THE DEVELOPMENT AND VALIDATION OF A NOVEL GENETIC-BASED WARFARIN DOSING NOMOGRAM

DISSERATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Robert S. Kidd, Pharm.D., M.S.

* * * * *

The Ohio State University
2008

Dissertation Committee:
Professor James Dalton, Adviser
Professor William Hayton
Associate Professor Tom Schmittgen
Associate Professor Daren Knoell

Approved by

Adviser
Graduate Program in Pharmacy
ABSTRACT

Warfarin has been used as an anticoagulant for various thromboembolic disorders for over 60 years with an estimated two million new prescriptions per year. However, due to a very narrow therapeutic index and a large inter-patient variability in dose requirements, its use results in more serious adverse events annually than any other medication. Recent advances in the field of pharmacogenomics have resulted in a focus on the development of warfarin dosing algorithms that include genetic and non-genetic information. Several of these algorithms have proved very promising in research settings, but their application in clinical practice has not been fully validated. One of difficulties with the current genetic-based algorithms is that they are multi-factor equations derived from regression analysis. The goal of this research is to develop and evaluate a simple genetic-based warfarin dosing algorithm that would be practical to use in a typical clinical practice setting. The result of this research is a validated, tabular nomogram that does not require any calculations to predict a practical warfarin dosage regimen.
Dedicated to Leslie, Eli and Maddy
ACKNOWLEDGMENTS

First and foremost, I wish to thank my adviser and mentor, James Dalton, for his support and patience in a long and winding pathway to the completion of my degree.

I am also grateful to Tom Schmittgen, Daren Knoell, Duxin Sun and William Hayton for their willingness to serve on my dissertation committee and for their support during the process. I also wish to thank David Newton, Arthur Harralson, and Tracy Fitzsimmons of Shenandoah University for their encouragement and support.

Finally, I would like to acknowledge and thank the co-authors of the articles that have derived from this dissertation including: Arthur Straughn, Marvin Meyer, Joyce Blaisdell, Joyce Goldstein, James Dalton, Timothy Curry, Susan Gallagher, Timi Edeki, Andrea Redman, Leslie Dickmann, Denise Ritchie, Yuen Hon, Mark Johnson, Craig Richard and Arthur Harralson.
VITA

September 23, 1968 .......................... Born – Alcoa, TN

1990 ........................................ B.S. Biochemistry and Cellular Biology
University of Tennessee

1995 ........................................ Pharm.D.
University of Tennessee

1998 ........................................ M.S. Pharmaceutical Sciences
University of Tennessee

1998 - 2003 ................................. Assistant Professor
Biopharmaceutical Sciences
Shenandoah University

2003 – present .............................. Associate Professor
Biopharmaceutical Sciences
Shenandoah University
PUBLICATIONS

Research Publications


**FIELDS OF STUDY**

Major Field: Pharmacy
TABLE OF CONTENTS

Abstract .................................................................................................................. ii
Dedication ............................................................................................................. iii
Acknowledgments ................................................................................................ iv
Vita .......................................................................................................................... v
List of Tables ......................................................................................................... xi
List of Figures ......................................................................................................... xiii

Chapters:

1. Introduction ....................................................................................................... 1
2. Pharmacokinetics of chlorpheniramine, phenytoin, glipizide and nifedipine
   in an individual homozygous for the CYP2C9*3 allele ..................................... 11
   2.1 Introduction .................................................................................................... 11
   2.2 Methods ......................................................................................................... 12
      2.2.1 Pharmacokinetic data analysis............................................................... 15
      2.2.2 Statistical analysis................................................................................. 15
      2.2.3 Genotyping............................................................................................ 17
   2.3 Results ............................................................................................................. 17
   2.4 Discussion ...................................................................................................... 32
3. Identification of a novel allele of \(\textit{CYP2C9}\) in an African-American exhibiting toxicity to phenytoin and subsequent evaluation in a patient receiving warfarin ..... 38

3.1 Introduction  .............................................................................................................. 38

3.2 Methods ................................................................................................................. 39

3.3 Results ..................................................................................................................... 41

3.4 Discussion ............................................................................................................... 47

3.5 \(\textit{CYP2C9}*6\) and warfarin .................................................................................. 49

4. The development and validation of a simple genetic-based warfarin nomogram.... 53

4.1 Introduction .............................................................................................................. 53

4.2 Methods .................................................................................................................. 54

4.2.1 Genotyping ........................................................................................................ 55

4.2.2 Statistical analysis ............................................................................................. 56

4.3 Results ..................................................................................................................... 57

4.4 Discussion ............................................................................................................... 66

5. The presence of the apolipoprotein E (APOE) E4 allele increases stable warfarin dose requirements in patients that do not possess common variant alleles of vitamin K epoxide complex 1 and cytochrome P450 2C9 ............................. 70

5.1 Introduction .............................................................................................................. 70

5.2 Methods .................................................................................................................. 73
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Example warfarin dosing algorithms that include <em>CYP2C9</em> and/or <em>VKOR</em> genotype data</td>
</tr>
<tr>
<td>2.1</td>
<td>Statistical comparisons used</td>
</tr>
<tr>
<td>2.2</td>
<td>Chlorpheniramine pharmacokinetic parameters (mean ±SD). Statistical comparisons were made using the definitions in Table 2.1</td>
</tr>
<tr>
<td>2.3</td>
<td>Nifedipine pharmacokinetic parameters (mean ±SD). Statistical comparisons were made using the definitions in Table 2.1</td>
</tr>
<tr>
<td>2.4</td>
<td>Glipizide pharmacokinetic parameters (mean ±SD). Statistical comparisons were made using the definitions in Table 2.1</td>
</tr>
<tr>
<td>2.5</td>
<td>Phenytoin pharmacokinetic parameters (mean ±SD). Statistical comparisons were made using Student's t-test at a 5% level of significance</td>
</tr>
<tr>
<td>3.1</td>
<td>Actual plasma phenytoin concentrations over the course of 41 days in the study subject with day 1 as the day of presentation with phenytoin toxicity, and expected phenytoin concentrations in a theoretical <em>CYP2C9</em>/<em>I</em>/<em>I</em> and <em>CYP2C19</em>/<em>I</em>/<em>I</em> extensive metabolizer with the same initial phenytoin concentration</td>
</tr>
<tr>
<td>4.1</td>
<td>Study populations’ characteristics</td>
</tr>
<tr>
<td>4.2</td>
<td><em>CYP2C9</em> allele frequency in the development and validation cohorts</td>
</tr>
</tbody>
</table>
4.3  *CYP2C9* genotype prevalence and associated mean daily warfarin dose in the development and validation cohorts. ..........................................................59

4.4  *VKORC1* allele frequency in the development and validation cohorts. ...........60

4.5  *VKORC1* +1542 haplotype prevalence and associated mean daily warfarin dose in the development and validation cohorts. .................................60

4.6  Mean daily warfarin dose by *CYP2C9* and *VKORC1* haplotype in the development cohort.................................................................61

5.1  Patient characteristics stratified by *APOE* group........................................77

6.1  Nomogram based warfarin induction..........................................................96
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Plasma concentration versus time profile for chlorpheniramine in normal</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>individuals, extensive metabolizers (EM’S); open circles, and a CYP2C9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>poor metabolizer (2C9 PM); closed circles. All individuals received a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>single oral dose (1 mg)</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Plasma concentration versus time profile for nifedipine study 2 in</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>normal individuals, (EM’S); open circles, and a CYP2C9 poor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>metabolizer, 2C9 PM; closed circles. All individuals received a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>single oral dose (two 30 mg tablets) of nifedipine. Error bars represent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the standard deviation of the mean plasma concentration observed at each</td>
<td></td>
</tr>
<tr>
<td></td>
<td>time</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Plasma concentration versus time profile for glipizide, in normal</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>individuals, extensive metabolizers (EM’S); open circles, and a CYP2C9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>poor metabolizer, 2C9 PM; closed circles. All individuals received a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>single oral dose (10 mg) of glipizide. Error bars represent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the standard deviation of the mean plasma concentration observed at each</td>
<td></td>
</tr>
<tr>
<td></td>
<td>time</td>
<td></td>
</tr>
</tbody>
</table>
2.4 Blood glucose concentration versus time profile in normal individuals (extensive metabolizers; open circles) and a CYP2C9 poor metabolizer (poor metabolizer; closed circles). All individuals received a single oral dose (10 mg) of glipizide. Error bars represent the standard deviation of the mean blood concentration observed at each time.

2.5 Plasma concentration versus time profile for phenytoin in normal individuals (extensive metabolizers; open circles) and a CYP2C9 poor metabolizer (poor metabolizer; closed circles). All individuals received a single oral dose (100 mg) of phenytoin on four separate occasions. Error bars represent the standard deviation of the mean plasma concentration observed at each time.

2.6 CYP2C9 genotyping. Agarose gels of PCR products amplified from genomic DNA were digested with restriction enzymes as indicated. Lanes are identified as M (DNA markers), lane 1 (poor metabolizer of interest in this paper), lane 2 (intermediate metabolizer) from Sullivan-Klose et al. (1996), and lane 3 (homozygous CYP2C9*1 wild-type control). The genotypes of the samples are indicated below each lane. The top gel demonstrates that NsiI digested the PCR products of the CYP2C9*1 and CYP2C19*2 alleles containing Ile359 into fragments of 134 bp (denoted by arrow) and 31 bp but did not digest those from.
the \textit{CYP2C9*3} alleles. The middle gel demonstrates that \textit{KpnI} digested the products of the \textit{CYP2C9*3} alleles into fragments of 135 bp (denoted by arrow) and 30 bp but did not digest products of the other two alleles. The bottom gel shows that \textit{AvaII} digested the PCR products of the \textit{CYP2C9*1} and \textit{CYP2C9*3} alleles containing Arg$^{144}$ while PCR products from the \textit{CYP2C9*2} allele (Cys$^{144}$) remained uncut (denoted by arrow).

3.1 The $t$ is the time (days) between any two phenytoin plasma concentration, $K_m$ is the Michaelis-Menten constant (\(\mu\text{g/ml}\)), $C_1$ is the larger phenytoin plasma concentration (\(\mu\text{g/ml}\)), $C_2$ is the smaller phenytoin concentration (\(\mu\text{g/ml}\)), $V_d$ is the volume of distribution (l) and $V_{\text{max}}$ is the maximal elimination rate (mg/day).

3.2 Phenytoin plasma concentration versus time profile in the study subject (●) and expected concentrations in a CYP2C9 extensive metabolizer, EM (■)……

3.3 Schematic depiction of CYP2C9 818delA allele genotyping test. Includes specific PCR amplification of exon 5 and restriction map of \textit{Mnl I} sites. Arrows indicate the primers. A. \textit{CYP2C9*1}, *2 or *3. B. \textit{CYP2C9 818delA}; the bp deletion at the end of exon 5 creates a new \textit{Mnl I} site generating fragments of 82 and 21 bp seen only in restriction digests from the new variant allele.
3.4 *CYP2C9 818delA* genotyping test. PCR products amplified from genomic DNA were digested with the restriction enzyme *Mnl I* and electrophoresed on agarose gels. The first lane contains molecular weight markers, lane 1 contains uncut sample and lanes 2 through 5 contain *Mnl I* digested PCR products from individuals with the genotypes indicated below each lane. The gel demonstrates that *Mnl I* digested the PCR products of the *CYP2C9 818delA* alleles into fragments of 217 bp and 82 bp (denoted by arrow). PCR products of *CYP2C9*1 alleles were digested into fragments of 217 bp and 104 bp. Lane 5 shows that *Mnl I* digested the PCR products of the *CYP2C9*1 and *CYP2C9 818delA* alleles into a combination of fragments of 217 bp, 104 bp and 82 bp (denoted by arrow).

4.1 The front of the Kidd-Harralson warfarin dosing nomogram card……………..63

4.2 The reverse side of the Kidd-Harralson warfarin dosing nomogram card……….64

4.3 Predicted and actual warfarin dose requirements in the validation cohort of 53 patients (*r = 0.73; P < 0.001*)…………………………………………………………..65

5.1 Weekly warfarin dose requirements (mean ± SEM) for all patients compared to the no *APOE*E4 allele, the *APOE*E3E3 and *APOE*E4 allele groups. Numbers of patients in each group are shown within each bar. There were no significant differences among any of the groups…………..79
5.2 Weekly warfarin dose requirements (mean ± SEM) by APOE group in $CYP2C9^{*1*1}$ patients, and patients with one or more $CYP2C9$ variant allele. Numbers of patients in each group are shown within each bar. There was a significant difference in warfarin dose requirements between $CYP2C9^{*1*1}$ APOE*E4 carriers compared to $CYP2C9^{*1*1}$ patients with no APOE*E4 allele (*p < 0.05). ………………………………………………………………………………………………………81

5.3 Weekly warfarin dose requirements (mean ± SEM) by APOE group in VKORC1 Group B/B patients, and VKORC1 B/A and A/A patients. Numbers of patients in each group are shown within each bar. There were significant differences in warfarin dose requirements between VKORC1 Group B/B APOE*E4 carriers compared to VKORC1 Group B/B APOE*E3E3 patients (*p < 0.001) and VKORC1 Group B/B patients With no APOE*E4 allele (†p< 0.001)…………………………………………………………………………………83

5.4 Weekly warfarin dose requirements (mean ± SEM) by APOE group in patients with both $CYP2C9^{*1*1}$ and VKORC1 Group B/B genotypes, and patients with one or more variant allele of both $CYP2C9$ and VKORC1. Numbers of patients in each group are shown within each bar. There were significant differences between warfarin dose requirements between $CYP2C9^{*1*1}$ and VKORC1 Group B/B patients who were APOE*E4 carriers compared to $CYP2C9^{*1*1}$ and VKORC1 Group B/B genotypes
who were APOE*E3E3 patients (’p < 0.01) and CYP2C9*1*1 and
VKORC1 Group B/B patients with no APOE*E4 allele (†p < 0.01).
CHAPTER 1

INTRODUCTION

Warfarin is the most widely prescribed oral anticoagulant, and it is used for the prophylaxis or treatment of thromboembolic disorders including venous thrombosis and pulmonary embolism, to reduce the risk of embolism in atrial fibrillation and prosthetic heart valves, and after a myocardial infarction [1]. Warfarin is often initiated at a five mg once daily dose, and then adjusted over time to achieve optimal anticoagulation for each patient as measured by the international normalized ratio (INR) [2, 3, 4]. The final maintenance dose has wide inter-patient variability, with some patients requiring a sixteen-fold higher dose than other patients [5].

Achieving a safe and effective warfarin maintenance dose can take weeks or months after the initiation of therapy. During this dose adjustment period the patient is most susceptible to unwanted clotting due to insufficient anticoagulation, and to excess bleeding events due to excessive anticoagulation [5, 6, 7]. The risk of bleeding during the first month of therapy is a significant concern, especially for outpatients not having daily INR testing [2, 8]. Zhang et al reported that 2,634 (14.7%) of 17,895 patients who took warfarin were diagnosed with a hemorrhagic event within one week of filling the warfarin prescription [7]. This study also documented that there was no relationship
between average daily warfarin dose and the incidence of hemorrhagic events which demonstrates that warfarin dose requirements are highly patient specific.

Numerous variables have been associated with warfarin dose requirements including age, diet, herbal products, other medications (e.g., amiodarone), comorbid conditions (e.g., liver disease), prosthetic heart valve, factors related to body mass (body mass index, body surface area, height, weight, gender), and race [5]. Recent investigations have highlighted the significant role of genetic factors which may be responsible for up to approximately 40% of warfarin dose variability [9, 10, 11]. The two most studied genetic factors that affect warfarin dose requirements are the enzymes cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKOR) [12].

CYP2C9 is the primary enzyme responsible for inactivating S-warfarin, the enantiomer with the majority of warfarin’s pharmacological activity [13]. Numerous single nucleotide polymorphisms (SNPs) in the gene encoding for CYP2C9 have been correlated with reduced enzyme activity, and decreased ability to metabolize warfarin. Up to approximately 20% of the warfarin dose variability among patients has been correlated with the metabolic capacity of CYP2C9 [9, 14]. In particular, the CYP2C9*2 and CYP2C9*3 variant alleles have been shown to cause a 30% and 95% decrease in enzymatic activity of CYP2C9, respectively [15, 16]. CYP2C9 variant alleles have also been correlated with higher levels of circulating active S-warfarin, excess anticoagulation, higher INR values, increased incidence of hemorrhage, and lower than average warfarin maintenance doses [17, 18, 19, 20].
The pharmacological target for warfarin is inhibition of the enzyme, VKOR. This enzyme reduces vitamin K 2,3-epoxide to the active vitamin K hydroquinone which is a required cofactor in the production of several procoagulation factors including clotting factors II, VII, IX, and X. Numerous studies have identified several SNPs in the genetic sequence for vitamin K epoxide reductase complex 1 (VKORC1) that have been correlated with increased responsiveness to warfarin and altered warfarin dose requirements [21, 22, 23, 24, 25, 26, 27, 28, 29, 30]. In most of these studies, the VKORC1 variant alleles were more highly correlated with warfarin dose requirement than CYP2C9 variants. Overall, up to approximately 40% of the dose variability among patients may be due to the metabolic status of VKORC1.

Based on the overwhelming data in the literature, the package insert for Coumadin® was updated in August 2007 to reflect the impact of CYP2C9 and VKORC1 pharmacogenomics on warfarin dose requirements. This information is contained in a new subheading in the “Pharmacokinetics” section titled “Pharmacogenomics” and it includes the following language:

“A meta-analysis of 9 qualified studies including 2775 patients (99% Caucasian) was performed to examine the clinical outcomes associated with CYP2C9 gene variants in warfarin-treated patients. In this meta-analysis, 3 studies assessed bleeding risks and 8 studies assessed daily dose requirements. The analysis suggested an increased bleeding risk for patients carrying either the CYP2C9*2 or CYP2C9*3 alleles. Patients carrying at least one copy of the CYP2C9*2 allele required a mean daily
warfarin dose that was 17% less than the mean daily dose for patients homozygous for the CYP2C9*1 allele. For patients carrying at least one copy of the CYP2C9*3 allele, the mean daily warfarin dose was 37% less than the mean daily dose for patients homozygous for the CYP2C9*1 allele.

In an observational study, the risk of achieving INR >3 during the first 3 weeks of warfarin therapy was determined in 219 Swedish patients retrospectively grouped by CYP2C9 genotype. The relative risk of over anticoagulation as measured by INR >3 during the first 2 weeks of therapy was approximately doubled for those patients classified as *2 or *3 compared to patients who were homozygous for the *1 allele.

Warfarin reduces the regeneration of vitamin K from vitamin K epoxide in the vitamin K cycle, through inhibition of vitamin K epoxide reductase (VKOR), a multiprotein enzyme complex. Certain single nucleotide polymorphisms in the VKORC1 gene (especially the -1639G>A allele) have been associated with lower dose requirements for warfarin. In 201 Caucasian patients treated with stable warfarin doses, genetic variations in the VKORC1 gene were associated with lower warfarin doses. In this study, about 30% of the variance in warfarin dose could be attributed to variations in the VKORC1 gene alone; about 40% of the variance in warfarin dose could be attributed to variations in VKORC1 and CYP2C9 genes combined. About 55% of the variability in warfarin dose could be
explained by the combination of VKORC1 and CYP2C9 genotypes, age, height, body weight, interacting drugs, and indication for warfarin therapy in Caucasian patients. Similar observations have been reported in Asian patients.  


Additionally, in the “DOSAGE AND ADMINISTRATION SECTION” the following statement was added:

“The lower initiation doses should be considered for patients with certain genetic variations in CYP2C9 and VKORC1 enzymes…” [31]

This dosage statement clearly does not provide the prescriber with specific dosing recommendation based a patient’s CYP2C9 and VKORC1 genotype. Consequently, the prescriber is not likely to accurately and efficiently use the CYP2C9 and VKORC1 genotype information to determine an appropriate warfarin dosing regimen even if the genetic information is available to them. Therefore, a practical method for prescribers to determine appropriate warfarin dose requirements based on CYP2C9 and VKORC1 genetic information is obviously needed.

Several studies have investigated the individual contribution of various factors to warfarin dose variability and created mathematical models to explain warfarin dose variability. The combination of VKORC1 and/or CYP2C9 genotype data with non-genetic data including age, weight, height, race, target INR, indication, BSA, gender, prosthetic heart valve presence, and/or concurrent medications has been shown to explain up to 63% of the variability of warfarin doses [9, 10, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43]. These factor have been used to develop
genomic-based algorithms are equation-based regression models as shown in Table 1.1 [9, 10, 24, 25, 29, 41, 42, 43].
<table>
<thead>
<tr>
<th>Reference</th>
<th>Published Algorithm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sconce et al 2005 [9]</td>
<td>( Dose = 0.628 - 0.0135 \text{(Age in years)} - 0.240 (CYP2C9<em>2) - 0.370 (CYP2C9</em>3) - 0.241 (VKORC1) + 0.0162 \text{(Height in cm).} ) CYP2C9: input 0, 1, or 2 for the number of *2 and *3 alleles within the patient’s genotype; VKORC1: input 1 for GG, 2 for GA, and 3 for AA. VKORC1 = 1639 G&gt;A.</td>
</tr>
<tr>
<td>Tham et al 2006 [10]</td>
<td>( 10^{(0.838 - 0.005 \times \text{Age in years} + 0.003 \times \text{Weight in kg} - 0.189 \times CYP2C9<em>3 - 0.283 \times VKORC1)} ). Exp is the exponential function of the warfarin dose and CYP2C9</em>3, VKORC1CC, and VKORC1TC are coded as 1 if present and 0 if absent. VKORC1 = 381 T&gt;C.</td>
</tr>
<tr>
<td>Kamali et al 2004 [24]</td>
<td>8.09 – 0.06 x Age in years (if no CYP2C9<em>3 allele), or 8.05 – 0.06 x Age in years – 1.12 (if 1 or 2 CYP2C9</em>3 alleles).</td>
</tr>
<tr>
<td>Gage et al 2004 [25]</td>
<td>( \exp(0.385 - 0.0083 \times \text{age in years} + 0.498 \times \text{BSA} - 0.341 \times \text{amiodarone} + 0.378 \times \text{Target INR} - 0.208 \times \text{CYP2C9<em>2} - 0.350 \times \text{CYP2C9</em>3} - 0.341 \times \text{amiodarone} + 0.378 \times \text{Target INR} - 0.125 \times \text{simvastatin} - 0.075 \times \text{female}). ) Exp is the e(^{-}) (inverse natural log); BSA is in m(^2); the SNPs are coded 0 if absent, 1 if heterozygous, and 2 if homozygous; race is 1 if white (0 otherwise); female is 1 if not male (0 otherwise); target INR is the desired INR; and amiodarone and simvastatin are 1 if the patient is taking that drug (0 otherwise).</td>
</tr>
<tr>
<td>Takahashi et al 2006 [29]</td>
<td>Patients with homozygous wild-type genotype for both CYP2C9 and VKORC1: daily dose = 6.6 – 0.035 x (age in years) + 0.031 x (weight in kg). For patients with either heterozygous or homozygous variant of CYP2C9, the daily dose was reduced by 1.7 or 2.8 mg, respectively. For patients with either heterozygous or homozygous variant of VKORC1, the daily dose was further reduced by 1.3 or 2.9 mg, respectively. CYP2C9 = *2/*3/*11, VKORC1 = 1639 G&gt;A.</td>
</tr>
<tr>
<td>Zhu et al 2007 [41]</td>
<td>ln (dose) = 1.35-0.008(age in years)+0.116(sex)+0.004(weight in lbs)-0.376(VKORC1-AA)+0.271(VKORC1-GG)-0.307(CYP2C9<em>2)-0.318(CYP2C9</em>3). Sex, input 1 for male (0 otherwise); VKORC1-AA, input 1 for AA (0 otherwise); VKORC1-GG, input 1 for GG (0 otherwise); CYP2C9, input 0, 1, or 2 for the number of *2 and *3 alleles. VKORC1 = 1173 C&gt;T.</td>
</tr>
<tr>
<td>Miao et al 2007 [42]</td>
<td>6.22-0.011(age in years)+0.017(weight in kg)-0.775(CYP<em>3)-3.397(VKORC1-x1)–4.803(VKORC1-x2). CYP</em>3: input 1 for *1/*3, input 0 for *1/*1. VKORC1-x1: input 1 for GA (0 otherwise). VKORC1-x2: input 1 for AA (0 otherwise). VKORC1 = 1639 G&gt;A.</td>
</tr>
<tr>
<td>Anderson et al 2007 [43]</td>
<td>1.64 + ( \exp[3.984 + <em>1</em>1(0) + <em>1</em>2(-0.197) + <em>1</em>3(-0.360) + <em>2</em>3(-0.947) + <em>2</em>2(-0.265) + <em>3</em>3(-1.892) + Vc-CT(-0.304) + Vc-TT(-0.569) + Vc-CC(0) + age(-0.009) + \text{male sex}(0.094) + \text{female sex}(0.003)] ). Equation yields weekly dose, divide by seven for predicted dose in mg/day. Reduce dose by 25% if on amiodarone. Vc refers to VKORC1 with variants CT, TT, or CC; ( \exp ) is the exponential to base ( e ); *1, *2, *3 refer to CYP2C9wild-type (*1) or variant (*2, *3) genotypes, respectively.</td>
</tr>
</tbody>
</table>

Table 1.1: Example warfarin dosing algorithms that include CYP2C9 and/or VKOR genotype data.
These studies were all performed to assess which genetic and non-genetic factors were best correlated with warfarin dose requirements, and then to use these significant factors to develop an algorithm to predict warfarin dosage regimens that are correlated with actual stable warfarin dose requirements. These algorithms have the potential to increase the efficiency of reaching the required maintenance doses and therefore reducing potential adverse drug events [12]. However, each one requires a dose to be calculated based on genetic and non-genetic factors in the form of a multi-factor equation. The result of the calculation is also a theoretical dose that must be rounded to a practical dose at the discretion of the prescriber based on the dosage strengths available.

The primary aim of this work was to develop and validate a simple genetic-based warfarin dosing nomogram that would be more practical for use in a typical clinical setting. Several criteria were determined to be critical for this nomogram. First and foremost, the nomogram must predict a practical warfarin dose that is strongly correlated with patients’ stable dose requirements. Additionally, the predicted dose should not be a theoretical dose that requires additional clinical judgment to convert it to a practical dose based on the dosage strengths available. For example, if an equation predicted a dose requirement of 1.487 mg per day, some prescribers may select one mg per day for the initial dosage regimen, other prescribers may select two mg per day, and a third group may select to alternate one and two mg per day. Second, it must be simple enough that the average practitioner would be willing to use it. Third, there should be the reasonable expectation that the dose will be correctly determined by the prescriber. Fourth, based on the current literature and the new Coumadin® package insert, it should include both
CYP2C9 and VKORC1 genotype. Finally, any additional factor(s) found to be significantly correlated with warfarin dose requirements should be included, but only if these factors can be incorporated without excessively complicating the nomogram.

My interest in warfarin pharmacogenomics began with three CYP2C9 studies that are included in chapters two and three, and it was this research that led to the idea to develop the genetic-based warfarin dosing nomogram. Chapter four describes the development and validation of this novel genetic-based warfarin dosing nomogram which includes both CYP2C9 and VKORC1 genotype. Chapter five describes my continued research in this area with the analysis of apolipoprotein E genotypes and their correlation with warfarin dose requirements. Finally, chapter six summarizes my research in this area to date, and outlines the next phase of my research.
CHAPTER 2

PHARMACOKINETICS OF CHLORPHENIRAMINE, PHENYTOIN, GLIPIZIDE AND NIFEDIPINE IN AN INDIVIDUAL HOMOZYGOUS FOR THE CYP2C9*3 ALLELE\textsuperscript{1}


2.1 Introduction

The genesis of my interest in pharmacogenomics began in 1997, and this first study involving CYP2C9 has led me to the current project of the development and validation of a simple genetic-based warfarin dosing nomogram. Our laboratories commonly performed pharmacokinetic studies in healthy volunteers to examine the bioequivalence of generic drug products. We identified an individual who displayed marked alterations in phenytoin and glipizide pharmacokinetics, but no differences in nifedipine and chlorpheniramine pharmacokinetics. These studies provide convincing evidence that the oxidative metabolism of glipizide is also performed by CYP2C9, and that the CYP2C9*3 allele is responsible for the poor metabolizer phenotype of phenytoin and glipizide.

A 29 year old male Caucasian who participated in six bioequivalence studies over a period of several years displayed severe hypoglycemia after a single dose of glipizide. A retrospective analysis of previous clinical pharmacokinetic studies in which this
individual participated was undertaken to determine if he exhibited significantly different pharmacokinetic and pharmacodynamic parameters compared with the other participants in each study. In each instance, the study was reviewed and approved by the University of Tennessee Institutional Review Board. Before entry into each study, potential participants received a complete blood and urine analysis, an electrocardiogram, and a physical exam. Individuals were excluded for any abnormal findings.

2.2 Methods

The first study was a comparative, crossover study of three chlorpheniramine dose levels (1, 2 and 4 mg) in 24 healthy volunteers including the study individual. On four separate occasions, each individual received a single oral dose of chlorpheniramine or placebo under fasted conditions. Studies were performed with a one week washout period between doses. Serial blood samples (7 ml each) were obtained immediately before and for up to 48 h after each dose. Blood samples were centrifuged immediately, and the plasma layer stored frozen (0°C) until analysis. Samples were analyzed for chlorpheniramine plasma concentrations using a proprietary gas chromatographic method by PPD Pharmaco (Richmond, VA, USA). Standard curves were linear over a range of 0.25-50.0 ng/ml. The assay precision, expressed as the percentage coefficient of variation (%CV), ranged from 6.8% for the lowest standard to 3.1% for the highest standard.

The second study was a four-way crossover study comparing the bioavailability of different lots of Dilantin® (100 mg phenytoin, Parke-Davis Pharmaceuticals, Ann Arbor, MI, USA). On four separate occasions, 24 healthy volunteers including the study
individual received a single 100 mg dose of phenytoin under fasted conditions. Studies were performed with a one week washout period between doses. Serial blood samples (8 ml each) were obtained immediately before and up to 73 h after each dose. Plasma phenytoin concentrations were determined utilizing a reversed phase high pressure liquid chromatographic (HPLC) assay developed in our laboratories. Briefly, an aliquot of plasma (1 ml) was alkalinized and extracted with 8 ml of methylene chloride. The organic phase was evaporated and the sample was reconstituted with 0.1 ml of mobile phase. The mobile phase contained 25% (v/v) acetonitrile in 0.05 M sodium phosphate buffer (pH 4.4) at a flow rate of 1 ml/min. One bottle (20 ml) of octane sulfonic acid (Low UV PIC B8, Waters Corp., Milford, MA, USA) was added to each litre of mobile phase. The stationary phase was a C_{18} reversed phase column (NovaPak, 3.9 x 150 mm, Waters Corp) and the analytes were monitored with ultraviolet detection at 205 nm. Standard curves were linear over a range of 0.05-2.6 µg/ml using hexobarbital as the internal standard. The assay precision (%CV) ranged from 13% for the lowest standard to 5% for the highest standard.

The third study was a three-way crossover bioavailability study comparing Adalat® CC (Bayer, New Haven, CT, USA) and two experimental formulations of nifedipine under fed conditions. Twelve healthy volunteers including the study individual received two 30 mg tablets of the appropriate nifedipine formulation, after a standard high fat breakfast, on three separate occasions. Studies were performed with a one week washout period between doses. Serial blood samples (10 ml each) were obtained immediately before and up to 35 h after each dose. The fourth study was also a three-way
crossover bioavailability study of nifedipine in 12 healthy volunteers. In this study, each participant received two-30 mg tablets of either Procardia XL® (Pfizer, New York, NY, USA) or two different experimental nifedipine formulations under fed conditions. Serial blood samples (10 ml each) were obtained immediately before and up to 49 h after each dose. The fifth study was a four-way crossover bioavailability study of nifedipine in 12 healthy volunteers. On separate occasions, each volunteer and the study individual received two 30 mg tablets of either Procardia XL® or three different experimental formulations of nifedipine under fasted conditions. Serial blood samples were obtained immediately before and up to 49 h after each dose. For all nifedipine studies, samples were analyzed for plasma nifedipine concentrations using a proprietary gas chromatographic method by PPD Pharmaco. Standard curves were linear over a range of 1.0-200 ng/ml. The assay precision (%CV) ranged from 10.3% for the lowest standard to 3.7% for the highest standard.

The sixth study was a four-way crossover bioavailability study of glipizide. On separate occasions, 24 healthy volunteers and the study individual received a single 10 mg oral dose of either Glucotrol XL® (Pfizer, New York, NY, USA) or an experimental glipizide formulation under both fasted and fed conditions. Studies were performed with a one week washout period between doses. Serial blood samples (10 ml each) were obtained immediately before and up to 35 h after each dose. Samples were analysed for plasma glipizide concentrations using a proprietary reversed phase HPLC method by PPD Pharmaco. Standard curves were linear over a range of 20-1000 ng/ml. The assay precision (%CV) ranged from 11.3% for the lowest standard to 4.7% for the highest
2.2.1 Pharmacokinetic data analysis

Pharmacokinetic parameters were determined for individual subjects in each study using standard methods. The maximum plasma concentration ($C_{\text{max}}$) and time at which it was achieved ($T_{\text{max}}$) were obtained by direct inspection of the individual plasma concentration-time profiles. The terminal slope of the natural logarithm (ln) concentration versus time plot was calculated by linear least squares regression, and the half-life calculated as 0.693 divided by the absolute value of slope. The area under the plasma concentration-time curve (AUC) from time zero to the last sampling time ($t_{\text{last}}$) was calculated by the linear trapezoid rule. The AUC from $t_{\text{last}}$ to time infinity ($AUC_{t_{\text{last}}-\infty}$) was calculated as the concentration at $t_{\text{last}}$ divided by the absolute value of the slope. The total AUC from time zero to infinity ($AUC_{0-\infty}$) was calculated as the sum of $AUC_{0-t_{\text{last}}}$ and $AUC_{t_{\text{last}}-\infty}$. The oral clearance ($CL_0$) was determined by dividing the dose by the $AUC_{0-\infty}$.

2.2.2 Statistical analysis

Statistical tests were performed to identify differences between the pharmacokinetic parameters ($C_{\text{max}}$, $T_{\text{max}}$, half-life, $CL_0$ and $AUC_{0-\infty}$) for each individual and the mean pharmacokinetic parameters of the other participants in each study. In most cases, this required the comparison of a single value for each individual with the mean values of the remaining participants in each study. To do so, the pharmacokinetic parameters of the remaining participants were listed in ascending order and the
interquartile range (IQR) calculated as the difference between the third and first quartiles in these individuals. The study individual was then considered as either not significantly different, an outlier, or an extreme outlier based on the equations listed in Table 2.1 [44].

<table>
<thead>
<tr>
<th>Statistical Definition</th>
<th>Manner of comparison of each pharmacokinetic parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extreme upper outlier</td>
<td>Individual &gt; (IQR*3.0) + 3rd quartile</td>
</tr>
<tr>
<td>Upper outlier</td>
<td>Individual &gt; (IQR*1.5) + 3rd quartile</td>
</tr>
<tr>
<td>Not different</td>
<td>1st Quartile - (IQR<em>1.5) &lt; individual &lt; (IQR</em>1.5) + 3rd quartile</td>
</tr>
<tr>
<td>Lower outlier</td>
<td>Individual &lt; 1st quartile - (IQR*1.5)</td>
</tr>
<tr>
<td>Extreme upper outlier</td>
<td>Individual &lt; 1st quartile - (IQR*3.0)</td>
</tr>
</tbody>
</table>

Table 2.1: Statistical comparisons used.

Where possible, mean pharmacokinetic parameter values in the study individual were calculated and used for comparison. For example, the study individual received four separate doses of phenytoin. Because the three lots of phenytoin were found to be bioequivalent, the mean pharmacokinetic parameters of the individual (n=4) were compared with the mean pharmacokinetic parameters of the remaining participants in the study (n=92). For the chlorpheniramine study, a statistical comparison was made between the study individual and the remaining participants at each of the three dose levels. For each nifedipine study, a statistical comparison was made between the study individual and the remaining participants when receiving the innovator product under the same fasted or fed condition. In the glipizide study, the individual was dropped from the study because of severe adverse effects before receiving the innovator product. In this instance, a statistical comparison was made between this individual and the remaining participants when receiving the same generic product.
Statistical comparisons, where possible, were made using Student's t-test at a 5% level of significance.

2.2.3 Genotyping

The individual identified as a phenotypic poor metabolizer of phenytoin and glipizide in the studies described above subsequently provided informed consent for genotyping. The study was reviewed and approved by the University of Tennessee Institutional Review Board and the National Institutes for Environmental Health Sciences Institutional Review Board. A single 20 ml peripheral blood sample was collected in sterile glass tubes containing ethylenediaminetetraacetic acid as an anticoagulant. The sample was immediately transferred to polypropylene tubes and frozen (0°C) before analysis. *CYP2C9*1 and *CYP2C9*3 genotyping tests were performed by mismatch polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) tests and *CYP2C9*2 by PCR-RFLP [45]. Genotyping tests for the *CYP2C19* alleles were performed as follows:

*CYP2C19*2, *CYP2C9*19*3, *CYP2C19*4, *CYP2C19*5, and *CYP2C19*6 genotyping were performed with previously published methods [46, 47, 48, 49]. Other individuals participating in the six pharmacokinetic studies were not available for genotyping.

2.3 Results

Figure 2.1 shows the chlorpheniramine plasma concentration-time profiles for the
study individual compared with the remaining participants in the study for the 1 mg dose which is representative of all three dose levels.

Figure 2.1: Plasma concentration versus time profile for chlorpheniramine in normal individuals, extensive metabolizers (EM’s); open circles, and a CYP2C9 poor metabolizer (2C9 PM); closed circles. All individuals received a single oral dose (1 mg) of chlorpheniramine. Error bars represent the standard deviation of the mean plasma concentration observed at each time.
Table 2.2 provides the resulting chlorpheniramine pharmacokinetic parameters for the study individual compared with the remaining participants for each of the three dose levels. The study individual did not show statistically significant differences for any of the calculated pharmacokinetic parameters for any chlorpheniramine dose.

<table>
<thead>
<tr>
<th></th>
<th>1 mg</th>
<th>2 mg</th>
<th>4 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/l)</td>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>AUC&lt;sub&gt;0-48h&lt;/sub&gt; (µg*h/l)</td>
</tr>
<tr>
<td>Normal (n=23)</td>
<td>1.98 ± 0.83</td>
<td>3.0 ± 1.4</td>
<td>34.5 ± 21.6</td>
</tr>
<tr>
<td>PM (n=1)</td>
<td>1.97</td>
<td>4.0</td>
<td>36.9</td>
</tr>
<tr>
<td>Outlier lower limit</td>
<td>0.41</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Outlier upper limit</td>
<td>3.13</td>
<td>6.5</td>
<td>77.9</td>
</tr>
<tr>
<td>Outlier?</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

Table 2.2: Chlorpheniramine pharmacokinetic parameters (mean ± S.D.). Statistical comparisons were made using the definitions in Table 2.1.

Figure 2.2 compares the nifedipine plasma concentration-time profile for the study individual compared with the remaining 11 participants in the second study which is representative of all three studies. The two concentration-time profiles are virtually super-imposable.
Figure 2.2: Plasma concentration versus time profile for nifedipine study 2 in normal individuals, (EM’S); open circles, and a CYP2C9 poor metabolizer, 2C9 PM; closed circles. All individuals received a single oral dose (two 30 mg tablets) of nifedipine. Error bars represent the standard deviation of the mean plasma concentration observed at each time.
Table 2.3 provides the resulting nifedipine pharmacokinetic parameters for the study individual compared with the 11 remaining participants in each of the three nifedipine studies. The study individual showed no statistically significant differences for any of the calculated pharmacokinetic parameters for each nifedipine study.
### Table 2.3: Nifedipine pharmacokinetic parameters (mean ± S.D.). Statistical comparisons were made using the definitions in Table 2.1.
Figure 2.3 and 2.4 provide the glipizide plasma concentrations and the blood glucose levels for the study individual compared with the other 23 participants in this study. Here, the two concentration-time profiles are significantly different.
Figure 2.3: Plasma concentration versus time profile for glipizide, in normal individuals, extensive metabolizers (EM’S); open circles, and a CYP2C9 poor metabolizer, 2C9 PM; closed circles. All individuals received a single oral dose (10 mg) of glipizide. Error bars represent the standard deviation of the mean plasma concentration observed at each time.
Figure 2.4: Blood glucose concentration versus time profile in normal individuals (extensive metabolizers; open circles) and a CYP2C9 poor metabolizer (poor metabolizer; closed circles). All individuals received a single oral dose (10 mg) of glipizide. Error bars represent the standard deviation of the mean blood concentration observed at each time.
Table 2.4 provides the resulting glipizide pharmacokinetic parameters for the study individual compared to the other 23 participants. The study individual showed significant differences in all calculated pharmacokinetic parameters except $T_{\text{max}}$ for the single glipizide dose. In this instance, the study individual also showed significant adverse effects from the single dose of glipizide which would be expected from the extremely low blood glucose levels seen in Fig. 2.4. He complained of being hungry, feeling weak and faint, and was observed to have a rapid heart rate, trembling hands, and was sweating and pale. His blood pressure was slightly elevated, and an electrocardiogram was normal. He temporarily responded to food, juices and glucose solutions, but his feeling of weakness soon returned until his blood sugar finally stabilized approximately 48 h after the dose.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>$C_{\text{max}}$ (µg/l)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>Half-life (h)</th>
<th>CL$_{\text{oral}}$ (l/h)</th>
<th>AUC$_{0-\infty}$ (µg*h/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=23)</td>
<td>451.8 ± 81.3</td>
<td>16.5 ± 7.6</td>
<td>5.3 ± 2.0</td>
<td>2.16 ± 0.31</td>
<td>4712 ± 707</td>
</tr>
<tr>
<td>PM (n=1)</td>
<td>1270</td>
<td>15.0</td>
<td>10.7</td>
<td>0.389</td>
<td>25686</td>
</tr>
<tr>
<td>Outlier limit</td>
<td>490</td>
<td>47.3</td>
<td>9.8</td>
<td>1.35</td>
<td>6352</td>
</tr>
<tr>
<td>Extreme outlier limit</td>
<td>531</td>
<td>69.6</td>
<td>13.2</td>
<td>0.7</td>
<td>7679</td>
</tr>
<tr>
<td>Outlier?</td>
<td>Extreme</td>
<td>No</td>
<td>Yes</td>
<td>Extreme</td>
<td>Extreme</td>
</tr>
</tbody>
</table>

Table 2.4: Glipizide pharmacokinetic parameters (mean ± S.D.). Statistical comparisons were made using the definitions in Table 2.1.
Figure 2.5 provides the phenytoin plasma concentrations for the study individual compared with the other 23 participants in this study. Here, the two concentration-time profiles are significantly different.
Fig. 2.5: Plasma concentration versus time profile for phenytoin in normal individuals (extensive metabolizers; open circles) and a CYP2C9 poor metabolizer (poor metabolizer; closed circles). All individuals received a single oral dose (100 mg) of phenytoin on four separate occasions. Error bars represent the standard deviation of the mean plasma concentration observed at each time.
Table 2.5 provides the resulting phenytoin pharmacokinetic parameters for the study individual compared with the other 23 participants. The resulting parameters agree with reported data for normal individuals, and the study individual showed a decrease in clearance that was comparable to a previously reported decrease in clearance of tolbutamide, another CYP2C9 substrate, in an individual homozygous for the CYP2C9*3 allele [50]. The study individual showed significant differences in all calculated pharmacokinetic parameters for the phenytoin doses. Despite the extremely low clearance, no adverse experience was reported by the individual.

![Phenobarbital pharmacokinetic parameters](image)

Table 2.5: Phenytoin pharmacokinetic parameters (mean ± S.D.). Statistical comparisons were made using Student's t-test at a 5% level of significance.

Genotyping studies revealed that this individual was homozygous for the CYP2C9*3 allele and the results are shown in Fig. 2.6. The 165-bp amplified PCR fragment from the study individual was completely digested by KpnI, but not by NsiI, indicating that he was homozygous for the defective CYP2C9*3 allele. In contrast, lane 3 shows that the wild-type allele (CYP2C9*1) is completely digested by NsiI and not by NsiI.
*KpnI.* An intermediate metabolizer who was *CYP2C9*<sup>2*3</sup> is shown in lane two as a positive control individual [45]. The bottom gel shows that the study individual is homozygous for Arg 144, as would be expected for the *CYP2C9*<sup>*3</sup> allele. The study individual was also genotyped for *CYP2C19* variant alleles. Genotyping tests indicated that he was homozygous for the wild-type *CYP2C19*<sup>*1</sup> (data not shown). The DNA of the study individual did not contain any of the examined *CYP2C19* alleles (*CYP2C19*<sup>*2</sup>, *CYP2C919*<sup>*3</sup>, *CYP2C19*<sup>*4</sup>, *CYP2C19*<sup>*5</sup> or *CYP2C19*<sup>*6</sup>).
Figure 2.6: CYP2C9 genotyping. Agarose gels of PCR products amplified from genomic DNA were digested with restriction enzymes as indicated. Lanes are identified as M (DNA markers), lane 1 (poor metabolizer of interest in this paper), lane 2 (intermediate metabolizer) [45], and lane 3 (homozygous CYP2C9*1 wild-type control). The genotypes of the samples are indicated below each lane. The top gel demonstrates that NsiI digested the PCR products of the CYP2C9*1 and CYP2C19*2 alleles containing Ile\(^{359}\) into fragments of 134 bp (denoted by arrow) and 31 bp but did not digest those from the CYP2C9*3 alleles. The middle gel demonstrates that KpnI digested the products of the CYP2C9*3 alleles into fragments of 135 bp (denoted by arrow) and 30 bp but did not digest products of the other two alleles. The bottom gel shows that AvaII digested the PCR products of the CYP2C9*1 and CYP2C9*3 alleles containing Arg\(^{144}\) while PCR products from the CYP2C9*2 allele (Cys\(^{144}\)) remained uncut (denoted by arrow).
2.4 Discussion

Our studies examined the pharmacokinetics of several known cytochrome P450 substrates in healthy human volunteers, including known substrates for CYP2C9 (phenytoin), CYP2D6 (chlorpheniramine), and CYP3A4 (nifedipine). We identified a single individual who demonstrated normal drug clearance for CYP2D6 and CYP3A4 substrates, but significantly diminished drug clearance for CYP2C9 substrates. Our pharmacokinetic data closely agree with previously reported tolbutamide clearance values in an individual homozygous for CYP2C9*3. Miners et al. showed that the clearance of tolbutamide in an individual was 22% of that observed in normal individuals, while the clearance of theophylline (CYP1A2) and debrisoquine (CYP2D6) did not differ compared with normal individuals [50]. This individual was later shown to be homozygous for CYP2C9*3 [45]. In comparison, we showed that phenytoin and glipizide clearances in our poor metabolizer, who was also homozygous for CYP2C9*3, were 21% and 18% of normal individuals, respectively. These studies confirm previous in vitro data suggesting that glipizide is a substrate for CYP2C9 [51].

Theoretically, the observed increases in phenytoin and glipizide plasma concentrations after a single oral dose could be a result of enhanced bioavailability and/or reduced systemic clearance. However, significant increases in bioavailability are impossible for these two drugs, given their near complete absorption and small first-pass metabolism after oral administration. For example, an increase in bioavailability from 0.95 to 1.0 would only explain 5% of the over 400% increase in AUC₀₋∞ observed for
both phenytoin and glipizide. Because both drugs are minimally eliminated by the kidneys (ie. less than 5%), the observed increase in plasma concentrations is almost certainly a result of the significant decrease in systemic hepatic clearance.

Genotyping studies confirmed the presence of two \textit{CYP2C9} variant alleles in this individual. Thus, our data conclusively demonstrate that the \textit{CYP2C9*3} variant can cause a drastic decrease in phenytoin and glipizide clearance, and is responsible for this individual poor metabolizer phenotype. One previous person, identified as a poor metabolizer of tolbutamide, was genotyped and completely sequenced [45]. A second, preliminary, study identified two individuals as poor metabolizers of losartan [52]. One of these individuals was subsequently identified as a poor metabolizer of tolbutamide. In a third study, another individual identified as a poor metabolizer of S-warfarin was also shown to be homozygous for the \textit{CYP2C9*3} allele [53]. In the present study, the \textit{CYP2C9*3} allele was responsible for a dramatic decrease in clearance of the two \textit{CYP2C9} substrates, while having no effect on his clearance of CYP3A4 or CYP2D6 substrates.

Studies with recombinant proteins have shown that the Leu$^{359}$ substitution in the \textit{CYP2C9*3} allele produces an enzyme with a higher $K_m$ and lower intrinsic clearance for both tolbutamide and S-warfarin 7-hydroxylation, and lower phenytoin hydroxylation than \textit{CYP2C9*1} [45, 54]. The amino acid change is within a putative substrate binding site [55]. At least five individuals homozygous for the \textit{CYP2C9*3} variant have now been clearly shown to be poor metabolizers of tolbutamide, losartan, S-warfarin, and phenytoin and glipizide in the present study [45, 52, 53]. The effects of the \textit{CYP2C9*2} allele on
catalytic activity towards CYP2C9 substrates have been more conflicting. Negligible to small decreases in $V_{\text{max}}$ for tolbutamide hydroxylation have been reported [45, 54, 56]. However, using warfarin as a substrate, the results have been more variable. Kaminsky et al. reported that the CYP2C9*2 allele had no effect on S-warfarin hydroxylation while Rettie et al. reported an approximate six-fold decrease [56, 57]. The Arg144Cys substitution in CYP2C9*2 does not reside in any known substrate binding site. A recent report suggests that the results depend on the cDNA expression system, and NADPH:cytochrome P450 oxidoreductase ratios [58]. The CYP2C9*2 allele was proposed to affect the interaction between the P450 and the reductase.

The seriousness of this poor metabolizer phenotype and the occurrence of serious adverse events during oral hypoglycemic therapy have not been previously documented. In this case, a single 10 mg dose of glipizide, which is the equivalent of 2000 mg of tolbutamide, caused severe hypoglycemia in this CYP2C9 poor metabolizer. Because of the seriousness of severe hypoglycemia associated with the administration of a single dose of an oral hypoglycemic documented here, a simple widely available test for this polymorphism needs to be developed. Application of the Hardy-Weinberg principle to the data of Sullivan-Klose et al. leads to an estimation of 1:278 for the poor metabolizer phenotype in the Caucasian-American population [45]. A second study estimated a frequency of 0.078 for the CYP2C9*3 allele in 457 case-control patients in a lung cancer study [59]. Application of the Hardy-Weinberg principle to these data suggests 1:166 Caucasians might be homozygous for CYP2C9*3. These studies agree with earlier estimates that poor metabolizers of phenytoin represent approximately 1:500 by
application of the Hardy-Weinberg principle [60, 61]. Assuming an estimate of 1:278 for the \textit{CYP2C9}*3 allele suggests that over 2000 patients per year in the USA alone are at risk for severe adverse events associated with the administration of oral hypoglycemics [62]. In our study, the individual was confined to our research facilities, received glucose solutions, and was frequently monitored for changes in blood sugar. In the normal situation of an individual being started on an oral hypoglycemic, it is highly unlikely any of these extreme precautions would be taken. In addition to oral hypoglycemics, although not determined in this study, the potential for severe adverse events is obvious with the therapeutic use of warfarin and phenytoin in a \textit{CYP2C9} poor metabolizer. Steward et al. reported a grossly elevated anticoagulant response and an increased S:R enantiomeric ratio for warfarin in an individual homozygous for the \textit{CYP2C9}*3 allele [53]. Furuya et al. also showed that individuals heterozygous for the \textit{CYP2C9}*2 allele require significantly lower doses of warfarin to maintain therapeutic anticoagulation and prevent adverse effects [63]. Odani et al. also reported that individuals heterozygous for the \textit{CYP2C9}*3 allele have a $V_{\text{max}}$ for phenytoin 33% lower than normal individuals [64]. Finally, using a novel scoring method, both warfarin and phenytoin have been calculated to be among the most pharmacogenetically hazardous drugs [65].

Others have recognized the need for an \textit{in vivo} probe for \textit{CYP2C9} activity. The use of phenytoin urinary metabolic ratios was previously examined, but was found not to be useful in predicting \textit{CYP2C9} phenotype [66]. Tolbutamide half-life and urinary metabolic ratios have also been examined as potential probes for \textit{CYP2C9} phenotype [67]. Tolbutamide urinary metabolic ratios were not effective, but tolbutamide half-life
was able to distinguish between poor metabolizers and extensive metabolizers. However, the equipment and techniques used to assay for tolbutamide are not available to most clinicians and therefore do not constitute an ideal probe for CYP2C9. Odani et al. suggested that a polymerase chain reaction based-genetic test would be useful for the determination of a patient's CYP2C9 genotype [64]. However, equipment and techniques for this test are not available to most clinicians. There is a need for a fast, safe, inexpensive, readily available test that can distinguish between poor metabolizers and extensive metabolizers. Based on the findings of this study, future research in our laboratory will examine the development of an *in vivo* probe which will meet these criteria.

In summary, the poor metabolizer phenotype for CYP2C9 substrates is caused by the Leu\(^{359}\) mutation. An extremely large decrease in clearance was directly linked to the presence of the Leu\(^{359}\) mutation. Other known allele such as CYP2C9*2 may also alter clearance of substrates such as warfarin to a certain degree, but the substitution of a Leu for Ile in the CYP2C9 protein is the major determinant of clearance for tolbutamide, phenytoin and glipizide, all of which are CYP2C9 substrates. This mutation in CYP2C9 did not affect the clearance of drugs not metabolized by CYP2C9, specifically CYP3A4 (nifedipine), CYP2D6 (chlorpheniramine) or CYP1A2 (theophylline) substrates. Individuals homozygous for the CYP2C9*3 allele demonstrate extremely poor clearance of the CYP2C9 substrates phenytoin and glipizide. This poor clearance can be clinically very dangerous if these individuals are not identified before drug administration. A single dose of glipizide in this individual decreased his blood sugar to below the limits of
quantification (20 mg/dl) even with scheduled ingestions of glucose solutions. Finally, the development of a fast, simple, and inexpensive phenotyping or genotyping test for CYP2C9 to be used before the administration of phenytoin, warfarin and oral hypoglycemics could prevent many severe adverse events associated with their use in CYP2C9 poor metabolizers.
CHAPTER 3

IDENTIFICATION OF A NOVEL ALLELE OF CYP2C9 IN AN AFRICAN-
AMERICAN EXHIBITING TOXICITY TO PHENYTOIN AND SUBSEQUENT
EVALUATION IN A PATIENT RECEIVING WARFARIN\textsuperscript{1,2}


3.1 Introduction

The initial study subject was a 64-year-old, 173 cm, 69 kg (64 kg ideal body weight) female African-American who was placed on oral phenytoin (Dilantin®) 100 mg three times per day after a five day hospital stay for status epilepticus. She had a history of diabetes, asthma, stroke, depression, hypertension and alcohol abuse, but denied alcohol use since 1989. The study subject was not taking any medications known to significantly inhibit the clearance of phenytoin. She also did not have clinical findings consistent with hepatic disease despite a history of alcoholism. Her alkaline phosphatase (AlkPhos) was within normal limits at 94 units/l, and her aspartate aminotransferase (AST) was mildly elevated at 85 units/l. The subject had normal albumin levels of 4.1 g/dl. Her serum creatinine and blood urea nitrogen (BUN) were normal at 0.7 mg/dl and 11.0 mg/ dl, respectively. Thirteen days after discharge from the hospital she presented with complaints of slurred speech, mental confusion, memory loss and inability to stand. These symptoms had progressively worsened over the previous week. At the time of admission she was also receiving albuterol two puffs every four hours, loratidine 10 mg once daily, metformin 500 mg twice daily, pancreatase 1 g once daily, levothyroxine 100
µg once daily, and Premarin® 0.625 mg once daily.

3.2 Methods

Plasma phenytoin concentrations were determined utilizing fluorescence polarization on an Abbott AxSYM® system. Liquimmune® Assayed Immunoassay Controls Level 1, 2 and 3 from Medical Analysis System were run each morning prior to sample analysis. The control means (acceptable ranges) were 6.0 (4.8-7.2), 13.3 (10.7-16.0) and 32 (22-40) µg/ml, respectively. The sensitivity of the assay was 1.0 µg/ml. Cross-reactivity was tested with the phenytoin metabolite, 5-\textit{p}-hydroxyphenyl-5-phenylhydantoin (HPPH) and HPPH-glucuronide. HPPH at a concentration of 5 µg/ml resulted in a less than 3 µg/ml change in reported phenytoin concentrations and HPPH-glucuronide at a concentration of 100 µg/ml resulted in a less than 7 µg/ml change in reported phenytoin concentrations.

Pharmacokinetic parameters were determined using standard methods. The elimination slope of natural logarithm concentration versus time plot was determined by linear least squares regression, and the half-life was calculated as 0.693 divided by the absolute value of the slope. Calculated phenytoin concentrations were determined using Figure 3.1.
Figure 3.1: The $t$ is the time (days) between any two phenytoin plasma concentration, $K_m$ is the Michaelis-Menten constant ($\mu$g/ml), $C_1$ is the larger phenytoin plasma concentration ($\mu$g/ml), $C_2$ is the smaller phenytoin concentration ($\mu$g/ml), $V_d$ is the volume of distribution (l) and $V_{\text{max}}$ is the maximal elimination rate (mg/day).

Plasma phenytoin concentrations in a theoretical $CYP2C9*1*1$ and $CYP2C19*1*1$ extensive metabolizer (EM) were calculated using mean parameters of $V_{\text{max}} = 6.07$ mg/kg/day, $K_m = 4.00$ $\mu$g/ml and $V_d = 0.65$ l/kg. The identified time frame between concentrations and an initial concentration of 49.5 $\mu$g/ml were known, so calculated concentrations were determined by successive iterations of the equation to reach the desired time difference. The area under the plasma concentration-time curve (AUC) from time zero to the last sampling time ($t_{\text{last}}$) was calculated by the linear trapezoid rule. The AUC from $t_{\text{last}}$ to time infinity ($\text{AUC}_{t_{\text{last}}-\infty}$) was calculated by dividing the concentration at time $t_{\text{last}}$ by the absolute value of the elimination slope. The AUC from time zero to time infinity ($\text{AUC}_{0-\infty}$) was calculated as the sum of $\text{AUC}_{0-t_{\text{last}}}$ and $\text{AUC}_{t_{\text{last}}-\infty}$.

The genomic DNA from the study subject was examined for $CYP2C9$ and $CYP2C19$ variant alleles after pharmacokinetic evidence indicated that she was
phenotypically a poor metabolizer of phenytoin. The genotyping study was reviewed by the Shenandoah University Institutional Review Board and the National Institutes of Environmental Health Sciences Institutional Review Board. Informed consent was given before collection of a single 20 ml peripheral blood sample in a sterile glass tube containing ethylenediaminetetraacetic acid as an anticoagulant and frozen at 0°C prior to analysis. Genotyping for CYP2C9*2 and CYP2C9*3 alleles was performed by previously described methods [45]. Genotyping tests for the CYP2C19 alleles were performed as previously described: CYP2C19*2, CYP2C19*3, CYP2C19*4, CYP2C19*5, CYP2C19*6, CYP2C19*7 and CYP2C19*8 [47, 48, 49, 68, 69].

3.3 Results

Table 3.1 provides the serial plasma phenytoin concentrations samples obtained from the study subject and the calculated concentration in the theoretical CYP2C9 EM over the course of 41 days. Figure 3.2 compares the phenytoin plasma concentration-time profile for the study individual and a theoretical CYP2C9 EM with the same initial plasma phenytoin concentration of 49.5 μg/ml.
<table>
<thead>
<tr>
<th>Days after presentation with phenytoin toxicity</th>
<th>Actual plasma phenytoin concentration (µg/ml)</th>
<th>Expected plasma phenytoin concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>49.5</td>
<td>49.5</td>
</tr>
<tr>
<td>Day 1 (2nd level)</td>
<td>40.3</td>
<td>47.3</td>
</tr>
<tr>
<td>Day 2</td>
<td>38.2</td>
<td>38.8</td>
</tr>
<tr>
<td>Day 3</td>
<td>38.8</td>
<td>30.5</td>
</tr>
<tr>
<td>Day 4</td>
<td>41.2</td>
<td>22.4</td>
</tr>
<tr>
<td>Day 5</td>
<td>38.9</td>
<td>14.7</td>
</tr>
<tr>
<td>Day 6</td>
<td>32.8</td>
<td>7.9</td>
</tr>
<tr>
<td>Day 7</td>
<td>32.1</td>
<td>2.8</td>
</tr>
<tr>
<td>Day 9</td>
<td>32.8</td>
<td>*BLQ</td>
</tr>
<tr>
<td>Day 10</td>
<td>30.0</td>
<td>BLQ</td>
</tr>
<tr>
<td>Day 11</td>
<td>25.1</td>
<td>BLQ</td>
</tr>
<tr>
<td>Day 12</td>
<td>24.9</td>
<td>BLQ</td>
</tr>
<tr>
<td>Day 14</td>
<td>22.0</td>
<td>BLQ</td>
</tr>
<tr>
<td>Day 15</td>
<td>21.2</td>
<td>BLQ</td>
</tr>
<tr>
<td>Day 16</td>
<td>5.3</td>
<td>BLQ</td>
</tr>
<tr>
<td>Day 41</td>
<td>5.3</td>
<td>BLQ</td>
</tr>
</tbody>
</table>

*BLQ (Below the lower limit of quantitation)

Table 3.1: Actual plasma phenytoin concentrations over the course of 41 days in the study subject with day 1 as the day of presentation with phenytoin toxicity, and expected phenytoin concentrations in a theoretical *CYP2C9*/*1*/*1* and *CYP2C19*/*1*/*1* extensive metabolizer with the same initial phenytoin concentration.
Figure 3.2: Phenytoin plasma concentration versus time profile in the study subject (●) and expected concentrations in a CYP2C9 extensive metabolizer, EM (■).

The linear least squares regression of ln concentration versus time points resulted in an elimination rate constant (ke) of 0.054 days\(^{-1}\) or 0.0023 h\(^{-1}\) \((r^2 = 0.98)\). The half-life was calculated to be 12.9 days or 310 h. The AUCs were 895 and 153 µg x day/ml for the study subject and the theoretical CYP2C9 EM, respectively.

Genotyping studies indicated that the study subject did not carry either of the known defective CYP2C9 alleles (CYP2C9*2 or CYP2C9*3). Although CYP2C19 has been shown to contribute to the metabolism of phenytoin to a minor extent, genotyping tests indicated the study subject carried no tested CYP2C19 variant alleles (CYP2C19*2,
CYP2C19*3, CYP2C19*4, CYP2C19*5, CYP2C19*6, CYP2C19*7 and CYP2C19*8). Subsequent PCR amplification and direct sequencing of the -1197 bp upstream region, and all of the exons and intron-exon junctions of CYP2C9 revealed the individual was homozygous for a previously unreported mutation, a deletion of base pair 818 near the 3’-end of exon 5 of the cDNA (818delA). A restriction fragment length polymorphism-PCR genotype test was developed to detect the new CYP2C9 818delA allele. The deletion creates a new Mnl I site in the variant allele. A CYP2C9 intron 4 specific forward primer (5’-CAGAGCTTGGTATATGGTATG-3’) paired with an intron 5 specific reverse primer (5’-GTAAACACAGAACTAGTCAAC-3’) was used to amplify CYP2C9 in a method similar to that described for CYP2C19*2 and *3 [68]. A 321 bp product is produced from all CYP2C9 alleles that do not contain the deletion, whereas a 320 bp product is formed from the CYP2C9 818delA allele (Figure 3.3). PCR products were restricted with Mnl I and resolved on a 4% agarose gel. Figure 3 shows the results of the new genotyping test for the CYP2C9 818delA allele. Fragments of 217 and 104 bp are generated from the digestion of the 321 bp PCR products from the CYP2C9*1, *2 and *3 alleles. Fragments of 217, 82 and 21 bp are generated from the digestion of PCR products from the CYP2C9 818delA allele. A diffuse 21 bp fragment cannot be visualized on the gel.
Figure 3.3: Schematic depiction of CYP2C9 818delA allele genotyping test. Includes specific PCR amplification of exon 5 and restriction map of Mnl I sites. Arrows indicate the primers. A. CYP2C9*1, *2 or *3. B. CYP2C9 818delA; the bp deletion at the end of exon 5 creates a new Mnl I site generating fragments of 82 and 21 bp seen only in restriction digests from the new variant allele.
Figure 3.4: CYP2C9 818delA genotyping test. PCR products amplified from genomic DNA were digested with the restriction enzyme Mnl I and electrophoresed on agarose gels. The first lane contains molecular weight markers, lane 1 contains uncut sample and lanes 2 through 5 contain Mnl I digested PCR products from individuals with the genotypes indicated below each lane. The gel demonstrates that Mnl I digested the PCR products of the CYP2C9 818delA alleles into fragments of 217 bp and 82 bp (denoted by arrow). PCR products of CYP2C9*1 alleles were digested into fragments of 217 bp and 104 bp. Lane 5 shows that Mnl I digested the PCR products of the CYP2C9*1 and CYP2C9 818delA alleles into a combination of fragments of 217 bp, 104 bp and 82 bp (denoted by arrow).
To determine the frequency of the new CYP2C9 818delA allele in African-Americans, 79 African-Americans from middle Tennessee were genotyped. Genotyping of CYP2C alleles in these individuals had been approved by the Meharry Medical College Institutional Review Board. One of 158 alleles contained the CYP2C9 818delA mutation giving a frequency of 0.6% (95% confidence limits 0.1 to 3.5%) for the CYP2C9 818delA allele in this population of African-Americans. One hundred seventy-two French Caucasian pre-existing samples from a case-control study of tobacco-related cancers in smokers from a study by Benhamou et al., 1997 [70] were genotyped to determine the frequency in a Caucasian group. No CYP2C9 818delA mutations were found in the 344 alleles giving a frequency of 0.0% (95% confidence limits from 0 to 1.1%). This allele has been named CYP2C9*6 in accordance with the international CYP allele nomenclature committee.

3.4 Discussion

CYP2C9 is a clinically important enzyme that metabolizes many therapeutic agents including phenytoin and warfarin, both of which have narrow therapeutic indices. Coding polymorphisms have been reported which alter the metabolism of a number of CYP2C9 substrates to varying extents by altering the kinetics and/or efficiency of the enzyme. However, this study is the first description of a null mutation in CYP2C9 that would prevent the protein from being expressed. This point is supported by the fact that the AUC of the study subject is 5.8 times that of the theoretical extensive metabolizer. Since AUC and clearance are inversely related, the clearance of the study subject is
approximately 17% that of the theoretical extensive metabolizer. Therefore, we estimate
CYP2C9 accounts for approximately 83% of phenytoin clearance in normal individuals,
and the value seen in the study subject devoid of any CYP2C9 protein (17% of normal)
represents only pathways not dependent on CYP2C9, such as CYP2C19. The substantial
reduction in the clearance of phenytoin in this individual is consistent with the presence
of a homozygous CYP2C9 null mutation. The incidence of this polymorphism is
estimated to be 0.6% (0.1 to 3.5%) in African-Americans. However, the incidence of
another CYP2C9 polymorphism has been found to differ 10-fold in African-Americans in
different regions of the country as a result of the heterogeneity of the African-American
population in the United States [71]. Individuals heterozygous for the CYP2C9*6 allele
may also be at great potential risk for toxicity from a number of drugs metabolized by
CYP2C9. In addition, compound heterozygotes (e.g. CYP2C9*2*6 and CYP2C9*3*6)
would also be at increased risk of toxicity.

There are many conditions other than genetic polymorphisms that could alter the
clearance of phenytoin including enzyme induction or inhibition, hepatic disease and
altered protein binding. However, as previously noted the study subject had no routine
clinical signs that would predict such poor phenytoin clearance. The study subject’s
medications were reviewed, and loratadine and metformin were discontinued to avoid
possible drug interactions. There was no subsequent change in the clearance of
phenytoin, although loratadine has been shown to activate CYP2C9 mediated
tolbutamide hydroxylation in vitro [72].

Serious clinical effects of pharmacogenetic polymorphisms of CYP2C9 have been
widely reported for patients taking glipizide, warfarin and phenytoin. Kidd et al. reported that an individual homozygous for the \( CYP2C9^*3 \) allele exhibited life-threatening hypoglycemia from a single ten mg dose of glipizide [73]. Steward et al., reported grossly elevated anticoagulant response to warfarin in an individual homozygous for the \( CYP2C9^*3 \) allele [53]. Other studies have shown that individuals with the \( CYP2C9^*2 \) or \( CY2C9^*3 \) alleles have smaller dose requirements of warfarin and are at an increased risk of severe and even life-threatening bleeding complications during anticoagulation therapy [74, 75].

In the present study, after less than two weeks of therapy, a standard starting dosage regimen for phenytoin of 100 mg three times daily produced plasma phenytoin concentrations almost 2.5 times that which is considered to be the maximum therapeutic concentration of 20 \( \mu \)g/ml. Due to the number of potentially toxic substrates of the CYP2C9 enzyme, this study documents the need for simple, inexpensive and widely available tests for pharmacogenetic polymorphisms of this enzyme. It also demonstrates the potential for severe toxicity from the administration of standard phenytoin doses to a patient homozygous for a previously unknown null mutation of \( CYP2C9 \). Severe complications would also be predicted in individuals carrying this allele after treatment with other CYP2C9 substrates with narrow therapeutic indices including the commonly used clinical drugs such as warfarin and glipizide.

3.5 \( CYP2C9^*6 \) and warfarin

After the completion of this study, a second study subject was identified. This subject was 77 year old African-American male smoker, who had been stabilized on
a weekly warfarin dose of only ten mg. He was being treated with warfarin for recurrent venous thromboembolism and a cerebrovascular accident. The patient's medical history was significant for hypertension, coronary artery disease, benign prostatic hypertrophy, osteoarthritis, anemia, and diverticulosis. The only medication he was taking besides warfarin was terazosin which has not been shown to interact significantly with warfarin. The patient had normal liver function and had albumin levels within the normal range.

The patient had been followed by the institution's anticoagulation clinic since starting warfarin therapy in 1992. He presented to his initial appointment in August 1992 with an INR of 12.37 after receiving warfarin doses between 7.5 and 20 mg per day while an in-patient and 5 mg per day upon discharge. Even when the weekly warfarin dose was decreased to 17.5 mg, the INR remained elevated at 10.79. Subsequent dosage adjustments revealed that a weekly warfarin dose within the narrow range of nine to ten mg was optimal for this patient.

Due to this patient's consistently low warfarin requirements, sample of blood was collected and S- and R-warfarin concentrations were determined, as well as, \textit{CYP2C9} genotyping. The patient's INR was 2.42 at this time. Using a previously published method [76], it was determined that the patient's warfarin S:R ratio was 3.2:1. This is over six times the expected ratio of 0.5:1, and provides clear evidence of impaired S-warfarin clearance. With this high degree of impairment in S-warfarin metabolism, this patient would certainly be expected to have at least one \textit{CYP2C9} variant allele. However, genetic analysis of the \textit{CYP2C9*2} locus by restriction
fragment length polymorphism-PCR and of the CYP2C9*3 and *5 loci by previously published methods [77] revealed neither the presence of CYP2C9*2 nor CYP2C9*3. CYP2C9*6 genotyping was then performed by restriction fragment length polymorphism-PCR [78]. He was found to be homozygous for the CYP2C9*6 allele.

This is the first report of warfarin dose requirement in a patient with the CYP2C9*6 allele. At that time, clinic-based studies evaluating CYP2C9 genetic polymorphisms and warfarin parameters have only reported results of the CYP2C9*1, CYP2C9*2, and CYP2C9*3 genotypes with exception of a single study [77]. This patient's warfarin dose was approximately 70% less than a warfarin dose of 35 mg per week. This patient had been initiated on a five mg per day maintenance dose after receiving higher loading doses which resulted in markedly elevated INRs. If this patient's impaired CYP2C9 metabolic capacity had been known at the time of therapy initiation, he could have been started on a much lower dose of warfarin.

There are several consistencies between this CYP2C9*6 homozygote taking warfarin and the population in which CYP2C9*6 was first studied [78]. There is a racial parallel in that CYP2C9*6 has only been found in African-Americans. The patient in which CYP2C9*6 was first identified had been taking phenytoin, another CYP2C9 substrate with a narrow therapeutic index. After taking phenytoin 100 mg three times daily for 13 days, she presented with slurred speech, mental confusion, memory loss, and the inability to stand. Her phenytoin level of 49.5 μg/ml indicates severely impaired CYP2C9 enzyme function [78]. The patient in the
present case had a warfarin S:R ratio more than six times higher than expected, also indicating severely impaired S-warfarin metabolism. However, at the time of blood collection he did not manifest symptoms of warfarin toxicity and had an INR within the therapeutic range. Although he had had markedly elevated INRs during initiation of therapy, there is no documentation of bleeding episodes.

Although warfarin is commonly initiated at a dose of five mg per day, clinicians should be aware that some patients will require much lower doses due to the presence of CYP2C9 genetic polymorphisms including CYP2C9*6. While adjusting the dose downward in these patients, the risk remains for bleeding episodes and elevated INRs. If patients were genotyped CYP2C9 variant alleles before starting warfarin and other CYP2C9 substrates, the delays in determining the optimal dose could be minimized and the potential for adverse events may be decreased.
4.1 Introduction

A recent report published by the American Enterprise Institute - Brookings Joint Center for Regulatory Studies and authored by three Food and Drug Administration officials estimated the effect of CYP2C9 genetic testing on the incidence of adverse drug events and health care costs [79]. The report estimated that incorporating pharmacogenetic testing into the initiation of warfarin therapy could prevent 85,000 serious bleeding events, 17,000 strokes and save $1.1 billion annually in the United States alone. The authors also acknowledged that the report may underestimate the full benefits of pharmacogenetic testing prior to the initiation of warfarin therapy because it did not consider the additional value of VKOR genotype testing.

As previously discussed, there are numerous genetic-based warfarin dosing algorithms in the literature. However, all require the dose to be calculated based on a number of genetic and non-genetic factors in the form of a multi-factorial equation. The aim of this study was to develop a simple genetic-based warfarin dosing nomogram that would be more practical for use in a typical clinical setting. Several criteria were determined to be critical for this nomogram. First and foremost, the nomogram must
predict a practical warfarin dose that is strongly correlated with patients’ stable dose requirements. Additionally, the predicted dose should not be a theoretical dose that requires additional clinical judgment to convert it to a practical dose based on the dosage strengths available. For example, if an equation predicted a dose requirement of 1.487 mg per day, some prescribers may select one mg for the initial dosage regimen, other prescribers may select two mg day, and a third group may select to alternate one and two mg per day. Second, it must be simple enough that the average practitioner would be willing to use it. Third, there should be the expectation that the dose will be correctly determined by the prescriber. Fourth, based on the current literature and the new Coumadin® package insert, it should include both CYP2C9 and VKORC1 genotype. Finally, any additional factor(s) found to be significantly associated with dose requirements should be included, but only if these factors can be incorporated without excessively complicating the nomogram.

4.2 Methods

Subjects were recruited from a single ambulatory care clinic over a six week period. Inclusion criteria were that each subject must be 18 years old or older and currently receiving warfarin. The subjects signed an informed consent and agreed to have an additional ten ml of blood drawn for genotype analysis at the time of a scheduled appointment for an International Normalized Ratio (INR) check. Demographic and clinical data were subsequently gathered from the patients’ medical records including age, race, gender, weight, height, indication, concurrent amiodarone or aspirin use, INR
and stable warfarin dose. The stable warfarin dose was defined as three consecutive office visits at least three weeks apart on the same dose yielding a therapeutic INR. A second cohort of unrelated patients was recruited from two ambulatory care clinics for validation of the nomogram. This study was approved by the Institutional Review Board at Shenandoah University prior to its commencement.

4.2.1 Genotyping

Genomic DNA (gDNA) was isolated from the blood samples using a Qiagen BioSprint 15 semi-automated DNA isolation instrument (Qiagen Inc, Chatsworth, CA) following manufacturers guidelines, and then frozen at -20°C until the time of genotyping. The genotyping was performed on an Applied Biosystems 7300 real-time PCR (Applied Biosystems, Foster City, CA) with commercially available, validated Applied Biosystems TaqMan® Drug Metabolism Genotyping Assays for CYP2C9*2, CYP2C9*3, CYP2C9*7, CYP2C9*8, CYP2C9*12 and CYP2C9*13; and Applied Biosystems TaqMan® SNP Genotyping Assays for VKORC1 +1542 G>C (rs8050894) and +2255 C>T (rs2359612). These VKORC1 designations refer to the nucleotide position numbered from the A in the ATG initiation codon of AY587020. Each assay included non-labeled primers and one wild-type and one variant allele specific fluorescent TaqMan® labeled oligonucleotide probes. PCR was performed with a reaction volume of 10 μl, including 4.75 μl of TaqMan® Universal PCR Master Mix, 0.5 μl of 20X DME Genotyping Assay Mix, 3.75 μl of DEPC H₂O, and 1.0 μl of gDNA. The PCR cycling conditions were as follows: 1 cycle of 50°C for 2 minutes, followed by 1
cycle of 95°C for 10 minutes and 50 cycles of 92°C for 15 seconds and 60°C for 90 seconds.

Selected samples were sequenced to confirm real time PCR analysis, and served as positive controls. DNA (50 ng) was amplified by PCR using Taq polymerase (Promega, Madison, WI) and intron-specific primers flanking exons 2, 3, 7 or 9 of CYP2C9 or the intronic region harboring SNPs in VKORC1. PCR conditions were as follows: 95°C for 2 min, 95°C for 1 min, 50°C for 1 min, 72°C for 1 min (35 cycles), and 72°C for 7 min. PCR products were purified using a GENECLEAN Spin Kit (MP Biomedicals, Solon, OH) and cycle-sequenced using BigDye terminator technology (Applied Biosystems, Foster City, CA). For sequencing, the same forward and reverse primers were used as for PCR. Sequencing conditions were: 96°C for 5 min, 96°C for 30 s, 50°C for 10 s, and 60°C for 4 min (35 cycles). Purified extension products were resolved by capillary electrophoresis on an ABI 310 Genetic Analyzer and files analyzed using Chromas© v1.45.

4.2.2 Statistical Analysis

Hardy-Weinberg equilibrium was tested by allele counting and $\chi^2$ analysis. Stepwise regression was used to identify genetic and non-genetic factors that significantly contribute to stable warfarin dose requirements, and correlation coefficients were determined for each factor. Pearson correlation analysis was used to assess the nomogram in the validation cohort, and the absolute prediction error and prediction error were also calculated. A P value of less than 0.05 was considered to be statistically
significant. All statistical analyses were performed using SPSS for Windows version 15.0 software (SPSS Inc., Chicago, IL).

4.3 Results

Complete data were obtained from a total of 265 patients in the development cohort and 53 in the validation cohort. Characteristics of the two study cohorts are presented in Table 4.1. All patients in the development cohort were Caucasian, and 51 of the 53 (96.2%) patients in the validation cohort were also Caucasian. The remaining two patients were Mediterranean and African American. In the development cohort, 55% percent of the patients were male and 45% were female, and 74% were male and 26% female in the validation cohort. The most common indication for warfarin in both the development cohort (56%) and in the validation cohort (55%) was atrial fibrillation.

![Table 4.1: Study populations’ characteristics.](image)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Development Cohort Mean±S.D. (n=265)</th>
<th>Validation Cohort Mean±S.D. (n=53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>age (yrs)</td>
<td>72.8±10.9</td>
<td>72.2±11.0</td>
</tr>
<tr>
<td>weight (kg)</td>
<td>85.4±21.1</td>
<td>89.1±17.4</td>
</tr>
<tr>
<td>height (cm)</td>
<td>170±10</td>
<td>175±10</td>
</tr>
<tr>
<td>body surface area (m²)</td>
<td>2.0±0.3</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>warfarin (mg/day)</td>
<td>4.1±1.8</td>
<td>4.5±1.9</td>
</tr>
</tbody>
</table>
CYP2C9 variant alleles were identified in 40.8% of the patients in the development cohort and in 34.0% of the patients of the validation cohort. The individual allele frequencies are shown in Table 4.2, and the prevalence of the resulting individual CYP2C9 genotypes are presented in Table 4.3 along with the associated mean warfarin daily doses for both cohorts. Both cohorts of patients demonstrated significantly higher mean daily warfarin dose requirements for CYP2C9*1*1 patients compared to each one of the other five CYP2C9 genotypes presented in Table 4.3. The CYP2C9 genotype frequencies did not demonstrate any deviation from Hardy-Weinberg equilibrium in either the development cohort (P = 0.90) or the validation cohort (P = 0.25).

<table>
<thead>
<tr>
<th>CYP2C9 Alleles</th>
<th>Development Cohort Allele Frequency No. (%)</th>
<th>Validation Cohort Allele Frequency No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1</td>
<td>385 (78.0%)</td>
<td>87 (82.1%)</td>
</tr>
<tr>
<td>*2</td>
<td>68 (12.8%)</td>
<td>13 (12.3%)</td>
</tr>
<tr>
<td>*3</td>
<td>37 (7.0%)</td>
<td>6 (5.7%)</td>
</tr>
<tr>
<td>*7</td>
<td>0 (0%)</td>
<td>not evaluated</td>
</tr>
<tr>
<td>*8</td>
<td>0 (0%)</td>
<td>not evaluated</td>
</tr>
<tr>
<td>*12</td>
<td>4 (0.8%)</td>
<td>not evaluated</td>
</tr>
<tr>
<td>*13</td>
<td>0 (0%)</td>
<td>not evaluated</td>
</tr>
</tbody>
</table>

Table 4.2: CYP2C9 allele frequency in the development and validation cohorts.
<table>
<thead>
<tr>
<th>CYP2C9 Genotype</th>
<th>Development Cohort Prevalence No. (%)</th>
<th>Validation Cohort Prevalence No. (%)</th>
<th>Development Cohort Mean ± S.D. Daily Warfarin Dose (mg)</th>
<th>Validation Cohort Mean ± S.D. Daily Warfarin Dose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>1</em>1</td>
<td>157 (59.2%)</td>
<td>35 (66.0)</td>
<td>4.6±1.8</td>
<td>4.8±1.9</td>
</tr>
<tr>
<td><em>1</em>2</td>
<td>66 (24.9%)</td>
<td>12 (22.6%)</td>
<td>3.8±1.4</td>
<td>4.4±1.8</td>
</tr>
<tr>
<td><em>1</em>3</td>
<td>27 (10.2%)</td>
<td>5 (9.4%)</td>
<td>3.3±1.2</td>
<td>3.4±2.1</td>
</tr>
<tr>
<td><em>1</em>12</td>
<td>4 (1.6%)</td>
<td>0 (0.0%)</td>
<td>3.3±1.1</td>
<td>-</td>
</tr>
<tr>
<td><em>2</em>2</td>
<td>1 (0.4%)</td>
<td>0 (0.0%)</td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td><em>2</em>3</td>
<td>6 (2.3%)</td>
<td>1 (1.9%)</td>
<td>2.0±0.4</td>
<td>3.2</td>
</tr>
<tr>
<td><em>3</em>3</td>
<td>4 (1.5%)</td>
<td>0 (0.0%)</td>
<td>1.1±0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.3: CYP2C9 genotype prevalence and associated mean daily warfarin dose in the development and validation cohorts.

VKORC1 +1542 and +2255 were found to be in linkage disequilibrium in the development cohort ($r^2 = 1.0$). Therefore, only VKORC1 +1542 was evaluated in the validation cohort and only data for VKORC1 +1542 is presented. One or more VKORC1 variant allele was identified in 60.4% of the patients in the development cohort and in 67.9% of the patients of the validation cohort. The individual allele frequencies are shown in Table 4.4, and the prevalence of the resulting individual VKORC1 genotypes are presented in Table 4.5 with the associated mean warfarin daily doses for both cohorts of patients. The VKORC1 genotype frequencies did not demonstrate any deviation from Hardy-Weinberg equilibrium in either the development cohort ($P = 0.25$) or the validation cohort ($P = 0.75$).
### Table 4.4: VKORC1 allele frequency in the development and validation cohorts.

<table>
<thead>
<tr>
<th>VKORC1 Alleles</th>
<th>Development Cohort Allele Frequency No. (%)</th>
<th>Validation Cohort Allele Frequency No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1542 G</td>
<td>332 (62.6%)</td>
<td>56 (52.8%)</td>
</tr>
<tr>
<td>1542 C</td>
<td>198 (37.4%)</td>
<td>50 (47.2%)</td>
</tr>
<tr>
<td>2255 C</td>
<td>332 (62.6%)</td>
<td>-</td>
</tr>
<tr>
<td>2255 T</td>
<td>198 (37.4%)</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 4.5: VKORC1 +1542 haplotype prevalence and associated mean daily warfarin dose in the development and validation cohorts.

<table>
<thead>
<tr>
<th>VKORC1 +1542</th>
<th>Development Cohort Prevalence No. (%)</th>
<th>Validation Cohort Prevalence No. (%)</th>
<th>Development Cohort Mean Daily Warfarin Dose (mg) ± S.D.</th>
<th>Validation Cohort Mean Daily Warfarin Dose (mg) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>105 (39.6%)</td>
<td>17 (32.1%)</td>
<td>5.0±1.9</td>
<td>6.0±1.8</td>
</tr>
<tr>
<td>GC</td>
<td>122 (46.0%)</td>
<td>22 (41.5%)</td>
<td>3.9±1.5</td>
<td>4.4±1.5</td>
</tr>
<tr>
<td>CC</td>
<td>38 (14.3%)</td>
<td>14 (26.4%)</td>
<td>2.7±1.1</td>
<td>3.0±1.4</td>
</tr>
</tbody>
</table>

Patients were then assigned into two VKORC1 haplotype groups based on VKORC1 1542 and 2255 genotypes. The VKORC1 Group BB was comprised of patients who possessed two wild-type alleles at positions 1542 and 2255 [21]. One additional theoretical haplotype, H6, could also be represented by this combination, but it was not identified among 186 European-Americans in a previous study exploring the effect of VKORC1 haplotypes on the response to warfarin by examining eight additional VKORC1
SNPs [21]. The VKORC1 Group AA was comprised of patients who possessed two variant alleles at both positions 1542 and 2255. Again, one additional haplotype, H5, could also be represented by this combination, but again it was only identified in a single patient in the same study by examining eight additional VKORC1 SNPs. Finally, VKORC1 Group AB was comprised of patients who possessed one variant allele at position 1542 and 2255. Table 4.6 presents the combined result of VKORC1 haplotype and CYP2C9 genotypes on mean warfarin dose requirements.

<table>
<thead>
<tr>
<th>VKORC1 Haplotype</th>
<th>CYP2C9 Genotype</th>
<th><em>1</em>1</th>
<th><em>1</em>2</th>
<th><em>1</em>3</th>
<th><em>1</em>12</th>
<th><em>2</em>2</th>
<th><em>2</em>3</th>
<th><em>3</em>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td></td>
<td>5.5±1.9</td>
<td>4.7±1.3</td>
<td>4.0±1.3</td>
<td>4.6</td>
<td>2.7</td>
<td>2.3</td>
<td>1.1±0.3</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>65</td>
<td>23</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>AB</td>
<td></td>
<td>4.4±1.5</td>
<td>3.5±1.2</td>
<td>2.8±0.8</td>
<td>3.1±1.2</td>
<td>-</td>
<td>1.9±0.5</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>68</td>
<td>33</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td>2.9±1.1</td>
<td>2.4±1.1</td>
<td>2.4±0.9</td>
<td>2.5</td>
<td>-</td>
<td>2.50</td>
<td>-</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td>24</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.6: Mean daily warfarin dose (mg) ± S.D. by CYP2C9 and VKORC1 haplotype in development cohort.

In addition to the significant association between daily warfarin dose and CYP2C9 and VKORC1 variant alleles, lower warfarin dose requirements were associated with increasing age (r = -0.25), weight (r = 0.24), height (r = 0.26) and body surface area (r = 0.27). The novel genetic-based warfarin dosing nomogram was developed in a tabular form as shown in Figure 4.1. The CYP2C9*1*1 and *1*2 genotypes and the
*CYP2C9*2*2 and *2*3 genotypes were combined in the nomogram since practical warfarin dose were not significantly different between the two genotypes. Age was include as a significant factor in only *CYP2C9*1*1 and *1*2 patients. Height was also selected as another factor for inclusion into the nomogram, but only in *CYP2C9*1*1 and *1*2 patients. Although body surface area was found to be more highly correlated with the warfarin dose requirements, it was not selected since it would normally require an additional calculation to determine body surface area from weight and height. For enhanced clinical utility, the nomogram was designed to be printed on cardstock (i.e. 3”x5” or 2”x3”) to allow additional information to be incorporated on the reverse side (Figure 4.2).
### Warfarin Dose in mg/day

<table>
<thead>
<tr>
<th>VKORC1</th>
<th>CYP2C9</th>
<th><em>1</em>1</th>
<th><em>1</em>3</th>
<th><em>2</em>2</th>
<th><em>3</em>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>1</em>1</td>
<td><em>1</em>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>1</em>2</td>
<td><em>2</em>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>3</em>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| BB | 5 mg† | 4 mg | 2.5 mg | 1 mg |
| AB | 4 mg† | 2.5 mg | 2 mg | 1 mg |
| AA | 3 mg† | 2.5 mg | 2 mg | 0.5 mg |

Approximate time to max. effect for a given dosage regimen:
- 5-7 days
- 10-14 days
- 14-21 days
- 28+ days

Dose adjustments for CYP2C9*1*1 or CYP2C9*1*2 genotypes ONLY (yellow column):
- Age ≥ 65 years old - subtract 1.0 mg
- Height ≤ 155 cm (5’1”) - subtract 1.0 mg
- Height ≥ 175 cm (5’9”) - add 1.0 mg

© 2007 Kidd and Harralson

Figure 4.1: The front of the warfarin dosing nomogram card.
Cytochrome P450 2C9 (CYP2C9)
CYP2C9 is primarily responsible for the metabolism of the more active S-enantiomer of warfarin. The presence of CYP2C9*2 and/or CYP2C9*3 has been shown to decrease the clearance of S-warfarin and will result in a longer warfarin half-life. Therefore, a longer period of time will be required for the concentration of warfarin to accumulate to a steady-state concentration and produce the maximal effect from a given dosage regimen. Other CYP2C9 alleles are known to exist at lower frequencies and in different ethnic populations, and some of these may also result in a decreased clearance of S-warfarin. For example, CYP2C9*6 may be substituted in the chart for CYP2C9*3 if present.

Vitamin K Epoxide Reductase Complex 1 (VKORC1)
Warfarin’s pharmacologic activity is the result of an inhibition of vitamin K epoxide reductase (VKOR). Several VKORC1 alleles -4931 (381T>C), -1639 (3673G>A), +1173 (6484C>T), +1542 (6853G>C), and +2255 (7566C>T) are associated with lower warfarin dose requirements, and have been demonstrated to be in linkage disequilibrium. Therefore, genotyping results for any of these VKORC1 alleles may be used for VKORC1 haplotype determination: VKORC1 BB represents the presence of two wild-type alleles, AB represents one wild-type and one variant allele, and AA represents two variant alleles.

Disclaimer: This chart serves as a guideline for warfarin induction based on the factors assessed. Other factors may significantly alter warfarin dose requirements (eg drug interactions), and dosage adjustments may be required. Certain patient sub-populations may require larger doses than are predicted by this nomogram, appropriate clinical judgment should be used in these patients. Specific patient care decisions including the determination of an appropriate warfarin induction dose are the responsibility of the patient’s healthcare provider.

Figure 4.2: The reverse side of the warfarin dosing nomogram card.

Correlation analysis showed a highly significant correlation between the predicted dose and the actual dose in the development cohort (r = 0.62; P < 0.001) The nomogram was subsequently evaluated in an unrelated cohort of 53 patients. Figure 4.3 shows the highly significant relationship found between the predicted dose from the nomogram, and the stable dose requirements in this validation cohort (r = 0.73; P < 0.001). The absolute prediction error in the validation cohort was 1.13 mg per day, and the prediction error was -0.75 mg per day. This relationship was not significant different than the correlation when CYP2C9 genotype (eg *1*1 and *1*2; and *2*2 and *2*3 were not grouped for simplification of the nomogram (r = 0. ; P< 0.00)
Figure 4.3: Predicted and actual warfarin dose requirements in the validation cohort of 53 patients ($r = 0.73; P < 0.001$).
4.4 Discussion

As seen in both the development and the validation cohort, the stable warfarin dose required to achieve a therapeutic INR are highly variable between patients. In the development cohort several genetic and non-genetic factors were found to be significantly correlated with warfarin dose requirements. Four of these factors were selected to be incorporated into the novel genetic-based warfarin dosing nomogram. This nomogram was developed in a tabular form that does not require any calculations to predict a practical warfarin dose based on the patient’s CYP2C9 genotype, VKORC1 genotype, age and height. The CYP2C9 variant alleles *2 and *3 were included in the nomogram based on their frequency in the evaluated Caucasian population, their documented impact on warfarin metabolism and dose requirements, and availability of testing. As noted on the reverse side of the nomogram, other CYP2C9 variant alleles can be incorporate or substituted in the nomogram for CYP2C9*2 and *3.

The nomogram also incorporates a VKORC1 haplotype which was based on VKORC1 genotype at position +1542 which was found to be in linkage disequilibrium with +2255 in this study. The VKORC1 variants +1542 and +2255 have also been shown to be in linkage disequilibrium with several other variants alleles, including -4931 (381T>C), -1639 (3673G>A), and +1173 (6484C>T) [21]. Therefore, VKORC1 genotyping results from any of these variant alleles will yield the same dose prediction.

The nomogram was found to predict warfarin doses that were highly correlated with patients’ actual stable warfarin doses when evaluated in a second cohort of patients. The correlation was similar to those of other warfarin dosing nomogram currently in the
literature [9, 10, 24, 25, 29, 41, 42, 43]. However, this warfarin dosing nomogram is unique compared to the other genetic-based warfarin dosing nomograms because it does not require any calculations. Equation-based nomograms first require the calculation of a theoretical dose from a multi-factor regression equation. This theoretical dose must then be rounded to a practical dose that can actually be administered to the patient. For example, if an equation predicted a dose requirement of 1.487 mg per day, some prescribers may select one mg per day for the initial dosage regimen, other prescribers may select two mg per day, and a third group may select to alternate one and two mg per day. In comparing the correlations between this nomogram and others, it should be noted that the equation based nomograms use the predicted theoretical dose to evaluate the models accuracy compared to the practical dose used to assess the accuracy of this model. Additionally, this nomogram takes advantage of the differential effect of variant alleles in one enzyme in the presence or absence of variant alleles in the other enzyme. For example, the VKORC1 AA haplotype results in approximately a two mg per day reduction in dose requirements in a CYP2C9*1*1 individual compared to a CYP2C9*1*1 and VKORC1 BB individual. However, the same VKORC1 AA haplotype has a relatively minor effect on dose requirement of 0.5 mg per day or less when a patient has two CYP2C9 variant alleles.

Although shown to be highly accurate, on average the nomogram tends to underestimate dose requirements, as noted by the prediction error of -0.75 mg day. One reason for the underestimation is that the maximum predicted dose for this nomogram is six mg per day. In the development cohort, 85% (34 out of 40) of the predicted doses
that deviated from the actual doses by more than two mg per day were an under-prediction of actual dose. The mean daily dose in this group was 7.1 ± 1.4 mg (range 4.3 to 11.4) with only a single patient requiring less than five mg per day. Based on this finding and the fact that more than 40% of the variability in dose requirements is still not explained with this nomogram, we are currently investigating additional factors that may be predictive of the larger dosage requirements. Some of these factors may include APOE genotype, the presence of other VKORC1 variant alleles, vitamin K intake and alcohol consumption. We also did not distinguish for target INR which is higher in certain patients particularly those with prosthetic heart valves. Additionally, future optimization of the nomogram for low frequency genotypes (eg CYP2C9*3*3 and VKORC1 AA) may allow for more accurate dose predictions in these patients.

In addition to dose prediction, the bottom row of the table is designed to assist prescribers in timing dosage adjustments with further explanation of the reverse side of card as follows:

“The presence of CYP2C9*2 and/or CYP2C9*3 has been shown to decrease the clearance of S-warfarin and will result in a longer warfarin half-life. Therefore, a longer period of time will be required for the concentration of warfarin to accumulate to a steady-state concentration and produce the maximal effect from a given dosage regimen.”
It may not be readily apparent to a prescriber that the presence of *CYP2C9* variant alleles would lengthen the amount of time required to reach a maximal effect from a dosage regimen. Since patients with *CYP2C9* variant alleles may have their dosage adjusted on a schedule that is more appropriate for patient who has normal half-life. For example, a *CYP2C9*<sup>2*3</sup> patient may continue to accumulate warfarin and have increasing INRs for two to three weeks after a new dose is initiated. If this new dose resulted in a subtherapeutic INR after one week, a prescriber may be inclined to increase the dose at that time to increase the INR into the therapeutic range. However, the dose may have been appropriate had a longer period of time been allowed to elapse prior to another dosage adjustment. In this example, the premature dosage increase could result in a supratherapeutic INR after the maximal effect is finally reached in two to three more weeks.

In conclusion, the newly developed nomogram has been shown to predict practical warfarin doses that are highly correlated with stable warfarin dose required to achieve therapeutic INRs. Similar to some warfarin algorithms in the literature, it incorporates both *CYP2C9* and *VKORC1* genotype in addition to non-genetic factors. There are two main advantages of this nomogram. First, it does not require any calculations for a dose prediction. Second, all dose predictions are practical and therefore can be prescribed without further consideration of how to round the theoretical dose to a practical one. Future studies are planned to prospectively evaluate the use of the dosing nomogram to initiate warfarin therapy and to compare outcomes with a control group of patients who are initiated conventionally.
CHAPTER 5

THE PRESENCE OF THE APOLIPOPROTEIN E (APOE) E4 ALLELE INCREASES STABLE WARFARIN DOSE REQUIREMENTS IN PATIENTS THAT DO NOT POSSESS COMMON VARIANT ALLELES OF VITAMIN K EOPOXIDE COMPLEX 1 AND CYTOCHROME P450 2C9

5.1 Introduction

Warfarin’s pharmacologic activity is the result of an inhibition of vitamin K epoxide reductase which is the rate limiting step in the vitamin K dependent activation system of several clotting factors. Vitamin K is an essential cofactor required for the activation of the clotting factors II, VII, IX and X; and the procoagulant proteins C, S and Z. Warfarin specifically targets vitamin K epoxide reductase complex subunit 1 (VKORC1). Recent studies have shown that VKORC1 genotype is more strongly correlated with warfarin dose requirements than CYP2C9 genotype [21, 22, 23, 24, 25, 26, 27, 28, 29, 30]. The major dietary form of vitamin K found in the bloodstream is phylloquinone, also known as vitamin K1. In the bloodstream, vitamin K1 is extensively bound to chylomicrons and chylomicron remnants. Apolipoprotein E (APOE) is responsible for the uptake of these vitamin K-rich lipoproteins through receptor-mediated endocytosis in the liver and other tissues. Three common alleles of APOE have been
identified: \(E2, E3\) and \(E4\). Plasma vitamin K1 levels have been shown to be highest in those individuals with an \(APOE*E2\) allele, intermediate in those with the wild-type \(APOE*E3\) allele, and the lowest in individuals possessing an \(APOE*E4\) allele [80, 81]. Some investigators hypothesize that this difference may be due to the presence of an \(APOE*E4\) allele which results in a more efficient uptake of these vitamin K-rich lipoproteins from the bloodstream into the liver. The presence of the \(APOE*E4\) allele could then counteract the anticoagulant effect of warfarin by increasing the availability of vitamin K in the liver, increasing the activation of vitamin K dependent clotting factors, and resulting in higher warfarin dose requirements to achieve a therapeutic international normalized ratio (INR). This increased hepatic uptake of vitamin K could be considered analogous to the common clinical use of vitamin K administration which counteracts the anticoagulant effect of warfarin by increasing vitamin K availability to the liver and enhancing vitamin K dependent clotting factor synthesis.

Accordingly, Kohnke et al. found the presence of two \(APOE*E4\) alleles were correlated significantly with higher warfarin dose requirements of 56.9 mg per week compared to the presence of either one or no \(APOE*E4\) alleles which were 34.3 and 34.6 mg per week, respectively [82]. The \(APOE*E4\) allele frequency was reported to be 19.1% in this Swedish study population. In a subsequent study, Kohnke et al did not find a significant association between the presence of \(APOE*E4\) alleles and warfarin dose requirements in an Italian population, but the \(APOE*E4\) allele frequency was only 6.9% [83]. In studying phenprocoumon, another coumarin anticoagulant structurally related to warfarin, Visser et al. found an approximately 50% increase in dose requirements for
patients with the $APOE^{*}E4E4$ genotype compared to patients with the $APOE^{*}E3E3$ genotype [84].

In contrast to these studies, other researchers hypothesized that the presence of the $APOE^{*}E4$ allele and its associated acceleration of the uptake of the vitamin K-rich chylomicrons results in a more efficient and rapid metabolism and elimination of this vitamin K source [81]. Therefore, less vitamin K would be available for the vitamin K dependent synthesis of the clotting factors, and should therefore result in lower warfarin dose requirements. Visser et al also reported acenocoumarol, another coumarin anticoagulant structurally related to warfarin, had marginally lower dose requirements in $APOE^{*}E4$ carriers of 3.4 mg per week compared to patients with the $APOE^{*}E3E3$ genotype which would support this second hypothesis [84]. It was noted that this dose reduction was comparable to the dose reduction associated with the $CYP2C9^{*}1^{*}3$ genotype. Sconce et al examined $APOE$ genotype, fasted plasma vitamin K concentrations and warfarin dose requirements. No significant differences were found in $APOE$ genotype and fasted plasma vitamin K concentrations. However, they did find that the presence of at least one $APOE^{*}E4$ allele was associated with significantly lower warfarin dose requirements (3.3±1.9 mg/day) compared to the $APOE^{*}E3E3$ genotype (4.0±1.8 mg/day) [84]. An additional study found that $APOE$ genotype contributed to less than five percent of the warfarin dose variability and therefore was not considered to substantially alter warfarin dose requirements [86].

Overall, the previous studies of $APOE$ genotype and warfarin dose requirements are inconsistent. This discrepancy may be the result of the presence of $CYP2C9$ and/or
VKORC1 variant alleles and their potential confounding effect on warfarin dose requirements. Although some of the previous studies have controlled for CYP2C9 variability, none controlled for VKORC1 which has an even greater effect on warfarin dose requirements. The presence of CYP2C9 and VKORC1 variant alleles in prior studies may have obscured the significance of APOE on warfarin dose requirements. Therefore, the primary aim of this project was to evaluate the association between the presence of the APOE*E4 allele and stable warfarin dose requirements in patients who do not possess a common variant allele of VKORC1 or CYP2C9.

5.2 Methods

This study was approved by the Shenandoah University Institutional Review Board. The patients were from a cohort of 265 warfarin patients previously identified for the creation of a warfarin dosing nomogram. These patients were recruited from a single ambulatory care clinic over a six week period. Patients, who were 18 years old or older and currently receiving warfarin, were recruited into the study. They signed an informed consent and agreed to have an additional ten milliliters of blood drawn for genotype analysis at the time of a scheduled appointment for an INR check. Clinical data including age, gender, weight, concomitant use of amiodarone or aspirin, and INR were obtained. A stable warfarin dose, which was defined as three consecutive office visits at least three weeks apart on the same dose yielding a therapeutic INR, was also recorded.
5.2.1 Genotyping

A volume of 200 µl of EDTA-anticoagulated venous blood was combined with 500 µl tissue lysis buffer (Qiagen), 20 µl proteinase K (Qiagen) and 4 µl of RNAase A (Qiagen) and incubated at 56°C for 1 hour in a shaker-incubator at 900 rpm. Genomic DNA (gDNA) was subsequently extracted from each sample using a BioSprint DNA Blood Kit (Qiagen) and a BioSprint 15 workstation (Qiagen). The samples were then frozen at a temperature of -20°C until the time of genotyping.

Samples were previously genotyped for CYP2C9*2, *3, and *12; and VKORC1 rs8050894 (1542 G>C) and rs2359612 (2255 C>T). The designations 1542 and 2255 refer to the nucleotide positions numbered from the A in the ATG initiation codon of AY587020. The samples were subsequently genotyped for APOE rs429358 and rs7412; and APOE*E2, E3, and E4 alleles were assigned. All genotyping assays were performed by allelic discrimination using real-time PCR 5’ nuclease assays (Applied Biosystems). The assay included non-labeled primers and fluorescent TaqMan® labeled oligonucleotide probes, one wild-type and one variant allele specific (Applied Biosystems). PCR was performed with a reaction volume of 10 µl, including 4.75 µl of TaqMan® Universal PCR Master Mix, 0.5 µl of 20X DME Genotyping Assay Mix, 3.75 µl of DEPC H₂O, and 1.0 µl of genomic DNA. The PCR cycling conditions were as follows: 1 cycle of 50°C for 2 minutes, followed by 1 cycle of 95°C for 10 minutes and 50 cycles of 92°C for 15 seconds and 60°C for 90 seconds. Appropriate negative controls were also run. Allelic discrimination was carried out by measuring fluorescence intensity at the endpoint by an ABI 7300 Real Time PCR System (Applied Biosystems).
The results of the measurements were evaluated by use of SDS software Version 1.3 (Applied Biosystems), and the genotype was determined. The APOE*E4 carrier group was defined as the APOE*E2E4, APOE*E3E4 and APOE*E4E4 genotypes. The no APOE*E4 allele group was comprised of all the remaining APOE genotypes including the APOE*E3E3 genotype. Finally, the APOE*E3E3 genotype was comprised only of those APOE*E3E3 patients.

Patients were divided into two VKORC1 haplotype groups based on VKORC1 1542 and 2255 genotypes which correspond to 6853 and 7566 in some literature [21]. The VKORC1 Group B/B was comprised of patients who possessed two wild-type alleles at positions 1542 and 2255 [21]. One additional theoretical haplotype, H6, could also be represented by this combination, but it was not identified among 186 European-Americans in a previous study exploring the effect of VKORC1 haplotypes on the response to warfarin. The VKORC1 Group A/A was comprised of patients who possessed two variant alleles at both positions 1542 and 2255. Again, one additional haplotype, H5, could also be represented by this combination, but it was only identified in a single patient in the same study by genotyping for eight additional SNPs in VKORC1. Finally, VKORC1 Group A/B was comprised of patients who possessed one variant allele at either position 1542 or 2255.

5.2.2 Statistical analysis

The sample size required for each group was estimated to be 52 patients in order to detect at 25% difference in dose with a power of 80 percent and a significance level of
five percent. This effect size would correspond to a 1.25 mg per day or 8.75 mg per week warfarin dose difference compared to an empiric five mg per day or 35 mg per week dose, respectively.

The primary analysis evaluated the association between the presence of the APOE*E4 allele and the warfarin dose requirements in CYP2C9*1*1 patients, VKORC1 Group B/B patients, and patients possessing both CYP2C9*1*1 and VKORC1 Group B/B. Comparative analyses were also done to evaluate the association between the presence of the APOE*E4 allele and warfarin dose requirements in all the remaining patients that were not in each primary analysis group. Levene’s test for equality of variances was first evaluated, and then an independent-samples t test was used to compare groups for equality of means. A Bonferroni correction was utilized for each comparison, since the APOE*E4 carrier group was compared to two different groups: APOE*E3E3 genotypes and the no APOE*E4 allele group. Hardy-Weinberg equilibrium was tested by allele counting and \( \chi^2 \) analysis. The statistical analysis was performed using SPSS for Windows version 15.0 software (SPSS Inc).

5.3 Results

Of the 265 patients with complete data in the original cohort, 159 patients were CYP2C9*1*1, 106 patients were assigned to VKORC1 Group B/B, and 67 were both CYP2C9*1*1 and VKORC1 Group B/B. Table 1 summarizes the patient characteristics for the entire study population and stratified by APOE analysis group. There were no
significant differences in age, weight, height, body surface area (BSA), gender or ethnicity among the groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients (n=265)</th>
<th>No APOE*E4 allele (n=212)</th>
<th>APOE*E3E3 (n=176)</th>
<th>APOE*E4 carrier (n=53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs) mean ± SD</td>
<td>72.7 ± 10.90</td>
<td>73.3 ± 10.60</td>
<td>73.5 ± 10.70</td>
<td>70.4 ± 11.84</td>
</tr>
<tr>
<td>Weight (kg) mean ± SD</td>
<td>85.4 ± 21.17</td>
<td>84.5 ± 20.57</td>
<td>84.6 ± 20.38</td>
<td>89.2 ± 23.31</td>
</tr>
<tr>
<td>Height (cm) mean ± SD</td>
<td>170 ± 10.1</td>
<td>170 ± 10.2</td>
<td>171 ± 10.3</td>
<td>170 ± 9.8</td>
</tr>
<tr>
<td>BSA (m²) mean ± SD</td>
<td>2.0 ± 0.27</td>
<td>2.0 ± 0.27</td>
<td>2.0 ± 0.27</td>
<td>2.0 ± 0.28</td>
</tr>
<tr>
<td>Females, n (% in group)</td>
<td>120 (45.3%)</td>
<td>95 (44.8%)</td>
<td>72 (40.9%)</td>
<td>25 (47.2%)</td>
</tr>
</tbody>
</table>

Table 5.1: Patient characteristics stratified by APOE group.

Of the 53 APOE*E4 carriers; 44 APOE*E3E4, six APOE*E4E4, and three APOE*E2E4 genotypes were identified. This represents an APOE*E4 allele frequency of 10.9% in our study population. The CYP2C9*2 and *3 allele frequencies were 12.8% and 7.0%, respectively; and the VKORC1 1542 and 2255 allele frequencies were both 37.4%. All alleles and genotypes were in Hardy-Weinberg equilibrium.

Figure 5.1 presents the mean weekly warfarin dose requirements in all 265 patients (28.8 ± 12.32 mg per week) by APOE group. There were no significant differences in mean weekly warfarin dose requirements between APOE*E4 carriers (32.1
± 15.73 mg per week) and either the APOE*E3E3 genotype or the no APOE*E4 allele group, 28.0 ± 11.30 (p = 0.086) and 27.9 ± 11.20 mg per week (p = 0.078), respectively.
Figure 5.1: Weekly warfarin dose requirements (mean ± SEM) for all patients compared to the no APOE*E4 allele, the APOE*E3E3 and APOE*E4 allele groups. Numbers of patients in each group are shown within each bar. There were no significant differences among any of the groups.

Figure 5.2 presents the mean weekly warfarin dose requirements by APOE group in the 159 CYP2C9*1*1 patients and the remaining 106 patients who possess one or more CYP2C9 variant allele. In the CYP2C9*1*1 patients, there was no significant difference in mean weekly warfarin dose requirements between APOE*E4 carriers (36.8 ± 15.87 mg per week) and the APOE*E3E3 genotype (31.3 ± 11.42 mg per week) after the Bonferroni correction (p = 0.028). However, there was a significantly higher mean weekly warfarin dose requirements between APOE*E4 carriers (36.8 ± 15.87 mg per week) and the no APOE*E4 allele group, 30.8 ± 11.31 (p = 0.013). Patients with one or more CYP2C9 variant alleles had lower mean dose requirements compared to
CYP2C9*1*1 patients, but within this subpopulation possessing one or more CYP2C9 variant alleles there were no significant differences in mean weekly warfarin dose requirements among APOE*E4 carriers (22.8 ± 10.75 mg per week) compared to both the APOE*E3E3 genotype and the no APOE*E4 allele group, 23.2 ± 9.27 (p = 0.868) and 23.9 ± 9.72 mg per week (p = 0.676), respectively.
Figure 5.2: Weekly warfarin dose requirements (mean ± SEM) by APOE group in CYP2C9*1*1 patients, and patients with one or more CYP2C9 variant allele. Numbers of patients in each group are shown within each bar. There was a significant difference in warfarin dose requirements between CYP2C9*1*1 APOE*E4 carriers compared to CYP2C9*1*1 patients with no APOE*E4 allele (*p < 0.05).
Figure 5.3 illustrates the mean weekly warfarin dose requirements by \textit{APOE} group in 106 \textit{VKORC1} Group B/B patients compared to 159 \textit{VKORC1} Group A/B and A/A patients. In \textit{VKORC1} Group B/B group, there were significantly higher mean weekly warfarin dose requirements among \textit{APOE*E4} carriers (42.4 ± 14.55 mg per week) compared to both the \textit{APOE*E3E3} genotype and the no \textit{APOE*E4} allele group, 32.1 ± 11.36 (p < 0.001) and 32.3 ± 11.28 mg per week (p < 0.001), respectively. \textit{VKORC1} Group A/B and A/A patients required lower doses compared to \textit{VKORC1} Group B/B, but within \textit{VKORC1} Group A/B and A/A there were no significant differences in mean weekly warfarin dose requirements among \textit{APOE*E4} carriers (22.1 ± 9.12 mg per week) compared to either the \textit{APOE*E3E3} genotype or the no \textit{APOE*E4} allele group, 25.4 ± 10.51 (p = 0.141) and 25.3 ± 10.33 mg per week (p = 0.141), respectively.
Figure 5.3: Weekly warfarin dose requirements (mean ± SEM) by APOE group in VKORC1 Group B/B patients, and VKORC1 B/A and A/A patients. Numbers of patients in each group are shown within each bar. There were significant differences in warfarin dose requirements between VKORC1 Group B/B APOE*E4 carriers compared to VKORC1 Group B/B APOE*E3E3 patients (*p < 0.001) and VKORC1 Group B/B patients with no APOE*E4 allele (†p < 0.001).
Figure 5.4 presents the mean weekly warfarin dose requirements by APOE group in 67 CYP2C9*1*1 and VKORC1 Group B/B patients, and 67 patients with one or more CYP2C9 variant allele and VKORC1 Group A/B or A/A. In the CYP2C9*1*1 and VKORC1 Group B/B patients, there were significantly higher mean weekly warfarin dose requirements among APOE*E4 carriers (45.8 ± 14.54 mg per week) compared to both the APOE*E3E3 genotype and the no APOE*E4 allele group, 35.1 ± 11.00 (p = 0.002) and 35.2 ± 10.66 mg per week (p = 0.001), respectively. Patients with one or more CYP2C9 variant alleles and VKORC1 Group A/B or A/A required lower doses compared to CYP2C9*1*1 and VKORC1 Group B/B patients, but there were no significant differences in mean weekly warfarin dose requirements among APOE*E4 carriers (18.8 ± 10.30 mg per week) compared to both the APOE*E3E3 genotype and the no APOE*E4 allele group, 21.1 ± 8.07 (p = 0.417) and 21.3 ± 7.87 mg per week (p = 0.359), respectively.
Figure 5.4: Weekly warfarin dose requirements (mean ± SEM) by APOE group in patients with both CYP2C9*1*1 and VKORC1 Group B/B genotypes, and patients with one or more variant allele of both CYP2C9 and VKORC1. Numbers of patients in each group are shown within each bar. There were significant differences between warfarin dose requirements between CYP2C9*1*1 and VKORC1 Group B/B patients who were APOE*E4 carriers compared to CYP2C9*1*1 and VKORC1 Group B/B genotypes who were APOE*E3E3 patients (⁎ p < 0.01) and CYP2C9*1*1 and VKORC1 Group B/B patients with no APOE*E4 allele († p < 0.01).
Although not an original objective of this study, it was observed during the data analysis that the group of patients that possessed a CYP2C9*3 allele appeared to contain a relatively high number of APOE*E3E4 genotypes. A Pearson $\chi^2$ analysis was used to evaluate the frequency of the APOE*E3E4 genotype in patients possessing at least one CYP2C9*3 allele compared to patients who did not possess a CYP2C9*3 allele. Among the 35 patients whom possessed one or more CYP2C9*3 alleles, ten (28.6%) were also APOE*E3E4 genotype compared to only 34 APOE*E3E4 genotype patients (14.8%) of the remaining 230 patients whom did not possess a CYP2C9*3 allele ($p = 0.041$).

5.4 Discussion

In this study, the presence of a one or more APOE*E4 alleles was associated with increased warfarin dose requirements in patients wild-type for both VKORC1 and CYP2C9. The presence of the APOE*E4 allele was also associated with increased warfarin dose requirements in patients wild-type for VKORC1, irrespective of CYP2C9 genotype. The APOE*E4 allele was also associated with increased warfarin dose requirements in CYP2C9*1*1 patients compared to the no APOE*E4 allele group, but not the APOE*E3E3 genotype. No other analyses yielded a significant association between the presence of the APOE*E4 allele and warfarin dose requirements. Our study also detected a novel association between the APOE*E3E4 genotype and the CYP2C9*3 allele that may explain in part the discrepancies between the presence of the APOE*E4 allele and warfarin dose requirements in previous studies. In this study population, the APOE*E3E4 genotype was associated significantly with the CYP2C9*3 allele, with
patients having the \textit{CYP2C9*3} allele being 2.3 times more likely to be the \textit{APOE*E3E4} genotype than patients without the \textit{CYP2C9*3} allele. Since the presence of the \textit{CYP2C9*3} allele would decrease warfarin dose requirements, this association may offset the effect of the \textit{APOE*E3E4} genotype on warfarin dose requirements in certain patients.

Several studies have identified an increase in dose requirements in patients with the \textit{APOE*E4E4} genotype. \textit{CYP2C9*1*1} patients with the \textit{APOE*E4E4} genotype required significantly higher mean warfarin doses compared to patients who had only one or no \textit{APOE*E4} alleles in a Swedish population [82]. Similarly, Visser et al. found an approximately 50\% increase in phenprocoumon dose requirements, another coumarin anticoagulant structurally related to warfarin, in patients with the \textit{APOE*E4E4} genotype compared to patients with the \textit{APOE*E3E3} genotype [84]. In a subsequent study, Kohnke et al. were not able to find a significant association between the \textit{APOE} genotype and warfarin dose requirements in an Italian population [83]. However, they did not identify any patients with the \textit{APOE*E4E4} genotype, and the \textit{APOE*E4} allele frequency was very low compared to the Swedish population. The \textit{APOE*E4} allele frequency in our study was higher than that found in the Italian population, but lower than in the Swedish population. These prior studies did not report dose requirements in the presence of a single \textit{APOE*E4} allele, nor investigate the potentially confounding effects of \textit{VKORC1} and \textit{CYP2C9} variant alleles.

Visser et al. found a significant effect of the \textit{APOE*E4E4} genotype in all patients regardless of \textit{CYP2C9} genotype as previously noted, and reported that the dose reduction was comparable to the \textit{CYP2C9*1*3} genotype [84]. As described above, our study
identified an association between the CYP2C9*3 allele and the APOE*E3E4 genotype. This association could explain the finding of lower dose requirements in presence of the APOE*E4 allele. Since the Visser et al results were in all patients irrespective of CYP2C9 genotype, the decrease dose requirements could be due to the associated CYP2C9*3 allele instead of the APOE*E4 allele. Additionally, numerous studies have shown VKORC1 genotype to be more strongly associated with warfarin dose requirements than CYP2C9 genotype. In our study, when evaluating CYP2C9*1*1 patients without controlling for VKORC1 genotype, there were no significant differences between APOE*E4 carriers compared to the APOE*E3E3 genotype, but the difference was significant when compared to the no APOE*E4 allele group. When the same group of CYP2C9*1*1 patients were analyzed and patients possessing one or more variant alleles of VKORC1 were removed, it was shown that the APOE*E4 carriers had significantly higher warfarin dose requirements of over 10 mg per week in comparison to both the APOE*E3E3 genotype patients and the no APOE*E4 allele group.

Visser et al also reported acenocoumarol, another coumarin anticoagulant structurally related to warfarin, demonstrated lower dose requirements in APOE*E4 carriers compared to patients with the APOE*E3E3 genotype [84]. Again, this analysis was performed in all patients irrespective of CYP2C9 genotype, and a confounding association between APOE*E3E4 genotype and CYP2C9*3 could have skewed the results. The analyses presented in the Visser acenocoumarol study also did not control for VKORC1 genotype. As a result, the findings cannot be compared directly to our data because of the potential effects of CYP2C9 or VKORC1 genotype. Sconce et al reported
that APOE*E4 carriers require a lower mean warfarin dose compared to patients with the APOE*E3E3 genotype [85]. This finding was based on the incorporation the APOE genotype into their previously published model which utilizes CYP2C9 genotype including the CYP2C9*3 allele and VKORC1 genotype, in addition to age and height. Finally, one additional study reported no significant correlation between the presence of the APOE genotype and warfarin dose requirements based on the incorporation of APOE genotype into their warfarin dosing model [86]. This model also already utilized both CYP2C9 genotype and VKORC1 genotype, in addition to age, gender, body surface area, presence or absence of a prosthetic heart valve, and diabetes. Based on their findings, it was suggested that APOE genotype has a clinically insignificant effect on these warfarin dosing models after considering other factors including CYP2C9 and VKORC1 genotype. However, these last two findings of a slightly lower warfarin dose requirement and an insignificant correlation may be the result of the association between the APOE*E3E4 genotype and the CYP2C9*3 allele. The significance of the APOE genotype in certain patients may be obscured in studies that do not control for CYP2C9 and VKORC1 genotypes due to these two enzymes’ significant contribution to warfarin dose requirements.

The novel identification of an association between the APOE*E3E4 genotype and the CYP2C9*3 allele should be examined in existing warfarin patient databases and also considered in future studies of the association between warfarin dose requirements and APOE genotype. However, this association was only shown in a 100% Caucasian population in the present study and may not be found in other ethnic groups. Although
no direct association is apparent, it is interesting to note that the *APOE* gene is located on the long arm of chromosome 19 at position 13.2, and the *CYP2A6* gene, in which variant alleles have been associated with altered metabolism of S-warfarin, also resides on 19q13.2 [87]. In addition to the discovery of this novel association, the overall findings of our study suggest *APOE* genotype can have a clinically significant effect on warfarin dose requirements in certain patient populations. In the absence of *VKORC1* variant alleles, the *APOE*/*E4* carriers required a significantly higher mean warfarin dose to achieve a therapeutic INR compared to both the *APOE*/*E3*/*E3* genotype and the no *APOE*/*E4* allele group. However, *APOE* genotype was not significantly associated with warfarin dose in patients who possessed one or more variant alleles of *VKORC1*.

In conclusion, this study used a unique approach to examine whether the presence of the *APOE*/*E4* allele would have a significant effect on warfarin dose requirements in individuals who do not possess a variant allele of *VKORC1* or *CYP2C9*. It was found that in these patients, the *APOE*/*E4* allele was associated with higher warfarin dose requirements. This was also true in all *VKORC1* group B/B patients regardless of *CYP2C9* genotype. It is reasonable to assume the *APOE* genotype may have limited clinical importance in patients that possess a variant allele of *VKORC1* due to the significant impact of *VKORC1* variant allele(s) on warfarin dose requirements. This study demonstrated that in the absence of *VKORC1* variant allele(s), the *APOE*/*E4* allele can have a significant effect on stable warfarin dose requirements. In our patient population almost 40% of the patients were *VKORC1* Group B/B, and the presence of the *APOE*/*E4* allele in *VKORC1* Group B/B patients, regardless of *CYP2C9* genotype, was
shown to be strongly associated with higher mean warfarin dose requirements of more than ten mg per week. Therefore, the determination of APOE genotype may assist in the determination of an appropriate warfarin maintenance dose in a significant percentage of patients who do not possess a variant allele of VKORC1.
CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

6.1 Genetic-Based Warfarin Dosing Nomogram

To date, we have achieved a primary goal of this research which was to develop and validate a simple genetic-based warfarin dosing nomogram. The nomogram incorporates the genetic factors of CYP2C9 and VKORC1 genotype and the non-genetic factors of age and height. Other factors could be incorporated into the nomogram to further improve its correlation, but these additional factors would also increase the complexity of the nomogram. We firmly believe that to initially penetrate the average clinician’s pharmacotherapeutic decision making process that a genetic-based nomogram should be as simple as possible. In the future, as genetic testing becomes more common, other factors may be incorporated that could increase the correlation of the nomogram. These factors may include apolipoprotein E (APOE) genotype, other less common CYP2C9 and VKORC1 variant alleles, and/or other factors.

One example is the nomogram’s limitations with high dose patients, since the largest predicted dose is six mg per day. Applying the nomogram to the development
cohort resulted in only 40 (15.1%) of the predicted doses differing from the stabilized dose by more two mg per day. Thirty-four (85%) of these 40 were the result of an under-prediction of the stabilized dose. The mean daily dose in this group was 7.1 ± 1.4 mg (range 4.3 to 11.4), and only one patient in this group required less than five mg per day.

From the APOE study, it appears that the presence of the APOE*E4 allele may be one important factor associated with increased warfarin dose requirements. However, the incorporation of APOE genotype into the nomogram would require an additional genetic test.

Newly identified VKORC1 variant alleles have also associated with increased warfarin dose requirements including VKORC1 +497 (rs2884737) and +3730 (rs7294) [88]. We are in the process of evaluating our cohort of patients for these VKORC1 alleles to determine if their presence may be correlated with warfarin dose requirements in our patients. If we find a positive association, the nomogram table could be modified in the future to include VKORC1 genotypes that are correlated with increased warfarin dose requirements. However, this would also require additional genetic testing, and would increase the complexity of the nomogram. In conclusion, at this point in time we believe that the current nomogram offers the best compromise between accuracy and clinical utility.

Another potential limitation is that the nomogram was developed in a Caucasian population and was validated in a primarily Caucasian population. Different ethnics groups often possess CYP2C9 and VKORC1 variant alleles at different frequencies. For example, Mushiroda et al. found the CYP2C9*3 allele frequency in a Japanese population
was only approximately one-third the frequency in our Caucasian cohort [89]. Additionally, the *VKORC1* variant allele frequency was almost two and half times greater in the Japanese cohort compared to our Caucasian cohort. Additionally, some variant alleles have only been identified in certain ethnic groups. For example, *CYP2C9*\( ^{*6} \) has only been found in African-Americans, and not in a Caucasian population [78]. These factors may limit the applicability of the nomogram in groups other than Caucasians.

6.2 Prospective Evaluation of the Nomogram

The next phase of this research will be to prospectively evaluate the use of the validated warfarin dosing nomogram to initiate warfarin therapy and to compare outcomes with a control group of patients who are initiated conventionally. The study will be open to all adult patients with a diagnosis deep vein thrombosis or pulmonary embolism who are to be initiated on warfarin therapy and are entering The George Washington University Medical Faculty Associates, Inc (MFA) or George Washington University Hospital, and will be subsequently followed at the MFA. Those individuals agreeing to participate in the study will be asked to sign the consent form, provide a buccal swab and fill out a demographic sample sheet that contains the following information: age, gender, ancestry of the individual back to their grandparents, other medications and a study identification number. Patients will be randomized into a nomogram based group and a control group which will be initiated conventionally. Patient outcomes including number of days within the target INR and the frequency of warfarin-related adverse events during the first 30 days of treatment will be compared between the groups. A pharmacoeconomic
analysis will also be performed to assess the cost-effectiveness of genetic testing of *CYP2C9* and *VKORC1* for the initiation of warfarin therapy.

6.2.1 Methods

Patients with the diagnosis of deep vein thrombosis or pulmonary embolism in whom warfarin is scheduled to be initiated will be invited to participate in the study. Only patients less than 18 years of age and/or a baseline INR of > 1.4 will be excluded. Informed consent and buccal swabs will be collected from all participants at the time of initial visit. Additional data collected that will be collected includes patient age, height, gender, ethnicity, baseline laboratory values including INR, hematocrit, hemoglobin, and liver function tests.

DNA will be isolated and analyzed as previously described. Samples will be genotyped for *CYP2C9* and *VKORC1* variant alleles using real-time PCR and the TaqMan© assays as previously described. After genotyping is completed, the *CYP2C9* and *VKORC1* genotype will be reported along with the study identification number. The anticipated turnaround time for the analysis is no more than 48 hours. Only Drs. Barbour or Wasserman will be able to access the patient record and associate the study number with the patient, and therefore the patients’ genotype and medical history will only be known to the two primary care providers.

All patients will receive a five mg warfarin dose on days one and two. An INR will be obtained on day three, and the dosage adjustment will be based on INR for the control group. For the nomogram dosing group, the results of these tests will then be
relayed to the physician, and not a specific dose recommendation. The physician will use the results of the genetic testing, in conjunction with the patients’ age and height, to determine the dose of warfarin for that patient. The nomogram dosing group will be initiated as outlined in Table 6.1. Repeat INRs will be obtained on days one, three, eight to ten, and then weekly thereafter until two therapeutic INRs are obtained. All patients will be maintained on low molecular weight heparin until the target INR is reached.

<table>
<thead>
<tr>
<th>Day</th>
<th>INR</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baseline INR</td>
<td>5 mg</td>
</tr>
<tr>
<td>2</td>
<td>&lt; 1.5</td>
<td>7.5 - 10 mg (7.5 mg for &gt; 65 years-old or &lt; 5’1”)</td>
</tr>
<tr>
<td></td>
<td>1.5 – 1.9</td>
<td>5 mg for one day, then nomogram dose</td>
</tr>
<tr>
<td></td>
<td>2.0 - 3.0</td>
<td>2.5 mg for one day, then nomogram dose</td>
</tr>
<tr>
<td></td>
<td>&gt; 3.0 to 4.0</td>
<td>hold 1 dose, repeat INR, then nomogram dose</td>
</tr>
<tr>
<td></td>
<td>&gt; 4.0</td>
<td>hold 2 doses, repeat INR, then nomogram dose</td>
</tr>
</tbody>
</table>

Table 6.1: Nomogram based warfarin induction.

Once the nomogram based dose is initiated, the subsequent adjustments in dose will be determined on the basis of the INR. The control group will follow the same initial induction pattern, but dosage adjustment will be based only on INR values.

6.2.2 Statistical Analysis

The primary end points will be the number of days within the target INR range and the frequency of warfarin related adverse events during the first 30 days of treatment. Demographic and baseline clinical characteristics will be compared using $\chi^2$ and Mann-
Whitney tests for categorical and continuous variables, respectively. Deviation of allele frequencies from Hardy-Weinberg equilibrium will be tested by the $\chi^2$ test. The number of days within the target range will be compared between the nomogram group and the control patients using an unpaired Student t-test. The frequency of warfarin related adverse events in the two groups will be compared using the $\chi^2$ test. Two-sided significance tests will be used, and a P value of less than 0.05 will be considered significant. SPSS v.15 software will be used for all analyses (SPSS, Inc. Chicago, Illinois). A sample size of 125 patients in each group will be needed to detect a 15% difference in the number of days within target INR range. The estimated sample size will provide a power of 0.90 with a two-sided test and an $\alpha = 0.05$.

6.2.3 Pharmacoeconomic Analysis

In order to assess the potential policy and economic challenges of translating warfarin pharmacogenomic testing into wider clinical practice, we will perform two analyses: 1) cost-effectiveness analysis of the use of the proposed genetic testing intervention compared to currently used clinical approaches; and 2) assessment of the potential for equitable translation and diffusion of this technology by examining the disparities which may arise when using pharmacogenomic testing in the prescribing of warfarin. The cost effectiveness analysis will guided by the recommendations of the Panel on Cost-effectiveness in Health and Medicine and current studies providing specific guidelines for analyzing the cost effectiveness of pharmacogenomics. Specific focus will be placed on comparing the time to therapeutic INR, and stabilization within
INR goal and frequency of adverse events associated with therapy in the nomogram and control groups. The potential for equitable translation will be determined by dividing patients into groups based on the types of insurance they have. The research team will assess whether these various insurance providers are currently paying for pharmacogenomic tests (e.g. HER-2) in order to determine whether they are likely to cover the warfarin tests. Subsequently, the research team will look for associations between the various demographic characteristics of the patients and whether their genetic tests are likely to be reimbursed in order to determine if disparities may arise from the incorporation of this test into the patient population being analyzed.

6.3 Conclusions

Two of the major obstacles for integrating genetic information into pharmacotherapeutic decision making is related to the general lack of randomized, prospective studies examining the predictive power of genetic biomarkers and the assessment of the economical benefits associated with their application. The study will address these issues with regard to warfarin therapy using the validated genetic-based warfarin dosing nomogram. Warfarin is an ideal candidate for early adoption of widespread genetic based dosing for numerous reasons. It has a very narrow therapeutic index, a wide inter-patient variability in dose requirements, and genetic variability in at least two genes are significantly associated with altered dosage requirements. Additionally, several million prescriptions for warfarin are prescribed annually, therefore the impact of these results could have a significant impact on clinical practice worldwide.
BIBLIOGRAPHY


30. Crawford DC, Ritchie MD, Rieder MJ. Identifying the genotype behind the phenotype: a role model found in VKORC1 and its association with warfarin dosing. Pharmacogenomics. 2007; 8: 487-496.


85. Sconce EA, Daly AK, Khan TI, Wynne HA, Kamali F. APOE genotype makes a small contribution to warfarin dose requirements. Pharmacogenet Genomics 2006; 16: 609-611.

