). Differences in patient populations across the various studies could influence the varying results on genetic variants associated with bleeding risk, since patients were ascertained from a community setting in the Roth et al study (Roth et al., 2014) compared to

patients being treated only at anti-coagulation clinics (Higashi, 2002; Limdi et al., 2009; Tomek et al., 2013). Roth et al found a novel association between *CYP4F2*3* and decreased risk of major bleeding, however this finding warrants replication in independent samples (Roth et al., 2014).

1.3 Gene-environment interactions on risk of serious bleeding

Despite the research focus on the initiation period, additional factors could contribute to bleeding risk beyond the first year of warfarin therapy. To our knowledge, there have been no studies investigating longer-term exposure (6+ months) of warfarin and associated gene-environment interactions on risk of serious bleeding events in a community setting due to power limitations. "Understanding [gene-environment] interactions can increase the power, accuracy, and precision of detecting effects from both environmental exposures and genetic contribution" as well as elucidate a clearer picture "of biological mechanisms and pathways in assessing disease risk (Austin, 2013)." Thus, exploring gene-environment interactions should be considered to provide further explanations for effects modifying the genetic effects present on bleeding risk in warfarin users, while investigating the differences in previous findings on bleeding risk. There are two particular environmental factors that warrant exploration. First, a gap in the literature exists for studies investigating the influence of pharmacogenetics interacting with environmental and patient factors associated with major bleeding risk in warfarin users. Most studies to date included warfarin users from urban anticoagulation clinics at academic medical centers (Roth et al., 2014), which may not be representative of the care settings of many warfarin patients.

Environmental factors affect warfarin metabolism and have varying associated risks on excessive bleeding or cerebrovascular clotting and stroke (Moyer et al., 2009; Mueller, Patel,

Halawa, Dumitrascu, & Dawson, 2014). It is critical in utilizing all of the known patient data to assess environmental risk contributions and decrease risk of adverse bleeding events.

Second, obesity may be important because it has been found to be associated with increased risk of hemorrhagic bleeding in women and men; with women who have a body mass index greater than 29 kg/m^2 having a relative risk of 1.90 (95% CI: 1.28-2.82) increased risk of stroke (Rexrode et al., 1997) and men with a body mass index greater than 30 having an adjusted relative risk of 2.0 (95% CI: 1.48-2.71) increased risk of stroke (Kurth et al., 2002). Body mass index (BMI) is an index of weight-to-height commonly used to classify underweight, normal, overweight, and obesity categories in patients (WHO, 2014). Wallace et al compared initial warfarin response in obese patients versus non-obese patients and found that compared to normal weight, obese and morbidly obese patients required a significantly longer median time to achieve therapeutic INR (Wallace et al., 2013). This has implications of increasing patients' bleeding risk due to the majority of hemorrhaging events occurring when INRs are outside of the therapeutic window (Oake, Fergusson, Forster, & Walraven, 2007). This comparison also found a decreased initial response to warfarin in the obese and morbidly obese patients compared to normal weight. Mueller et al found that for each 1-point increase in BMI, the average weekly dose of warfarin increased which could affect risk of major bleeding event in patients (Mueller et al., 2014). As adapted from the World Health Organization BMI Index, obesity is defined as BMI ≥ 30.0 kg/m^2, not obese is BMI <30.0 kg/m^2 (WHO, 2014).

No studies have investigated the gene-obesity interaction that may be associated with risk of serious bleeding in patients as obesity could modify the genetic effects that are well established.

1.4 Care setting

Care setting is an additional environmental contributor that could be associated with serious bleeding risk. In their meta-analysis, Baker et al found that in the United States, the time in therapeutic range is around 51% in community-based settings and 63% in specialized anticoagulation clinics (Baker, Cios, Sander, & Coleman, 2009), which suggests implications for adverse bleeding events if time is spent outside of the therapeutic range. For atrial fibrillation patients, the proportion of time spent within the therapeutic INR range was 55% (95% CI: 51%-58%), with patients in anticoagulation clinics spending on average 63% (95% CI: 58%-68%) compared to patients in the usual care community practice spending 11% (95% CI: 2%-20%) less time in therapeutic INR range. Further, numerous studies have been conducted to determine in which care settings optimal care for warfarin therapy takes place, and while anticoagulation clinics have been shown to have lower bleeding rates, it is not entirely clear which alternative settings may be able to provide comparable care (Baker et al., 2009; Bussey et al., 2013; Rossiter, Soor, Telner, Aliarzadeh, & Lake, 2013; Salinger et al., 2009). Persistence on warfarin therapy was found to be suboptimal due to discontinuation of warfarin use within 90 days of initiation in long term care residents with atrial fibrillation, despite following the clinical practice guidelines to prevent stroke in these patients (Patel, Reardon, Nelson, Philpot, & Neidecker, 2013). Patient safety is an imperative for anticoagulation management and detailed information needs to be provided to patients when they are transferring between settings, including outpatient, emergency, inpatient, subacute, and long-term care (Deitelzweig, 2013). Type of care setting could modify previously well-established genetic effects on serious bleeding. We investigated whether an interaction exists between genetic status and type of care setting utilized (specialty or non-specialist) on risk of major bleeding events.

The objective of this study is to address the evidence gap by exploring gene-environment interactions that may be associated with bleeding risk. Variation in initial response to warfarin in obese and morbidly obese has motivated this study in analyzing associations with adverse events in warfarin users. The potential association between genetic risk factors, environmental factors, patient clinical characteristics, and risk of major bleeding events prompted us to examine the interaction between obesity and genetic status, and risk of major bleeding events in a group of warfarin patients, controlling for race, age, and sex. We wanted to better understand the health outcomes of obese and non-obese patients taking warfarin and receiving care in a particular type of care setting using a case-control study design. We hypothesized that an interaction exists between the presence of having at least one genetic variant in *CYP2C9* (*2, rs17998523 or *3, rs1057910), *VKORC1* (*1173,* rs993448), and/or *CYP4F2* (*3, rs2108622) and obesity on risk of major bleeding events in warfarin users. We further hypothesized that an interaction exists between the presence of having at least one genetic variant in *CYP2C9* (*2, rs17998523 or *3, rs1057910), *VKORC1* (*1173,* rs993448), and/or *CYP4F2* (*3, rs2108622) and type of care setting associated with risk of major bleeding events in warfarin users.

2. METHODS

This analysis is based on a previous case-control study conducted by Roth et al (Roth et al., 2014). These data were identified, collected, and validated on 265 warfarin patients with a major bleeding event.

2.1 Study sample

Cases were identified as men and women, age 18 years and older with an inpatient diagnosis of a bleeding event with no major bleeding event in the year prior to the index date (i.e., reference date) between January 1, 2005 and October 1, 2010, and estimated to be using

warfarin at or within three days of the bleed. The validated *International Classification of Diseases, Ninth Revision* algorithm was used to identify major bleeding event using established methods and the following classification for "major bleeding" followed: if bleeding event was clinically apparent and resulted in hospitalization, hemoglobin dropped >2mg/dl, and/or >2 units of packed red blood cells were transfused (Roth et al., 2014). The Roth et al study provides further details on additional requirements that defined major bleeding events. Cases were enrolled in Group Health (GH) Cooperative, a nonprofit integrated health-care system that insures and offers comprehensive medical care for ~650,000 patients across Washington state (Roth et al., 2014). Pharmacy records were used to determine warfarin use as an estimate by looking at the number of days supply, with participants required to be within three days of the event. Cases were eligible for this analysis if they were enrolled in GH for at least the previous year and had no major bleeding event recorded prior to the index date.

Controls were men and women age 18 years and older, enrolled in GH, and had no prior bleeding events as determined in the automated databases. Controls were frequency matched on index (bleeding) date between January 1, 2005 and April 1, 2011. A reference date (diagnosis date of case) was used for collection of information that allowed for the comparison of case and control data from the same time frame. Controls were eligible if they were enrolled in GH for at least one year prior to reference date and used warfarin therapy at or within three days of the reference date. There were 265 cases and 305 controls. Additional information on the cases and controls is described in a previous study (Roth et al., 2014).

To collect information that was not available in the GH automated databases, study patients provided supplemental information (about clinical and demographic characteristics) by completing a 44-item self-report survey.

2.2 Statistical analyses

Deviation of genotype frequencies from Hardy–Weinberg equilibrium were tested by applying the Hardy–Weinberg model to the data (Rodriguez, Gaunt, & Day, 2009). The $\chi 2$ goodness-of-fit test was used to compare expected vs. observed frequency of *CYP2C9*, *VKORC1*, and *CYP4F2* genotypes in controls and defined significant deviation as a test resulting in $p < 0.05$.

Statistical analyses were performed using the STATA Statistical Package, Version 13.0 (STATA, Austin, TX). Case and control demographics were compared using $\chi 2$ tests, Fisher's exact test for categorical variables, and the Student's t-test for continuous variables, following established methods in the literature (McDonald, 2009).

2.3 Logistic regression analysis

Models were constructed using logistic regression to estimate the risk of major bleeding associated with the presence of main effects for genetic variants and environmental risk factors, expressed as univariate and multivariate odds ratios with 95% confidence intervals. Similar to previous studies, our analyses grouped heterozygous and homozygous variant patients in a single "variant" category due to the small number of homozygous variants (Higashi, 2002; Limdi & Veenstra, 2011; Roth et al., 2014). Categorical data are presented as the count (percentage). A p-value of statistical significance was defined as <0.05.

2.4 Univariate logistic regression analysis of the association of risk factors on risk of serious bleeding in warfarin users

The univariate model included each of the following terms as a predictor of interest on risk of major bleeding: presence of a *CYP2C9* or *VKORC1* variant (binary), obesity (binary), presence *CYP4F2*3* (binary), age at index date (continuous), sex (binary), specialty care setting (binary), and race (white/other, binary).

2.5 Gene-obesity interaction tabulation

Using a 2x4 tabulation, we determined interaction of genetics and obesity based on the multiplicative scale analyzing the odds ratio (OR) if patient was obese and genetic variant present (G^+, E^+) not being equal to the OR of non-obese and genetic variant present (G^+, E^-) multiplied by the OR of obese and no genetic variant present (G^-, E^+) (Botto & Khoury, 2001; Austin, 2013). This method was also used to test whether a greater effect of obesity in individuals with the associated risk genotype (*CYP2C9/VKORC1* or *CYP4F2*) existed or whether a smaller effect of obesity in individuals with the associated risk genotype (*CYP2C9/VKORC1* or *CYP4F2*) was present. Equal ORs for these comparison groups (G^+, E^+ vs. G^+, E^- and G^-, E^+) would indicate no interaction. A greater effect would exhibit a synergistic interaction and a smaller effect would represent an antagonistic interaction (Austin, 2013). The following equations were used:

$OR_{G+E+} = OR_{G+E-} \times OR_{G-E=}$ (no interaction)

$OR_{G+E+} > OR_{G+E-} \times OR_{G-E=}$ (synergistic interaction)

$OR_{G+E+} < OR_{G+E-} \times OR_{G-E=}$ (antagonistic interaction)

2.6 Logistic regression analysis of the association of risk factors and gene-obesity interactions on risk of major bleeding

A multivariate logistic regression model was constructed and included both interaction terms as the predictors of interest on bleeding risk. In model 1, each of the estimates for risk of serious bleeding was adjusted for potential confounders, including presence of a *CYP2C9* or *VKORC1* variant (binary), obesity (binary), presence *CYP4F2*3* (binary), age at index date (continuous), male, and race (white/other, binary). These covariates were selected based on

patient characteristics previously adjusted for in earlier studies on outcomes in warfarin users (Higashi, 2002; Meyer zu Schwabedissen et al., 2006; Roth et al., 2014).

2.7 Gene-care setting interaction tabulation

Interaction of genetics and care setting was determined based on the multiplicative scale analyzing the odds ratio (OR) if patient received care at a specialized care setting and genetic variant present (G^+, E^+) not being equal to the OR of non-specialized care setting and genetic variant present (G^+, E^-) multiplied by the OR of specialized care setting and no genetic variant present (G^-, E^+) (Botto & Khoury, 2001; Austin, 2013). This method was also used to test whether a greater effect of care setting in individuals with the associated risk genotype (*CYP2C9/VKORC1* or *CYP4F2*) existed or whether a smaller effect of care setting in individuals with the associated risk genotype (*CYP2C9/VKORC1* or *CYP4F2*) was present. Equal ORs for these comparison groups (G^+, E^+ vs. G^+, E^-, and G^-, E^+) would indicate no interaction. A greater effect would exhibit a synergistic interaction and a smaller effect would represent an antagonistic interaction (Austin, 2013). The same equations used for the gene-obesity analysis were used for the gene-care setting analysis.

2.8 Logistic regression analysis of the association of risk factors and gene-care setting interactions on risk of major bleeding

A multivariate logistic regression model was constructed and included both interaction terms as the predictors of interest on bleeding risk. In model 2, estimates for risk of serious bleeding were adjusted for presence of a *CYP2C9* or *VKORC1* variant (binary), care setting (binary), presence *CYP4F2*3* (binary), age at index date (continuous), male, and race (white/other, binary). The interaction terms were generated from the gene and environment data.

2.9 Exposure variables

Two genetic terms were included in the model due to the contrasting bleeding risk of the variants investigated in our study confer. That is, variants in *CYP2C9 (*2, *3)* have been associated with an increased bleeding risk and *VKORC1 (1173)* trends toward an increased bleeding risk (Higashi, 2002; Lindh, Lundgren, Holm, Alfredsson, & Rane, 2005; Meckley et al., 2008), while *CYP4F2*3* has been associated with a decreased bleeding risk (Roth et al, 2014). We generated the first genetic term by combining the presence of any *CYP2C9* risk variant or presence of the *VKORC1* risk variant into a binary variable to increase the power for our study. To investigate genetic associations of *CYP4F2*3,* we included a binary term in the model for presence of *CYP4F2* variant and a subsequent interaction term with obesity.

We generated a binary variable for obesity, defining obesity as BMI $\geq$ 30.0 kg/m^2 and not obese as BMI <30.0 kg/m^2, respectively, according to the WHO body mass index cut-off points (WHO, 2014).

The overall association of genetic variants of interest on bleeding complications of warfarin therapy and interactions with BMI (obese and not obese) was evaluated. Care setting (specialized anticoagulation clinic vs. other) was evaluated as well by generating a binary variable using care setting data of specialist care setting (anticoagulation clinic) and non-specialist care setting (primary care physician, cardiologist, or other). The care setting data was collected by the self-report survey.

3. RESULTS

3.1 Study sample characteristics

Table 1 provides descriptive statistics of the study sample characteristics. The study sample, among cases and controls, had an average age at index date of 71.1 and 69.5 years, respectively. Among cases, 47% were obese and among controls, 58% were obese (P=0.01). The

sample was comprised of 50.6% and 57.7% males and 49.4% and 42.3% females, among cases and controls, respectively. Most participants were Caucasian, 93% and 95%, among cases and controls, respectively. Cases and controls did not differ significantly by race or care setting. Race information was not reported and thus there was missing data for four cases and three controls.

3.2 Hardy-Weinberg Equilibrium Testing

The observed *CYP2C9*2/*3, VKORC1,* and *CYP4F2*3* genotype frequency distributions were in Hardy-Weinberg equilibrium in the study controls (Table 2). There was no significant difference in the genotype distributions for any of the SNPs between cases and controls (Table 2).

3.3 Univariate logistic regression analysis of the association between risk factors and risk of serious bleeding in warfarin users

Table 3 displays the univariate model with the results from the association between risk factors and risk of serious bleeding. Univariate analyses demonstrated that obese patients compared to non-obese patients had a decreased risk of serious bleeding event with an odds ratio (OR) of 0.65 (95% Confidence Intervals CI: 0.46-0.90), which was statistically significant (p= 0.01). Patients with *CYP4F2*3* compared to those without *CYP4F2*3* had a decreased risk of serious bleeding event with an OR of 0.71 (95% CI: 0.51-0.98, p=0.04). In this univariate model, patients treated at an anticoagulation clinic (specialty care setting) compared to patients not treated at an anticoagulation clinic had a decreased risk of serious bleeding event with an OR of 0.64 (95% CI: 0.46-0.89, p=0.009). The following risk factors were not statistically significant: *CYP2C9* or *VKORC1* variant present, age at index date, sex, and race (Table 3).

3.4 Gene-environment interactions tabulation for obesity

Table 4 displays the results from the gene-obesity interaction tabulation of the risk on serious bleeding. The OR_{G+E+} (0.67) is similar to the product of the OR_{G-E+} and OR_{G+E-} (0.59),

indicating there is no interaction for *CYP2C9/VKORC1* and obesity on risk of serious bleeding on a multiplicative scale. Based on the tabulation in Table 4, the results show that there may be an interaction for *CYP4F2* and obesity because the OR_{G+E+} (0.49) is dissimilar from the product of the OR_{G-E+} and OR_{G+E-} (0.24). The ORG_{G+E+} (obese and genetic variant present) is statistically significantly different (p=0.002) from the OR_{G-E-} (the referent group).

3.5 Multivariate logistic regression analysis of the association between risk factors, gene-obesity interactions, and risk of serious bleeding in warfarin users for obesity

Table 5 displays Model 1 results of the multivariate logistic regression analysis of the association between risk factors and risk of serious bleeding. The odds ratio for presence of a *CYP2C9* or *VKORC1* variant (OR: 1.01) was not statistically significant. When holding all other risk factors included in the model constant, obese patients compared to non-obese patients had a statistically significant decreased risk of serious bleeding with an OR of 0.42 (95% CI: 0.20-0.89, p=0.02). Obese patients who have a *CYP2C9* or *VKORC1* variant present compared to non-obese patients who do not have one of these variants have an increased risk of serious bleeding event with an odds ratio of 1.23, but this result was not statistically significant, demonstrating no interaction. Holding all other risk factors constant, patients with *CYP4F2*3* compared to those without *CYP4F2*3* had a statistically significant decreased risk of serious bleeding event with an OR of 0.49 (95% 0.30-0.82), with p-value of 0.01. The odds of serious bleeding in obese patients with *CYP4F2*3* is around 2 times greater (OR: 1.99; 95% CI: 1.0-3.95) than the odds of serious bleeding among non-obese and without *CYP4F2*3*, with a statistically significant p-value of 0.049. Male patients had a decreased risk of serious bleeding event with an OR of 0.71, which was statistically significant (95% CI: 0.50, 0.99; p=0.049). The ORs for age at index date and race were not statistically significant, showing no main effect associated with risk of bleeding event.

3.6 Gene-environment interactions tabulation for care setting

Table 6 displays the results from the gene-care setting interaction tabulation of the risk on serious bleeding. The OR_{G+E+} (0.67) is similar to the product of the OR_{G-E+} and OR_{G+E-} (0.56), indicating there is no interaction between *CYP2C9/VKORC1* and care setting on risk of serious bleeding. The results show that there may be an interaction for *CYP4F2*3* and care setting because the OR_{G+E+} (1.0) is dissimilar to the product of the OR_{G-E+} and OR_{G+E-} (0.68). However, this potential interaction for *CYP4F2*3* and care setting is unlikely because the OR is 1.0 for both OR_{G+E+} and OR_{G-E-}, thus, there is no difference in risk associated with the referent group of no genetic and environmental exposure present (OR: 1.0).

3.7 Multivariate logistic regression analysis for care setting

Table 7 displays Model 2 results of the multivariate logistic regression analysis of the association of risk factors and risk for serious bleeding in warfarin users. The odds ratio for presence of a *CYP2C9* or *VKORC1* variant (OR: 1.02) was not statistically significant. When holding all other risk factors included in this model constant, obese patients compared to non-obese patients had a statistically significant decreased risk of serious bleeding with an OR of 0.66 (95% CI: 0.46-0.94, p=0.02). Patients who have a *CYP2C9* or *VKORC1* variant present and who were treated at a specialty care setting (anticoagulation clinic) have a slightly increased risk of serious bleeding event with an odds ratio of 1.15, but this result confirmed no interaction because it was not statistically significant. Patients who have a *CYP4F2*3* and who were treated at a specialty care setting (anticoagulation clinic) odds ratio of 0.87, but this result demonstrated no interaction because it was not statistically significant (Table 7). When holding all other risk factors included in the model constant the ORs for the following terms were not statistically

significant: main effect of specialty care setting, main effect of presence of *CYP4F2*3,* age at index date, sex, and race.

4. DISCUSSION

We analyzed a case-control study to investigate gene-environment interaction effects on risk of serious bleeding in warfarin users in a community setting. No interaction was found between *CYP2C9/VKORC1* and obesity. A statistically significant interaction was found between *CYP4F2* x obesity, however, the p-value of 0.049 would not hold its statistical significance to Bonferroni correction for multiple comparisons (Simes, 1986). No statistically significant interaction was found between *CYP2C9*/*VKORC1* x care setting or between *CYP4F2* x care setting.

These findings are important because among this population, the gene-environment interaction of *CYP4F2*3* and obesity conferred a clinically significant (but borderline statistically significant) increased risk of serious bleeding event among warfarin patients. However, when those genetic (*CYP4F2*3)* and environmental (obesity) factors are considered separately, each confers a statistically significant decreased risk of serious bleeding event and is protective. Using the multivariate model, it appears that being both obese and having *CYP4F2*3* attenuate the protective effects of only having one of those risk factors. The mechanisms for this potential effect merit exploration, including both the interplay between inflammatory markers and/or vitamin K stores in obese patients and their risk of bleeding. The main effect of *CYP4F2*3* and obesity on major bleeding show a decreased risk and any effects related to these two factors that have not been accounted for result in an increased risk displayed in the interaction term. These varying associations on bleeding risk imply further investigation into

understanding whether the main effect is an artifact of the interaction since the association of the interaction is in the opposite direction of the factors not dependent on the other one.

Our findings may provide evidence for understanding some of the variability in published findings for associations between pharmacogenomic markers and risk of serious bleeding events in warfarin patients.

From our findings care setting did not seem to have an effect on risk of serious bleeding and future research should include investigating additional environmental risk factor interactions in patients.

There are several limitations of our study worth noting. Determining the type of care setting for anticoagulation management was a limitation in this study, since this information was gathered through self-report surveys and automated records. Automated records existed for patients in the integrated group practice plan and for those in network plans, so self-report bias is worth noting for interpretations of these results. Where variables were missing for patients in the network plans, data was ascertained from pharmacy and electronic medical records (Roth et al., 2014). Strength of these findings is that the data was analyzed from the largest known to date case control study in warfarin pharmacogenomics with exposure data collected. While the study was the largest of its kind, the sample sizes still limited the power to be able to detect interactions in general and a greater sample size would increase the power for this type of study. A study approach that might provide increased power to investigate gene-environment interactions associated with bleeding risk in warfarin users might make use of a large claims database or bigger health maintenance organization system.

We found an interaction between *CYP4F2**3 and obesity on risk of serious bleeding in warfarin users. Potential areas for future research include exploring different environmental

factors on outcomes for warfarin users, such as distance to provider, cost of travel to clinic visits in a rural setting, gene-diet interactions, and other factors that may contribute to bleeding risk for warfarin users. These findings need to be validated in a larger population to inform future clinical guidelines in order for warfarin users to receive the optimal benefit.

TABLES

Table 1: Clinical and demographic characteristics by case - control status.

Variable	Cases (n=265)	Control (n=305)	p-value¥
Automated Data Variables			
Age in Years at Index Date, Mean (SD)	71.1 (12.7)	69.5 (11.2)	0.12
	n (column %)		
Body Mass Index (BMI)			**0.01**
Not obese (<30.0 kg/m^2)	140 (53%)	128 (42%)	
Obese (≥30.0 kg/m^2)	125 (47%)	177 (58%)	
Male/Female	134 (50.6%)/ 131 (49.4%)	176 (57.7%)/ 129 (42.3%)	0.09
Self-Report Survey Variables			
	n (column %)		
Race			0.65
American Indian or Alaska Native	3 (1%)	1 (.3%)	
Asian	3 (1%)	6 (2%)	
Black/African American	7 (3%)	5 (2%)	
Native Hawaiian or Other Pacific Islander	1 (1%)	1 (.3%)	
White Caucasian	243 (93%)	287 (95%)	
Other	4 (1%)	2 (.7%)	
Care Setting			0.14
Anticoagulation Clinic	111 (48.4%)	161 (57.3%)	
Primary Care	71 (31%)	77 (27.4%)	
Cardiologist	20 (8.7%)	23 (8.2%)	
Other	27 (11.8%)	20 (7.1%)	

Note: Bold p-value indicates statistically significant differences between cases and controls at α=0.05
¥Based on χ2 tests, Fisher's exact test, Student's t-test

Table 2: Observed Vs. Expected Frequency of SNP genotypes and tests for Hardy-Weinberg Equilibrium in controls (adapted from *Roth et al* 2014).

Variable	Observed in Cases		Observed in Controls		H-W Expected in Controls	χ^2, p-value
	N	%	N	%	%	
All Patients	265	64.70	302	35.30	-	
***CYP2C9**2 Status**						
C:C	201	75.80	245	81.10	80.50	χ^2=1.29
C:T	58	21.90	52	17.20	18.40	p=0.26
T:T	6	2.30	5	1.70	1.10	
***CYP2C9**3 Status**						
A:A	235	88.70	256	84.80	85.00	χ^2=0.44
A:C	30	11.30	45	14.90	14.40	p=0.51
C:C	0	0.00	1	0.30	0.60	
***CYP4F2**3 Status**						
G:G	154	57.70	151	50.00	50.00	χ^2<0.01
G:A	97	36.60	125	41.40	41.40	p=0.94
A:A	14	5.30	26	8.60	8.60	
***VKORC1* 1173 Status**						
G:G	98	37.00	117	38.70	38.30	χ^2=0.09
G:A	126	47.50	140	46.30	47.40	p=0.76
A:A	41	15.50	45	14.90	14.50	

H-W=Hardy-Weinberg Equilibrium

Note: No chi-squared two-sided test at α=0.05 was significant for difference between observed and expected counts

Table 3: Univariate Logistic Regression Analysis of the Association between Risk Factors and Risk of Serious Bleeding in Warfarin Users.

Risk factors	OR (95% CI) Univariate model	p-value
CYP2C9 or *VKORC1* variant present	1.08 (0.75-1.6)	0.68
Obese (BMI>30 kg/m^2)	**0.65 (0.46-0.90)**	**0.01**
*CYP4F2*3*	**0.71 (0.51-0.98)**	**0.04**
Age at index date	1.01 (0.99-1.03)	0.12
Sex	0.75 (0.54-1.04)	0.09
Specialty Care setting	**0.64 (0.46-0.89)**	**0.009**
Race	0.69 (0.36-1.32)	0.23

Note: Bold p-value indicates statistically significant at α=0.05
Odd ratio (OR); Confidence interval (CI)

Table 4: Analysis of obesity, presence of pharmacogenetic alleles, and risk of serious bleeding.

	High-risk genotype present (*CYP2C9* or *VKORC1* allele)		High-risk genotype absent (No *CYP2C9* or *VKORC1* allele)	
	Obese	Not obese	Obese	Not obese
Cases	93	104	32	36
Controls	127	95	50	33
ORs	OR_{G+E+} =0.67	OR_{G+E-} =1.0	OR_{G-E+} =0.59	OR_{G-E-} =1.0 (REF)
	95% CI: (0.45, 1.0) p-value= 0.05	95% CI: (0.34, 1.0) p-value=0.06	95% CI: (0.29, 1.18) p-value=0.14	

	High-risk genotype present (*CYP4F2* allele)		High-risk genotype absent (No *CYP4F2* allele)	
	Obese	Not obese	Obese	Not obese
Cases	64	47	61	93
Controls	90	64	87	64
ORs	OR_{G+E+} =0.49	OR_{G+E-} =0.51	OR_{G-E+} =0.48	OR_{G-E-} =1.0 (REF)
	95% CIs: (0.31, 0.76) **p-value= 0.002**	95% CIs: (0.30, 0.85) **p-value= 0.009**	95% CIs: (0.30, 0.78) **p-value= 0.002**	

Significant *p*-values are in bold.

Table 5: Multivariate Logistic Regression Analysis, Including Gene-Obesity Interaction, of the Association of Risk Factors and Risk of Serious Bleeding in Warfarin Users

Risk factors	OR (95% CI) Multivariate model	p-value
CYP2C9 or *VKORC1* variant present	1.01 (0.57-1.79)	0.97
Obese (BMI>30 kg/m^2)	**0.42 (0.20-0.89)**	**0.02**
CYP2C9 or *VKORC1* X obesity	1.23 (0.57-2.69)	0.60
*CYP4F2*3*	**0.49 (0.30-0.82)**	**0.01**
*CYP4F2*3* X obesity	**1.99 (1.0-3.95)**	**0.049**
Age at index date	1.01 (0.99-1.02)	0.36
Sex	**0.71 (0.50-0.99)**	**0.049**
Race	0.74 (0.39-1.47)	0.39

Significant *p*-values are in bold.

Table 6: Analysis of Care Setting, Presence of Pharmacogenetic Alleles, and Risk of Serious Bleeding

	High-risk genotype present (*CYP2C9* or *VKORC1* allele)		High-risk genotype absent (No *CYP2C9* or *VKORC1* allele)	
	Specialty Care setting	Not specialty care setting	Specialty Care setting	Not specialty care setting
Cases	88	109	27	41
Controls	121	101	45	38
ORs	OR_{G+E+} =0.67	OR_{G+E-} =1.0	OR_{G-E+} =0.56	OR_{G-E-} =1.0 (REF)
	95% CIs: (0.45, 1.01) p-value=0.05	95% CIs: (0.58,1.7) p-value=0.99	95% CIs: (0.28, 1.12) p-value=0.10	

	High-risk genotype present (*CYP4F2* allele)		High-risk genotype absent (No *CYP4F2* allele)	
	Specialty Care setting	Not specialty	Specialty Care setting	Not specialty
Cases	47	64	68	86
Controls	44	71	83	83
ORs	OR_{G+E+} =1.0	OR_{G+E-} =0.87	OR_{G-E+} =0.79	OR_{G-E-} =1.0 (REF)
	95% CIs: (0.62, 2.09) p-value=0.59	95% CIs: (0.54,1.4) p-value=0.57	95% CIs: (0.49, 1.23) p-value=0.32	

Table 7: Multivariate Logistic Regression Analysis Including Gene-Care Setting Interaction, of the Association of Risk Factors and Risk for Serious Bleeding in Warfarin Users

	OR (95% CI)	
Risk factors	**Multivariate model**	**p-value**
CYP2C9 or *VKORC1* variant present	1.02 (0.60-1.7)	0.90
Obese (BMI>30 kg/m^2)	**0.66 (0.46-0.94)**	**0.02**
Specialty Care setting	0.6 (0.29-1.2)	0.17
CYP2C9 or *VKORC1* X Care setting	1.16 (0.54-2.5)	0.70
*CYP4F2*3*	0.76 (0.48-1.2)	0.27
*CYP4F2*3* X Care setting	0.86 (0.44-1.7)	0.70
Age at index date	1.01 (0.99-1.0)	0.40
Sex	0.71 (0.50-1.0)	0.05
Race	0.74 (0.38-1.5)	0.53

Significant *p*-values are in bold.

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