Genetic and Environmental Factors Affecting Hemostasis in Yup'ik People

Nicholas T. Au

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2014

Reading Committee:

Allan E. Rettie, Chair

Kenneth E. Thummel

Kent L. Kunze

Program Authorized to Offer Degree: Pharmacy – Medicinal Chemistry

University of Washington

Abstract

Genetic and Environmental Factors Affecting Hemostasis in Yup'ik People

Nicholas T. Au

Chair of the Supervisory Committee:
Professor Allan E. Rettie
Department of Medicinal Chemistry

Fish oil has been widely studied for its protective effects against heart disease. These effects are attributed to ω3 PUFA content, which are also found in sea mammals in addition to fish. The mechanism by which ω3 PUFAs provide cardiovascular benefits remain unclear but experimental data have alluded to effects on hemostasis. ω3 PUFAs may also modify bleeding risk based on anecdotal reports in the literature. The studies presented in this dissertation examine the effect of ω3 PUFA consumption on measures of hemostasis among Yup'ik study participants, for whom fish and marine mammals are a dietary staple. Measures of clotting potential included clotting factors II and V, PT/INR, PTT, and sP-selectin. These data were assessed for an association with $\delta^{15}N$, a validated biomarker of ω3 PUFA intake. We found that the platelet biomarker, sP-selectin, correlated strongly and inversely with $\delta^{15}N$. This supports earlier findings of diminished platelet activation in Greenland Inuits and suggests that platelet activity varies with ω3 PUFA content in blood. There was also an unexpected bimodal distribution in plasma clotting factor V activity where approximately 11% of samples had values below 30%. While we validated the factor V activity data by measuring factor V protein concentrations by ELISA, the low values were not produced during repeat visits by some of the same individuals. This observation warrants investigation.

We then explored whether low vitamin K status is evident in Yup'ik people. Given that the main dietary source of vitamin K is green leafy vegetables, which are only intermittently available in the remote sub-Arctic regions where the Yup'ik live, and that vitamin K is necessary for the production of

vitamin K-dependent clotting factors, it is plausible that this population may be more prone to a hypocoagulable state because of limited vitamin K intake. We further hypothesized that Yup'ik people may also harbor unique polymorphisms in the genes of vitamin K cycle-associated enzymes that modify vitamin K status. Acute vitamin K status was determined by directly quantifying vitamin K concentrations in plasma using a new stable-label, LC-MS assay, while chronic vitamin K status was assessed by measuring concentrations of PIVKA-II in plasma. We then analyzed these data for statistically significant associations with genetic variants of the vitamin K hydroxylases, *CYP4F2* and *CYP4F11*, as well as *VKORC1*, which catalyzes the recycling of the vitamin in the liver. Additionally, we measured the vitamin K content of tundra greens that are part of the traditional Yup'ik diet to determine if these foods are a significant, dietary source of vitamin K. While no significant associations with *CYP4F11* and *VKORC1* were found, we observed that the allele frequency of *CYP4F2*3* was more than twice that of other world populations. Because *CYP4F2*3* codes for an enzyme with low catalytic activity, this SNP may have a role in conserving vitamin K levels in the body. This hypothesis was supported by the observation that carriers of *CYP4F2*3* possessed significantly higher plasma phylloquinone levels compared to wild-type participants. This SNP also associated with a reduced likelihood of having chronically low vitamin K status (PIVKA-II levels above 2 ng/mL). Furthermore, the vitamin K content of tundra greens were very similar to that of market vegetables measured in this study, suggesting that tundra greens are an excellent seasonal source of vitamin K.

Finally, based on the association between *CYP4F2*3* and vitamin K status in Yup'ik people, we explored the extent to which the genetic profile of CYP4F enzymes plays a role in altering hepatic stores of vitamin K. First, we quantified the vitamin K content of liver tissue obtained from the UW School of Pharmacy Human Liver Bank. Despite measuring a wide range of phylloquinone levels, we were not able find any association of *CYP4F2*3* genotype with hepatic vitamin K content due to likely to a lack of dietary control in samples from these liver donors, as well as low numbers of the variant homozygote genotype in this largely Caucasian population. Therefore, we next measured hepatic vitamin K levels in livers of *cyp4f14* knockout mice in comparison to *cyp4f14* wild-type control mice. Cyp4f14 is a reported

vitamin E ω-hydroxylase in mice, and is a candidate for the murine ortholog of CYP4F2. Although this study did not reveal differences in hepatic vitamin K content between *cyp4f14* knockouts and controls, there was a substantial increase in hepatic phylloquinone content in female mice compared to males. This difference in hepatic vitamin K content between sexes has also been previously reported for vitamin E, but more studies are needed to understand these findings and implications for human health.

Overall, the data from this thesis shed new light on the genetic and environmental factors that contribute to variability in coagulation and vitamin K status of Yup'ik people. These factors may be particularly important considerations when prescribing anticoagulant and antiplatelet medications or treating medical conditions involving hemostasis in this population. Continued research in this area is warranted to validate and extend our observations.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	arachidonic acid
AI/AN	American Indian and Alaska Native
ALA	α-linolenic acid
APCI	atmospheric pressure chemical ionization
CANHR	Center for Alaska Native Health Research
CI	confidence interval
COX	cyclooxygenase
CYPP450	cytochrome P450
$\delta^{15}N$	$^{15}N/^{14}N$ isotopic ratio
D5D	Δ5-desaturase
D6D	Δ6-desaturase
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ESI	electrospray ionization
F5F8D	combined factor V and factor VIII deficiency
FBS	fetal bovine serum
G6PD	glucose-6-phosphate dehydrogenase
GGCX	γ-glutamyl carboxylase
Gla	γ-carboxyglutamate
GLA	γ-linolenic acid
Glu	glutamic acid
HDL	high density lipoprotein
HER2	human epidermal growth factor receptor 2
HPLC	high performance liquid chromatography
INR	international normalized ratio
IQR	interquartile range
LA	linoleic acid
LC-MS	liquid-chromatography-mass spectrometry
LOX	lipoxygenase
LT	leukotrienes
MK4	menaquinone-4

MK4-d7	deuterated menaquinone-4
MK4O	menaquinone-4 epoxide
MK4O-d7	deuterated menaquinone-4-epoxide
MRM	multiple reaction monitoring
NQO	NADPH quinone oxidoreductase
OC	oral contraceptive
OR	odds ratio
PBS	phosphate buffered saline
PCI	percutaneous coronary intervention
PG	prostaglandins
PIVKA-II	vitamin K absence or antagonism-factor II
PRP	platelet-rich plasma
PT	prothrombin Time
PTT	partial thromboplastin time
PUFA	polyunsaturated fatty acids
RBC	red blood cell
SD	standard deviation
sP-selectin	soluble P-selectin
TF	tissue factor
TX	thromboxanes
UPLC	ultra performance liquid chromatography
VK1	phylloquinone
VK1-d7	deuterated phylloquinone
VKDP	vitamin K-dependent proteins
VKH2	vitamin K1 dihydroquinone
VKO	phylloquinone epoxide
VKO	vitamin k epoxide
VKO-d7	deuterated phylloquinone epoxide
VKOR/VKORC1	vitamin K epoxide reductase
vWF	von Willebrand’s factor

LIST OF FIGURES

Figure Number Page

LIST OF TABLES

Table Number Page

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to those individuals who have helped make his effort possible through their contributions:

To Dr. Allan E. Rettie, for his careful guidance throughout my training, encouragement to "see the big picture", emphasis on independent thinking, creative ideas, and enlightening scientific discussions;

To Dr. Kenneth E. Thummel, for providing me with an opportunity to work on a multidisciplinary project with Alaska Native people and serving on the reading and supervisory committees;

To Dr. Rheem A. Totah, for her valuable advice throughout my training and for serving on the supervisory committee;

To Dr. Kent L. Kunze, for serving on the supervisory and reading committee;

To Dr. Melissa A. Austin, for her statistical and epidemiological guidance on this project, as well as serving on the supervisory committee;

To Dr. Gail D. Anderson and Dr. Dave L. Veenstra, for serving on the supervisory committee;

To Dr. Bert Boyer, Scarlett Hopkins, Jynene Black, Dr. Diane O'Brien, and the Yup'ik people for their contributions to this work and providing me with an opportunity to collaborate with CANHR/UAF;

To. Dr. Morayma Reyes, Joanne Estergreen, and Ann McTee for their contribution with sample analysis at the UW Department of Laboratory Medicine;

To Mr. Dale Whittington and Dr. Ross Lawrence, for their help and guidance in the mass spectrometry facility;

To Dr. Timothy Thornton, for providing me insight on statistical analysis in this project;

To Dr. Fred Farin and Jessie Tsai, for their contributions to this work;

To Dr. Sarah Booth for providing menaquinone standards for the vitamin K assay;

To Dr. Matt McDonald, for his help with synthesizing vitamin K standards and training me to perform extractions with biological samples, and to my other fellow Rettie labmates, Amanda Johnson, Dr. Kayte Edson, Dr. Oliver Parkinson, Dr. Jamil Haque, and Dr. Aaron Teitelbaum, for their support and friendship;

To Dr. Sidney D. Nelson for providing me with my first opportunity to work in a laboratory setting and believing in my ability to complete the joint Pharm.D./Ph.D. program;

To my fellow project colleagues, Tove Ryman, Allie Fohner, and Joe Yracheta, for giving me greater insight to other aspects of the PGRN projects;

To my Pharm.D./Ph.D. colleague, Dr. Jean Dinh, for her support and undergoing the rigorous pharmacy and graduate school training with me;

To the Department of Medicinal Chemistry, Pharmacogenomics Research Network, and ITHS TL1 Multidisciplinary Predoctoral Clinical Research Training Program for their financial support;

To my brothers of the Kappa Psi Pharmaceutical Fraternity, Inc., for their lifelong fellowship and support;

Finally, to my parents Roger and Joanna and my brother Darren, for their love, support, and encouragement.

DEDICATION

To my parents Roger and Joanna and my brother Darren,

For their love, support, and encouragement.

Chapter 1

The ω3 PUFAs, Vitamin K, and Coagulation

1.1 Pharmacogenetics

Defined in 1959 by Friedrich Vogel, the term "pharmacogenetics" refers to the study of genetic variability in response to a pharmacologic therapy. The aim of pharmacogenetic research is to accurately predict which patients with a particular condition would respond best to a specific medical therapy or are at greatest risk of experiencing adverse effects. Although clinical, behavioral, and environmental factors are well-known sources of patient variability, pharmacogenetic research continues to provide important insights on drug response especially as new agents enter the market. Medical practices are generally empirical in nature with a "trial-and error" approach to prescribing medications. However, it is estimated that medications are only 50-60% efficacious for those prescribed and that the unpredictability of adverse drug reactions contributes to its status in the U.S. as a major leading cause of death (1, 2). An individual's genome is thought to account for 20-95% of the variation observed in drug disposition and effects, highlighting the potential for inter-individual variability in medication response (3). With the characterization of the human genome in 2003 and as continual advancements are made in genetics to reduce the cost of genotyping, pharmacogenetics offers the potential for greatly enhanced personalized medicine.

Varying degrees of inter-individual response to environmental agents was documented as early as 510 B.C. by the Greek philosopher and mathematician Pythagoras. He noted that some individuals, experienced a potentially fatal reaction after consuming fava beans (4). Over two millennia later researchers eventually discovered that his observations could be attributed to a hereditary abnormality of the glucose-6-phosphate dehydrogenase (G6PD), an enzyme that helps protect red blood cells (RBCs) from oxidative stress (5). People with G6PD deficiency are at risk of experiencing hemolytic anemia after consuming fresh fava beans or inhaling the pollen of fava bean plants, a reaction often referred to as

"favism". It is now recognized that G6PD deficiency affects approximately 400 million people worldwide and is considered to be the most commonly inherited enzyme deficiency (6). In addition to cautionary measures with legume foods, G6PD deficiency has important pharmacogenetic considerations because a broad list of medications that includes several antibiotics, analgesics, and antimalarials can also cause hemolytic anemia in these individuals.

One of the earliest metabolic observations that would eventually lead to discovering the pharmacogentic influence of cytochrome P450 (CYPP450) enzymes involved debrisoquine and sparteine. In 1977, researcher's at St. Mary's Hospital in London reported a bimodal distribution of the ratio between excreted debrisoquine and its 4-hydroxy metabolite in 94 healthy volunteers, which implicated the presence of differential metabolism in their sample population (7). A separate group in 1979 from Bonn, Germany noted that 5% of their study volunteers excreted 100% of the administered dose of sparteine as the parent drug (8). Subsequent studies would eventually attribute the poor metabolism of these drugs to defective alleles of *CYP2D6* (9, 10). The major phenotypes that classify the degree of metabolism by CYP2D6 today are poor metabolizers, intermediate metabolizers, extensive metabolizers, and ultra-rapid metabolizers (11). Furthermore, these findings would eventually rise to discovering the involvement of other CYP450s polymorphisms that cause inter-individual variability in the metabolism and disposition of xenobiotics (12).

The direct application of pharmacogenetic testing to avoid potential adverse reactions and optimize efficacy has impacted several areas of clinical practice. For example, prior to prescribing the anti-HIV drug, abacavir, patients are genetically tested for the human leukocyte antigen B (*HLA-B*57:01*) variant to reduce the risk of developing an immune-mediated hypersensitivity reaction when exposed to the drug (13). Genetic testing for human epidermal growth factor receptor 2 (HER2) overexpression, which occurs in 15-20% of all breast cancers, has also proven to be useful by identifying optimal responders to the HER2-binding monoclonal antibody, trastuzumab (14). A more recent example that has led to much discussion in the clinical community is the interaction of clopidogrel and *CYP2C19* loss-of-function alleles. Clopidogrel is a thienopyridine prodrug that requires hepatic biotransformation

by CYP2C19 and several other CYP450 drug metabolizing enzymes to form an active thiol metabolite that selectively and irreversibly inhibits the platelet ADP receptor, P2Y12, thus reducing platelet activation and aggregation (15). Clopidogrel active metabolite formation is an inefficient process due to the presence of a competing hydrolytic pathway that results in hydrolysis of at least 85% of the parent drug to an inactive carboxylic acid metabolite (16, 17). Therefore, factors that reduce the capacity for conversion of clopidogrel to its active thiol metabolite may confer increased risks for serious adverse cardiovascular events among clopidogrel-treated patients with acute coronary syndromes undergoing percutaneous coronary intervention (PCI). Several studies have suggested that *CYP2C19*2* status plays a role in dictating health outcomes of clopidogrel-treated patients. In a study of patients under 45 years old given clopidogrel (N = 259), the **2* allele was found to be in independent predictor of prognosis after the first myocardial infarction (18). In a larger study (N = 2208) of patients with acute myocardial infarction who received clopidogrel, those carrying any two *CYP2C19* loss-of-function alleles (**2, *3, *4,* or **5*) had a higher rate of cardiovascular events than non-carriers. Furthermore, among those who underwent PCI during hospitalization, the rate of cardiovascular events among patients with two *CYP2C19* loss-of-function alleles was 3.58 times the rate among than non-carriers. Pharmacokinetic studies have also found lower AUC, C_{max}, and platelet responsiveness in patients with a *CYP2C19*2* allele (19, 20). Pharmacogenetic studies of clopidogrel and *CYP2C19* genotype status prompted the FDA to issue a black box warning for the drug in 2010 noting that the *CYP2C19* genetic polymorphism plays a major role in its bioactivation and efficacy (21). However, pharmacogenetic testing for *CYP2C19* is not required and randomized clinical trials are still needed to determine whether genotyping prior to prescribing would improve health outcomes in clopidogrel-treated patients. This might be expected to be particularly important for Asian patients where the prevalence of *CYP2C19* poor metabolizers is about 5-fold higher than White populations (22).

1.2 Ethnic Pharmacogenetic Variability

There is substantial evidence that pharmacogenetic variation is extremely diverse across ethnic groups (23). One of the earliest examples where pharmacologic response was found to vary across ethnicities was in regards to cardiovascular therapy with beta-blocker antihypertensives. It had been observed as early as the 1980s that African-American patients responded less favorably to β-blockers than Caucasians (24). Evidence from subsequent genetic research have shown that the reduced β-blocker efficacy in African-Americans may be related to variation in the G-protein receptor kinase 5 (*GRK5*) genes. A non-synonymous N-terminal polymorphism (*GRK5-Leu41*) encodes for a variant kinase that decreases β-1 adrenergic receptor signaling, mimicking a "natural" β-blocker effect. The *GRK5-Leu41* variant is found in approximately 40% of African-Americans, but is very rare (<1%) of European-Americans. This may partly explain why the use of β-blockers in some African-Americans patients has not led to increased survival rates in heart failure as with other populations (25, 26).

Characterizing pharmacogenetic differences across ethnicities involves clinical studies performed in large populations, but there is a notable lack of data for minority groups. Also, there is a clear underrepresentation of rural and indigenous populations in genetic and environmental studies where accessibility to, and frequency of, clinical monitoring may be limited compared to those living in urban settings. American Indian and Alaska Native (AI/AN) populations in particular have rarely been included in this type of research despite the fact that nearly 5.2 million AI/AN people live in the United States (27). Studies in AI/AN people have been challenged by the perception that past health research has provided little benefit to indigenous populations. There is also a history of mistrust in the use of research data, particularly genetic information, as this has implications for identity and shared heritage (28). A full understanding of genetic variation in AI/AN communities is essential if pharmacogenetic testing is to reach its optimal clinical utility in patients from these populations. It has been shown that extrapolation of pharmacogenetic findings across different ethnic populations may not be appropriate and that further efforts must be made to include data on AI/AN people (29).

1.3 Gene-Environment Interactions

Given the limited availability of genetic data in AI/AN people, it is plausible that chronic disease patterns in this population have both a genetic and environmental basis and that some genetic factors may not be detectable in the absence of environmental influences. The observation of an effect of genotype on a disease risk that varies among different environmental exposures (or vice versa) is defined as a "gene-environment interaction" and has been characterized in a number of clinically relevant cases (30). For example, the "alcohol flush reaction" in which an individual's face turns red upon alcohol consumption is due to reduced activity of the aldehyde dehydrogenase enzyme in *ALDH2*2* carriers, and commonly encountered in East Asian populations (31). Also, it was also shown that young women who carried a Factor V Leiden mutation and used oral contraceptives (OC) were at greater risk of developing deep vein thrombosis compared to non-carriers of Factor V Leiden who used OCs as well as carriers of Factor V Leiden who did not use OCs (32). Only recently has the availability of high throughput genotyping technologies allowed assessment of genetic variation in studies of environmental risk, yet not all genetic studies have provided sufficient detail on lifestyle and environmental factors that could also affect disease risk. The difficulty in assessing the large magnitude of potential environmental and genetic effects coupled with the need to use both reliable epidemiologic and biological data also further complicates the identification of gene-environment interactions. Nevertheless, studying gene–environment interactions in AI/AN people is critical for fully characterizing new and existing biological pathways. This information is expected to help improve the accuracy and precision in the assessment of both genetic and environmental influences on disease and drug response.

1.4 The Native Alaskan Yup'ik People

The Yup'ik people are one group among the AI/AN that participate in community-based participatory research in pharmacogenetics (33). Their community includes over 20,000 members living in approximately 50 remote Alaskan communities bordering the Bering Sea. While the lives of Yup'ik

people were shaped by Russian exploration into Alaska (and eventual Westernization) throughout the 19th century, much of their historical culture is evident in current social and dietary practices. A notable characteristic of the Yup'ik people is the high intake of fish and other marine animals, relative to the non-Native Alaskan population. This dependency on marine food sources originated from a semi-nomadic lifestyle where food was hunted or gathered rather than obtained from a common market. This diet is thought to contribute to the low prevalence of chronic diseases previously reported in the Yup'ik (34, 35). The Yup'ik people were one of the last populations to experience Westernization and over the last few decades, have shifted away from traditional subsistence-based diet towards a more Western-influenced commercial diet characterized by a high content of saturated fats, sugar, and refined foods and low fiber content (36). The "Western" diet has been associated with an increase in the burden of certain chronic diseases, including obesity, cardiovascular disease, and type 2 diabetes in much of the developed world for reasons still not entirely understood (37). Though many Yup'ik people today incorporate a mixed traditional-Western diet, this change in dietary pattern has coincided with an increase in prevalence of chronic diseases observed (38-40). The health consequences of a Westernizing diet and the benefit of a traditional diet on chronic disease are not entirely clear, but warrant further investigation of the specific benefits/risks of individual foods sources.

Initial characterization of the subsistence-based diet in circumpolar populations and its relation to human health was studied in the 1970's by Bang et al. (41). Even more so than today, a generation ago Greenlandic Inuits lived a lifestyle as Arctic hunters and fishermen where the diet predominantly consisted of marine animals and fish, and was low in carbohydrates. This diet was thought to contribute to the low serum cholesterol, triglyceride and β-lipoprotein levels observed in this population relative to Danish people (42). This prompted the investigators to explore factors in the Greenland Inuit diet that may explain their generally low incidence of cardiovascular death, which constitutes approximately 3.5% of all deaths despite having a life-span greater than 60 years (41, 43). In a small study of 24 Greenland Inuit participants, Dyerberg and Bang et al. observed that Eskimo participants had bleeding times (a platelet-dependent measure of coagulation) that were nearly twice that of Danish controls and they also

exhibited decreased platelet aggregation (41). The researchers hypothesized that this "naturally hypocoagulated" state in the Greenlandic people could be attributed to their high dietary consumption of fish, which is rich in polyunsaturated fatty acids (PUFAs). The possible hypocoagulative effects of PUFAs in Greenlandic Inuits were reported as early as the 1940s and the potential of these dietary components for preventing heart disease has stimulated much investigation over the last 30 years (44). It was similarly reported nearly a decade after the findings of Bang et al. that cardiovascular mortality in Alaska Native people was 33% lower than non-native people (34). The reduced amount of atherosclerotic lesions found in the coronary arteries of Alaska Native people was also suggestive of a lipid-lowering and antithrombotic effect of a high PUFA diet in these populations (45).

1.5 Polyunsaturated Fatty Acids

As studies were conducted to assess the impact of shifting dietary patterns on health, considerable interest has been placed on the PUFAs and their essential fatty acid precursors, α-linolenic acid (ALA) and linoleic acid (LA). PUFAs are a class of long-chain fatty acids containing two or more double bonds with compound nomenclature based on the length of the carbon chain and position of the last double bond away from the terminal methyl group (e.g. ω3, ω6 etc.). ALA, through a series of enzymatically-driven desaturation (Δ5 and Δ6-desaturase) and elongation reactions, gives rise to the formation of the biologically active ω3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 1.1). These ω3 PUFAs are involved in many physiological roles that ultimately promote anti-inflammatory and anti-thrombotic effects (46). In contrast, LA is converted to the ω6 PUFA, arachidonic acid (AA), through the same set of enzymes involved in EPA/DHA formation from ALA. AA is a precursor to a family of eicosanoids involved in cell-signaling and inflammation. These include prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs). The main source of ω6 fatty acids is from vegetable and seed oils (i.e, corn, sunflower seed, safflower seed) whereas ω3 fatty acids derive mainly from intake of fish, flaxseed oil, canola, and walnuts.

Analysis of data from anthropological studies suggest two important points about evolution of the human diet with regard to fatty acid content; 1) that humans evolved consuming a diet that was much lower in saturated and trans fatty acids, and 2) the early diet contained roughly equal amounts of ω6 and ω3 PUFAs (47, 48). In the last 150 years, increased consumption by many world populations of ω6 fatty acids in the Western diet due to the greater intake of vegetable oils, decreased intake of fish, and industrial production of meat rich in ω6 but poor in ω3 fatty acids has led to a dramatic increase in the ω6/ω3 fatty acid ratio to approximately 10:1 and even upwards of 30:1 (49). A higher ω6/ω3 fatty acid ratio is thought to contribute to the higher incidence of chronic illnesses observed today because of the competition between ω6 and ω3 fatty acids for the AA pathway (50). ALA and LA acid utilize the same elongase and desaturase enzymes and the concentrations of these fatty acids drive the pathway to favor formation of their respective PUFAs. The higher amounts of ω6 fatty acids found in the Western diets promotes the production of AA-derived eicosanoids in larger quantities relative to those formed from ω3 fatty acids. In excess amounts, these AA-derived eicosenoids promote platelet aggregation, thrombus formation, cell proliferation, and inflammation.

The opposite physiologic effect is observed after the consumption of fish oil. EPA and DHA have slightly different effects on the AA pathway, but ultimately lead to reduced inflammation. EPA is an inhibitor of the enzyme Δ5-desaturase (D5D), which converts dihomo-γ-linolenic acid (DGLA) to AA. EPA also competes with AA as a substrate for cyclooxygenase (COX) or lipoxygenase (LOX). These enzymes normally convert AA into the proinflammatory 2-series TXs, PGs, and 4-series LTs, but will preferentially convert EPA to the less potent 3-series TXs, PGs, and 5-series LTs (51). DHA, on the other hand, inhibits Δ6-desaturase (D6D), which converts γ-linolenic acid (GLA) to DGLA in a step upstream of AA formation. Unlike other ω6 fatty acids, DGLA can be metabolized to the anti-inflammatory 1-series PGs and TXs by COX enzymes. However, these observations, in addition to the slow conversion of DGLA to AA mediated by Δ5-desaturase, may not fully explain the beneficial effects of DHA supplementation (52). Instead, DHA may play more important roles in affecting cell membrane structure and function than EPA because it is a slightly larger molecule and possesses a higher degree of

unsaturation (22 carbons and 6 double bonds vs. 20 carbons and 5 double bonds in EPA) (53). Consequently, DHA can incorporate more efficiently into membranes to reduce lipid peroxidation and has also been found to disrupt the composition of lipid rafts, which are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids (54, 55). Since lipid rafts serve as platforms for proteins involved in cell-signaling, DHA may interrupt signaling pathways mediated by inflammatory cytokines (56). Despite these differences, both EPA and DHA can similarly displace AA after incorporation into the membranes of platelets and vascular cells, which decreases the availability of AA to be liberated from phospholipid membranes by phospholipase A2 to produce inflammatory eicosanoids (57, 58). Other mechanisms reported for EPA/DHA effects include altering the expression of transcription factors (i.e. NF-κB) and interacting with nuclear receptors (e.g. PPAR) involved in mediating their expression, which could impact inflammatory response and lipid metabolism (59). Anti-arrhythmic effects of ω3 PUFAs have also been shown to occur by reducing membrane electrical excitability and activity of voltage-gated Na^+ channels in cardiomyocytes (60). DHA and EPA can also be metabolized by COX and LOX enzymes into lipid mediators known as resolvins and protectins. These metabolites have been demonstrated in vivo in animals to have immunomodulatory effects that both guard against and promote recovery from inflammatory states (61). Overall, these findings demonstrate multiple mechanisms by which ω3 PUFAs may provide health benefits from dietary consumption. Furthermore, the ingestion of fish or fish oil are direct sources of ω3 PUFAs, thus bypassing the competition against ω6 PUFAs for enzymes involved in the AA pathway. Since humans cannot synthesize ALA or LA, and the conversion of ALA to EPA and DHA is very limited (5% for EPA and <0.5% for DHA), obtaining an adequate amount of PUFAs from food sources is essential to minimizing the production of inflammatory markers (62).

Cold-water fish and marine animals make up the core component of the Yup'ik diet that is enriched in ω3 PUFAs. One study reported an average dietary ω3 PUFA consumption of 3-4 g/day in Alaska Native people compared to the average consumption of 0.2 g/day by the American population (63). Early studies in the Greenland Inuit associated high dietary ω3 PUFA consumption with

hypocoagulability and further support for fish oils precipitating facile bleeding can be found in ongoing case reports of abnormal responses to the oral anticoagulant warfarin linked to ω3 PUFAs consumption (64). This awareness in the clinical community results in routine counseling of warfarin patients about the risk of bleeding events when taking oral anticoagulants and ω3 PUFAs concurrently, especially those patients with a history of acute bleeding episodes (i.e. hemorrhagic stroke). Controlled clinical trials including non-circumpolar populations however, have been inconclusive. For example, one small study (N = 40) that evaluated the effects of fish oil (4.0 g/day) on bleeding time over a 4-week period noted only one incidental case of minor nasal bleeding among the 22 study volunteers who were concurrently receiving oral anticoagulants (65). Another study of 610 study volunteers who received Omacor (DHA and EPA in fish oil) 4.0 g/day and either warfarin or aspirin for 12 months found only a modest increase in bleeding time (66). It is important to note that natural dietary sources of ω3 PUFAs may provide enhanced bioavailability and a wider spectrum of the bioactive agents compared to commercially available ω3 PUFA products that may contain only one active ingredient. These factors are important sources of variability that could be unaccounted for in clinical studies. Despite the extensive research on ω3 PUFAs over the last 30 years, there is still room to explore other environmental and genetic influences that may play a role in mediating a dietary impact on the manifestation of chronic disease, especially in underserved populations. Given the remote geographical location of the Yup'ik and our focus on hypocoagulability in this population, potentially restricted access to fresh green vegetables high in vitamin K is an important factor to consider. Together, with the ω3 PUFAs, there may also be other factors in the traditional Yup'ik diet or genome that confer their resistance against cardiovascular disease.

1.6 Vitamin K

The term "vitamin K" encompasses a group of fat-soluble vitamins that contain a common 2-methyl-1,-4-naphthoquinone nucleus with varying side chains at the C-3 position (67). Vitamin K exists naturally in two main forms, either as phylloquinone (vitamin K1) or the menaquinones (vitamin K2).

Phylloquinone contains a phytyl side chain and is the main circulating form in humans. The menaquinones are characterized by a series of unsaturated isoprenyl side-chain units and are designated MK-n based on the number of isoprenyl units. Menadione (vitamin K3) is a synthetic form of vitamin K that, unlike phylloquinone and the menaquinones, does not have an aliphatic side chain at C-3 (Figure 1.2). Vitamins K1 and K2 have similar physical and chemical properties. They are thermally stable, highly lipophilic (log P ~ 9) and readily soluble in many non-polar organic solvents, but insoluble in water. These compounds can be degraded by light and alkaline conditions (68, 69). Phylloquinone exists as an oil at room temperature whereas menadione and the menaquinones are solids.

The liver is the major organ in which most vitamin K compounds are stored. Both phylloquinone and the menaquinones play important physiological roles in modulating soft-tissue calcification and bone metabolism (70). However, the most well characterized role of vitamin K is in hemostasis through the γ-carboxylation of various vitamin K-dependent coagulation factors. As a key component of the vitamin K cycle (Figure 1.3), vitamin K is reduced to its hydroquinone that serves as the essential cofactor for the enzyme γ-glutamyl carboxylase (GGCX). This integral membrane enzyme is located in the endoplasmic reticulum and catalyzes γ-carboxylation of glutamate residues on vitamin K-dependent proteins (VKDP) to γ-carboxyglutamate (Gla). These negatively-charged Gla residues bind calcium ions at the N-terminus of the VKDP, inducing a conformational change that exposes hydrophobic residues. The Gla domain hydrophobic patch inserts into the interstitial region of the lipid bilayer, which helps facilitate anchoring of the Gla protein to phospholipid membranes containing phosphatidylserine during the initiation of the clotting cascade (71). Full carboxylation is required for activity of vitamin K-dependent proteins. Vitamin K 2,3-epoxide is a by-product of carboxylation that must be reduced back to the hydroquinone by Vitamin K epoxide reductase (VKOR) to initiate further cycles of Gla protein formation. Reduction of the epoxide to the hydroquinone involves two sequential reduction steps, both of which can be catalyzed by VKOR.

Phylloquinone is present in all plants and cyanobacteria where it serves as a cofactor for photosystem-I-mediated electron transport by chlorophyll during photosynthesis (72). Since chlorophyll

imparts green pigmentation, green leafy vegetables such as spinach (293–441 µg/100 g) and broccoli (76.6–136 µg/100 g) tend to have the highest phylloquinone content in contrast to vegetables that lack green color, such as carrots (3.9–14.8 µg/100 g) and potatoes (0.8-2.7 µg/100 g) (69). The overall phylloquinone content in vegetables can vary considerably based on geographical growth location, climate, soil condition, and stage of maturation. This can also be affected by the how the sample is prepared (i.e. raw or cooked) and which part of the plant is analyzed (i.e. inner vs. outer leaves) (73).

Unlike the sources of phylloquinone, the menaquinones are found in various fermented foods and cheese, as well eggs, meats and milk. These are synthesized in bacteria and participate in the redox reactions of bacterial respiration in the cytoplasmic membrane. Though menaquinones are known to stimulate vitamin-K-dependent carboxylase activity in vitro, phylloquinone appears to be the preferred substrate for Gla-protein formation (74). The serum half-life of menaquinones, particularly in those with more isoprenyl units, is generally longer than phylloquinone and may allow for greater extrahepatic tissue uptake (75). Colonic flora are known to specifically synthesize higher order menaquinones (i.e. MK-10 and above) and are thought to contribute to the overall menaquinone content in human liver (76, 77). Data from initially-germ free rats colonized with specific bacterial organisms showed that the types of menaquinone produced were a function of the bacterial strain. In the fecal matter of these rats, MK-9, MK-10, MK-11, was detected from *B. vulgatus* colonized rats, while MK-7 and MK-8 were present in the feces of rats colonized with *E.coli*.(78). Menaquinone concentrations in the livers of these rats were also reflective of the major menaquinone detected in feces: MK-10 in the *B. vulgatus* group and MK-8 in the *E. coli* group. Usui et al. reported that the menaquinones accounted for approximately 90% of the total hepatic vitamin K content in humans and speculated that they may be absorbed following synthesis by intestinal bacteria (79). However, the absorption of bacterial menaquinones in the human colon is expected to be poor due to the absence of bile salts normally found in the small intestine, which are necessary for vitamin K uptake (80). Menaquinones are also tightly associated with the bacterial cytoplasmic membrane, which could decrease their availability for absorption. Overall, the question of the precise source of human menaquinones needs further exploration.

Although menadione does not support γ-carboxylase activity, it is often listed as a vitamin K supplement or "source of vitamin K" in animal feed because it can be converted to other forms of vitamin K. Early work in the 1960's using chickens and rats demonstrated that the conversion of radiolabeled phylloquinone to MK-4 occurred through cleavage and replacement of ^{14}C-labeled side chain with an unlabeled geranylgeranyl side chain (81). Menadione was suspected to be an intermediate metabolite in this conversion pathway because urinary excretion of menadione increased after oral (but not subcutaneous) administration of phylloquinone, MK-4, and MK-7 in human volunteers (82). Okano et al. showed that, in mice, oral administration of phylloquinone and menadione that had both the ring and side chains labeled with deuterium, led to an increase in the concentration of MK-4 lacking deuterium in the side chain (83). The Okano group later identified a prenyltransferase enzyme, UBIAD1, as the MK-4 biosynthetic enzyme in humans following specific inhibition of UBIAD1 synthesis with short interfering RNA against the *UBIAD1* gene in osteoblast-like MG-63 cells (84). These researchers hypothesized that side-chain removal occurs after intestinal absorption of phylloquinone to release menadione in the bloodstream, where it is then transferred to peripheral tissues and subsequently prenylated by UBIAD1 to form MK-4 (85). However, this conversion mechanism is still under much debate.

During the commercial hydrogenation of polyunsaturated oils to produce trans fatty acids, phylloquinone present in vegetable oils undergoes saturation of the 2',3' double bond in the phytyl side chain to form dihydrophylloquinone (86). Since hydrogenated oils are found in commercially processed foods, fried foods, baked goods, and margarine, dihydrophylloquinone is also present in these sources and is detectable in human plasma (87). Dihydrophylloquinone was able to counteract warfarin-induced prolongation of prothrombin time (PT) in rats, suggesting that it is a cofactor for γ-carboxylation of Gla-residues on vitamin K-dependent coagulation factors (88). However, in contrast to phylloquinone, administration of dihydrophylloquinone did not to restore levels of PIVKA-II (protein-induced by vitamin K absence or antagonist II), a biomarker of vitamin K deficiency, to baseline in human study volunteers taking a controlled vitamin K restricted diet reported and was not as well absorbed (89). This suggests that dihydrophylloquinone has only a minor effect in promoting γ-carboxylation of VKDP in humans and

that the process of commercial hydrogenation decreases the effective contribution of food sources to vitamin K status.

Our knowledge regarding the absorption of oral vitamin K mostly derives from phylloquinone since it is the dominant circulating K vitamer. Phylloquinone is solubilized into mixed micelles after oral intake with the aid of bile and pancreatic secretions (90). Phylloquinone is then taken up into lymph lacteals after incorporation into chylomicrons, drains into larger lymphatic vessels and eventually enters the blood system via the thoracic duct. Once in the circulation, these chylomicrons acquire apolipoprotein C and E from high density lipoprotein (HDL). Endothelial lipoprotein lipase removes the triglycerides of chylomicrons and converts them into chylomicron remnants. In the liver, chylomicron remnants are taken up by receptor-mediated endocytosis and then degraded by lysosomes. Their lipids are then repackaged and released into circulation as VLDL and ultimately LDL for further transport to extrahepatic tissues. Phylloquinone can be detected in plasma 20-30 minutes after ingestion and peak levels are attained after 2-4 hours. The oral bioavailability of phylloquinone is approximately 40-80% (91, 92). The efficiency of absorption depends on the vehicle with which it is co-administered. The absorption of phylloquinone from vegetables was reported to be 1.5 times slower than when administered as a pharmaceutical formulation. This is not surprising because absorption of phylloquinone from plants would require more time for digestive breakdown of cellular membranes. The concurrent intake of butter was shown to increase the absorption of phylloquinone from spinach by nearly 3-fold, but had an even stronger effect on increasing the rate of menaquinone-4 entering the circulation (93). This is likely explained by the stimulation of bile salt release from the presence of fats in butter. Phylloquinone is rapidly cleared from plasma, and only 10-20% of the peak level remains after 24 hours (91).

Information about vitamin K and hemostasis has been frequently derived from studies with the oral anticoagulant drug, warfarin [3α-acetonylbenzyl-4-hydroxycoumarin], which exerts its therapeutic effects by inhibiting the recycling of vitamin K hydroquinone (94). Warfarin's inhibitory actions on VKOR decrease the formation of activated clotting factors, thereby minimizing the risk of potentially fatal clots in the vascular system. Although a highly efficacious anticoagulant, warfarin is known for

potentiating the risk of adverse bleeding events and patients must be monitored clinically to minimize these risks. Adequate anticoagulation control is achieved through monitoring the international normalized ratio (INR), a clinical parameter that assesses the prolongation of prothrombin time induced by warfarin. Maintaining an "in-range" INR is challenging due to a host of factors that include drug-drug and drug-food interactions, acute and chronic illness, as well as noncompliance (95-97). While much of the instability in INR is managed by regular clinic visits, overlaid on these issues is the question of genetic variability affecting warfarin dose requirements and the vitamin K cycle. Hereditary resistance to warfarin was recognized over 40 years ago, but it was not until the mid-late 1990s that the first studies appeared associating polymorphisms with warfarin sensitivity in the P450 enzyme, *CYP2C9*, which is primarily responsible for the metabolic clearance of the more active S-enantiomer of the drug (98-101). These reports stimulated much activity in the field which continued to gain momentum with the more recent discovery of common variation in the *VKORC1* gene as a major determinant of therapeutic warfarin dose (102, 103). A relatively common *VKORC1* gene haplotype consisting of 5 linked mutations (in European-Americans) is associated with reduced hepatic mRNA expression of the VKOR enzyme, which ultimately necessitates a lower warfarin dose to achieve a specified target INR. The "low dose" *VKORC1* haplotype is common among Asians (89%) and less frequent in Europeans (37%) and African-Americans (14%). Due to strong linkage disequilibrium between haplotype SNPs, the *-1639 G>A* SNP alone can classify individuals into high and low warfarin dose groups (104).

Recently, a second P450 gene, *CYP4F2*, has been shown to influence warfarin dose (105). Specifically, the *CYP4F2*3* (*V433M*) allele contributes to warfarin dose requirements, in that patients with a variant *TT* genotype required 1.0 mg/day more warfarin than those with a *CC* genotype. The minor "low activity" variant allele has a frequency of 30% in European Americans and Asians and 7% in African-Americans. The Rettie laboratory reported that CYP4F2 could act as vitamin K hydroxylase and suggested that the *V433M* variant conferred diminished hepatic enzyme activity towards reduced vitamin K1 as a result of changes in protein stability rather than alterations in the intrinsic function of the enzyme. Very recently CYP4F11, an "orphan" P450 enzyme, was identified as a second vitamin K hydroxylase,

however, the effect of variation in the *CYP4F11* gene on warfarin dose has yet to be explored (106). Known genetic variation in the *CYP2C9, VKORC1,* and *CYP4F2* genes accounts for ~40% of the inter-patient differences in warfarin dose requirements among European populations and other readily accessible clinical factors (e.g., age, gender, weight, diet, concomitant medications) account for another 20%. However, these known factors explain far less of the variability in warfarin dose requirements in Africans and African-Americans, demonstrating marked inter-racial differences in the factors controlling warfarin response (107, 108).

While data showing a modulating effect of ω3 PUFAs on the vitamin K cycle in humans is unclear, the evidence is available from animal studies. Rats treated with a diet enriched in EPA had lowered levels of vitamin K-dependent coagulation factors II and X (by 70% and 50% respectively) (109). In humans, fish oil also reduced factors VII and X, albeit to a modest extent (110-112). Because each of these coagulation factors depends on vitamin K-mediated γ-carboxylation for their activity, these results may point to an antagonistic effect of ω3 PUFAs on the vitamin K pathway. Also, given that the main dietary source of vitamin K is green leafy vegetables, which are only intermittently available in the remote sub-Arctic regions where the Yup'ik people live, it is plausible that this population may be more prone to a hypocoagulable state because they have low intake of vitamin K. There is currently little information about vitamin K status among indigenous groups of the Arctic regions, but it is possible that those who live in rural communities and adhere to a traditional diet could be especially prone to low vitamin K in blood, particularly during seasons when traditional non-marine dietary constituents such as "tundra greens" and seaweed are unavailable.

1.7 Assessment of Vitamin K Status

Much of the early investigations on vitamin K established its role in the regulation of coagulation protein activity. There is ongoing research looking into the role of vitamin K in physiological functions outside of hemostasis. Short-chain menaquinones (MK-3, MK-4, and MK-5), but not phylloquinone, are

reported to induce apoptosis in vitro in several cancer cell lines by inducing DNA damage, depolarization of mitochondrial membranes, and activation of the caspase 3 enzyme (113). Vitamin K has also been reported to play a role in maintaining bone mineral density by participating in the γ-carboxylation of osteocalcin, a VKDP secreted by osteoblasts. The intake of menaquinones may also promote activation of matrix Gla-protein that inhibits vascular calcification in the prevention of coronary heart disease (114).

Methods to determine vitamin K status have broadened the understanding of vitamin K's role in several physiological pathways. Direct assessment of vitamin K can be accomplished by analyzing blood or urine. Plasma phylloquinone levels are most commonly quantified since it is the predominant vitamin form in the diet of developed countries. However, very sensitive methods are required for plasma analysis because population levels range from 0.22 to 8.88 nmol/L with fasting levels averaging close to 1 nmol/L (115, 116). Over the years, numerous chromatographic techniques have been used to evaluate vitamin K disposition in vivo. Early chromatographic methods involved thin-layer chromatography to separate vitamin K compounds from their 2,3-epoxy derivatives, but complete separation proved to be unachievable (117). Gas-liquid chromatography methods have also been used but are limited by the low volatility of the K vitamers, long retention times, and very high column temperatures required for analyte elution that could lead to compound degradation (118). High performance liquid chromatography (HPLC) based methods replaced many of these shortcomings and offered reasonable separation and detection. Reversed-phase liquid chromatography with UV detection has been utilized to quantify phylloquinone added to infant formulas and milk with a lower limit of detection at 1 ng per injection (119). Improved sensitivity of vitamin K quantitation was accomplished with HPLC fluorescence detection via post-column chemical (platinum or zinc) reduction which converted vitamin K into its fluorescent hydroquinone form (120-122). The limit of sensitivity for this method was reported to be as low as 25 pg of phylloquinone per injection in the analysis of animal tissues, plasma, and food. However, the presence of sample impurities, even after sample purification steps, still presented difficulties for identification of appropriate internal standards for these assays, especially when working with low plasma levels of vitamin K. Liquid-chromatography-mass spectrometry (LC-MS) offers greater sensitivity and

specificity than previously mentioned methods when used in the multiple reaction monitoring (MRM) mode (123-125). Although LC-MS methods are more expensive for routine use, a key advantage of this method is the ability to use stable labeled isotopic standards, which are often deuterated. Isotopically labeled internal standards with similar chemical characteristics to the analyte can help minimize mass detector fluctuations, provide for similar elution patterns and improve quantitation. Limits of detection for LC/MS based assays were reported to be approximately 20 pg per injection (125).

Plasma phylloquinone concentration reflects dietary intake over the previous 24 hours, but is not an accurate indicator of long-term vitamin K status. PIVKA-II is a des-carboxylated form of prothrombin (clotting factor II) that is elevated in plasma under physiological conditions of vitamin K deficiency. PIVKA-II is inactive as a clotting factor because it lacks sufficient Gla-proteins bind to calcium and promote coagulation. It has a half-life of approximately 60 hours and concentrations are typically determined with an ELISA by a monoclonal antibody to PIVKA-II (126, 127). This biomarker is generally undetectable in the majority of healthy individuals and any values above 2 ng/mL may be considered to have subclinical vitamin K deficiency (128). In warfarin patients, PIVKA-II levels can be a biomarker for the anticoagulative effect because values increase sharply after 3 days of warfarin therapy and peak between days 4-7 (129). Aside from coagulation, PIVKA-II levels are known to be elevated in patients with gastric cancer, hepatocarcinoma, and alcoholic liver disease (130-132).

1.8 Hemostasis and the Clotting Cascade

Hemostasis is the physiological process by which the body works to prevent blood loss. It involves a number of components in the blood that interact to ultimately form the fibrin mesh in a blood clot. The sequence of events in hemostasis are categorized as; 1) vasoconstriction, 2) primary hemostasis (platelet-plug formation), and 3) secondary hemostasis (clotting cascade). The initiation of primary hemostasis is accomplished by interactions between blood vessel wall components and platelets, which are small cellular fragments of megakaryocytes formed in the bone marrow that both aggregate and

release stored granules that help facilitate the clotting process (133). Normally, platelets freely circulate through the blood vessel in their inactive states. The endothelium also maintains this inactive state by secreting products which inhibit platelet activation, such as nitric oxide and prostaglandin I2 (prostacyclin) (134). Following injury to the vessel wall, local vasoconstriction occurs to reduce blood flow, allowing platelets to adhere to the exposed subendothelial surface. Endothelial cells then release von Willebrand's factor (vWF), a large, multimeric glycoprotein that interacts with platelet glycoprotein 1b receptors and promotes platelet adhesion to the exposed subendothelial collegen. The interaction between platelets, vWF, and collagen causes platelets to swell and aggregate, ultimately leading to the secretion of intracellular granules. These granules contain calcium, vWF, ADP, P-selectin and serotonin that further stimulate activation of other platelets and clotting factors (135). Activated platelets also synthesize and release thromboxane A2 (TXA2) and platelet activating factor, both of which are potent platelet agonists and vasoconstrictors (136). Once the platelet monolayer has formed, additional platelets are recruited by fibrinogen and VWF using platelet glycoprotein IIb/IIIa receptors.

The initial response of platelets is often not enough to stop blood flow at the site of injury. To prevent dislodging by the high viscosity of the blood, the platelet plug is reinforced with fibrin mesh generated from secondary hemostasis (137, 138). The formation of fibrin occurs through a complex chain of reactions between clotting factors known as the clotting cascade (Figure 1.4) (139). Most clotting factors are produced in the liver and circulate in the plasma as the inactive forms. Clotting factors II, VII, IX, and X, activated protein C, and protein S specifically require vitamin K for synthesis. Clotting is driven by two separate routes; the intrinsic and extrinsic pathways, both of which converge to a common pathway of thrombin generation and fibrin polymerization. The extrinsic pathway is initiated from the exposure of clotting factors to tissue factor (TF) (140). As with vWF, TF normally remains within in the subendothelium and is released upon tissue damage. TF complexes with factor VIIa in the presence of calcium on phospholipid surfaces of platelets and other cells. This TF/VIIa complex activates Factor IX to IXa in the intrinsic pathway as well as promotes activation of factor X to Xa. Factor Xa only converts a small amount of factor II (prothrombin) to factor IIa (thrombin) due to the presence of lipoprotein-

associated tissue factor pathway inhibitor (141). However, this initial reaction is enough to amplify the coagulation response in several ways. Thrombin activates factors V, VIII, and IX in a positive feedback mechanism that accelerates thrombin formation. Thrombin also interacts with platelet receptor GpIb to promote platelet activation, aggregation, and granule release. The ultimate purpose of thrombin is to cleave soluble fibrinogen into fibrin monomer that then forms insoluble fibrin polymers. Thrombin also activates factor XIII, which covalently crosslinks the fibrin polymers into a fibrin mesh which helps stabilize the clot (139).

Negative regulation of coagulation response is achieved through several mechanisms especially as wound healing occurs. Circulating levels of antithrombin, a serine protease inhibitor, inactivates serine protease clotting factors (i.e factors IIa, IXa, Xa, XIa, and XIIa) and is potentiated by heparin (142, 143). Thrombin, through interaction with thrombomodulin on endothelial surfaces, triggers the activity of APC and its cofactor protein S, which in a negative feedback loop inactivates factors Va and VIIIa (144). The endothelium also produces tissue plasminogen activator (t-PA), which proteolytically cleaves plasminogen into plasmin. The primary role of plasmin is to dissolve the fibrin mesh of the thrombus into soluble fragments (145).

1.9 Measures of Coagulation and Clotting Activity

A wide variety of clinical tests are available to aid in the diagnosis, management, and monitoring of patients with bleeding disorders, or to aid in evaluating patients with thrombotic or fibrinolytic abnormalities. These tests are generally done in controlled laboratory settings because results are heavily influenced by sample collection, processing, and storage. Without proper handling, samples may undergo hemolysis, accumulate clots, or contain fibrin strands that render the sample unusable for analysis.

One of the most routinely used tests in the clinical setting is prothrombin time (146, 147). This test is commonly used to measure the efficacy of warfarin therapy because it is affected by vitamin K status. The PT measures the time necessary to generate fibrin after activation of factor VII. It is an assessment of the extrinsic and common pathways of the clotting cascade because it is initiated by the addition of TF. A thromboplastin reagent containing TF and phospholipid is first added to initiate a complex with factor VII. Calcium chloride solution is then added to replace the plasma calcium previously chelated by the citrate in the blood collection tube. As the factor VII-tissue thromboplastin complex activates factor X, the coagulation cascade proceeds into the common pathway and eventually to fibrin formation. The PT is timed in seconds (determined photo-optically) from when the calcium chloride is added to the plasma until the sample clots. The PT is commonly reported as the international normalized ratio since PT measurements vary depending on the commercial source of thromboplastin reagent that instrument used (148). The INR is calculated by dividing the test PT value by the established mean PT value of the normal population, which is typically 12 seconds. This value is then raised to the power of the International Sensitivity Index, which corrects for the relative strength of the thromboplastin reagent and that is provided by the manufacturer. By normalizing the PT as INR, the problems associated with inter-laboratory variability in the PT is reduced.

In contrast, the partial thromboplastin time (PTT) measures the time required for fibrin generation after initiation of the intrinsic pathway and is a direct measure of heparin activity (147). The PTT reagent containing contact activator (kaolin, silica, ellagic acid, or celite) and platelet phospholipid substitute is first added to the platelet-poor plasma sample. During the incubation period with this reagent, the intrinsic pathway is initiated by conversion of factor XII to XIIa, which converts factor XI to XIa. However, the cascade cannot continue since the subsequent conversion of factor IX to IXa requires calcium. After the addition of calcium chloride to the test sample, the clotting cascade proceeds to completion and clotting time is simultaneously recorded by measuring change the optical density of fibrin formation. Aside from pharmacologic influences, the PTT (as well as PT) can be prolonged when a plasma sample is deficient in clotting factors II, XII, IX, XI, VIII, X, V, and/or fibrinogen. Abnormal

results can also occur in the presence of a circulating inhibitor to any of the intrinsic pathway factors (149).

The PTT is also used in measuring clotting factor activity of sample plasma. The principal use of a coagulation factor assay is to assess ability of the sample plasma to normalize the prolonged clotting time of factor deficient plasma. A calibration curve is first created by preparing serial dilutions (e.g. doubling 1/10, 1/20, 1/40 etc.) of a standard commercial reference pooled plasma mixed with an equal volume of specific factor-deficient plasma, which contains normal levels of all other clotting factors but is deficient in the specific clotting factor being assayed. The PTT is performed on each dilution to create a standard curve designated with a reference factor activity of 100%. The same procedure is done with the test sample plasma and the percent activity is compared relative to the standard curve. Most samples from healthy individuals that have clotting factor activities >60% and values <30% may be indicative of clotting factor deficiency or hemophilia (150-152).

Platelet function tests serve as an alternative method of assessing hemostatic function. Appropriate platelet response is essential to preventing blood loss and diseases that over or under-compensate platelet function can lead to excess bleeding or tissue injury. Determining platelet activity can provide insight on vascular disease progression, inflammatory status, and bleeding tendencies that are otherwise not detected by other coagulation assays. Early platelet function tests of bleeding time involved inflating a blood pressure cuff on the forearm whereby the clinician would make small cuts in the lower arm to measure the time until bleeding is halted (153). A more current and commonly employed test requires a blood sample to obtain platelet-rich plasma (PRP) after centrifugation (154). A platelet aggregating agent such as ADP or collagen is added to the PRP sample and aggregation activity is interpreted by a photometer. Platelet counts are another useful diagnostic tool to assess bleeding disorders. An anticlotting agent, typically EDTA, is added to the blood sample and counting can be accomplished manually under a microscope or automatically by electronic instruments (155). However, a major limitation of these methods is that fresh samples are required and must be analyzed within hours after collection.

P-selectin is a cell adhesion molecule released from platelets that promotes cell-to-cell contact at sites of tissue injury and inflammation (156). Since P-selectin is stored in the α-granules of platelets and only mobilized to the plasma membrane upon stimulation, it has been used widely in clinical studies as a biomarker of platelet activation (157). Measurement of soluble P-selectin (sP-selectin) levels from plasma is accomplished by ELISA and unlike other platelet function assays, can be determined from frozen samples. This is particularly important for studies such as ours where samples are collected in the field (remote Alaskan villages) and need to be shipped to a distant locale for analysis. Similarly to platelets, studies have suggested that sP-selectin may be a useful biomarker of endothelial dysfunction in cardiovascular and chronic inflammatory disease. Higher levels of sP-selectin have been shown to be associated with increased risk of future cardiovascular events, obesity, and hypercholesterolima (158-160). Moreover, sP-selectin can be affected by the consumption of ω3 PUFAs. Eschen et al. reported a dose-dependent decrease of sP-selectin levels in healthy study volunteers after 12 weeks of ω3 PUFA supplementation (161). A smaller study by McEwen et al. similarly observed reduced sP-selectin expression in platelets from healthy volunteers after four weeks of 640 mg daily ω3 PUFA supplementation (162). A study by Moertl et al. involving 36 chronic heart failure patients found that P-selectin levels decreased after 12 weeks of 4 g/day ω3 PUFA treatment but not with 1 g/day (163). These findings support a role for sP-selectin as an inflammatory biomarker, but its level may be reflective of dietary practices involving ω3 PUFAs consumption.

1.10 Scope of Thesis

The overall goal of this thesis is to characterize unique gene and environment interactions that influence blood coagulation processes in Yup'ik people. The Westernization of the Yup'ik culture has introduced many changes in their dietary patterns and until recently, little scientific research has been done to understand how these changes impact health patterns in this population. Our collaborators at the University of Alaska Fairbanks previously initiated major research efforts to study dietary and

environmental aspects of Yup'ik lifestyle practices, and to build trust-based relationships conducive to original health-related research in this community. The work presented here expands these efforts by focusing on mechanisms underlying their historical bleeding phenotype. This has involved a thorough characterization in the Yup'ik people, of the numerous population coagulation indices and polymorphisms in genes/enzymes of the vitamin K cycle described above, together with an assessment of the impact of this population's high dietary intake of ω3 PUFAs due to their traditional marine animal-based (fish, seal etc.) diet. In Chapter 2, we describe associations between a high ω3 PUFA intake and several plasma coagulation indices including platelet activity markers and both vitamin K-dependent and independent clotting factors. Chapter 3 reports an evaluation of the vitamin K status in the Yup'ik, through the development of highly sensitive stable-isotope labeled mass spectrometry assays for quantification of the vitamin K content of study participant plasma as well as the "tundra greens" that are regularly consumed in the Yup'ik diet. Finally, in Chapter 4, we assess potential interactions between vitamin K status and vitamin K cycle polymorphisms and measure hepatic vitamin K levels from two sources. We report measurements from human liver samples from the UW School of Pharmacy Liver Bank to determine whether an association exists between common *CYP4F2* and *VKORC1* polymorphisms and liver stores of vitamin K. Additionally, we assess whether hepatic vitamin K content in mice differs between wild-type and knockouts of *Cyp4f14*, a mouse ortholog of *CYP4F2*. Results from these studies have greatly expanded our knowledge of the interplay between key clinical, disease and environmental modifiers in the context of underlying genetic variation that may contribute to health outcomes in this remote, population which is underserved in terms of contemporary application of genetic information to improving health outcomes.

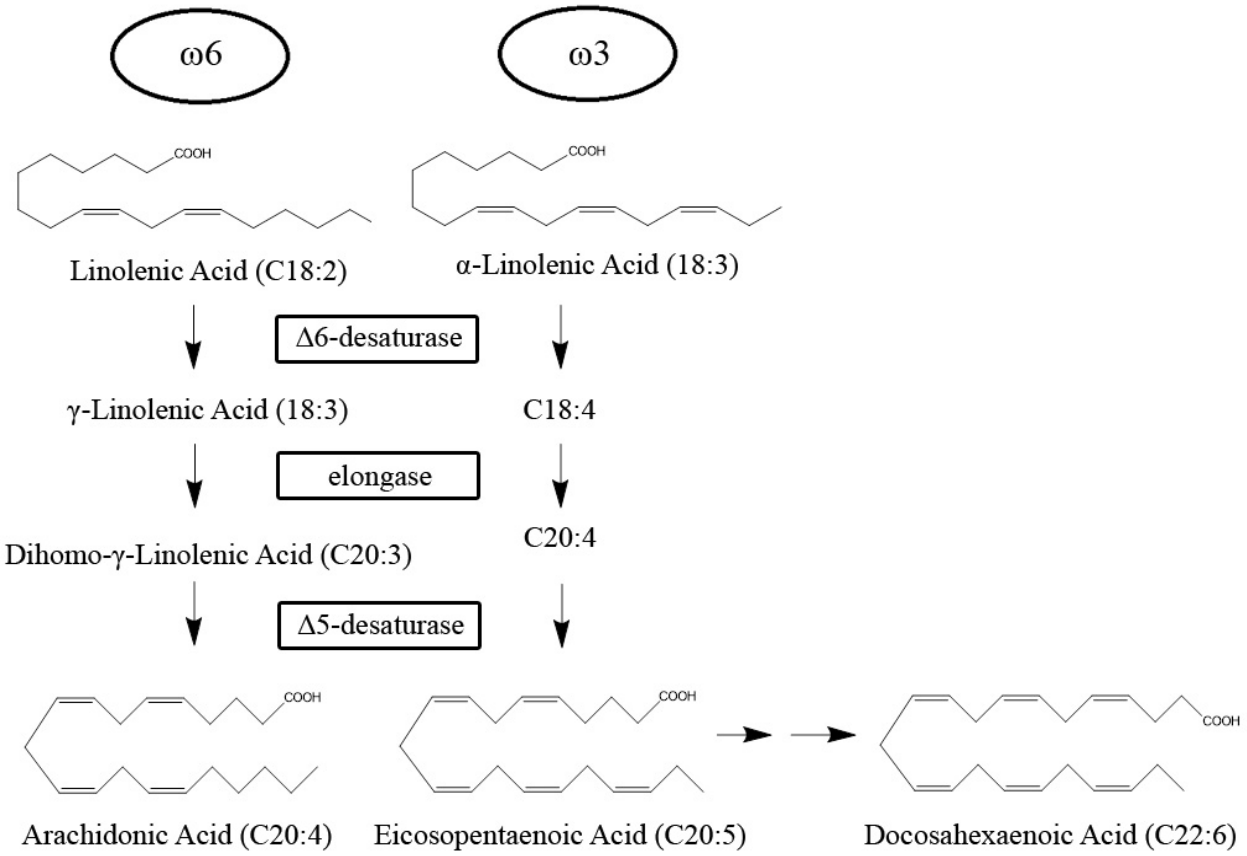

Figure 1.1. The metabolism of linolenic acid into arachidonic acid. This metabolic pathway utilizes the same set of enzymes in the formation of eicosopentaenoic acid and docosahexaenoic acid from α-linolenic acid.

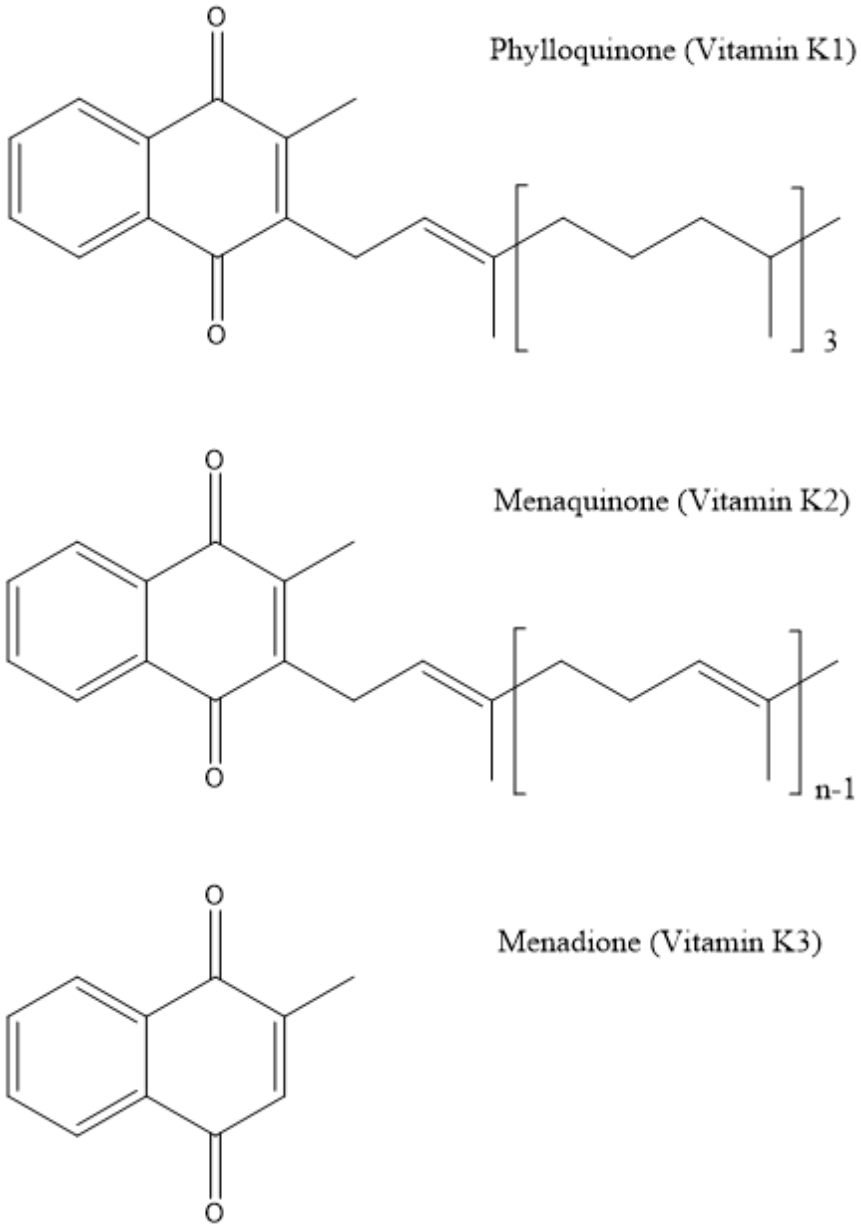

Figure 1.2. The chemical structures of phylloquinone, menaquinones, and menadione. Phylloquinone possesses a phytyl side chain whereas menaquinones vary in the length of the isoprenyl chain. Menadione must be chemically synthesized and does not contain an aliphatic side chain.

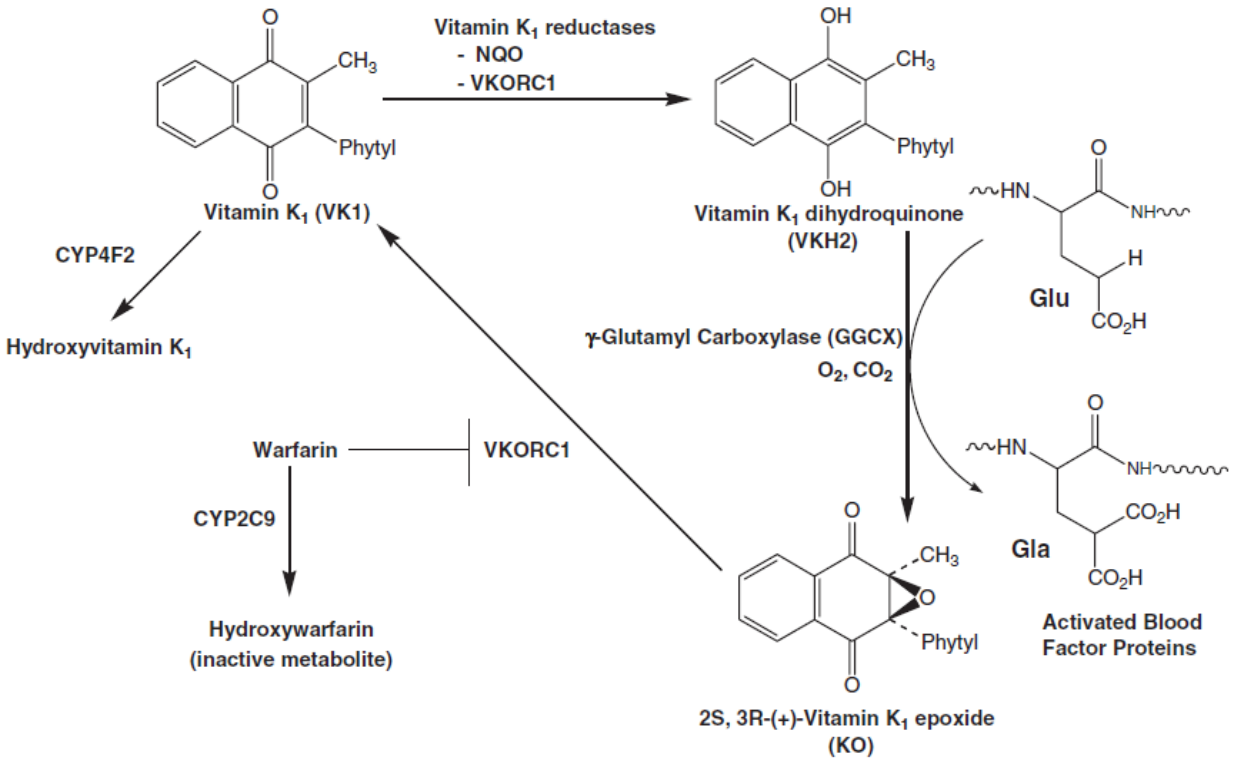

Figure 1.3. The vitamin K cycle. Vitamin K1 dihydroquinone (VKH2) is the essential cofactor to γ-glutamyl carboxylase, the enzyme responsible for activating several blood factor proteins through the carboxylation of key Glu residues on those proteins, which initiates the blood coagulation cascade response. During this process, VKH2 is oxidized to vitamin K epoxide (VKO), which must then undergo two $2e^-$ reductions to regenerate the active cofactor. Vitamin K1 epoxide reductase (VKORC1), the warfarin target enzyme, is known to catalyze at least the first of these reductions, generating VK1 from VKO. VKORC1 is also the likely biological reductant in the conversion of VK1 back to VKH2, but it is possible that other enzymes, such as NADPH quinone oxidoreductase (NQO), might be involved in this step. Variants of *CYP2C9*, *CYP4F2*, and *VKORC1* have been reported to influence dosing requirements of warfarin patients (164).

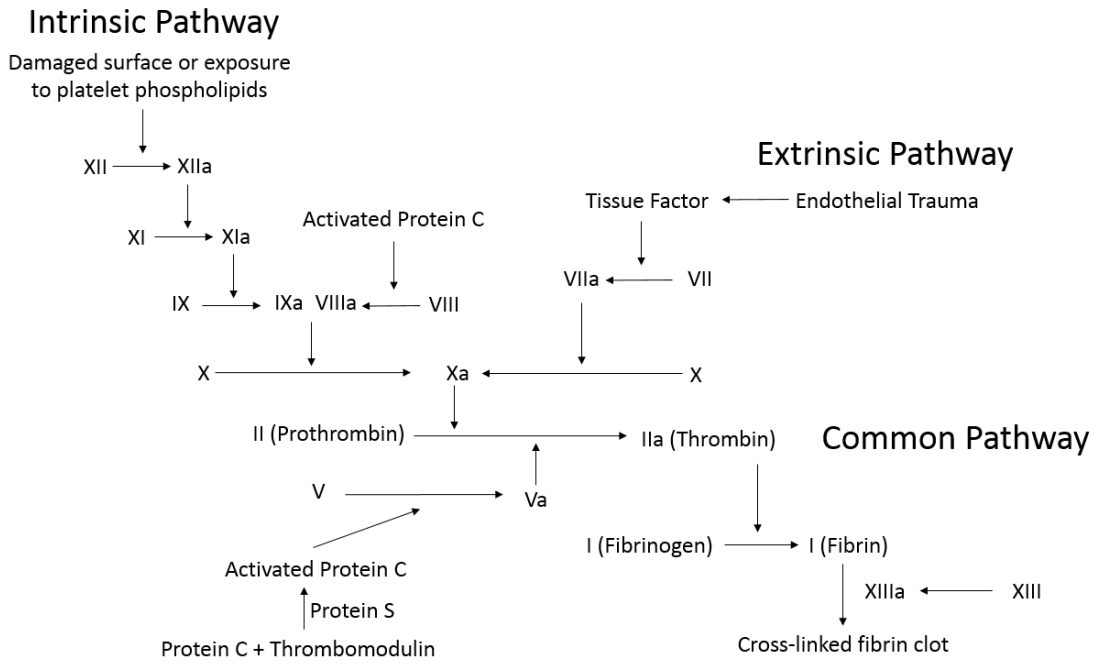

Figure 1.4. The components of the clotting cascade. The generation of thrombin can be divided into the intrinsic and extrinsic pathways that converge to the common pathway. Activated protein C and its cofactor, protein S, act as a negative feedback mechanism to the clotting cascade through selective inactivation of factors Va and VIIIa.

1.11 Notes to Chapter 1

1. Ventola, C. L. (2011) Pharmacogenomics in clinical practice: reality and expectations, *P & T : a peer-reviewed journal for formulary management 36*, 412-450.

2. Vogenberg, F. R., Barash, C. I., and Pursel, M. (2010) Personalized medicine: part 2: ethical, legal, and regulatory issues, *P & T : a peer-reviewed journal for formulary management 35*, 624-642.

3. Kalow, W., Tang, B. K., and Endrenyi, L. (1998) Hypothesis: comparisons of inter- and intra-individual variations can substitute for twin studies in drug research, *Pharmacogenetics 8*, 283-289.

4. Nebert, D. W. (1999) Pharmacogenetics and pharmacogenomics: why is this relevant to the clinical geneticist?, *Clinical genetics 56*, 247-258.

5. Alving, A. S., Carson, P. E., Flanagan, C. L., and Ickes, C. E. (1956) Enzymatic deficiency in primaquine-sensitive erythrocytes, *Science 124*, 484-485.

6. Peters, A. L., and Van Noorden, C. J. (2009) Glucose-6-phosphate dehydrogenase deficiency and malaria: cytochemical detection of heterozygous G6PD deficiency in women, *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 57*, 1003-1011.

7. Mahgoub, A., Idle, J. R., Dring, L. G., Lancaster, R., and Smith, R. L. (1977) Polymorphic hydroxylation of Debrisoquine in man, *Lancet 2*, 584-586.

8. Eichelbaum, M., Spannbrucker, N., Steincke, B., and Dengler, H. J. (1979) Defective N-oxidation of sparteine in man: a new pharmacogenetic defect, *European journal of clinical pharmacology 16*, 183-187.

9. Skoda, R. C., Gonzalez, F. J., Demierre, A., and Meyer, U. A. (1988) Two mutant alleles of the human cytochrome P-450db1 gene (P450C2D1) associated with genetically deficient metabolism of debrisoquine and other drugs, *Proceedings of the National Academy of Sciences of the United States of America 85*, 5240-5243.

10. Kagimoto, M., Heim, M., Kagimoto, K., Zeugin, T., and Meyer, U. A. (1990) Multiple mutations of the human cytochrome P450IID6 gene (CYP2D6) in poor metabolizers of debrisoquine. Study of the functional significance of individual mutations by expression of chimeric genes, *The Journal of biological chemistry 265*, 17209-17214.

11. Beverage, J. N., Sissung, T. M., Sion, A. M., Danesi, R., and Figg, W. D. (2007) CYP2D6 polymorphisms and the impact on tamoxifen therapy, *Journal of pharmaceutical sciences 96*, 2224-2231.

12. Samer, C. F., Lorenzini, K. I., Rollason, V., Daali, Y., and Desmeules, J. A. (2013) Applications of CYP450 testing in the clinical setting, *Molecular diagnosis & therapy 17*, 165-184.

13. Martin, M. A., and Kroetz, D. L. (2013) Abacavir pharmacogenetics--from initial reports to standard of care, *Pharmacotherapy 33*, 765-775.

14. Kumler, I., Tuxen, M. K., and Nielsen, D. L. (2014) A systematic review of dual targeting in HER2-positive breast cancer, *Cancer treatment reviews 40*, 259-270.

15. Kazui, M., Nishiya, Y., Ishizuka, T., Hagihara, K., Farid, N. A., Okazaki, O., Ikeda, T., and Kurihara, A. (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite, *Drug metabolism and disposition: the biological fate of chemicals 38*, 92-99.

16. Lins, R., Broekhuysen, J., Necciari, J., and Deroubaix, X. (1999) Pharmacokinetic profile of 14C-labeled clopidogrel, *Seminars in thrombosis and hemostasis 25 Suppl 2*, 29-33.

17. Ancrenaz, V., Daali, Y., Fontana, P., Besson, M., Samer, C., Dayer, P., and Desmeules, J. (2010) Impact of genetic polymorphisms and drug-drug interactions on clopidogrel and prasugrel response variability, *Current drug metabolism 11*, 667-677.

18. Collet, J. P., Hulot, J. S., Pena, A., Villard, E., Esteve, J. B., Silvain, J., Payot, L., Brugier, D., Cayla, G., Beygui, F., Bensimon, G., Funck-Brentano, C., and Montalescot, G. (2009) Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study, *Lancet 373*, 309-317.

19. Brandt, J. T., Close, S. L., Iturria, S. J., Payne, C. D., Farid, N. A., Ernest, C. S., 2nd, Lachno, D. R., Salazar, D., and Winters, K. J. (2007) Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel, *Journal of thrombosis and haemostasis : JTH 5*, 2429-2436.

20. Hulot, J. S., Bura, A., Villard, E., Azizi, M., Remones, V., Goyenvalle, C., Aiach, M., Lechat, P., and Gaussem, P. (2006) Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects, *Blood 108*, 2244-2247.

21. Gladding, P., Panattoni, L., Webster, M., Cho, L., and Ellis, S. (2010) Clopidogrel pharmacogenomics: next steps: a clinical algorithm, gene-gene interactions, and an elusive outcomes trial, *JACC. Cardiovascular interventions 3*, 995-1000.

22. Man, M., Farmen, M., Dumaual, C., Teng, C. H., Moser, B., Irie, S., Noh, G. J., Njau, R., Close, S., Wise, S., and Hockett, R. (2010) Genetic variation in metabolizing enzyme and transporter genes: comprehensive assessment in 3 major East Asian subpopulations with comparison to Caucasians and Africans, *Journal of clinical pharmacology 50*, 929-940.

23. Meyer, U. A., and Zanger, U. M. (1997) Molecular mechanisms of genetic polymorphisms of drug metabolism, *Annual review of pharmacology and toxicology 37*, 269-296.

24. Veiga, R. V., and Taylor, R. E. (1986) Beta blockers, hypertension, and blacks--is the answer really in?, *Journal of the National Medical Association 78*, 851-856.

25. Liggett, S. B., Cresci, S., Kelly, R. J., Syed, F. M., Matkovich, S. J., Hahn, H. S., Diwan, A., Martini, J. S., Sparks, L., Parekh, R. R., Spertus, J. A., Koch, W. J., Kardia, S. L., and Dorn, G. W., 2nd. (2008) A GRK5 polymorphism that inhibits beta-adrenergic receptor signaling is protective in heart failure, *Nature medicine 14*, 510-517.

26. Cresci, S., Dorn, G. W., 2nd, Jones, P. G., Beitelshees, A. L., Li, A. Y., Lenzini, P. A., Province, M. A., Spertus, J. A., and Lanfear, D. E. (2012) Adrenergic-pathway gene variants influence beta-blocker-related outcomes after acute coronary syndrome in a race-specific manner, *Journal of the American College of Cardiology 60*, 898-907.

27. Humes, K. R., Jones, N. A., Ramirez, R. R., and United States. Bureau of the Census. (2011) *Overview of race and Hispanic origin : 2010*, U.S. Dept. of Commerce, Economics and Statistics Administration, U.S. Census Bureau, Washington, D.C.

28. Pacheco, C. M., Daley, S. M., Brown, T., Filippi, M., Greiner, K. A., and Daley, C. M. (2013) Moving forward: breaking the cycle of mistrust between American Indians and researchers, *American journal of public health 103*, 2152-2159.

29. Fohner, A., Muzquiz, L. I., Austin, M. A., Gaedigk, A., Gordon, A., Thornton, T., Rieder, M. J., Pershouse, M. A., Putnam, E. A., Howlett, K., Beatty, P., Thummel, K. E., and Woodahl, E. L. (2013) Pharmacogenetics in American Indian populations: analysis of CYP2D6, CYP3A4, CYP3A5, and CYP2C9 in the Confederated Salish and Kootenai Tribes, *Pharmacogenetics and genomics 23*, 403-414.

30. Ottman, R. (1996) Gene-environment interaction: definitions and study designs, *Prev Med 25*, 764-770.

31. Irons, D. E., Iacono, W. G., Oetting, W. S., and McGue, M. (2012) Developmental Trajectory and Environmental Moderation of the Effect of ALDH2 Polymorphism on Alcohol Use, *Alcohol Clin Exp Res*.

32. Vandenbroucke, J. P., Koster, T., Briet, E., Reitsma, P. H., Bertina, R. M., and Rosendaal, F. R. (1994) Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation, *Lancet 344*, 1453-1457.

33. Mohatt, G. V., Plaetke, R., Klejka, J., Luick, B., Lardon, C., Bersamin, A., Hopkins, S., Dondanville, M., Herron, J., and Boyer, B. (2007) The Center for Alaska Native Health Research Study: a community-based participatory research study of obesity and chronic disease-related protective and risk factors, *International journal of circumpolar health 66*, 8-18.

34. Middaugh, J. P. (1990) Cardiovascular deaths among Alaskan Natives, 1980-86, *American journal of public health 80*, 282-285.

35. Adler, A. I., Boyko, E. J., Schraer, C. D., and Murphy, N. J. (1994) Lower prevalence of impaired glucose tolerance and diabetes associated with daily seal oil or salmon consumption among Alaska Natives, *Diabetes care 17*, 1498-1501.

36. Bersamin, A., Zidenberg-Cherr, S., Stern, J. S., and Luick, B. R. (2007) Nutrient intakes are associated with adherence to a traditional diet among Yup'ik Eskimos living in remote Alaska Native communities: the CANHR Study, *International journal of circumpolar health 66*, 62-70.

37. Bersamin, A., Luick, B. R., King, I. B., Stern, J. S., and Zidenberg-Cherr, S. (2008) Westernizing diets influence fat intake, red blood cell fatty acid composition, and health in remote Alaskan Native communities in the center for Alaska Native health study, *Journal of the American Dietetic Association 108*, 266-273.

38. Kuhnlein, H. V., Receveur, O., Soueida, R., and Egeland, G. M. (2004) Arctic indigenous peoples experience the nutrition transition with changing dietary patterns and obesity, *The Journal of nutrition 134*, 1447-1453.

39. McLaughlin, J. B., Middaugh, J. P., Utermohle, C. J., Asay, E. D., Fenaughty, A. M., and Eberhart-Phillips, J. E. (2004) Changing patterns of risk factors and mortality for coronary heart disease among Alaska Natives, 1979-2002, *Jama 291*, 2545-2546.

40. Murphy, N. J., Schraer, C. D., Thiele, M. C., Boyko, E. J., Bulkow, L. R., Doty, B. J., and Lanier, A. P. (1995) Dietary change and obesity associated with glucose intolerance in Alaska Natives, *Journal of the American Dietetic Association 95*, 676-682.

41. Dyerberg, J., and Bang, H. O. (1979) Haemostatic function and platelet polyunsaturated fatty acids in Eskimos, *Lancet 2*, 433-435.

42. Bang, H. O., Dyerberg, J., and Nielsen, A. B. (1971) Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos, *Lancet 1*, 1143-1145.

43. Bang, H. O., Dyerberg, J., and Sinclair, H. M. (1980) The composition of the Eskimo food in north western Greenland, *The American journal of clinical nutrition 33*, 2657-2661.

44. Bang, H. O., and Dyerberg, J. (1980) The bleeding tendency in Greenland Eskimos, *Danish medical bulletin 27*, 202-205.

45. Newman, W. P., Middaugh, J. P., Propst, M. T., and Rogers, D. R. (1993) Atherosclerosis in Alaska Natives and non-natives, *Lancet 341*, 1056-1057.

46. Harnack, K., Andersen, G., and Somoza, V. (2009) Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids, *Nutrition & metabolism 6*, 8.

47. Simopoulos, A. P. (1999) Essential fatty acids in health and chronic disease, *The American journal of clinical nutrition 70*, 560S-569S.

48. Eaton, S. B., and Konner, M. (1985) Paleolithic nutrition. A consideration of its nature and current implications, *The New England journal of medicine 312*, 283-289.

49. (1988) Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. The Expert Panel, *Archives of internal medicine 148*, 36-69.

50. Gomez Candela, C., Bermejo Lopez, L. M., and Loria Kohen, V. (2011) Importance of a balanced omega 6/omega 3 ratio for the maintenance of health: nutritional recommendations, *Nutricion hospitalaria 26*, 323-329.

51. Das, U. N. (2005) Can COX-2 inhibitor-induced increase in cardiovascular disease risk be modified by essential fatty acids?, *The Journal of the Association of Physicians of India 53*, 623-627.

52. Johnson, M. M., Swan, D. D., Surette, M. E., Stegner, J., Chilton, T., Fonteh, A. N., and Chilton, F. H. (1997) Dietary supplementation with gamma-linolenic acid alters fatty acid content and eicosanoid production in healthy humans, *The Journal of nutrition 127*, 1435-1444.

53. Hashimoto, M., Hossain, S., Yamasaki, H., Yazawa, K., and Masumura, S. (1999) Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells, *Lipids 34*, 1297-1304.

54. Zerouga, M., Jenski, L. J., and Stillwell, W. (1995) Comparison of phosphatidylcholines containing one or two docosahexaenoic acyl chains on properties of phospholipid monolayers and bilayers, *Biochimica et biophysica acta 1236*, 266-272.

55. Stillwell, W., and Wassall, S. R. (2003) Docosahexaenoic acid: membrane properties of a unique fatty acid, *Chemistry and physics of lipids 126*, 1-27.

56. Stillwell, W., Shaikh, S. R., Zerouga, M., Siddiqui, R., and Wassall, S. R. (2005) Docosahexaenoic acid affects cell signaling by altering lipid rafts, *Reproduction, nutrition, development 45*, 559-579.

57. Khairallah, R. J., Kim, J., O'Shea, K. M., O'Connell, K. A., Brown, B. H., Galvao, T., Daneault, C., Des Rosiers, C., Polster, B. M., Hoppel, C. L., and Stanley, W. C. (2012) Improved mitochondrial function with diet-induced increase in either docosahexaenoic acid or arachidonic acid in membrane phospholipids, *PloS one 7*, e34402.

58. Arterburn, L. M., Hall, E. B., and Oken, H. (2006) Distribution, interconversion, and dose response of n-3 fatty acids in humans, *The American journal of clinical nutrition 83*, 1467S-1476S.

59. Adkins, Y., and Kelley, D. S. (2010) Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids, *The Journal of nutritional biochemistry 21*, 781-792.

60. Leaf, A., Kang, J. X., Xiao, Y. F., and Billman, G. E. (2003) Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils, *Circulation 107*, 2646-2652.

61. Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G., and Moussignac, R. L. (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals, *The Journal of experimental medicine 196*, 1025-1037.

62. Plourde, M., and Cunnane, S. C. (2007) Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements, *Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 32*, 619-634.

63. Ebbesson, S. O., Risica, P. M., Ebbesson, L. O., and Kennish, J. M. (2005) Eskimos have CHD despite high consumption of omega-3 fatty acids: the Alaska Siberia project, *International journal of circumpolar health 64*, 387-395.

64. McClaskey, E. M., and Michalets, E. L. (2007) Subdural hematoma after a fall in an elderly patient taking high-dose omega-3 fatty acids with warfarin and aspirin: case report and review of the literature, *Pharmacotherapy 27*, 152-160.

65. Smith, P., Arnesen, H., Opstad, T., Dahl, K. H., and Eritsland, J. (1989) Influence of highly concentrated n-3 fatty acids on serum lipids and hemostatic variables in survivors of myocardial infarction receiving either oral anticoagulants or matching placebo, *Thrombosis research 53*, 467-474.

66. Eritsland, J., Arnesen, H., Gronseth, K., Fjeld, N. B., and Abdelnoor, M. (1996) Effect of dietary supplementation with n-3 fatty acids on coronary artery bypass graft patency, *The American journal of cardiology 77*, 31-36.

67. Holmes, M. V., Hunt, B. J., and Shearer, M. J. (2012) The role of dietary vitamin K in the management of oral vitamin K antagonists, *Blood reviews 26*, 1-14.

68. Lambert, W. E., Vanneste, L., and De Leenheer, A. P. (1992) Enzymatic sample hydrolysis and HPLC in a study of phylloquinone concentration in human milk, *Clinical chemistry 38*, 1743-1748.

69. Booth, S. L., and Sadowski, J. A. (1997) Determination of phylloquinone in foods by high-performance liquid chromatography, *Methods in enzymology 282*, 446-456.

70. Shearer, M. J., and Newman, P. (2008) Metabolism and cell biology of vitamin K, *Thrombosis and haemostasis 100*, 530-547.

71. Huang, M., Rigby, A. C., Morelli, X., Grant, M. A., Huang, G., Furie, B., Seaton, B., and Furie, B. C. (2003) Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins, *Nat Struct Biol 10*, 751-756.

72. Gross, J., Cho, W. K., Lezhneva, L., Falk, J., Krupinska, K., Shinozaki, K., Seki, M., Herrmann, R. G., and Meurer, J. (2006) A plant locus essential for phylloquinone (vitamin K1) biosynthesis originated from a fusion of four eubacterial genes, *The Journal of biological chemistry 281*, 17189-17196.

73. Ferland, G. (2012) The discovery of vitamin K and its clinical applications, *Annals of nutrition & metabolism 61*, 213-218.

74. Buitenhuis, H. C., Soute, B. A., and Vermeer, C. (1990) Comparison of the vitamins K1, K2 and K3 as cofactors for the hepatic vitamin K-dependent carboxylase, *Biochimica et biophysica acta 1034*, 170-175.

75. Schurgers, L. J., and Vermeer, C. (2000) Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations, *Haemostasis 30*, 298-307.

76. Conly, J. M., and Stein, K. (1992) The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis, *Progress in food & nutrition science 16*, 307-343.

77. Ramotar, K., Conly, J. M., Chubb, H., and Louie, T. J. (1984) Production of menaquinones by intestinal anaerobes, *The Journal of infectious diseases 150*, 213-218.

78. Kindberg, C., Suttie, J. W., Uchida, K., Hirauchi, K., and Nakao, H. (1987) Menaquinone production and utilization in germ-free rats after inoculation with specific organisms, *The Journal of nutrition 117*, 1032-1035.

79. Usui, Y., Tanimura, H., Nishimura, N., Kobayashi, N., Okanoue, T., and Ozawa, K. (1990) Vitamin K concentrations in the plasma and liver of surgical patients, *The American journal of clinical nutrition 51*, 846-852.

80. Lipsky, J. J. (1988) Antibiotic-associated hypoprothrombinaemia, *The Journal of antimicrobial chemotherapy 21*, 281-300.

81. Martius, C. (1961) The metabolic relationships between the different K vitamins and the synthesis of the ubiquinones, *The American journal of clinical nutrition 9(4)Pt 2*, 97-103.

82. Thijssen, H. H., Vervoort, L. M., Schurgers, L. J., and Shearer, M. J. (2006) Menadione is a metabolite of oral vitamin K, *The British journal of nutrition 95*, 260-266.

83. Okano, T., Shimomura, Y., Yamane, M., Suhara, Y., Kamao, M., Sugiura, M., and Nakagawa, K. (2008) Conversion of phylloquinone (Vitamin K1) into menaquinone-4 (Vitamin K2) in mice: two possible routes for menaquinone-4 accumulation in cerebra of mice, *The Journal of biological chemistry 283*, 11270-11279.

84. Nakagawa, K., Hirota, Y., Sawada, N., Yuge, N., Watanabe, M., Uchino, Y., Okuda, N., Shimomura, Y., Suhara, Y., and Okano, T. (2010) Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme, *Nature 468*, 117-121.

85. Okano, T., Nakagawa, K., and Kamao, M. (2009) [In vivo metabolism of vitamin K: in relation to the conversion of vitamin K1 to MK-4], *Clinical calcium 19*, 1779-1787.

86. Davidson, K. W., Booth, S. L., Dolnikowski, G. G., and Sadowski, J. A. (1996) The conversion of phylloquinone to 2′,3′-dihydrophylloquinone during hydrogenation of vegetable oils, *Journal of agricultural and food chemistry 44*, 980-983.

87. Booth, S. L., Davidson, K. W., Lichtenstein, A. H., and Sadowski, J. A. (1996) Plasma concentrations of dihydro-vitamin K1 following dietary intake of a hydrogenated vitamin K1-rich vegetable oil, *Lipids 31*, 709-713.

88. Sato, T., Ozaki, R., Kamo, S., Hara, Y., Konishi, S., Isobe, Y., Saitoh, S., and Harada, H. (2003) The biological activity and tissue distribution of 2',3'-dihydrophylloquinone in rats, *Biochimica et biophysica acta 1622*, 145-150.

89. Booth, S. L., Lichtenstein, A. H., O'Brien-Morse, M., McKeown, N. M., Wood, R. J., Saltzman, E., and Gundberg, C. M. (2001) Effects of a hydrogenated form of vitamin K on bone formation and resorption, *The American journal of clinical nutrition 74*, 783-790.

90. Shearer, M. J., McBurney, A., and Barkhan, P. (1974) Studies on the absorption and metabolism of phylloquinone (vitamin K1) in man, *Vitamins and hormones 32*, 513-542.

91. Barkhan, P., and Shearer, M. J. (1977) Metabolism of vitamin K1 (phylloquinone) in man, *Proceedings of the Royal Society of Medicine 70*, 93-96.

92. Olson, R. E. (1984) The function and metabolism of vitamin K, *Annual review of nutrition 4*, 281-337.

93. Gijsbers, B. L., Jie, K. S., and Vermeer, C. (1996) Effect of food composition on vitamin K absorption in human volunteers, *The British journal of nutrition 76*, 223-229.

94. Au, N., and Rettie, A. E. (2008) Pharmacogenomics of 4-hydroxycoumarin anticoagulants, *Drug Metab Rev 40*, 355-375.

95. Demirkan, K., Stephens, M. A., Newman, K. P., and Self, T. H. (2000) Response to warfarin and other oral anticoagulants: effects of disease states, *Southern medical journal 93*, 448-454; quiz 455.

96. Holbrook, A. M., Pereira, J. A., Labiris, R., McDonald, H., Douketis, J. D., Crowther, M., and Wells, P. S. (2005) Systematic overview of warfarin and its drug and food interactions, *Arch Intern Med 165*, 1095-1106.

97. Kimmel, S. E., Chen, Z., Price, M., Parker, C. S., Metlay, J. P., Christie, J. D., Brensinger, C. M., Newcomb, C. W., Samaha, F. F., and Gross, R. (2007) The influence of patient adherence on anticoagulation control with warfarin: results from the International Normalized Ratio Adherence and Genetics (IN-RANGE) Study, *Arch Intern Med 167*, 229-235.

98. Furuya, H., Fernandez-Salguero, P., Gregory, W., Taber, H., Steward, A., Gonzalez, F. J., and Idle, J. R. (1995) Genetic polymorphism of CYP2C9 and its effect on warfarin maintenance dose requirement in patients undergoing anticoagulation therapy, *Pharmacogenetics 5*, 389-392.

99. Steward, D. J., Haining, R. L., Henne, K. R., Davis, G., Rushmore, T. H., Trager, W. F., and Rettie, A. E. (1997) Genetic association between sensitivity to warfarin and expression of CYP2C9*3, *Pharmacogenetics 7*, 361-367.

100. Aithal, G. P., Day, C. P., Kesteven, P. J., and Daly, A. K. (1999) Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications, *Lancet 353*, 717-719.

101. O'Reilly, R. A., and Aggeler, P. M. (1965) Coumarin anticoagulant drugs: hereditary resistance in man, *Fed Proc 24*, 1266-1273.

102. Wadelius, M., Chen, L. Y., Downes, K., Ghori, J., Hunt, S., Eriksson, N., Wallerman, O., Melhus, H., Wadelius, C., Bentley, D., and Deloukas, P. (2005) Common VKORC1 and GGCX polymorphisms associated with warfarin dose, *Pharmacogenomics J 5*, 262-270.

103. Rieder, M. J., Reiner, A. P., Gage, B. F., Nickerson, D. A., Eby, C. S., McLeod, H. L., Blough, D. K., Thummel, K. E., Veenstra, D. L., and Rettie, A. E. (2005) Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose, *The New England journal of medicine 352*, 2285-2293.

104. Limdi, N. A., Wadelius, M., Cavallari, L., Eriksson, N., Crawford, D. C., Lee, M. T., Chen, C. H., Motsinger-Reif, A., Sagreiya, H., Liu, N., Wu, A. H., Gage, B. F., Jorgensen, A., Pirmohamed, M., Shin, J. G., Suarez-Kurtz, G., Kimmel, S. E., Johnson, J. A., Klein, T. E., and Wagner, M. J. (2010) Warfarin pharmacogenetics: a single VKORC1 polymorphism is predictive of dose across 3 racial groups, *Blood 115*, 3827-3834.

105. Caldwell, M. D., Awad, T., Johnson, J. A., Gage, B. F., Falkowski, M., Gardina, P., Hubbard, J., Turpaz, Y., Langaee, T. Y., Eby, C., King, C. R., Brower, A., Schmelzer, J. R., Glurich, I., Vidaillet, H. J., Yale, S. H., Qi Zhang, K., Berg, R. L., and Burmester, J. K. (2008) CYP4F2 genetic variant alters required warfarin dose, *Blood 111*, 4106-4112.

106. Edson, K. Z., Prasad, B., Unadkat, J. D., Suhara, Y., Okano, T., Guengerich, F. P., and Rettie, A. E. (2013) Cytochrome P450-dependent catabolism of vitamin K: omega-hydroxylation catalyzed by human CYP4F2 and CYP4F11, *Biochemistry 52*, 8276-8285.

107. Limdi, N. A., Arnett, D. K., Goldstein, J. A., Beasley, T. M., McGwin, G., Adler, B. K., and Acton, R. T. (2008) Influence of CYP2C9 and VKORC1 on warfarin dose, anticoagulation attainment and maintenance among European-Americans and African-Americans, *Pharmacogenomics 9*, 511-526.

108. Limdi, N. A., Beasley, T. M., Crowley, M. R., Goldstein, J. A., Rieder, M. J., Flockhart, D. A., Arnett, D. K., Acton, R. T., and Liu, N. (2008) VKORC1 polymorphisms, haplotypes and haplotype groups on warfarin dose among African-Americans and European-Americans, *Pharmacogenomics 9*, 1445-1458.

109. Leray, C., Wiesel, M. L., Freund, M., Cazenave, J. P., and Gachet, C. (2001) Long-chain n-3 fatty acids specifically affect rat coagulation factors dependent on vitamin K: relation to peroxidative stress, *Arteriosclerosis, thrombosis, and vascular biology 21*, 459-465.

110. Oosthuizen, W., Vorster, H. H., Jerling, J. C., Barnard, H. C., Smuts, C. M., Silvis, N., Kruger, A., and Venter, C. S. (1994) Both fish oil and olive oil lowered plasma fibrinogen in women with high baseline fibrinogen levels, *Thrombosis and haemostasis 72*, 557-562.

111. Shahar, E., Folsom, A. R., Wu, K. K., Dennis, B. H., Shimakawa, T., Conlan, M. G., Davis, C. E., and Williams, O. D. (1993) Associations of fish intake and dietary n-3 polyunsaturated fatty acids with a hypocoagulable profile. The Atherosclerosis Risk in Communities (ARIC) Study, *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association 13*, 1205-1212.

112. Marckmann, P., Bladbjerg, E. M., and Jespersen, J. (1997) Dietary fish oil (4 g daily) and cardiovascular risk markers in healthy men, *Arteriosclerosis, thrombosis, and vascular biology 17*, 3384-3391.

113. Nishimaki, J., Miyazawa, K., Yaguchi, M., Katagiri, T., Kawanishi, Y., Toyama, K., Ohyashiki, K., Hashimoto, S., Nakaya, K., and Takiguchi, T. (1999) Vitamin K2 induces apoptosis of a novel cell line established from a patient with myelodysplastic syndrome in blastic transformation, *Leukemia 13*, 1399-1405.

114. Geleijnse, J. M., Vermeer, C., Grobbee, D. E., Schurgers, L. J., Knapen, M. H., van der Meer, I. M., Hofman, A., and Witteman, J. C. (2004) Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: the Rotterdam Study, *The Journal of nutrition 134*, 3100-3105.

115. Sadowski, J. A., Hood, S. J., Dallal, G. E., and Garry, P. J. (1989) Phylloquinone in plasma from elderly and young adults: factors influencing its concentration, *The American journal of clinical nutrition 50*, 100-108.

116. Azharuddin, M. K., O'Reilly, D. S., Gray, A., and Talwar, D. (2007) HPLC method for plasma vitamin K1: effect of plasma triglyceride and acute-phase response on circulating concentrations, *Clinical chemistry 53*, 1706-1713.

117. Donnahey, P. L., Burt, V. T., Rees, H. H., and Pennock, J. F. (1979) High-performance liquid chromatography of menaquinone-4, 2,3-epoxymenaquinone-4, demethylmenaquinone-4 and related compounds, *Journal of chromatography 170*, 272-277.

118. Parrish, D. B. (1980) Determination of vitamin K in foods: a review, *Critical reviews in food science and nutrition 13*, 337-352.

119. Indyk, H. E., Littlejohn, V. C., Lawrence, R. J., and Woollard, D. C. (1995) Liquid chromatographic determination of vitamin K1 in infant formulas and milk, *Journal of AOAC International 78*, 719-723.

120. Kamao, M., Suhara, Y., Tsugawa, N., and Okano, T. (2005) Determination of plasma Vitamin K by high-performance liquid chromatography with fluorescence detection using Vitamin K analogs

as internal standards, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 816*, 41-48.

121. Wang, L. Y., Bates, C. J., Yan, L., Harrington, D. J., Shearer, M. J., and Prentice, A. (2004) Determination of phylloquinone (vitamin K1) in plasma and serum by HPLC with fluorescence detection, *Clinica chimica acta; international journal of clinical chemistry 347*, 199-207.

122. Haroon, Y., Bacon, D. S., and Sadowski, J. A. (1986) Liquid-chromatographic determination of vitamin K1 in plasma, with fluorometric detection, *Clinical chemistry 32*, 1925-1929.

123. Suhara, Y., Kamao, M., Tsugawa, N., and Okano, T. (2005) Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry, *Analytical chemistry 77*, 757-763.

124. Ducros, V., Pollicand, M., Laporte, F., and Favier, A. (2010) Quantitative determination of plasma vitamin K1 by high-performance liquid chromatography coupled to isotope dilution tandem mass spectrometry, *Analytical biochemistry 401*, 7-14.

125. Fu, X., Peterson, J. W., Hdeib, M., Booth, S. L., Grusak, M. A., Lichtenstein, A. H., and Dolnikowski, G. G. (2009) Measurement of deuterium-labeled phylloquinone in plasma by high-performance liquid chromatography/mass spectrometry, *Analytical chemistry 81*, 5421-5425.

126. Belle, M., Brebant, R., Guinet, R., and Leclercq, M. (1995) Production of a new monoclonal antibody specific to human des-gamma-carboxyprothrombin in the presence of calcium ions. Application to the development of a sensitive ELISA-test, *Journal of immunoassay 16*, 213-229.

127. Chawla, D., Deorari, A. K., Saxena, R., Paul, V. K., Agarwal, R., Biswas, A., and Meena, A. (2007) Vitamin K1 versus vitamin K3 for prevention of subclinical vitamin deficiency: a randomized controlled trial, *Indian pediatrics 44*, 817-822.

128. Holden, R. M., Morton, A. R., Garland, J. S., Pavlov, A., Day, A. G., and Booth, S. L. (2010) Vitamins K and D status in stages 3-5 chronic kidney disease, *Clinical journal of the American Society of Nephrology : CJASN 5*, 590-597.

129. Lee, W., Chung, H. J., Kim, S., Jang, S., Park, C. J., Chi, H. S., Chun, S., and Min, W. K. (2010) PIVKA-II is a candidate marker for monitoring the effects of the oral anticoagulant warfarin, *Clinical biochemistry 43*, 1177-1179.

130. Miskad, U. A., Yano, Y., Nakaji, M., Kishi, S., Itoh, H., Kim, S. R., Ku, Y., Kuroda, Y., and Hayashi, Y. (2001) Histological study of PIVKA-II expression in hepatocellular carcinoma and adenomatous hyperplasia, *Pathology international 51*, 916-922.

131. Oshiro, Y., Takada, Y., Enomoto, T., Fukao, K., Ishikawa, S., and Iijima, T. (2004) A resected case of metachronous liver metastasis from lung cancer producing alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II), *Hepato-gastroenterology 51*, 1144-1147.

132. Ohhira, M., Ohtake, T., Saito, H., Ikuta, K., Tanaka, K., Tanabe, H., Kawashima, T., Fujimoto, Y., Naraki, T., Ono, M., and Kohgo, Y. (1999) Increase of serum des-gamma-carboxy prothrombin in alcoholic liver disease without hepatocellular carcinoma, *Alcoholism, clinical and experimental research 23*, 67S-70S.

133. Projahn, D., and Koenen, R. R. (2012) Platelets: key players in vascular inflammation, *Journal of leukocyte biology 92*, 1167-1175.

134. Mitchell, J. A., Ali, F., Bailey, L., Moreno, L., and Harrington, L. S. (2008) Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium, *Experimental physiology 93*, 141-147.

135. Blair, P., and Flaumenhaft, R. (2009) Platelet alpha-granules: basic biology and clinical correlates, *Blood reviews 23*, 177-189.

136. Chignard, M., Le Couedic, J. P., Vargaftig, B. B., and Benveniste, J. (1980) Platelet-activating factor (PAF-acether) secretion from platelets: effect of aggregating agents, *British journal of haematology 46*, 455-464.

137. Hawiger, J. (1987) Formation and regulation of platelet and fibrin hemostatic plug, *Human pathology 18*, 111-122.

138. Budzynski, A. Z. (1986) Fibrinogen and fibrin: biochemistry and pathophysiology, *Critical reviews in oncology/hematology 6*, 97-146.

139. Adams, R. L., and Bird, R. J. (2009) Review article: Coagulation cascade and therapeutics update: relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants, *Nephrology (Carlton) 14*, 462-470.

140. Marlar, R. A., Kleiss, A. J., and Griffin, J. H. (1982) An alternative extrinsic pathway of human blood coagulation, *Blood 60*, 1353-1358.

141. Butenas, S., and Mann, K. G. (2002) Blood coagulation, *Biochemistry. Biokhimiia 67*, 3-12.

142. Quinsey, N. S., Greedy, A. L., Bottomley, S. P., Whisstock, J. C., and Pike, R. N. (2004) Antithrombin: in control of coagulation, *The international journal of biochemistry & cell biology 36*, 386-389.

143. Walsh, P. N., and Ahmad, S. S. (2002) Proteases in blood clotting, *Essays in biochemistry 38*, 95-111.

144. Esmon, C. T. (2005) Coagulation inhibitors in inflammation, *Biochemical Society transactions 33*, 401-405.

145. Schaller, J., and Gerber, S. S. (2011) The plasmin-antiplasmin system: structural and functional aspects, *Cellular and molecular life sciences : CMLS 68*, 785-801.

146. Yim, J. M., Albers, G. W., and Vlasses, P. H. (1996) Anticoagulant therapy monitoring with international normalized ratio at US academic health centers, *The Annals of pharmacotherapy 30*, 1390-1395.

147. Raber, M. N. (1990) Coagulation Tests, In *Clinical Methods: The History, Physical, and Laboratory Examinations* (Walker, H. K., Hall, W. D., and Hurst, J. W., Eds.) 3rd ed., Boston.

148. Vanscoy, G. J., and Krause, J. R. (1991) Warfarin and the international normalized ratio: reducing interlaboratory effects, *DICP : the annals of pharmacotherapy 25*, 1190-1192.

149. Kamal, A. H., Tefferi, A., and Pruthi, R. K. (2007) How to interpret and pursue an abnormal prothrombin time, activated partial thromboplastin time, and bleeding time in adults., *Mayo Clin Proc 82*, 864-873.

150. Dufner, G. S., and Marbet, G. A. (2002) [Factor XIII in man: a review], *Hamostaseologie 22*, 11-19.

151. Spreafico, M., and Peyvandi, F. (2009) Combined Factor V and Factor VIII Deficiency, *Seminars in thrombosis and hemostasis 35*, 390-399.

152. Goodeve, A. C., Rosen, S., and Verbruggen, B. (2010) Haemophilia A and von Willebrand's disease, *Haemophilia : the official journal of the World Federation of Hemophilia 16 Suppl 5*, 79-84.

153. Finazzi, G., Budde, U., and Michiels, J. J. (1996) Bleeding time and platelet function in essential thrombocythemia and other myeloproliferative syndromes, *Leukemia & lymphoma 22 Suppl 1*, 71-78.

154. Rand, M. L., Leung, R., and Packham, M. A. (2003) Platelet function assays, *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis 28*, 307-317.

155. Hervig, T., Haugen, T., Liseth, K., Kjeldsen-Kragh, J., Scott, C. S., and Johannessen, B. (2004) The platelet count accuracy of platelet concentrates obtained by using automated analysis is influenced by instrument bias and activated platelet components, *Vox sanguinis 87*, 196-203.

156. Gearing, A. J., and Newman, W. (1993) Circulating adhesion molecules in disease, *Immunology today 14*, 506-512.

157. Kamath, S., Blann, A. D., and Lip, G. Y. (2001) Platelet activation: assessment and quantification, *European heart journal 22*, 1561-1571.

158. Ridker, P. M., Buring, J. E., and Rifai, N. (2001) Soluble P-selectin and the risk of future cardiovascular events, *Circulation 103*, 491-495.

159. Schneider, D. J., Hardison, R. M., Lopes, N., Sobel, B. E., and Brooks, M. M. (2009) Association between increased platelet P-selectin expression and obesity in patients with type 2 diabetes: a BARI 2D (Bypass Angioplasty Revascularization Investigation 2 Diabetes) substudy, *Diabetes care 32*, 944-949.

160. Davi, G., Romano, M., Mezzetti, A., Procopio, A., Iacobelli, S., Antidormi, T., Bucciarelli, T., Alessandrini, P., Cuccurullo, F., and Bittolo Bon, G. (1998) Increased levels of soluble P-selectin in hypercholesterolemic patients, *Circulation 97*, 953-957.

161. Eschen, O., Christensen, J. H., De Caterina, R., and Schmidt, E. B. (2004) Soluble adhesion molecules in healthy subjects: a dose-response study using n-3 fatty acids, *Nutrition, metabolism, and cardiovascular diseases : NMCD 14*, 180-185.

162. McEwen, B. J., Morel-Kopp, M. C., Chen, W., Tofler, G. H., and Ward, C. M. (2013) Effects of omega-3 polyunsaturated fatty acids on platelet function in healthy subjects and subjects with cardiovascular disease, *Seminars in thrombosis and hemostasis 39*, 25-32.

163. Moertl, D., Berger, R., Hammer, A., Hutuleac, R., Koppensteiner, R., Kopp, C. W., and Steiner, S. (2011) Dose-dependent decrease of platelet activation and tissue factor by omega-3 polyunsaturated fatty acids in patients with advanced chronic heart failure, *Thrombosis and haemostasis 106*, 457-465.

164. McDonald, M. G., Rieder, M. J., Nakano, M., Hsia, C. K., and Rettie, A. E. (2009) CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant, *Molecular pharmacology 75*, 1337-1346.

Chapter 2

ω3 PUFA Effects on Coagulation in Alaska Native People

2.1 The Effects of ω3 PUFAs on Hemostasis

High consumption of ω3 polyunsaturated fatty acids (ω3 PUFAs) commonly found in the oils of cold-water fish are associated with improved health effects in several chronic disease states (1-4). The benefits of a high ω3 PUFA diet have been the subject of substantial research, beginning with the work by Dyerberg and Bang in the 1980s (5). These investigators reported that the Greenland Inuit, who consumed very high levels of ω3 PUFAs deriving from fish and marine animals, had prolonged bleeding times and decreased platelet aggregation relative to Danish controls. This observation was thought to contribute to the relatively low incidence of cardiovascular death observed in this population. Since then, ω3 PUFAs have been reported to have a plethora of biological effects relating to cardiovascular physiology and studies detailing their consumption in humans have supported a beneficial role in cardiac health (6-8). An important physiological mechanism that may explain the antiplatelet effects of ω3 PUFAs reported in clinical studies involves the displacement of arachidonic acid from platelets and vascular cell membranes by the major ω3 PUFA components of fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This modulates the availability of arachidonic acid as a substrate for phospholipase A2, resulting in decreased production of the potent platelet aggregating eicosanoid, thromboxane A2 (TXA2), and increased production of the less potent thromboxane, TXA3 (9-11).

In addition to these cardiovascular benefits, some reports in rats suggest that ω3 PUFAs may precipitate bleeding diathesis through interfering with clotting factor activity, though it is unclear if the mechanism is vitamin K-dependent. One study reported that vitamin K-dependent clotting factors II and VII-X were reduced by 70% in those animals fed a purified fish oil diet (MaxEPA) for 6 weeks, but returned to basal levels after administration of vitamin K (12). Another study reported decreased levels of both vitamin K-dependent (prothrombin and factor VII) and vitamin K-independent (factor V) clotting

factors after giving fish oil to rats fed a vitamin K-restricted diet (13). In humans, however, the evidence for ω3 PUFA effects on hemostatic measures of coagulation is not strong (14-16). The association between ω3 PUFAs and bleeding events described in recent studies have generally involved patients taking ω3 PUFAs concurrently with medications that have antithrombotic and/or anticoagulative effects. For example, a small study (N = 40) that evaluated the effects of fish oil (4 g/day) on bleeding time over a 4-week period noted only one incidental case of minor nasal bleeding among the 22 study volunteers who were concurrently receiving oral anticoagulants (17). Another larger study of 610 study volunteers who received Omacor (DHA and EPA in fish oil) 4 g/day and either warfarin or aspirin for 12 months found only a modest increase in bleeding time in study volunteers who consumed fish oil (18).

It is important to note that dietary sources of ω3 PUFAs may provide enhanced bioavailability and a wider spectrum of the bioactive agents compared to commercially available ω3 PUFA products that contain only one active ingredient and no food matrix. This could be an important source of variability that is unaccounted for in many clinical studies. To resolve these inconsistencies, studies involving populations where ω3 PUFAs are a substantial part of the diet are needed. The Yup'ik people represent one such group among the indigenous Alaska Native population that consume high amounts of naturally available ω3 PUFAs from their traditional marine-based diet. Though Westernization of the Yup'ik population has led to many recent changes in their cultural and dietary practices, fish and other marine animals continue to be a staple of Yup'ik diet patterns (19, 20). The effect of ω3 PUFAs on hemostastic function has not been previously characterized in this population, but may be an important consideration when they are prescribed medications that have anticoagulative or antithrombotic effects. Furthermore, ω3 PUFAs could be an important modifier of cardiovascular health in the Yup'ik.

In this study, we evaluated the relationship between ω3 PUFA intake and various indices of coagulation to explore the hemostatic effects of ω3 PUFA intake in the Yup'ik. Based on the reported antiplatelet effects of ω3 PUFAs in humans and the reduction in clotting factor activity in rats fed a purified fish oil diet, we assessed platelet function, clotting factor activity and coagulation status. The specific parameters measured were soluble P-selectin (sP-selectin), clotting factors II, V, fibrinogen,

prothrombin time (PT), International Normalized Ratio (INR), and partial thromboplastin time (PTT). While many studies have relied on self-reporting of ω3 PUFA intake by study participants or utilized complicated methods for extraction and quantitative analysis of ω3 PUFAs from plasma, this study capitalized on the $^{15}N/^{14}N$ isotopic ratio ($\delta^{15}N$) in red blood cell (RBC) membranes as a biomarker of dietary ω3 PUFA intake. This method was developed by O'Brien et al. and is now established as a rapid, medium throughput assay for assigning ω3 PUFA intake status (21). Importantly, RBCs provide a more stable and informative measure of ω3 PUFA intake than serum analysis because they reflect dietary intake over 1–3 months.

2.2 Study Design and Analytical Procedures

Study Recruitment and Sample Collection

Data were gathered by the Center for Alaska Native Health Research (CANHR) as part of a Northwest-Alaska Pharmacogenetic Research Network study. It was a cross-sectional, community-based, participatory research study of genetic and nutritional factors affecting blood coagulation in Yup'ik people living in the Yukon–Kuskokwim region of Southwestern Alaska (22). In total, 772 study participants were recruited across ten communities between October 2010 and November 2013, where four were considered inland (greater than 5 miles away from the coast) and six were coastal (defined as within 5 miles of the coastal water). Participants were excluded from analysis if they self-reported their ethnicity as non-native Alaskans or 'other'. Demographic data on age, sex, and community location were recorded. Dietary questionnaire data were also collected as part of a parallel health outcomes study, described by Nash et al. (23). Blood was collected into EDTA tubes and processed at the site of collection using a portable centrifuge. Samples were centrifuged for 15 minutes at 2500 rpm (RCF = 887 g). Plasma, serum, lymphocytes, and RBCs were isolated and stored in aliquots at -20^{o}C in a portable freezer. All samples were shipped to the University of Alaska Fairbanks within seven days and stored there at -80^{o}C.

Samples for platelet function, clotting factor activity, and coagulation status measurements were sent to the University of Washington Medicinal Chemistry Department and subsequently transferred to the Department of Laboratory Medicine for these analyses. The study was conducted according to the guidelines in the Declaration of Helsinki and all procedures involving human study volunteers were approved by the Institutional Review Boards at the University of Alaska Fairbanks, the University of Washington, and the Yukon–Kuskokwim Health Corporation Human Studies Committee. Informed consent was obtained from all study participants prior to participation in the study.

$\delta^{15}N$ Stable Nitrogen Isotope Analysis

RBC samples were prepared and analyzed for $\delta^{15}N$ at the Alaska Stable Isotope Facility by continuous-flow isotope ratio mass spectrometry, with a Costech ECS4010 Elemental Analyzer (Costech Scientific Inc, Valencia, CA) interfaced to a Finnigan Delta Plus XP isotope ratio mass spectrometer via the Conflo III interface (Thermo-Finnigan Inc, Bremen, Germany), as previously described by O'Brien et al. (21). Briefly, aliquots of RBCs were removed and autoclaved for 20 minutes at 121°C to destroy blood-borne pathogens, apportioned into tin capsules (3.5 x 3.75 mm) and oven dried at 60°C to a final weight of 0.2–0.4 mg. Neither autoclaving nor the use of EDTA-treated tubes affect RBC carbon or nitrogen isotope ratios (24). The relationship between EPA and $\delta^{15}N$ has been reported to be positive and linear ($EPA = 1.04(\delta^{15}N) - 0.67$, $R^2 = 0.70$, $p < 0.0001$), while that between DHA and $\delta^{15}N$ was found to be exponential non-linear and fitted as $y = a\,(1\text{-}e^{-r\,x})$ ($p < 0.0001$) (21). Nitrogen isotope ratios are represented in delta notation as 'permil' relative to international standards: $[\delta^{15}N = (R_{sample} - R_{standard})/R_{standard}] \times 1000‰$, where R is the ratio of heavy to light isotope, and the standard is atmospheric nitrogen. An integer value difference of one for $\delta^{15}N$ represents a 0.1% difference in the amount of nitrogen-15 isotope in the sample relative to atmospheric nitrogen, which has a nitrogen-15 abundance of 0.366%. Multiple peptone working standards ($\delta^{15}N = 7.0$, $n = 128$) were analyzed concurrently to assess

analytical accuracy and precision, measured as the standard deviation (SD) of these analyses. Accuracy was within 0.1‰ and precision was within 0.2‰ (23).

Measures of Coagulation

We included several coagulation indices to assess hemostatic function in this study. All coagulation assays were performed at the University of Washington Department of Laboratory Medicine. Since samples were collected in remote regions where clinical laboratories are unavailable, platelet aggregation tests that require analysis within a few hours after collection were not feasible. Therefore, we used a surrogate marker for platelet activation, soluble P-selectin (sP-selectin), to assess the effect of ω3 PUFA intake on platelets because samples could be frozen rapidly at the point of collection for subsequent analysis in Seattle. sP-selectin is a cell adhesion molecule released from platelets that promotes cell-to-cell contact at sites of tissue injury and inflammation. Since sP-selectin is stored in the alpha granules of platelets and only mobilized to the plasma membrane upon stimulation, it has been used widely in clinical studies as a biomarker of platelet activation (25). A commercially available sP-selectin ELISA kit (R&D systems) was used, which contained microplates pre-coated with monoclonal antibodies specific for sP-selectin. Samples, standards, and controls were pipetted into the wells together with a polyclonal antibody specific for sP-selectin, which had been conjugated to horseradish peroxidase. After a 1-hour incubation at room temperature followed by a wash step to remove unbound antibodies, tetramethylbenzidine substrate was added to each well and incubated for an additional 15 minutes at room temperature before adding stop solution. Readings were performed at 450 nm using a microplate reader and sample concentrations were determined using a standard curve. We further validated that sP-selectin was an appropriate biomarker for platelet activity in a small subset of our samples (N = 170) by measuring counts of the platelet microparticle, CD41+. Methods for measuring CD41+ by flow-cytometry are described elsewhere (26).

The STA-Compact coagulation analyzer (Diagnostica Stagl), an electromagnetic-mechanical clot detection system, was used to measure PT and PTT. Citrated plasma was pipetted into a cuvette containing a steel ball. After briefly heating samples at 37ºC, thromboplastin and calcium chloride was added to the plasma activating the conversion of fibrinogen to fibrin. When the oscillation of the steel ball in the cuvette was stopped by clot formation, the observed clotting time was recorded in seconds. For measuring PTT, a reagent containing particulate activator (silica) for contact phase activation and a standardized amount of cephalin (platelet substitute) was pipetted into the plasma. After a short incubation period, calcium chloride was added to the sample to allow the conversion of fibrinogen to fibrin.

Vitamin K-dependent clotting factor II as well as vitamin K-independent clotting factor V were measured to assess any potential effects of ω3 PUFAs on those elements of the clotting cascade. A subset of samples was assessed for combined factor V and factor VIII deficiency (F5F8D) (see below for additional details on this facet of this study). Clotting factor activity was tested by the PT (II and V) and PTT (VIII) measurements. The percent activity of each plasma clotting factor was determined from a standard curve (log-based) prepared using dilutions of Unicalibrator and a normal plasma pool (Diagnostica Stago) containing known levels of each clotting factor. Sample plasma was mixed in equal parts with specific factor-deficient plasma, which contained normal levels of all other clotting factors but was deficient in the specific clotting factor being assayed. The PT/PTT measurements were recorded for each sample dilution by the analyzer and then converted into percent activity based on the standard curve. Fibrinogen concentration, quantitatively assessed by the Clauss clotting method, was used to determine whether clots found in samples would affect coagulation assays. Hemolyzed samples were excluded from sP-selectin analysis. Furthermore, samples containing clots were excluded if fibrinogen concentrations were < 150 ng/mL. The reference ranges of coagulation tests measured in our study are represented in Table 2.1.

Statistical Analysis

All statistical analyses were performed using STATA (version 11.0 SE). We evaluated the relationship between ω3 PUFA intake ($\delta^{15}N$) and coagulation indices (sP-selectin, activities of clotting factors II and V, fibrinogen, PT/INR, and PTT) using a multivariate linear regression model. These variables were natural log-transformed to improve normality prior to statistical analysis. $\delta^{15}N$ was treated as the independent variable and measures of coagulation were treated as dependent variables. Study participant age and sex were included as covariates. A two-sample t-test was used to compare $\delta^{15}N$ between groups and a community's geographical status (inland vs. coastal). Differences in natural log-transformed coagulation variables were also compared between and geographical status groups using the two sample t-test. A p-value ≤ 0.05 was considered statistically significant for all statistical tests. Only data that were complete with $\delta^{15}N$ and passed quality controls were included in the final analysis with each coagulation variable.

2.3 Results

Distribution Normality of Variables

The reference ranges for the coagulation variables measured in our study are given in Table 2.1. These ranges were used to identify outliers in our data and determine those that needed further quality control analysis. The characteristics of $\delta^{15}N$ and coagulation parameters from samples analyzed in this study are presented on Table 2.2. Natural log transformation of the coagulation variables generally improved the normality of that data (Figure 2.1), as indicated by the skewness and kurtosis values. Only natural log-transformed dependent (coagulation) variables were used in regression analysis while $\delta^{15}N$ was treated as the independent variable and was not transformed.

Study Participant Demographics

Demographic characteristics of Yup'ik study participants are shown in Table 2.3. The average $\delta^{15}N$ was 8.73 ± 1.28‰ in our study, which was significantly higher than that of a previously characterized population that did not include Alaska Native people (7.4 ± 0.4‰, $p < 0.001$) (27). The average age in our study was 36.7 ± 18.1 years and 48.6% of participants were women. Participant age correlated positively with $\delta^{15}N$ ($p < 0.001$, $R^2 = 0.330$) (Figure 2.2) and women tended to have higher $\delta^{15}N$ values than men ($p < 0.001$) (Figure 2.3). ω3 PUFA intake also differed significantly by a community's geographical status, with coastal communities exhibiting higher $\delta^{15}N$ values compared to inland communities ($\delta^{15}N$ = 9.06 ± 1.45 for coastal vs. 8.38 ± 0.93‰ for inland, $p < 0.001$, Figure 2.4). These results are in accordance with previous findings on dietary trends and traditional food intake in the Yup'ik (23). The associations of natural log-transformed coagulation variables with age and sex are described in Table 2.4. Significant differences in coagulation variables after stratifying by geographical status were only observed with natural log PTT ($p < 0.001$). Therefore, geographical status was included as a covariate in the regression model for natural log PTT but not with other measures of coagulation.

Regression Analysis

The results of the multivariate regression analysis before and after natural log-transformation, as well as after the adjustment of age and sex, are described in Table 2.5. Natural log-transformed sP-selectin levels varied inversely with $\delta^{15}N$, indicating that lower platelet activity correlated significantly with higher ω3 PUFA consumption ($p < 0.001$, $R^2 = 0.038$) (Figure 2.5). This association further improved after adjusting for age and sex ($p < 0.001$, $R^2 = 0.092$). CD41+ counts were also found to positively correlate with sP-selectin levels (Figure 2.6), which validated the sP-selectin data ($p = 0.001$, $R^2 = 0.069$). Natural log-transformed fibrinogen levels were initially found to have a positive association with $\delta^{15}N$ ($p = 0.003$), but the association was lost ($p = 0.863$) after adjusting for age and sex, suggesting evidence of confounding (Figure 2.7).

The correlations of factors II, factor V, PT, INR, and PTT with $\delta^{15}N$ before and after natural log transformation are displayed in Figures 2.8 and 2.12. While no significant associations were observed with either factor II or V and $\delta^{15}N$, the associations was positive PTT ($p = 0.005$) after adjusting for age and sex, but not for PT ($p = 0.737$) or INR ($p = 0.356$). Since a community's geographical status was found to be associated with both $\delta^{15}N$ and natural log PTT ($p < 0.001$) we included geographical status as a covariate here to $\delta^{15}N$ in addition to age and sex. The association with natural log PTT was no longer significant ($p = 0.481$, $R^2 = 0.088$), indicating that geographical status was a confounder to these observations with PTT.

Factor V Bimodality

Interestingly, we observed some evidence of bimodality in the factor V data, highlighted by the rectangular box in Figure 2.9. Approximately 18% of our samples displayed clotting factor activity at or below the lower reference range of 50% activity, although the occurrence of low factor V activity is considered very rare (28). We categorized the data based on factor V activity less than or equal to 30% (i.e., low activity) or greater than 30% and then compared regression results between these two groups (Table 2.6). We chose to use 30% as the activity threshold since factor V measurements at or below this value can be considered clinically actionable, which included approximately 10% of the samples. Since the skewness and kurtosis values for factor V did not improve after natural-log transformation, regression was performed on both untransformed and natural log-transformed data. A negative and significant association was observed between factor V and $\delta^{15}N$ for the > 30% activity group ($p = 0.016$, $R^2 = 0.020$) but not for ≤ 30% activity group ($p = 0.231$, $R^2 = 0.026$) after adjusting for age and sex. This association was also significant for natural log factor V after adjusting for the covariates age and sex in the > 30% group ($p = 0.036$, $R^2 = 0.015$) and not significant for ≤ 30% group ($p = 0.474$, $R^2 = 0.017$). No significant associations in the univariate analysis were found between either factor V or natural log factor V and $\delta^{15}N$.

We further assessed for differences in PT, INR and PTT between these two groups (Table 2.7 and Figure 2.13). We observed that these three test values were slightly increased in this subset of samples with low factor V activity (≤ 30%). These results were statistically significant before and after natural-log transformation ($p < 0.001$ for PT, INR and $p = 0.013$ for PTT). This indicates that the low factor V activity observed in some study participants may result in a phenotype with clinically measurable changes. However, it should be noted that only a proportion of low factor V samples had prolonged PT/INR and PTT values above the reference ranges (24% for PT/INR, 60% for PTT, and 14% with both prolonged PT/INR and PTT). We verified the integrity of the factor V assay by correlating factor V activity with factor V antigen levels (Figure 2.14) and observed a positive correlation ($p = 0.001$, $R^2 = 0.332$, $N = 39$). We also explored the possibility of a combined factor V factor VIII (F5F8) deficiency, a very rare phenotype which has been reported in some individuals with low factor V activity (29, 30). However, further testing did not support this possibility based on the observation that approximately 77% of low factor V activity samples (≤ 30%) had factor VIII activity values above the reference range of 50% for factor VIII (Figure 2.15).

2.4 Discussion

The purpose of this study was to determine whether a high dietary intake of ω3 PUFAs from the traditional marine-based diet of the Yup'ik people could be a factor contributing to their historical tendency towards bleeding diathesis. Our most significant finding is that high ω3 PUFA intake, as measured by $\delta^{15}N$, was strongly associated with the platelet biomarker, sP-selectin. The measured levels of sP-selectin were validated by the positive correlation with the CD41+ platelet microparticle counts. The observed inverse association between sP-selectin and $\delta^{15}N$ provides evidence that a platelet-inhibition effect is enhanced by greater consumption of ω3 PUFAs. A few other studies have similarly reported that sP-selectin decreases only after high doses ω3 PUFAs were consumed. For example, one study in CHF patients observed sP-selectin reduction from Omacor (commercially available form of EPA/DHA) dosed

at 4 g/day but not 1 g/day after 12 weeks (31). Another study reported that sP-selectin was only reduced by ingestion of 6.6 g/day ω3 PUFA in healthy volunteers (32). Furthermore, an elevated level of sP-selectin is observed in inflammatory disease states and is considered a risk factor for cardiovascular events (32-34). Overall, our data provide the most comprehensive evidence yet obtained that ω3 PUFA intake from a natural, as opposed to supplemented, diet is a major modifier of platelet activity. A beneficial association of marine food intake (via $\delta^{15}N$) with blood pressure and adiponectin was recently reported in the Yup'ik and the inverse association with sP-selectin observed in this study provides an additional mechanism by which a marine–based diet may lower the incidence of chronic and inflammatory disease state in this population (35).

We observed no significant association between vitamin K-dependent factor II and $\delta^{15}N$, but found a modestly significant and inverse association with vitamin K-independent factor V activity after the adjustment for age and sex in the > 30% activity group. Our original analysis also included the measurement of vitamin K-dependent factor IX to further explore possible vitamin K-dependent effects of ω3 PUFA intake in addition to factor II. However, we were not able to validate factor IX activity with factor IX antigen levels and therefore excluded factor IX from our analysis. Despite the lack of association with $\delta^{15}N$, factor II activity proved to be more reliable for our study, likely due its longer half-life of 65 hours as opposed to 45 hours for factor IX, which should increase sample stability. In addition to the animal studies described earlier, the mechanism by which ω3 PUFAs reduce clotting factor levels in humans has not been clear since effects on lowering both vitamin K-dependent and vitamin K-independent clotting factors have been reported. For example, a study involving 25 male study volunteers given 3 g of ω3 PUFAs daily for 4 weeks showed reduced levels of thrombin, fibrinogen, and factor V in plasma (36). Another double-blind, cross-over study of 20 healthy study participants using olive oil as placebo, found that 6 g of daily fish oil intake alone lowered factors V and VII (vitamin K-dependent) in addition to triglycerides, but both fish oil and olive oil reduced levels of fibrinogen and factor X (vitamin K-dependent). Although our data are limited by the fact that only the > 30% activity group exhibited an inverse association between factor V activity and ω3 PUFA intake, the observation of factor V levels ≤

30% activity is quite uncommon in other human studies reported to date, reflecting the rarity of factor V deficiency (30). Furthermore, we did not find evidence that greater ω3 PUFA intake led to prolongation of PT/INR or PTT. The most likely mechanism for modifying any bleeding risk in our study population is through the inhibitory effects of ω3 PUFAS on platelet activity, and to some extent, vitamin K-independent effects by reducing factor V levels. However, it remains to be determined if this risk is any different for patients on anticoagulant or antithrombotic therapy.

The bimodality in the distribution of factor V activity was an unexpected observation. We validated these results using an ELISA-based assay to confirm activity levels on samples that were unexpectedly low (≤ 30% activity), in order to rule out the possibility of errors introduced during the sample collection process. The incidence of isolated factor V deficiency is considered to be a rare, inherited coagulopathy (1 heterozygote in 1,000 people and 1 homozygote among 1,000,000 people) and notable prolongation of both the PT and PTT are a characteristic of individuals affected by this condition (28, 30). We found only partial support for this phenotype since only a small proportion (14%) of our low factor V samples had both PT/INR and PTT values above the reference ranges. F5F8D is another rare coagulopathy known to result in low factor V levels, but our results do not support this condition based on the finding that most of the low factor V samples did not have low factor VIII activity. Furthermore, there have been no anecdotal reports of bleeding episodes among Yup'ik study participants of previous CANHR studies (22, 35). While it is possible that Yup'ik people may harbor their own unique genetic variations that contribute to these differences, when we analyzed a small number of study participants (N = 7) with low factor V levels who participated in repeat community visits approximately two years apart, factor V activity in these samples was within the 'normal' range. This implicates an environmental, rather than genetic, basis for the low factor V activity levels measured in some Yup'ik plasma samples collected for the current study. Since high factor V levels are considered to be a risk factor for thrombotic events, particularly in individuals with Factor V Leiden, an environmental effect

that lowers factor V levels in the Yup'ik may assist in cardiovascular protection, in addition to the clear antiplatelet effects conferred by a high ω3 PUFA diet (37).

Finally, the level of fibrinogen, a soluble glycoprotein that is converted to fibrin by thrombin during clot formation, was found to have an initial positive association with ω3 PUFA intake in our study. However, this relationship was no longer significant after adjusting for age and sex, indicating that it could not be correctly characterized without adjustment for confounding. A few small studies have reported decreased fibrinogen levels in response to ω3 PUFA intake and it is possible that this is another mechanism by which ω3 PUFAs may exert antithrombotic effects on the hemostatic system (36, 38, 39). Our current data do not support this hypothesis for fibrinogen. However, since fibrinogen originally served as a quality control variable in our study, not all samples that underwent coagulation tests were measured for fibrinogen. Therefore, our study may not be adequately designed to test for an association between fibrinogen and ω3 PUFA intake.

A limitation of this study is that coagulation assays could not be performed at the time of sample collection due to the unavailability of clinical laboratories in the Alaskan communities where study participants were recruited. This likely affected the integrity of some plasma samples, as indicated by the presence of clots and absence of fibrin strands in some of the analyzed samples. It should also be noted that both sP-selectin and $\delta^{15}N$ are biomarkers for our true parameters of interest, namely platelet activity and EPA/DHA levels, and that any associations obtained using these parameters are indirectly inferred. Nonetheless, both of these biomarkers have been extensively validated and used by other researchers, so we can have confidence in the robustness of the associations observed in this study. A final limitation was that our study design was cross-sectional. Therefore, we were not able to establish causality or exclude the possibility of confounding from other dietary or lifestyle factors.

In summary, we found that $\delta^{15}N$ is highly correlated with the platelet biomarker sP-selectin in Yup'ik people living in the Yukon-Kuskokwim delta and that ω3 PUFA intake could potentially have vitamin K-independent effects on factor V levels and other measures of bleeding. These data support an important role for ω3 PUFAs in reducing the risk of cardiovascular and/or inflammatory disease states,

which has been reported from other studies on EPA/DHA (40-42). The low levels of clotting factor V in a small subset of samples may also be an additional mechanism of cardiovascular protection in some individuals, and warrants further investigation.

Clinical Parameter	Reference Range	Relationship to Coagulation
sP-selectin	20-44 ng/mL	Biomarker of platelet activation
Clotting factor II	50-115%	Vitamin K-dependent clotting factor
Clotting factor V	50-150%	Vitamin K-independent clotting factor
Fibrinogen	150-400 mg/dL	Precursor to fibrin during clot formation
PT	10.7-15.6 sec	Clotting time based on extrinsic pathway
INR	0.8-1.3	Normalized ratio of prothrombin time
PTT	22-35 sec	Clotting time based on intrinsic pathway

Table 2.1. Reference ranges of coagulation parameters analyzed in this study. Ranges were defined by the University of Washington Department of Laboratory Medicine and are based on data from a population of non-Alaska Native people.

	Untransformed values					
Variable	Mean ± SD	Median (IQR)	Range	Skewness	Kurtosis	N
$\delta^{15}N$ (‰)	8.73 ± 1.28	8.44 (1.58)	6.10-14.50	1.06	4.26	734
sP-selectin (ng/mL)	34.6 ± 11.4	33.2 (14.3)	11.3-80.4	0.72	3.62	717
Clotting factor II (%)	107.4 ± 20.1	103.0 (22.0)	35.0-180.0	0.80	4.01	709
Clotting factor V (%)	81.6 ± 32.0	87.0 (38.0)	3.5-211.0	-0.47	3.05	706
Fibrinogen (ng/mL)	344.8 ± 106.0	333.0 (124.0)	142-842	0.70	4.37	358
PT (sec)	12.7 ± 1.61	12.7 (1.40)	8.0-25.0	1.91	14.2	723
INR	1.01 ± 0.17	1.00 (0.20)	0.6-2.5	2.47	19.3	722
PTT (sec)	33.8 ± 10.4	32.0 (6.0)	14-199	9.88	148.1	449
	Natural log-transformed values					
Variable	Mean ± SD	Median (IQR)	Range	Skewness	Kurtosis	
$\delta^{15}N$ (‰)	2.16 ± 0.14	2.13 (0.18)	1.81-2.67	0.66	3.28	
sP-selectin (ng/mL)	3.49 ± 0.33	3.50 (0.43)	2.43-4.39	-0.27	3.19	
Clotting factor II (%)	4.66 ± 0.18	4.63 (0.21)	3.55-5.19	-0.11	5.70	
Clotting factor V (%)	4.27 ± 0.60	4.47 (0.46)	1.25-5.35	-1.87	6.53	
Fibrinogen (ng/mL)	5.79 ± 0.32	5.81 (0.37)	4.84-6.74	-0.40	3.44	
PT (sec)	2.54 ± 0.12	2.54 (0.11)	2.19-3.46	0.64	7.31	
INR	$-4.02 \times 10^{-4} \pm 0.15$	0.00 (0.20)	-0.51-0.92	0.69	7.22	
PTT (sec)	3.50 ± 0.20	3.47 (0.18)	2.64-5.29	2.23	21.2	

Table 2.2. Characteristics of $\delta^{15}N$ and coagulation parameters from samples analyzed in this study. Variables were natural-log transformed to improve the normality of the frequency distributions. Skewness and kurtosis values are given for data before and after natural-log transformation of variables. For simplicity, only natural-log transformed dependent (coagulation) variables were used in regression analysis. $\delta^{15}N$ was used as the independent variable and was not transformed. A variable with symmetrical distribution has a skewness of zero. The kurtosis for a standard normal distribution is three.

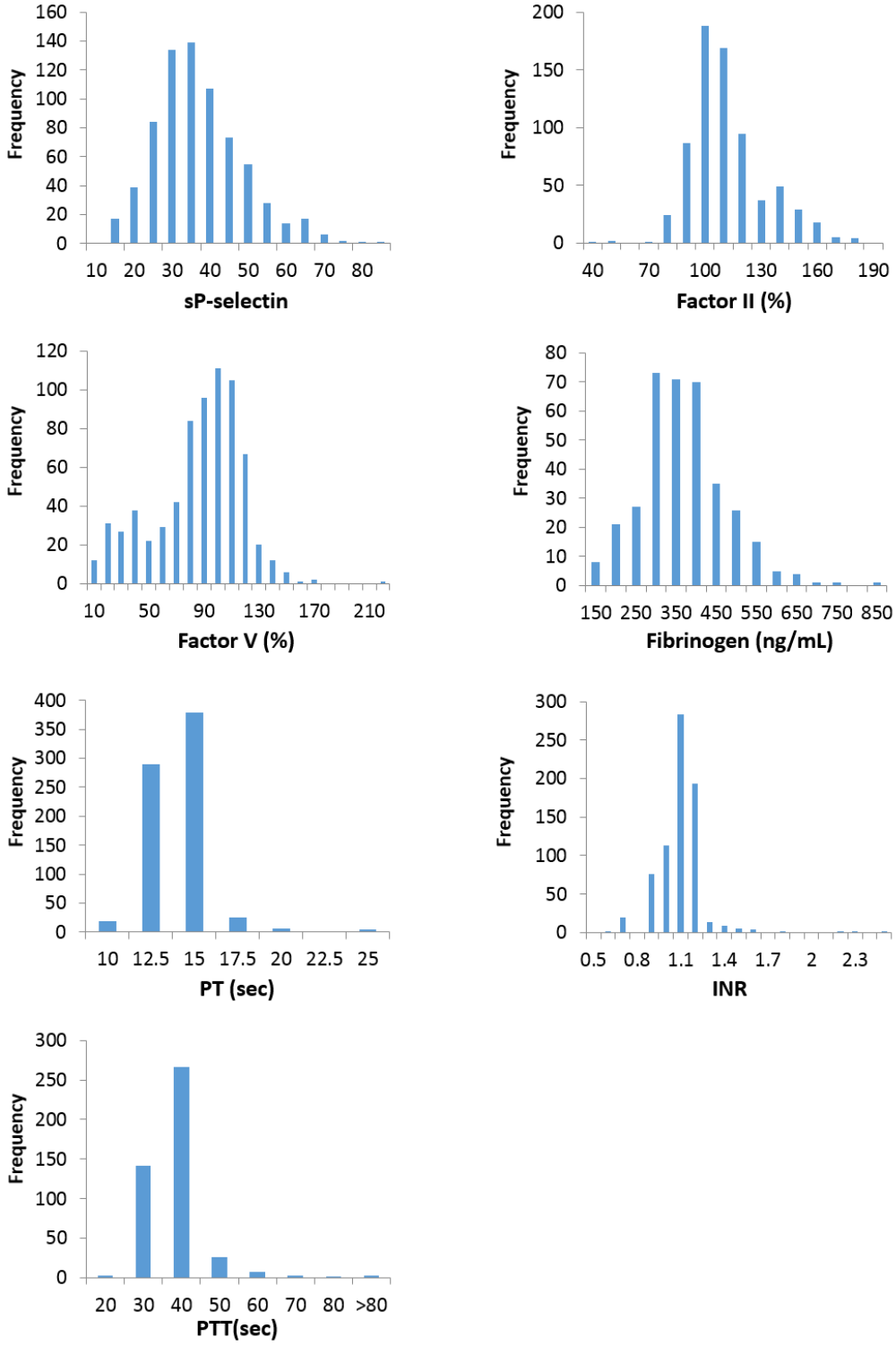

Figure 2.1. Distributions of natural-log transformed coagulation variables. Since the original data were not normally distributed, natural-log transformation was performed to improve normality prior to statistical analysis.

	All	Men	Women
Sample size	734	388	357
Age (mean ± SD)	36.7 ± 18.1	34.5 ± 17.4	39.2 ± 18.6
Range of ages	13-93	14-85	13-93
$\delta^{15}N$ (mean ± SD)	8.74 ± 1.28	8.39 ± 1.09	9.13 ± 1.36
Range of $\delta^{15}N$	6.10-14.51	6.10-13.21	6.59-14.51
	Coastal	**Inland**	
Number of men	204	184	
Number of women	183	163	
All $\delta^{15}N$ (mean ± SD)	9.06 ± 1.45	8.38 ± 0.93	
Men $\delta^{15}N$ (mean ± SD)	8.64 ± 1.26	8.11 ± 0.78	
Women $\delta^{15}N$ (mean ± SD)	9.51 ± 1.52	8.70 ± 0.98	

Table 2.3. Sex, age, and community status demographics of the Yup'ik study participants. Coastal communities were considered to be within 5 miles from the coast and inland communities were located greater than 5 miles away. See Figures 2.2-2.4 for details of the relationships for age, sex and geographical status with $\delta^{15}N$ status.

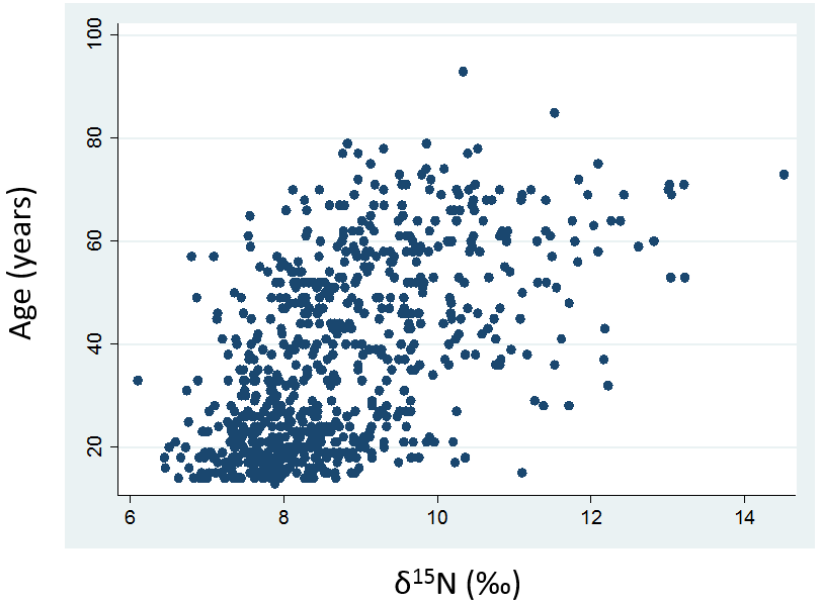

Figure 2.2. The positive correlation between age and $\delta^{15}N$ status in Yup'ik people. Higher $\delta^{15}N$ values indicate greater intake of ω3 PUFAs ($N = 734$, $p < 0.001$, $R^2 = 0.559$).

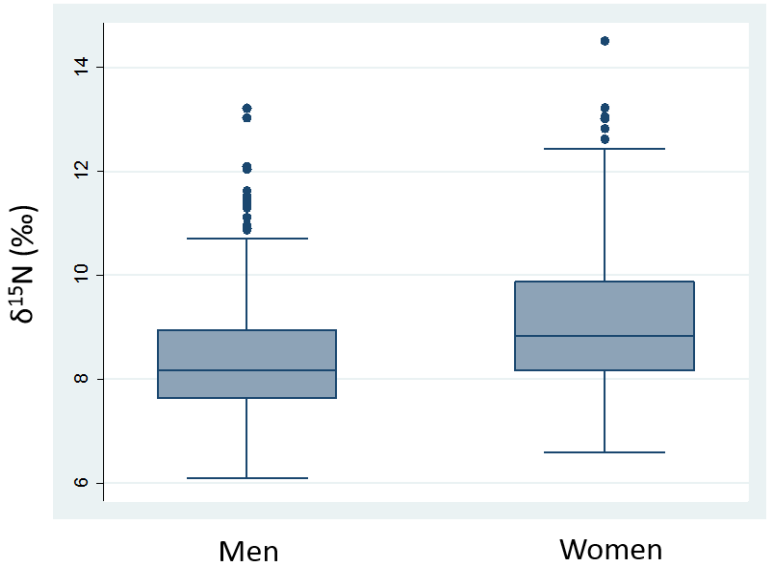

Figure 2.3. Sex differences in $\delta^{15}N$ values among Yup'ik people. Women tended to have higher $\delta^{15}N$ values, suggesting that their ω3 PUFA intake was greater compared to men ($p < 0.001$). Median (IQR) $\delta^{15}N$ for men was 7.94 (1.00) and for women was 8.44 (1.85).

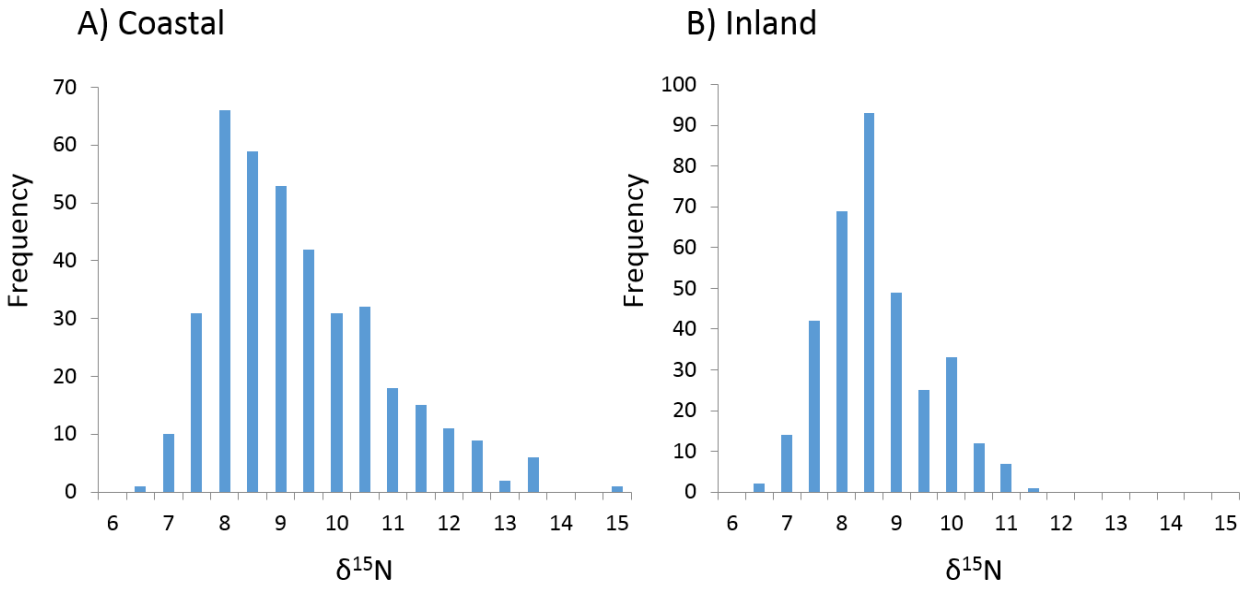

Figure 2.4. Distribution of $\delta^{15}N$ values from our population of Yup'ik study participants stratified by, A) coastal and B) inland communities. Coastal communities generally exhibited higher $\delta^{15}N$ values (mean ± SD, 9.06 ± 1.45) than inland communities (8.38 ± 0.93), $p < 0.001$.

Natural-log transformed variable	Association with age (p-value)	Association with sex (p-value)
sP-selectin	0.523	< 0.001
Clotting factor II	0.032	0.015
Clotting factor V	0.004	0.227
Fibrinogen	< 0.001	0.137
PT	0.014	0.025
INR	0.013	0.029
PTT	0.094	0.011

Table 2.4. The association of natural log-transformed coagulation variables with age and sex. The associations with age were determined by univariate linear regression while associations with sex were analyzed by a two-sample t-test.

Variable	Untransformed			Natural-log transformed			Natural-log transformed and adjusted for age and sex		
	P-value (unadjusted)	R-value	R^2	P-value (adjusted)	R-value	R^2	P-value (adjusted)	R-value	R^2
sP-selectin	< 0.001	-0.197	0.039	< 0.001	-0.195	0.038	< 0.001	-0.304	0.092
Clotting factor II	0.151	0.054	0.003	0.186	0.050	0.003	0.614	-0.117	0.014
Clotting factor V	0.133	0.051	0.003	0.050	0.073	0.005	0.865	0.113	0.013
Fibrinogen	0.003	0.159	0.025	0.003	0.158	0.025	0.863	0.257	0.066
PT	0.227	0.045	0.002	0.117	< 0.001	0.003	0.737	0.117	0.014
INR	0.513	0.024	0.001	0.266	-0.045	0.002	0.356	0.122	0.015
PTT	0.203	0.064	0.004	0.390	0.041	0.002	*0.481	*0.297	*0.088

Table 2.5. Statistical significance and correlation coefficients from a multivariate regression analysis on coagulation variables with $\delta^{15}N$. Data are represented before and after natural log transformation, as well as after further adjustment for age and sex. The significance level was set at $\alpha = 0.05$. R-values measure the strength and the direction of a linear relationship between two variables and R^2 indicates how well data fit a statistical model (i.e. goodness-of-fit). *Natural log PTT was adjusted for geographical status in addition to age and sex.

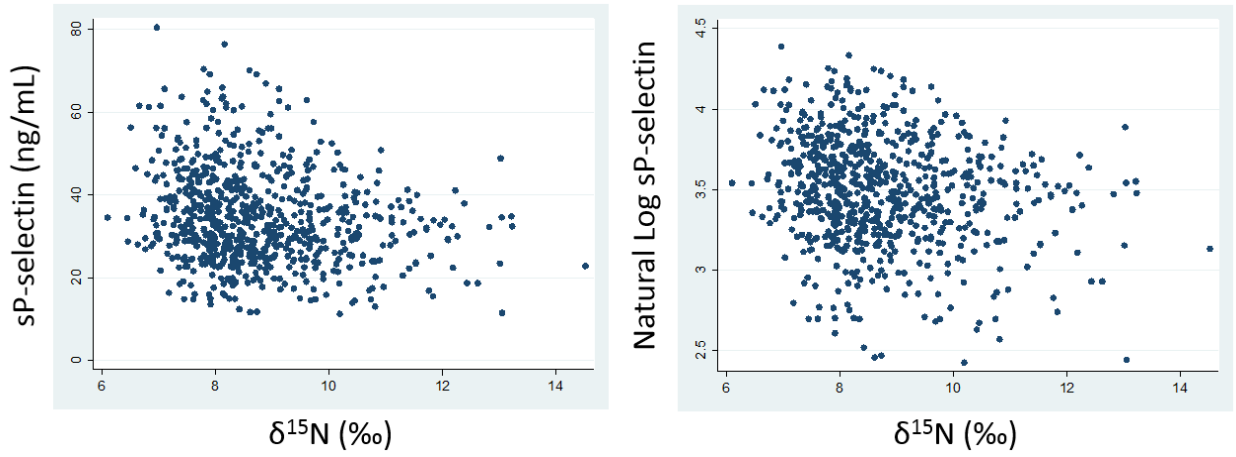

Figure 2.5. The correlation between sP-selectin and $\delta^{15}N$, before and after natural log transformation. The negative association in the non-transformed model was significant ($p < 0.001$, $R^2 = 0.039$) and improved further after natural log-transformation and adjusting for age and sex ($p < 0.001$, $R^2 = 0.092$).

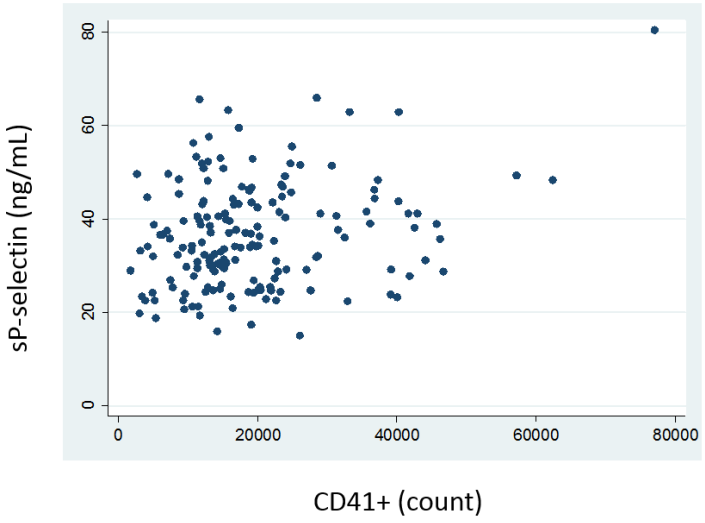

Figure 2.6. The positive correlation between sP-selectin and CD41+ microparticles from the plasma samples of Yup'ik study participants. CD41+ microparticles are derived from platelets and were measured from our samples to validate that sP-selectin was an appropriate platelet biomarker in our study ($p = 0.001$, $R^2 = 0.069$, $N = 170$).

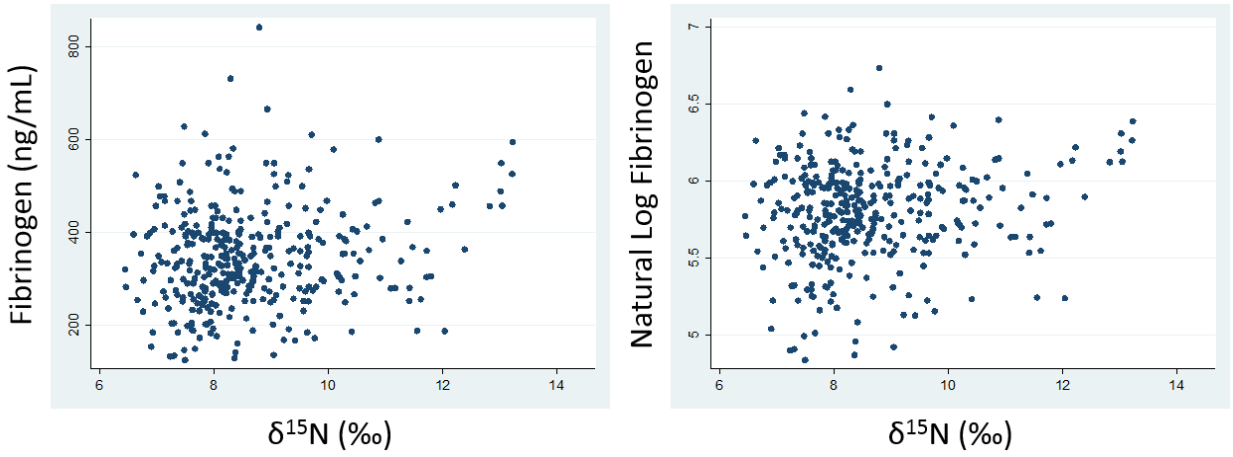

Figure 2.7. Correlation between fibrinogen and $\delta^{15}N$ before and after natural log transformation. Though the unadjusted association of natural log fibrinogen appears to be a positive and significant relationship ($p = 0.003$, $R^2 = 0.025$), the association was lost after adjusting for age and sex ($p = 0.863$, $R^2 = 0.066$), indicating evidence of confounding.

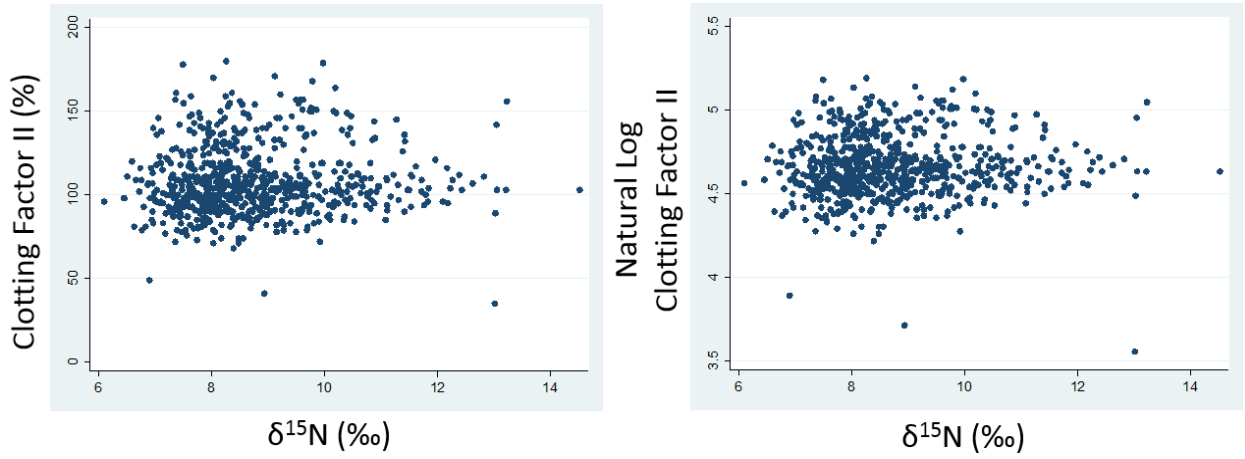

Figure 2.8. Correlation between clotting factor II activity and $\delta^{15}N$ before and after natural log transformation. Clotting factor II is a vitamin K-dependent protein and the lack of any association before ($p = 0.186$, $R^2 = 0.003$) or after ($p = 0.614$, $R^2 = 0.014$) the adjustment of age and sex with the natural-log transformed variable suggests that ω3 PUFA intake does not affect clotting factor activity through a vitamin K-dependent mechanism.

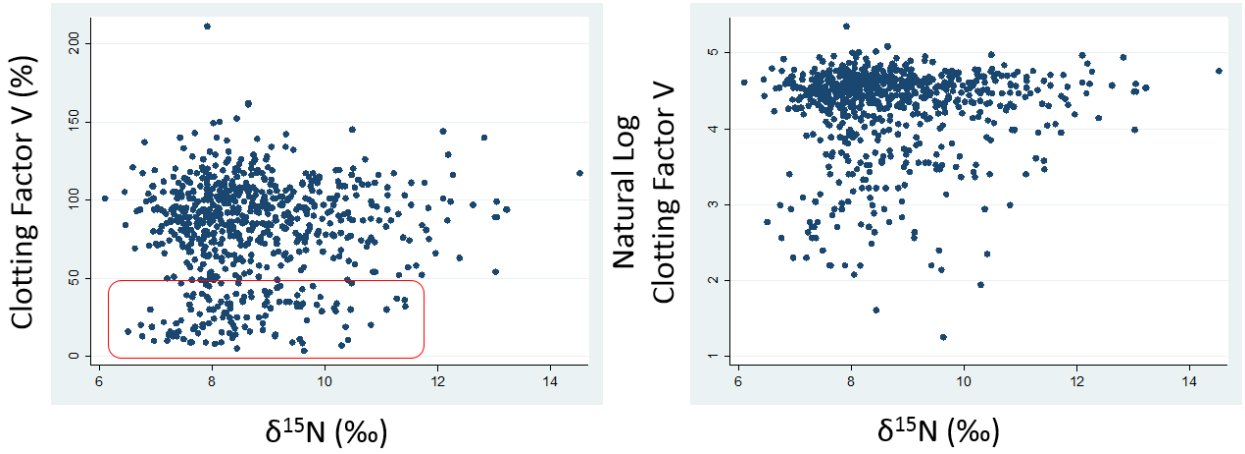

Figure 2.9. The correlation between factor V and $\delta^{15}N$, before and after natural log transformation. Bimodality of factor V data could be seen where approximately 18% (highlighted by the rectangular box) of our samples had factor V activity measured to be less than 50%. The association with $\delta^{15}N$ was further explored by categorizing factor V data into groups of either >30% or ≤ 30% activity (explored later in Table 6).

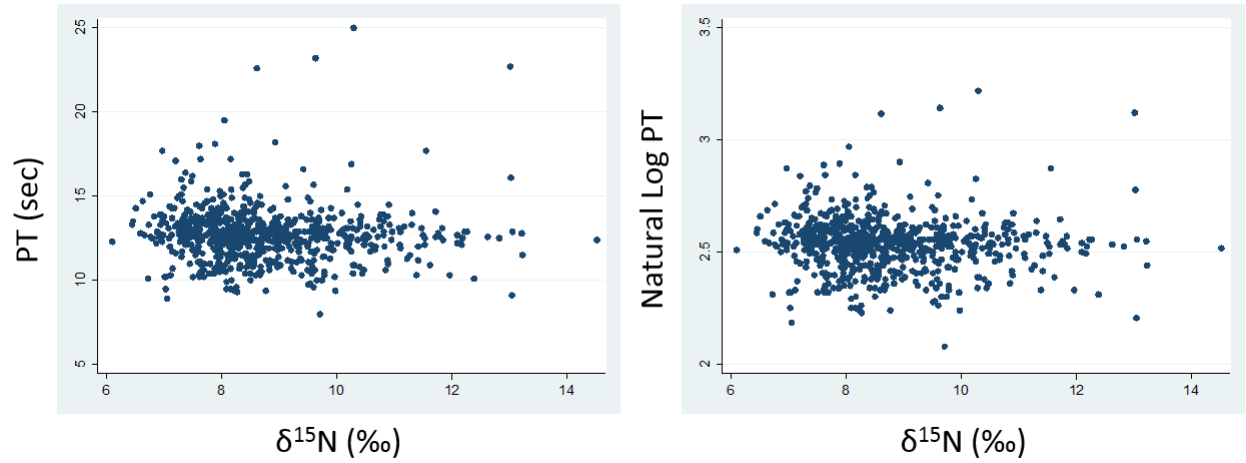

Figure 2.10. The correlation between PT and $\delta^{15}N$, before and after natural log transformation. No significant association of natural log PT with $\delta^{15}N$ was observed before ($p = 0.117$ $R^2 = 0.003$), and after ($p = 0.737$, $R^2 = 0.014$) the adjustment with age and sex.

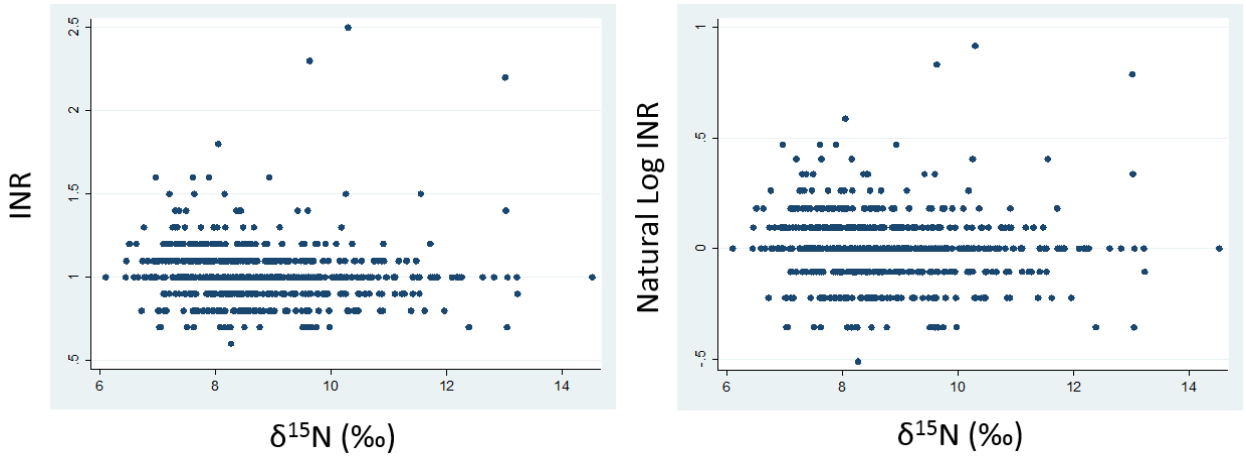

Figure 2.11. The correlation between INR and $\delta^{15}N$, before and after natural log transformation. No significant association of natural log INR with $\delta^{15}N$ was observed before, ($p = 0.266$, $R^2 = 0.002$) and after ($p = 0.356$, $R^2 = 0.015$) the adjustment for age and sex.

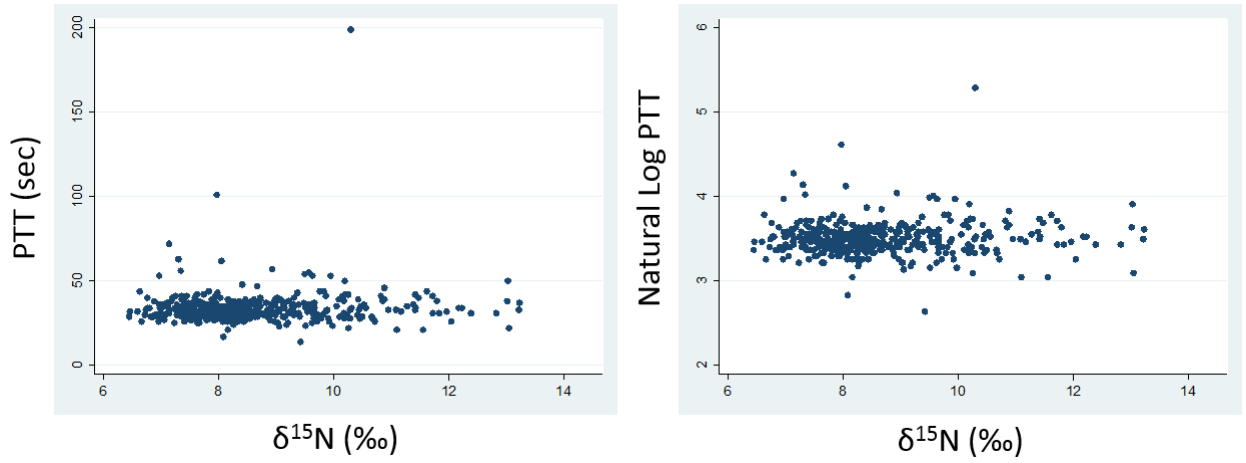

Figure 2.12. The correlation between PTT and $\delta^{15}N$, before and after natural log transformation. A significant association was observed after the adjustment with age and sex ($p = 0.005$, $R^2 = 0.036$). However, the significance was lost after the inclusion of geographical status as a covariate to the regression model in addition to age and sex ($p = 0.481$, $R^2 = 0.088$).

	Factor V > 30%					Factor V ≤ 30%				
Mean ± SD	88.6 ± 25.2					18.2 ± 7.11				
N	636					70				
	Skewness	**Kurtosis**	**P-value**	**R-value**	**R^2**	**Skewness**	**Kurtosis**	**P-value**	**R-value**	**R^2**
Untransformed	-0.132	3.66				-0.009	2.03			
Unadjusted			0.492	-0.026	0.001			0.473	0.087	0.008
Adjusted for age and sex			0.016	-0.142	0.020			0.231	0.162	0.026
Natural log-transformed	-1.13	4.17				-0.888	3.51			
Unadjusted			0.351	-0.037	0.001			0.998	0.000	0.000
Adjusted for age and sex			0.036	-0.122	0.015			0.474	0.131	0.017

Table 2.6. Multivariate regression analysis results between factor V activity and $\delta^{15}N$ before and after natural log-transformation. The analysis was stratified by groups of factor V activity (> 30% or ≤ 30%). Regression was performed on both the untransformed and natural log-transformed factor V variable since skewness and kurtosis value did not improve after natural log transformation.

	Factor V > 30%			Factor V ≤ 30%			
Variable	**Mean ± SD**		**N**	**Mean ± SD**		**N**	**P-value**
Factor V (%)	88.6 ± 25.2		636	18.2 ± 7.11		70	<0.001
PT (sec)	12.6 ± 1.52		673	14.2 ± 2.66		69	<0.001
INR	1.00 ± 0.17		674	1.17 ± 0.31		69	<0.001
PTT (sec)	32.7 ± 4.59		391	41.2 ± 27.2		43	0.013
Regression with $\delta^{15}N$	**P-value**	**R-value**	**R^2**	**P-value**	**R-value**	**R^2**	
Unadjusted	0.492	-0.026	0.001	0.473	0.087	0.008	
Adjusted for age and sex	0.016	-0.142	0.020	0.231	0.162	0.026	

Table 2.7. Differences in PT, INR, and PTT between samples with clotting factor V activity > 30% and those that were ≤ 30%. Differences between the two groups were analyzed by two sample t-test for both untransformed and natural log-transformed values of PT, INR, and PTT. P-values were the same for both the untransformed and natural-log transformed variables.

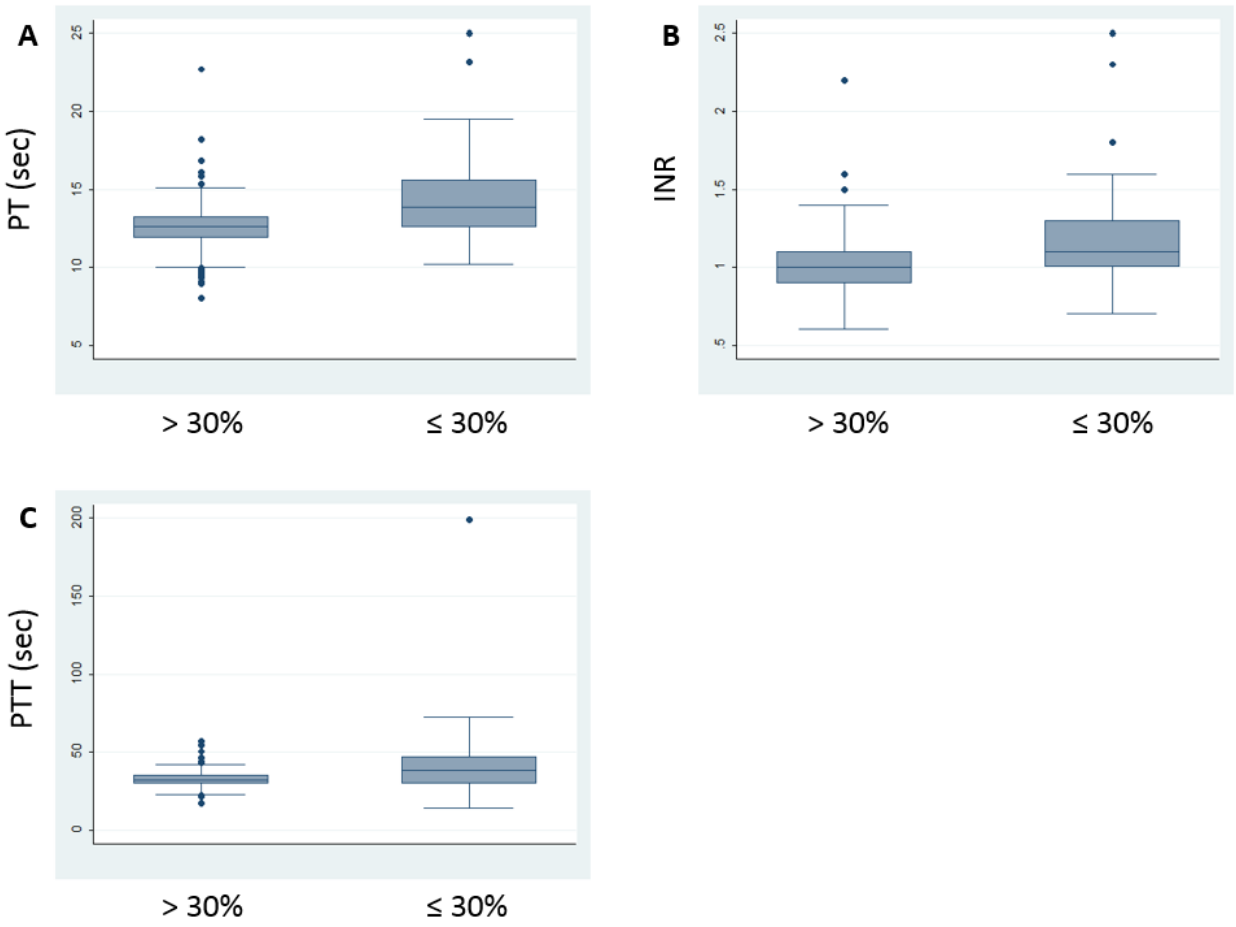

Figure 2.13. Box-and-whisker plot of PT, INR, and PTT values for plasma samples based on clotting factor V activity. The center line of each box represents the median, while upper and lower ends of the box represent the interquartile ranges. The outer whisker lines represent the maximum (top) and minimum (bottom) values, excluding outliers. Dotted points are outliers, defined as 1.5 times the upper and lower interquartile limits. Values are represented in Table 2.7.

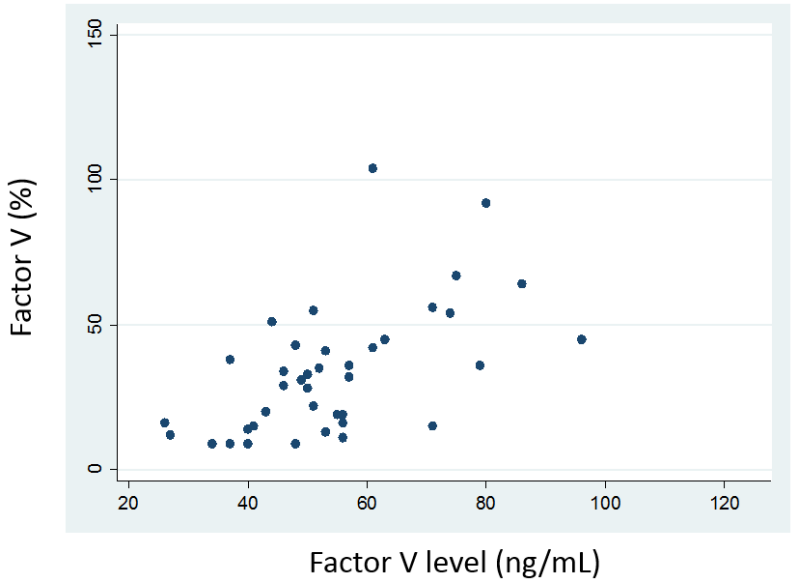

Figure 2.14. Factor V activity correlated positively with factor V levels from ELISA assay ($p < 0.001$, $R^2 = 0.332$, N= 39) in samples that were low in factor V (≤ 30%). The ELISA was used to validate the factor V activity levels of study samples by directly quantifying concentrations of factor V protein.

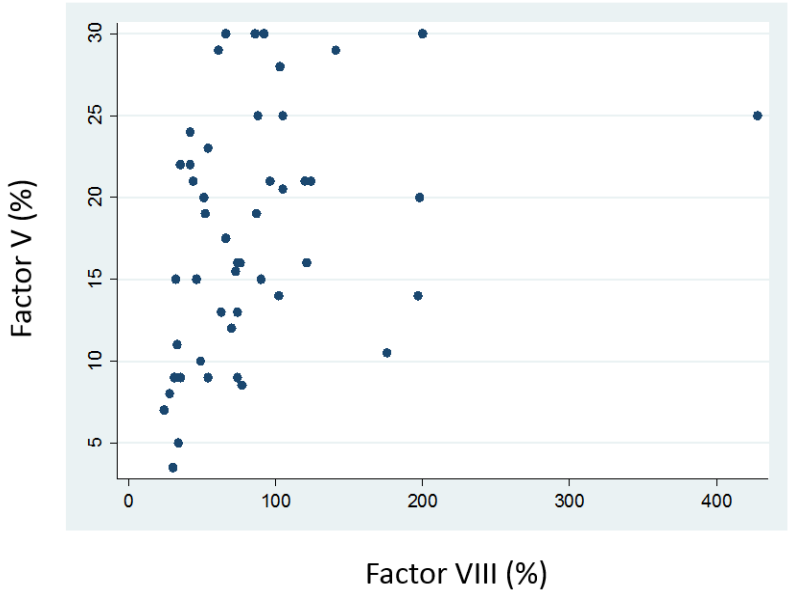

Figure 2.15. The correlation between factor V activity and factor VIII activity. Despite a positive correlation ($p = 0.018$, $R^2 = 0.116$), we did not find compelling evidence of F5F8D in our samples since approximately 77% of samples had factor VIII activity values above the lower reference of 50% for factor VIII.

2.5 Notes to Chapter 2

1. Bucher, H. C., Hengstler, P., Schindler, C., and Meier, G. (2002) N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials, *The American journal of medicine 112*, 298-304.

2. Mohammadi, E., Rafraf, M., Farzadi, L., Asghari-Jafarabadi, M., and Sabour, S. (2012) Effects of omega-3 fatty acids supplementation on serum adiponectin levels and some metabolic risk factors in women with polycystic ovary syndrome, *Asia Pacific journal of clinical nutrition 21*, 511-518.

3. Vecka, M., Dusejovska, M., Stankova, B., Zeman, M., Vavrova, L., Kodydkova, J., Slaby, A., and Zak, A. (2012) N-3 polyunsaturated fatty acids in the treatment of atherogenic dyslipidemia, *Neuro endocrinology letters 33 Suppl 2*, 87-92.

4. Lemaitre, R. N., King, I. B., Mozaffarian, D., Kuller, L. H., Tracy, R. P., and Siscovick, D. S. (2003) n-3 Polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study, *The American journal of clinical nutrition 77*, 319-325.

5. Dyerberg, J., and Bang, H. O. (1979) Haemostatic function and platelet polyunsaturated fatty acids in Eskimos, *Lancet 2*, 433-435.

6. Daviglus, M. L., Stamler, J., Orencia, A. J., Dyer, A. R., Liu, K., Greenland, P., Walsh, M. K., Morris, D., and Shekelle, R. B. (1997) Fish consumption and the 30-year risk of fatal myocardial infarction, *The New England journal of medicine 336*, 1046-1053.

7. Yamagishi, K., Iso, H., Date, C., Fukui, M., Wakai, K., Kikuchi, S., Inaba, Y., Tanabe, N., and Tamakoshi, A. (2008) Fish, omega-3 polyunsaturated fatty acids, and mortality from cardiovascular diseases in a nationwide community-based cohort of Japanese men and women the JACC (Japan Collaborative Cohort Study for Evaluation of Cancer Risk) Study, *Journal of the American College of Cardiology 52*, 988-996.

8. (1999) Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico, *Lancet 354*, 447-455.

9. Calder, P. C. (2001) Polyunsaturated fatty acids, inflammation, and immunity, *Lipids 36*, 1007-1024.

10. Khairallah, R. J., Kim, J., O'Shea, K. M., O'Connell, K. A., Brown, B. H., Galvao, T., Daneault, C., Des Rosiers, C., Polster, B. M., Hoppel, C. L., and Stanley, W. C. (2012) Improved mitochondrial function with diet-induced increase in either docosahexaenoic acid or arachidonic acid in membrane phospholipids, *PloS one 7*, e34402.

11. Arterburn, L. M., Hall, E. B., and Oken, H. (2006) Distribution, interconversion, and dose response of n-3 fatty acids in humans, *The American journal of clinical nutrition 83*, 1467S-1476S.

12. Andriamampandry, M., Freund, M., Wiesel, M. L., Rhinn, S., Ravanat, C., Cazenave, J. P., Leray, C., and Gachet, C. (1998) Diets enriched in (n-3) fatty acids affect rat coagulation factors dependent on vitamin K, *Comptes rendus de l'Academie des sciences. Serie III, Sciences de la vie 321*, 415-421.

13. Nieuwenhuys, C. M., Feijge, M. A., Vermeer, C., Hennissen, A. H., Beguin, S., and Heemskerk, J. W. (2001) Vitamin K-dependent and vitamin K-independent hypocoagulant effects of dietary fish oil in rats, *Thrombosis research 104*, 137-147.

14. Oosthuizen, W., Vorster, H. H., Jerling, J. C., Barnard, H. C., Smuts, C. M., Silvis, N., Kruger, A., and Venter, C. S. (1994) Both fish oil and olive oil lowered plasma fibrinogen in women with high baseline fibrinogen levels, *Thrombosis and haemostasis 72*, 557-562.

15. Shahar, E., Folsom, A. R., Wu, K. K., Dennis, B. H., Shimakawa, T., Conlan, M. G., Davis, C. E., and Williams, O. D. (1993) Associations of fish intake and dietary n-3 polyunsaturated fatty acids with a hypocoagulable profile. The Atherosclerosis Risk in Communities (ARIC) Study, *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association 13*, 1205-1212.

16. Marckmann, P., Bladbjerg, E. M., and Jespersen, J. (1997) Dietary fish oil (4 g daily) and cardiovascular risk markers in healthy men, *Arteriosclerosis, thrombosis, and vascular biology 17*, 3384-3391.

17. Smith, P., Arnesen, H., Opstad, T., Dahl, K. H., and Eritsland, J. (1989) Influence of highly concentrated n-3 fatty acids on serum lipids and hemostatic variables in survivors of myocardial infarction receiving either oral anticoagulants or matching placebo, *Thrombosis research 53*, 467-474.

18. Eritsland, J., Arnesen, H., Gronseth, K., Fjeld, N. B., and Abdelnoor, M. (1996) Effect of dietary supplementation with n-3 fatty acids on coronary artery bypass graft patency, *The American journal of cardiology 77*, 31-36.

19. Bersamin, A., Zidenberg-Cherr, S., Stern, J. S., and Luick, B. R. (2007) Nutrient intakes are associated with adherence to a traditional diet among Yup'ik Eskimos living in remote Alaska Native communities: the CANHR Study, *International journal of circumpolar health 66*, 62-70.

20. Ryman, T. K., Austin, M. A., Hopkins, S., Philip, J., O'Brien, D., Thummel, K., and Boyer, B. B. (2014) Using exploratory factor analysis of FFQ data to identify dietary patterns among Yup'ik people, *Public health nutrition 17*, 510-518.

21. O'Brien, D. M., Kristal, A. R., Jeannet, M. A., Wilkinson, M. J., Bersamin, A., and Luick, B. (2009) Red blood cell delta15N: a novel biomarker of dietary eicosapentaenoic acid and docosahexaenoic acid intake, *The American journal of clinical nutrition 89*, 913-919.

22. Mohatt, G. V., Plaetke, R., Klejka, J., Luick, B., Lardon, C., Bersamin, A., Hopkins, S., Dondanville, M., Herron, J., and Boyer, B. (2007) The Center for Alaska Native Health Research Study: a community-based participatory research study of obesity and chronic disease-related protective and risk factors, *International journal of circumpolar health 66*, 8-18.

23. Nash, S. H., Bersamin, A., Kristal, A. R., Hopkins, S. E., Church, R. S., Pasker, R. L., Luick, B. R., Mohatt, G. V., Boyer, B. B., and O'Brien, D. M. (2012) Stable nitrogen and carbon isotope ratios indicate traditional and market food intake in an indigenous circumpolar population, *The Journal of nutrition 142*, 84-90.

24. Wilkinson, M. J., Yai, Y., and O'Brien, D. M. (2007) Age-related variation in red blood cell stable isotope ratios (delta13C and delta15N) from two Yupik villages in southwest Alaska: a pilot study, *International journal of circumpolar health 66*, 31-41.

25. Kamath, S., Blann, A. D., and Lip, G. Y. (2001) Platelet activation: assessment and quantification, *European heart journal 22*, 1561-1571.

26. Chandler, W. L. (2013) Microparticle counts in platelet-rich and platelet-free plasma, effect of centrifugation and sample-processing protocols, *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis 24*, 125-132.

27. Kraft, R. A., Jahren, A. H., and Saudek, C. D. (2008) Clinical-scale investigation of stable isotopes in human blood: delta13C and delta15N from 406 patients at the Johns Hopkins Medical Institutions, *Rapid communications in mass spectrometry : RCM 22*, 3683-3692.

28. Asselta, R., and Peyvandi, F. (2009) Factor V deficiency, *Seminars in thrombosis and hemostasis 35*, 382-389.

29. Huang, J. N., and Koerper, M. A. (2008) Factor V deficiency: a concise review, *Haemophilia : the official journal of the World Federation of Hemophilia 14*, 1164-1169.

30. Lippi, G., Favaloro, E. J., Montagnana, M., Manzato, F., Guidi, G. C., and Franchini, M. (2011) Inherited and acquired factor V deficiency, *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis 22*, 160-166.

31. Moertl, D., Berger, R., Hammer, A., Hutuleac, R., Koppensteiner, R., Kopp, C. W., and Steiner, S. (2011) Dose-dependent decrease of platelet activation and tissue factor by omega-3 polyunsaturated fatty acids in patients with advanced chronic heart failure, *Thrombosis and haemostasis 106*, 457-465.

32. Eschen, O., Christensen, J. H., De Caterina, R., and Schmidt, E. B. (2004) Soluble adhesion molecules in healthy subjects: a dose-response study using n-3 fatty acids, *Nutrition, metabolism, and cardiovascular diseases : NMCD 14*, 180-185.

33. Ridker, P. M., Buring, J. E., and Rifai, N. (2001) Soluble P-selectin and the risk of future cardiovascular events, *Circulation 103*, 491-495.

34. Katayama, M., Handa, M., Araki, Y., Ambo, H., Kawai, Y., Watanabe, K., and Ikeda, Y. (1993) Soluble P-selectin is present in normal circulation and its plasma level is elevated in patients with thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome, *British journal of haematology 84*, 702-710.

35. Boyer, B. B., Mohatt, G. V., Plaetke, R., Herron, J., Stanhope, K. L., Stephensen, C., and Havel, P. J. (2007) Metabolic syndrome in Yup'ik Eskimos: the Center for Alaska Native Health Research (CANHR) Study, *Obesity (Silver Spring) 15*, 2535-2540.

36. Vanschoonbeek, K., Feijge, M. A., Paquay, M., Rosing, J., Saris, W., Kluft, C., Giesen, P. L., de Maat, M. P., and Heemskerk, J. W. (2004) Variable hypocoagulant effect of fish oil intake in humans: modulation of fibrinogen level and thrombin generation, *Arteriosclerosis, thrombosis, and vascular biology 24*, 1734-1740.

37. Tans, G., Nicolaes, G. A., and Rosing, J. (1997) Regulation of thrombin formation by activated protein C: effect of the factor V Leiden mutation, *Seminars in hematology 34*, 244-255.

38. Gajos, G., Zalewski, J., Rostoff, P., Nessler, J., Piwowarska, W., and Undas, A. (2011) Reduced thrombin formation and altered fibrin clot properties induced by polyunsaturated omega-3 fatty acids on top of dual antiplatelet therapy in patients undergoing percutaneous coronary intervention (OMEGA-PCI clot), *Arteriosclerosis, thrombosis, and vascular biology 31*, 1696-1702.

39. Vernaglione, L., Cristofano, C., and Chimienti, S. (2008) Omega-3 polyunsaturated fatty acids and proxies of cardiovascular disease in hemodialysis: a prospective cohort study, *Journal of nephrology 21*, 99-105.

40. Simopoulos, A. P. (1999) Essential fatty acids in health and chronic disease, *The American journal of clinical nutrition 70*, 560S-569S.

41. Colussi, G., Catena, C., Baroselli, S., Nadalini, E., Lapenna, R., Chiuch, A., and Sechi, L. A. (2007) Omega-3 fatty acids: from biochemistry to their clinical use in the prevention of cardiovascular disease, *Recent patents on cardiovascular drug discovery 2*, 13-21.

42. Calder, P. C. (2006) Polyunsaturated fatty acids and inflammation, *Prostaglandins, leukotrienes, and essential fatty acids 75*, 197-202.

Chapter 3

Impact of Vitamin K Cycle Polymorphisms on Acute and Chronic Vitamin K Status in Alaska Native People

3. 1 Relationship Between Genes of the Vitamin K Cycle Enzymes and Vitamin K Status

The generic term "vitamin K" encompasses a group of lipid-soluble vitamins that are most well-recognized for their role in coagulation. Vitamin K acts as a cofactor to the enzyme γ-glutamyl carboxylase (GGCX), which catalyzes the posttranslational carboxylation of glutamic acid (Glu) to γ-carboxy glutamic acid (Gla) on vitamin K-dependent clotting factors. Vitamin K 2,3-epoxide is a by-product of this process that must be converted back to its hydroquinone form by vitamin K epoxide reductase (VKOR) to be utilized again by GGCX, completing the vitamin K cycle (1). In this way, hepatic vitamin K is recycled for use. Genetic polymorphism in the *VKORC1* gene, such as the *1173 T>C* SNP, is an important source of inter-individual variation in the maintenance dose of the vitamin K antagonist drug, warfarin (2, 3). Vitamin K can also be catabolized and eliminated as chain-shortened acid metabolites following initial metabolic oxidation by the vitamin K hydroxylase, CYP4F2 (4). The *CYP4F2*3* (V433M) allele is associated with higher warfarin dose requirements, presumably due to decreased capacity to eliminate vitamin K and greater availability of the hydroquinone to support GGCX activity (5, 6). CYP4F11, another recently identified vitamin K hydroxylase, has not been studied in relation to warfarin dose but may also contribute to vitamin K status (4). Humans cannot synthesize vitamin K and must obtain it from dietary sources. It exists naturally in two main forms, either as phylloquinone (vitamin K1), commonly found in plants, or menaquinones (vitamin K2), which are found in meats and fermented foods (7). Phylloquinone is the predominant form of vitamin K present in the diet of westernized cultures (8).

Vitamin K status is generally assessed in the clinical setting indirectly through the use of functional clotting assays and direct measurement of the vitamin K in body fluids is less common. For

example, PIVKA-II (protein-induced by vitamin K absence or antagonist-II) is the undercarboxylated form of vitamin K-dependent clotting factor II. It is considered a chronic measure of long-term vitamin K status with values greater than 2 ng/mL taken to be an indicator of subclinical deficiency (9, 10). The prothrombin time (PT), alternatively expressed as the international normalized ratio (INR), is a clinical measure of clotting time that assesses the efficacy of the oral anticoagulant warfarin. The PT/INR value in warfarin patients is strongly affected by vitamin K intake (11). In contrast to PIVKA-II, direct measurement of the phylloquinone concentration in plasma reflects dietary intake over the past 24 hours (12). However, the measurement of phylloquinone from serum or plasma is not performed routinely. It requires highly sensitive analytical methods as well as extensive sample preparation prior to detect and quantify the low nanomolar concentration of vitamin K1 in plasma.

Vitamin K status may be an important factor affecting the coagulation status among the Yup'ik people of Southwest Alaska. Historical accounts of bleeding tendency in Greenlandic Inuits were summarized by Dyerberg and Bang in the 1980's, and included episodes of epistaxis, haemopthysis, urinary tract bleeding, obstetrical bleeding and submucosal bleeding (13, 14). Though the investigators attributed these symptoms to the high consumption of ω3 PUFAs in this population that lead to antagonism of platelet activity (discussed previously in Chapter 2), vitamin K status was never explored in their studies. Given that the main dietary source of vitamin K is green leafy vegetables, which are only intermittently available in the remote sub-Arctic regions where the Yup'ik people live, it is plausible that this population may have been more historically prone to a hypocoagulable state because they have low intake of vitamin K, particularly during seasons when traditional sources such as "tundra greens" and seaweed are unavailable. There is currently little information about vitamin K status among indigenous groups of the Arctic regions and no information is available for the Yup'ik people about the genetic variation of vitamin K recycling and catabolizing enzymes, specifically variation in *VKORC1*, *CYP4F2,* and *CYP4F11*.

In this study we quantified the vitamin K plasma levels from Yup'ik study participants and stratified those concentrations by the common vitamin K cycle polymorphisms; *VKORC1-1173 T>C*

(rs9934438), *CYP4F2*3* (C>T SNP, V433M, rs2108622), *CYP4F2 W12G* (A>C, rs3093105), *CYP4F11 R276C* (G>A, rs8104361), and *CYP4F11 N446D* (A>G SNP, rs106046) genotypes. Additionally, we used a case-control study with logistic regression analysis to determine if these genes influence long-term vitamin K status, as determined from PIVKA-II measurements. Lastly, we analyzed the vitamin K content of tundra greens that are regularly consumed in the traditional Yup'ik diet. Tundra greens are collected seasonally in Alaska and may be an important source of vitamin K in addition to, or alternative to, commercially available food sources. By assessing short and long-term status of vitamin K in the Yup'ik people and measuring levels from tundra greens, we have established, for the first-time, base-line measures of vitamin K availability and status in this population.

3.2 Experimental Procedures

Reagents and Standards

Standards of phylloquinone (VK1) and menaquinone-4 (MK-4) were commercially available from Supleco (catalog no. 47773 and 47774 respectively). Phylloquinone epoxide (VKO) and menaquinone-4 epoxide (MK-4O) were synthesized by the method of Dowd (15). Briefly, 5 mg of VK1 and 7.5 mg of hydrogen peroxide was added to a round-bottom flask containing 2.5 mL of absolute ethanol and 0.3 mL of 3 M aqueous sodium bicarbonate. The mixture was heated at 75^{o}C for 1 hour with stirring and then quenched with 10 mL of water. The solution was extracted three times with 10 mL of diethyl ether, dried over magnesium sulfate and filtered. The filtrate was concentrated under vacuum and then redissolved in 1 mL methanol. The solution was then further purified using a Beckman Coulter Ultrasphere ODS 5 µm 10 mm x 25 cm C18 Reversed Phase Semi-Preparative column (part no. 235328) and methanol as the mobile phase.

Deuterated phylloquinone (VK1-d7) was commercially available from Aldrich (catalog no. catalog no. 705470). Deuterated menaquinone-4 (MK-4-d7), a gift from Dr. Matthew McDonald, was synthesized by the method of Suhara (16). Deuterated phylloquinone epoxide (VKO-d7) was synthesized

from VK1-d7 and deuterated menaquinone-4 epoxide (MK-4O-d7) was synthesized from MK-4-d7 by the method of Dowd (15). All standards were prepared in methanol and characterized spectrophotometrically and chromatographically before use. The molar extinction coefficient of VK1 and MK-4 is 19000 M^{-1} at 248 nm and 30800 M^{-1} at 226 nm for VKO and MK-4O, respectively (17, 18).

LC-MS conditions

All standards and samples were analyzed on a Waters TQ-S mass spectrometer connected to a Waters Acquity I-Class ultra performance liquid chromatography (UPLC) system with an atmospheric pressure chemical ionization (APCI) source operating in negative mode. The APCI probe temperature was set to 650ºC. The corona voltage was set to 30 µA and the desolvation gas flow was 500 L/hr. The temperature-controlled UPLC column compartment was set to 50ºC. Chromatographic separation of analytes was achieved on a Waters Acquity UPLC BEH phenyl 1.7 μm, 2.1 x 50 mm column (part no. 186002884) with a Phenomenex SecurityGuard Cartridge System (part no. KJ0-4282) equipped with a 4.0 x 2.0 mm C8 SecurityGuard cartridge (part no. AJ0-4289). The mobile phase consisted of solvent A (water) and solvent B (0.05% formic acid in methanol). A linear gradient was run as follows: 85% solvent B at 0 minutes to 100% solvent B at 3 minutes with a flow rate of 0.35 mL/min. Elution continued with Solvent B at 100% for 3 more minutes before re-equilibration back to 85% solvent B over a final 2 minutes. The cycle was complete at 8 minutes.

Assessment of Vitamin K Status in the Yup'ik

Data were gathered in collaboration with the Center for Alaska Native Health Research (CANHR) as part of a Northwest-Alaska Pharmacogenetic Research Network study described previously in Chapter 2. Out of 772 study participant samples collected, 755 had measureable PIVKA-II data and a smaller subset of 211 plasma samples were analyzed for vitamin K content. All study participants fasted for 12 hours prior to blood draws and all samples were frozen at -80ºC prior to work-up. All plasma

samples were prepared in duplicates prior to LC-MS analysis and extracted using modified method originally described by Paroni et al (19). Briefly, to each 0.5 mL plasma sample, 2 ng of VK1-d7, 2 ng MK-4-d7, 4 ng VKO-d7, and 4 ng MK-4O-d7 were added. Ethanol (2 mL) was added followed by 1 minute of vigorous mixing. The samples were then centrifuged at 3,000 rpm (1,932 g) for 5 minutes and the supernatant was transferred into a separate tube. Samples were then extracted using 1.0 mL Oasis HLB Polymeric SPE columns (part no. WAT094225, Waters). The column cartridges were initially conditioned with 1.0 mL of methanol and 1.0 mL of water. The samples were loaded onto the columns and then washed with 1.0 mL of water, 1.0 mL of 50:50 (v/v) water/methanol, and 1.0 mL 20:80 (v/v) water/methanol. The columns were dried for 1 minute and finally eluted with 1 mL of acetonitrile/isopropanol/dichloromethane (70:10:20). This eluate was evaporated under nitrogen and the residue was dissolved in 100 μL of methanol for analysis of 15 μL aliquots that were automatically injected into the LC/MS system. PIVKA-II was measured by the UW Department of Laboratory Medicine as part of a test panel to assess coagulation status; the method is described in Chapter 2. The kit was commercially available from Diagnostica Stago (Asserachrom PIVKA-II, cat. no. 00631). Hemolyzed samples were excluded from data analysis and the limit of quantitation for the PIVKA-II assay was 2 ng/mL.

Tundra Green Preparation

All samples of tundra greens were obtained from areas near the Yup'ik village study sites where plasma had been collected from study participants. Since different parts of tundra greens may be consumed by Yup'ik people, the leaf and stems (where available) were separately analyzed as two sets of duplicates before and after boiling for 15 minutes in water to reflect varied preparation of these foods prior to consumption. For comparison, we also measured vitamin K content of tundra greens in a separate set of commercially available 'reference' vegetables, which included broccoli, spinach, iceberg lettuce, celery, basil, red onion, and carrot. Samples were stored at -80ºC after collection and then freeze-dried

under vacuum prior to extraction. To each 20 mg aliquot of freeze-dried tundra greens, 10 ng of VK1-d7 and 20 ng VKO-d7 was added. After the addition of 0.5 mL of water, each sample was homogenized with a Bertin Technologies Precellys 24 tissue homogenizer utilizing 2.8 mm ceramic beads (MO BIO Laboratories, Inc., catalog number 3114-325). Water (4.0 mL) and 4.0 mL of 2-propanol/hexane (3:2, v/v) were added and, after vigorous vortexing for 1 minute, samples were centrifuged at 3000 rpm (1500g) for 5 minutes. The upper hexane layer was removed and the extraction step was repeated with 4 mL of hexane. After drying the isolated supernatant layer under nitrogen, samples were reconstituted in 500 µL of hexane and applied to Bond Elut 500 mg silica SPE columns (part no. 12113036, Varian). These cartridges were conditioned initially with 4.0 mL of ethyl ether/hexane (4:96, v/v) and then with 4.0 mL of hexane. After loading the concentrated vegetable extracts, the columns were washed with 4.0 mL of hexane, and eluted with 8.0 mL of ether in hexane (4:96, v/v). The eluate was evaporated under nitrogen and the residue was dissolved in 100 µL of methanol solution for analysis of 15 µL aliquots by LC/MS.

Validation Procedure and Quantification

Due to presence of residual levels of VK1 in human plasma, fetal bovine serum (FBS) was used instead as the medium for preparation of standard curves. Standard curves were also generated using phosphate-buffered saline (PBS). FBS or PBS was spiked with stock solutions ranging from 0.1 to 3.0 ng/mL of VK1, VKO, MK-4, and MK-4O. In creating a standard curve to analyze VK1 and VKO from tundra greens, samples containing 20 mg of freeze-dried red onion (which contains negligible levels of vitamin K) in 0.5 mL of water were spiked with stock solutions ranging from 5 to 500 ng of VK1 and 0.5 to 50 ng of VKO. All samples were extracted and analyzed by LC-MS to obtain peak-area ratios for each analyte compared to its deuterated internal standard. Plasma samples were excluded if variation in measured vitamin K between duplicates was greater than 20%. Twelve archived plasma samples from known warfarin patients (where plasma levels of vitamin K analytes were expected to be elevated) were

also used to further validate the assay. These data were compared to twelve other archived plasma samples from non-warfarin patients.

Genotyping of CYP4F2, CYP4F11, and VKORC1

The SNPs for *CYP4F2*3* (C>T SNP, V433M, rs2108622), *CYP4F2 W12G* (A>C, rs3093105), *CYP4F11 R276C* (G>A, rs8104361), *CYP4F11 N446D* (A>G SNP, rs106046) and *VKORC1-1173 T>C* (rs9934438) were analyzed using TaqMan SNP Genotyping Assays (Applied Biosystems, Inc.) on 96.96 Dynamic Genotyping Arrays (Fluidigm). Dynamic Arrays were primed and loaded on the Fluidigm HX and thermo-cycled on the Fluidigm FC1 controller. End-point fluorescence was read on a BioMark™ Real-Time PCR System (Fluidigm) and analyzed using SNP Genotyping Analysis software (Fluidigm). Samples with total call rates below 70% were excluded from further analysis.

Statistical Analysis

All statistical analyses were performed using STATA (version 11.0 SE). We compared vitamin K analyte levels in plasma between warfarin and non-warfarin patients using a two-sample t-test. To evaluate the association between plasma vitamin K levels and *CYP4F2, CYP4F11*, and *VKORC1* genotypes, we used a multivariate gene-dosing model regression model that included the covariates sex, age, and geographical status of the study community from which the plasma samples were obtained. Geographical status was defined as coastal if study participants lived within 5 miles of the coast, otherwise their status was assigned as inland. Genotype categories were classified for all genes as having zero, one, or two copies of the variant allele. A χ^2 goodness-of-fit test was used to determine if the observed genotype frequencies for *CYP4F2, CYP4F11*, and *VKORC1* SNPs differed significantly from the expected frequencies under the assumptions of Hardy-Weinberg Equilibrium.

A logistic regression model was used to estimate the odds ratio (OR) of the association between genotype and long-term vitamin K status. The outcome was dichotomous and categorized as either

PIVKA-II ≥ 2 ng/mL (low vitamin K status) or PIVKA-II < 2 ng/mL (normal vitamin K status). The predictor variables of interest were genotype (0, 1, or 2 copies of variant allele), age (continuous), sex, (binary) and whether the study community was located in a coastal or inland region (binary). The OR and confidence interval (CI) of 95% were reported. The overall fit of the model to the data was evaluated using the likelihood ratio test ($\chi 2$) and goodness-of-fit test (Pearson χ^2). A two-tailed p-value less than 0.05 was considered significant for all statistical tests. Not all genotyped samples passed analytical quality controls and only participant samples with a complete data were analyzed statistically.

3.3 Results

Development and Validation of the LC-MS assay

The LC-MS conditions and MRM transitions for each vitamin K analyte are summarized in Table 3.1 and representative chromatograms are shown in Figure 3.1. No ion overlap of unlabeled compounds into the channels monitoring the deuterated standards above background was observed, within the experimental concentration ranges. The linearity of response for all analytes in plasma was tested by spiking standards of VK1, VKO, MK-4, and MK-4O into 0.5 mL PBS and 0.5 mL of FBS in concentrations that ranged from 0.1 ng/mL to 2 ng/mL (Figures 3.2-3.5). The linearity of response for all analytes in tundra greens was assessed by spiking VK1 and VKO standards into samples containing 0.5 mL water with 20 mg of freeze-dried red onion in concentrations that ranged from 5 ng to 500 ng for VK1 and 0.5 to 50 ng for VKO (Figure 3.6). L inear regression was used to compare measurements obtained by LC-MS to expected concentrations of calibration standards. Standard curves for both PBS and FBS were linear with R^2 values of 0.99. For the standard curve derived from red onions, the R^2 values were also 0.99 for both VK1 and VKO.

The VK1, VKO, MK-4, and MK-4O plasma concentrations were compared between warfarin (N = 12) and non-warfarin (N = 12) patients as a separate validation of the LC-MS assay. The mean, median, standard deviation (SD) and interquartile range (IQR) for each analyte between warfarin and

non-warfarin patients are presented in Table 3.2. Though VK1 and MK-4 did not differ significantly between the two groups, VKO and MK-4O were elevated to 1.70 ± 1.09 ng/mL and 0.14 ± 0.08 ng/mL, respectively, in warfarin patients compared to non-detectable amounts of these analytes in non-warfarin patients (Figures 3.7 and 3.8). This is expected because inhibition of VKOR by warfarin hinders the reduction of epoxide in the vitamin K cycle and increases its concentrations in plasma. Indeed, this seminal observation, first made 40 years ago, laid the basis for the early understanding of the role that the vitamin K cycle plays in the mechanism of coumarin anticoagulation (20).

CYP4F2, CYP4F11 and VKORC1 SNP Allele Frequencies

The allele frequencies of *CYP4F2, CYP4F11*, and *VKORC1* SNPs observed in Yup'ik people relative to other ethnic populations are displayed in Table 3.3. Genetic information from non-Yup'ik populations were obtained from the International HapMap Project, which include CEU: Utah residents with ancestry from northern and western Europe, YRI: Yoruba in Ibadan, Nigeria, and JPT: Japanese in Tokyo (21). The *CYP4F2*3* allele was observed at a much higher frequency in Yup'ik people (0.485) relative to CEU (0.232), YRI (0.058) and JPT populations (0.235). In contrast, the variant allele frequencies for both *CYP4F11* SNPs; *R276C* (0.008) and *N446D* (0.134), were lower in Yup'ik people compared to other ethnic groups. The *VKORC1-1173 T>C* allele (0.21) was at a frequency most similar to Asians (0.09) and would be predicted to contribute significantly to a low warfarin dose phenotype in this population. The genotype frequencies for the SNPs *CYP4F2, CYP4F11* and *VKORC1* are displayed in Table 3.4. There were no significant deviations from Hardy-Weinberg Equilibrium observed for genotypes of *CYP4F2*3* ($p = 0.414$), *CYP4F2 W12G* (Fisher's Exact $p = 0.218$), *CYP4F11 R276C* ($p = 1.00$), and *CYP4F11 N446D* ($p = 0.350$), but this was significantly different for *VKOR-1173 T>C* SNP ($p < 0.001$). Because of the low variant allele frequency for *CYP4F2 W12G* and *CYP4F11 R276C*, these SNPs were excluded from further analysis.

Vitamin K Levels in Plasma

The distribution of plasma VK1 levels in the Yup'ik before and after natural log transformation are represented in Figure 3.9. The normality of the distribution (skewness = 2.94, kurtosis = 13.78) improved after natural log transformation (skewness = 0.42, kurtosis 3.10). Plasma VKO, MK-4, and MK-4O were detected in only trace amounts below the limit of quantitation (< 0.1 ng/mL) and therefore, were excluded from detailed analysis of plasma data obtained from study participants. We observed no differences in natural log VK1 levels in plasma between sexes ($p = 0.112$) or geographical status ($p = 0.134$), but found a modest positive association with age ($p = 0.043$, $R^2 = 0.0197$).

The mean ± SD of VK1 for all plasma samples (N = 211) measured was 0.46 ± 0.42 ng/mL (Table 3.5, Figures 3.10-3.12). When these results were stratified by *CYP4F2*3* genotype, the mean values were generally higher for study participants genotyped as *CYP4F2 *3/*3* (0.62 ± 0.56 ng/mL, N = 54) compared to those who were **1/*3* (0.43 ± 0.35, N = 98) or **1/*1* (0.39 ± 0.33 ng/mL, N = 53). When these differences were compared using natural log transformed values, the difference relative to the *1/*1 genotype was significant for *3/*3 ($p = 0.003$) but not for *1/*3 ($p = 0.396$). Furthermore, *CYP4F2*3* genotype associated positively with natural log VK1 levels in plasma ($p = 0.002$, $R^2 = 0.047$) even after adjusting for age, sex, and geographical status ($p = 0.004$, $R^2 = 0.0779$). When comparing VK1 levels across *CYP4F11 N446D* genotype, the values were highest (0.92 ± 1.12, N = 3) for the GG genotype compared to the AG (0.40 ± 0.29, N = 38) and AA (0.48 ± 0.45, N =132) genotypes, but did not reach statistical significance in the regression model before ($p = 0.855$, $R < 0.001$) and after ($p = 0.992$, $R^2 = 0.060$) statistical adjustment. In comparisons based on *VKORC1-1173 T>C* genotype status, the measured mean VK1 levels were higher (0.75 ± 0.88 ng/mL, N = 9) for the CC genotype relative to the TC (0.47 ± 0.48 ng/mL, N = 51) and TT (0.45 ± 0.35 N = 145) genotypes. However, under the regression model we did not find any statistically significant associations between natural log VK1 and *VKORC1-1173 T>C* genotype ($p = 0.816$, $R^2 < 0.001$). These associations remained insignificant after the adjustment of age, sex, and geographical status ($p = 0.996$, $R^2 = 0.039$).

Vitamin K Content of Tundra Greens

A list of tundra greens that were collected from field visits by investigators are found on Table 3.6. The measured VK1 and VKO contents in micrograms per gram of freeze-dried plant material are listed in Table 3.7. Leaves were found to generally have higher VK1 content compared to stems. Boiling decreased the phylloquinone content by an average of 46.4% (5.49-71.0%) for leaves and 58.0% (45.1-75.3%) for stems. The VK1 levels in tundra greens were similar to amounts measured from raw, freeze-dried reference vegetables (Table 3.8). Small amounts of VKO were detected in non-boiled samples, but were present in much lower concentrations compared to VK1.

PIVKA-II status

Among the 755 study participants in which PIVKA-II was measured, 272 (36%) had PIVKA-II ≥ 2 ng/mL. The distribution of PIVKA-II values in our study is represented in Figure 3.12 and the descriptive statistics of *CYP4F2*, *CYP4F11*, and *VKORC1* genotypes from the logistic regression analysis are presented in Table 3.9. A significant proportion of men tended to have low PIVKA-II status (40.4%) compared to women (31.2%, Pearson $\chi^2 = 6.92$, $p = 0.009$). No significant differences were observed in PIVKA-II status between coastal and inland communities ($p = 0.082$).

The *CYP4F2*3* allele was found to confer lower odds (OR = 0.80, 95% CI: 0.65 - 0.99, p = 0.04) of low vitamin K status and this association improved after the adjustment with age, sex, and geographical status (OR = 0.77, 95% CI: 0.62 - 0.95, p = 0.016) (Table 3.10). When homozygotes and heterozygotes were individually compared to wild-type, the association was the stronger for *3/*3 (OR = 0.59, 95% CI: 0.38 - 0.91, p = 0.017) than in *1/*3 (OR = 0.75, 95% CI: 0.52 - 1.08, p = 0.120). When *CYP4F2 N446D* was analyzed, the unadjusted OR was 1.03 (95% CI: 0.74 - 1.43, p = 0.873) but remained insignificant (OR = 1.03, 95% CI: 0.74 - 1.44, p = 0.850) after adjusting for age, sex and geographical status (Table 3.11). For *VKORC1-1173 T>C* genotype, the unadjusted OR was 0.90 (95% CI: 0.70 - 1.14, p = 0.382) and the adjusted OR was 0.89 (95% CI: 0.68 - 1.12, p = 0.285), but were both

insignificant (Table 3.12). We also did not find any significant difference in natural log VK1 between participants based on PIVKA-II status ($p = 0.557$).

3.4 Discussion

Many members of the Yup'ik people living in Southwest Alaska incorporate into their lifestyle a subsistence-based diet that is high in marine animals and fish, which are significant sources of ω3 polyunsaturated fatty acids (PUFAs). In Chapter 2, it was shown that higher ω3 PUFA consumption (through the surrogate biomarker, $\delta^{15}N$) affects hemostasis, likely through modifying platelet activity. The contribution of subsistence food from the Yup'ik diet to vitamin K status has not been previously explored but may also impact hemostasis through vitamin K-dependent pathways involving genetic and environmental factors. It is well recognized that maintaining a consistent amount of vitamin K in the diet is important for warfarin patients to stabilize the INR during anticoagulation therapy. Therefore, other genetic and environmental factors that alter vitamin K status could be important clinical considerations when prescribing anticoagulant drugs or other medications that impact the vitamin K cycle.

We evaluated the effect of *CYP4F2*3, CYP4F11 N446D,* and *VKORC1-1173 T>C* both acute (plasma VK1) and long-term (PIVKA-II) vitamin K status in our study population. A significant finding of the genotyping results is that the frequency of the *CYP4F2*3* allele is notably higher than the frequencies reported for other studied populations and is associated with plasma VK1 and PIVKA-II concentration. CYP4F2 has been shown to act as an ω-hydroxylase to VK1 and MK-4, and variation in the *CYP4F2* gene (**3, V433M*) is associated with a higher warfarin dose requirement of approximately 1 mg/day (4, 5). The CYP4F2 protein encoded by the *CYP4F2*3* allele exhibits reduced capacity to metabolize VK1, secondary to a decrease in steady-state levels of the hepatic enzyme (6). Our observation that *CYP4F2*3* genotype positively associates with plasma VK1 concentrations and that these levels are increased in study participants carrying the *3/*3 genotype relative to *1/*3 and *1/*1 provides supporting evidence that *CYP4F2*3* may help conserve vitamin K in the liver by slowing its

hepatic metabolism. This end result could also potentially prolong the serum half-life of VK1, which is approximately 7.5 hours (22). A recent study has shown that *CYP4F2*3* genotype influences α-tocopherol (vitamin E) levels in plasma (23). The investigators found that among study participants receiving vitamin E, the *CYP4F2*3* SNP polymorphism was significantly associated with higher α-tocopherol levels after 48 weeks of treatment relative to baseline levels (23). Given that both vitamin E and vitamin K are fat-soluble vitamins, the results found in our study may allude a broader role for *CYP4F2* in fat-soluble vitamin metabolism.

Though CYP4F11 has also been reported to be a vitamin K hydroxylase, the lack of any significant association between *CYP4F11 N446D* and VK1 in plasma, in addition to its much lower allele frequency compared to *CYP4F2*3,* suggests that the *CYP4F2* has the more dominant role in our study population in affecting acute vitamin K status (4). In comparing differences in plasma VK1 based on *VKORC1-1173 T>C* status, the genotype frequencies observed in our study did not meet the assumptions of Hardy-Weinberg Equilibrium. This suggests that there could be false positive or negative genotypes of *VKORC1-1173 T>C* assigned to our samples. Furthermore, our study was not powered to test for differences between *VKORC1-1173 T>C* (as well as *CYP4F2 W12G* and *CYP4F11 R276C*) genotype groups, because of low frequency of the minor genotype. Variation among intronic SNPs of *VKORC1* have been associated with altered *VKORC1* mRNA expression levels, in addition to warfarin dose requirements (2, 24). It is plausible that greater hepatic levels of the VKOR protein in the CC genotype could result in more "recycling" of vitamin K, potentially elevating vitamin concentrations in plasma. A separate study involving the measurement of plasma vitamin K in populations with greater frequency of the *VKORC1-1173 T>C* variant allele would be needed to confirm this. It is important to note that while the results with *CYP4F2*3* were statistically significant, the mean change in plasma VK1 levels observed between genotype groups was relatively small, given that population reference ranges for plasma VK1 levels have been reported to be as great as 0.22 to 8.88 nmol/L (25, 26).

The measured vitamin K content of the tundra greens collected from study communities was similar to the panel of reference vegetables. In both cases, the vitamin content was higher for vegetables

and parts of the plant (i.e. leaves) with greater green pigmentation, as expected since vitamin K is concentrated in chloroplasts (27, 28). Because tundra greens were replete in vitamin K they may serve as an important source of vitamin K in remote Alaskan regions with limited access to fresh produce. However, since tundra greens are collected mainly during the summer seasons, seasonality is a factor that could affect vitamin K status, but that was not assessed in our study. Furthermore, we did not measure the vitamin K content of marine foods and animals, which are a staple of the Yup'ik diet.

The potential of the *CYP4F2*3* allele to maintain higher levels of VK1 in plasma may have an impact on chronic vitamin K status. Both plasma vitamin K and PIKVA-II are influenced by dietary vitamin K intake, but PIVKA-II is a more commonly measured biomarker of vitamin K status due to its longer half-life relative to serum phylloquinone, reported to be 45 hours in infants and approximately 3 days in adults (29-32). Data from our logistic regression model suggest that the *CYP4F2*3* SNP reduces the likelihood of having low vitamin K status, and this effect was most evident in the *3/*3 homozygotes relative to the **1/*1* genotype. Similar to our results with plasma VK1, PIVKA-II status did not appear to be influenced by *CYP4F11 N446D* and *VKOR-1173 T>C* genotypes. The observed effects of the *CYP4F2*3* genotype on vitamin K status appear to be most evident in the variant **3/*3* homozygotes, which suggests that *CYP4F2* plays a significant in role in the elimination of vitamin K even with only 1 copy of the presumably more functional allele.

A limitation of measuring VK1 from plasma is that concentrations reflect dietary intake over the past 24 hours (33). Since our study participants fasted only 12 hours prior to blood draws, using plasma VK1 concentrations alone to assess vitamin K status may not accurately reflect the contribution of the *CYP4F2*3* allele to overall vitamin K status. Another limitation is that PIVKA-II concentrations below 2 ng/mL cannot be accurately quantified and the majority of our PIVKA-II measurements (~64%) fall below this threshold, thus hindering an evaluation of the association between low vitamin K status and *CYP4F2*3*, *CYP4F11 N446D*, and *VKORC1-1173 T>C* genotype. Although sample integrity could be another major concern, samples were analyzed for both plasma VK1 and PIVKA-II upon first thaw after storage in -20ºC and those manifesting likely significant sources of variability (i.e. clots and hemolysis)

were excluded to improve the robustness of study data. We were also only able to measure vitamin K levels from plasma whereas *CYP4F2*3*, *CYP4F11 N446D*, and *VKORC1-1173 T>C* could also influence hepatic vitamin K levels (explored in chapter 4). Lastly, our study was also cross-sectional in design and could not therefore conclude causality of our results. Future pharmacokinetic studies measuring potential differences in serum half-life and changes in concentration over time of vitamin K among *CYP4F2*3* genotyped individuals would be needed to confirm the effect of CYPF2*3 on vitamin K status.

In summary, our data suggest that the *CYP4F2*3* polymorphism contributes to both acute and long-term variation in vitamin K status. Specifically, high frequency of the *CYP4F2*3* allele observed in Yup'ik people suggests that preservation of the low activity vitamin K hydroxylase allele could be a safe guard mechanism against low vitamin K status. This could reduce the risk of a hypocoagulative state in a tundra environment with less seasonal and dietary availability of vitamin K. While this may play a less significant role today, given westernization in the lifestyle of Yup'ik people over the past few decades and greater commercial access to market foods, these findings are likely still be important pharmacogenetic considerations when prescribing anticoagulant medications and treating chronic disease.

Analyte	Parent (*m/z*)	Daughter (*m/z*)	Cone (V)	Collision (V)	Retention Time (min)
VK1	450.6	185.1	58	30	3.56
MK-4	444.6	185.2	56	28	3.03
VKO	466.5	187.0	30	22	3.33
MK-4O	460.5	187.2	30	19	2.79
VK1d7	457.7	185.1	50	28	3.55
MK-4d7	451.7	192.2	56	28	3.02
VKOd7	473.5	194.2	46	22	3.31
MK-4Od7	467.5	194.0	30	19	2.77

Table 3.1. LC-MS conditions and MRM transitions of each vitamin K analytical standard. The conditions were similar between non-deuterated and deuterated standards, but each was optimized for response on the Xevo TQ-S instrument.

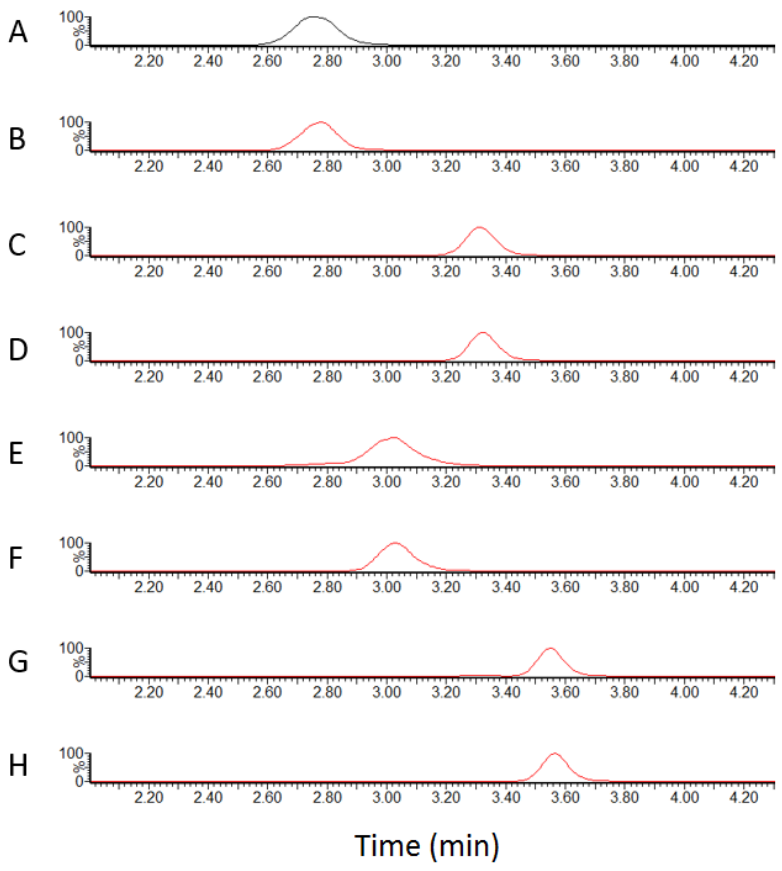

Figure 1.1. LC-MS chromatograms of a standard mix containing; A) MK-4O-d7, B) MK-4O, C) VKO-d7, D) VKO, E) MK-4-d7, F) MK-4, G) VK1-d7 and H) VK1.

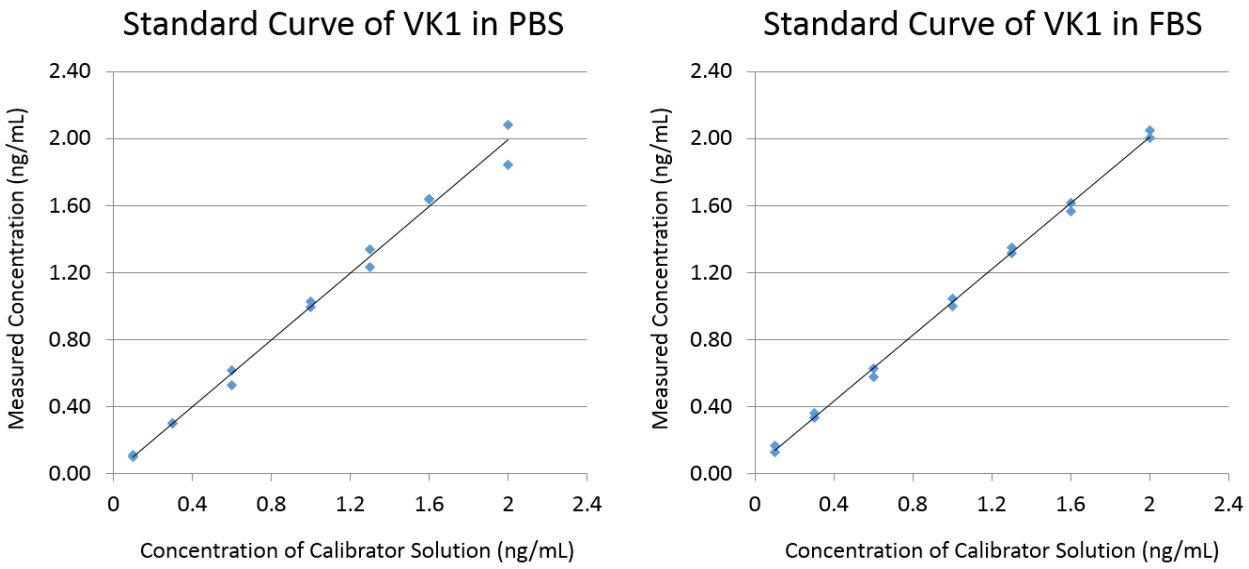

Figure 3.2. Standard curves comparing the measured concentration of VK1 to the expected concentration of the calibrator solution. Both PBS (phosphate buffered saline) and FBS (fetal bovine serum) were used as matrices and response was linear with R^2 values of >0.99, indicating that matrix effects of protein binding at these concentrations did not significantly affect the standard curves. The equations for the standard curves were y = 0.33x – 0.0014 for PBS and y = 0.33x + 0.11 for FBS.

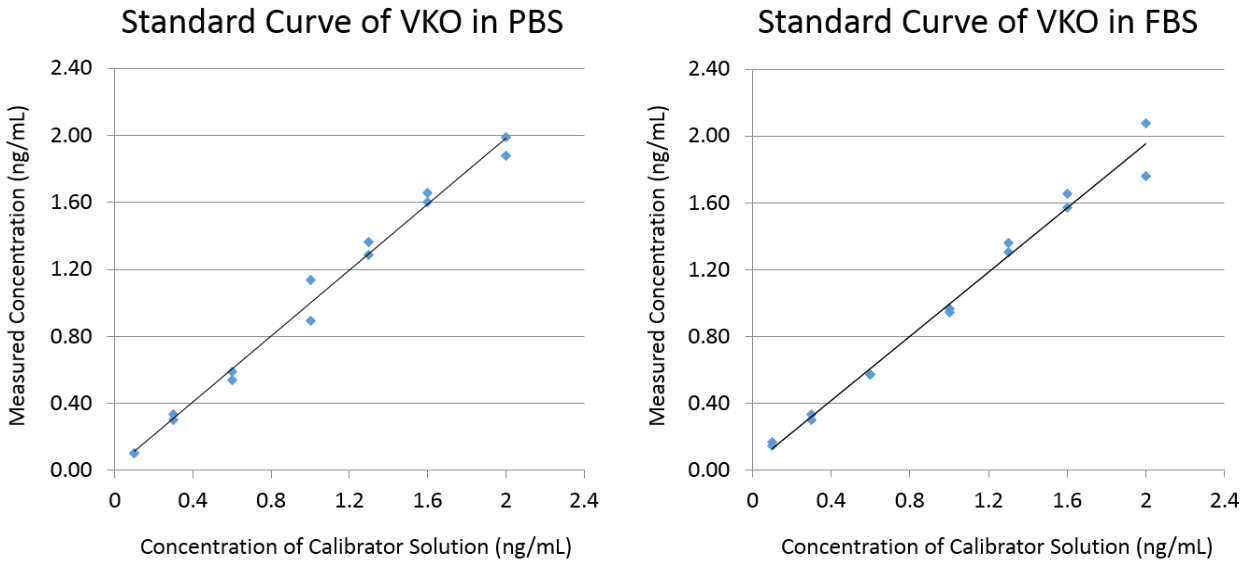

Figure 3.3. Standard curves comparing the measured concentration of VKO to the expected concentration of the calibrator solution. Both PBS and FBS were used as matrices and was response was linear with R^2 values of 0.99. The equations for the standard curves were y = 0.15x – 0.017 for PBS and y = 0.14x + 0.020 for FBS.

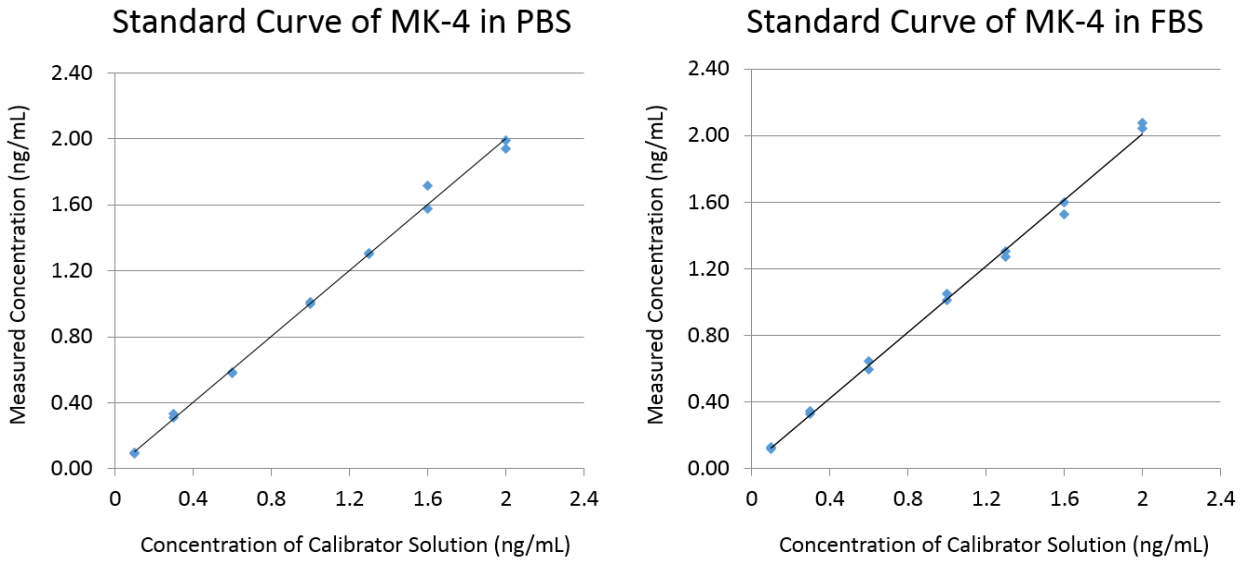

Figure 3.4. Standard curves comparing the measured concentration of MK-4 to the expected concentration of the calibrator solution. Both PBS and FBS were used as matrices and response was linear with R^2 values of 0.99. The equations for the standard curves were $y = 0.38x - 4.0 \times 10^{-4}$ for PBS and $y = 0.38x + 0.007$ for FBS.

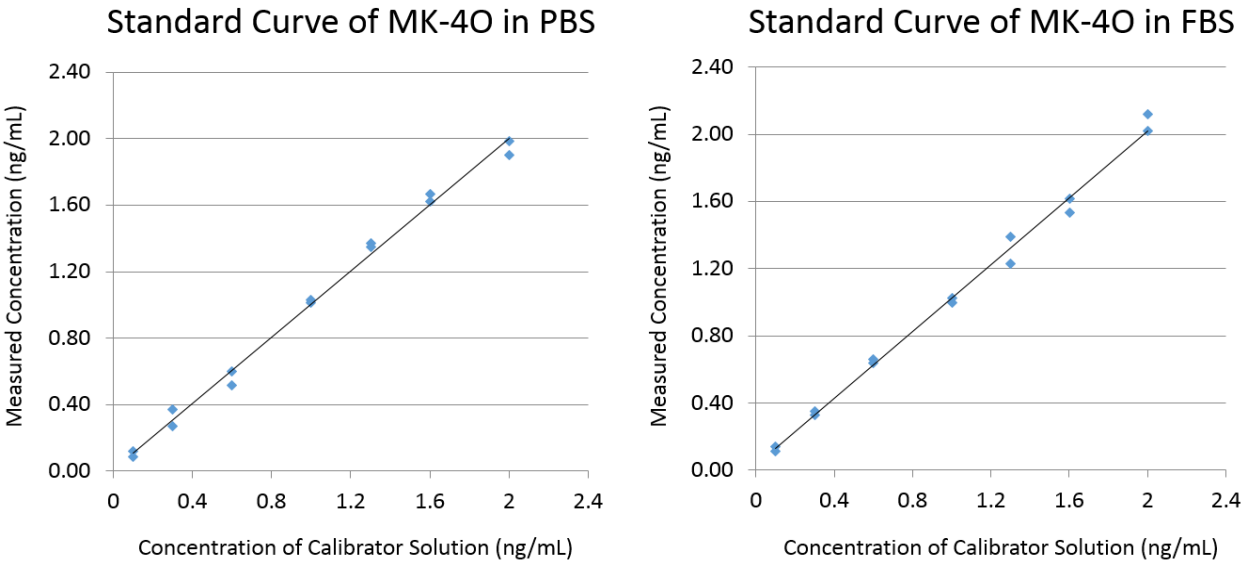

Figure 3.5. Standard curves comparing the measured concentration of MK-4O to the expected concentration of the calibrator solution. Both PBS and FBS were used as matrices and response was linear with R^2 values of 0.99. The equations for the standard curves were $y = 0.16x - 0.005$ for PBS and $y = 0.16x - 0.002$ for FBS.

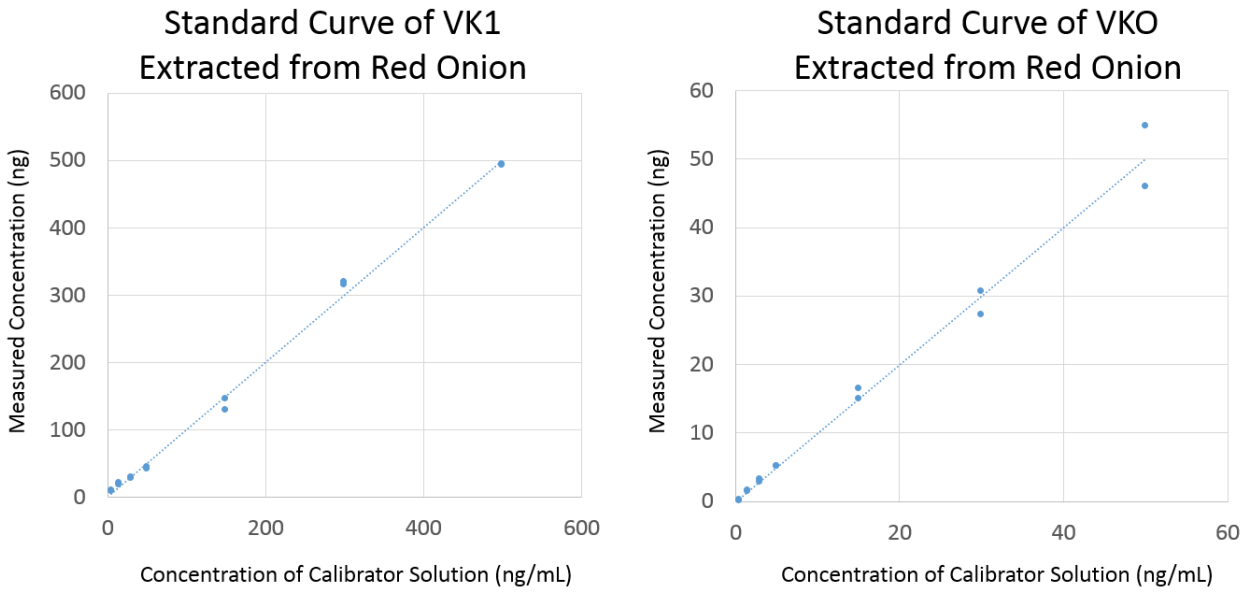

Figure 3.6. Standard curves comparing the measured concentration of VK1 and VKO to the expected concentration of the calibrator solution using 20 mg of freeze-dried red onion as the matrix. Both curves were linear with R^2 values of 0.99. Trend-line equations were $y = x + 4 \times 10^{-14}$ for VK1 and $y = x$ for VKO.

	No Warfarin		**Warfarin**	
Analyte	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)
VK1 (ng/mL)	1.43 ± 1.92	0.72 (1.39)	0.98 ± 0.46	1.00 (0.67)
VKO (ng/mL)	< 0.10	< 0.10	1.70 ± 1.09	1.55 (1.66)
MK-4 (ng/mL)	< 0.10	< 0.10	< 0.10	< 0.10
MK-4O (ng/mL)	< 0.10	< 0.10	0.14 ± 0.08	0.16 (0.15)

Table 3.2. Vitamin K analyte concentrations in the plasma of non-warfarin and warfarin patients. Values are represented here as the mean ± SD and median (IQR) in ng/mL.

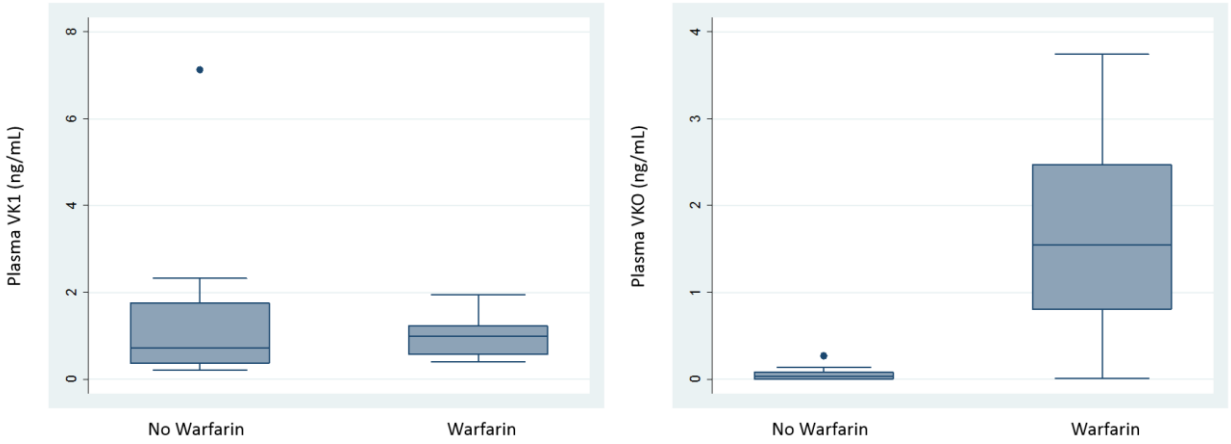

Figure 3.7. Box-and-whisker plots comparing VK1 and VKO levels between archived plasma samples of non-warfarin and warfarin patients. The top and bottom of the darkened boxes represent the 75th and 25th percentiles, respectively. The median is represented by the middle line. Whiskers are the maximum and minimum values excluding outliers. The outliers are identified as closed circles. While no significant difference was observed for VK1 in plasma ($p = 0.435$), VKO levels were significantly higher in warfarin patients ($p < 0.001$).

Figure 3.8. Box-and-whisker plots comparing MK-4 and MK-4O levels between archived plasma samples of non-warfarin and warfarin patients. While no significant difference was observed for MK-4 in plasma ($p = 0.3572$), MK-4O levels were significantly higher in warfarin patients ($p = 0.001$).

Gene	Rs Number	SNP	Yup'ik Variant Allele Frequency	CEU Variant Allele Frequency	YRI Variant Allele Frequency	JPT Variant Allele Frequency
CYP4F2	2108622	V433M	0.485	0.232	0.058	0.235
	3093105	W12G	0.025	–	–	0.034
CYP4F11	8104361	R276C	0.008	0.258	0.167	0.023
	1060463	N446D	0.134	0.385	0.568	0.710
VKORC1	28940302	1173T>C	0.210	0.602	0.978	0.099

Table 3.3. Genetic variation of *CYP4F2, CYP411*, and *VKORC1* SNPs in the Yup'ik people in comparison to other ethnicities. Numbers were obtained from NCBI and variant allele frequencies from the HapMap Project database (Thorisson GA, Smith AV, Krishnan L, Stein LD. The International HapMap Project Web site. Genome Res 2005;15:1592-3), accessed July 15, 2014. Dash denotes the absence of variant allele frequencies in the HapMap database for those populations. Abbreviations are CEU: Utah residents with ancestry from northern and western Europe, YRI: Yoruba in Ibadan, Nigeria, JPT: Japanese in Tokyo.

Gene	SNP	Copies of Variant Allele			P-value
		0	1	2	
CYP4F2	V433M	209	356	187	0.414
	W12G	711	35	3	0.218
.*CYP4F11*	R276C	738	12	0	1.00
	N446D	488	154	15	0.350
VKORC1	-1173 T>C	496	206	50	< 0.001

Table 3.4. Frequencies of *CYP4F2, CYP411*, and *VKORC1* variant genotypes in the Yup'ik people. Genotype groups were categorized as having zero, one, or two copies of the variant allele. The p-values (χ^2) test whether the genotype frequencies are significantly different from the expectations of Hardy-Weinberg Equilibrium. This was only significant for *VKORC1 -1173 T>C*, indicating that false positive or negative genotypes of *VKORC1-1173 T>C* could have been assigned to our samples.

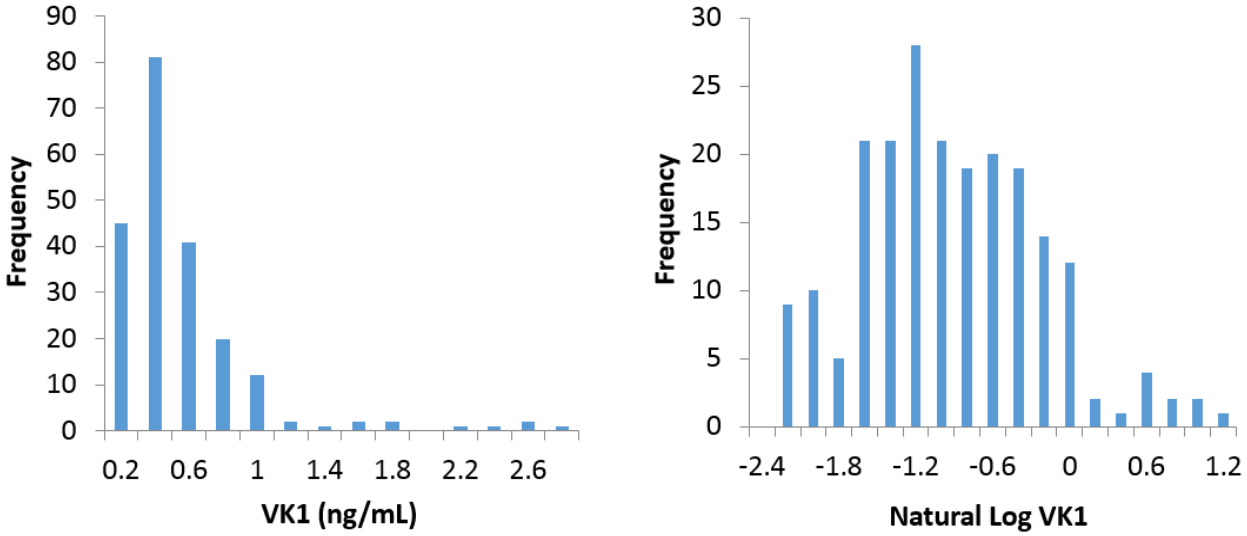

Figure 3.9. Distribution of VK1 levels measured from plasma of Yup'ik study participants before and after natural log transformation. A measurement is counted into a particular bin if it is equal to or less than the bin number down to the last bin. The values were skewed towards the left near lower values, indicating non-normal distribution. Natural log transformation improved normality for statistical testing.

		Non-Transformed VK1 (ng/mL)		Natural Log Transformed VK1		Regression Analysis			
Group	N	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	Unadjusted		Adjusted for age, sex, and geographical status	
						P-value	R-value	P-value	R-value
All	211	0.46 ± 0.42	0.34 (0.35)	-1.03 ± 0.69	-1.08 (0.95)				
*CYP4F2*3*									
*1/*1	54	0.39 ± 0.33	0.30 (0.35)	-1.17 ± 0.68	-1.20 (1.04)	0.002	0.214	0.004	0.279
*1/*3	98	0.43 ± 0.35	0.32 (0.28)	-1.07 ± 0.65	-1.16 (0.80)				
*3/*3	53	0.62 ± 0.56	0.43 (0.42)	-0.76 ± 0.72	-0.86 (0.92)				
CYP4F11 N446D									
AA	132	0.48 ± 0.45	0.35(0.38)	-1.02 ± 0.72	-1.06 (1.03)	0.742	0.028	0.984	0.246
AG	38	0.40 ± 0.29	0.31 (0.28)	-1.14 ± 0.65	-1.17 (0.84)				
GG	3	0.92 ± 1.12	0.29 (1.96)	-0.60 ± 1.21	-1.24 (2.14)				
VKORC1-1173 T>C									
TT	145	0.45 ± 0.35	0.35 (0.35)	-1.02 ± 0.66	-1.04 (0.92)	0.072	0.179	0.986	0.200
TC	51	0.47 ± 0.48	0.31 (0.30)	-1.07± 0.72	-1.17 (0.89)				
CC	9	0.75 ± 0.88	0.32 (0.54)	-0.82 ± 1.07	-1.14 (1.27)				

Table 3.5. Mean, standard deviations (SD), median, and interquartile ranges (IQR) of VK1 before and after natural log transformation according to *CYP4F2*3*, *CYP4F11 N446D*, and *VKORC1 -1173 T>C* genotype. Multivariate linear regression was used to test for associations between natural log transformed VK1 values and genotype. The results were further adjusted for potential confounding by age, sex, and geographical status.

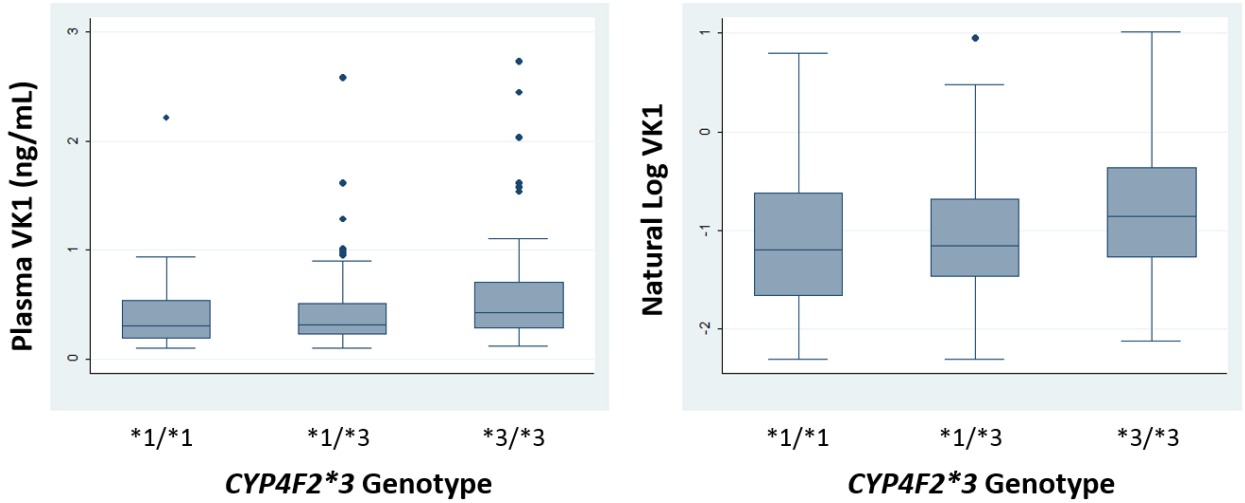

Figure 3.10. Box-and-whisker plots comparing plasma VK1 levels from Yup'ik study participants stratified by *CYP4F2*3* genotype. Actual values are displayed in Table 3.3. Due to the non-normal distribution of VK1, only the natural log transformed values were used for statistical testing in multivariate regression analysis.

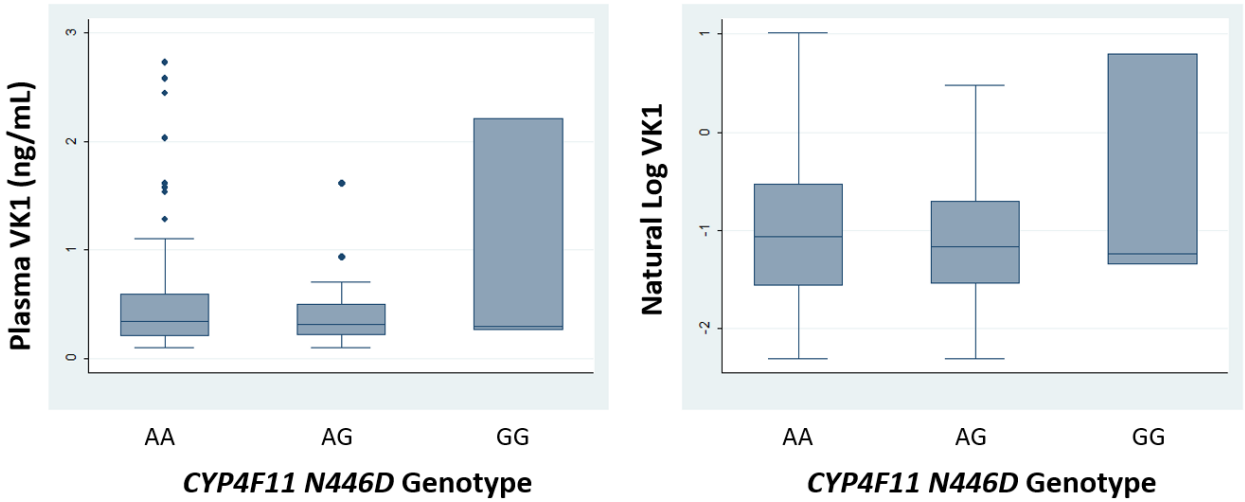

Figure 3.11. Box-and-whisker plots comparing plasma VK1 levels from Yup'ik study participants stratified by *CYP4F11 N446D* genotype. Actual values are displayed on Table 3.3. Due to the non-normal distribution of VK1, only the natural log transformed values were used for statistical testing in multivariate regression analysis.

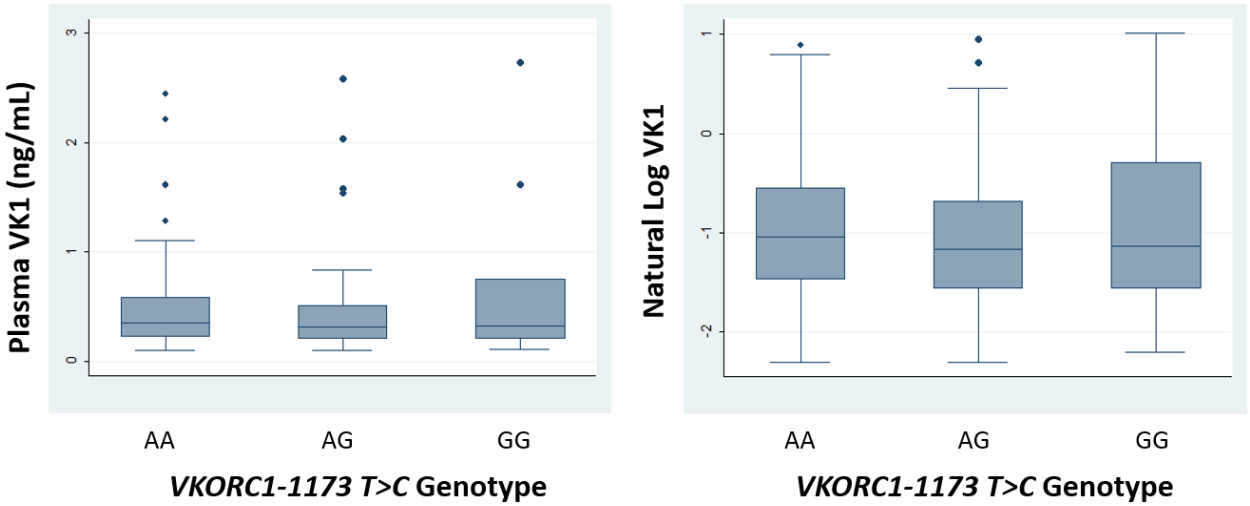

Figure 3.12. Box-and-whisker plots comparing plasma VK1 levels from Yup'ik study participants stratified by *VKORC1-1173 T>C* genotype. Actual values are displayed on Table 3.3. Due to the non-normal distribution of VK1, only the natural log transformed values were used for statistical testing in multivariate regression analysis.

Latin Name	Family	Common Name	Native Name(s)	Harvest Location	Preparation	Uses
Angelica lucida	Celery/Apiaceae	Wild celery	Ikituq (Yup'ik);Ikiitug (Cup'ik)	Tundra	Raw	Food
Arenaria peploides L.	Caryophyllaceae	Beach greens, seabeach, sandwort	Tukulleggar (Cup'ik)	Beach	Raw; Boiled, served with butter	Food
Artemisia tilesii	Aster/ Asteraceae	Wormwood	Caiggluk (Yup'ik); Neqnial‴ngur (Cup'ik)	Bluff by ocean	Boiled for tea; chewed raw	Medicinal
Ledum palustris ssp groenlandicum	Heath/ Ericaceae	Labrador tea	Ayuq (Yup'ik); Ayu (Cup'ik)	Bluff by river	Boiled for tea, add sprigs to black tea while steeping	Food, tea; medicinal tea
Hippuris vulgaris	Horsetail/ Equisetaceae	Mares Tail	Pugtassar (Cup'ik-dead that float on pond in the spring); Tayaruq (Yup'ik)	Pond	Boiled	Food
Rumex arcticus	Buckwheat/ Polygonaceae	Sourdock; arctic dock	ciwassar (Cup'ik); quagciq (Yup'ik)	Tundra	Raw; boiled	Food
Ranunculus Pallasii	Buttercup, crowfoot/ Ranunculaceae	Pallas Buttercup	Kapukaraq (Yup'ik); aaggulunguar (Cup'ik)	Pond	Boiled	Food
Caltha palustris	Caltha palustris	Marsh Marigold	Wivlug (Cup'ik)	Pond	Leaves eaten	Food

Table 3.6. A list of traditional tundra greens normally consumed in the diet of Yup'ik people that were collected from rural communities where plasma samples were obtained. Tundra greens are eaten either raw or boiled and are gathered seasonally.

Latin Name	VK1 (µg/g) (Mean ± SD)				VKO (µg/g) (Mean ± SD)	
	Leaves	Stem	Boiled Leaves	Boiled Stem	Leaves	Stem
Angelica lucida	2.73 ± 0.51	-	2.58	-	< 0.075	-
Arenaria peploides L.	11.0 ± 1.21	2.46 ± 0.81	6.78	0.83	< 0.075	< 0.075
Artemisia tilesii	21.7 ± 4.66	5.13 ± 0.79	6.29	1.97	0.35 ± 0.08	0.09 ± 0.05
Ledum palustris ssp groenlandicum	9.23 ± 1.32	1.86 ± 0.91	5.85	0.46	0.92 ± 0.12	0.26 ± 0.06
Hippuris vulgaris	27.2 ± 4.52	10.5 ± 2.94	13.3	4.48	0.26 ± 0.10	0.20 ± 0.07
Rumex arcticus	31.2 ± 3.29	-	13.0	-	2.24 ± 0.47	0.73 ±0.08
Ranunculus Pallasii	37.7 ± 8.90	3.76 ± 0.36	18.1	2.16	0.16 ± 0.04	0.28 ± 0.15
Caltha palustris	48.2 ± 8.50	9.71 ± 1.67	19.9	5.33	< 0.075	< 0.075

Table 3.7. The VK1 and VKO content of tundra greens. Measurements were made on both the leaves and stems from each plant, where available. Separate measurements were made on samples that had been boiled for 15 minutes in water prior to freeze-drying to provide a comparison to the vitamin K content of raw samples. VKO was not detected in boiled samples. The lower limit of quantitation for VK1 was (0.75 µg/g) and for VKO was (0.075 µg/g) per sample based on standard curves generated from 20 mg of freeze-dried red onion. Data for boiled samples were measured as an average of two samples.

Vegetable type	VK1 (µg/g) (Mean)	VKO (µg/g) (Mean)
Broccoli (bud)	17.6	0.38
Broccoli (stalk)	6.59	0.10
Spinach (leaves)	25.6	0.20
Spinach (stem)	6.13	<0.075
Lettuce (leaves)	7.87	3.62
Celery (leaves)	25.0	5.62
Celery (stalk)	4.94	0.54
Basil (leaves)	64.8	1.92
Basil (stem)	5.57	<0.075
Carrot (root)	1.65	0.26
Red Onion (whole)	0.15	<0.075

Table 3.8. The VK1 and VKO content of raw, freeze-dried reference vegetables. Standards were obtained from a commercially available market in the Seattle area. Analysis was performed with one set of duplicate samples. Data were obtained as an average of two samples.

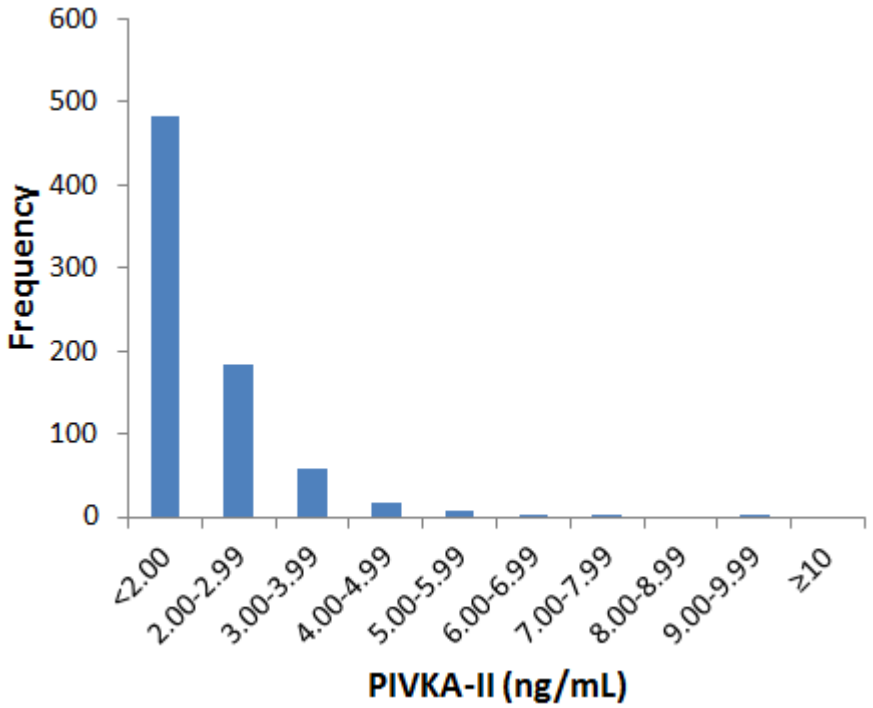

Figure 3.13. Distribution of PIVKA-II levels from the population of Yup'ik study participants (N = 753). A measurement is counted in a particular bin if it is equal to or less than the bin number down to the last bin. Approximately 36% of samples had levels above 2 ng/mL. Two values that were significant outliers (> 10 ng/mL) were measured as 1618 and 1708 ng/mL.

Group	Sample Size	Age (years)	Age Range	% Women	% with PIVKA-II ≥ 2 ng/mL
All	755	36.7 ± 18.1	13-93	47.55%	36.03%
*CYP4F2*3* Genotype					
*1/*1	202	34.4 ± 18.4	14-93	48.5%	41.6%
*1/*3	351	37.4 ± 17.9	14-77	46.7%	35.6%
*3/*3	181	37.9 ± 17.6	14-79	47.0%	31.5%
CYP4F11 N446D Genotype					
AA	484	37.5 ± 18.1	14-85	48.3%	33.7%
AG	140	35.7 ± 17.3	14-73	47.9%	33.6%
GG	16	28.4± 16.7	14-79	50.0%	37.5%
VKOR 1173 T > C Genotype					
TT	478	36.4 ± 18.1	14-79	47.5%	37.0%
TC	207	37.2 ± 17.6	14-79	46.4%	36.7%
CC	52	38.7 ± 19.4	14-93	48.1%	28.8%

Table 3.9. Study participant demographics in relation to vitamin K cycle genotypes and PIVKA-II level. Data are from samples that have complete information on genotype status (excluding no calls) and PIVKA-II levels that passed quality control standards.

Predictor	Odds Ratio	Standard Error	Z	P-value	95% CI
*CYP4F2*3*	0.77	0.085	-2.40	0.016	0.62 - 0.95
Age	1.00	0.004	-0.72	0.470	0.99 - 1.01
Sex	0.67	0.106	-2.52	0.012	0.49 - 0.92
Geographic Status	0.73	0.116	-1.96	0.050	0.54 - 1.00
Model Evaluation	**χ2**	**Degrees of Freedom**		**P-value**	
Likelihood Ratio Test	15.4	4		0.004	
Goodness of Fit (Pearson χ^2)	412.1	418		0.572	

Table 3.10. Descriptive statistics for the logistic regression of PIVKA-II status and *CYP4F2*3* genotype. Odd ratios, standard errors, z-statistics, p-values, and 95% confidence intervals (CI) are represented. The p-value of 0.004 indicates that the model is statistically significant. The p-value of 0.527 for the goodness-of-fit test indicates that the model fits reasonably well to the data.

Predictor	Odds Ratio	Standard Error	Z	P-value	95% CI
CYP4F11 N446D	1.03	0.175	0.19	0.850	0.74 - 1.44
Age	1.00	0.005	-0.72	0.471	0.99 - 1.01
Sex	0.70	0.118	-2.14	0.032	0.50 - 0.97
Geographic Status	0.73	0.124	-1.83	0.067	0.53 - 1.02
Model Evaluation	**χ2**	**Degrees of Freedom**		**P-value**	
Likelihood Ratio Test	8.73	4		0.068	
Goodness of Fit (Pearson χ^2)	329	308		0.189	

Table 3.11. Descriptive statistics for the logistic regression of PIVKA-II status and *CYP4F11 N446D* genotype. Odd ratios, standard errors, z-statistics, p-values, and 95% confidence intervals (CI) are represented. The p-value of 0.068 indicates that the model was not statistically significant even though the p-value of 0.189 for the goodness-of-fit test indicated that the model fit well to the data.

Predictor	Odds Ratio	Standard Error	Z	P-value	95% CI
VKORC1-1173 T>C	0.87	0.111	-1.07	0.285	0.68 - 1.12
Age	1.00	0.004	-1.01	0.315	0.99 - 1.00
Sex	0.67	0.105	-2.57	0.010	0.49 - 0.91
Geographic Status	0.75	0.117	-1.83	0.067	0.55 - 1.02
Model Evaluation	**χ2**	**Degrees of Freedom**		**P-value**	
Likelihood Ratio Test	12.23	4		0.016	
Goodness of Fit (Pearson χ^2)	397	374		0.203	

Table 3.12. Descriptive statistics for the logistic regression of PIVKA-II status and *VKORC1-1173 T>C* genotype. Odd ratios, standard errors, z-statistics, p-values, and 95% confidence intervals (CI) are represented. The p-value of 0.016 indicates that the model is statistically significant. The p-value of 0.203 for the goodness-of-fit test indicates that the model fits reasonably well to the data.

3.5 Notes to Chapter 3

1. Rettie, A. E., and Tai, G. (2006) The pharmocogenomics of warfarin: closing in on personalized medicine, *Molecular interventions 6*, 223-227.

2. Rieder, M. J., Reiner, A. P., Gage, B. F., Nickerson, D. A., Eby, C. S., McLeod, H. L., Blough, D. K., Thummel, K. E., Veenstra, D. L., and Rettie, A. E. (2005) Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose, *The New England journal of medicine 352*, 2285-2293.

3. Takahashi, H., Wilkinson, G. R., Nutescu, E. A., Morita, T., Ritchie, M. D., Scordo, M. G., Pengo, V., Barban, M., Padrini, R., Ieiri, I., Otsubo, K., Kashima, T., Kimura, S., Kijima, S., and Echizen, H. (2006) Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans, *Pharmacogenetics and genomics 16*, 101-110.

4. Edson, K. Z., Prasad, B., Unadkat, J. D., Suhara, Y., Okano, T., Guengerich, F. P., and Rettie, A. E. (2013) Cytochrome P450-dependent catabolism of vitamin K: omega-hydroxylation catalyzed by human CYP4F2 and CYP4F11, *Biochemistry 52*, 8276-8285.

5. Caldwell, M. D., Awad, T., Johnson, J. A., Gage, B. F., Falkowski, M., Gardina, P., Hubbard, J., Turpaz, Y., Langaee, T. Y., Eby, C., King, C. R., Brower, A., Schmelzer, J. R., Glurich, I., Vidaillet, H. J., Yale, S. H., Qi Zhang, K., Berg, R. L., and Burmester, J. K. (2008) CYP4F2 genetic variant alters required warfarin dose, *Blood 111*, 4106-4112.

6. McDonald, M. G., Rieder, M. J., Nakano, M., Hsia, C. K., and Rettie, A. E. (2009) CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant, *Molecular pharmacology 75*, 1337-1346.

7. Holmes, M. V., Hunt, B. J., and Shearer, M. J. (2012) The role of dietary vitamin K in the management of oral vitamin K antagonists, *Blood reviews 26*, 1-14.

8. Shearer, M. J. (1995) Vitamin K, *Lancet 345*, 229-234.

9. Meguro, T., and Yamada, K. (1982) A simple and rapid test for PIVKA-II in plasma, *Thrombosis research 25*, 109-114.

10. Bakhshi, S., Deorari, A. K., Roy, S., Paul, V. K., and Singh, M. (1996) Prevention of subclinical vitamin K deficiency based on PIVKA-II levels: oral versus intramuscular route, *Indian pediatrics 33*, 1040-1043.

11. Demirkan, K., Stephens, M. A., Newman, K. P., and Self, T. H. (2000) Response to warfarin and other oral anticoagulants: effects of disease states, *Southern medical journal 93*, 448-454; quiz 455.

12. Booth, S. L., and Suttie, J. W. (1998) Dietary intake and adequacy of vitamin K, *The Journal of nutrition 128*, 785-788.

13. Bang, H. O., and Dyerberg, J. (1980) The bleeding tendency in Greenland Eskimos, *Danish medical bulletin 27*, 202-205.

14. Dyerberg, J., and Bang, H. O. (1979) Haemostatic function and platelet polyunsaturated fatty acids in Eskimos, *Lancet 2*, 433-435.

15. Dowd, P., Ham, S. W., Marchand, A. P., and Rajapaksa, D. (1992) Mechanism of Epoxidation of Vitamin K with Basic Hydrogen Peroxide, *J. Org. Chem. 57*, 3514-3516.

16. Suhara, Y., Hanada, N., Okitsu, T., Sakai, M., Watanabe, M., Nakagawa, K., Wada, A., Takeda, K., Takahashi, K., Tokiwa, H., and Okano, T. (2012) Structure-activity relationship of novel menaquinone-4 analogues: modification of the side chain affects their biological activities, *Journal of medicinal chemistry 55*, 1553-1558.

17. Sommer, P., and Kofler, M. (1966) Physicochemical properties and methods of analysis of phylloquinones, menaquinones, ubiquinones, plastoquinones, menadione, and related compounds, *Vitamins and hormones 24*, 349-399.

18. Bevans, C. G., Krettler, C., Reinhart, C., Tran, H., Kossmann, K., Watzka, M., and Oldenburg, J. (2013) Determination of the warfarin inhibition constant Ki for vitamin K 2,3-epoxide reductase complex subunit-1 (VKORC1) using an in vitro DTT-driven assay, *Biochimica et biophysica acta 1830*, 4202-4210.

19. Paroni, R., Faioni, E. M., Razzari, C., Fontana, G., and Cattaneo, M. (2009) Determination of vitamin K1 in plasma by solid phase extraction and HPLC with fluorescence detection, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 877*, 351-354.

20. Sadowski, J. A., and Suttie, J. W. (1974) Mechanism of action of coumarins. Significance of vitamin K epoxide, *Biochemistry 13*, 3696-3699.

21. Thorisson, G. A., Smith, A. V., Krishnan, L., and Stein, L. D. (2005) The International HapMap Project Web site, *Genome research 15*, 1592-1593.

22. Schurgers, L. J., and Vermeer, C. (2002) Differential lipoprotein transport pathways of K-vitamins in healthy subjects, *Biochimica et biophysica acta 1570*, 27-32.

23. Athinarayanan, S., Wei, R., Zhang, M., Bai, S., Traber, M. G., Yates, K., Cummings, O. W., Molleston, J., Liu, W., and Chalasani, N. (2014) Genetic polymorphism of cytochrome P450 4F2, vitamin E level and histological response in adults and children with nonalcoholic fatty liver disease who participated in PIVENS and TONIC clinical trials, *PloS one 9*, e95366.

24. Gong, I. Y., Schwarz, U. I., Crown, N., Dresser, G. K., Lazo-Langner, A., Zou, G., Roden, D. M., Stein, C. M., Rodger, M., Wells, P. S., Kim, R. B., and Tirona, R. G. (2011) Clinical and genetic determinants of warfarin pharmacokinetics and pharmacodynamics during treatment initiation, *PloS one 6*, e27808.

25. Azharuddin, M. K., O'Reilly, D. S., Gray, A., and Talwar, D. (2007) HPLC method for plasma vitamin K1: effect of plasma triglyceride and acute-phase response on circulating concentrations, *Clinical chemistry 53*, 1706-1713.

26. Sadowski, J. A., Hood, S. J., Dallal, G. E., and Garry, P. J. (1989) Phylloquinone in plasma from elderly and young adults: factors influencing its concentration, *The American journal of clinical nutrition 50*, 100-108.

27. Booth, S. L., and Sadowski, J. A. (1997) Determination of phylloquinone in foods by high-performance liquid chromatography, *Methods in enzymology 282*, 446-456.

28. Oostende, C., Widhalm, J. R., and Basset, G. J. (2008) Detection and quantification of vitamin K(1) quinol in leaf tissues, *Phytochemistry 69*, 2457-2462.

29. Greer, F. R., Marshall, S. P., Severson, R. R., Smith, D. A., Shearer, M. J., Pace, D. G., and Joubert, P. H. (1998) A new mixed micellar preparation for oral vitamin K prophylaxis: randomised controlled comparison with an intramuscular formulation in breast fed infants, *Archives of disease in childhood 79*, 300-305.

30. Kishi, K., Sonomura, T., Mitsuzane, K., Nishida, N., Kimura, M., Satoh, M., Yamada, R., Kodama, N., Kinoshita, M., Tanaka, H., and et al. (1992) Time courses of PIVKA-II and AFP levels after hepatic artery embolization and hepatic artery infusion against hepatocellular carcinoma: relation between the time course and tumor necrosis, *Radiation medicine 10*, 189-195.

31. Jones, D. Y., Koonsvitsky, B. P., Ebert, M. L., Jones, M. B., Lin, P. Y., Will, B. H., and Suttie, J. W. (1991) Vitamin K status of free-living subjects consuming olestra, *The American journal of clinical nutrition 53*, 943-946.

32. Suttie, J. W., Mummah-Schendel, L. L., Shah, D. V., Lyle, B. J., and Greger, J. L. (1988) Vitamin K deficiency from dietary vitamin K restriction in humans, *The American journal of clinical nutrition 47*, 475-480.

33. Sokoll, L. J., Booth, S. L., O'Brien, M. E., Davidson, K. W., Tsaioun, K. I., and Sadowski, J. A. (1997) Changes in serum osteocalcin, plasma phylloquinone, and urinary gamma-carboxyglutamic acid in response to altered intakes of dietary phylloquinone in human subjects, *The American journal of clinical nutrition 65*, 779-784.

Chapter 4

Hepatic Vitamin K Content as a Function of *CYP4F* Genotype

4.1 Phylloquinone and Menaquinone Content in Human Liver

The liver is the primary organ in humans for the absorption and storage of vitamin K where both phylloquinone (vitamin K1) and menaquinones (vitamins K2) appear to be present, although only a handful of studies have been performed (1-3). Early work using semi-quantitative analytical methods suggested that phylloquinone, the major circulatory form of vitamin K in plasma, accounts for a significant proportion of the total vitamin K content in the liver. For example, a study in 1972 by Duello et al. identified phylloquinone, MK-4 and MK-7 through MK-11 using large amounts of tissue (> 5 kg) and concluded that phylloquinone accounts for approximately 50% of the hepatic vitamin K content of human liver (1). Another study that analyzed hepatic vitamin K content from 8 infants and 3 adults also concluded that phylloquinone was the predominant form in the liver, though menaquinone content increased with age and long-chain menaquinones were also detected (2). However, Usui et al., who studied surgical tissue from Japanese patients reached the opposite conclusion that phylloquinone makes up only about 10% of the hepatic vitamin K content (3). These latter investigators found further that hepatic vitamin K levels were highly variable and the very long-chain menaquinones, such as MK-10 and MK-11, accounted for the majority of total K vitamer content. Concentrations of phylloquinone from plasma of study subjects were also rapidly depleted under a vitamin K-restricted diet or under fasting conditions, which was conjectured to explain the high menaquinone content remaining in the liver relative to phylloquinone.

Most vitamin K is absorbed through the upper intestine, through the duodenum and jejunum, with less being absorbed from the ileum and colon (4-6). Vitamin K is incorporated into chylomicron particles in the small intestine before being secreted into the circulatory system through lymphatic vessels and eventually taken up by the liver. It is believed that liver uptake phylloquinone involves an active

transport process while menaquinones are absorbed via passive diffusion (4, 5). The source of long-chain menaquinones found in hepatic stores is widely believed to derive from intestinal microflora (7). In support of this, a report by Conly and Stein demonstrated that total menaquinone content from post-mortem liver samples was significantly reduced in donors who were exposed to broad spectrum antimicrobials prior to death, but no difference was observed in phylloquinone levels (8). The reduced gut floral production and absorption of vitamin K after antibiotic treatment is a possible explanation for why prolongation of the INR occurs in warfarin patients exposed to antibiotics (9). However, this hypothesis is still under debate given that absorption of vitamin K from the colon, where intestinal bacteria reside, is poor and that tight binding of menaquinones to bacterial cytoplasmic membranes may limit their bioavailability (10-12). Interestingly, despite almost fifty years of intensive investigation of this subject, the specific enzymes and proteins involved in the hepatic uptake, transport, storage, and elimination of vitamin K remain largely unknown, although it seems likely that some of these processes are modulated by the vitamin K-cycle associated enzymes (13).

Based on i) our results from Chapter 3 regarding the high allele frequency of *CYP4F2*3* in Yup'ik people and its association with elevated levels of plasma vitamin K, as well as lower levels of PIVKA-II, and ii) the recent report identifying human hepatic CYP4F2 and CYP4F11 as vitamin K hydroxylases it seems likely that variation in the *CYP4F2* and *CYP4F11* genes could have a critical role in vitamin K homeostasis (14). A probable mechanistic pathway is that CYP4F2 and CYP4F11 (important collateral enzymes to the vitamin K cycle) because they alone initiate P450-mediated phylloquinone (K1) catabolism to the pharmacologically inactive acid metabolites, which are ultimately eliminated in the urine as glucuronides.

Therefore, in this chapter, the role that these enzymes play in vitamin K homeostasis is further explored by analyzing vitamin K content of genotyped liver tissue. We first developed a robust extraction procedure for vitamin K from human liver samples archived in the University of Washington School of Pharmacy Human Liver Bank. These human liver samples have been genotyped for the *CYP4F2 V433M* and *CYP4F11 N446D,* as well as *VKORC1-1639 G>A,* which is in complete linkage disequilibrium with

the *-1173 T>C* SNP (15, 16). This will allow us to analyze the association between these SNPs and hepatic vitamin K levels. Additionally, we analyzed the vitamin K content from *cyp4f14* knockout mice. Murine *cyp4f14* (a possible ortholog to human *CYP4F2*) is a vitamin E ω-hydroxylase and may play a similar role to *CYP4F2* (and/or *CYP4F11*) in the metabolism of vitamin K (17, 18). Efforts were made to establish assays for MK-1-MK-13, but ultimately we focused on quantifying phylloquinone, MK-4, and their respective epoxides in liver samples from both humans and rats by LC/MS/MS with stable isotope-labeled internal standards.

4.1 Methods

Reagents and Standards

As described in chapter 3, the standards of phylloquinone (VK1) and menaquinone-4 (MK-4) were commercially available while phylloquinone epoxide (VKO) and MK-4 epoxide (MK-4O) were synthesized by the method of Dowd (19). Deuterated phylloquinone (VK1-d7) was commercially available from Aldrich (catalog no. 705470). Deuterated MK-4 (MK-4-d7), a gift from Dr. Matthew McDonald, was synthesized by the method of Suhara (20). Deuterated phylloquinone epoxide (VKO-d7) was synthesized from VK1-d7 and deuterated MK-4 epoxide (MK-4O-d7) was synthesized from MK-4-d7 by the method of Dowd (19). MK-7 (catalog no. sc-218691) and MK-9 (catalog no. sc-211788) were commercially available from Santa Cruz Biotechnology. Analytical standards for menaquinones 1, 2, 3, 5, 6, 8, 10, 11, 12, and 13 were received as gifts from Dr. Sarah Booth at Tufts University. All standards were prepared in methanol and characterized spectrophotometrically and chromatographically before use.

LC-MS analysis

All standards and samples were analyzed on a Waters TQ-S mass spectrometer connected to a Waters Acquity I-Class UPLC system with an atmospheric pressure chemical ionization (APCI) source,

as described in Chapter 3. Initial development of analytical standards was performed in positive ion mode, but negative ion was ultimately favored for greater compound sensitivity and response of vitamin K compounds. The total run time for the assay was extended to 13 minutes to account for elution of long-chain menaquinone compounds.

CYP4F14 Knock-Out Mice Livers

Six whole livers from *cyp4f14* null (-/-) mice and six livers from *cyp4f14* control (+/+) mice were received as gifts from Dr. Parker at Cornell University. The protocol for creating *cyp4f14* null mice is described in Bardowell et al. (21). Mice were fed a 6-week diet of soybean oil enriched chow prior to mice were deeply anesthetized by isoflurane inhalation and exsanguinated *via* cardiac puncture. Liver tissue samples were flash-frozen in liquid nitrogen and stored at −80°C prior to shipping to the University of Washington for analysis of vitamin K content.

Liver Sample Preparation

All human and mouse liver samples had been stored at -80°C. Samples of human liver (n = 51) from hospitalized donors and their genotyping results were obtained from the University of Washington School of Pharmacy Human Liver Bank (Seattle, WA). An aliquot (50-100 mg wet weight) of each liver sample was weighed in duplicate. To each sample, 2 ng of VK1-d7, 2 ng of MK-4-d7, 4 ng VKO-d7, and 4 ng of MK-4O-d7 was added. Samples were then homogenized with a Bertin Technologies Precellys 24 tissue homogenizer utilizing 2.8 mm ceramic beads (MO BIO Laboratories, Inc., catalog number 3114-325). Four mL of 2-propanol/hexane (3:2, v/v) was added and, after vigorous vortexing for 1 minute, samples were centrifuged at 3000 rpm (1500g) for 5 minutes. The upper hexane layer was removed and the extraction step was repeated with 4 mL of hexane. After drying the isolated supernatant layer under nitrogen, samples were reconstituted in 500 µL of hexane and applied to Bond Elut 500 mg silica SPE columns (part no. 12113036, Varian). These cartridges were conditioned initially with 4 mL of ethyl

ether/hexane (4:96, v/v) and then with 4 mL of hexane. After loading the concentrated extracts, the columns were washed with 4 mL of hexane, and eluted with 8 mL of ether in hexane (4:96, v/v). The eluate was evaporated under nitrogen and the residue was dissolved in 100 μL of methanol solution for analysis of 15 μL aliquots by LC/MS.

Validation Procedure and Quantification

Reference standard curves were generated using phosphate-buffered saline (PBS). Stock solutions ranging from 0.5 to 3.0 ng of VK1, VKO, MK-4, and MK-4O were spiked into 500 μL of PBS. In creating a standard curve to assess matrix effects, individual liver samples were first screened and the one with the lowest endogenous vitamin K content was selected to prepare a liver homogenate that was spiked with stock solutions ranging from 0.5 to 3.0 ng of each vitamin K standard. All samples were extracted and analyzed by LC-MS as described above to obtain peak-area ratios for each analyte compared to its deuterated internal standard. MK-4-d7 served as the internal standard for MK-7 and MK-9.

Statistical Analysis

Among mouse livers, comparisons were made between cyp4f14 control and null animals as well as between males and females using the two-sample t-test with a significance level of $\alpha = 0.05$. For human livers, we used a linear regression model with age and sex as covariates to evaluate any association between hepatic vitamin K content and genotypes of *CYP4F2*3*, *CYP4F11 D446N*, and *VKORC1-1639 G>A*. Each genotype was individually classified as having 0, 1, or 2 copies of the minor allele. Vitamin K values were natural log-transformed to improve normality of the data. We excluded livers with phylloquinone content greater than 100 ng/g due to its suspected administration in the hospital prior to tissue collection.

4.3 **Results**

Assay Development

LC-MS quantitation of vitamin K was first investigated using APCI positive mode based on other published reports (22-24). Although electrospray ionization (ESI) was considered, the APCI source appeared to be less susceptible to matrix effects than did ESI. MK-1 through MK-10 eluted from the column by 13 minutes at high organic concentration (near 100% methanol). Figure 4.1 provides a representative chromatogram illustrating their detection in APCI positive mode. We also attempted to optimize LC-MS conditions for MK-11, MK-12, and MK-13, but were not able to observe any distinguishable peaks for daughter ions. This is likely due to the fact that the ionization potentials of menaquinones decreased as the chain-length increased. Furthermore, due to analytical standard sample limitations, with the exception of commercially available MK-4, MK-7, and MK-9, we were not able to optimize these compounds in APCI negative mode. The MRM transitions of each menaquinone standard in APCI positive mode is displayed on Table 4.1 and their chromatograms are shown on Figure 4.1.

APCI negative ion mode was ultimately employed for the analysis of hepatic VK1, VKO, MK-4, and MK-4O largely because our primary analyte of interest, VK1, exhibited more than a two-fold greater response (peak-area ratio) in this mode compared to APCI positive ion. The MRM transitions for VK1, VKO, MK-4, MK-4O, and their respective deuterated standards are discussed in Chapter 3. The MRM transition in APCI negative mode for MK-7 was 648.723 > 185.126 and for MK-9 it was 784.67 > 185.07. The linearity of response for all analytes in plasma was tested by spiking standards of VK1, VKO, MK-4, and MK-4O, MK-7, and MK-9 into 0.5 mL PBS and 0.5 mL of liver homogenate in concentrations that ranged from 0.5 ng to 3 ng/mL (Figures 4.2-4.7). The limit of quantitation was 1 ng/g tissue for both VK1 and VKO. Linear regression was used to compare measurements obtained by LC-MS to expected concentrations of calibration standards. Standard curves for both PBS and liver homogenate were linear with R^2 values greater than 0.99 for VK1 and VKO. Since the R^2 values, slope, and intercept values were similar for each individual metabolite between standard curves for PBS and

liver homogenate, the PBS standard curve was used to estimate analyte concentrations. In the case of MK-7 and MK-9, slopes were similar between PBS and homogenate but the accuracy of the estimates was not as strong as the other analytes. The respective R^2 values were 0.891 and 0.886, but decreased slightly to 0.869 and 0.839 in liver homogenate. The limit of detection was 1 ng/g. However, since only VK1 was measurable within the limits of quantitation for the majority of samples, further analysis focused only on VK1. Trace amounts of MK-7 and MK-9 were identified, and very little or no MK-4 could be observed. We found VKO only in livers HL-146 (1.22 ng/g), HL-156 (37.1 ng/g), and HL-166 (50.8 ng/g).

Phylloquinone Content of Human Liver and Association with Genotype

The VK1 content of human livers, demographic information, and genotype information are provided in Table 4.2. Four liver samples (HL-127, HL-146, HL-156 and HL-156) exhibited very high vitamin K levels compared to the rest of the sample analyzed. From hospital records, two liver donors (HL-146 and HL-156) were known to have been treated with vitamin K prior to death, while two other liver samples (HL-127 and HL-166) did not have available medication data that allowed us to exclude this possibility, and so we suspect that these livers were indeed treated with vitamin K in the hospital setting. Therefore, we excluded hepatic VK1 values above 100 ng/g prior to statistical analysis. The average VK1 content, excluding outliers above 100 ng/g, was 12.6 ± 14.2 ng/g (28.0 ± 31.6 pmol/g) with a range of 1.44 - 71.67 ng/g (3.20 – 159.3 pmol/g). A comparison of hepatic VK1 content between genotypes is shown in Figures 4.8-4.10 and results are displayed in Table 4.3. Upon regression analysis, we observed no significant association between natural log VK1 levels and *CYP4F2 V433M*, *CYP4F11 N446D*, and *VKORC1-1639 G>A* genotype before or after the adjustment for age and sex. However, the frequency of variant genotypes was low for each of these SNPs among available liver samples. We did not observe any significant associations of natural log VK1 with age (0.195) or differences between sex ($p = 0.523$).

Phylloquinone Content of Mouse Liver and Association with Cyp4f14 Status

The differences in vitamin K content of mouse livers between *cyp4f14* control and null mice stratified by sex are represented in Figure 4.11, with mean ± standard deviation (SD) values presented in Table 4.4. We did not observe any significant differences in VK1 content between control and null mice for both sex groups. However, there were significant differences in VK1 levels between male and female mice where mean ± SD (in ng/g) values for males was 32.1 ± 8.13 and for females was 101.0 ± 25.0 ($p < 0.001$).

4.4 Discussion

The main goal of this study was to evaluate human hepatic vitamin K content with respect to *CYP4F2 V433M*, *CYP4F11 N446D*, and *VKORC1-1639 G>A* genotype. Assay development converged on final analysis of only VK1, due to limitations on menaquinone analytical standard availability and low levels of all other relevant analytes in these banked human tissue samples. The hepatic VK1 content from the Liver Bank samples was not found to vary significantly across the different genotypes, but this is likely due to several limitations discussed below.

First, donor information regarding medication exposure was either absent or very limited. For example, among four liver samples that had unusually high VK1 content, only two were noted to have been exposed to vitamin K in the hospital, while medication information on the other two donors was not recorded. In fact, comprehensive medication information was available for only half of the liver samples analyzed and so this could be a significant source of variability. More importantly, the study was completely uncontrolled with regard to dietary information about recent vitamin K intake by the donors and it seems likely that this would be a major reason underlying the wide ranges in VK1 content even in the samples remaining after exclusion of those with values > 100 ng/g.

The Liver Bank population is composed largely of Caucasian donors and so the frequencies of the alleles of interest differ substantially from those found in the population of Yup'ik people, especially for

*CYP4F2*3*. Consequently, our human Liver Bank sample population contained very low numbers of *CYP4F2*3* homozygotes, which meant that the analysis was underpowered for determining differences in vitamin K content between *CYP4F2*3* genotypes.

In a broader sense, the study was limited by an inability to quantitate longer chain menaquinones due to technical limitations in addition to the limited availability of standards. We noticed that the ionization efficiency in APCI positive mode for menaquinones decreased as the chain length increased. While we were not able to explore this observation for APCI negative mode, this suggests that APCI ionization may not be a suitable detection method for longer-chain menaquinones.

On a positive note, our mean ± SD values of 28.0 ± 31.6 pmol/g for hepatic VK1 is similar to the reported 28.0 ± 4.3 pmol/g a study of hospitalized patients by Usui et al., this demonstrating our assay's ability to reproduce earlier findings (3). Our UPLC-based assay also has faster run-times compared to the retention times reported in the Usui study (i.e., ~60 minute retention time for MK-10), and so provides for higher throughput. Finally, the current assay utilizes highly sensitive and specific MRM detection methods to quantify analytes in human liver instead of the post-column electrochemical reduction methods used previously.

To address some of the study limitations discussed above, we switched to a mouse model that provided control over dietary intake of vitamin K. The *cyp4f14* knockout mouse was developed by Parker and co-workers to probe the effects of CYP4F family P450s on vitamin E metabolism and disposition (17). Here we have begun to extend this work to vitamin K because, in humans CYP4F2 is implicated in the metabolism of both vitamin E and vitamin K. However, we found that hepatic VK1 content did not differ between *cyp4f14* null mice and controls. Although Bardowell et al. reported decreased metabolic capacity of γ-, δ-, and α-tocopherol (TOH) in *cyp4f14* knockout mice (17), our results suggest that *cyp4f14* is unlikely to have a critical role in the metabolism of VK1 in mice. However, our finding that hepatic VK1 content was significantly higher in female mice are reflective of similar observations by Bardowell et al., wherein levels of vitamin E were higher in several tissues including plasma (γ-, δ-TOH), lung (α-, γ-, δ-TOH), kidney (γ-, δ-TOH), liver (α-, γ-, δ-TOH), heart (α-

TOH), brain (α-, γ-, δ-TOH), and fat (δ-TOH) (21). Higher hepatic concentrations of both phylloquinone and menaquinone in female rats compared to males were also reported by Huber et al. (25). The reasons for these gender differences in vitamin K content (as well as for vitamin E) are unknown, but warrants further investigation. Interestingly, we did not observe any differences in hepatic vitamin K content between sexes for human liver samples, but other study variables discussed above may have clouded any intrinsic gender differences.

In summary, these studies resulted in the development of a highly sensitive and specific assay for the quantitation of VK1 in liver samples, although high inter-individual variability precluded a robust analysis of the genotype effects we had wished to explore. Larger and more controlled studies are needed to determine whether SNPs of *CYP4F2*, *CYP4F11*, and *VKORC1* affect human hepatic vitamin K content. Liver storage of vitamin K and vitamin E have clear gender-associated associations in mice (and to some extent rats) that also warrant further study to elucidate the underlying mechanisms.

Analyte	**Parent (*m/z*)**	**Daughter (*m/z*)**	**Retention Time (min)**
MK-1	243.1	187	1.35
MK-2	309.2	187	1.95
MK-3	376.2	187	2.71
MK-4	445.2	187	3.46
MK-5	513.4	187	4.89
MK-6	581.5	187	6.34
MK-7	648.6	187	7.67
MK-8	717.9	187	8.78
MK-9	786.2	187	9.74
MK-10	853.7	187	10.92

Table 4.1. The MRM transitions of menaquinone standards in APCI positive mode. MK-4, MK-7, and MK-9 were later optimized in APCI negative mode in addition to VK1, VKO, MK-4, and MK-4O. Conditions were optimized on the Xevo TQ-S instrument.

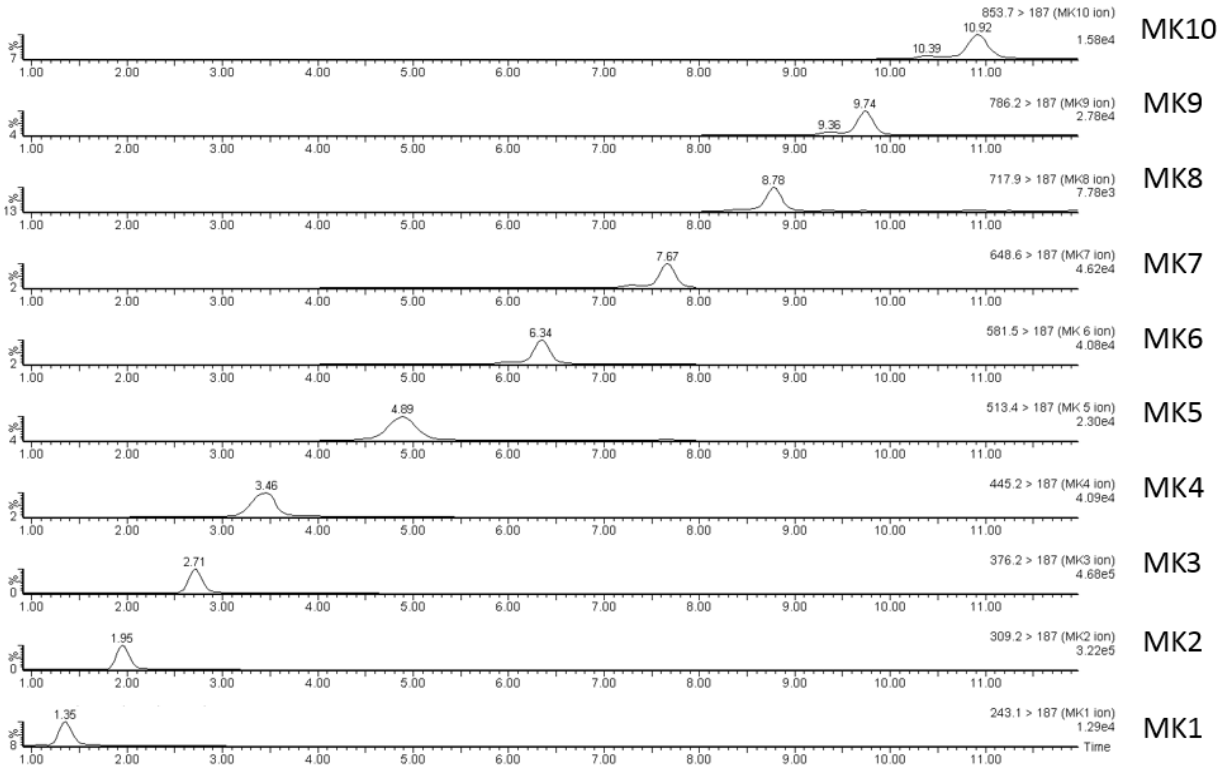

Figure 4.1. Chromatogram of menaquinone standards analyzed in APCI positive mode. The total run time was 13 minutes. While APCI negative mode was ultimately used for these analyses, not all menaquinone analytes were pursued beyond positive mode due to limited availability of chemical standards.

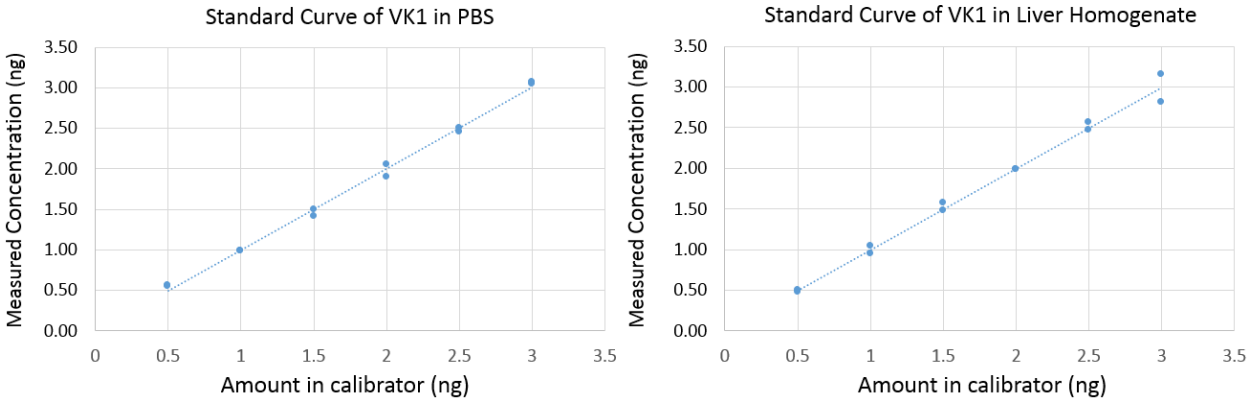

Figure 4.2. Standard curves comparing the measured concentration of VK1 to the expected concentration of the calibrator solution. Both PBS (phosphate buffered saline) and liver homogenate were used as matrices and response was linear with R^2 values of > 0.99, indicating that matrix effects of protein binding at these concentrations did not significantly affect the standard curves. The equation for the standard curves were 0.56 – 0.035 for both PBS and 0.55 + 0.029 liver homogenate.

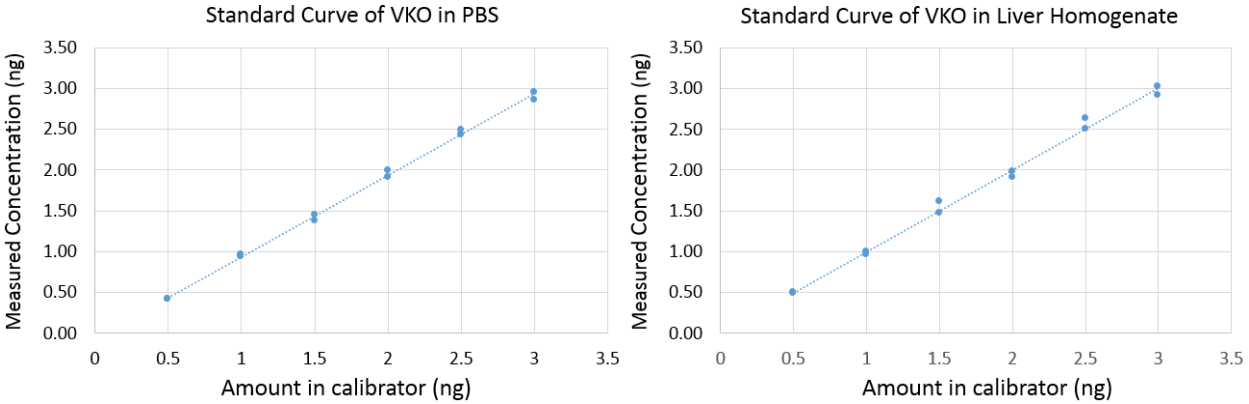

Figure 4.3. Standard curves comparing the measured concentration of VKO to the expected concentration of the calibrator solution. Both PBS (phosphate buffered saline) and liver homogenate were used as matrices and response was linear with R^2 values of > 0.99, indicating that matrix effects of protein binding at these concentrations did not significantly affect the standard curves. The equation for the standard curves were y = 0.17 + 0.029 for PBS and 0.17 + 0.026 for liver homogenate.

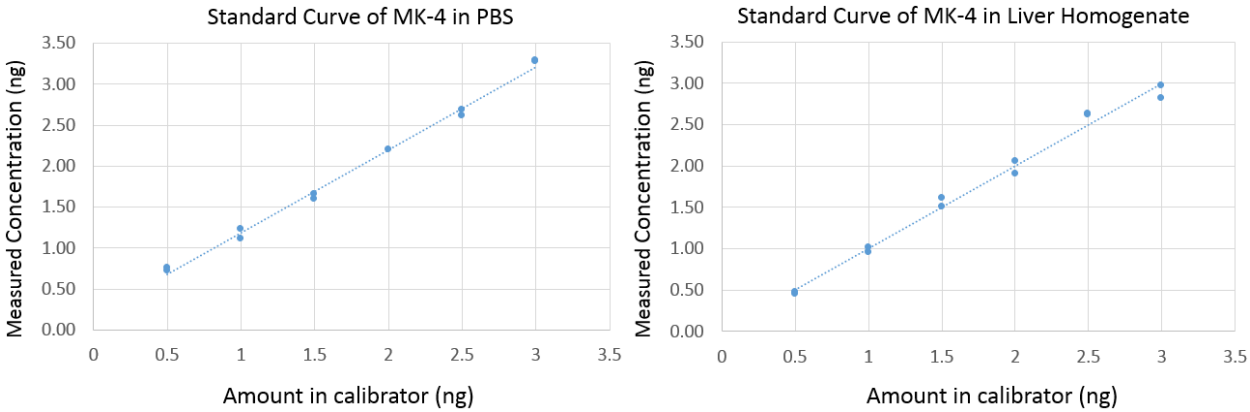

Figure 4.4. Standard curves comparing the measured concentration of MK-4 to the expected concentration of the calibrator solution. Both PBS (phosphate buffered saline) and liver homogenate were used as matrices and response was linear with R^2 values of 0.976 for PBS and 0.990 for liver homogenate , indicating that matrix effects of protein binding at these concentrations did not significantly affect the standard curves. The equation for the standard curves were 0.52 – 0.15 for PBS and 0.60 + 0.014 liver homogenate.

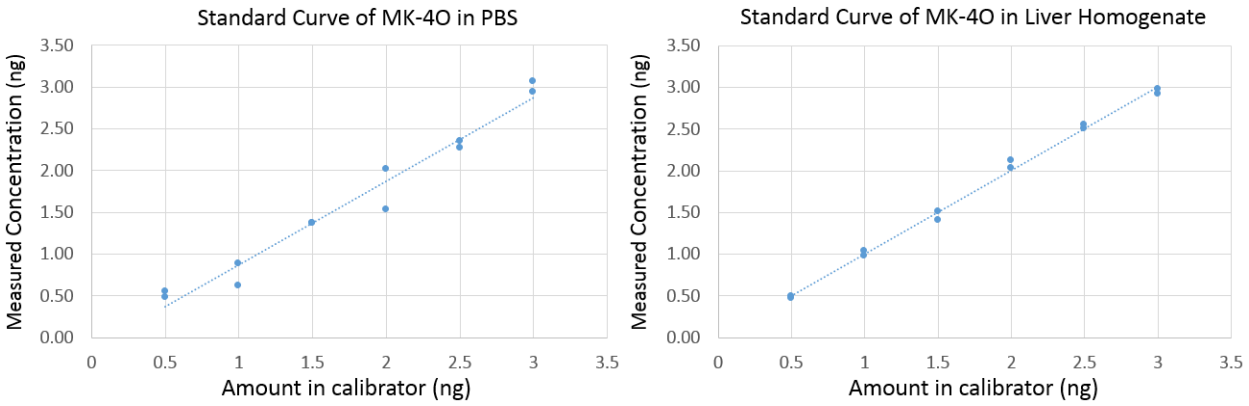

Figure 4.5. Standard curves comparing the measured concentration of MK-4O to the expected concentration of the calibrator solution. Both PBS (phosphate buffered saline) and liver homogenate were used as matrices and response was linear with R^2 values of 0.967 for PBS and 0.996 for liver homogenate, indicating that matrix effects of protein binding at these concentrations did not significantly affect the standard curves. The equation for the standard curves were 0.32 – 0.023 for PBS and 0.32 + 0.011 liver homogenate.

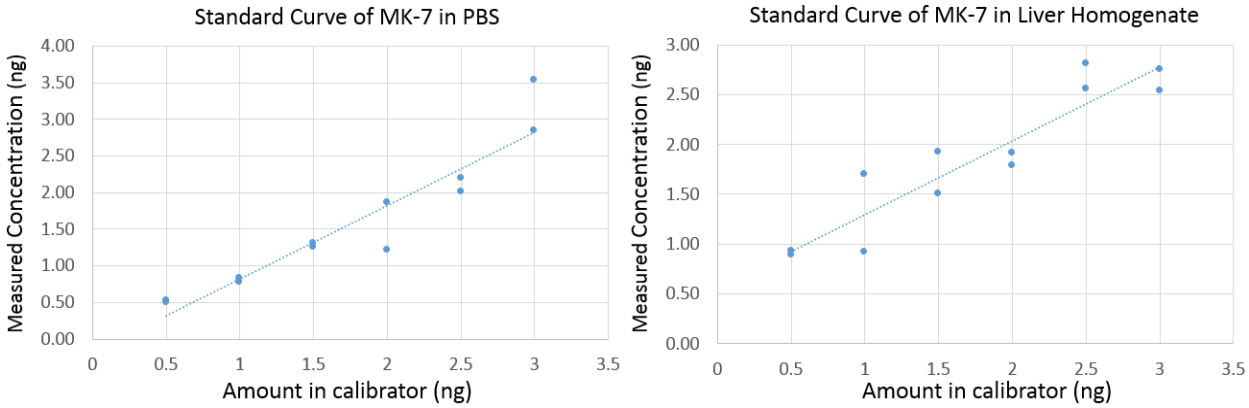

Figure 4.6. Standard curves comparing the measured concentration of MK-7 to the expected concentration of the calibrator solution. MK-4-d7 was used as the internal standard. Both PBS (phosphate buffered saline) and liver homogenate were used to compare matrix effects. In PBS, the response was linear with R^2 values of 0.89 for PBS and 0.87 for liver homogenate. The equations for the standard curves were 0.76 – 0.081 for PBS and 0.74 + 0.56 for liver homogenate.

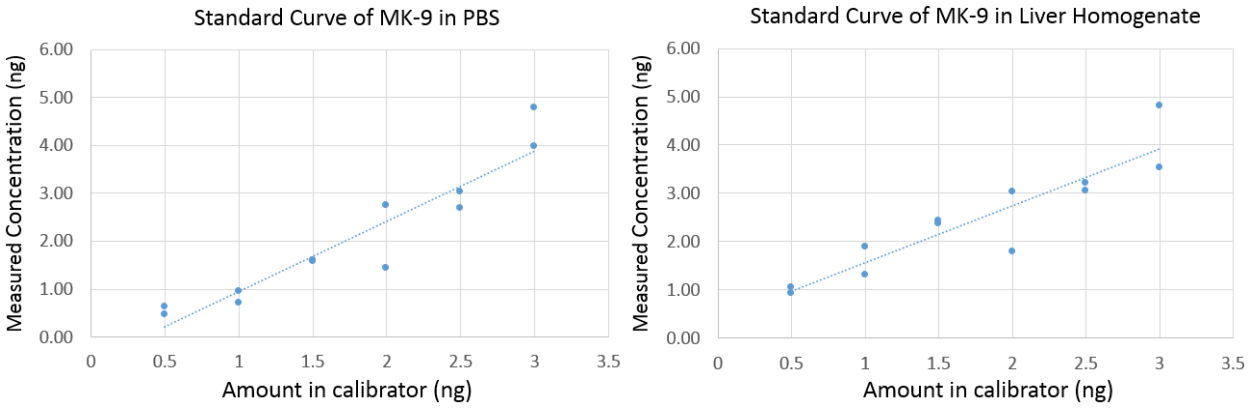

Figure 4.7. Standard curves comparing the measured concentration of MK-9 to the expected concentration of the calibrator solution. MK-4-d7 was used as the internal standard. Both PBS (phosphate buffered saline) and liver homogenate were used to compare matrix effects. In PBS, the response was linear with R^2 values of 0.886 for PBS and 0.839 for liver homogenate. The equations for the standard curves were 0.66 – 0.008 for PBS and 0.62 + 0.095 for liver homogenate.

Liver ID	Age	Sex	*CYP4F11 N446D (A>G)*	*VKORC1-1639 (G>A)*	*CYP4F2*3 V433M (C>T)*	VK1 (ng/g)	VK1 (pmol/g)
HL-102	21	M	AA	AA	TC	9.62	21.38
HL-103	15	F	AA	AG	CC	< 1.00	< 2.22
HL-104	32	M	-	GG	CC	5.9	13.11
HL-105	21	M	-	AA	TC	4.06	9.02
HL-106	45	F	AG	GG	TC	< 1.00	< 2.22
HL-108	42	F	AG	AG	TC	16.89	37.54
HL-109	46	M	AG	AG	TC	23.88	53.08
HL-111	28	M	AG	GG	TC	3.94	8.77
HL-113	9	F	AG	AG	TC	2.58	5.73
HL-114	19	M	AG	AA	TC	5.52	12.27
HL-115	52	F	AG	AG	TC	18.55	41.23
HL-118	25	M	GG	GG	TC	43.84	97.43
HL-119	24	M	AG	GG	TC	2.08	4.63
HL-120	45	F	AG	AG	TC	3.03	6.74
HL-121	59	F	-	AG	TC	27.96	62.14
HL-124	51	F	AG	AA	CC	6.44	14.32
HL-125	32	M	GG	GG	TC	2.98	6.63
HL-127	38	M	GG	AG	TC	120.93	268.74
HL-128	51	M	GG	AA	TT	18.26	40.57
HL-130	49	M	AG	AG	TC	3.36	7.47
HL-131	62	F	AA	GG	CC	3.56	7.91
HL-133	45	F	AG	GG	TC	20.64	45.87

HL-134	7	M	AA	AG	CC	31.44	69.86
HL-135	45	F	GG	AG	CC	2.26	5.03
HL-136	39	M	AG	GG	TC	4.86	10.79
HL-137	11	M	-	GG	TC	4.77	10.61
HL-138	9	F	AA	AA	CC	3.01	6.69
HL-139	15	F	AA	GG	CC	14.3	31.78
HL-141	59	M	AG	AG	TC	71.67	159.26
HL-142	49	F	AA	GG	CC	2.68	5.95
HL-143	48	M	AA	GG	TC	< 1.00	< 2.22
HL-144	68	F	AA	GG	CC	1.87	4.17
HL-145	38	M	AA	AG	CC	1.44	3.19
HL-146	10	M	AG	AG	CC	209.72	466.05
HL-147	70	F	AG	AG	TC	8.28	18.4
HL-148	60	F	AA	GG	CC	44.85	99.67
HL-149	63	F	AG	AG	TT	12.5	27.77
HL-150	30	M	-	GG	CC	6.35	14.11
HL-152	64	F	AG	GG	CC	8.84	19.65
HL-153	59	F	AG	GG	CC	4.68	10.4
HL-154	26	M	AA	AG	CC	6.76	15.02
HL-155	21	M	AG	AG	CC	4.19	9.31
HL-156	44	M	AG	GG	TC	2651.47	5892.14
HL-157	41	F	GG	GG	CC	2.6	5.78
HL-158	59	M	AA	AA	TC	12.09	26.86
HL-159	53	F	AG	GG	CC	13.02	28.94
HL-160	67	M	GG	GG	CC	24.06	53.46
HL-161	53	M	-	GG	CC	4.56	10.13
HL-162	56	F	GG	AG	TT	5.43	12.07
HL-163	55	M	AG	AG	CC	8.47	18.82
HL-164	50	F	-	GG	TC	7.27	16.17
HL-165	61	M	-	AG	CC	13.68	30.4
HL-166	59	F	AG	AG	TT	1180.62	2623.6
HL-167	44	M	GG	AG	CC	12.83	28.52
HL-168	43	M	AA	GG	CC	1.69	3.75
HL-169	57	M	GG	AG	CC	21.08	46.84
HL-170	50	F	AA	GG	CC	9.78	21.74
HL-172	28	M	-	GG	CC	50.38	111.95

Table 4.2. The age, sex, genotypes, and VK1 content of liver samples obtained from the UW School of Pharmacy Human Liver Bank. Dashes denote missing genotype information for the particular liver. The limit of quantitation for VK1 was 1 ng/g of liver tissue. Two livers, HL-146 and HL-156, were known to be administered vitamin K in the hospital prior to collection of the organ.

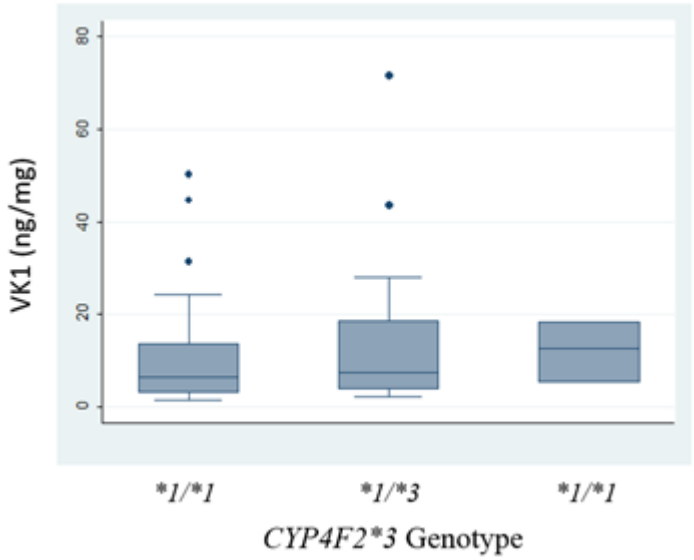

Figure 4.8. Box-and-whisker plots comparing hepatic VK1 content across *CYP4F2*3* genotype. The top and bottom of the darkened boxes represent the 25th and 75th percentiles, respectively. The median is represented by the middle line, whiskers are the 95% CI, and outliers are shown as closed circles. Actual values are displayed in Table 4.3. Outlier samples that have hepatic VK1 values above 100 ng/g were excluded from the statistical analysis due to suspected administration of vitamin K in the hospital setting.

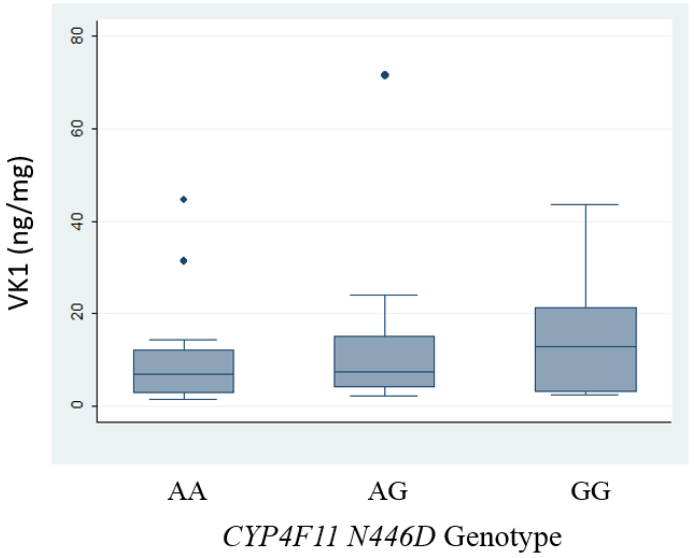

Figure 4.9. Box-and-whisker plots of hepatic VK1 content stratified by *CYP4F11 N446D* genotype. Actual values are displayed on Table 4.3. Outlier samples with hepatic VK1 values above 100 ng/g were excluded from statistical analysis.

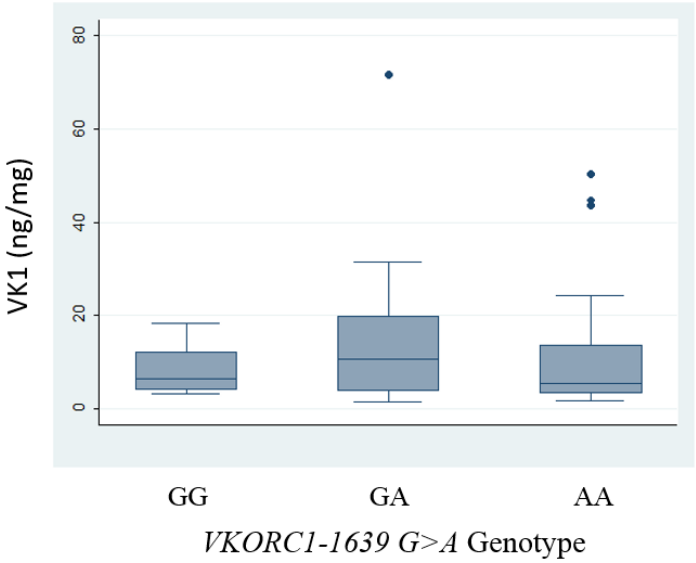

Figure 4.10. Box-and-whisker plots of hepatic VK1 content stratified by *VKORC1-1639 G>A* genotype. Actual values are displayed on Table 4.3. Outlier samples with hepatic VK1 values above 100 ng/g were excluded from statistical analysis.

		Non-transformed VK1 (ng/mL)		**Natural log transformed VK1**		**Regression Analysis**			
Group	N	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	Unadjusted		Adjusted for age and sex	
						P-value	R-value	P-value	R-value
All	51	12.6 ± 14.2	6.76 (13.3)	2.06 ± 0.97	1.91 (1.56)				
*CYP4F2*3*									
**1/*1*	27	11.5 ± 12.8	6.44 (10.7)	1.96 ± 0.99	1.86 (1.51)				
**1/*3*	21	14.2 ± 17.0	7.27 (14.6)	2.14 ± 1.00	1.98 (1.55)	0.399	0.121	0.401	0.271
**3/*3*	3	12.1 ± 6.43	12.5 (12.8)	2.37 ± 0.62	2.52 (1.21)				
CYP4F11 N446D									
AA	13	11.0 ± 13.1	6.76 (9.41)	1.82 ± 1.11	1.91 (1.51)				
AG	20	12.2 ± 15.4	7.36 (10.9)	2.06 ± 0.89	1.99 (1.29)	0.350	0.148	0.575	0.269
GG	9	14.8 ± 13.8	12.8 (18.1)	2.22 ± 1.10	2.55 (1.96)				
VKORC1-1639 G>A									
GG	24	12.1 ± 14.5	5.38 (10.4)	1.95 ± 1.02	1.68 (1.43)				
GA	20	14.8 ± 16.1	10.5 (16.0)	2.22 ± 1.03	2.33 (1.66)	0.694	0.057	0.649	0.252
AA	7	8.43 ± 5.36	6.44 (8.03)	1.96 ± 0.63	1.86 (1.09)				

Table 4.3. The means, standard deviations (SD), median, and interquartile ranges (IQR) for human hepatic VK1 content stratified by genotype. P-values and R-values were obtained from multivariate regression before and after the adjustment for age and sex, where $p \leq 0.05$ was considered statistically significant. Outlier samples with hepatic VK1 content values above 100 ng/g were excluded due to suspected administration of vitamin K.

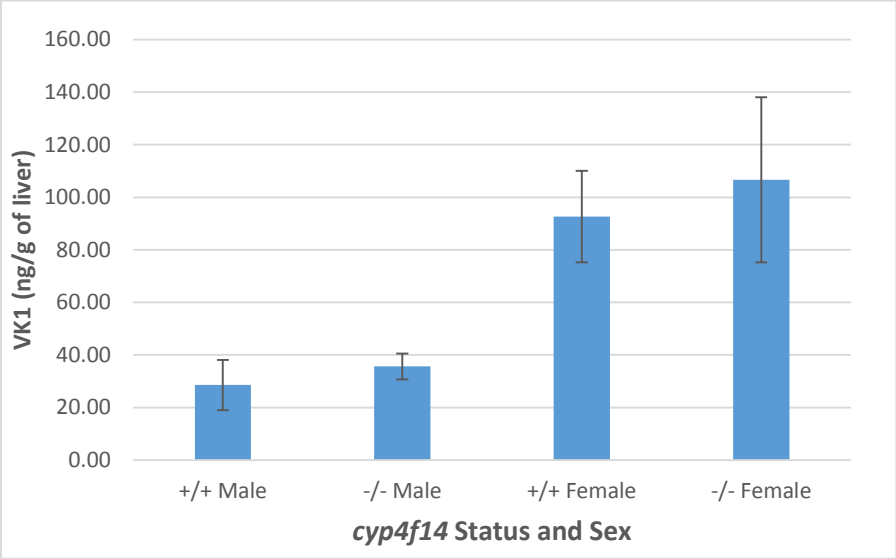

Figure 4.11. Differences in VK1 levels measured from livers of mice stratified by *cyp4f14* status and sex. The +/+ denotes control mice and -/- denotes cyp4f14 null mice. Null mice had slightly higher hepatic VK1 content compared to controls mice, but females had significantly greater VK1 levels compared to males (< 0.001).

Group	Mean ± SD	P-value
+/+ Male	28.6 ± 9.54	0.139
-/- Male	35.6 ± 4.95	
+/+ Female	92.7 ± 17.5	0.460
-/- Female	106.6 ± 31.4	
All +/+	62.0 ± 37.4	0.582
All -/-	71.1 ± 42.9	
All Males	32.1 ± 8.13	< 0.001
All Females	101.0 ± 25.0	

Table 4.4. The mean ± SD values of VK1 content in the livers of *cyp4f14* knockout (+/+) and control mice (-/-). VKO, MK-4, and MK-4O were below detectable levels (< 1 ng/g) in mouse livers. While there were relatively little difference in VK1 levels between control and knock-out genotypes of *cyp4f14*, the differences in VK1 between sexes were much more pronounced. P-values were obtained using a two-sample t-test and $p \leq 0.05$ was considered statistically significant.

4.5 Notes to Chapter 4

1. Duello, T. J., and Matschiner, J. T. (1972) Characterization of vitamin K from human liver, *The Journal of nutrition 102*, 331-335.

2. Kayata, S., Kindberg, C., Greer, F. R., and Suttie, J. W. (1989) Vitamin K1 and K2 in infant human liver, *Journal of pediatric gastroenterology and nutrition 8*, 304-307.

3. Usui, Y., Tanimura, H., Nishimura, N., Kobayashi, N., Okanoue, T., and Ozawa, K. (1990) Vitamin K concentrations in the plasma and liver of surgical patients, *The American journal of clinical nutrition 51*, 846-852.

4. Hollander, D. (1973) Vitamin K1 absorption by everted intestinal sacs of the rat, *The American journal of physiology 225*, 360-364.

5. Hollander, D., Muralidhara, K. S., and Rim, E. (1976) Colonic absorption of bacterially synthesized vitamin K2 in the rat, *The American journal of physiology 230*, 251-255.

6. Kindberg, C., Suttie, J. W., Uchida, K., Hirauchi, K., and Nakao, H. (1987) Menaquinone production and utilization in germ-free rats after inoculation with specific organisms, *The Journal of nutrition 117*, 1032-1035.

7. Bentley, R., and Meganathan, R. (1982) Biosynthesis of vitamin K (menaquinone) in bacteria, *Microbiological reviews 46*, 241-280.

8. Conly, J., and Stein, K. (1994) Reduction of vitamin K2 concentrations in human liver associated with the use of broad spectrum antimicrobials, *Clinical and investigative medicine. Medecine clinique et experimentale 17*, 531-539.

9. Rice, P. J., Perry, R. J., Afzal, Z., and Stockley, I. H. (2003) Antibacterial prescribing and warfarin: a review, *British dental journal 194*, 411-415.

10. Stafford, D. W. (2005) The vitamin K cycle, *Journal of thrombosis and haemostasis : JTH 3*, 1873-1878.

11. Conly, J. M., and Stein, K. (1992) The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis, *Progress in food & nutrition science 16*, 307-343.

12. Beulens, J. W., Booth, S. L., van den Heuvel, E. G., Stoecklin, E., Baka, A., and Vermeer, C. (2013) The role of menaquinones (vitamin K(2)) in human health, *The British journal of nutrition 110*, 1357-1368.

13. Shearer, M. J., and Newman, P. (2008) Metabolism and cell biology of vitamin K, *Thrombosis and haemostasis 100*, 530-547.

14. Edson, K. Z., Prasad, B., Unadkat, J. D., Suhara, Y., Okano, T., Guengerich, F. P., and Rettie, A. E. (2013) Cytochrome P450-dependent catabolism of vitamin K: omega-hydroxylation catalyzed by human CYP4F2 and CYP4F11, *Biochemistry 52*, 8276-8285.

15. Rieder, M. J., Reiner, A. P., Gage, B. F., Nickerson, D. A., Eby, C. S., McLeod, H. L., Blough, D. K., Thummel, K. E., Veenstra, D. L., and Rettie, A. E. (2005) Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose, *The New England journal of medicine 352*, 2285-2293.

16. Bodin, L., Verstuyft, C., Tregouet, D. A., Robert, A., Dubert, L., Funck-Brentano, C., Jaillon, P., Beaune, P., Laurent-Puig, P., Becquemont, L., and Loriot, M. A. (2005) Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity, *Blood 106*, 135-140.

17. Bardowell, S. A., Duan, F., Manor, D., Swanson, J. E., and Parker, R. S. (2012) Disruption of mouse cytochrome p450 4f14 (Cyp4f14 gene) causes severe perturbations in vitamin E metabolism, *The Journal of biological chemistry 287*, 26077-26086.

18. Sontag, T. J., and Parker, R. S. (2002) Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status, *The Journal of biological chemistry 277*, 25290-25296.

19. Dowd, P., Ham, S. W., Marchand, A. P., and Rajapaksa, D. (1992) Mechanism of Epoxidation of Vitamin K with Basic Hydrogen Peroxide, *J. Org. Chem. 57*, 3514-3516.

20. Suhara, Y., Hanada, N., Okitsu, T., Sakai, M., Watanabe, M., Nakagawa, K., Wada, A., Takeda, K., Takahashi, K., Tokiwa, H., and Okano, T. (2012) Structure-activity relationship of novel menaquinone-4 analogues: modification of the side chain affects their biological activities, *Journal of medicinal chemistry 55*, 1553-1558.

21. Bardowell, S. A., Ding, X., and Parker, R. S. (2012) Disruption of P450-mediated vitamin E hydroxylase activities alters vitamin E status in tocopherol supplemented mice and reveals extra-hepatic vitamin E metabolism, *Journal of lipid research 53*, 2667-2676.

22. Suhara, Y., Kamao, M., Tsugawa, N., and Okano, T. (2005) Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry, *Analytical chemistry 77*, 757-763.

23. Karl, J. P., Fu, X., Dolnikowski, G. G., Saltzman, E., and Booth, S. L. (2014) Quantification of phylloquinone and menaquinones in feces, serum, and food by high-performance liquid chromatography-mass spectrometry, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 963*, 128-133.

24. Song, Q., Wen, A., Ding, L., Dai, L., Yang, L., and Qi, X. (2008) HPLC-APCI-MS for the determination of vitamin K(1) in human plasma: method and clinical application, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 875*, 541-545.

25. Huber, A. M., Davidson, K. W., O'Brien-Morse, M. E., and Sadowski, J. A. (1999) Gender differences in hepatic phylloquinone and menaquinones in the vitamin K-deficient and -supplemented rat, *Biochimica et biophysica acta 1426*, 43-52.

Chapter 5

General Conclusions and Future Directions

5.1. General Conclusions

The diet of the Yup'ik people of southwestern Alaska is characterized by a high consumption of fish and marine animals (1). These traditional foods are rich sources of ω3 PUFA and studies in both humans and animals have demonstrated effects of ω3 PUFAs on cardiovascular physiology and hemostasis (2-4). While higher ω3 PUFA intakes are associated with reduced risk of cardiovascular disease, some reports have suggested that greater intake may also potentiate anticoagulative effects in individuals taking oral anticoagulants. However, the extent to which ω3 PUFAs affect the hemostatic system in humans, particularly when consumed from natural dietary sources, remains unclear. In chapter 2, we explored the effects of ω3 PUFA intake on hemostasis in Yup'ik people in an observational study that spanned four years. We found an inverse correlation between the platelet biomarker, sP-selectin, and $\delta^{15}N$, a highly quantifiable indicator of ω3 PUFAs consumption. Interestingly, the association between higher ω3 PUFAs intake and reduced platelet activation in this population appears to be a result of variation in the degree to which study participants adhere to a traditional Yup'ik diet that relies on ω3 PUFA-rich fish and marine mammals. This observation supports the prior findings of Dyerberg and Bang in the 1980s that platelet aggregation was reduced and bleeding times were prolonged in Greenland Inuit study volunteers (5). However, we found no evidence that ω3 PUFA consumption had a significant effect on vitamin K-dependent clotting factor II and only a modest, inverse association with vitamin K-independent clotting factor V was observed. There was also no association between ω3 PUFA intake and clinical measures of coagulation, PTT and PT/INR. Collectively, these findings suggest that ω3 PUFAs are unlikely to strongly impact coagulation status or bleeding risk in the Yup'ik population. Evidence for any effect of ω3 PUFA intake on hemostasis appears to be driven by altered platelet activity, as indicated by the sP-selectin data.

In chapter 3, we assessed the vitamin K status of Yup'ik people since it may be a significant modifier of hemostasis in this population. We used plasma vitamin K levels as a measure of acute vitamin K status and the biomarker PIVKA-II as an indicator of chronic vitamin K status. We then determined whether these two measures differed across various genotypes for enzymes involved in the vitamin K cycle. Specific SNPs included in the analysis were *CYP4F2*3, CYP4F11 N446D,* and *VKORC1 -1173 T>C.* While fasting plasma vitamin K levels did not greatly differ from levels reported in other population studies, we found that nearly one-third of Yup'ik people in our study population had measureable PIKVA-II levels at or greater than 2 ng/mL, indicative of low vitamin K status. Another significant finding of these analyses was the notably high allele frequency of *CYP4F2*3*, which approached 50%. Previous studies have identified the CYP4F2 enzyme as a vitamin K hydroxylase and variation in the **3 (V433M)* allele is associated with higher warfarin dose requirements (6, 7). In Yup'ik people the *CYP4F2*3* genotype was significantly associated with higher plasma VK1 concentrations and with a reduced likelihood of having PIVKA-II levels above 2 ng/mL. In contrast, the *CYP4F11 N446D* and *VKORC1-1173 T>C* alleles did not exhibit any significant associations with plasma vitamin K levels or PIVKA-II in our study. However, the allele frequencies of these SNPs in Yup'ik people were much lower than the frequency of *CYP4F2*3*, resulting in a lower power to detect possible associations with vitamin K status for these particular variants.

To probe sources of vitamin K in the Yup'ik diet, we next analyzed the vitamin K content of tundra greens collected in the Yukon-Kuskokwim river delta. We found that all tundra greens examined contained vitamin K in amounts comparable to commercially available market vegetables, indicating that these foods could serve as a significant source of vitamin K in remote regions of Alaska where residents have limited access to fresh produce. Since tundra greens are collected mainly during the summer, there are likely other sources of vitamin K found in other traditional foods, such as fish and mammalian meats, which may also contain significant amounts of vitamin K.

We suspected that the *CYP4F2*3* allele promoted conservation of hepatic vitamin K stores and evaluated this hypothesis in Chapter 4 by quantifying vitamin K content of banked (non-Yup'ik) human

liver samples with respect to *CYP4F2 V433M*, *CYP4F11 N446D*, and *VKORC1-1639 G>A* genotype. Although we measured a wide range of phylloquinone levels, no association of genotype with hepatic vitamin K content was observed. This is likely due to a lack of control for factors that could influence hepatic vitamin K levels in the samples analyzed, such as dietary intake and administered medications. There were also only a few variant homozygote genotypes among the available human liver samples, and these low numbers likely hindered our ability to fully evaluate any association between genotype and hepatic vitamin K levels.

We further explored whether the *cyp4f14* gene, a potential murine ortholog of CYP4F2, was a modifier of vitamin K status in mice. However, when we measured hepatic vitamin K levels from livers of *cyp4f14* knockout mice in comparison to *cyp4f14* wild-type control mice, we did not find significant differences in hepatic vitamin K content between *cyp4f14* knockouts and controls, although there was trend towards higher levels in the null animals. Interestingly, there was a very notable increase in hepatic phylloquinone content in female mice compared to males, but the reasons behind this sex difference remain unknown.

5.2 Future Directions

It is important to delineate the molecular mechanism(s) of ω3 PUFA effects on platelet function, including possible effects on altered membrane lipid composition, platelet receptor function, and inflammatory eicosanoid production. Enrichment of membrane ω3 PUFA concentrations could affect platelet activity by changing membrane biophysical properties and the function of embedded glycoprotein receptors critical to the initiation and platelet activation and platelet aggregation. In addition, by displacing arachidonic acid in platelet and vascular cell membranes, enrichment with ω3 PUFAs could alter the production of thromboxanes (TBXA2, TBXA3) and prostaglandins (PGI2, PGI3), molecules that have positive and negative effects on the activation process (2-4). Specifically, reduced concentrations of TBXA2, in favor of less active TBXA3, will lower the platelet activation state. PGI2 and PGI3 appear to

be equipotent inhibitors of platelet activation (8). Thus, a lower TBXA2/PGI2+PGI3 ratio should favor reduced platelet activation. To explore a causal relationship between ω3 PUFA and platelet activation, future studies could determine whether or not individuals with extremely high $\delta^{15}N$ also exhibit one or more of the following in comparison to individuals with an extremely low $\delta^{15}N$: a higher concentrations of platelet membrane ω3 PUFAs (EPA and DHA); a lower platelet TBXA2/TBXA3 concentration ratio; and a lower TBX2/PGI2 + PGI3 concentration ratio. An evaluation of any associations between $\delta^{15}N$ and plasma eicosanoids would be an important first step to elucidating molecular mechanisms underlying the beneficial effects of the traditional Yup'ik diet.

It would also be of interest to determine if the $\delta^{15}N$ value is associated with altered response to treatment with the anti-platelet drug, aspirin. Resistance to aspirin treatment is a well-documented phenomenon and it seems plausible that ω3 PUFA intake could be a modifying dietary factor in the variable patient response to this widely used drug (9-11). Ultimately, one could even envision studies to determine the value of $\delta^{15}N$ testing in patients receiving antiplatelet therapy to reduce the risk of adverse side effects. This approach also lends itself to a pharmacogenomic investigation because genetic variation in the platelet membrane protein, PEAR1 (platelet endothelial aggregation receptor 1), has been shown to associate with greater platelet response to collagen-related peptide exposure and higher sP-selectin levels after ADP activation (12, 13). It is conceivable that high dietary consumption of ω3 PUFAs may modify the *PEAR1* SNP associations by altering the function of the platelet membrane receptor or downstream events, such as thromboxane and prostaglandin synthesis. Resequencing of the *PEAR1* gene in Alaska Native people and determining its association with platelet function might further identify modifiers to antithrombotic therapy, since *PEAR1* SNPs appear to influence platelet aggregation in aspirin-treated individuals (14, 15).

Finally, while it seems clear that the *CY4F2*3* allele plays a significant role in maintaining vitamin K status in Yup'ik people, an important remaining goal is to determine whether the mechanism for this effect involves altered intra-hepatic levels of vitamin K. The investigations conducted here with archived human tissue highlight the need for a tightly controlled population, so human studies may not be

feasible. Measurement of vitamin K levels in liver tissue from *cyp4f14* null mice appeared to be a promising avenue, but preliminary studies were disappointing, although a trend towards higher phylloquinone levels in these knockout mice was apparent. It is possible that other murine liver P450s catalyze vitamin K catabolism. Therefore, an important next step is to identify which of the remaining hepatic isoforms are the major contributors to vitamin K metabolism in mouse liver microsomes in order to better target the complement of cyp4f isoforms to delete in a new murine model for *in vivo* studies.

5.3 Notes to Chapter 5

1. Bersamin, A., Zidenberg-Cherr, S., Stern, J. S., and Luick, B. R. (2007) Nutrient intakes are associated with adherence to a traditional diet among Yup'ik Eskimos living in remote Alaska Native communities: the CANHR Study, *International journal of circumpolar health 66*, 62-70.

2. Calder, P. C. (2001) Polyunsaturated fatty acids, inflammation, and immunity, *Lipids 36*, 1007-1024.

3. Khairallah, R. J., Kim, J., O'Shea, K. M., O'Connell, K. A., Brown, B. H., Galvao, T., Daneault, C., Des Rosiers, C., Polster, B. M., Hoppel, C. L., and Stanley, W. C. (2012) Improved mitochondrial function with diet-induced increase in either docosahexaenoic acid or arachidonic acid in membrane phospholipids, *PloS one 7*, e34402.

4. Arterburn, L. M., Hall, E. B., and Oken, H. (2006) Distribution, interconversion, and dose response of n-3 fatty acids in humans, *The American journal of clinical nutrition 83*, 1467S-1476S.

5. Dyerberg, J., and Bang, H. O. (1979) Haemostatic function and platelet polyunsaturated fatty acids in Eskimos, *Lancet 2*, 433-435.

6. Caldwell, M. D., Awad, T., Johnson, J. A., Gage, B. F., Falkowski, M., Gardina, P., Hubbard, J., Turpaz, Y., Langaee, T. Y., Eby, C., King, C. R., Brower, A., Schmelzer, J. R., Glurich, I., Vidaillet, H. J., Yale, S. H., Qi Zhang, K., Berg, R. L., and Burmester, J. K. (2008) CYP4F2 genetic variant alters required warfarin dose, *Blood 111*, 4106-4112.

7. McDonald, M. G., Rieder, M. J., Nakano, M., Hsia, C. K., and Rettie, A. E. (2009) CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant, *Molecular pharmacology 75*, 1337-1346.

8. Kobzar, G., Mardla, V., Jarving, I., and Samel, N. (2001) Comparison of anti-aggregatory effects of PGI2, PGI3 and iloprost on human and rabbit platelets, *Cellular physiology and biochemistry :*

international journal of experimental cellular physiology, biochemistry, and pharmacology 11, 279-284.

9. Emani, S., Trainor, B., Zurakowski, D., Baird, C. W., Fynn-Thompson, F. E., Pigula, F. A., and Emani, S. M. (2014) Aspirin unresponsiveness predicts thrombosis in high-risk pediatric patients after cardiac surgery, *The Journal of thoracic and cardiovascular surgery 148*, 810-814; discussion 814-816.

10. Mao, Y., Lei, L., Su, J., Yu, Y., Liu, Z., and Huo, Y. (2014) Regulators of G protein signaling are up-regulated in aspirin-resistant platelets from patients with metabolic syndrome, *Die Pharmazie 69*, 371-373.

11. Xu, Z. H., Jiao, J. R., Yang, R., Luo, B. Y., Wang, X. F., and Wu, F. (2012) Aspirin resistance: clinical significance and genetic polymorphism, *The Journal of international medical research 40*, 282-292.

12. Nanda, N., Bao, M., Lin, H., Clauser, K., Komuves, L., Quertermous, T., Conley, P. B., Phillips, D. R., and Hart, M. J. (2005) Platelet endothelial aggregation receptor 1 (PEAR1), a novel epidermal growth factor repeat-containing transmembrane receptor, participates in platelet contact-induced activation, *The Journal of biological chemistry 280*, 24680-24689.

13. Jones, C. I., Bray, S., Garner, S. F., Stephens, J., de Bono, B., Angenent, W. G., Bentley, D., Burns, P., Coffey, A., Deloukas, P., Earthrowl, M., Farndale, R. W., Hoylaerts, M. F., Koch, K., Rankin, A., Rice, C. M., Rogers, J., Samani, N. J., Steward, M., Walker, A., Watkins, N. A., Akkerman, J. W., Dudbridge, F., Goodall, A. H., and Ouwehand, W. H. (2009) A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways, *Blood 114*, 1405-1416.

14. Herrera-Galeano, J. E., Becker, D. M., Wilson, A. F., Yanek, L. R., Bray, P., Vaidya, D., Faraday, N., and Becker, L. C. (2008) A novel variant in the platelet endothelial aggregation receptor-1 gene is associated with increased platelet aggregability, *Arteriosclerosis, thrombosis, and vascular biology 28*, 1484-1490.

15. Wurtz, M., Nissen, P. H., Grove, E. L., Kristensen, S. D., and Hvas, A. M. (2014) Genetic Determinants of On-Aspirin Platelet Reactivity: Focus on the Influence of PEAR1, *PloS one 9*, e111816.

LIST OF REFERNCES

(1988). "Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. The Expert Panel." Arch Intern Med **148**(1): 36-69.

(1999). "Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico." Lancet **354**(9177): 447-455.

Adams, R. L. and R. J. Bird (2009). "Review article: Coagulation cascade and therapeutics update: relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants." Nephrology (Carlton) **14**(5): 462-470.

Adkins, Y. and D. S. Kelley (2010). "Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids." J Nutr Biochem **21**(9): 781-792.

Adler, A. I., E. J. Boyko, et al. (1994). "Lower prevalence of impaired glucose tolerance and diabetes associated with daily seal oil or salmon consumption among Alaska Natives." Diabetes Care **17**(12): 1498-1501.

Aithal, G. P., C. P. Day, et al. (1999). "Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications." Lancet **353**(9154): 717-719.

Alving, A. S., P. E. Carson, et al. (1956). "Enzymatic deficiency in primaquine-sensitive erythrocytes." Science **124**(3220): 484-485.

Ancrenaz, V., Y. Daali, et al. (2010). "Impact of genetic polymorphisms and drug-drug interactions on clopidogrel and prasugrel response variability." Curr Drug Metab **11**(8): 667-677.

Andriamampandry, M., M. Freund, et al. (1998). "Diets enriched in (n-3) fatty acids affect rat coagulation factors dependent on vitamin K." C R Acad Sci III **321**(5): 415-421.

Arterburn, L. M., E. B. Hall, et al. (2006). "Distribution, interconversion, and dose response of n-3 fatty acids in humans." Am J Clin Nutr **83**(6 Suppl): 1467S-1476S.

Asselta, R. and F. Peyvandi (2009). "Factor V deficiency." Semin Thromb Hemost **35**(4): 382-389.

Athinarayanan, S., R. Wei, et al. (2014). "Genetic polymorphism of cytochrome P450 4F2, vitamin E level and histological response in adults and children with nonalcoholic fatty liver disease who participated in PIVENS and TONIC clinical trials." PLoS One **9**(4): e95366.

Au, N. and A. E. Rettie (2008). "Pharmacogenomics of 4-hydroxycoumarin anticoagulants." Drug Metab Rev **40**(2): 355-375.

Azharuddin, M. K., D. S. O'Reilly, et al. (2007). "HPLC method for plasma vitamin K1: effect of plasma triglyceride and acute-phase response on circulating concentrations." Clin Chem **53**(9): 1706-1713.

Bakhshi, S., A. K. Deorari, et al. (1996). "Prevention of subclinical vitamin K deficiency based on PIVKA-II levels: oral versus intramuscular route." Indian Pediatr **33**(12): 1040-1043.

Bang, H. O. and J. Dyerberg (1980). "The bleeding tendency in Greenland Eskimos." Dan Med Bull **27**(4): 202-205.

Bang, H. O., J. Dyerberg, et al. (1971). "Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos." Lancet **1**(7710): 1143-1145.

Bang, H. O., J. Dyerberg, et al. (1980). "The composition of the Eskimo food in north western Greenland." Am J Clin Nutr **33**(12): 2657-2661.

Bardowell, S. A., X. Ding, et al. (2012). "Disruption of P450-mediated vitamin E hydroxylase activities alters vitamin E status in tocopherol supplemented mice and reveals extra-hepatic vitamin E metabolism." J Lipid Res **53**(12): 2667-2676.

Bardowell, S. A., F. Duan, et al. (2012). "Disruption of mouse cytochrome p450 4f14 (Cyp4f14 gene) causes severe perturbations in vitamin E metabolism." J Biol Chem **287**(31): 26077-26086.

Barkhan, P. and M. J. Shearer (1977). "Metabolism of vitamin K1 (phylloquinone) in man." Proc R Soc Med **70**(2): 93-96.

Belle, M., R. Brebant, et al. (1995). "Production of a new monoclonal antibody specific to human des-gamma-carboxyprothrombin in the presence of calcium ions. Application to the development of a sensitive ELISA-test." J Immunoassay **16**(2): 213-229.

Bentley, R. and R. Meganathan (1982). "Biosynthesis of vitamin K (menaquinone) in bacteria." Microbiol Rev **46**(3): 241-280.

Bersamin, A., B. R. Luick, et al. (2008). "Westernizing diets influence fat intake, red blood cell fatty acid composition, and health in remote Alaskan Native communities in the center for Alaska Native health study." J Am Diet Assoc **108**(2): 266-273.

Bersamin, A., S. Zidenberg-Cherr, et al. (2007). "Nutrient intakes are associated with adherence to a traditional diet among Yup'ik Eskimos living in remote Alaska Native communities: the CANHR Study." Int J Circumpolar Health **66**(1): 62-70.

Beulens, J. W., S. L. Booth, et al. (2013). "The role of menaquinones (vitamin K(2)) in human health." Br J Nutr **110**(8): 1357-1368.

Bevans, C. G., C. Krettler, et al. (2013). "Determination of the warfarin inhibition constant Ki for vitamin K 2,3-epoxide reductase complex subunit-1 (VKORC1) using an in vitro DTT-driven assay." Biochim Biophys Acta **1830**(8): 4202-4210.

Beverage, J. N., T. M. Sissung, et al. (2007). "CYP2D6 polymorphisms and the impact on tamoxifen therapy." J Pharm Sci **96**(9): 2224-2231.

Blair, P. and R. Flaumenhaft (2009). "Platelet alpha-granules: basic biology and clinical correlates." Blood Rev **23**(4): 177-189.

Bodin, L., C. Verstuyft, et al. (2005). "Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity." Blood **106**(1): 135-140.

Booth, S. L., K. W. Davidson, et al. (1996). "Plasma concentrations of dihydro-vitamin K1 following dietary intake of a hydrogenated vitamin K1-rich vegetable oil." Lipids **31**(7): 709-713.

Booth, S. L., A. H. Lichtenstein, et al. (2001). "Effects of a hydrogenated form of vitamin K on bone formation and resorption." Am J Clin Nutr **74**(6): 783-790.

Booth, S. L. and J. A. Sadowski (1997). "Determination of phylloquinone in foods by high-performance liquid chromatography." Methods Enzymol **282**: 446-456.

Booth, S. L. and J. W. Suttie (1998). "Dietary intake and adequacy of vitamin K." J Nutr **128**(5): 785-788.

Boyer, B. B., G. V. Mohatt, et al. (2007). "Metabolic syndrome in Yup'ik Eskimos: the Center for Alaska Native Health Research (CANHR) Study." Obesity (Silver Spring) **15**(11): 2535-2540.

Brandt, J. T., S. L. Close, et al. (2007). "Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel." J Thromb Haemost **5**(12): 2429-2436.

Bucher, H. C., P. Hengstler, et al. (2002). "N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials." Am J Med **112**(4): 298-304.

Budzynski, A. Z. (1986). "Fibrinogen and fibrin: biochemistry and pathophysiology." Crit Rev Oncol Hematol **6**(2): 97-146.

Buitenhuis, H. C., B. A. Soute, et al. (1990). "Comparison of the vitamins K1, K2 and K3 as cofactors for the hepatic vitamin K-dependent carboxylase." Biochim Biophys Acta **1034**(2): 170-175.

Butenas, S. and K. G. Mann (2002). "Blood coagulation." Biochemistry (Mosc) **67**(1): 3-12.

Calder, P. C. (2001). "Polyunsaturated fatty acids, inflammation, and immunity." Lipids **36**(9): 1007-1024.

Calder, P. C. (2006). "Polyunsaturated fatty acids and inflammation." Prostaglandins Leukot Essent Fatty Acids **75**(3): 197-202.

Caldwell, M. D., T. Awad, et al. (2008). "CYP4F2 genetic variant alters required warfarin dose." Blood **111**(8): 4106-4112.

Chandler, W. L. (2013). "Microparticle counts in platelet-rich and platelet-free plasma, effect of centrifugation and sample-processing protocols." Blood Coagul Fibrinolysis **24**(2): 125-132.

Chawla, D., A. K. Deorari, et al. (2007). "Vitamin K1 versus vitamin K3 for prevention of subclinical vitamin deficiency: a randomized controlled trial." Indian Pediatr **44**(11): 817-822.

Chignard, M., J. P. Le Couedic, et al. (1980). "Platelet-activating factor (PAF-acether) secretion from platelets: effect of aggregating agents." Br J Haematol **46**(3): 455-464.

Collet, J. P., J. S. Hulot, et al. (2009). "Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study." Lancet **373**(9660): 309-317.

Colussi, G., C. Catena, et al. (2007). "Omega-3 fatty acids: from biochemistry to their clinical use in the prevention of cardiovascular disease." Recent Pat Cardiovasc Drug Discov **2**(1): 13-21.

Conly, J. and K. Stein (1994). "Reduction of vitamin K2 concentrations in human liver associated with the use of broad spectrum antimicrobials." Clin Invest Med **17**(6): 531-539.

Conly, J. M. and K. Stein (1992). "The production of menaquinones (vitamin K2) by intestinal bacteria and their role in maintaining coagulation homeostasis." Prog Food Nutr Sci **16**(4): 307-343.

Cresci, S., G. W. Dorn, 2nd, et al. (2012). "Adrenergic-pathway gene variants influence beta-blocker-related outcomes after acute coronary syndrome in a race-specific manner." J Am Coll Cardiol **60**(10): 898-907.

Das, U. N. (2005). "Can COX-2 inhibitor-induced increase in cardiovascular disease risk be modified by essential fatty acids?" J Assoc Physicians India **53**: 623-627.

Davi, G., M. Romano, et al. (1998). "Increased levels of soluble P-selectin in hypercholesterolemic patients." Circulation **97**(10): 953-957.

Davidson, K. W., S. L. Booth, et al. (1996). " The conversion of phylloquinone to 2′,3′-dihydrophylloquinone during hydrogenation of vegetable oils." J Agric Food Chem **44**: 980-983.

Daviglus, M. L., J. Stamler, et al. (1997). "Fish consumption and the 30-year risk of fatal myocardial infarction." N Engl J Med **336**(15): 1046-1053.

Demirkan, K., M. A. Stephens, et al. (2000). "Response to warfarin and other oral anticoagulants: effects of disease states." South Med J **93**(5): 448-454; quiz 455.

Donnahey, P. L., V. T. Burt, et al. (1979). "High-performance liquid chromatography of menaquinone-4, 2,3-epoxymenaquinone-4, demethylmenaquinone-4 and related compounds." J Chromatogr **170**(1): 272-277.

Dowd, P., S. W. Ham, et al. (1992). "Mechanism of Epoxidation of Vitamin K with Basic Hydrogen Peroxide." J. Org. Chem. **57**: 3514-3516.

Ducros, V., M. Pollicand, et al. (2010). "Quantitative determination of plasma vitamin K1 by high-performance liquid chromatography coupled to isotope dilution tandem mass spectrometry." Anal Biochem **401**(1): 7-14.

Duello, T. J. and J. T. Matschiner (1972). "Characterization of vitamin K from human liver." J Nutr **102**(3): 331-335.

Dufner, G. S. and G. A. Marbet (2002). "[Factor XIII in man: a review]." Hamostaseologie **22**(1): 11-19.

Dyerberg, J. and H. O. Bang (1979). "Haemostatic function and platelet polyunsaturated fatty acids in Eskimos." Lancet **2**(8140): 433-435.

Eaton, S. B. and M. Konner (1985). "Paleolithic nutrition. A consideration of its nature and current implications." N Engl J Med **312**(5): 283-289.

Ebbesson, S. O., P. M. Risica, et al. (2005). "Eskimos have CHD despite high consumption of omega-3 fatty acids: the Alaska Siberia project." Int J Circumpolar Health **64**(4): 387-395.

Edson, K. Z., B. Prasad, et al. (2013). "Cytochrome P450-dependent catabolism of vitamin K: omega-hydroxylation catalyzed by human CYP4F2 and CYP4F11." Biochemistry **52**(46): 8276-8285.

Eichelbaum, M., N. Spannbrucker, et al. (1979). "Defective N-oxidation of sparteine in man: a new pharmacogenetic defect." Eur J Clin Pharmacol **16**(3): 183-187.

Emani, S., B. Trainor, et al. (2014). "Aspirin unresponsiveness predicts thrombosis in high-risk pediatric patients after cardiac surgery." J Thorac Cardiovasc Surg **148**(3): 810-814; discussion 814-816.

Eritsland, J., H. Arnesen, et al. (1996). "Effect of dietary supplementation with n-3 fatty acids on coronary artery bypass graft patency." Am J Cardiol **77**(1): 31-36.

Eschen, O., J. H. Christensen, et al. (2004). "Soluble adhesion molecules in healthy subjects: a dose-response study using n-3 fatty acids." Nutr Metab Cardiovasc Dis **14**(4): 180-185.

Esmon, C. T. (2005). "Coagulation inhibitors in inflammation." Biochem Soc Trans **33**(Pt 2): 401-405.

Ferland, G. (2012). "The discovery of vitamin K and its clinical applications." Ann Nutr Metab **61**(3): 213-218.

Finazzi, G., U. Budde, et al. (1996). "Bleeding time and platelet function in essential thrombocythemia and other myeloproliferative syndromes." Leuk Lymphoma **22 Suppl 1**: 71-78.

Fohner, A., L. I. Muzquiz, et al. (2013). "Pharmacogenetics in American Indian populations: analysis of CYP2D6, CYP3A4, CYP3A5, and CYP2C9 in the Confederated Salish and Kootenai Tribes." Pharmacogenet Genomics **23**(8): 403-414.

Fu, X., J. W. Peterson, et al. (2009). "Measurement of deuterium-labeled phylloquinone in plasma by high-performance liquid chromatography/mass spectrometry." Anal Chem **81**(13): 5421-5425.

Furuya, H., P. Fernandez-Salguero, et al. (1995). "Genetic polymorphism of CYP2C9 and its effect on warfarin maintenance dose requirement in patients undergoing anticoagulation therapy." Pharmacogenetics **5**(6): 389-392.

Gajos, G., J. Zalewski, et al. (2011). "Reduced thrombin formation and altered fibrin clot properties induced by polyunsaturated omega-3 fatty acids on top of dual antiplatelet therapy in patients undergoing percutaneous coronary intervention (OMEGA-PCI clot)." Arterioscler Thromb Vasc Biol **31**(7): 1696-1702.

Gearing, A. J. and W. Newman (1993). "Circulating adhesion molecules in disease." Immunol Today **14**(10): 506-512.

Geleijnse, J. M., C. Vermeer, et al. (2004). "Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: the Rotterdam Study." J Nutr **134**(11): 3100-3105.

Gijsbers, B. L., K. S. Jie, et al. (1996). "Effect of food composition on vitamin K absorption in human volunteers." Br J Nutr **76**(2): 223-229.

Gladding, P., L. Panattoni, et al. (2010). "Clopidogrel pharmacogenomics: next steps: a clinical algorithm, gene-gene interactions, and an elusive outcomes trial." JACC Cardiovasc Interv **3**(10): 995-1000.

Gomez Candela, C., L. M. Bermejo Lopez, et al. (2011). "Importance of a balanced omega 6/omega 3 ratio for the maintenance of health: nutritional recommendations." Nutr Hosp **26**(2): 323-329.

Gong, I. Y., U. I. Schwarz, et al. (2011). "Clinical and genetic determinants of warfarin pharmacokinetics and pharmacodynamics during treatment initiation." PLoS One **6**(11): e27808.

Goodeve, A. C., S. Rosen, et al. (2010). "Haemophilia A and von Willebrand's disease." Haemophilia **16 Suppl 5**: 79-84.

Greer, F. R., S. P. Marshall, et al. (1998). "A new mixed micellar preparation for oral vitamin K prophylaxis: randomised controlled comparison with an intramuscular formulation in breast fed infants." Arch Dis Child **79**(4): 300-305.

Gross, J., W. K. Cho, et al. (2006). "A plant locus essential for phylloquinone (vitamin K1) biosynthesis originated from a fusion of four eubacterial genes." J Biol Chem **281**(25): 17189-17196.

Harnack, K., G. Andersen, et al. (2009). "Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids." Nutr Metab (Lond) **6**: 8.

Haroon, Y., D. S. Bacon, et al. (1986). "Liquid-chromatographic determination of vitamin K1 in plasma, with fluorometric detection." Clin Chem **32**(10): 1925-1929.

Hashimoto, M., S. Hossain, et al. (1999). "Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells." Lipids **34**(12): 1297-1304.

Hawiger, J. (1987). "Formation and regulation of platelet and fibrin hemostatic plug." Hum Pathol **18**(2): 111-122.

Herrera-Galeano, J. E., D. M. Becker, et al. (2008). "A novel variant in the platelet endothelial aggregation receptor-1 gene is associated with increased platelet aggregability." Arterioscler Thromb Vasc Biol **28**(8): 1484-1490.

Hervig, T., T. Haugen, et al. (2004). "The platelet count accuracy of platelet concentrates obtained by using automated analysis is influenced by instrument bias and activated platelet components." Vox Sang **87**(3): 196-203.

Holbrook, A. M., J. A. Pereira, et al. (2005). "Systematic overview of warfarin and its drug and food interactions." Arch Intern Med **165**(10): 1095-1106.

Holden, R. M., A. R. Morton, et al. (2010). "Vitamins K and D status in stages 3-5 chronic kidney disease." Clin J Am Soc Nephrol **5**(4): 590-597.

Hollander, D. (1973). "Vitamin K1 absorption by everted intestinal sacs of the rat." Am J Physiol **225**(2): 360-364.

Hollander, D., K. S. Muralidhara, et al. (1976). "Colonic absorption of bacterially synthesized vitamin K2 in the rat." Am J Physiol **230**(2): 251-255.

Holmes, M. V., B. J. Hunt, et al. (2012). "The role of dietary vitamin K in the management of oral vitamin K antagonists." Blood Rev **26**(1): 1-14.

Huang, J. N. and M. A. Koerper (2008). "Factor V deficiency: a concise review." Haemophilia **14**(6): 1164-1169.

Huang, M., A. C. Rigby, et al. (2003). "Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins." Nat Struct Biol **10**(9): 751-756.

Huber, A. M., K. W. Davidson, et al. (1999). "Gender differences in hepatic phylloquinone and menaquinones in the vitamin K-deficient and -supplemented rat." Biochim Biophys Acta **1426**(1): 43-52.

Hulot, J. S., A. Bura, et al. (2006). "Cytochrome P450 2C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects." Blood **108**(7): 2244-2247.

Humes, K. R., N. A. Jones, et al. (2011). Overview of race and Hispanic origin : 2010. Washington, D.C., U.S. Dept. of Commerce, Economics and Statistics Administration, U.S. Census Bureau.

Indyk, H. E., V. C. Littlejohn, et al. (1995). "Liquid chromatographic determination of vitamin K1 in infant formulas and milk." J AOAC Int **78**(3): 719-723.

Irons, D. E., W. G. Iacono, et al. (2012). "Developmental Trajectory and Environmental Moderation of the Effect of ALDH2 Polymorphism on Alcohol Use." Alcohol Clin Exp Res.

Johnson, M. M., D. D. Swan, et al. (1997). "Dietary supplementation with gamma-linolenic acid alters fatty acid content and eicosanoid production in healthy humans." J Nutr **127**(8): 1435-1444.

Jones, C. I., S. Bray, et al. (2009). "A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways." Blood **114**(7): 1405-1416.

Jones, D. Y., B. P. Koonsvitsky, et al. (1991). "Vitamin K status of free-living subjects consuming olestra." Am J Clin Nutr **53**(4): 943-946.

Kagimoto, M., M. Heim, et al. (1990). "Multiple mutations of the human cytochrome P450IID6 gene (CYP2D6) in poor metabolizers of debrisoquine. Study of the functional significance of individual mutations by expression of chimeric genes." J Biol Chem **265**(28): 17209-17214.

Kalow, W., B. K. Tang, et al. (1998). "Hypothesis: comparisons of inter- and intra-individual variations can substitute for twin studies in drug research." Pharmacogenetics **8**(4): 283-289.

Kamal, A. H., A. Tefferi, et al. (2007). "How to interpret and pursue an abnormal prothrombin time, activated partial thromboplastin time, and bleeding time in adults." Mayo Clin Proc **82**(7): 864-873.

Kamao, M., Y. Suhara, et al. (2005). "Determination of plasma Vitamin K by high-performance liquid chromatography with fluorescence detection using Vitamin K analogs as internal standards." J Chromatogr B Analyt Technol Biomed Life Sci **816**(1-2): 41-48.

Kamath, S., A. D. Blann, et al. (2001). "Platelet activation: assessment and quantification." Eur Heart J **22**(17): 1561-1571.

Karl, J. P., X. Fu, et al. (2014). "Quantification of phylloquinone and menaquinones in feces, serum, and food by high-performance liquid chromatography-mass spectrometry." J Chromatogr B Analyt Technol Biomed Life Sci **963**: 128-133.

Katayama, M., M. Handa, et al. (1993). "Soluble P-selectin is present in normal circulation and its plasma level is elevated in patients with thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome." Br J Haematol **84**(4): 702-710.

Kayata, S., C. Kindberg, et al. (1989). "Vitamin K1 and K2 in infant human liver." J Pediatr Gastroenterol Nutr **8**(3): 304-307.

Kazui, M., Y. Nishiya, et al. (2010). "Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite." Drug Metab Dispos **38**(1): 92-99.

Khairallah, R. J., J. Kim, et al. (2012). "Improved mitochondrial function with diet-induced increase in either docosahexaenoic acid or arachidonic acid in membrane phospholipids." PLoS One **7**(3): e34402.

Kimmel, S. E., Z. Chen, et al. (2007). "The influence of patient adherence on anticoagulation control with warfarin: results from the International Normalized Ratio Adherence and Genetics (IN-RANGE) Study." Arch Intern Med **167**(3): 229-235.

Kindberg, C., J. W. Suttie, et al. (1987). "Menaquinone production and utilization in germ-free rats after inoculation with specific organisms." J Nutr **117**(6): 1032-1035.

Kishi, K., T. Sonomura, et al. (1992). "Time courses of PIVKA-II and AFP levels after hepatic artery embolization and hepatic artery infusion against hepatocellular carcinoma: relation between the time course and tumor necrosis." Radiat Med **10**(5): 189-195.

Kobzar, G., V. Mardla, et al. (2001). "Comparison of anti-aggregatory effects of PGI2, PGI3 and iloprost on human and rabbit platelets." Cell Physiol Biochem **11**(5): 279-284.

Kraft, R. A., A. H. Jahren, et al. (2008). "Clinical-scale investigation of stable isotopes in human blood: delta13C and delta15N from 406 patients at the Johns Hopkins Medical Institutions." Rapid Commun Mass Spectrom **22**(22): 3683-3692.

Kuhnlein, H. V., O. Receveur, et al. (2004). "Arctic indigenous peoples experience the nutrition transition with changing dietary patterns and obesity." J Nutr **134**(6): 1447-1453.

Kumler, I., M. K. Tuxen, et al. (2014). "A systematic review of dual targeting in HER2-positive breast cancer." Cancer Treat Rev **40**(2): 259-270.

Lambert, W. E., L. Vanneste, et al. (1992). "Enzymatic sample hydrolysis and HPLC in a study of phylloquinone concentration in human milk." Clin Chem **38**(9): 1743-1748.

Leaf, A., J. X. Kang, et al. (2003). "Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils." Circulation **107**(21): 2646-2652.

Lee, W., H. J. Chung, et al. (2010). "PIVKA-II is a candidate marker for monitoring the effects of the oral anticoagulant warfarin." Clin Biochem **43**(13-14): 1177-1179.

Lemaitre, R. N., I. B. King, et al. (2003). "n-3 Polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study." Am J Clin Nutr **77**(2): 319-325.

Leray, C., M. L. Wiesel, et al. (2001). "Long-chain n-3 fatty acids specifically affect rat coagulation factors dependent on vitamin K: relation to peroxidative stress." Arterioscler Thromb Vasc Biol **21**(3): 459-465.

Liggett, S. B., S. Cresci, et al. (2008). "A GRK5 polymorphism that inhibits beta-adrenergic receptor signaling is protective in heart failure." Nat Med **14**(5): 510-517.

Limdi, N. A., D. K. Arnett, et al. (2008). "Influence of CYP2C9 and VKORC1 on warfarin dose, anticoagulation attainment and maintenance among European-Americans and African-Americans." Pharmacogenomics **9**(5): 511-526.

Limdi, N. A., T. M. Beasley, et al. (2008). "VKORC1 polymorphisms, haplotypes and haplotype groups on warfarin dose among African-Americans and European-Americans." Pharmacogenomics **9**(10): 1445-1458.

Limdi, N. A., M. Wadelius, et al. (2010). "Warfarin pharmacogenetics: a single VKORC1 polymorphism is predictive of dose across 3 racial groups." Blood **115**(18): 3827-3834.

Lins, R., J. Broekhuysen, et al. (1999). "Pharmacokinetic profile of 14C-labeled clopidogrel." Semin Thromb Hemost **25 Suppl 2**: 29-33.

Lippi, G., E. J. Favaloro, et al. (2011). "Inherited and acquired factor V deficiency." Blood Coagul Fibrinolysis **22**(3): 160-166.

Lipsky, J. J. (1988). "Antibiotic-associated hypoprothrombinaemia." J Antimicrob Chemother **21**(3): 281-300.

Mahgoub, A., J. R. Idle, et al. (1977). "Polymorphic hydroxylation of Debrisoquine in man." Lancet **2**(8038): 584-586.

Man, M., M. Farmen, et al. (2010). "Genetic variation in metabolizing enzyme and transporter genes: comprehensive assessment in 3 major East Asian subpopulations with comparison to Caucasians and Africans." J Clin Pharmacol **50**(8): 929-940.

Mao, Y., L. Lei, et al. (2014). "Regulators of G protein signaling are up-regulated in aspirin-resistant platelets from patients with metabolic syndrome." Pharmazie **69**(5): 371-373.

Marckmann, P., E. M. Bladbjerg, et al. (1997). "Dietary fish oil (4 g daily) and cardiovascular risk markers in healthy men." Arterioscler Thromb Vasc Biol **17**(12): 3384-3391.

Marlar, R. A., A. J. Kleiss, et al. (1982). "An alternative extrinsic pathway of human blood coagulation." Blood **60**(6): 1353-1358.

Martin, M. A. and D. L. Kroetz (2013). "Abacavir pharmacogenetics--from initial reports to standard of care." Pharmacotherapy **33**(7): 765-775.

Martius, C. (1961). "The metabolic relationships between the different K vitamins and the synthesis of the ubiquinones." Am J Clin Nutr **9(4)Pt 2**: 97-103.

McClaskey, E. M. and E. L. Michalets (2007). "Subdural hematoma after a fall in an elderly patient taking high-dose omega-3 fatty acids with warfarin and aspirin: case report and review of the literature." Pharmacotherapy **27**(1): 152-160.

McDonald, M. G., M. J. Rieder, et al. (2009). "CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant." Mol Pharmacol **75**(6): 1337-1346.

McEwen, B. J., M. C. Morel-Kopp, et al. (2013). "Effects of omega-3 polyunsaturated fatty acids on platelet function in healthy subjects and subjects with cardiovascular disease." Semin Thromb Hemost **39**(1): 25-32.

McLaughlin, J. B., J. P. Middaugh, et al. (2004). "Changing patterns of risk factors and mortality for coronary heart disease among Alaska Natives, 1979-2002." JAMA **291**(21): 2545-2546.

Meguro, T. and K. Yamada (1982). "A simple and rapid test for PIVKA-II in plasma." Thromb Res **25**(1-2): 109-114.

Meyer, U. A. and U. M. Zanger (1997). "Molecular mechanisms of genetic polymorphisms of drug metabolism." Annu Rev Pharmacol Toxicol **37**: 269-296.

Middaugh, J. P. (1990). "Cardiovascular deaths among Alaskan Natives, 1980-86." Am J Public Health **80**(3): 282-285.

Miskad, U. A., Y. Yano, et al. (2001). "Histological study of PIVKA-II expression in hepatocellular carcinoma and adenomatous hyperplasia." Pathol Int **51**(12): 916-922.

Mitchell, J. A., F. Ali, et al. (2008). "Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium." Exp Physiol **93**(1): 141-147.

Moertl, D., R. Berger, et al. (2011). "Dose-dependent decrease of platelet activation and tissue factor by omega-3 polyunsaturated fatty acids in patients with advanced chronic heart failure." Thromb Haemost **106**(3): 457-465.

Mohammadi, E., M. Rafraf, et al. (2012). "Effects of omega-3 fatty acids supplementation on serum adiponectin levels and some metabolic risk factors in women with polycystic ovary syndrome." Asia Pac J Clin Nutr **21**(4): 511-518.

Mohatt, G. V., R. Plaetke, et al. (2007). "The Center for Alaska Native Health Research Study: a community-based participatory research study of obesity and chronic disease-related protective and risk factors." Int J Circumpolar Health **66**(1): 8-18.

Murphy, N. J., C. D. Schraer, et al. (1995). "Dietary change and obesity associated with glucose intolerance in Alaska Natives." J Am Diet Assoc **95**(6): 676-682.

Nakagawa, K., Y. Hirota, et al. (2010). "Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme." Nature **468**(7320): 117-121.

Nanda, N., M. Bao, et al. (2005). "Platelet endothelial aggregation receptor 1 (PEAR1), a novel epidermal growth factor repeat-containing transmembrane receptor, participates in platelet contact-induced activation." J Biol Chem **280**(26): 24680-24689.

Nash, S. H., A. Bersamin, et al. (2012). "Stable nitrogen and carbon isotope ratios indicate traditional and market food intake in an indigenous circumpolar population." J Nutr **142**(1): 84-90.

Nebert, D. W. (1999). "Pharmacogenetics and pharmacogenomics: why is this relevant to the clinical geneticist?" Clin Genet **56**(4): 247-258.

Newman, W. P., J. P. Middaugh, et al. (1993). "Atherosclerosis in Alaska Natives and non-natives." Lancet **341**(8852): 1056-1057.

Nieuwenhuys, C. M., M. A. Feijge, et al. (2001). "Vitamin K-dependent and vitamin K-independent hypocoagulant effects of dietary fish oil in rats." Thromb Res **104**(2): 137-147.

Nishimaki, J., K. Miyazawa, et al. (1999). "Vitamin K2 induces apoptosis of a novel cell line established from a patient with myelodysplastic syndrome in blastic transformation." Leukemia **13**(9): 1399-1405.

O'Brien, D. M., A. R. Kristal, et al. (2009). "Red blood cell delta15N: a novel biomarker of dietary eicosapentaenoic acid and docosahexaenoic acid intake." Am J Clin Nutr **89**(3): 913-919.

O'Reilly, R. A. and P. M. Aggeler (1965). "Coumarin anticoagulant drugs: hereditary resistance in man." Fed Proc **24**(6): 1266-1273.

Ohhira, M., T. Ohtake, et al. (1999). "Increase of serum des-gamma-carboxy prothrombin in alcoholic liver disease without hepatocellular carcinoma." Alcohol Clin Exp Res **23**(4 Suppl): 67S-70S.

Okano, T., K. Nakagawa, et al. (2009). "[In vivo metabolism of vitamin K: in relation to the conversion of vitamin K1 to MK-4]." Clin Calcium **19**(12): 1779-1787.

Okano, T., Y. Shimomura, et al. (2008). "Conversion of phylloquinone (Vitamin K1) into menaquinone-4 (Vitamin K2) in mice: two possible routes for menaquinone-4 accumulation in cerebra of mice." J Biol Chem **283**(17): 11270-11279.

Olson, R. E. (1984). "The function and metabolism of vitamin K." Annu Rev Nutr **4**: 281-337.

Oostende, C., J. R. Widhalm, et al. (2008). "Detection and quantification of vitamin K(1) quinol in leaf tissues." Phytochemistry **69**(13): 2457-2462.

Oosthuizen, W., H. H. Vorster, et al. (1994). "Both fish oil and olive oil lowered plasma fibrinogen in women with high baseline fibrinogen levels." Thromb Haemost **72**(4): 557-562.

Oshiro, Y., Y. Takada, et al. (2004). "A resected case of metachronous liver metastasis from lung cancer producing alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II)." Hepatogastroenterology **51**(58): 1144-1147.

Ottman, R. (1996). "Gene-environment interaction: definitions and study designs." Prev Med **25**(6): 764-770.

Pacheco, C. M., S. M. Daley, et al. (2013). "Moving forward: breaking the cycle of mistrust between American Indians and researchers." Am J Public Health **103**(12): 2152-2159.

Paroni, R., E. M. Faioni, et al. (2009). "Determination of vitamin K1 in plasma by solid phase extraction and HPLC with fluorescence detection." J Chromatogr B Analyt Technol Biomed Life Sci **877**(3): 351-354.

Parrish, D. B. (1980). "Determination of vitamin K in foods: a review." Crit Rev Food Sci Nutr **13**(4): 337-352.

Peters, A. L. and C. J. Van Noorden (2009). "Glucose-6-phosphate dehydrogenase deficiency and malaria: cytochemical detection of heterozygous G6PD deficiency in women." J Histochem Cytochem **57**(11): 1003-1011.

Plourde, M. and S. C. Cunnane (2007). "Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements." Appl Physiol Nutr Metab **32**(4): 619-634.

Projahn, D. and R. R. Koenen (2012). "Platelets: key players in vascular inflammation." J Leukoc Biol **92**(6): 1167-1175.

Quinsey, N. S., A. L. Greedy, et al. (2004). "Antithrombin: in control of coagulation." Int J Biochem Cell Biol **36**(3): 386-389.

Raber, M. N. (1990). Coagulation Tests. Clinical Methods: The History, Physical, and Laboratory Examinations. H. K. Walker, W. D. Hall and J. W. Hurst. Boston.

Ramotar, K., J. M. Conly, et al. (1984). "Production of menaquinones by intestinal anaerobes." J Infect Dis **150**(2): 213-218.

Rand, M. L., R. Leung, et al. (2003). "Platelet function assays." Transfus Apher Sci **28**(3): 307-317.

Rettie, A. E. and G. Tai (2006). "The pharmocogenomics of warfarin: closing in on personalized medicine." Mol Interv **6**(4): 223-227.

Rice, P. J., R. J. Perry, et al. (2003). "Antibacterial prescribing and warfarin: a review." Br Dent J **194**(8): 411-415.

Ridker, P. M., J. E. Buring, et al. (2001). "Soluble P-selectin and the risk of future cardiovascular events." Circulation **103**(4): 491-495.

Rieder, M. J., A. P. Reiner, et al. (2005). "Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose." N Engl J Med **352**(22): 2285-2293.

Ryman, T. K., M. A. Austin, et al. (2014). "Using exploratory factor analysis of FFQ data to identify dietary patterns among Yup'ik people." Public Health Nutr **17**(3): 510-518.

Sadowski, J. A., S. J. Hood, et al. (1989). "Phylloquinone in plasma from elderly and young adults: factors influencing its concentration." Am J Clin Nutr **50**(1): 100-108.

Sadowski, J. A. and J. W. Suttie (1974). "Mechanism of action of coumarins. Significance of vitamin K epoxide." Biochemistry **13**(18): 3696-3699.

Samer, C. F., K. I. Lorenzini, et al. (2013). "Applications of CYP450 testing in the clinical setting." Mol Diagn Ther **17**(3): 165-184.

Sato, T., R. Ozaki, et al. (2003). "The biological activity and tissue distribution of 2',3'-dihydrophylloquinone in rats." Biochim Biophys Acta **1622**(3): 145-150.

Schaller, J. and S. S. Gerber (2011). "The plasmin-antiplasmin system: structural and functional aspects." Cell Mol Life Sci **68**(5): 785-801.

Schneider, D. J., R. M. Hardison, et al. (2009). "Association between increased platelet P-selectin expression and obesity in patients with type 2 diabetes: a BARI 2D (Bypass Angioplasty Revascularization Investigation 2 Diabetes) substudy." Diabetes Care **32**(5): 944-949.

Schurgers, L. J. and C. Vermeer (2000). "Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations." Haemostasis **30**(6): 298-307.

Schurgers, L. J. and C. Vermeer (2002). "Differential lipoprotein transport pathways of K-vitamins in healthy subjects." Biochim Biophys Acta **1570**(1): 27-32.

Serhan, C. N., S. Hong, et al. (2002). "Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals." J Exp Med **196**(8): 1025-1037.

Shahar, E., A. R. Folsom, et al. (1993). "Associations of fish intake and dietary n-3 polyunsaturated fatty acids with a hypocoagulable profile. The Atherosclerosis Risk in Communities (ARIC) Study." Arterioscler Thromb **13**(8): 1205-1212.

Shearer, M. J. (1995). "Vitamin K." Lancet **345**(8944): 229-234.

Shearer, M. J., A. McBurney, et al. (1974). "Studies on the absorption and metabolism of phylloquinone (vitamin K1) in man." Vitam Horm **32**: 513-542.

Shearer, M. J. and P. Newman (2008). "Metabolism and cell biology of vitamin K." Thromb Haemost **100**(4): 530-547.

Simopoulos, A. P. (1999). "Essential fatty acids in health and chronic disease." Am J Clin Nutr **70**(3 Suppl): 560S-569S.

Skoda, R. C., F. J. Gonzalez, et al. (1988). "Two mutant alleles of the human cytochrome P-450db1 gene (P450C2D1) associated with genetically deficient metabolism of debrisoquine and other drugs." Proc Natl Acad Sci U S A **85**(14): 5240-5243.

Smith, P., H. Arnesen, et al. (1989). "Influence of highly concentrated n-3 fatty acids on serum lipids and hemostatic variables in survivors of myocardial infarction receiving either oral anticoagulants or matching placebo." Thromb Res **53**(5): 467-474.

Sokoll, L. J., S. L. Booth, et al. (1997). "Changes in serum osteocalcin, plasma phylloquinone, and urinary gamma-carboxyglutamic acid in response to altered intakes of dietary phylloquinone in human subjects." Am J Clin Nutr **65**(3): 779-784.

Sommer, P. and M. Kofler (1966). "Physicochemical properties and methods of analysis of phylloquinones, menaquinones, ubiquinones, plastoquinones, menadione, and related compounds." Vitam Horm **24**: 349-399.

Song, Q., A. Wen, et al. (2008). "HPLC-APCI-MS for the determination of vitamin K(1) in human plasma: method and clinical application." J Chromatogr B Analyt Technol Biomed Life Sci **875**(2): 541-545.

Sontag, T. J. and R. S. Parker (2002). "Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status." J Biol Chem **277**(28): 25290-25296.

Spreafico, M. and F. Peyvandi (2009). "Combined Factor V and Factor VIII Deficiency." Semin Thromb Hemost **35**(4): 390-399.

Stafford, D. W. (2005). "The vitamin K cycle." J Thromb Haemost **3**(8): 1873-1878.

Steward, D. J., R. L. Haining, et al. (1997). "Genetic association between sensitivity to warfarin and expression of CYP2C9*3." Pharmacogenetics **7**(5): 361-367.

Stillwell, W., S. R. Shaikh, et al. (2005). "Docosahexaenoic acid affects cell signaling by altering lipid rafts." Reprod Nutr Dev **45**(5): 559-579.

Stillwell, W. and S. R. Wassall (2003). "Docosahexaenoic acid: membrane properties of a unique fatty acid." Chem Phys Lipids **126**(1): 1-27.

Suhara, Y., N. Hanada, et al. (2012). "Structure-activity relationship of novel menaquinone-4 analogues: modification of the side chain affects their biological activities." J Med Chem **55**(4): 1553-1558.

Suhara, Y., M. Kamao, et al. (2005). "Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry." Anal Chem **77**(3): 757-763.

Suttie, J. W., L. L. Mummah-Schendel, et al. (1988). "Vitamin K deficiency from dietary vitamin K restriction in humans." Am J Clin Nutr **47**(3): 475-480.

Takahashi, H., G. R. Wilkinson, et al. (2006). "Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans." Pharmacogenet Genomics **16**(2): 101-110.

Tans, G., G. A. Nicolaes, et al. (1997). "Regulation of thrombin formation by activated protein C: effect of the factor V Leiden mutation." Semin Hematol **34**(3): 244-255.

Thijssen, H. H., L. M. Vervoort, et al. (2006). "Menadione is a metabolite of oral vitamin K." Br J Nutr **95**(2): 260-266.

Thorisson, G. A., A. V. Smith, et al. (2005). "The International HapMap Project Web site." Genome Res **15**(11): 1592-1593.

Usui, Y., H. Tanimura, et al. (1990). "Vitamin K concentrations in the plasma and liver of surgical patients." Am J Clin Nutr **51**(5): 846-852.

Vandenbroucke, J. P., T. Koster, et al. (1994). "Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation." Lancet **344**(8935): 1453-1457.

Vanschoonbeek, K., M. A. Feijge, et al. (2004). "Variable hypocoagulant effect of fish oil intake in humans: modulation of fibrinogen level and thrombin generation." Arterioscler Thromb Vasc Biol **24**(9): 1734-1740.

Vanscoy, G. J. and J. R. Krause (1991). "Warfarin and the international normalized ratio: reducing interlaboratory effects." DICP **25**(11): 1190-1192.

Vecka, M., M. Dusejovska, et al. (2012). "N-3 polyunsaturated fatty acids in the treatment of atherogenic dyslipidemia." Neuro Endocrinol Lett **33 Suppl 2**: 87-92.

Veiga, R. V. and R. E. Taylor (1986). "Beta blockers, hypertension, and blacks--is the answer really in?" J Natl Med Assoc **78**(9): 851-856.

Ventola, C. L. (2011). "Pharmacogenomics in clinical practice: reality and expectations." P T **36**(7): 412-450.

Vernaglione, L., C. Cristofano, et al. (2008). "Omega-3 polyunsaturated fatty acids and proxies of cardiovascular disease in hemodialysis: a prospective cohort study." J Nephrol **21**(1): 99-105.

Vogenberg, F. R., C. I. Barash, et al. (2010). "Personalized medicine: part 2: ethical, legal, and regulatory issues." P T **35**(11): 624-642.

Wadelius, M., L. Y. Chen, et al. (2005). "Common VKORC1 and GGCX polymorphisms associated with warfarin dose." Pharmacogenomics J **5**(4): 262-270.

Walsh, P. N. and S. S. Ahmad (2002). "Proteases in blood clotting." Essays Biochem **38**: 95-111.

Wang, L. Y., C. J. Bates, et al. (2004). "Determination of phylloquinone (vitamin K1) in plasma and serum by HPLC with fluorescence detection." Clin Chim Acta **347**(1-2): 199-207.

Wilkinson, M. J., Y. Yai, et al. (2007). "Age-related variation in red blood cell stable isotope ratios (delta13C and delta15N) from two Yupik villages in southwest Alaska: a pilot study." Int J Circumpolar Health **66**(1): 31-41.

Wurtz, M., P. H. Nissen, et al. (2014). "Genetic Determinants of On-Aspirin Platelet Reactivity: Focus on the Influence of PEAR1." PLoS One **9**(10): e111816.

Xu, Z. H., J. R. Jiao, et al. (2012). "Aspirin resistance: clinical significance and genetic polymorphism." J Int Med Res **40**(1): 282-292.

Yamagishi, K., H. Iso, et al. (2008). "Fish, omega-3 polyunsaturated fatty acids, and mortality from cardiovascular diseases in a nationwide community-based cohort of Japanese men and women the

JACC (Japan Collaborative Cohort Study for Evaluation of Cancer Risk) Study." J Am Coll Cardiol **52**(12): 988-996.

Yim, J. M., G. W. Albers, et al. (1996). "Anticoagulant therapy monitoring with international normalized ratio at US academic health centers." Ann Pharmacother **30**(12): 1390-1395.

Zerouga, M., L. J. Jenski, et al. (1995). "Comparison of phosphatidylcholines containing one or two docosahexaenoic acyl chains on properties of phospholipid monolayers and bilayers." Biochim Biophys Acta **1236**(2): 266-272.

VITA

Nicholas Ta-Wae Au was born on April 10th, 1984 in Seattle, Washington to Roger Au and Joanna Li. He graduated from Roosevelt High School in June of 2002, and in September of that year, entered the University of Washington in Seattle as an undergraduate student. While there, he earned his Pharm.D. in June of 2009 and continued on with his graduate studies in the University of Washington Department of Medicinal Chemistry. Nicholas received the Doctor of Philosophy degree in Medicinal Chemistry in the fall of 2014.