Identification of dietary and biological factors influencing vitamin K metabolism

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Abstract

Vitamin K is an essential vitamin required for multiple physiological functions. There are two known forms of vitamin K: (1) phylloquinone, the predominant dietary source found in green leafy vegetables and vegetable oils; and (2) menaquinones, which vary in length and saturation of the side chain and are found in animal byproducts and fermented foods. Of the menaquinones, menaquinone-4 is unique in that it is endogenously produced from dietary phylloquinone. In establishing recommendations for the U.S. population, it is important to capture trends in dietary patterns and changes in consumption that may influence dietary intake. However, the large inter-individual variability in vitamin K status is not entirely explained by dietary intakes. Non-dietary factors, including age and sex, need to be addressed in order to better define current recommendations. Endogenous menaquinone-4 production is linked to cholesterol metabolism. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are potent inhibitors of endogenous cholesterol synthesis and could potentially limit the conversion of phylloquinone to menaquinone-4 in tissues. The overall objective of this thesis project was to characterize the dietary and non-dietary factors influencing vitamin K status and metabolism, with a focus on the conversion of phylloquinone to menaquinone-4.

In order to meet this objective, we examined the National Health and Nutrition Examination Survey 2011-2012, a large publically-available data set to examine usual dietary intakes of phylloquinone in U.S. adults. The usual phylloquinone intakes of adults in the U.S. were consistent with the previous report of NHANES data from 1988-1994, and vegetables continue to be the primary dietary contributors to phylloquinone intake. However, we found that the food sources of phylloquinone appear to be shifting because mixed dishes, such as macaroni and cheese, sandwiches, and pizza are the second highest contributor to phylloquinone intake. We then utilized a C57Bl6 mouse model to determine the effects of diet manipulation, sex, age, and statin treatment on vitamin K status and metabolism. Female mice had higher tissue concentrations of both phylloquinone and menaquinone-4 in response to adequate dietary phylloquinone concentrations compared to their male counterparts. Older mice had higher concentrations of phylloquinone in the liver, compared to young mice. Menaquinone-4 formation in kidney was reduced in response to atorvastatin treatment, which may be indicative of tissue-specific function. Statin treatment did not affect menaquinone-4 formation in intestine or brain.

Collectively, the findings of this thesis work indicate that dietary source, sex, age, and statins influence vitamin K status and metabolism. Characterizing factors that influence vitamin K metabolism will help identify additional functions of vitamin K, and ultimately help guide recommendations and establish potential therapeutic uses and targets of vitamin K.

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1. Introduction

The fat-soluble vitamin K is an essential cofactor for the carboxylation of vitamin K-dependent proteins that are involved in various physiological processes, including clotting, bone formation, and regulation of calcification (1). Vitamin K occurs in two forms: (a) phylloquinone, which is the predominant dietary form found in green leafy vegetables and vegetable oils (2); and (b) menaquinones, which differ structurally from phylloquinone in the length and saturation of their side chain, and are found primarily in animal-based and fermented foods (3). Whereas evidence suggests menaquinone-4 is formed endogenously from dietary phylloquinone, all other menaquinones are a product of bacterial synthesis (4). Menaquinone-4 appears to have unique properties beyond its function as an enzyme cofactor (5,6). However the contribution of menaquinones, including menaquinone-4, to human health is not well understood.

The current dietary recommendations for vitamin K are limited to Adequate Intakes (AI) set at 120 ug and 90 ug/d of dietary phylloquinone for adult men and women, respectively (7). Current recommendations are based on median self-reported dietary phylloquinone intakes for U.S. adults using National Health and Nutrition Examination Survey (NHANES) data collected in 1988-1994, and food composition databases available at that time. Menaquinones had not been systematically quantified in the food supply at the time of the development of the AI for vitamin K, and dietary contribution of menaquinones to total vitamin K intake is still not well characterized. Thus development of the AI was limited to phylloquinone, even though all forms of vitamin K function as an enzymatic cofactor. There is insufficient knowledge regarding vitamin K metabolism and bioavailability, a lack of a clinically-defined biomarker of vitamin K status, and currently no established disease outcomes linked to vitamin K insufficiency to

establish an estimated average requirement, hence a Recommended Dietary Allowance (RDA). Currently, vitamin K is the only essential fat-soluble vitamin that lacks an RDA.

In order to establish an RDA, it is critical to characterize biological variables that may influence vitamin K dietary requirements and status. Given the reliance on dietary phylloquinone intakes in establishing AI for the US population, it is important to capture trends in consumption and to identify subgroups most likely to be at risk of low vitamin K intakes. Finally, the impact of diet and other biological factors on vitamin K metabolism needs to be better characterized.

To address these gaps, usual vitamin K intakes and dietary vitamin K sources were estimated across a range of age groups in both men and women using current phylloquinone food composition data as applied to intake data obtained from the NHANES 2011-2012 cohort. To better understand the physiological relevance of vitamin K adequacy or insufficiency, and further explore sex-specific and age-specific differences in vitamin K metabolism, a mouse model was then optimized to examine the role of these biological factors in response to dietary vitamin K manipulation. This model provided an opportunity to further study the impact of biological variables on tissue-specific regulation of different vitamin K forms, including menaquinone-4. Mammals, including humans, have the capacity to convert dietary phylloquinone to menaquinone-4 in multiple tissues (8–11). Endogenous menaquinone-4 production is linked to cholesterol metabolism because it requires an isoprenoid side chain, geranylgeranyl pyrophosphate (GGPP), which is synthesized from isopentenyl pyrophosphate (IPP) an intermediate in the cholesterol synthesis pathway (12). The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are potent inhibitors of endogenous cholesterol synthesis. Therefore statins inhibit isoprenoid intermediates, including

GGPP, as well as the biosynthesis of cholesterol (13). To examine the cross talk between endogenous cholesterol synthesis and vitamin K metabolism, statins were used to antagonize menaquinone-4 formation. These studies will provide insight into the conversion of phylloquinone to menaquinone-4 and to help identify additional functions that may be unique to menaquinone-4.

In summary, the overall goal of this project was to: (a) estimate current intakes of phylloquinone in the U.S. population; (b) identify how diet, sex, and age contribute to changes in vitamin K status; and (c) better understand endogenous menaquinone-4 formation in mammals by antagonizing the formation of cholesterol precursors in mice using statins.

Hypothesis 1: U.S. adults have higher intake of phylloquinone when compared to the current Adequate Intake recommendations due to inclusion of updated food composition databases.

Specific Aims:

1.1 To estimate the amount of phylloquinone consumed in the diet of U.S. adults as a whole, and in men and women across different age groups.

1.2 To estimate the contribution of different food groups to phylloquinone intake.

1.2.1 To characterize the contribution of different food groups in individuals with high or low vegetable intake (≥ 2 cups, < 2 cups vegetables/day).

1.2.2 To characterize the contribution of different mixed dishes to phylloquinone intake.

Hypothesis 2: There are sex-specific differences in response to dietary vitamin K manipulation in a rodent model.

Specific Aims:

2.1 To identify the effect of sex and diet on circulating, tissue, and fecal vitamin K concentrations in C57Bl/6 mice during dietary vitamin K manipulation.

2.2 Characterize key enzyme expression in the vitamin K metabolic pathway in response to dietary vitamin K manipulation.

Hypothesis 3: Endogenous cholesterol synthesis is a rate-determining step for the conversion of dietary phylloquinone to menaquinone-4.

Specific Aims:

3.1 Analyze the effect of HMG-CoA reductase inhibitors in the C57Bl/6 mouse model on the conversion of dietary phylloquinone to menaquinone-4.

3.1.1 Determine the effect of a statin intervention on the conversion of dietary phylloquinone to menaquinone-4 in young (4mo) and older (20 mo) C57Bl6 mice utilizing deuterium-labeled phylloquinone as a substrate provided in the diet.

3.1.2 Examine the effect of statin intervention on the production of cholesterol precursors, including isopentenyl pyrophosphate (IPP), geranylgeranyl pyrophosphate (GGPP), and tissues concentrations of cholesterol in both young (4mo) and older (20 mo) C57Bl6 mice.

3.2 Characterize gene expression of key enzymes in vitamin K and cholesterol metabolic pathways in response to interruption of the cholesterol precursors through use of HMG-CoA reductase inhibitors.

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2. Literature Review

Vitamin K is a fat-soluble vitamin comprising multiple forms that fall into two general categories; (1) phylloquinone; and (2) menaquinones. All forms are characterized by the 2-methyl -1, 4- naphthoquinone ring. Phylloquinone contains a phytyl side chain, whereas menaquinones (MK-n) contain a polyisoprenyl side chain where n indicates the number of isoprenoid units (1–3). All known vitamin K forms share bioactivity as enzyme cofactors responsible for activating multiple vitamin K-dependent proteins (4). Emerging evidence suggests novel physiological roles for vitamin K in human health and disease (5–7). However, efforts to elucidate these roles are limited by our current understanding of vitamin K requirements and metabolism.

Vitamin K function

Vitamin K is required for post-translational modifications of glutamic acid to form γ-carboxyglutamate (gla) residues present in certain proteins (**Figure 1**). These proteins are referred to as vitamin K-dependent proteins (8). The number of vitamin K-dependent proteins identified continues to increase, and it is now evident that these proteins have important roles in physiological processes beyond coagulation, such as bone metabolism and regulation of calcification **(Table 1)** (4,8). All vitamin K forms function as an enzyme cofactor for posttranslational modification of vitamin K-dependent proteins, although their relative enzymatic activity varies in accordance with the side chain length (9–11). The active form of vitamin K required for the post-translational glutamate carboxylase (ggcx) is the reduced form (**Figure 1**). The glutamate carboxylase is responsible for the addition of a second carboxyl group to the glutamate amino acid residues, resulting in protein functionality. The vitamin K epoxide

reductase (VKOR) reduces the quinone (dietary form) of vitamin K, and recycles the oxidized 2,3-epoxide vitamin K, for the cycle to continue (2,4). VK antagonists (i.e. warfarin) target the VKOR enzyme, thus inhibiting vitamin K recycling, which reduces the amount of vitamin K available for carboxylation of coagulation factors II, VII, IX, X and protein C and S (12). Recently, a second gene, VKORC1L1, has been found to support the activity of VKOR, respond to vitamin K antagonists, and is present in extra-hepatic tissues (13). However, VKORC1L1 is unique in that it appears to be 50-fold more resistant to the antagonists, which may explain the low susceptibility of extra-hepatic tissues to vitamin K antagonists and the corresponding lack of effects on vitamin K-dependent proteins (13).

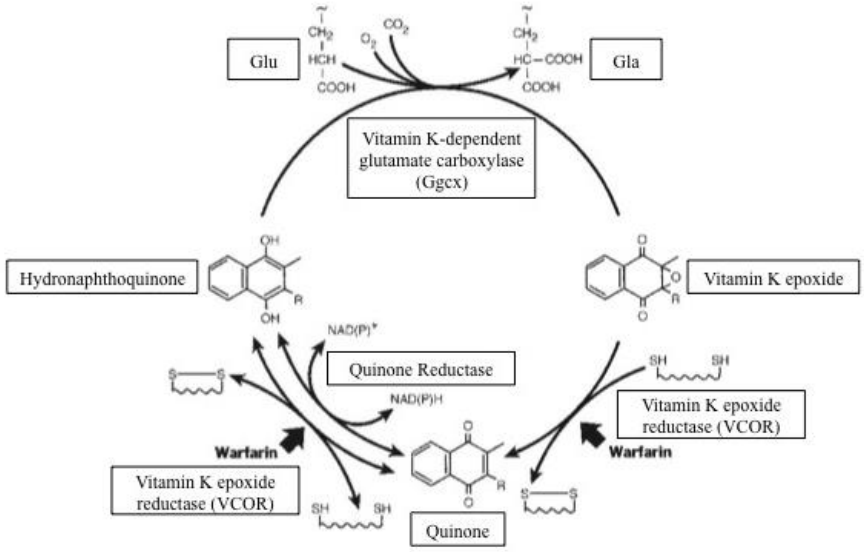

Figure 1. Vitamin K cycle. Reprinted from *Modern Nutrition in Health and Disease*, 11th edition, A. Catharine Ross et al., Figure 20.4 Tissue metabolism of vitamin K. pp. 312, Copyright (2014), with permission from Lippincott Williams & Wilkins.

Table 1. Characterized vitamin K dependent proteins.

Protein	Primary Action	Tissue Location
Prothrombin (factor II)	Pro-coagulant	Liver
Factor VII	Pro-coagulant	Liver
Factor IX	Pro-coagulant	Liver
Factor X	Pro-coagulant	Liver
Protein C	Anti-coagulant	Liver
Protein S	Anti-coagulant	Liver
Protein Z	Anti-coagulant	Liver
Osteocalcin	Bone mineralization	Bone
Periostin	Extra-cellular matrix mineralization	Bone
Matrix Gla protein	Calcification regulator	Bone, cartilage, other soft tissues
Gla-rich proteins	Calcification regulator	Universal
Gas 6	Ligand for tyrosine kinase Axl	Universal

Absorption, transport, and metabolism of vitamin K

The intestinal absorption of lipids, packaging into chylomicrons, their secretion into the lymphatic lacteals, and entry into systemic circulation that characterize lipid metabolism are also applicable to vitamin K, due to its lipophilic properties (14,15). There is more known on the metabolism of phylloquinone than menaquinones because phylloquinone is the predominant form in the diet and circulation. Interestingly, there are very few menaquinones forms in circulation except in response to supplemental doses in excess of dietary intakes (16). No specific transport proteins have been identified for vitamin K. Instead, the lipophilic nature of vitamin K forms allows them to be transported by lipoproteins. Almost all of phylloquinone is carried by triglyceride-rich lipoproteins (TRL), which is comprised of chylomicron remnants and very low-density lipoproteins (VLDL) (17–21). The transport of vitamin K forms has been demonstrated through the use of radiolabeled and stable isotopes in feeding studies. Clearance of labeled phylloquinone from plasma occurs within the first 8 to 10 hours of ingestion (18–21).

Clearance of menaquinone-4 is similar to phylloquinone, in that it occurs rapidly and is predominantly associated with the TRL. However, in contrast to phylloquinone, after 8 hours the largest proportion of menaquione-4 is associated with LDL (19). Few studies have examined the mechanisms of cellular uptake of vitamin K. The evidence is limited to *in vitro* studies, but they have demonstrated the LDL receptor (LDLR) and LDL receptor related protein 1 (LRP) play a predominate role in vitamin K uptake (22,23). Tissue specificity and distribution of vitamin K forms suggest that storage may be related to tissue-specific requirements or function (24).

All forms of vitamin K are catabolized in the liver by a common degradation pathway resulting in 5- and 7-carbon compounds that are secreted in urine as glucuronides (4,25,26). A characteristic of vitamin K metabolism is the CYP4F2 enzyme which degrades both vitamin K and other isoprenoids, including vitamin E. CYP4F2 is a cytochrome P450 enzyme that was recently identified as a candidate gene for possible determinants of circulating phylloquinone concentrations in a genome-wide association study of three community-based cohorts (27).

Clinical effects of inadequate intake

Clinically significant vitamin K deficiency results in vitamin K-responsive hypoprothrombinemia, increased prothrombin time, and in severe cases adverse bleeding events (28). Experimentally-induced, clinical vitamin K deficiency studies are extremely rare because of the ethical issues related to the risk of induced bleeding. Udall et al. (1965) fed healthy adults a diet containing approximately 10 µg of phylloquinone per day for three weeks, after which there was a significant increase in prothrombin time, but still within the normal range for optimal clotting (29). Certain populations, specifically individuals on broad-spectrum antibiotic therapy, are thought to be more prone to vitamin K deficiency. For example, patients on neomycin

receiving phylloquinone deficient intravenous fluids had increased prothrombin time that subsequently responded to phylloquinone supplementation (30). As a result of this study, they concluded that the minimal daily requirement was between 0.3 and 1.0 µg per kilogram body weight of phylloquinone, which served as the basis for the original international dietary recommendation (30,31). In more recent studies, there was no effect on prothrombin time in healthy adults fed vitamin K-restricted diets containing 5 and 10 µg phylloquinone per day for more than two weeks (32,33). Thus the limited studies conducted have demonstrated that even a significant restriction in vitamin K intake will not impair normal hemostatic control in healthy adults. What is not known is the impact of vitamin K restriction on vitamin K functions, independent of clotting.

Dietary sources of vitamin K and current recommendations

Primary sources of phylloquinone are dark green leafy vegetables (34), along with phylloquinone-rich plant oils that are incorporated into many food products (4). Phylloquinone is the predominant dietary vitamin K form, and has been the focus of most vitamin K metabolic studies to date. In contrast to phyllquinone, which is entirely plant-based and abundant in the food supply, menaquinones are found in limited animal-based foods, such as dairy and meats, as well as fermented foods (4,35,36). The food composition data for the menaquinones are incomplete and therefore it is possible there are multiple dietary sources of menaquinones that are currently not captured in current dietary assessment studies.

Vitamin K is also produced by gut bacteria, which complicates assessment of contributors to overall vitamin K status in humans. Medium and long-chain menaquinones (defined as menaquinone-6 or higher) act as electron carriers in prokaryotic respiration (1). They are synthesized by specific anaerobic bacteria, some of which occupy the human gut.

Menaquinone fecal concentrations correlate with the abundance of these bacterial species (1,4). Evidence supporting the absorption and metabolism of bacterially-synthesized menaquinones from the gut is limited. In comparison to other menaquinones, there is evidence suggesting that menaquione-4 is primarily formed from dietary phylloquinone by means of tissue-specific conversion (1,37,38). How this conversion occurs and what unique functions menaquinones may have that explain this unique tissue-specific conversion is an emerging area of research.

The current U.S. Dietary Reference Intakes for vitamin K are represented by Adequate Intake (AI) values based on our current knowledge of phylloquinone intakes only, as the menaquinone content of foods in limited and not incorporated into the recommendation. The current AI for adult men and women are 120 µg and 90 µg per day respectively (31). However, phyllquinone intake varies among age groups and geographic location (4,39,40). International dietary recommendations vary by approximately 2-fold, as some countries continue to use the original RDA of 1.0 ug phylloquinone/kilogram bodyweight. For example, the current recommendation for New Zealand, Australia, Germany, Switzerland, and Austria is 60 µg per day for women and 70 µg per day for men, which is approximately half the recommendation in the United States (35). A Recommended Dietary Allowance (RDA) cannot be established due to insufficient knowledge regarding vitamin K's bioavailability, transport, excretion, and the lack of a robust biomarker that is validated for subclinical requirements independent of clotting, which are critical to establishing dietary requirements for humans.

Factors influencing vitamin K status

Observational and intervention studies have been conducted to characterize dietary and non-dietary biological factors that influence vitamin K status in humans, including diet, sex, and age. Feeding studies have demonstrated that circulating phylloquinone, a common biomarker

used to evaluate vitamin K status, responds to dietary intake of vitamin K (41–43). With the diversification of the food supply and changes in food preparation techniques, plant-based oils have become a more noticeable food source of vitamin K (44). The complexity of food matrices and the fat content of foods influence vitamin K bioavailability. Studies have demonstrated that post-prandial circulating concentrations of phylloquinone are significantly higher after intake of phylloquinone-fortified oil or vegetables with added fat compared to vegetables alone (45,46). Jones et al. showed the bioavailability of phylloquinone in meals containing fast food and refined cereals, with lower than average intake of fruits, vegetables, and whole grains, is more than twice as high when compared to meal patterns with higher than average intake of fruits, vegetables, whole grains, fish, and dairy (47). Collectively these studies suggest that the phylloquinone obtained from a meal with phylloquinone-rich oil has greater bioavailability than phylloquinone obtained from fruits and vegetables. When considering the American diet as a whole, foods prepared with phylloquinone-rich plant oils could have the ability to contribute substantially to daily vitamin K intake given Americans' low consumption of vegetables, including phylloquinone-rich vegetables, such as dark leafy greens (48,49). As such, trends in contributors to vitamin K intake should be considered when evaluating status and defining future recommendations.

Non-dietary factors, such as age and sex, have been shown to be associated with vitamin K status, although that data are inconsistent. Self-reported phylloquinone intake varies by sex, with women reporting higher intakes compared to men (40,43,50). However, these sex-specific differences in phylloquinone intake are not reflected in circulating measures of phylloquinone (41,43). Some studies have reported lower phylloquinone intake with age, especially in adults aged > 65 years (41,51,52). In contrast, a recent report in Irish adults found a decrease in self-

reported intake among young adults ages 18-35 years compared to intakes for the same age group reported a decade before, which may be attributed to lower intake of green vegetables (40).

Animal models of vitamin K metabolism

The majority of preclinical studies in the vitamin K field rely on use of a single species and single sex rodent model, specifically the rat (53–59); few studies to date have included both male and female rats (60–62). There is evidence supporting sex-specific differences in rats, such that females are reported to be more resistant to vitamin K deficiency compared to males (63,64), which has been hypothesized to be attributable to increased coprophagy in females (65), increased vitamin K requirement in males (63,66), specific effects of sex hormones on vitamin K metabolism (67,68), and differences in the biosynthesis of menaquinones by gut bacteria and corresponding absorption (68). There is limited information available regarding the sex-specific responses to vitamin K dietary manipulation or vitamin K metabolism in mice. The C57BL6 genetic background is the most commonly used strain for transgenic models and although transgenic mouse models for studying key enzymes in vitamin K metabolism and vitamin K dependent proteins have been developed, none have compared responses between sexes (54,60,61). A recent examination of sex and diet effects in male and female C57Bl6 mice found that male and female mice respond differently to dietary phylloquinone manipulation. It is on a sufficient phylloquinone diet that female mice had significantly higher tissue concentrations, which may be attributed to the role of estrogen and potential vitamin K requirements for reproduction (62). Sex-specific differences have not been well characterized in humans, and although there is a suggestive sex effect, the data are inconsistent (69). This further supports the need to include both sexes in vitamin K studies using preclinical models.

An aging research initiative through the National Institutes of Health (NIH) has deemed the use of aging mouse models critical to expanding geroscience research and better understanding age-related chronic disease pathologies (70). Aging results in a distinctive systemic environment that may alter diet or drug efficacy that would not be observed in a younger animal. Therefore, it is critical to incorporate aging models, a strong recommendation that may increase time and cost but will yield stronger results and improve translatability to human physiology (70). The role of age on diet-induced changes in tissue concentrations of vitamin K has been investigated in rats of various ages (i.e. 3, 12, 24 months). Rats were fed different vitamin K diets for approximately one month and found that during aging, menaquinone-4 concentrations in extra-hepatic tissues (heart and kidney) of both male and female rats decreases (71). This selective change in distribution suggest a tissue-specific dynamic in metabolism that is affected not only by diet but by age. In comparison, some studies have examined the role of lifetime dietary vitamin K exposure on tissue concentrations of vitamin K in an aging rat model. In this study, it was found that the ratio of menaquinone-4 to total vitamin K concentration increased in older age, which may reflect an increased demand for menaquinone-4 (72). Additionally, despite high lifelong dietary phylloquinone intake, tissue concentrations of menaquinone-4 at 20 months were lower (72). The decrease in menaquinone-4 may be indicative of reduced conversion from dietary phylloquinone or increased utilization. More extensive animal studies incorporating both sex and age into study design to characterize differences in metabolism will help identify mechanisms modulating tissue-specific requirements, and later clarify sex and age related differences of vitamin K status in humans.

Diet intervention studies in rodent models including rats and mice have been used to better examine vitamin K metabolism. Whereas vitamin K is primarily obtained in the

phyllquinone form in the human diet, the two primary forms used in domestic and laboratory chow are phyllquinone or menadione (73). The current recommendation for rodents is 1.0mg phyllquinone /kg diet (73). Adequacy is commonly defined as the amount of vitamin K needed to maintain normal levels of vitamin K dependent clotting factors (73). Bleeding is the sign of frank deficiency and can occur in a rodent within 9 to 21 days of exposure to a vitamin K free diet (74). Although the recommended form of vitamin K is phyllquinone, the more common form in rodent diets is menadione, which is a synthetic derivative that lacks the aliphatic side chain and acts as a pro-vitamin. Due to the lower cost, water-solubility, and diet stability, it is often used in rodent diets. Absorption of phylloquinone occurs in the proximal intestine through the formation of chylomicrons in the intestine which enter lymphatic circulation and ultimately phylloquinone accumulation in the liver (73). In contrast, menadione is extremely polar resulting in efficient and passive absorption, direct entry into circulation, and rapid metabolism. Additionally, this small compound can pass the blood brain barrier, whereas phylloquinone cannot (73,75). These differences between phylloquinone and menadione absorption and transport have implications when studying the effect of vitamin K in rodent models. Not only are they metabolized differently, menadione has limited value in preclinical absorption studies because of its extreme toxicity when administered in high doses (76), and lack of translatability to vitamin K forms found in the human diet.

Stable isotopes used in human feeding studies have been successfully applied to animal models. The use of isotopes allows us to observe the absorption and utilization of dietary phylloquinone, and subsequent conversion to menaquinone-4 to mimic the human condition. In a male rat model using a phylloquinone and menadione-free diet supplemented with deuterium-labeled phylloquinone (37,77), labeled menaquinone-4 was detected in various tissues

demonstrating the dietary phylloquinone was the primary labeled source of menaquinone-4 (37,77). The selective tissue distribution and production of labeled menaquinone-4 suggests a specific function of menaquinone-4 in extra-hepatic tissues that extends beyond the well-established function of vitamin K as an enzyme cofactor. Okano et al. (78) also utilized stable isotopes to directly measure conversion of phylloquinone to menaquinone-4 in the mouse cerebra. Menaquinone-4 was reportedly much higher than phylloquinone in all tissues in mice, which is inconsistent with the other rodent studies in which there was a distinct tissue-specific distribution of menaquinone-4. Unfortunately these authors fed a normal chow diet and did not take the presence of MD in the chow into account in their interpretation of their results. Hence, the labeled dietary phylloquinone contributed to the tissue concentrations of menaquinone-4, but due to the presence of MD in the chow diet, MD was continually being utilized in the conversion of menaquinone-4, which resulted in high menaquinone-4 concentrations in liver, which is not observed when the animals are exclusively fed phylloquinone. Animal models have been utilized to better understand vitamin K metabolism and tissue specific properties, but the breadth of diet manipulations, variations in vitamin K source and concentration, have resulted in difficulties interpreting and/or replicating studies.

Conversion of dietary phylloquinone and menadione to menaquinone-4

A theory of enzymatic conversion of dietary phylloquinone to endogenous menaquinone-4 has been proposed (79,80). According to this theory, the phytyl side chain of phylloquinone is removed and a geranylgeranyl pyrophosphate (GGPP) side chain is donated to form menaquinone-4. *Ubiad1* was identified as a human prenyltransferase enzyme responsible for the geranylgeranyl side chain addition step of the conversion process from phylloquinone to menaquinone-4 (80). Although *ubiad1* was originally proposed to function as the enzyme

responsible for both steps in the conversion, subsequent evidence regarding its role as the enzyme responsible for the phytyl side chain removal have not been forthcoming. However, the characterization of *ubiad1* as a key enzyme in vitamin K metabolism and the unique tissue distribution of menaquinone-4 have led to the question of whether menaquinone-4 is locally synthesized from phylloquinone, or if it is transported to the target tissue f such as the liver or intestine as a precursor (ie menadione) or in the form of menaquinone-4 (81).

To test the hypothesis that the side chain is removed and replaced, a rodent study was conducted that administered tritium labeled phylloquinone, followed by measurement in extra-hepatic tissues. These tissues were found to contain unlabeled phylloquinone primarily from the diet, in addition to labeled menaquinone-4 on the napthoquinone ring with an unlabeled side chain (82). These data confirm that phylloquinone is converted to menaquinone-4 through side chain cleavage and GGPP addition (82). Additional studies in rodent models have demonstrated tissue specific conversion of dietary phylloquinone to menaquinone-4 through the use of deuterium-labeled phylloquinone. Okano et al. (78) used deuterium-labeled phylloquinone carrying the label on the naphthoquinone ring but not the phytyl side chain. The labeled phylloquinone was given orally to mice, and the naphthoquinone ring-labeled menaquinone-4 was detected in brain. Furthermore, when mice were orally fed deuterium-labeled phylloquinone on both the ring and the side chain, there was no detectable menaquinone-4 labeled on both the ring and the geranylgeranyl side chain, which indicates that the phytyl side chain of phylloquinone is replaced by geranylgeranyl side chain to produce menaquinone-4 (78).

The current theory supports a two-step reaction in the endogenous production of menaquinone-4, which results in the naphthoquinone ring intermediate, menadione. Due to previous limitations and inability to measure menadione, justification for the two-step reaction

was incomplete. In a study by Al Rajabi et al. (37), deuterium-labeled collard greens were provided as the source of labeled phylloquinone to male Fischer 344 rats. Labeled phylloquinone and labeled menaquinone-4 were detected in tissues within 7 days of intake. The molecular weight of the labeled menaquinone-4 indicated that the menaquinone-4 carried the deuterium label on the naphthoquinone ring, but not on the side chain, which confirms side chain removal for menaquinone-4 formation. In addition, labeled menadione originating from labeled phylloquinone was detected in serum and urine, providing evidence that menadione is an intermediate in the conversion of dietary phylloquinone to tissue menaquinone-4 (37).

Manipulating the vitamin K forms in the diet has also demonstrated that the conversion of phylloquinone to menaquinone-4 is a unidirectional reaction. In rodent models provided menaquinone-4 as the primary form of vitamin K in the diet, there was no phylloquinone detected in serum or liver (24,83). In addition, altering the concentrations of menaquinone-4 provided in the diet does not alter tissue concentrations of menaquinone-4, further supporting the hypothesis that conversion begins with isolation of the naphthoquinone ring derived from dietary phylloquinone or provided as menadione (83).

These findings demonstrate that mammals are capable of converting phylloquinone and menadione to menaquinone-4, and that menaquinone-4 is the preferred form in certain tissues. These studies also support the hypothesis that the conversion requires removal of the phytyl side chain on phylloquinone and addition of the geranylgeranyl side chain to the menadione intermediate producing menaquinone-4. In addition, these results demonstrate that the accumulation of menaquinone-4 occurs primarily in extra-hepatic tissues.

There is abundant evidence for the origin of the napthoquinone ring during the endogenous production of menaquinone-4. What is lacking is the evidence for the origin of the side chain added during synthesis of menaquinone-4. Specifically, that menaquinone-4 requires the isoprenoid side chain GGPP, which is synthesized from isopentenyl pyrophosphate (IPP), an intermediate in the endogenous cholesterol synthesis pathway (**Figure 2**). Briefly, the pathway converts acetyl-CoA to mevalonate and then to IPP via a number of unidirectional enzymatic steps (84). A rate-determining step in the pathways is the conversion of hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate via HMG-CoA reductase. HMG-CoA reductase inhibitors (statins) deplete cells of mevalonate-derived isoprenoid compounds, including GGPP (84,85). Therefore, HMG-CoA reductase inhibitors could potentially inhibit menaquinone-4 synthesis by depleting cells of GGPP, one of the two substrates required for menaquinone-4 formation. As statins are often utilized in preclinical models, they could become an effective tool to examine the interaction between vitamin K metabolism and endogenous cholesterol synthesis to advance our knowledge on menaquinone-4 production and function.

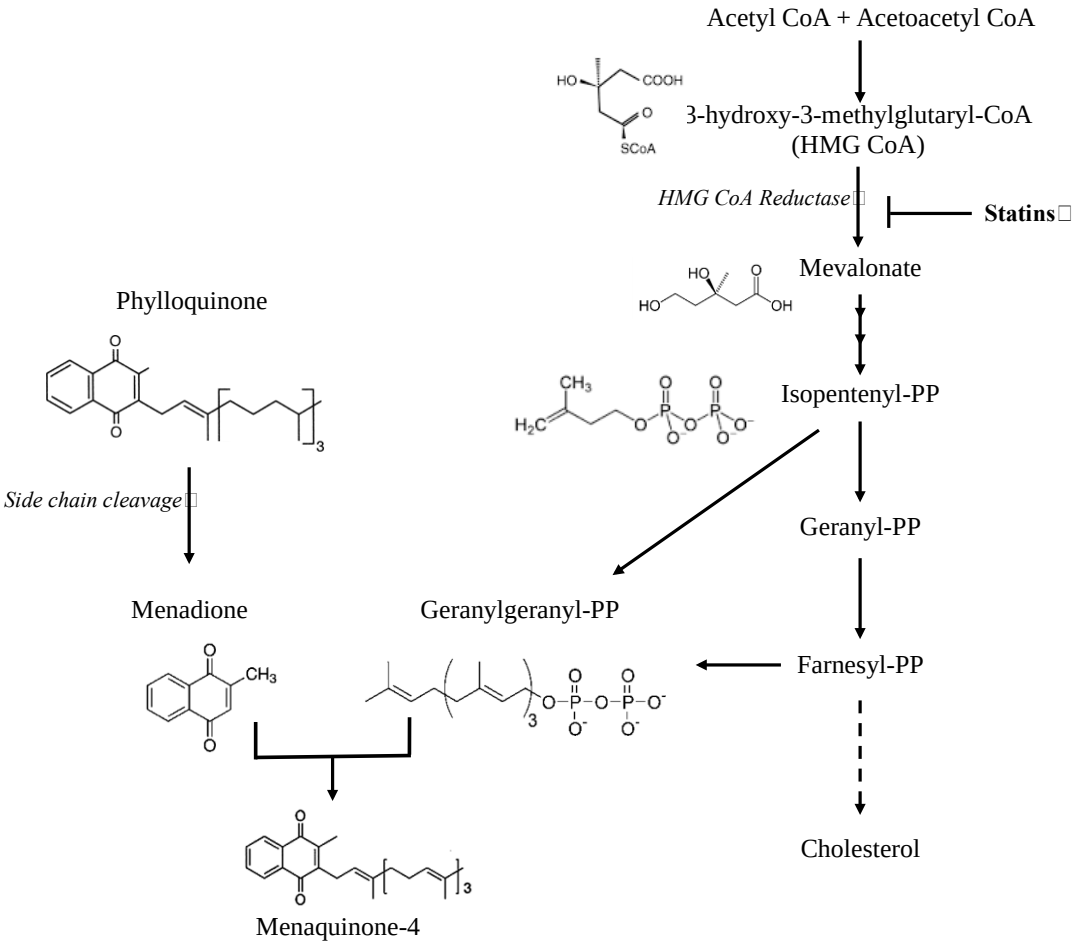

Figure 2. Menaquinone-4 biosynthesis pathway.

To date, there are limited indications of the mechanisms whereby specific tissues such as brain, pancreas, and kidney accumulate menaquinone-4, or why the accumulation varies in response to diet manipulation and pharmacological interventions. There is evidence to suggest that menaquinone-4 has roles in cellular functions that are independent of its role as an enzyme cofactor (86–90). In certain tissues of mice, rats, and humans, the concentration of menaquinone-4 is several fold higher than that of phylloquinone (24,62,91,92). Further examination of the

conversion of phylloquinone to menaquinone-4 is necessary to identify the mechanism of conversion, factors that influence the conversion, and potential regulation of conversion.

Clinical implications

Further exploration into the alternative actions of vitamin K and vitamin K-dependent proteins have resulted in several hypotheses that link vitamin K to age-related chronic diseases, including cardiovascular disease (93–96), osteoarthritis (7,97–99), kidney disease (100–102), and coronary artery calcification (103–105). The mechanisms by which vitamin K or vitamin K-dependent proteins modulate disease progression are unclear. Vitamin K has been shown to have anti-inflammatory effects (106,107), which may be indicative of its role in age-relate chronic disease progression (108–112). However the role of different forms of vitamin K in various disease states is unknown. Recent evidence suggests an association between calcification and menaquinone-4 formation in the kidney. The kidney is a site of conversion and responds to dietary manipulation (62,91). These observations suggest the conversion of phylloquinone to menaquinone-4 in the kidney is a regulated process. In a rat model of chronic kidney disease, animals with apparent chronic kidney disease have significantly higher kidney menaquinone-4 concentrations. These higher concentrations may be indicative of changes in the efficiency of conversion of phylloquinone to menaquinone-4 in chronic kidney disease, or that utilization of menaquinone-4 may be impaired in the disease state, further exacerbating the condition (91). More recently, a few studies have found a relationship between vitamin K and kidney disease, but the mechanism behind this relationship is unclear (101, 113). Further studies are warranted to confirm the role of vitamin K and whether it translates to modifying chronic kidney disease progression.

The work detailed in this thesis directly addresses the need to better understand the contribution of dietary and non-dietary factors influence dietary vitamin K intake, vitamin K status, and the conversion of phylloquinone to menaquinone-4. The knowledge garnered will extend the current knowledge base, inform the design of future studies on vitamin K status and metabolism, and aid in identification of alternative functions of vitamin K.

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Manuscripts

3. Mixed dishes are a top contributor to phylloquinone intake in U.S. adults: data from the 2011-2012 NHANES

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4. Abbreviations: AI: Adequate Intake; AMPM: Automated Multiple Pass Method; BMI: body mass index; CDC: Center for Disease Control; FDA: Food and Drug Administration; FNDDS: Food and Nutrient Database of Dietary Studies; FPED: Food Patterns Equivalents Database; HNRCA: Human Nutrition Research Center on Aging; IQR: Interquartile Range; MEC: Mobile Examination Center; NHANES: National Health and Nutrition Examination Survey; NCHS: National Center for Health Statistics; NFNAP: National Food and Nutrient Analysis Program; USDA: United States Department of Agriculture; VKDP: vitamin K-dependent proteins;

Abstract

Background: Phylloquinone is the most abundant form of vitamin K in U.S. diets. Green vegetables are considered the predominant dietary source of phylloquinone. As our food supply diversifies and expands, the food groups that contribute to phylloquinone intake are also changing, which may change absolute intakes. Thus, it is important to identify the contributors to dietary vitamin K estimates to guide recommendations on intakes and food sources.

Objective: The purpose of this study was to estimate: (1) the amount of phylloquinone consumed in the diet of U.S. adults; (2) the contribution of different food groups to phylloquinone intake in individuals with high or low vegetable intake (≥ 2 cups, < 2 cups vegetables/day); and (3) to characterize the contribution of different mixed dishes to phylloquinone intake.

Methods: Usual phylloquinone intake was determined from NHANES 2011-2012 (≥ 20 y; 2092 men and 2214 women) and the National Cancer Institute Method utilizing a complex, stratified, multistage probability cluster sampling design.

Results: On average, 43.0% of men and 62.5% of women met the AI (120 and 90 μg/day, respectively) for phylloquinone, with the lowest self-reported intakes noted among men especially in the older age groups (51-70 and ≥71 y). Vegetables were the highest contributor to phylloquinone intake, contributing 60.0% in the high vegetable intake group and 36.1% in the low vegetable intake group. Mixed dishes were the second highest contributor to phylloquinone intake contributing 16.0% in the high vegetable intake group and 28.0% in the low vegetable intake group.

Conclusion: Self-reported phylloquinone intakes using updated food composition data applied to NHANES 2011-2012 reveal that men are not meeting the current AI whereas women are. Application of current food composition data confirms that vegetables continue to be the primary

dietary source of phylloquinone in the U.S. diet. However, mixed dishes and convenience foods have emerged as a previously unrecognized, but significant contributor to phylloquinone intake in the U.S. These findings emphasize the need for expansion of food composition databases that consider how mixed dishes are compiled and defined.

Keywords: Dietary assessment, National Health and Examination Survey (NHANES), nutrition counseling, phylloquinone, vitamin K, warfarin

Introduction

Vitamin K is a fat-soluble vitamin that is required for the post-translational carboxylation of vitamin K dependent proteins (VKDPs) involved in multiple physiological processes, including clotting, bone formation, and regulation of vascular calcification (1). The primary dietary form of vitamin K is phylloquinone. In the U.S. diet, phylloquinone is predominantly found in dark green leafy vegetables and vegetable oils (2). Menaquinones are secondary forms that differ in structure and dietary sources when compared to phylloquinone (3).

The current U.S. Dietary Reference Intakes (DRIs) recommend an Adequate Intake (AI) for vitamin K based on our current knowledge of phylloquinone intakes (4). The current AI for adult men and women is 120 μg and 90 μg/d, respectively (4). A Recommended Dietary Allowance (RDA) was not established because there is insufficient knowledge regarding vitamin K's bioavailability, transport, and excretion. Further, there is no single biomarker that reflects vitamin K status or adequate physiological function. Analysis of the Third National Health and Nutrition Examination Survey (NHANES III (1988-1994)) indicated that mean intakes of dietary vitamin K ranged from 89 to 117 μg/d for men (≥ 19 y), and 79 to 88 μg/d for women (≥ 19 y), thus providing a reference for setting the current AI recommendations (**Supplemental Table 1**) (4). More recently, it was reported that phylloquinone intakes have declined among Irish adults, particularly among younger adults (5). Estimated intakes of phylloquinone among U.S. adults have not been examined since the analysis of NHANES III so it is not known if these shifts are unique to Ireland or a more global phenomenon.

Food composition databases are a robust source of dietary pattern information and nutritional content, to which they play an integral role in diet assessment and recommendation. Over time, the databases have expanded or updated the nutrient profiles of combination meals,

restaurant foods, and culturally diverse foods. This provides a better resource to accurately capture an individual's dietary pattern and change over time. Advances in methodology have allowed for broader characterization of vitamin K in the food supply, including many of these mixed dishes. With the diversification of the food supply and changes in food preparation techniques, plant-based oils have become a more noticeable food source of vitamin K (6). Characterizing the contribution of these additional foods sources of vitamin K is critical for developing dietary recommendations for the U.S. population as a whole, but also for clinical populations such as individuals on vitamin K antagonist anticoagulant therapy (coumarin-based oral anticoagulants). The direct drug-nutrient interaction influences drug efficacy and dosing (7) and changes in dietary vitamin K intake can result in adverse events including over-anticoagulation or severe clotting (8). Individuals initiating or continuing therapy receive counseling to maintain a diet with consistent vitamin K intake to ensure anticoagulant stability, demonstrating the importance of accurately identifying dietary sources of vitamin K.

The purpose of this study was to: (1) characterize the usual intake of phylloquinone consumed in the diet of U.S. adults; (2) characterize dietary patterns by phylloquinone intake in individuals with high or low vegetable intake ($\geq$ 2 cups/cup equivalents < 2 cups/cup equivalents vegetables/day), and (3) identify the contribution of mixed dishes to phylloquinone intake, in the most recently available data on the current diets of American adults.

Methods

NHANES is a nationally representative, cross-sectional survey that samples civilian non-institutionalized U.S. resident using a complex, multistage, probability cluster design to sample participants. The survey is conducted by the U.S. Center for Disease Control (CDC) National

Center for Health Statistics, who obtain written informed consent for all participants and proxies. The survey protocol was approved by the National Center of Health Statistics research ethics review board.

In this study, demographic, health examination, and dietary survey data from 2011-2012 NHANES were assessed for adults (≥20 y; n=5,560). Individuals without two reliable dietary recalls (n=1,209) and extreme outliers (records with < 400 calories/d (n=44) or > 6,000 µg/d of phylloquinone n=1) were excluded. Thus this analysis includes data for 4,306 participants. Pregnant and lactating women were not excluded from the analysis as the recommendations for phylloquinone intake are the same for adult women and individuals in these life stage groups.

NHANES participants were asked to complete an in-person house-hold interview, during which demographic data (including age, race, sex, income, and education) were obtained using the Computer-Assisted Personal Interviewing system. Following the in-home interview, participants underwent a health examination in the Mobile Examination Center (MEC) by a trained health technician. Physical examination data recorded by the trained health technician and utilized in our analyses includes body mass index (BMI) only. The first 24-hour dietary recall was collected in the MEC and was conducted in-person via the validated Automated Multiple Pass Method (AMPM) which has been described elsewhere (9,10). The second dietary recall occurred 3 to 10 days after the MEC examination and was conducted via telephone using the same methodology.

Phylloquinone content of foods was obtained from the United States Department of Agriculture (USDA) 2011-2012 Food and Nutrition Database for Dietary Studies (FNDDS). The vitamin K values in the FNDDS are from the USDA National Nutrient Database for Standard Reference. These values are determined through routine analysis by the Vitamin K

Laboratory at Tufts University as part of the USDA National Food and Nutrient Analysis Project (NFNAP) (11). Classification of vegetable intake was determined using the USDA Food Patterns Equivalents Database (FPED) 2011-2012, that classifies reported foods into 37 corresponding food pattern components and totals daily intake in cup, ounce, teaspoon, gram, or count of specified food patterns. In this study, cups and/or cup equivalents of total vegetable intake were used to determine if a participant had a "high" or "low" vegetable intake on any given day. A study participant was classified as having a high vegetable intake if he or she consumed an average of two or more cup and/or cup equivalents of vegetables, and a low vegetable intake if he or she consumed an average of less than two cups and/or cup equivalents. Two cups of vegetables per day is the lowest level of recommended daily vegetable intake in this study's population (12). Food group classifications were determined according to the USDA What We Eat in America (WWEIA) 15 food groupings. Alcoholic beverages, water, sugars, and "other" food groups were combined into one "other" food group category and baby food/infant formula was not analyzed as no participants in this study population consumed these foods. Eleven food groups are reported (**Supplemental Table 2 and 3**). The 'mixed dishes' food group was further expanded for subgroup analysis. Due to the interaction between vitamin K and coumarin-based anticoagulant therapy stability, inclusion of substantial amounts of vitamin K in dietary supplements carry high risk of severe clinical implications including over-anticoagulation or severe clotting for individuals prescribed these medications (13). Thus availability of vitamin K supplements is very limited, and because they do not contribute substantially to vitamin K intakes, supplements were not included in these estimates (14).

NHANES dietary recalls report two days of a person's intake and therefore these data represent the participant's intake on any given day, and not the long-term habitual or usual intake

(15). To overcome this limitation, the National Cancer Institute developed a mixed effects model and quantile estimation procedure (16) to predict usual intake from repeated dietary recalls. The method also produces valid standard error estimates for complex survey data, allows for incorporation of subgroup effects as covariates, and uses a Box-Cox parameter for data which are difficult to transform to approximate normality (16).

The means and percentages presented in Table 1 reflect an adjustment for within-person variability with the use of the amount-only part of the National Cancer Institute Method to estimate usual intake distributions. Covariates included are weekend versus weekday, order of dietary recall, and total calories for nutrient estimation. Standard errors were approximated by Fay's Modified Balanced Repeated Replication technique using 16 sets of replicate weights with an initial perturbation factor of 0.7, to calculate appropriate weights for both the full and subgroup analyses. All weights remained as integers throughout the analysis. Replication weights were post-stratified to control totals computed from the initial sample weights.

All statistical analyses were performed using SAS v 9.4 (Cary, NC). Sample weights were used to account for the unequal probabilities of selection, non-participation by selected sample persons, dietary recall non-response, and differential allocation by day of the week for the dietary recall (16). Means and SEs were estimated for phylloquinone and are presented as μg/d for multiple demographic categories within males and females separately. Multiple pairwise t tests were used for all comparisons between population groups with df dictated by the NHANES sample design. The percentages and SEs of phylloquinone supplied by each food group, vegetables, and mixed dishes was compared between individuals with high or low vegetable intake on any given day by multiple pairwise t tests with df dictated by the NHANES sample design. Significance determined by $P < 0.01$.

Results

Men and women consumed 2,456 and 1,798 kcals/d respectively, and 117 µg phylloquinone/d for both sexes. Overall, usual phylloquinone intake is consistent among males and females by demographic category (**Table 1**). On average, 43% of men and 62.5% of women are meeting or exceeding the AI of 120 and 90 µg/day respectively. In men, the prevalence of phylloquinone intakes > 120 µg/day decreased with age, 45.4% of men 20-30 y meet or exceed the AI compared to 31.8% of men ≥71 y. There was no difference in AI prevalence by all other demographic categories. Phylloquinone intake in men range from 80.0- 195µg, representing men who eat < 2 cups/cup equivalents of vegetables per day and men who eat ≥ 2 cups/cup equivalents of vegetables per day. A similar relationship was observed in women, in whom phylloquinone intake ranged from 78-223µg, with lowest intake observed in individuals consuming < 2 cups/cup equivalents of vegetables per day, and highest among individuals who eat greater than 2 cups/cup equivalents of vegetables per day.

Comparisons of energy and phylloquinone intakes between vegetable intake groups were significantly different in men or women. In men, mean phylloquinone intake in high and low vegetable intake groups was 195 and 80.8 µg/d ($P < 0.01$), respectively. In women, mean phylloquinone intake in high and low vegetable intake groups was 223 and 78 µg/d ($P < 0.01$), respectively.

To characterize dietary sources of vitamin K, we examined the distribution of various food groups contributing to phylloquinone intake by high and low vegetable intake groups (**Table 2**). For both vegetable intake groups, vegetables, followed by mixed dishes contributed the most phylloquinone in the diet. In the high vegetable group, vegetables and mixed dishes

contributed 60.0% and 16.1% of total phylloquinone. For the low vegetable group, the contributions were 36.1% and 27.7% for vegetables and mixed dishes, respectively. Fats and oils and snacks and sweets were the next highest contributors with 5-10% of phylloquinone contribution in both groups. In the low vegetable intake group, protein foods also contributed 5.4% to total phylloquinone. All other food groups contributed <5% to total phylloquinone.

Vegetables are considered the predominant source of vitamin K in the diet and continue to be the greatest contributor to phylloquinone intake in both high and low vegetable intake groups. We examined the distribution of vegetable groups contributing to phylloquinone intake (**Figure 1**). In both high and low vegetable intake groups, dark green vegetables and lettuce were the top foods to contribute to phylloquinone intake. Unique to the low vegetable intake group, the contribution of white potatoes and string beans to phylloquinone intake from vegetables was two times greater in the low vegetable intake group compared to the high vegetable intake group (**Figure 1a**).

Mixed dishes were found to be the second largest food group contributing to dietary phylloquinone intake, thus we examined mixed dish food subgroups in greater detail that revealed a unique distribution within the categories by vegetable intake (**Figure 1b**). In individuals with high vegetable intake, soups accounted for 23.0% of mixed dishes' phylloquinone contribution followed by Asian dishes (such as fried rice with soy based sauce mixtures, lo/chow mein, egg rolls, dumplings, and sushi, 21.5%) and meat and poultry (17.9%). In individuals with low vegetable-intake, grain-based dishes (pasta/rice dishes, turnovers, and mac and cheese) contributed the highest phylloquinone contribution from mixed dishes (25.3%), which also had the largest percent difference between the vegetable intake groups. Sandwiches (burgers, chicken/turkey sandwiches, egg/breakfast sandwiches, and frankfurter sandwiches,

9.7%) and pizza (10%) were the next largest contributing food groups to phylloquinone from mixed dishes in individuals with low vegetable intake.

Discussion

This analysis revealed that the usual intake of phylloquinone across all demographic categories of Americans' is consistent with the current recommendations, although fewer men achieve the current AI, particularly in the older age groups, compared to the original estimates conducted in 1998-1994 (4,17). In comparison to a recent report from national surveys of Irish adults (5), we did not observe declines in overall phylloquinone intakes among younger U.S. adults or in women over the last 15 years. However, a decline in phylloquinone intake was also reported in the UK among British adults in 1986-87 and 2000-01. The percentage of adults meeting the recommended intake and average phylloquinone intake decreased from 1986-87 to 2000-01, which was attributed to a decline in total vegetable intake (18). Vegetables remain the predominant dietary source of phylloquinone in our analysis, though we did determine the novel observation that mixed dishes are prominent food sources of phylloquinone among U.S. adults. Given that most of these food sources were not captured in earlier surveys due to gaps in the nutrient databases at the time, it was unexpected that the inclusion of more comprehensive phylloquinone data from mixed dishes did not result in an overall net increase in phylloquinone intakes.

Categorizing individuals by vegetable intake further demonstrated vegetables are a major dietary source of phylloquinone, more specifically dark green vegetables and lettuce were the top two vegetable groups contributing most to phylloquinone intake. However, in the low vegetable intake group, white potatoes were a significant source of phylloquinone intake. The white potato

food group includes baked or boiled white potatoes, French fries and other fried white potatoes, and mashed potatoes. These foods are often accompanied by additional calories from oil, which is likely the source of phylloquinone in these dishes (20).

Our observations have identified mixed dishes as a significant contributor to dietary phylloquinone. Among those with low vegetable intake, grain-based dishes, pizza and sandwiches were major sources. Unique to these mixed dish groups are the moderate to high contributions to phylloquinone intake without any apparent phylloquinone-rich vegetables as a main ingredient, e.g. macaroni and cheese and burgers, further demonstrating the significant role of food preparation methods and ingredients in mixed dishes in terms of manipulating phylloquinone content in foods. Trends in dietary patterns from the 2015-2020 Dietary Guidelines are similar to our observations in the low vegetable intake group. The Dietary Guidelines for Americans report states that approximately 75 percent of the population are not meeting their recommended intake for vegetables, and more than half of the population is meeting or exceeding their total grain and protein food recommendations (12). Further demonstrating that although phylloquinone intake has not changed for the majority of U.S. adults, the distribution of foods contributing to total intake are shifting with the current eating patterns observed in the U.S.

In development of dietary recommendations, bioavailability of the nutrient is of consideration. Studies have shown the absorption of the fat-soluble phylloquinone, as measured by post-prandial serum concentration of phylloquinone, is significantly higher after intake of phylloquinone-fortified oil or vegetables with added fat compared to vegetables alone (21,22). Jones et al. showed the bioavailability of phylloquinone in meals containing fast food and refined cereals, with lower than average intake of fruits, vegetables, and whole grains, have more than

twice the bioavailability when compared to meal patterns with higher than average intake of fruits, vegetables, whole grains, fish, and dairy (23). Collectively these studies suggest that the phylloquinone obtained from a meal with phylloquinone-rich oil may have greater bioavailability than phylloquinone obtained from fruits and vegetables.

Surprisingly, among those with low vegetable intake, foods that may be classified as 'fast foods' or 'convenience meals' such as burgers, pizza, frankfurter sandwiches, and macaroni and cheese, were major sources of phylloquinone likely due to the addition of phylloquinone-rich oils, such as soybean and canola, used during food preparation. This challenges the assumption that phylloquinone intakes are markers of a healthy diet (24). Characterizing dietary patterns of phylloquinone intake and identifying uncommon food sources of vitamin K is important when setting recommendations for special populations, particularly individuals on vitamin K antagonist anticoagulants.

Current recommendations state that patients receiving vitamin K antagonist anticoagulation treatment maintain consistent intake of phylloquinone (25). In practice, this often translates to avoidance of phylloquinone-rich vegetables (26–28). Studies have shown that this results in a 35-46% lower mean intake of vitamin K as a result of lower green vegetable intake (26). The contribution of alternative sources of phylloquinone in this population is not well characterized even though dietary stability is critical to maintaining therapeutic control. The characterization of phylloquinone in mixed dishes, the continued use of phylloquinone-rich plant oils in food preparation, and possible increased bioavailability of phylloquinone from these sources warrants re-evaluation of current dietary recommendations and for individuals on vitamin K antagonist anticoagulants.

Strengths of this study include the use of a large nationally representative cohort of adults, and examination of both nutrient and food-level contributions of vitamin K to the diet. Moreover, we estimated usual intakes that account for measurement error, day-to-day variability, and within subject variability (as random error) in dietary assessment to the extent possible. This study has some limitations. Dietary data are limited by the accuracy and currency of the databases used to estimate nutrient intakes from food. As noted, phylloquinone is the most abundant form of vitamin K in the diet, but complete analysis of vitamin K intake was limited since menaquinones are not quantified in the FNDDS database. Some animal products are moderate sources of vitamin K from menaquinones content alone and will need to be considered when examining total vitamin K intake in future analyses (29). In addition, some foods with high vitamin K content may be episodically consumed and not captured in the two days of dietary recall. This study obtained food intake data via 24-hour recall, which has inherit errors in under-reporting. However, the AMPM has been validated for energy (total calories), and documented elsewhere (10,30). As previously stated, dietary supplements were not examined as part of this analysis.

This study demonstrates that as the U.S. diet continues to evolve and technological advances in food analysis continue, it is important to re-evaluate dietary sources of nutrients and corresponding changes in nutrient intakes. Green vegetables remain the predominant and rich source of dietary vitamin K, but the contribution of mixed dishes and convenience foods also has an important role with respect to dietary vitamin K, which may have led to previous underestimates in dietary vitamin K intakes in dietary surveys. The data presented here reinforces the value of continuing to update and improve data in our food composition databases to better capture vitamin K intake, and provide the necessary information for individuals to

achieve a more consistent dietary vitamin K intake pattern. This also has important clinical practice implications for dietitians and other health care providers who counsel patients initiating or continuing vitamin K antagonist anticoagulant therapy to consume a diet with consistent vitamin K intake. Future expansion of food composition databases must consider how mixed dishes are compiled and defined to better understand differences in current standardized mixed dishes compared to entering individual recipes. Within mixed dishes, oil is an extremely variable ingredient and can be difficult to capture, therefore the effect on vitamin K content may be altered significantly demonstrating the need for more comprehensive analysis of mixed dishes. Additional research is required to quantify all vitamin K forms including menaquinones in the U.S. food supply, and to determine the bioavailability of multiple vitamin K forms in conjunction with varied meal composition, providing insight into dietary requirements and physiological function of vitamin K.

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Table 1. Dietary phylloquinone and energy intakes adjusted for day-to-day variability in the diets of US adults (≥ 20 y) by age, BMI, race/ethnicity, income, education level, and vegetable intake, NHANES 2011-2012[1]

	Men				Women			
	n	Energy, kcal/d	phylloquinone, µg/d	> 120 µg/d, %	n	Energy, kcal/d	phylloquinone, µg/d	> 90µg/d, %
All adults	2092	2456 ± 33.9	117 ± 10.2	43.0	2214	1798 ± 28.6	117 ± 8.49	62.5
Age (yrs)								
20-30	413	2495 ± 63.7[a,b]	107 ± 8.86[a]	45.4	419	1874 ± 30.9[a]	101 ± 8.36[a]	66.1
31-50	703	2695 ± 158[a]	124 ± 5.59[b]	48.7	748	1865 ± 24.3[a]	109 ± 11.9[a]	63.4
51-70	681	2304 ± 74.2[b]	117 ± 26.6[a,b]	38.6	755	1757 ± 72.5[b]	136 ± 9.30[b]	61.8
≥ 71	295	1992 ± 46.3[c]	111 ± 11.9[a,b]	31.8	292	1587 ± 42.8[c]	111 ± 12.1[a]	56.2
BMI (kg/m^2)								
Underweight (<18.5)	54	2348 ± 215	87.6 ± 16.9[a]	47.2	77	1814 ± 120	93.2 ± 16.9[a]	60.3
Normal (18.5-24.9)	593	2501 ± 45.7	117 ± 24.3[a,b]	42.8	632	1834 ± 40.2	122 ± 12.7[b]	63.7
Overweight (25-29.9)	771	2416 ± 34.7	124 ± 12.1[b]	42.2	597	1765 ± 38.8	123 ± 12.2[a,b]	61.4
Obese (≥ 30)	674	2472 ± 97.2	108 ± 3.77 [a,b]	43.9	908	1790 ± 29.3	110 ± 6.04[a,b]	62.5
Race/ethnicity								
Non-Hispanic white	831	2494 ± 83.5[a]	98.0 ± 14.9[a]	43.6	848	1791 ± 54.4[a,c]	85.3 ± 8.93[a]	62.4
Non-Hispanic black	514	2479 ± 40.2[a]	115 ± 13.7[a]	42.2	626	1796 ± 34.5[a]	121 ± 10.0[b,e]	64.0
Non-Hispanic Asian	271	2372 ± 48.2[a,b]	131 ± 13.3[a,c]	36.1	267	1849 ± 24.7[a,b]	122 ± 16.6[b,c,e]	59.3
Mexican American/other Hispanic	407	2219 ± 59.8[b]	158 ± 22.1[b,c]	44.2	417	1678 ± 39.0[c]	140 ± 17.8[b,c,d]	61.5
Other race (multi-racial)	69	2398 ± 97.8[a,b]	109 ± 14.6[a,c]	36.7	56	1841 ± 100[a,c]	97.1 ± 13.9[a,e]	66.4
Annual Household Income								
Below (<$25,000)	577	2380 ± 57.5[a,b]	94.0 ± 9.05[a]	41.7	692	1780 ± 50.4	101 ± 5.23[a]	61.8
Above (≥$25,000)	1420	2485 ± 57.4[a]	123 ± 10.6[b]	43.4	1441	1801 ± 28.4	122 ± 12.0[b]	62.6
Not available	95	2137 ± 259[b]	97.9 ± 43.4[a]	40.6	81	1872 ± 127	124 ± 19.4[a,b]	64.3
Education Level [2]								
Did not complete high school	495	2379 ± 84.5	95.9 ± 12.1[a]	43.9	460	1658 ± 54.3[a]	83.4 ± 3.60[a]	58.8
High school graduate	457	2488 ± 116	104 ± 9.04[a,b]	44.1	430	1770 ± 46.9[b]	99.4 ± 8.07[a,c]	61.2
Some college	590	2462 ± 39.9	108 ± 4.41[b]	42.3	729	1803 ± 23.1[b]	114 ± 11.3[b,c]	63.0
College graduate	548	2472 ±43.3	146 ± 29.3[c]	42.4	594	1869 ± 56.4[b]	145 ± 10.8[d]	64.3
Vegetable intake[3]								
High (≥2 cups/d)	625	2805 ± 61.3[a]	195 ± 31.8[a]	50.9	546	2043 ± 33.5[a]	223 ± 16.7[a]	69.0
Low (<2 cups/d)	1467	2295 ± 36.1[b]	80.8 ± 3.36[b]	39.3	1668	1706 ± 33.3[b]	78.0 ± 3.47[b]	60.1

[1] Values are means ± SEs unless indicated otherwise. Labeled means in a column within a group without a common superscript letter are significantly different, $P \leq 0.01$. Multiple pairwise t tests were used for comparisons between population groups with df dictated by the NHANES sample design.

[2] 3 participants (2 males, 1 female) did not report education level.

[3] High vegetable intake defined as ≥ 2 cups or cup equivalents per day, low vegetable intake defined as < 2 cups or cup equivalents per day. Examples include 1 cup/cup equivalent equals 2 cups raw leafy vegetables, 1 cup chopped, cooked, or raw broccoli, peppers, or carrots. The Food and Nutrition Database for Dietary Studies defines cups and cup equivalents for various foods and are based on food pattern definitions used in the Dietary Guidelines for Americans (31). Due to the variability in foods, weight in grams varies by cooking method and food item. For vegetables, 1 cup in grams varies from 70 to 245 grams. Thus we categorized by cup/cup equivalents.

Table 2. Reported food group contribution to dietary phylloquinone in the diets of US adults (≥20 y) by high and low vegetable intake, NHANES 2011-2012[1,2,3]

	Contribution to total phylloquinone, %	Contribution to total phylloquinone, %
	High vegetable intake, ≥ 2 cups/d (n = 1,117)	Low vegetable intake, < 2 cups/d (n = 3,133)
Vegetables[4]	60.0 ± 3.0	36.1 ± 2.0*
Mixed dishes[5]	16.1 ± 1.8	27.7 ± 1.5*
Fats and oils[4]	6.4 ± 0.6	8.0 ± 0.6
Snacks and sweets[4]	5.0 ± 0.6	8.7 ± 0.4*
Beverages (non-alcoholic)[4]	3.4 ± 1.4	1.9 ± 0.4
Protein foods[4]	2.7 ± 0.2	5.4 ± 0.3*
Grains[4]	2.1 ± 0.3	4.6 ± 0.3*
Fruit[4]	1.8 ± 0.2	2.7 ± 0.3
Condiments and sauces[4]	1.6 ± 0.3	3.3 ± 1.0
Other[4]	0.5 ± 0.2	0.6 ± 0.1
Milk and dairy[4]	0.5 ± 0.1	0.9 ± 0.1*

[1] High vegetable intake defined as ≥ 2 cups or cup equivalents per day, low vegetable intake defined as < 2 cups or cup equivalents per day. Examples include 1 cup/cup equivalent equals 2 cups raw leafy vegetables, 1 cup chopped, cooked, or raw broccoli, peppers, or carrots. The Food and Nutrition Database for Dietary Studies defines cups and cup equivalents for various foods and are based on food pattern definitions used in the Dietary Guidelines for Americans (31). Due to the variability in foods, weight in grams varies by cooking method and food item. For vegetables, 1 cup in grams varies from 70 to 245 grams. Thus we categorized by cup/cup equivalents.

[2] Unlike the National Cancer Institute-adjusted statistics that represent long-term, usual dietary intake estimates presented in Table 1, the food intake statistics here are estimates on any given day. The above sample sizes reflect a categorization o the NHANES respondents based on their daily vegetable intake and describe the sample, not the population.

[3] Values are means ± SEs unless indicated otherwise. Multiple pairwise t-tests were used to compare phylloquinone contribution between high or low vegetable intake eaters within each food group.
*Different from high vegetable intake, $P < 0.01$.

[4] See supplemental table 2 for food groups.

[5] see supplemental table 3 for mixed dish sub-groups.

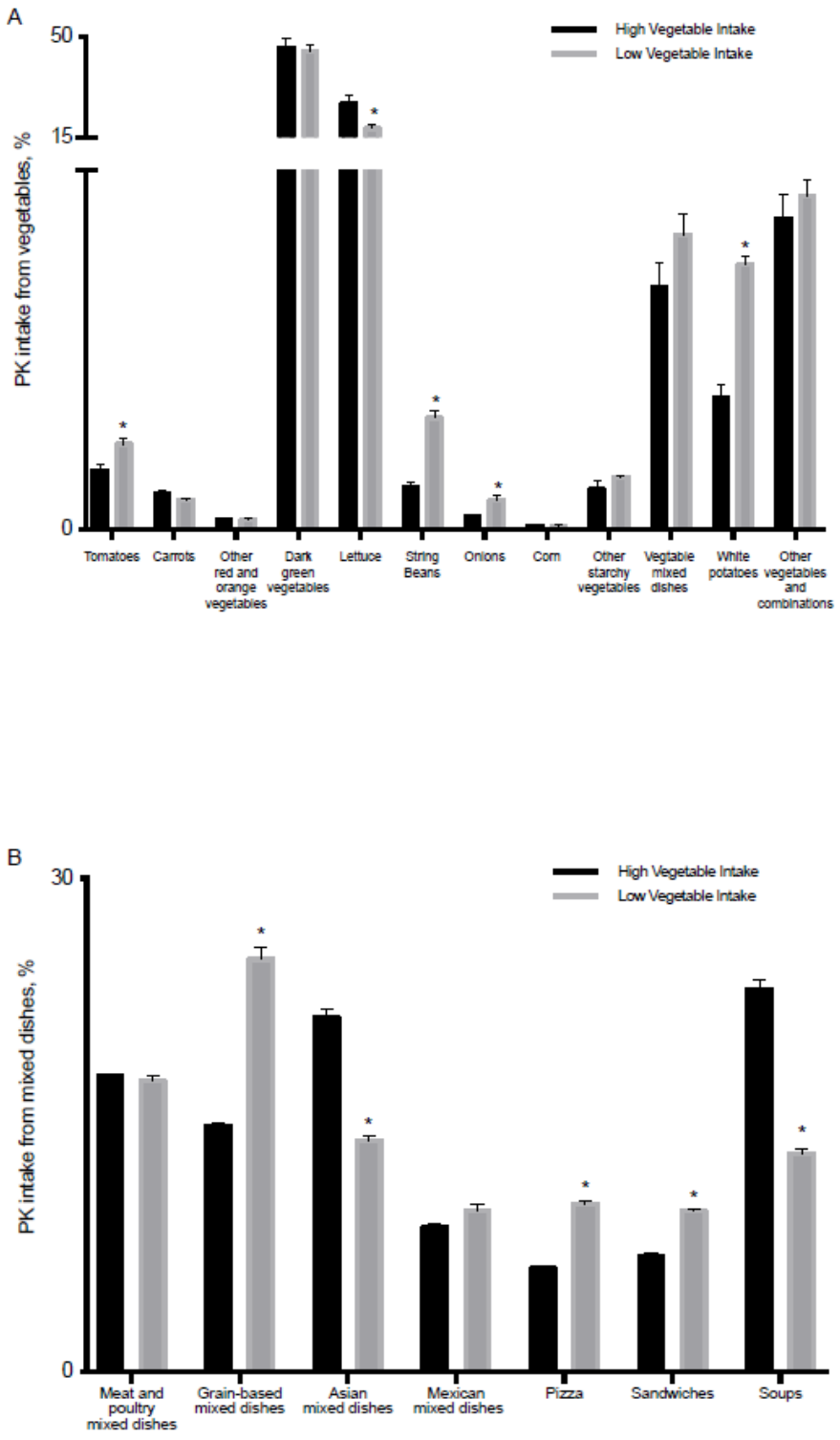

Figure 1. Contribution (%) of vegetable (A) and mixed dish (B) subgroups to dietary vitamin K intake in the diets of US adults (≥20y; high vegetable intake n=1,117; low vegetable intake n=3,133) NHANES by food group categories, NHANES 2011-2012. Data presented as mean ± SEs. *Significant difference between high and low vegetable intake, $P < 0.01$.

Online Supporting Material

Supplemental Table 1. Mean and SEs of vitamin K intake (ug/day) from food, NHANES III (1988-1994) [1,2]

	Men		Women	
	n	Phylloquinone, µg/d	n	Phylloquinone, µg/d
19-30 y	1,219	65.1 ± 3.6	1901	98 ± 14.6
31-50 y	2,533	125 ± 11.4	2,939	99.6 ± 3.3
51-70 y	1,942	120 ± 8.5	2,065	97.2 ± 4.4
≥ 71 y	1,255	97.8 ± 8.1	1,368	93.8 ± 4.3

[1] Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc: a report of the Panel on Micronutrients ... [et al.], Standing Commit[...] by Institute of Medicine (U.S.).Panel on Micronutrients Reproduced with permission of National Academy Press in the format Republish in a journal/magazine via Copyright Clearance Center.

[2] Data are limited to individuals who provided a complete and reliable 24-hour dietary recall on day 1. The means and SEs were computed using SAS PROC UNIVARIATE. For all other groups, data were adjusted using the Iowa State University Method. Mean, standard errors, and percentiles were obtained using C-Side. Standard errors were estimated via jackknife replication. Each standard error has 49 degrees of freedom. Food composition data are from the NDS-R Food and Nutrient Database, Version 30, 1999, Nutrition Coordinating Center, University of Minnesota.

Online Supporting Material

Supplemental Table 2. What We Eat In America Food Categories, USDA Agricultural Research Services. 2011-2012.[1]

Vegetables	Fats and Oils	Snacks and sweets	Beverages (non-alcoholic)	Protein foods	Grains	Fruit	Condiments and sauces	Milk and Dairy	Other
Vegetables excluding potatoes: Tomatoes Carrots Other red and orange vegetables Dark green vegetables Lettuce and lettuce salads String beans Onions Corn Other starchy vegetables Vegetable mixed dishes White potatoes: Baked boiled French fries Other fried white potatoes Mashed potatoes Potato mixtures	Butter and animal fats Margarine Cream cheese Sour cream Whipped cream Cream and cream substitutes Mayonnaise Salad dressings Vegetable oils	Savory snacks: Potato chips Tortilla, corn, or other chips Popcorn Pretzels Snack mix Crackers: Crackers Saltines Snack/meal bars: Cereal bars Nutrition bars Sweet bakery products: Cakes and pies Cookies and brownies Doughnuts, sweet rolls, pastries	100% juice: Citrus Apple Other fruit Vegetable Diet beverages: Diet soft drinks Diet sport and energy drinks Nutritional beverages Smoothies and grain drinks Coffee Tea	Meats: Beef Ground beef Pork Lamb goat Liver organ meats Poultry: Chicken, whole pieces Patties nuggets tenders Turkey Duck other Seafood: Fish Shellfish Eggs Eggs Omelets Cured meats/poultry: Cold cuts cured meats Bacon Frankfurters Sausages	Cooked grains: Rice Pasta Noodles Other cooked grains Breads, rolls, tortillas: Yeast breads Rolls buns Bagels English muffins Tortillas Quick breads and bread products: Biscuits Muffins Quick breads Pancakes Waffles French toast	Apples Bananas Grapes Peaches Nectarines Berries Citrus fruits Melons Dried fruits Other fruits and fruit salads	Tomato-based condiments Soy-based condiments Mustard Olives Pickles Pickled vegetables Pasta sauces (tomato based) Dips Gravies Other condiments and sauces	Milk: Whole Reduced fat Low fat Nonfat Flavored milk: Whole Reduced fat Low fat Nonfat Dairy drinks and substitutes: Milk shakes and other drinks Milk substitutes Cheese: Cheeses Cottage cheese Ricotta Yogurt: Regular Greek	Alcoholic beverages: Wine Beer Liquor and cocktails Water: Tap or bottled Flavored or carbonated Enhanced or fortified Sugars: Sugars Honey Sugar substitutes Jams Syrups Toppings Other: Protein and nutritional powders

Online Supporting Material

Supplemental Table 2. Continued.

Vegetables	Fats and Oils	Snacks and sweets	Beverages (non-alcoholic)	Protein foods	Grains	Fruit	Condiments and sauces	Milk and Dairy	Other
		Candy: Candy containing and not containing chocolates Other: Ice cream and frozen dairy desserts Pudding Gelatins Ices sorbets		Plant based protein foods: Beans Peas Legumes Nuts Seeds Processed soy products	Ready-to-eat cereals: High sugar ≥21.2g/100g Low sugar ≤21.2g/100g Cooked cereals: Oatmeal Grits Other cooked cereals				

[1] U.S. Department of Agriculture, Agricultural Research Service. 2015. What We Eat in America Food Categories 2011-2012. Available: **www.ars.usda.gov/nea/bhnrc/fsrg**

Online Supporting Material

Supplemental Table 3. What We Eat in America Mixed Dishes Food Category Subgroups. USDA Agricultural Research Services. 2011-2012.[1]

Meat, Poultry, seafood mixed dishes	Grain based mixed dishes	Asian mixed dishes	Mexican mixed dishes	Pizza	Sandwiches	Soups
Meat mixed dishes Poultry mixed dishes Seafood mixed dishes	Rice mixed dishes Pasta mixed dishes Macaroni and cheese Turnovers Other grain based items	Fried rice Lo/chow mein Stir-fry and soy based sauce mixtures Egg rolls Dumplings Sushi	Burritos Tacos Nachos Other Mexican mixed dishes	All variations of pizza	Burgers Chicken/turkey sandwiches Egg/breakfast sandwiches Frankfurter sandwiches Other sandwiches	All variations of soups

[1] U.S. Department of Agriculture, Agricultural Research Service. 2015. What We Eat in America Food Categories 2011-2012. Available: **www.ars.usda.gov/nea/bhnrc/fsrg**

4. Tissue concentrations of vitamin K and expression of key enzymes of vitamin K metabolism are influenced by sex and diet, but not housing in C57Bl6 mice

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4. Abbreviations: *Ggcx*: gamma glutamyl carboxylase; HPLC: high performance liquid chromatography; LC-APCI-MS: liquid chromatography atmospheric pressure chemical ionization mass spectrometry; MKn: menaquinones; MK4: menaquinone-4; PK: phylloquinone; *Vkorc1*: vitamin K expoxide reductase complex 1; *Vkorc1l1*: vitamin K epoxide reductase complex 1 like 1; *Ubiad1*: ubiA domain containing protein 1;

Abstract

Background: There has been limited characterization of biological variables that impact vitamin K metabolism. This gap in knowledge can limit the translation of data obtained from pre-clinical animal studies to future human studies.

Objective: The purpose of this study was to determine the effects of diet, sex and housing on serum, tissue, and fecal vitamin K concentrations and gene expression in C57BL6 mice during dietary vitamin K manipulation.

Methods: C57BL6 four month-old male and female mice were randomized to conventional or suspended-wire cages and fed control (1400±80 µg phylloquinone (PK) /kg) or deficient diet (31±0.45 µg PK/kg) for 28d in a factorial design. PK and menaquinone-4 (MK4) plasma and tissue concentrations were measured by HPLC. Long-chain menaquinones (MK-n) were measured in all matrices by LC-APCI-MS. Gene expression was quantified by RT-PCR in liver, brain, kidney, pancreas, and adipose tissue.

Results: Male and female mice respond differently to dietary manipulation in a tissue-dependent manner. In mice fed the control diet, females had 3x more MK4 in brain and mesenteric adipose tissue compared to males, and 100% greater PK concentrations in liver, kidney, and mesenteric adipose tissue compared with males. In mice fed the deficient diet, kidney MK4 concentrations were 4x greater in females compared to males, and there was no observed difference in other tissues. Males and females differed in the expression of: *Vkorc1* in mesenteric adipose tissue and pancreas; *Ubiad1* in kidney and brain. There was no effect of housing on serum, tissue, or fecal concentrations of any vitamin K form.

Conclusions: Vitamin K concentrations and expression of key metabolic enzymes differ between males and female mice and in response to dietary PK concentration. Identifying factors that may

impact study design and outcomes of interest is critical to optimize study parameters examining vitamin K metabolism in animal models.

Key words: coprophagy, *Ggcx*, long chain menaquinones, menaquinone-4, phylloquinone, suspended wire cages, *Ubiad1*, vitamin K, *Vkorc1*, *Vkorc1l1*

Introduction

Vitamin K is a fat-soluble vitamin characterized by the 2-methyl-1,4-naphthoquinone ring that exhibits anti-hemorrhagic activity and an isoprenoid side chain. The major dietary form, phylloquinone (PK), is found in dark green leafy vegetables and plant oils (1), and provides the basis for current dietary requirements (1,2). Menaquinones are a group of vitamin K derivatives that are named after the number of isoprenoid units in the side chain (3). Whereas evidence suggests that MK4 is formed from dietary PK by means of tissue-specific conversion by the enzyme ubiA prenyltransferase domain containing 1 (*ubiad1*), and may have unique functions among the known vitamin K forms (4–7), longer-chain menaquinones are a product of bacterial synthesis in the intestine (8,9). Bacterially-produced menaquinones act as electron carriers in cell respiration, transporting molecules across plasma membranes, and acting as an antioxidant preventing lipid oxidation (10). Dietary sources of menaquinones include dairy and meats, as well as fermented food products (3,10). The contribution of bacterially-synthesized menaquinones to overall vitamin K status in animal models is still uncertain (11), and the impact of menaquinones to human vitamin K requirements and status remains controversial.

There is growing awareness that there are sex-specific differences in response to specific interventions in animal models used for biomedical research (12). The majority of current preclinical studies in the vitamin K field rely on use of a single sex rodent model (13–18); few studies have included both male and female rodents (19,20). However in rats, females are reported to be more resistant to vitamin K deficiency compared to males (21,22), which has been hypothesized to be attributable to increased coprophagy in females (23), and increased vitamin K requirement in males (21,24), specific effects of sex hormones on vitamin K metabolism (25,26), and differences in the biosynthesis of menaquinones by gut bacteria and corresponding

absorption (26). The increased use of genetically modified mouse models and diet manipulation studies necessitates characterization of the biological variables affecting mouse models of vitamin K nutriture. This includes understanding sex-specific differences in vitamin K metabolism to gain insight into mechanistic activity of vitamin K and its alternative functions.

Coprophagy is a unique consideration in animal studies of vitamin K nutrition due to the high concentrations of bioavailable menaquinones produced by gut bacteria. To control for unanticipated menaquinone intake through coprophagy, suspended wire caging in combination with vitamin K deficient diets have been effectively used to create a vitamin K deficiency as defined by a decrease in prothrombin time (23,27). With recent NIH mandates to improve animal welfare (12,28), these caging options need to be evaluated to determine if they indeed account for differences in vitamin K metabolism across both sexes.

Our group has recently developed novel methods for accurately manipulating vitamin K in the mouse diet to match the predominant forms in the human diet (29), for measuring all known menaquinone forms (30), and for characterizing the effect(s) of diet and non-dietary biological factors, such as sex, on expression of genes involved in vitamin K recycling and function (31). This study utilized these techniques to determine the effects of sex, diet and caging on serum, tissue and fecal vitamin K concentrations and vitamin K metabolism related gene expression in C57Bl6 mice, a common animal model for genetic manipulation. We hypothesized that vitamin K concentrations in serum, liver, and extra-hepatic tissues would be significantly different between housing types. Additionally, we hypothesized a sex-specific difference in response to the dietary vitamin K manipulation between caging types.

Methods

Animals and Diets

C57BL/6NCrl VAF/Plus mice (4 mo of age, n = 64), obtained from Charles River Laboratory (Wilmington, MA), were acclimated on the AIN-93G diet (TD.94045, Harlan Teklad) in conventional caging for 1 week. We used a 2^3 factorial design to evaluate sex, diet and cage effects. Male and female mice were weight-matched and randomized to individual conventional [20.3 cm x 31.8 cm x 20.3 cm Zyfone plastic caging supplied with Biofresh cellolose bedding (Absorption Co, Ferndale, WA)] or suspended wire caging (20.3 cm x 25.4 cm x 19.1 cm). Within each cage and sex group, mice were randomly assigned to a control diet containing 1400 ± 80 µg PK/kg diet, or a deficient diet containing 31 ± 0.45 µg PK/kg diet (TD.120060, Harlan Teklad) for 4 weeks *ad libitum* resulting in 8 groups of 8 mice each. The experimental diet is a modification of TD.97053 enables replacement of regular corn oil with tocopherol-stripped corn oil and a PK source (**Supplemental Table 1**) (32). Tocopherol stripped corn oil is used due to a nutrient-nutrient interaction between vitamin K and vitamin E as reported by Tovar et al., 2006. This study demonstrated rats fed a vitamin E supplemented diet had significantly lower extra-hepatic tissue concentrations of vitamin K (33). To eliminate potential nutrient interactions in our study, diet was prepared with tocopherol stripped corn oil. Body weights were measured weekly. If significant weight loss was observed and mice showed clinical signs of dehydration, fluid replacement was initiated with 1 cc Ringer's lactate solution injected subcutaneously. Mice were maintained in AAALAC-accredited facilities with an environmentally controlled atmosphere (22ºC, 45% relative humidity, 15 air changes of 100% fresh hepa-filtered air per hour and a 12/12-hour light/dark cycle- 07:00 on). Animals were observed daily for clinical signs of distress or disease. At the end of the experiment, mice were

euthanized with carbon dioxide with secondary euthanasia ensured by subsequent cervical dislocation, followed by blood and tissue collection. Tissues of interest (brain, liver, kidney, pancreas, and mesenteric adipose tissue) were harvested and frozen immediately in liquid nitrogen and stored at -70ºC until time of analysis. All protocols were approved by the HNRCA Tufts University Animal Care and Use Committee.

Vitamin K Analysis

Tissues (0.10-0.20 g wet weight) were homogenized in PBS using a Powergen homogenizer (Fisher Scientific, Pittsburgh, PA). Plasma and tissue homogenate PK and MK4 were measured using reversed-phase HPLC (34).

Longer chain menaquinones (MK5 to MK13) concentrations were quantified in tissue and fecal samples by LC-APCI-MS (30).

Gene Expression

We profiled the expression of the following genes encoding enzymes involved in vitamin K metabolism: (1) vitamin K epoxide reductase complex subunit 1(*Vkorc1*); (2) vitamin K epoxide reductase complex subunit 1 like 1 (*Vkorc1l1*); (3) gamma glutamyl carboxylase (*Ggcx*); and (4) UbiA prenyl transferase domain-containing 1 (*Ubiad1*). Total RNA was isolated from tissues (kidney, liver, brain, mesenteric adipose tissue, and pancreas) using Trizol reagent and cDNA synthesized using Superscript III reverse transcriptase (Life technologies, Grand Island, NY). Pancreas tissue samples were not stored with RNAse inhibitor, increasing risk of degraded RNA and further lack of detectable expression. Real-time PCR was performed using SYBR green master mix and an ABI7300 thermocycler (Applied Biosystems, Foster City, CA). Primer

sequences were obtained from qPrimerDepot (35) or NCBI Primer Blast (**Supplemental Table 2**) (36). Relative expression was calculated using the 2-ΔΔCt method and statistical analyses were performed on ΔCt values *Gapdh* was used as the control gene.

Tail Clip Bleeding Assay (In vivo Bleeding Assay*)*

Tail bleeding time was measured by an *in vivo* bleeding assay on day 28 of the dietary intervention. Bleed time was defined as the start of bleeding to the cessation of bleeding (37).

Corticosterone Assay

Physiological responses to stress lead to the release of glucocorticoids, specifically corticosterone in mouse models (38). As a measure of stress in response to different caging conditions, total plasma corticosterone levels were measured by solid-phase enzyme linked immunosorbent assay (ELISA) kit (Alpco, Salem, NH). The intra and inter-assay coefficients of variation were 5.9% and 7.5% respectively.

Power Calculations and Statistical Analyses

Sample size calculations were based on the work of Metta and Johnson (22) who compared prothrombin (PT) time among male and female rats allowed coprophagy and not allowed coprophagy. power analysis was performed based on two-sample comparisons on the main effects for cage and sex. We determined that 4 mice per group would provide 80% power to detect a difference in liver vitamin K status between mice in conventional (allowed coprophagy) and wire-bottom cages (not allowed coprophagy), and 6 mice per group would

provide 90% power to detect a sex-specific difference in liver vitamin K status at α=0.05. Two extra mice in each group were added in case of death during the study.

The effects of cage, sex, and diet, on concentrations of PK and MK4 were analyzed by a three-way ANOVA model with interactions and a fixed effect for tissue PK and MK4 measurements. Results of the full model led to subgroup analysis within each specific tissue for the effects of sex and diet on PK and MK4 concentrations, where we used a two-way ANOVA and Tukey's HSD to examine sex by diet interactions. Pairwise comparisons of interest included male control (MC) vs. male deficient (MD); female control (FC) vs. female deficient (FD); MC vs FC; MD vs. FD. ANOVA was used to examine fluid replacement, change in body weight, and bleeding time between males and females in conventional and suspended wire cages. We assessed ANOVA model using diagnostics for assumptions of homogeneity of variance. No outliers were found that influence significance in the full model. Data were tested for normality by the Shapiro-Wilk test. Significance was determined by $P < 0.01$, and all analyses were carried out using SAS v 9.4 (Cary, NC). Data are reported as means ± SEM.

Results

Cage Effect

Overall, independent effects of diet ($P < 0.01$) and cage ($P < 0.01$) on body weight change in both male and female mice (sex effect $P = 0.37$) were observed. The deficient diet and wire-bottom cages were both associated with lower weight gain, the 3-way interaction between the effects of sex, cage type, and diet on weight change was not significant (P -interaction = 0.76) (**Table 1**). There was no diet by cage interaction (P -interaction = 0.92). Thirty-one of the 32 mice housed in suspended wire cages (97% of mice) required fluid replacement during the

28-day experimental period ($P < 0.01$) (**Supplemental Table 3**). In comparison, only two mice in the conventional cages required fluid replacement.

Analysis of PK and MKn tissue concentrations in the full statistical model showed no overall cage effect for any vitamin K form (main effect cage: PK $P = 0.09$; MK4 $P = 0.29$, long chain MKn all P values > 0.02). There was also no significant difference in bleeding time between suspended wire and conventional caging regardless of sex (data not shown, $P > 0.10$).

Due to the differences observed in weight gain between caging, and required fluid replacement of mice housed in suspended wire cages, we examined the effect of these environmental stressors as assessed by plasma corticosterone levels. Although mice in suspended wire caging showed differences in weight on the different diets, there was no significant difference in corticosterone levels between suspended wire cages or conventional cages (data not shown, $P = 0.87$).

Diet and Sex Effects

Because there was no effect of cage on concentrations of any vitamin K form, data were combined for cage types for all other analyses presented (**Table 2**). Results of the overall statistical model showed a significant difference of PK and MK4 concentrations related to tissue type ($P < 0.001$). Thus we chose to examine each tissue independently, to examine the effect of sex and diet on PK and MK4 concentrations. For all tissues, there was an observed diet effect in PK and MK4 concentrations (main effect of diet all P values < 0.01), except for PK concentrations in pancreas, which showed no effect ($P = 0.05$). There was an overall sex effect observed for PK and MK4 concentrations in each tissue (main effect of sex all P values < 0.01). The response to the diets differed by sex in most tissues (for all sex by diet interactions $p < 0.01$)

with mice on the control diet having higher tissue concentrations of PK and MK4. The only exception was in PK concentrations in pancreas (sex by diet interaction $P = 0.25$). Serum PK concentrations did not differ between males and females within either diet (main effect of sex $P = 0.91$; sex by diet interaction $P = 0.91$), PK concentrations did not differ between males and females on the deficient diet in any tissue ($P > 0.02$). MK4 concentrations were significantly different between males and females on the deficient diet in kidney only ($P < 0.01$) with female mice having higher concentrations. In contrast, females on the control diet had significantly higher PK and MK4 concentrations in all tissues (all P values < 0.01). Of the tissues analyzed, only kidney, liver, and fecal samples contained long chain menaquinones (**Table 2** and **3**). Within the deficient diet, there were no significant differences in MK9, MK10, or MK11 in kidney or liver between males and females (all P values > 0.02). Surprisingly, males had higher kidney MK9 and MK11 concentrations (all P values < 0.01), and in liver, females had significantly higher MK11 concentrations compared to males ($P < 0.01$). Long-chain menaquinones in kidney and liver were unaffected by diet (main effect of diet all P values > 0.12), however there was a significant sex by diet interaction in liver MK11 ($P < 0.01$).

Fecal PK, MK4 and detectable long chain menaquinone concentrations are presented in **Table 3**. For longer chain menaquinones (MK6-MK13), there was no effect of sex (all P values > 0.07), or diet (all P values > 0.06). There was no significant difference in menaquinone concentrations between males and females on the deficient diet (all P values > 0.28). In comparison, females on the control diet had significantly higher MK6, MK10, and MK11 compared to males (all P values < 0.01). For all other menaquinones, there was no difference in fecal concentrations by sex or diet ($P > 0.05$).

Gene Expression

Gene expression across different tissues varied and may be indicative of tissue specific regulation **(Figure 1)**. *Vkorc1* expression was significantly different between males and females in mesenteric adipose tissue and pancreas ($P < 0.03$), and in response to diet in pancreas and kidney ($P < 0.01$). Interestingly, *Vkorc1* in pancreas and kidney of both males and females had increased relative expression compared to liver *vkorc1*, which was consistently expressed across all groups. *Vkorc1l1* expression only had a significant sex and diet effect in kidney and mesenteric adipose tissue, ($P < 0.01$). Expression of *Ggcx* only had a significant sex by diet interaction in brain and liver ($P < 0.02$). *Ubiad1* expression was significantly different between males and females in kidney and brain ($P < 0.04$). There was a significant sex by diet interaction in liver ($P < 0.03$), and a diet effect observed in mesenteric adipose tissue ($P < 0.04$). Similar to *Ggcx*, *Ubiad1* was not expressed in pancreas.

Discussion

In this study of young C57Bl6 mice, there were significant sex-specific differences concentrations of PK, MK4 in all tissues examined, and in several long-chain menaquinone forms detected in liver and kidney. These results and supporting gene expression data indicate that males and females respond differently to dietary manipulation of vitamin K. As such, this study provides critical information about biological variables that could influence the function of vitamin K and its metabolites.

Sex specific differences in the tissue distribution of vitamin K forms has been documented in rats (24,39–42). However there is limited information available regarding the sex-specific responses to vitamin K dietary manipulation or vitamin K metabolism in mice. The

C57BL6 genetic background is the most commonly used strain for transgenic models and although transgenic mouse models for studying key enzymes in vitamin K metabolism and vitamin K dependent proteins have been developed, none have compared responses between sexes (14,19,20). We saw no difference in concentrations of PK and MK4 between males and females on the deficient diet in any tissue except pancreas. There may be a minimum vitamin K concentration for the individual tissues achieved with intakes sufficient to sustain coagulation that is similar for males and females, whereas only the higher intakes reveal sex-specific differences in tissue concentrations.

Collectively, these observations challenge the theories put forth based on rat studies that females are more resistant to vitamin K deficiency compared to males (21,22) because of possible increased vitamin K requirement in males (21,24). Instead, it is more plausible that the higher tissue concentrations observed in females in response to high vitamin K intakes may be attributed to the role of estrogen and potential vitamin K requirements for reproduction. Sex-specific differences have not been well characterized in humans. Although there is a suggestive effect of sex, the data are inconsistent (43). Which further supports the need to use of both sexes in vitamin K studies using preclinical models.

The extent to which bacterially-produced menaquinones contribute to vitamin K requirements and function in the liver is still unclear. In our study, it is assumed that the origin of hepatic and renal long chain menaquinones is attributable to coprophagy because phylloquinone was the exclusive dietary source of vitamin K and colonic absorption of bacterially-synthesized MKn was likely low. Despite characterizing eight bacterially-produced menaquinones in feces, we were only able to measure three long-chain menaquinone forms in peripheral tissues, and only in liver and kidney. Females had overall higher bacterially-produced menaquinone tissue

contents compared to males on the control diet, which may be indicative of sex-specific differences in gut microbiota and/or coprophagy, as reported for other species (23,26).

In our study, suspended wire caging had no effect on concentrations of tissue vitamin K forms or measures of vitamin K function. Mice can access cecal pellets and ingest the pellets as they are being excreted. Prevention of cecal pellet consumption requires alternative methods such as Elizabethan collars (44). Suspended wire caging however, resulted in poor health in the animals as indicated by more fluid replacement and less body weight gain. That there were no advantages to use of suspended wire cages in terms of limiting coprophagy lends further support for not using this caging type in mouse studies, as it does not significantly reduce or inhibit coprophagic behavior.

Vitamin K status reflects adequacy of requirements to support the biological function of post-translational modification of vitamin K-dependent proteins, which include proteins involved in coagulation and bone metabolism. *Vkorc1* is the rate-limiting step in the classic vitamin K cycle of protein post-translational modification. *Vkorc1l1*, the isomer to *vkorc1*, has demonstrated differential regulation of expression, tissue specific expression, and differences in vitamin K antagonist warfarin sensitivity (20,45–47), indicating that although the two enzymes are structurally similar, their functionality and regulation may be independent of one another. Our results suggest tissue-specific differences in expression and possible independent regulation of the two paralogs, reflected by the diet and sex effect on expression in different tissues. The consistent expression of *vkorc1* in liver, regardless of diet, may be indicative of a minimum threshold of vitamin K required to maintain coagulation. In contrast, the upregulation of *vkorc1* expression observed in kidney and pancreas of both males and females on the deficient diet may suggest a compensatory effect by extra-hepatic tissues for lower vitamin K intake.

A recently-identified enzyme involved in vitamin K metabolism is the *Ubiad1* gene coding for a prenyltransferase that enzymatically converts menadione to MK4 (6,48). The conversion is proposed to be a multistep process involving side chain cleavage of PK to the ring structure menadione, which is then readily converted to MK4 by ubiad1. The enzyme involved in side chain cleavage has yet to be identified but it thought to be localized to the intestine (5). The unique tissue distribution of PK and MK4 infers possible differences in regulation of conversion at the level of *Ubiad1*. Although expression of *Ubiad1* has been reported in mouse pancreas (48), we were unable to detect *Ubiad1* in this tissue. Interestingly, the pancreas also does not express *ggcx*, an enzyme critical to the vitamin K cycle. The lack of expression of *Ubiad1* in pancreas observed in our study suggests this tissue may not have the ability to produce or convert PK to MK4. However, the pancreas has high RNAase activity, to the extent that this tissue has been excluded in other studies examining vitamin K-related gene expression (20). Our sample preparation may have lead to significant RNA degradation resulting in undetectable levels of expression. Thus the origin of MK4 in pancreas cannot be determined solely by examining expression of *Ubiad1*.

Our data are consistent with prior studies that have demonstrated tissue-specific differences in MK4 and PK concentrations (4,41,49). A strength of this study is the exclusive use of PK as the dietary source of vitamin K, which mimics the human condition. Use of radiolabeled or stable isotopes in animal models have indicated that menadione, which is the predominant provitamin K form in rodent chow, also converts to MK4 (29,50,51). Menadione in the chow diet is continually being utilized in the conversion of MK4, which results in high MK4 concentrations in liver. In contrast studies in animals that are exclusively fed PK, results in accumulation of PK in the liver (4,52,53). Of note, menadione is not present in the food supply

nor is it allowed in the human diet due to concerns of hepatic toxicity (54). The purpose of this study was to examine the effect of inducing vitamin K deficiency using housing techniques and diet manipulation. We only used young animals in this study to determine if vitamin K deficiency was inducible. It is plausible that age is an important biological variable contributing to differences in response to dietary intervention, thus future studies may consider using aging mouse models to assess the role of age as a biological factor influencing vitamin K status. The inclusion of both male and female mice in our study is a substantial strength and is important when using preclinical models and the translatability to human conditions. Recent NIH mandates support the use of both sexes in animal studies to identify differences in response to various interventions and treatments (12). Our data support the need to use both sexes in vitamin K studies.

In conclusion, there are significant sex-specific differences in tissue concentrations of PK, MK4, and some long chain menaquinones. These results and concurrent sex-specific differences in gene expression of vitamin K-related genes indicate that male and female mice respond differently to dietary vitamin K manipulation. Recent discoveries of novel vitamin K dependent proteins that may function in multiple tissues other than liver highlight a need to understand tissue-specific differences in vitamin K metabolism, and identify potential sex specific differences in vitamin K dependent protein function or location. To test the hypothesis that tissue specificity may be indicative of vitamin K form-specific function, future research must consider sex-specific differences in preclinical animal models of vitamin K nutrition and metabolism.

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Table 1. Weight gain in male and female mice housed in either suspended or conventional caging fed a control or vitamin K deficient diet for 28 days.

	Male				Female						
Body Weight, g	Conventional		Suspended Wire		Conventional		Suspended Wire		*P*		
	D0	D28	D0	D28	D0	D28	D0	D28	Cage	Sex	Diet
Control	32.8±2.8	39.6±4.1	28.8±2.6	32.0±1.3	27.6±3.5	32.7±4.7	29.2±3.9	31.3±3.3	<0.01	0.37	<0.01
Deficient	31.3±2.0	35.3±3.9	33.0±2.9	32.7±0.9	27.1±3.6	30.4±4.6	24.9±1.4	25.6±1.7			

1. Values are mean ± SEM, n = 8 mice/group.
2. Overall P values reflect ANOVA for calculated change in bodyweight with cage, sex, and diet as factors.

Table 2. Serum and tissue PK, MK4, and detectable long-chain menaquinone concentrations of male and female mice fed a vitamin K control or deficient diet for 28 days.

Tissue	Vitamin K Form	Deficient Diet			Control Diet			*P*		
		Male	Female	*P*	Male	Female	*P*	Sex	Diet	Sex × diet interaction
Serum, pmol/mL	PK	ND	ND	-	0.80±0.2	0.90±0.2	0.88	0.91	<0.01†	0.91
	MK4	ND	ND	-	ND	ND	-	-	-	-
Liver, pmol/g	PK	8.3±2.1	15.4±3.9	0.04	26.0±6.5	51.0±12.7	<0.01*	<0.01†	<0.01†	<0.01†
	MK4	ND	ND	-	1.25±0.3	10.8±2.7	<0.01*	<0.01†	<0.01†	<0.01†
	MK9	33.1±8.3	5.8±1.4	0.05	37.1±9.3	15.8±3.9	0.14	0.02	0.48	0.72
	MK10	60.8±15.2	40.8±10.2	0.24	57.3±14.3	68.8±17.2	0.50	0.72	0.31	0.19
	MK11	351±87.8	200±49.9	0.02	244±60.9	401±100	0.01*	0.95	0.28	<0.01†
Kidney, pmol/g	PK	ND	ND	-	3.3±0.8	5.6±1.4	<0.01*	<0.01†	<0.01†	<0.01†
	MK4	2.0±0.5	10.4±2.6	<0.01*	16.3±4.1	122±30.6	<0.01*	<0.01†	<0.01†	<0.01†
	MK9	32.6±8.1	18.2±4.5	0.13	50.2±12.6	19.2±4.8	0.01*	<0.01†	0.17	0.22
	MK10	26.5±6.6	15.1±3.8	0.22	34.7±8.7	27.8±7.0	0.46	0.17	0.12	0.73
	MK11	48.3±12.1	28.9±7.2	0.02	52.2±13.1	25.0±6.2	<0.01*	<0.01†	0.99	0.48
Brain, pmol/g	PK	ND	ND	-	ND	ND	-	-	-	-
	MK4	12.3±3.1	19.5±4.9	0.19	27.2±6.8	111±27.7	<0.01*	<0.01†	<0.01†	<0.01†
Mesenteric adipose tissue, pmol/g	PK	3.75±0.9	7.0±1.8	0.29	10.1±2.5	25.0±6.2	<0.01*	<0.01†	<0.01†	0.01†
	MK4	6.75±1.7	8.4±2.1	0.72	10.2±2.5	44.4±11.1	<0.01*	<0.01†	<0.01†	<0.01†
Pancreas, pmol/g	PK	12.1±3.0	26.2±6.4	0.02	14.5±3.6	36.1±9.0	<0.01*	<0.01†	0.05	0.25
	MK4	40.6±10.1	46.7±11.7	0.89	127±31.7	337±84.3	<0.01*	<0.01†	<0.01†	<0.01†

1. Values are mean ± SEM, n = 16 mice/group.
2. ND: non-detectable. Concentration was below lower limit of detection for PK and MK4 (LLOD = 0.01 pmol/g) using an HPLC assay.
3. * Significant difference in Tukey HSD test between male and female mice within diet group at $P < 0.01$ adjusted alpha level.

4. † Indicates significant test within the ANOVA model for specific tissue and vitamin K form, at $P < 0.01$ adjusted significance level.

Table 3. Fecal menaquinone concentrations of male and female mice fed a control[2] or vitamin K deficient[3] diet for 28 days.

	Deficient Diet			Control Diet			*P*		
VK Form, pmol/g	Male	Female	*P*	Male	Female	*P*	Sex	Diet	Sex × diet interaction
PK	28.1±9.95	20.3±7.2	0.98	1920±678	2410±853	0.02	0.08	<0.01†	0.08
MK4	859±304	568±201	0.88	1080±381	1190±421	0.67	0.68	<0.01†	0.84
MK5	ND	ND	-	ND	ND	-	-	-	-
MK6	142±50.3	202±71.5	0.94	79.3±28.1	383±135	0.01*	0.07	0.75	0.06
MK7	44.4±15.7	55±19.4	0.95	29.1±10.3	ND	-	0.53	0.06	0.48
MK8	125±44.2	219±77.2	0.85	54.6±19.3	212±74.8	0.02	0.08	0.10	0.13
MK9	376±133	180±63.4	0.28	196±69.2	282±99.6	0.22	0.90	0.65	0.11
MK10	804±284	796±282	0.57	581±205	1210±429	0.01*	0.13	0.82	0.02
MK11	224±79.3	274±96.8	0.93	166±58.7	400±142	0.01*	0.07	0.92	0.05
MK12	13.8±4.9	23.3±8.2	0.52	15.2±5.4	32.8±11.6	0.07	0.08	0.59	0.39
MK13	78.1±27.6	59.4±21	0.87	60.3±21.3	104±36.8	0.19	0.41	0.40	0.30

1. Values are mean ± SEM, n = 8 mice/group.
2. ND: non-detectable. Concentration was below lower limit of detection for MK5 and MK7 (LLOD = 5.0 pmol/g) using an LC-MS assay.
3. * Significant difference in Tukey HSD test between male and female mice within diet group at $P < 0.01$ adjusted alpha level.
4. † Indicates significant test within the ANOVA model for specific tissue and vitamin K form, at $P < 0.01$ adjusted significance level.

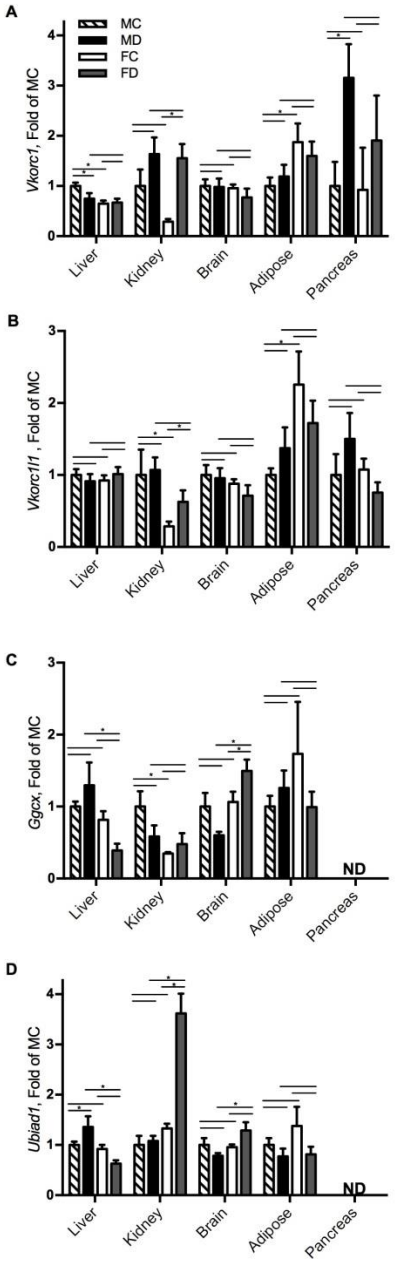

Figure 1. Gene expression of Vkorc1 (A), Vkorc1l1 (B), Ggcx (C), and Ubiad1 (D) in tissues of male and female C57Bl6 mice fed a PK deficient or control diet for 28d. Data are displayed as expression relative to the male control group and are presented as mean ± SEM, n = 16. Bars represent two-group comparisons of interest (MC to MD; FC to FD; MC to FC; MD to FD) with endpoints of each bar indicating the two groups being compared. Asterisk above a bar denotes significant difference at $P < 0.05$. ND: Not Detected with assay LLD < 20 copies/reaction in a 50 µL reaction, and LOQ at 98 copies/reaction. Vkorc1, vitamin K epoxide reductase complex 1; Vkorc1l1, vitamin K epoxide reductase complex 1 like 1; Ggcx, gamma glutamyl carboxylase; Ubiad1, ubiA domain containing protein 1.

Online Supporting Material

Supplemental Table 1. Composition of vitamin K deficient diet basal mix (Harlan Teklad TD 120060)

Ingredient	g/kg
Isolated Soy Protein	202.1
DL-Methionine	1.053
Sucrose	532.07
Corn Starch	157.904
Cellulose	52.63
Mineral Mix, AIN-76 (TD 170915)	36.84
Calcium Carbonate	11.84
p-Aminobenzoic Acid	0.116
Vitamin C, ascorbic acid, coated (97.5%)	1.074
Biotin	0.0004
Vitamin B-12 (0.1% in mannitol)	0.0316
Calcium Pantothenate	0.069
Choline Dihydrogen Citrate	3.68
Folic Acid	0.0021
Inositol	0.116
Niacin	0.1042
Pyridoxine HCl	0.0232
Riboflavin	0.0232
Thiamin HCl	0.0232
Vitamin A Palmitate (500,000 IU/g)	0.0421
Vitamin D3, Cholecalciferol (500,000 IU/g)	0.0046
d,l-α-Tocopherol Acetate (500 IU/g)	0.253
Macronutrient Distribution	% kcal from
Protein	20.5
Carbohydrate	79.0
Fat	0.5

Online Supporting Material

Supplemental Table 2. Primers for genes of interest.

Gene name	Gene symbol	mRNA Refseq accession#	Reverse primer	Forward primer
Vitamin K epoxide reductase complex subunit 1	*Vkorc1*	NM_178600.2	GGGAACTCAGCACCAGTAGG	CAGCATATTTGGTTGCCTGTT
Vitamin K expoxide reductase complex subunit 1 like 1	*Vkorc1l1*	NM_001001327.2	GTTCACAGCCCCAAACAAGT	GGCAATCTGGGCTATTTTGA
Gamma glutamyl carboxylase	*Ggcx*	NM_019802.5	GGCACGTGAGCATTCTTCTT	ATGGTTTGTTGGCCTTTCAG
ubiA prenyl transferase domain containing 1	*Ubiad1*	NM_027873.2	CCAGGGCCACATACTTGAAT	GTCCGCTCTGAAATTGGAAC
Glyceraldehyde 3 phosphate dehydrogenase	*Gapdh*	NM_008084.3	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT

Online Supporting Material

Supplemental Table 3. Fluid replacement in mice housed in conventional or suspended wire cages for 28 days.

	Male			Female		
Diet	Conventional	Suspended Wire	*P*	Conventional	Suspended Wire	*P*
Control	0/8	8/8	0.04	1/8	8/8	0.01
Deficient	1/8	8/8		0/8	7/8	

1. Data are presented as (number of animals that received fluid replacement)/(total n per group).
2. P values represent the results of the overall ANOVA model including cage, sex, and diet as factors.

5. Atorvastatin decreases renal menaquinone-4 formation in C57Bl6 male mice

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4. Abbreviations: APCI LC-MS: atmospheric pressure chemical ionization liquid chromatography mass spectrometry; FPP: Farnesyl pyrophosphate; GPP: Geranyl pyrophosphate; GGPP: Geranylgeranyl pyrophosphate; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; IPP: isopentenyl pyrophosphate; MK4: menaquinone-4;

Abstract

Background: Menaquinone-4 (MK4), a vitamin K metabolite, is converted from phylloquinone through a process that requires intermediates of endogenous cholesterol production. Recent evidence suggests MK4 formation is impaired in chronic kidney disease due to increase use of statins (HMG-CoA reductase inhibitors). However, this has yet to be demonstrated *in vivo*.

Objective: The purpose of this study was to determine the effect of statin treatment on MK4 formation in young and old male mice.

Methods: Four month (n=32) and 20-month old (n=32) C57BL6 male mice were randomly assigned to either a diet containing 300mg atorvastatin/kg diet and 2.67±0.33 mg phylloquinone/kg or a control diet containing 2.77±0.02 mg PK/kg for 8 weeks. During week 8, all mice received deuterium-labeled phylloquinone in the diet. Labeled and unlabeled phylloquinone and MK4 in in liver, kidney, brain, and intestine were measured by HPLC-MS. HMG-CoA reductase expression gene expression was quantified by reverse transcriptase-PCR. Tissue MK4 and phylloquinone concentrations were compared between statin treatment groups using general linear models.

Results: Mice given statins had lower total MK4 and deuterium-labeled MK4 kidney concentrations compared to mice not given statins (P = 0.02 and 0.04, respectively). In statin-treated mice, kidney MK4 concentration was reduced by 41% ($P \leq 0.05$), and percent deuterium-labeled MK4 was reduced by 46.5% compared to mice not given statins (p=0.001). MK4 concentrations did not differ between groups for any other tissue measured. There was no effect of statins on phylloquinone tissue concentrations. Hepatic HMG-CoA reductase expression increased in response to statin treatment by 1.5-fold in both young and old mice (all P values<0.05).

Conclusion: In male mice, statin treatment effectively reduced endogenous MK4 formation in the kidney, but not other organs. These observations are consistent with our hypothesis that cholesterol metabolism is involved in the generation of MK4. Further research is needed to understand potential regulatory mechanisms and the unique functions of MK4.

Keywords: cholesterol intermediates, geranylgeranyl pyrophosphate, menaquinones, phylloquinone, statins, vitamin K

Introduction

Vitamin K represents a group of vitamers essential for the carboxylation of vitamin K-dependent proteins involved in various physiological processes, including support of blood coagulation and regulation of calcification (1). There are two forms of vitamin K: (1) phylloquinone; and (2) menaquinones (MKn), where the n indicates the number of isoprenoid units in the side chain. All vitamin K forms are bioactive and provide the reducing agent for the carboxylation of vitamin K-dependent proteins (2,3). A unique characteristic of vitamin K metabolism is the tissue-specific differences in conversion of dietary phylloquinone to menaquinone-4 (MK4) (4). Furthermore, tissue concentrations of MK4 respond to dietary manipulation of phylloquinone. Identifying factors that influence the conversion of phylloquinone to MK4 will provide insight into the potential function of MK4, including regulation of pro-inflammatory cytokines, cell growth, and protection against oxidative stress (5–8).

Vitamin K metabolism and cellular cholesterol metabolism are linked because endogenous MK4 production requires an isoprenoid side chain and geranylgeranyl pyrophosphate (GGPP). GGPP is synthesized from isopentenyl pyrophosphate (IPP) and farnesyl pyrophosphate (FPP), linking (9). The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are potent inhibitors of endogenous cholesterol synthesis. Therefore, statins inhibit the biosynthesis of isoprenoid intermediates, including GGPP (10). *In vitro* studies have demonstrated statins regulate the key enzyme converting dietary phylloquinone to MK4, by limiting GGPP availability (11). In addition, supplementary GGPP rescues enzymatic activity and MK4 production, illustrating an interaction between cholesterol synthesis and MK4, and potential regulation of MK4 formation(11). The contribution

of cholesterol precursors to tissue concentrations of MK4 or the potential regulatory mechanisms involving enzymatic activity in both the vitamin K metabolic pathway and cholesterol synthesis pathway are not well understood. The objective of this study was to determine the effect of atorvastatin on endogenous production of MK4 in C57Bl6 male mice provided a diet with adequate concentrations of PK.

Methods

Animals and diets

20 month-old retired male breeders and 4 month-old C57BL/6NCrl VAF/Plus male mice were obtained from Charles River Laboratory (Wilmington, MA). Expanding on the previous C57Bl6 mouse model used for dietary manipulation studies (12), we chose to optimize an aging mouse model for comparison to the widely used young adult mice (13). All mice were acclimated on AIN-93G diet (TD.94045, Harlan Teklad) for 1 week, and transitioned to a control diet containing 2.77±0.02 mg phylloquinone /kg (TD.120060, Harlan Teklad). The experimental diet was a modification of TD.97053 (**Supplemental Table 1**). A 2x2 factorial design was used to evaluate age and diet effects. Male mice were weight-matched and randomized to a diet containing 300 mg atorvastatin/kg diet and 2.67±0.33 mg phylloquinone /kg or a control diet containing 2.77±0.02 mg phylloquinone /kg (TD.120060, Harlan Teklad) for 8 weeks *ad libitum*, resulting in 4 groups of 8 mice each. Atorvastatin dosing was targeted for less than 3mg/kg body weight/day, to minimize risk of liver toxicity during chronic exposure (14). During week 8, all mice received deuterium-labeled phylloquinone in the diet. Deuterium-labeled collard greens were hydroponically grown in a controlled environment at the USDA-Agricultural Research Service Children's Nutrition Research Center in Houston, Texas, as previously described (15).

Body weights were measured weekly. Mice were maintained in AAALAC-accredited facilities with an environmentally controlled atmosphere (22ºC, 45% relative humidity, 15 air changes of 100% fresh hepa-filtered air per hour and a 12/12-hour light/dark cycle). Animals were observed daily for clinical signs of distress or disease. At the end of the experiment, mice were euthanized with carbon dioxide and subsequent cervical dislocation, followed by tissue collection. Tissues of interest (brain, liver, intestine, and kidney) were harvested, frozen immediately in liquid nitrogen, and stored at -80ºC until time of analysis. All protocols were approved by the HNRCA Tufts University Animal Care and Use Committee.

Mass spectrometry

Tissues (0.10-0.20 g wet weight) were homogenized in PBS using a Powergen homogenizer (Fisher Scientific, Pittsburgh, PA). Concentrations of unlabeled phylloquinone, deuterium-labeled phylloquinone, unlabeled MK4, and deuterium-labeled MK4 were measured in tissue homogenates by atmospheric pressure chemical ionization LC-MS (APCI-LC/MS), as described elsewhere (16). Data were collected using Agilent Chemistation software (Version C.01.05). Efforts to measure isoprenoid intermediates including IPP, GPP, FPP, and GGPP by UPLC-MS/MS were attempted using a modified method of Henneman et al. 2011 (17), but we were unable to detect any of the isoprenoid intermediates in tissue.

RT-quantitative PCR

We profiled the expression of the following genes encoding enzymes involved in vitamin K and cholesterol metabolism: (1) vitamin K epoxide reductase complex subunit 1(*vkorc1*); (2) vitamin K epoxide reductase complex subunit 1 like 1 (*vkorc1l1*); (3) gamma glutamyl carboxylase (*ggcx*); (4) UbiA prenyl transferase domain-containing 1 (*ubiad1*); HMG-CoA Reductase (*hmgcr*); and LDL receptor (*ldlr*). All primer/probe sets for real-time PCR were

TaqMan gene expression assays (Applied Biosystems, Foster City, CA). Total RNA was isolated from tissues (kidney, liver, brain, and intestine) using Trizol reagent and the PureLink RNA Mini Kit (Ambion Life Technologies, Grand Island, NY) following the manufacturer's instructions. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using TaqMan Universal Master Mix II, no UNG and a QuantStudio 6 Flex System (Applied Biosystems, Foster City, CA). Relative expression was calculated using the 2-ΔΔCt method and statistical analyses were performed on ΔCt values. *Gapdh* was used as the control gene. All primer information is provided in **Supplemental Table 2**.

Plasma lipid concentrations

Plasma triglycerides were quantified using a kit from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Plasma total cholesterol was quantified using the Cholesterol/Cholesterol Ester Quantitation Kit from Abcam (Cambridge, MA). Both assays were carried out as per manufacturers' instructions.

Statistical analyses

Sample size calculations were based on previously reported data of deuterium-labeled phylloquinone tissue concentrations, using parameters of α=0.05; power=80%; effect size = 209 pmol/g and standard deviation =113.2 pmol/g for liver concentrations of phylloquinone. Accounting for multiple group comparisons, 8 animals were required per group. We initially evaluated the effects of age, statin treatment, and tissue and their interactions (for example age*tissue) on total concentrations and % labelled phylloquinone and MK4 using a three-way ANOVA model, With respect to total and % labelled phylloquinone, we detected an interaction between age and tissue. We next conducted tissue-specific analyses to determine the effect of

age on tissue phylloquinone. Because there was no effect of statin on tissue phylloquinone (all *P* values > 0.09), the statin treatment groups were collapsed. With respect to total and % labelled MK4, we detected an interaction between tissue and statin treatment. We next conducted tissue-specific analyses to determine the effect of statin treatment on tissue MK4. Because there was no effect of age on tissue MK4 (all *P* values > 0.54), the age groups were collapsed. The ANOVA model was assessed using diagnostics for assumptions of homogeneity of variance. No outliers were found that influenced significance in the full model. Data were tested for normality using the Shapiro-Wilk test. Significance was determined by $P < 0.05$, and all analyses were carried out using SAS v 9.4 (Cary, NC). Data are reported as means ± SEM.

Results

Body weight

Following eight weeks of atorvastatin treatment, there was no difference in body weight between atorvastatin treatment groups and the control groups ($P = 0.50$). Only changes in body weight were observed between the young and older mice, with older mice having significantly higher body weight (**Figure 1**, $P < 0.01$).

Tissue concentrations of phylloquinone

Total and percent labeled phylloquinone differed between age groups (all *P* values < 0.05), but not between atorvastatin treatment groups (all *P* values > 0.09). Older mice had significantly higher total phylloquinone in liver, but there was no significant difference in total phylloquinone concentrations between age groups in kidney and intestine (**Table 1**). Although older mice had approximately 40 percent more total phylloquinone, they had 50 percent less labeled phylloquinone in liver, and approximately 30 percent less labeled phylloquinone in

intestine when compared to young mice ($P < 0.01$). There were no differences observed in kidney percent labeled phylloquinone between young and old mice.

Tissue concentrations of menaquinone-4

Total and percent labeled MK4 differed by atorvastatin treatment ($P < 0.04$), but did not differ by age (all P values > 0.54). Total MK4 and percent deuterium labeled MK4 were reduced by approximately 40 percent in the kidney of atorvastatin-treated mice (**Table 2**). There was no effect of statin treatment on total MK4 (all P values > 0.31), or percent deuterium labeled MK4 (all P values > 0.78) in brain or intestine. There was no detectable MK4 in liver.

Circulating cholesterol and triglycerides

After eight weeks on atorvastatin, plasma total cholesterol was not significantly different in the atorvastatin-treated groups (**Supplemental Figure 1,** all P values > 0.52). Plasma triglycerides were reduced by approximately 20 percent in young mice treated with atorvastatin ($P = 0.004$). There was no effect of atorvastatin treatment on circulating triglycerides in older mice (**Supplemental Figure 2,** $P = 0.67$).

Gene expression

Because statins exert their pharmacological effect by inhibiting HMG-CoA reductase in the liver, we evaluated the effect of atorvastatin treatment on gene expression of *hmgcr* and *ldlr*, genes directly involved in cholesterol synthesis and recycling. In liver, atorvastatin increased expression of *hmgcr* in both young and old mice, compared to all mice without atorvastatin treatment (**Figure 2**). There was no effect of age or atorvastatin on hmgcr expression in any other tissue. *Ldlr* expression in kidney increased in older mice receiving atorvastatin compared to older mice not receiving atorvastatin. Older mice not treated with atorvastatin had increased *ldlr* expression in kidney compared to young mice not treated with atorvastatin. There was no effect

of age or statin treatment on *ldlr* expression in any other tissue. There was no effect of statin treatment or age in the expression of *vkorc1*, *vkorc1l1*, or *ggcx*, in any tissue (liver, kidney, brain, or intestine). Expression of *ubiad* in all older mice was reduced by approximately 50 percent compared to all younger mice in kidney. There was no effect of statin treatment on expression of *ubiad* in kidney.

Discussion

We evaluated the effect of atorvastatin treatment on MK4 production in young and old male mice to test the hypothesis that tissue-specific MK4 synthesis and cholesterol metabolism are linked. We hypothesized that atorvastatin would reduce MK4 in tissues specifically kidney, which is capable of converting dietary phylloquinone to MK4, and where both forms are present. In other tissues measured including brain, liver, and intestine, we saw no effect of statin treatment on MK4. In brain, where the sole vitamin K form is MK4, there was no effect of statin treatment which is indicative of the physiological properties of atorvastatin. Lipophilic statins including atorvastatin do not readily cross the blood brain barrier, unless it undergoes a chemical modification of lactonization increasing its permeability (18). Therefore the lack of an effect on MK4 is consistent with the inability of atorvastatin to enter the brain. In contrast to brain, where MK4 is the primary form, liver lacks the ability to convert dietary phylloquinone to MK4 resulting in phylloquinone as the primary form. Our results are consistent with previous studies as we were not able to detect MK4 (12,19). There are two hypothesized routes of conversion, one that takes place within a specific tissue, and an alternative route where the side chain from phylloquinone is removed within intestinal cells and the naphthoquinone ring is then transported to a destination tissue for MK4 formation (20,21). In our study, we examined intestinal concentrations of phylloquinone and MK4 and found no effect of atorvastatin on MK4.

Collectively, the data challenge current theories and indicate that conversion may not occur exclusively from phylloquinone.

Our finding that atorvastatin reduced MK4 formation in the kidney may be clinically relevant as recent evidence proposes an interaction between calcification associated with chronic kidney disease and MK4 formation. The kidney is a site of conversion, responds to dietary phylloquinone manipulation, and responds to statin treatment as previously demonstrated and supported by the results of our study (12,22). Recently, statin use in humans with chronic kidney disease was independently associated with calcification, suggesting that therapeutic doses of statins may accelerate calcification by depleting tissue concentrations of MK4, thus increasing risk of vascular calcification (23). In a rat model of chronic kidney disease with vascular calcification, animals with apparent chronic kidney disease have significantly higher kidney MK4 concentrations, which may be indicative of changes in the efficiency of conversion in chronic kidney disease or that utilization of MK4 may be impaired in the disease state, further exacerbating vascular calcification and disease pathology (22). However, the mechanisms by which vitamin K and/or vitamin K dependent proteins modulate disease progression are unclear.

The isoprenoid intermediate in the cholesterol synthesis pathway, GGPP, is required for endogenous synthesis of MK4, and has been demonstrated using *in vitro* models (11). Current hypotheses suggest the link between cholesterol synthesis and MK4 formation to be antagonistic such that, as MK4 formation increases, GGPP is depleted, which results in greater conversion of FPP to GGPP and ultimately reduces cholesterol synthesis. In contrast, a reduction in MK4 formation may shunt more GGPP to cholesterol synthesis and potentially lead to cholesterol accumulation. It is plausible the differences in MK4 concentrations observed are attributed to decreased concentrations of GGPP as a direct result of atorvastatin, and further exacerbated by

an increase in shunting of GGPP towards cholesterol synthesis to combat the atorvastatin interference. Tissue concentrations of cholesterol were not quantified nor were cholesterol intermediates/metabolites, limiting our interpretation that GGPP shunting occurs.

The conversion of MK4 is proposed to be a multi-step process involving side chain cleavage from dietary phylloquinone, and prenylation of the naphthoquinone ring to produce MK4 by the prenyltransferase *ubiad* (11,24–26). Nickerson et al. have reported that HMG-CoA reductase binds to *ubiad* impacting MK4 formation (9). As HMG-CoA reductase is the primary target for statins, recent *in vitro* studies have examined the role of various statins on *ubiad* activity and MK4 production. It was demonstrated that specific lipophilic statins are more likely to bind directly to *ubiad*, altering enzyme functionality, compared to aqueous statins that do not (11). However, the magnitude of these effects *in vivo* and potential regulatory mechanisms are unknown. We quantified relative gene expression of *ubiad* in various tissues, and saw a reduction in expression by approximately 50 percent in older mice compared to younger, but no effect of atorvastatin treatment. That an effect on gene expression in kidney did not change in response to atorvastatin yet there was a reduction in kidney MK4 suggests that *ubiad* is not responsible for regulating the conversion of dietary phylloquinone to MK4. This observation further supports our hypothesis that the cholesterol synthesis pathway, and not *ubiad*, is involved in MK4 formation. While the observation that there is an age-related change *ubiad* expression is novel, there was no corresponding age-related difference in MK4 formation so the significance of this observation is not known.

Strengths of this study include the use of a well-characterized mouse model that has previously been optimized for dietary PK interventions. Additionally, we studied older and younger mice which is consistent with current recommendations of the National Institutes of

Health to utilize aging animal models. Finally, we were able to induce changes in MK4 formation without deleterious effects of statin treatment, such as hepatic toxicity (27). However, the following limitations merit consideration. We were unable directly measure isoprenoid precursors in tissues, or tissue cholesterol concentrations. We limited our study to males, and generalizability to females is uncertain as sex specific differences in mice have been identified (12). The effect of atorvastatin treatment on plasma cholesterol was minimal, and may be related to the low dose of atorvastatin in the diet. Statins manipulate LDL cholesterol and have little to no effect on HDL cholesterol, the major component of circulating plasma lipoproteins in mice, which may explain our observations and also explain why statins are less effective in reducing total cholesterol in rodent models compared to humans (28,29).

In summary, we found atorvastatin can interfere MK4 formation in the kidney. However we have no direct evidence that cholesterol metabolism was modified through the use of atorvastatin. The cholesterol effect at this point in time is by inference only. The dynamic regulation of endogenous cholesterol synthesis and lack of an effect on cholesterol metabolism suggests that isoprenoid intermediates may be shunted away from MK4 formation to maintain cellular cholesterol concentrations, however the effect on tissue physiology and function remains unclear as the role of MK4 in kidney has yet to be determined. The clinical relevance of vitamin K forms, their function, and the conversion of phylloquinone to MK4 in different tissues has yet to be determined. Therefore, future studies are necessary to advance our understanding of vitamin K metabolism in the kidney and other tissues.

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Table 1. Effect Age on Tissue Concentrations of Total PK (pmol/g) and Percent Labeled PK (%) in young (4mo) and old (22mo) C57Bl6 Male Mice.

	Total PK (pmol/g)			Percent Labeled PK (%)		
Tissue	Young	Old	p-value [b]	Young	Old	p-value [b]
Kidney [a]	14.1±2.33	17.4±1.97	0.30	78.4±6.48	70.5±3.76	0.31
Liver [a]	16.4±1.44	25.0±2.42	0.005	48.1±5.00	21.3±4.83	<0.001
Intestine [a]	18.1±4.76	9.78±2.11	0.11	85.9±6.37	61.4±6.23	0.01

[a] Data presented as mean ± SEM, n = 16 mice/group.
[b] Significances at $p < 0.05$.

Table 2. Effect Atorvastatin on Tissue Concentrations of Total MK4 (pmol/g) and Percent Labeled MK4 (%) in young (4mo) and old (22mo) C57Bl6 Male Mice.

	Total MK4 (pmol/g)			Percent Labeled MK4 (%)		
Tissue	Statin +	Statin –	p-value [b]	Statin +	Statin –	p-value [b]
Kidney[a]	13.8±2.65	23.4±3.95	0.05	20.7±3.8	35.7±3.2	0.008
Brain[a]	13.9±1.49	19.7±1.75	0.31	19.8±3.5	19.7±1.8	0.96
Intestine[a]	2.88±0.81	3.62±0.74	0.51	22.9±5.6	24.7±3.5	0.78

[a] Data presented as mean ± SEM, n = 16 mice/group.
[b] Significances at $p < 0.05$.

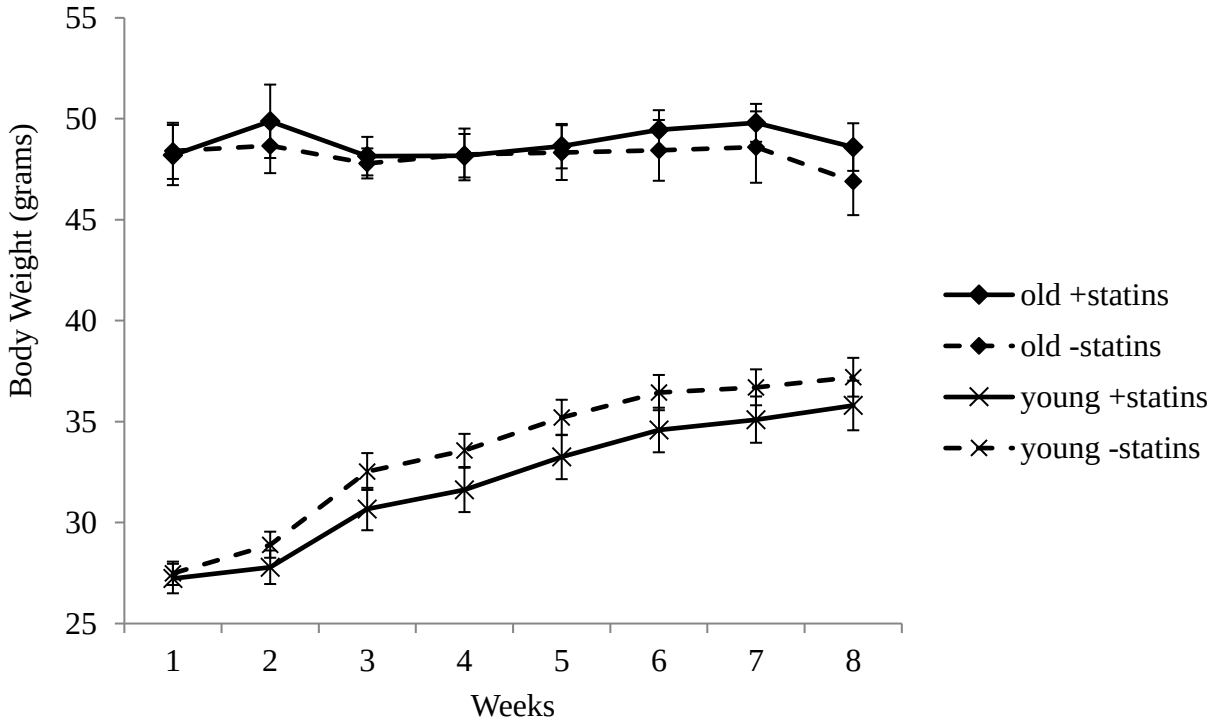


Figure 1. Effect of atorvastatin on weekly body weight in male C57Bl6 mice of 4mo and 22mo of age. Analyzed by repeated measures ANOVA. Data presented as means ± SEM, n = 8 mice/group. Significant difference between age groups ($p < 0.01$), no significant difference by atorvastatin treatment.

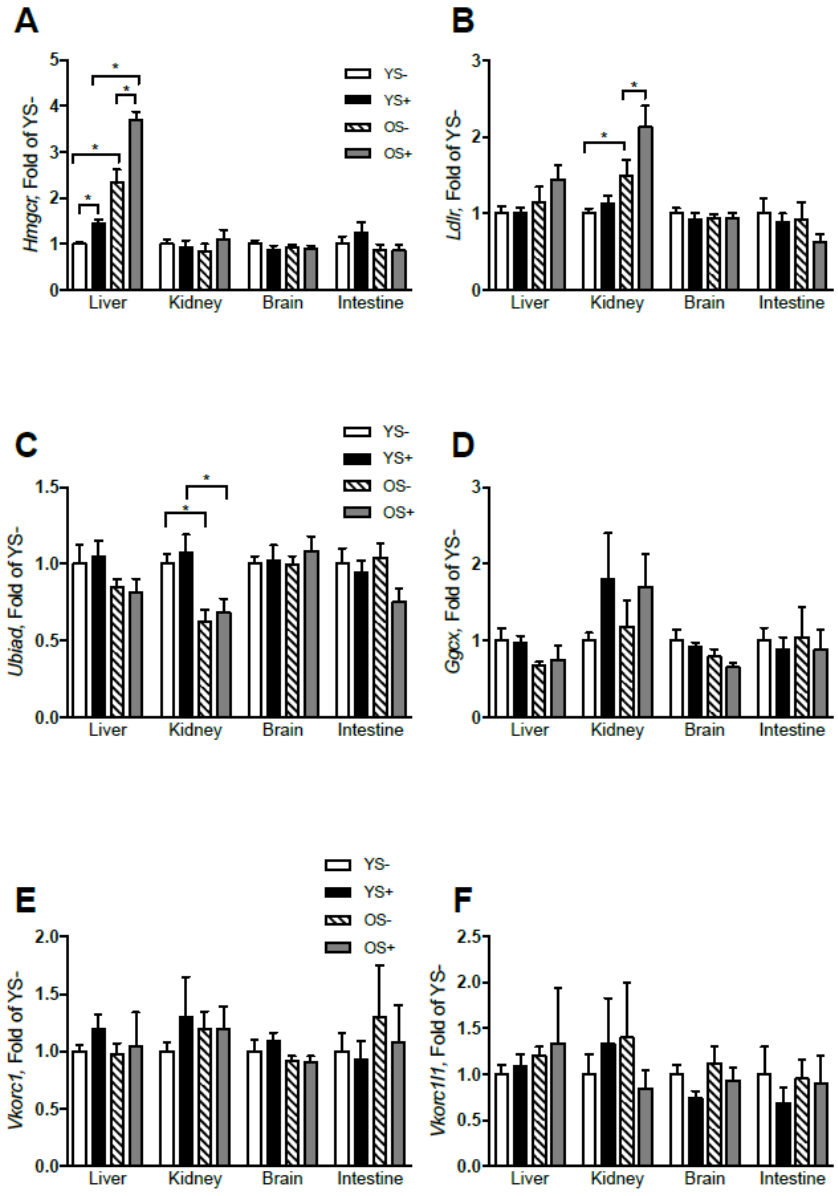


Figure 2. Gene expression of *hmgcr* (A), *ldlr* (B), *ubiad* (C), *ggcx* (D), *vkorc1* (E), and *vkorc1l1* (F) in tissues of male 4mo and 22mo old C57Bl6 mice on PK control diet with or without atorvastatin for 8 weeks. Data are displayed as expression relative to the young control diet group (YS-) and are presented as mean ± SEM, n = 8. Bars represent two-group comparisons of interest with endpoints of each bar indicating the two groups being compared. Asterisk above a bar denotes significant difference at $P < 0.05$. young on control diet, no atorvastatin (YS-), young + atorvastatin (YS+), old on control diet, no atorvastatin (OS-), old + atorvastatin (OS+). Limit of quantification (LOQ) at 98 copies/reaction. *vkorc1*, vitamin K epoxide reductase complex 1; *vkorc1l1*, vitamin K epoxide reductase complex 1 like 1; *ggcx*, gamma glutamyl carboxylase; *ubiad1*, ubiA domain containing protein 1; *hmgcr*: HMG-CoA reductase; *ldlr* : low density lipoprotein receptor.

Online Supporting Material

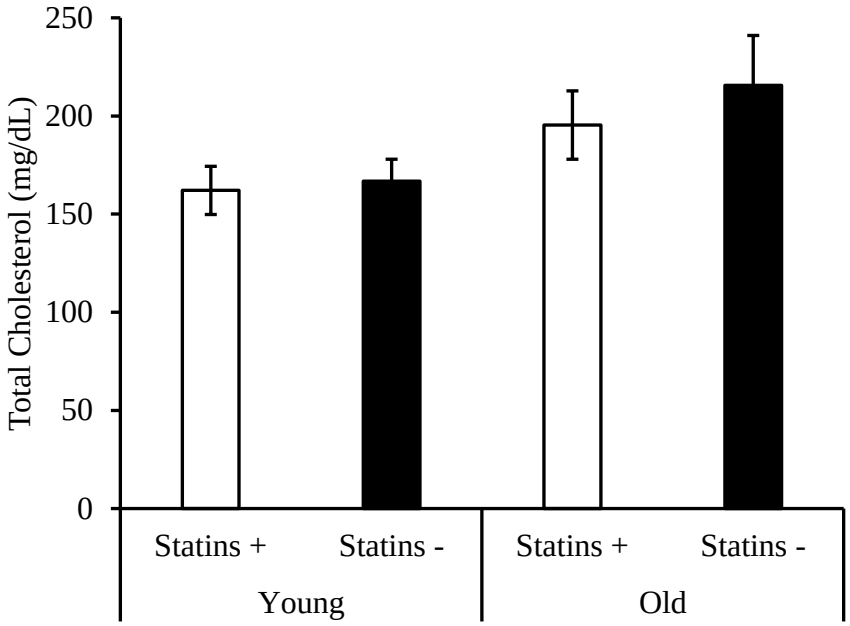
Supplemental Table 1. Composition of vitamin K deficient diet basal mix (Harlan Teklad TD 120060)

Ingredient	g/kg
Isolated Soy Protein	202.1
DL-Methionine	1.053
Sucrose	532.07
Corn Starch	157.904
Cellulose	52.63
Mineral Mix, AIN-76 (TD 170915)	36.84
Calcium Carbonate	11.84
p-Aminobenzoic Acid	0.116
Vitamin C, ascorbic acid, coated (97.5%)	1.074
Biotin	0.0004
Vitamin B12 (0.1% in mannitol)	0.0316
Calcium Pantothenate	0.069
Choline Dihydrogen Citrate	3.68
Folic Acid	0.0021
Inositol	0.116
Niacin	0.1042
Pyridoxine HCl	0.0232
Riboflavin	0.0232
Thiamin HCl	0.0232
Vitamin A Palmitate (500,000 IU/g)	0.0421
Vitamin D3, Cholecalciferol (500,000 IU/g)	0.0046
Vitamin E, DL-alpha Tocopherol Acetate (500 IU/g)	0.253
Macronutrient Distribution	% kcal from
Protein	20.5
Carbohydrate	79.0
Fat	0.5

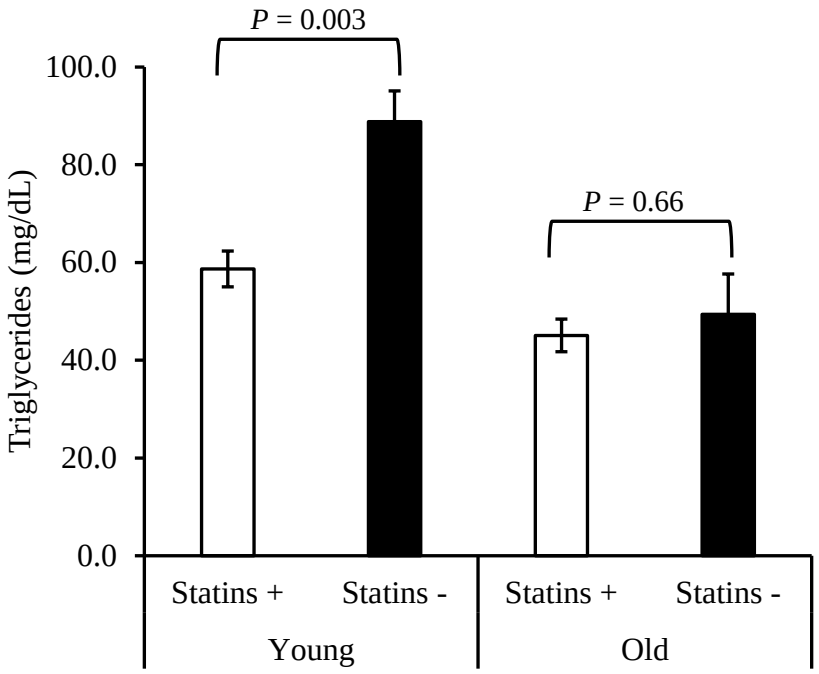
Supplemental Table 2. Primers for genes of interest.

Gene name	Gene symbol	Refseq accession#	Assay ID[1]
Vitamin K epoxide reductase complex subunit 1	*vkorc1*	NM_178600.2	Mm00724262_m1
Vitamin K expoxide reductase complex subunit 1 like 1	*vkorc1l1*	NM_001286382.1	Mm01197731_g1
Gamma glutamyl carboxylase	*ggcx*	NM_019802.5	Mm00517274_m1
ubiA prenyl transferase domain containing 1	*ubiad1*	NM_027873.2	Mm00503616_m1
Glyceraldehyde 3 phosphate dehydrogenase	*gapdh*	NM_008084.3;NM_001289726.1	Mm99999915_g1
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	*hmgcr*	NM_008255.2	Mm01282499_m1
low density lipoprotein receptor	*Ldlr*	NM_001252659.1;NM_001252658.1;NM_010700.3	Mm01177349_m1

[1]All primers supplied by Taqman (Life Technologies, Carlsbad, CA).



Supplemental Figure 1.
Circulating plasma total cholesterol concentrations in young (4mo) C57Bl6 mice, atorvastatin + (n=8) and atorvastatin – (n=8), and in old (20mo) C57Bl6 mice, atorvastatin + (n=8), and atorvastatin – (n=8). No significant difference in total cholesterol concentrations between groups, all *P* values > 0.52.



Supplemental Figure 2.
Circulating plasma triglyceride concentrations in young (4mo) C57Bl6 mice, statin + (n=8) and statin – (n=8), and in old (20mo) C57Bl6 mice, atorvastatin + (n=8), and atorvastatin – (n=8). * Significant difference between atorvastatin treatment in young mice only, $P = 0.003$, no difference between atorvastatin treatment in old mice, $P = 0.66$.

6. Summary and Discussion

Summary

All vitamin K forms share the function as an enzyme co-factor for y-glutamate carboxylase, providing functionality for various vitamin K dependent proteins (1). Emerging evidence suggests novel physiological roles for vitamin K extending beyond its traditional role in hemostasis. Vitamin K occurs in two forms: phylloquinone, which is the predominant dietary form found in green leafy vegetables and vegetable oils (1); and menaquinones, which differ structurally from phylloquinone in the length and saturation of their side chain, and are found primarily in animal-based and fermented foods (2). The current dietary recommendations for vitamin K are limited to Adequate Intakes (AI) set at 120 ug and 90 ug/d of dietary phylloquinone for adult men and women, respectively (3). Current recommendations are based on median self-reported dietary phylloquinone intakes for U.S. adults using National Health and Nutrition Examination Survey (NHANES) data collected in 1988-1994, and food composition databases available at that time. To date, menaquinones have not been systematically quantified in the food supply limiting the AI to phylloquinone, even though all forms of vitamin K function as an enzyme cofactor. Given the reliance on dietary phylloquinone intakes in establishing AI for the U.S. population, it is important to capture recent trends in consumption and to identify subgroups most like to be a risk of low phylloquinone intakes. Publically available datasets including current NHANES surveys provide great insight into dietary patterns and changes over time. Thus it is imperative to evaluate more recent surveys to identify trends in dietary patterns and their effect on phylloquinone intakes.

The contribution of dietary phylloquinone to vitamin K status has been well-studied. However, much of the inter-individual variability in vitamin K status is not explained by dietary

intakes. Non-dietary factors, including age, sex, and bioavailability, need to be addressed in order to identify additional functions, and ultimately better define current recommendations. Because current biomarkers of vitamin K status do not necessarily reflect individual tissue content, animal models are required to identify the physiological impact of these factors on vitamin K metabolism and status. Preclinical models have long enabled researchers to identify biological functions of specific nutrients, proteins, and more, as well as facilitated the discovery of various molecular mechanisms governing these biological functions. In order to characterize and understand the role of vitamin K adequacy or insufficiency, and further explore sex-specific and age-specific differences in vitamin K metabolism, we optimized the C57Bl6 mouse model for acute and chronic diet manipulation studies.

While phylloquinone is the predominant dietary form, menaquinones are primarily synthesized by gut bacteria potentially contributing to vitamin K status (2). However, the extent to which bacterially-synthesized or dietary menaquinones contribute to vitamin K nutriture is unclear. Menaquinone-4 is unique in that it is not bacterially synthesized and is produced endogenously through conversion of dietary phylloquinone (4,5). Menaquinone-4 appears to have unique functions beyond that of an enzyme co-factor. However, the mechanisms of action and their effect on vitamin K requirements have yet to be elucidated. Endogenous menaquinone-4 production is linked to cholesterol metabolism because it requires an isoprenoid side chain, geranylgeranyl pyrophosphate, which is synthesized from isopentenyl pyrophosphate an intermediate in the cholesterol synthesis pathway (6). The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are potent inhibitors of endogenous cholesterol synthesis. Therefore statins inhibit isoprenoid intermediates, including geranylgeranyl pyrophosphate, as well as the biosynthesis of cholesterol and potentially

menaqinone-4 (7). However, the effect of statins on tissue-specific conversion and menaquinone-4 formation is unclear.

The objective of this thesis research was to address these knowledge gaps by leveraging the National Health and Nutrition Examination Survey, a large publically available data set to examine usual dietary intakes of phylloquinone in U.S. adults, and by optimizing a mouse model to determine the physiological effects of diet manipulation, sex, and age on vitamin K status and metabolism. In addition, capitalizing on the model that was successfully used to examine sex-specific effects in response to diet manipulation, we used statins as a tool to antagonize menaquinone-4 formation.

To meet these objectives, analysis of the National Health and Nutrition Examination Survey from 2011-2012, the most recent dataset with dietary phylloquinone data, was evaluated to determine the usual dietary intake of phylloquinone in U.S. adults. This NHANES dataset is unique in that various food databases including the USDA What We Eat in America food database (WWEIA), the Food and Nutrition Database or Dietary Studies (FNDDS), and the Food Patterns Equivalents Database (FPED) are linked providing an extensive review of reported dietary information. Additionally, dietary phylloquinone was quantified for multiple dietary recalls. In comparison, the NHANES III dataset which was used to develop the current Dietary Reference Intakes (DRI), evaluated dietary phylloquinone as part of a supplementary analysis, applied available food composition databases which were limited in breadth of food items and lacked translatability to the Dietary Guidelines for Americans that included the food pyramid at the time, and utilized only a single dietary recall. Our study overcomes some of the limitations of the NHANES III dataset by using multiple food composition databases, and the National Cancer

Institute method to estimate usual dietary phylloquinone intake accounting for multiple dietary recalls.

This analysis has revealed that approximately 43% of men and 63% of women met the AI (120 and 90 µg/day, respectively) for phylloquinone, with declines since 1988-1994 noted among men especially in the older age groups (51-70 and ≥71 y). Vegetables were the predominant dietary source of phylloquinone. However, in individuals who consumed less than 2 cups/cup equivalents of vegetables per day, mixed dishes which included meat and poultry mixed dishes, grain based dishes, Asian mixed dishes, Mexican mixed dishes, pizza, sandwiches, and soups, were the second largest food group to contribute to dietary phylloquinone intake. Within mixed dishes, grain based dishes such as pasta/rice dishes, turnovers, and mac and cheese, were the predominant foods contributing to phylloquinone intake, followed by sandwiches and pizza. This analysis revealed that the usual intake of phylloquinone is consistent with current recommendations, but the food sources contributing to phylloquinone intake are changing. Our observations demonstrate the importance of diverse and comprehensive food databases in order to accurately capture dietary patterns. As dietary patterns shift, and alternative food sources become more significant contributors to dietary vitamin K, the impact of dietary menaquinones needs to be considered. Advancing the dietary recommendations for vitamin K requires examination of vitamin K bioavailability of all forms, and among various food matrices. This will provide greater insight in requirements and hopefully lead to development of a Recommended Dietary Allowance, a more robust recommendation within the DRIs.

To better understand the physiological relevance of vitamin K adequacy or insufficiency, and further explore sex-specific and age-specific differences in vitamin K status, the C57Bl6 mouse model was optimized to examine the role of these biological factors in response to dietary

vitamin K manipulation. Sex specific differences in the tissue distribution of vitamin K forms have been documented in rats (8–11). However there is limited information available regarding the sex-specific responses to vitamin K dietary manipulation or vitamin K metabolism in mice. In this study, female mice on the control diet with adequate dietary phylloquinone, had significantly higher phylloquinone and menaquinone-4 concentrations in adipose tissue, kidney, liver, pancreas, and brain compared to male mice. In contrast, on the deficient diet there were no sex-specific differences in phylloquinone or menaquinone-4 concentrations in any tissue except pancreas. There may be a minimum tissue-specific vitamin K requirement that is achieved with intakes sufficient to sustain coagulation in both male and female mice, whereas only the higher intakes reveal sex-specific differences. Sex-specific differences have not been well characterized in humans. Although there is a suggestive effect of sex, the data are inconsistent (12), which further supports the need to use both sexes in vitamin K studies using preclinical models.

Another biological factor that has been associated with differences in vitamin K status is age. However, it is often overlooked as a variable to consider in preclinical models. The use of aging mouse models is critical for understanding age-related disease pathologies as it improves translatability across species. But the increased time and cost of maintaining an aging mouse model deters most researchers from utilizing aged mice (13). Successful optimization of a mouse model that responded to dietary vitamin K manipulation provided an opportunity to further study the impact of age on tissue-specific regulation of different vitamin K forms. Previous studies in rats have shown and age-related change in vitamin K tissues concentrations, no data exist in mice (14). This was the first study to examine vitamin K status and tissue concentrations in an aged mouse model. Another benefit of the study design included the use of stable isotopes specifically deuterium labeled phylloquinone, which provide insight into absorption and tissue-specific

utilization. 4-month old and 22-month old mice were provided a diet with sufficient phylloquinone, and then later supplemented with deuterium-labeled phylloquinone to capture absorption and tissue-specific turnover of phylloquinone, and conversion of dietary phylloquinone to menaquinone-4. In contrast to previous studies in rats, older mice had significantly higher total phylloquinone in liver, and reduced total phylloquinone in intestine when compared to young 4-month old mice. The decreased concentration in liver and intestine deuterium labeled phylloquinone may be indicative of decreased utilization suggesting age-related and species-specific differences in metabolism.

To complement the examination of different biological factors on vitamin K status, the use of stable isotopes in conjunction with statins proved to be a powerful tool to manipulate menaquinone-4 formation. In the previous animal study, an additional arm was included to evaluate the effect of atorvastatin, a common lipophilic HMG-CoA reductase inhibitor, on the conversion of dietary phylloquinone to menaquinone-4. The primary action of statins is inhibition of HMG-CoA reductase in the cholesterol synthesis pathway. Reduced cholesterol synthesis results in increased recycling of LDL cholesterol and in turn lowers circulating LDL cholesterol levels, demonstrating the clinically significant role of statins in age-related chronic disease. Statins not only inhibit endogenous cholesterol synthesis but isoprenoid intermediates within the cholesterol synthesis pathway (15). The pleiotropic effects of statins independent of their lipid lowering function, are attributed to the inhibition of isoprenoids. The isoprenoid intermediate, geranylgeranyl pyrophosphate, is required for endogenous synthesis of menaquinone-4, linking cholesterol synthesis to vitamin K metabolism. Limited data exist examining the effect of statins on menaquinone-4 formation. This was the first study *in vivo* to demonstrate that mice on a diet containing atorvastatin and adequate concentrations of dietary

phylloquinone, reduced menaquinone-4 synthesis specifically in the kidney supporting previous *in vitro* studies (6). Statins are a useful tool to manipulate menaquinone-4 formation, and will assist in identifying alternative functions and tissue-specific requirements of menaquinone-4. Unfortunately, this study was limited by the inability to quantify isoprenoid intermediates in tissue, even though a successful detection method using UPLC-MS/MS was developed. Further method development and optimization is necessary to extract and quantify tissue concentrations of isoprenoid intermediates.

Discussion

Together all of the studies completed in this thesis demonstrate the need for greater inquiry into the bioavailability and metabolism of phylloquinone and menaquinones. The findings of the analysis of NHANES 2011-2012 illustrate that dietary patterns are changing. These changes are reflected in dietary vitamin K intake, as mixed dishes were found to be a significant dietary source, and may be attributed to individual preferences as well as changes in the food supply, only some of which may be captured in food composition databases. IT has recently been ascertained that menaquinones are in a variety of foods including meat, dairy, and fermented foods (2,16). Dietary assessment methods currently lack the ability to capture dietary menaquinones, and food composition databases have yet to systematically incorporate menaquinones as a vitamin K source. Thus current dietary intakes of vitamin K may be underestimated by inclusion of phylloquinone only. To justify the incorporation of menaquinones into dietary assessment and food composition databases, the bioavailability of various menaquinones needs to be assessed.

In humans, stable isotopes have previously been used to evaluate bioavailability (17–21). Circulating measures of vitamin K including phylloquinone or menaquinones (i.e. menaquinone-

7) were used as a transient measure of absorption and status. However, circulating concentrations may not be reflective of tissue uptake, thus supporting the use of preclinical models. The use of stable isotopes in preclinical models allows us to observe the absorption and utilization of dietary phylloquinone, and subsequent conversion to menaquinone-4 to mimic the human condition. Previous studies in rats given stable isotopes have examined the conversion of dietary phylloquinone to menaquinone-4 (5,22,23). There is limited information available for the effective use of vitamin K stable isotopes in mouse models. The C57BL6 genetic background is the most commonly used strain for transgenic models and although transgenic mouse models for studying key enzymes in vitamin K metabolism and vitamin K dependent proteins have been developed (24–26), none have used stable isotopes. This is the first study to use stable isotopes to examine the conversion of dietary deuterium-labeled phylloquinone to menaquinone-4 in mice. To further elucidate this conversion, HMG-CoA reductase inhibitors, more commonly known as statins were adopted as a tool to manipulate menaquinone-4. Few studies have demonstrated an interaction between statins and menaquinone-4 formation (4,6). Menaquinone-4 is unique in that an isoprenoid cholesterol precursor, geranylgeranyl pyrophosphate is required for synthesis, thus associating vitamin K and cholesterol metabolism. Current hypotheses suggest the link between cholesterol synthesis and menaquinone-4 formation to be antagonistic such that as menaquinone-4 formation increases, geranylgeranyl pyrophosphate is depleted which results in greater conversion of farnesyl pyrophosphate to geranylgeranyl pyrophosphate and ultimately reduces cholesterol synthesis. In contrast, a reduction in menaquinone-4 formation may shunt more geranylgeranyl pyrophosphate to cholesterol synthesis and potentially lead to cholesterol accumulation. As part of this thesis, we were able to manipulate menaquinone-4 formation in kidney with atorvastatin. It is plausible the differences in menaquinone-4 concentrations

observed are attributed to decreased concentrations of geranylgeranyl pyrophosphate as a direct result of atorvastatin, and further exacerbated by an increase in shunting of geranylgeranyl pyrophosphate towards cholesterol synthesis to combat the atorvastatin interference. However, our interpretation is limited since tissue concentrations of cholesterol or cholesterol synthesis intermediates were not quantified.

There is evidence to suggest that MK4 has roles in physiological functions that are independent of its role as an enzyme cofactor (27–31). In certain tissues the concentration of menaquinone-4 is several fold higher than that of phylloquinone (10,32–34), which may be indicative of tissue specific function in rodents and humans. The presence of vitamin K forms in extra-hepatic tissues has resulted in several hypotheses linking vitamin K to age-related chronic diseases including coronary artery calcification (35–41), osteoarthritis (42–45), and kidney disease (46–50). However, the mechanisms by which vitamin K and/or vitamin K dependent proteins modulate disease progression are unclear. Our finding that atorvastatin reduced menaquinone-4 formation in the kidney may be clinically relevant as recent evidence proposes an interaction between vitamin K, calcification, and kidney disease. The kidney is a site of conversion, responds to dietary phylloquinone manipulation, and responds to statin treatment as previously demonstrated and supported by the results of our study (32,33). These observations suggest the conversion in the kidney is a regulated process. In a rat model of chronic kidney disease with vascular calcification, animals with apparent chronic kidney disease have significantly higher kidney menaquinone-4 concentrations, which may be indicative of changes in the efficiency of conversion in chronic kidney disease or that utilization of menaquinone-4 may be impaired in the disease state, further exacerbating vascular calcification and disease pathology (33). More recently, statin use in humans with chronic kidney disease was

independently associated with calcification, suggesting that therapeutic doses of statins may accelerate calcification by depleting tissue concentrations of menaquinone-4, thus increasing risk of vascular calcification (47). The mechanism by which menaquinone-4 alters calcification has yet to be identified and warrants further investigation.

To supplement the observations linking vitamin K, calcification, and kidney disease, a recent study found that individuals with end stage renal disease (ESRD) and calciphylaxis, a necrotizing skin condition characterized by calcification, were more likely to be vitamin K deficient, indicated by low circulating phylloquinone, compared to individuals with ESRD only and independent of vitamin K antagonist therapy (51). The authors concluded that vitamin K deficiency may have a role in calcification progression in calciphylaxis, and that future research is needed to determine the therapeutic potential of vitamin K supplementation. Together, these studies further emphasize that vitamin K has a role in kidney. Whether the role is related to menaquinone-4 needs to be determined.

There is evidence to suggest that menaquinone-4 has roles in cellular functions that are independent of its role as an enzyme co-factor, such as acting as a nuclear receptor ligand, and preventing oxidative injury (27,29). However, these observations are limited to *in vitro* studies. In order to determine the potential functions of menaquinone-4, cellular localization studies are required to begin to identify the cellular function of menaquinone-4. Murine models of kidney disease are being used to characterize the mechanisms driving kidney dysfunction and resulting organ failure. Renal cell necrosis has been considered the most common etiology contributing to a decline in renal function, however the lack of correlation between necrosis and organ function implicates alternative forms of cell death as contributors to organ failure, more specifically, renal cell death can include apoptosis—programmed cell death (52). In murine models of kidney

injury, apoptosis correlates better with renal dysfunction than necrosis (53,54). Tissue dysfunction and eventual organ failure are a result of apoptotic dysregulation by excessive or defective cell death. We hypothesize that the potential mechanism of menaquinone-4 and cholesterol pathway intermediates in kidney may be related to regulation of apoptosis, as both have been independently associated with modulating apoptosis (28,55). Since we have demonstrated an interaction between menaquinone-4 and isoprenoid intermediates, the mechanism of action may involve both compounds. We hypothesize three pontential mechanisms modulating cellular apoptosis. These mechanisms are summarized in figure 1 below:

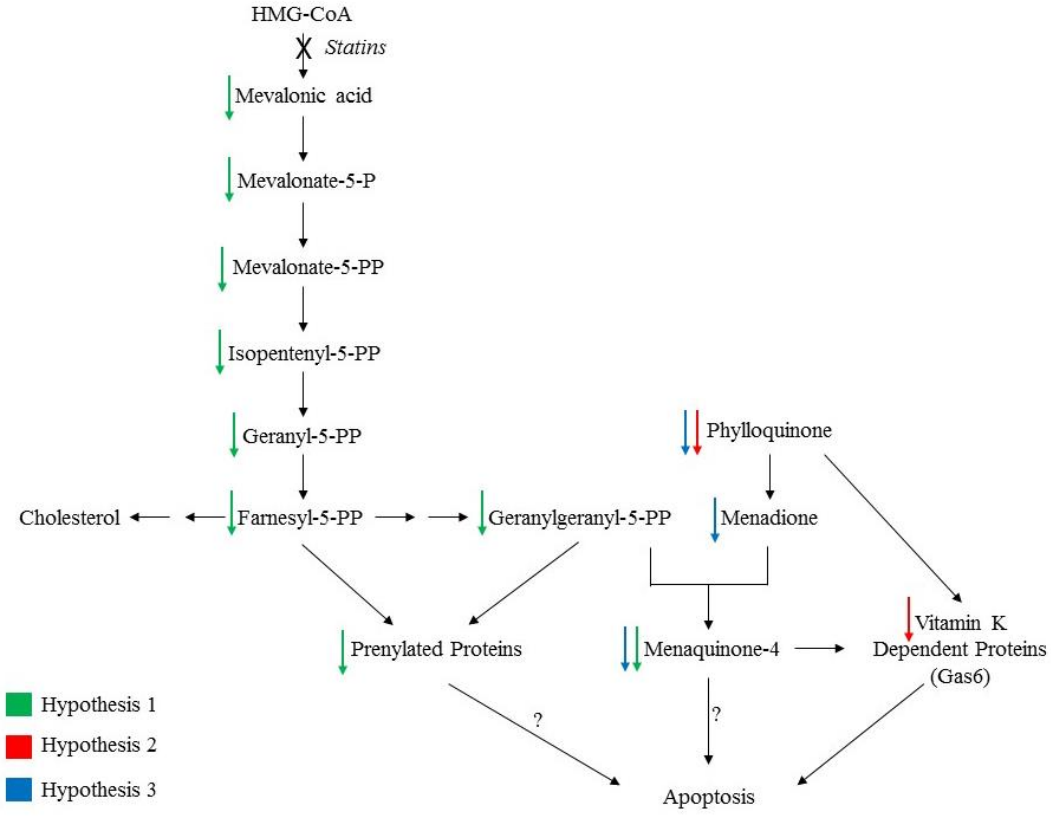


Figure 1. Proposed mechanisms of menaquinone-4 and isoprenoid compounds in apoptosis.

Results from our study demonstrated that statins can manipulate the isoprenoid intermediates, resulting in reduced production of menaquinone-4. We hypothesize the effect of statins on reducing isoprenoids can reduce protein prenylation and menaquinone-4 formation both of which may affect cellular apopotosis (hypothesis 1-green). What we could not achieve with our study design was direct evidence of either pathway. Therefore it is plausible that menaquinone-4 was reduced but has no effect on apoptois. One vitamin K-depedent protein gas6, has also been implicated in modulating cellular apoptosis (56). Both phylloquinone and menaquinone-4 are capable of acting as enzyme cofactors for the gamma-glutamyl carboxylase, which is responsible for carboxylating vitamin K dependent proteins providing functionality. We hypothesize that the depletion of phylloquinone or menaquinone-4 may reduce gas6 carboxylation and affect cellular apoptosis (hypothesis 2-red). The caveat to this hypothesis is that both vitamin K forms have equivalent bioactivity, so one cannot differentiate the relative contribution of each. Finally, menaquinone-4 requires the naphthoquinone ring from phylloquinone. Therefore a challenge to dietary phylloquinone will limit menadione availability for conversion to menaquinone-4, and ultimately affect apoptosis (hypothesis 3-blue). All of the hypothesized mechanisms may result in dysfunctional apoptosis contributing to the pathogenesis of kidney disease. Additional *in vitro* and *in vivo* studies are required to evaluate our hypothesized mechanisms, and the role in kidney injury and progression of kidney disease.

Current research has focused primarily on the conversion of phylloquinone to menaquinone-4. But we do not know to what extent, if at all, menaquinones are endogenously converted to menaquinone-4. Some evidence suggests that both phylloquinone and menaquinone-7 are broken down to the naphthoquinone ring (menadione) within intestinal cells and subsequently released into circulation (57,58). Stable isotopes would be useful to

conclusively determine if conversion from bacterially-synthesized menaquinones (from the gut or from a dietary source) to menadione and successively menaquinone-4 occurs. Coupling stable isotopes, preclinical animal models, and diet manipulation, will be useful to address questions of bioavailability and functionality, and ultimately help guide human studies to establish potential therapeutic uses and targets of vitamin K.

In summary, the collective findings of this thesis work indicate that dietary and non-factors influence vitamin K status and metabolism. Questions regarding bioavailability of menaquinones, alternative functions of various vitamin K forms, and the potential health effects, remain unanswered. However, these findings coupled with emerging evidence of roles for vitamin K in health and disease warrants further investigation.

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7. Appendices

7.1 Methods

Quantification of vitamin K in feces, serum and food by LC-APCI-MS

Purpose: To measure concentrations of phylloquinone (PK) and menaquinones (MK) 4 through 13 in feces, serum and food samples.

Sample Preparation

1. Sample storage
    a. Feces (dry or wet): -80°C to -20°C
    b. Food: -80°C
    c. Serum/plasma: -80°C
2. Fecal pre-processing
    a. Lyophilize ≥ 2 g wet wt. feces to a constant weight.
    b. Homogenize dried fecal sample into a fine powder using a mortar and pestle.
    c. Aliquot fecal powder into labeled cryovials for storage.

Vitamin K extraction (feces and food)

1. Select samples to be analyzed and defrost at room temperature. Include controls (e.g. baby food) in every run
2. Label 50 mL polypropylene centrifuge tubes
3. Stir or mix defrosted samples to ensure homogeneity
4. Weigh appropriate amount of sample and add to 50mL tubes
    a. Feces: 40 μg – 50 μg dry wt
    b. Food*: 200 μg – 500 μg
    c. To calculate the dilution factor, divide 1 by the exact weight of sample used so that calculated results will be returned in pmol/g or ng/g.
5. Add deuterium-labeled phylloquinone (5,6,7,8-d4, 2-methyl-d3) internal standard to each sample
6. Add 10 mL distilled water to each sample and vortex for 30 s
7. Add 15mL 2-propanol:hexane (3:2 v/v) to each sample
8. Vortex samples for 3 min
9. Sonicate each sample at continuous output 50% duty cycle, output control # 4 for 1 min
10. Vortex all samples for 3 min
11. Centrifuge at 20°C and 3000 rpm for 5 min
12. Aspirate the top layer of each tube using a Pasteur pipette and transfer to a labeled, glass 16 x 100 culture tube

Vitamin K extraction (serum/plasma)

1. Select samples to be analyzed and defrost at room temperature. Include high and low serum controls in every run
2. Pipette ≥ 250 μL of serum/plasma into 16 x 100 borosilicate screw cap culture tube*
3. Add deuterium-labeled phylloquinone (5,6,7,8-d4, 2-methyl-d3) internal standard to each sample
4. Add 0.5 mL ethanol to each tube and vortex vigorously for 5 s
5. Add 0.5 mL distilled water to each tube
6. Add 3 mL hexane to each tube. Cap tubes with Teflon lined caps and vortex vigorously for 3 min.

7. Centrifuge at 20°C and 3000 rpm for 5 min
8. Aspirate the top layer of each tube using a Pasteur pipette and transfer to a labeled, glass 16 x 100 culture tube

Solid phase extraction (feces, food, and serum/plasma)

1. Warm samples while evaporating hexane layer to dryness under a gentle nitrogen stream (≤ 10 psi)
2. Label a set of 16 x 100 culture tubes and put into Vac-Elute manifold rack. Place one 500 mg silica SPE column per sample on the Vac Elute SPS 24
3. Reconstitute each sample with appropriate amount of hexane and vortex each for 5 s:
    a. Feces: 4 mL hexane
    b. Food: 1-4 mL hexane*
    c. Serum/plasma: 0.5 mL hexane
4. Prepare 3.5% (3.5:96.5 v/v) diethyl ether in hexane (12 mL per sample required)
5. Set Vac-Elute to "WASTE"
6. Condition each column with 4mL of 3.5% diethyl ether in hexane and vacuum to dry
7. Condition each column with 4mL hexane and vacuum to dry
8. Add appropriate amount of sample to SPE column
    a. Feces: 1 mL
    b. Food: 0.5 mL – 1.0 mL*
    c. Serum/plasma: 0.5 mL
9. Vacuum to dry
10. Wash each column with 4mL hexane and vacuum to dry
11. Set Vac-Elute to "COLLECT"
12. Wash each column with 8mL 3.5% diethyl ether in hexane and vacuum to dry
13. Warm eluates while evaporating to dryness under a gentle nitrogen stream (<10 psi)
14. Reconstitute residue in 30 µL of methylene chloride and vortex for 5 s
15. Evaporate to dryness at 60°C in a hot water bath (10 min)
16. Reconstitute residue in 30 µL of methylene chloride and vortex for 5 s
17. Add appropriate amount of methanol to each tube and gently vortex for 5 s
    a. Feces: 170 µL methanol
    b. Food: 170 µL methanol*
    c. Serum/plasma: 90 µL methanol
18. Transfer solution to labeled amber HPLC vials. Cap each vial with an aluminum crimp cap
19. Centrifuge the sample vials at 20°C and 3,000 rpm for 5 min
20. Load samples into HPLC/MS system
21. Injection volume:
    a. Feces: 50 µL
    b. Food: 100 µL*
    c. Serum/plasma: 100 µL

HPLC system specifications

1. Instrument: Agilent 1260 HPLC
2. Column: Reversed-phase C_{18} analytical column; Kinetex 2.6 µm, 150 mm x 3.0 mm
3. Mobile phase:

   a. Solvent A: Methanol
   b. Solvent B: Methylene chloride
4. Solvent conditions:
   a. 0-2.5 min: isocratic; 100% methanol
   b. 2.5-10 min: linear gradient to 70% methanol and 30% methylene chloride
   c. 10-14 min: isocratic; 70% methanol and 30% methylene chloride
   d. 14-15.5 min: linear gradient to 100% methanol
   e. 15.5-20 min: isocratic; 100% methanol
5. Flow rate: 1.0 mL/min throughout
6. Calibration standard should include: deuterium-labeled PK, PK, and MK4 through MK13

MS system specifications

1. Instrument: Agilent 6130 Quadrupole MSD with atmospheric chemical ionization (APCI)
2. Ion source: Positive APCI
3. Spray chamber gas temperature: 350°C
4. Vaporizer temperature: 400°C
5. Drying nitrogen: 7.0 L/min
6. Nitrogen nebulizer pressure: 45 psig
7. Capillary voltage: 3800 V
8. Corona current: 5 μA
9. Selected ion monitoring mass-to-charge ratios (*m/z*):
   a. Deuterium-labeled PK: *m/z* 458
   b. PK: *m/z* 451
   c. MK4: *m/z* 445
   d. MK5: *m/z* 513
   e. MK6: *m/z* 582
   f. MK7: *m/z* 650
   g. MK8: *m/z* 718
   h. MK9: *m/z* 786
   i. MK10: *m/z* 854
   j. MK11: *m/z* 923
   k. MK12: *m/z* 991
   l. MK13: *m/z* 1059

**Note: these weights/volumes can be altered based on expected vitamin K concentrations in samples being analyzed. Lower concentrations necessitate greater starting weights/volumes and less dilution.*

Simultaneous measurement of phylloquinone and menaquinone-4 in human serum or plasma by Agilent HPLC with fluorimetric detection

Reference: Kenneth W. Davidson and James A Sadowski, Methods in Enzymology, Vol. 282, p408 1997.

Precipitation of proteins and extraction of plasma lipids:

1. pipet 0.250 mL of plasma into 16 X 100 borosilicate screw cap culture tubes
2. add 20 uL of internal standard ($K_{1(25)}$)
3. add 0.5 mL of ethanol to each tube
4. vortex vigorously for 5 seconds to denature proteins
5. add 0.5 mL of DI water to each tube
6. add 2.5 mL of hexane to each tube, cap tubes with Teflon lined caps.
7. vortex vigorously for 2 minutes
8. centrifuge for 5 minutes at 3,000 rpm
9. remove upper, hexane layer with a Pasteur pipet and aspirate into a clean tube
10. evaporate hexane layer to dryness in a centrifugational evaporator
11. add 0.5 mL of hexane to each tube
12. cap and store at –80^{o} C or proceed with SPE procedure

Solid-Phase Extraction (SPE) on Silica

1. prepare 3.5% ethyl ether in hexane (3.5 mL ether + 96.5 mL hexane), prepare 12 mL for each sample, use within 8 hours
2. place 3 mL (500 mg) silica SPE columns on the Vac Elute SPS 24, one column for each sample
3. wash each column with 4 mL of 3.5% ether/hexane
4. wash each column with 4 mL of hexane
5. pick up a sample in 0.5 mL of hexane and add it to a column
6. wash each column with 4 mL of hexane
7. elute vitamin K with 8 mL of 3.5% ether/hexane

8. collect eluate and evaporate ether/hexane to dryness in a centrifugational evaporator
9. if proceeding with HPLC procedure:

a. Redissolve sample for injection on the chromatograph. Reconstitute first in 30 □L of methylene chloride with vortexing for 5 seconds, quickly followed with 170 □L of methanol/aqueous solution (5.5 mL aqueous solution + methanol to give 1000 mL) – vortex another 5 seconds.

Aqueous solution =	2.0 M zinc chloride 1.0 M acetic acid 1.0 M sodium acetate

b. Transfer the reconstituted sample to an amber sample vial with conical insert. Cap each vial with Teflon lined rubber septum and an aluminum crimp cap. Centrifuge the sample vials at 3,000 rpm for 5 minutes to precipitate undissolved material. Program the Waters 2695 Separations Module to inject 150 □L of sample.

Procedure:

Prepare aqueous/methanol solution:
5.5 mL of aqueous solution (see SPE procedure step 7) qs to 1000 mL with absolute methanol.

Equilibrate the column and detector by pumping a 90:10 mixture of aqueous/methanol and methylene chloride at 0.60 mL/min through the column. The vacuum degasser is set to "normal". The 2695 is programmed to do the following gradient elution procedure:

Pump the 90:10 mixture at 0.60 mL/min from injection for the first 11.5 minutes
At 11.50 min change flow rate to 0.80 mL/min
At 11.50 min change the composition to 70:30
At 19.50 min change the composition to 90:10
At 23.50 min change the flow rate to 0.60 mL/min
At 24.0 min end the cycle

Excitation is performed at 244 nm and emission is monitored at 430 nm. Usually a calibration standard is injected with every six to ten samples in a run to compensate for changes in chromatographic conditions. Standard curves are prepared from each calibration injection. The fluorescence responses for vitamin K and for $K_{1(25)}$ are linear beyond normal physiological concentrations with the slope of the lines bisecting zero. We therefore routinely perform single-point calibration, forcing the slope of the line through zero. Quantitation is achieved by direct comparison of peak area ratios (K_1 to $K_{1(25)}$) generated from the calibration standard to those generated by the sample. An assay run generally consists of low and high controls plus 22 unknown samples (for a total of 24 samples). Peak integration and sample concentration calculations are performed using Empower Pro Empower 2 software from Waters Corporation.

Isoprenoid detection method by UPLC-MS/MS

UPLC-MS/MS Method
MS: negative ion electrospray, column placed close to ion source
Column temperature: Not controlled
Mobile phase temperature: not controlled
Column: Zorbax Rapid Resolution Column HT extend C18
Linear gradient:
Solution 1: (100mM NH_4HCO_3, 0.1% trimethylamine pH=9.09)
Solution 2: (acetonitrile/H_2O 4:1, 0.1% trimethylamine pH=10.5)

Time	% 100mM NH4HCO3, 0.1% TEA	% ACN:H2O, 0.1% TEA	Flow rate
0 min	80	20	0.35ml/min
5 min	0	100	0.35ml/min
10 min	0	100	0.35ml/min

Flush method:

Time	% 100mM NH4HCO3, 0.1% TEA	% Methanol	Flow rate
0 min	0	100	0.2 ml/min
25 min	100	0	0.3 ml/min
45 min	100	0	0.35 ml/min

Standard:

4 mL

	1'		vol added	amt added	final conc	1x
Final Standard	nmol/mL		ul	nmol	nmol/mL	nmol/ul
FPP	26.155		80.0	2092.43	523.11	0.52
GPP	31.826		64.0	2036.85	509.21	0.51
IPP	40.635		50.0	2031.76	507.94	0.51
		sum	194.0			
		water	3806.0			
		total vol	4000.0			

Calibration Curves:

IPP Calibration Curve

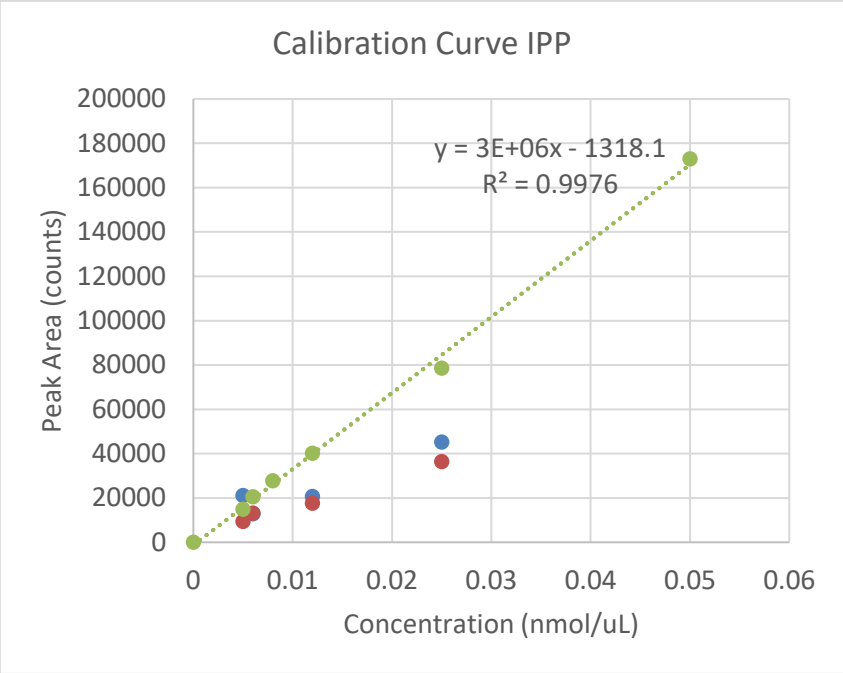
	IPP		GPP		FPP	
	Concentration	Area	Concentration	Area	Concentration	Area
100x	**0.005**	**14900**	**0.005**	**17800**	0.005	ND
80x	0.006	20500	0.006	21300	**0.007**	**21900**
60x	0.008	27700	0.008	39100	0.008	39800
40x	0.012	40100	0.013	67500	0.013	78300
20x	0.025	78500	0.026	151000	0.026	145000
10x	0.050	173000	0.051	321000	0.052	326000
5x	0.100	346000	0.100	644000	0.100	639000
1x	0.510	1870000	0.510	3440000	0.520	6190000

LLOD:

IPP: 5 pmol

GPP: 5 pmol

FPP: 7 pmol


Calibration Curve IPP
y = 3E+06x - 1318.1
R² = 0.9976
Peak Area (counts)
Concentration (nmol/uL)
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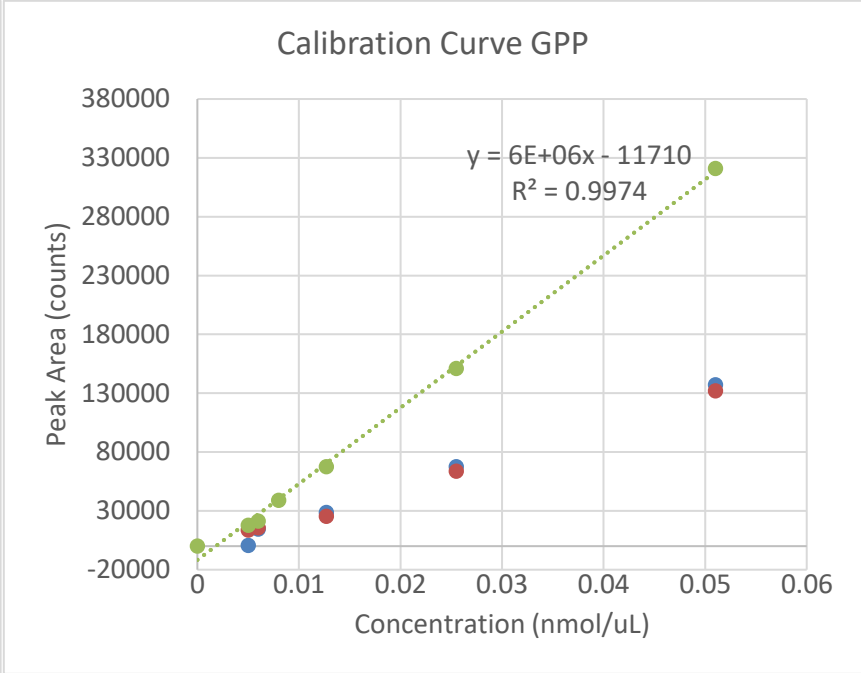
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
0.01
0.02
0.03
0.04
0.05
0.06


Calibration Curve GPP
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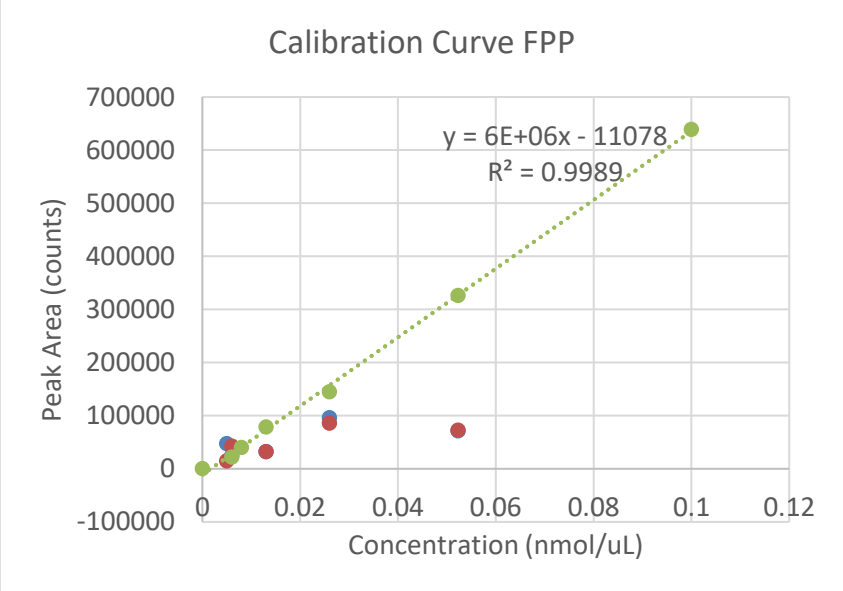
R² = 0.9974
Peak Area (counts)
Concentration (nmol/uL)
380000
330000
280000
230000
180000
130000
80000
30000
-20000
0
0.01
0.02
0.03
0.04
0.05
0.06


Calibration Curve FPP
y = 6E+06x - 11078
R² = 0.9989
Peak Area (counts)
Concentration (nmol/uL)
700000
600000
500000
400000
300000
200000
100000
0
-100000
0
0.02
0.04
0.06
0.08
0.1
0.12

Extraction Method

1. Weigh sample 50-75mg of tissue. Place in 2mL cryotube on ice.
2. Add 1mL of 2-propanol: 100 mM NH_4HCO_3 pH 7.8 (1:1 v/v) to sample in tube.
3. Homogenize sample on ice.
4. Transfer homogenate to 15mL conical tube.
5. Add 2ml propanol:hexane (40:60) to sample.
6. Sonicate on ice twice, 35 seconds.
7. Centrifuge for 5 min at 2000rpm and discard supernatant.
8. Pellet is re-suspended in 450 ul 2-propanol: 100 mM NH_4HCO_3 pH 7.8 (1:1 v/v) in 2mL round bottom Eppendorf tubes.
9. Vortex samples.
10. Add 750 ul acetonitrile for de-proteinization.
11. Keep samples on ice for 10 mins.
12. Centrifuge at 14,000g (small table top centrifuge -in cold room)
13. Transfer supernatant to 75 ml glass tube and dry under nitrogen stream at 40C (drying solution of acetonitrile and propanol:NH_4HCO_3).
14. Dissolve residue in 50 ul miliQ water
15. Inject less than 10 ul into HPLC-MS/MS.

To further clean samples tried C18 column:

1. Prepare C18 column with 1mL 2-propanol: 100 mM NH_4HCO_3 pull vacuum till solvent is completely through column.
2. Add sample from step 13. Transfer supernatant to column.
3. Switch to collection, elute with 8ml chloroform:methanol solution.

Chloroform: methanol preparations: 1%, 2.5%, 5%

Test with 1% Chloroform:Methanol
Standard added to column for all preparations

	IPP			GPP			FPP		
	Area	Concentration	Recovery	Area	Concentration	Recovery	Area	Concentration	Recovery
C18 test 1	14700	0.007	7.35	13600	0.004	4.53	20500	0.005	5.26
C18 test 2	15300	0.007	7.65	17800	0.005	5.93	16300	0.004	4.56
C18 1% chloroform	34900	0.017	17.45	63100	0.021	21.03	65900	0.012	12.83
C18 1% chloroform	35000	0.017	17.50	56700	0.018	18.90	42200	0.008	8.88

Additional chloroform:methanol tests

Sample	Spike Concentration	Injection vol	Injected concentration	IPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
5%	10	4	0.8	24000	0.034	4.220
5%	10	4	0.8	15300	0.022	2.770
2.50%	10	4	0.8	14200	0.021	2.586
2.50%	10	4	0.8	7860	0.012	1.530
2.5% 4ml	10	4	0.8	15000	0.022	2.720
2.5% 8ml	10	4	0.8	9700	0.015	1.836

Sample	Spike Concentration	Injection vol	Injected concentration	GPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
5%	10	4	0.8	9730	0.014	0.000
5%	10	4	0.8	4680	0.011	0.000
2.50%	10	4	0.8	9000	0.014	0.000
2.50%	10	4	0.8	2790	0.010	0.000
2.5% 4ml	10	4	0.8	676	0.008	0.001
2.5% 8ml	10	4	0.8	6350	0.012	0.000

Sample	Spike Concentration	Injection vol	Injected concentration	FPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
5%	10	4	0.8	46500	0.038	4.798
5%	10	4	0.8	17300	0.019	2.365
2.50%	10	4	0.8	23500	0.023	2.882
2.50%	10	4	0.8	26700	0.025	3.148
2.5% 4ml	10	4	0.8	11000	0.015	1.840
2.5% 8ml	10	4	0.8	13000	0.016	2.007

Due to the recovery issues associated with C18, we tried C18 filter pipette tips. Soaked the tip in mobile phase, loaded 100ul of 10x calibration standard onto tip, and added the standard to the vial.

Sample	Spike Concentration	Injection vol	Injected concentration	IPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
Zip tip	1	4	0.08	994	0.003	3.853

Sample	Spike Concentration	Injection vol	Injected concentration	GPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
Zip tip	1	4	0.08	52000	0.042	53.092

Sample	Spike Concentration	Injection vol	Injected concentration	FPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
Zip tip	1	4	0.08	169000	0.030	37.516

Next steps: evaluate extraction method
Chose to see if a filter tip would be better to help purify samples, but improve recovery.
Use 0.8um filter tip on a syringe, tested with spiked water first using 10x dilution, adding 20ul of 10x standard.

Sample	Spike Concentration	Injection vol	Injected concentration	IPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
water	0	4	0	0	0.000	#DIV/0!
water+filter	1	4	0.08	63100	0.086	107.363

Sample	Spike Concentration	Injection vol	Injected concentration	GPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
water	0	4	0	0	0.002	#DIV/0!
water+filter	1	4	0.08	78800	0.060	75.425

Sample	Spike Concentration	Injection vol	Injected concentration	FPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
water	0	4	0	0	0.002	#DIV/0!
water+filter	1	4	0.08	109000	0.080	100.065

Tested kidney sample with filter tip on syringe, spiked with 1x standard, added 20ul.

Sample	Spike Concentration	Injection vol	Injected concentration	IPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
kidney	0	4	0	0	0.000	#DIV/0!
Spike 1	10	4	0.8	819000	1.094	136.720
Spike 2	10	4	0.8	806000	1.076	134.553
Sample	Spike Concentration	Injection vol	Injected concentration	GPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
Kidney	0	4	0	0	0.002	#DIV/0!
spike 1	10	4	0.8	2140000	0.359	44.827
spike 2	10	4	0.8	2030000	0.340	42.536
Sample	Spike Concentration	Injection vol	Injected concentration	FPP	Concentration	Recovery
	nmol	ul	nmol	Area	nmol	%
kidney	0	4	0	46500	0.010	#DIV/0!
spike 1	10	4	0.8	4260000	0.712	88.981
spike 2	10	4	0.8	4120000	0.689	86.064

7.2 Additional Manuscripts

Vitamin K: dietary intake and requirements in different clinical conditions

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Abstract:

Purpose of review: Vitamin K is an enzyme cofactor for the carboxylation of vitamin K dependent proteins (VKDP). Functions include coagulation and regulation of calcification. Different clinical conditions may alter vitamin K requirements by affecting vitamin K status and VKDP carboxylation, which are reviewed here.

Recent findings: Vitamin K consumption greater than the current usual daily consumption to maintain health is indicated for prevention of vitamin K deficient bleeding (VKDB) in infants and for rescue of over-anticoagulation in patients on vitamin K-dependent oral anticoagulants (VKA). Additional vitamin K intake may be required in malabsorptive conditions such as cystic fibrosis and following bariatric surgery. Carboxylation of VKDP occurs in multiple extrahepatic tissues and has been implicated in soft tissue calcification and insulin resistance (IR), although the exact mechanisms have yet to be determined. Contribution of colonic flora to vitamin K requirements remains controversial.

Summary: With the increased incidence of VKDB and weight-loss surgical procedures, healthcare professionals need to monitor vitamin K status in certain patient populations. Future research on the roles of vitamin K in extrahepatic tissues as they pertain to chronic disease will provide insight in to the therapeutic potential of vitamin K and lead to the development of recommendations for specific clinical populations.

Key words: Vitamin K, phylloquinone, calcification, coagulation, warfarin

Introduction:

Dietary requirements for vitamin K were established decades ago based on its role as an enzyme cofactor for the ɣ-carboxylation of certain coagulation proteins. It is now recognized that vitamin K's role as an enzyme cofactor extends to multiple proteins involved in an array of biological functions, including regulation of tissue calcification [1,2**]. Although there is emerging evidence to suggest that the dietary requirements for vitamin K to promote arterial and joint health may be greater than was established for its role in coagulation, there are large gaps in knowledge about the optimal forms and doses of vitamin K required to achieve optimal health. Until these gaps in knowledge are addressed, recommendations for vitamin K intake will likely remain unchanged.

Dietary Sources of Vitamin K and Current Recommendations

Vitamin K is a fat-soluble vitamin comprising multiple forms that fall into two general categories: phylloquinone and menaquinones (MK-n; where the n indicate the number of isoprenoid units in the side chain), sometimes referred to as vitamin K_1 and vitamin K_2, respectively. This is an unfortunate oversimplification as it erroneously implies that all menaquinones are similar. Instead, menaquinones (of which there are more than 10 known forms) have unique sources and functions, particularly in extra-hepatic tissues [3*]. For example, whereas most menaquinones are produced by bacteria, MK-4 is not. Instead, evidence suggests that MK-4 is primarily formed from dietary phylloquinone by means of tissue-specific conversion [3*–5]. Mammalian homologs to bacterial enzymes involved in the production of MK-n have been used to better understand the conversion of phylloquinone to MK-4. Originally a prenyl transferase called UBIAD1 was identified as the single enzyme responsible for converting PK to MK-4 through multiple steps [6]. New evidence suggests that UBIAD1 is only responsible for one step in the pathway with an alternative yet-to-be identified enzyme for side chain cleavage of phylloquinone [7*]. The characterization of UBIAD1 and the unique tissue distribution of MK-4 have led to the question of whether MK-4 is locally synthesized from phylloquinone, or if it is transported to the target tissue from a site such as the liver or intestine as a precursor or MK-4 [7*]. Functions of UBIAD1 and MK-4 have expanded beyond vitamin K metabolism, and include roles in endothelial cell function, and cholesterol metabolism [8,9].

Medium and long-chain MK-n (defined as MK-6 or higher) act as electron carriers in prokaryotic respiration [3*]. They are synthesized by specific anaerobic bacteria, some of which occupy the human gut. MK-n fecal concentrations correlate with the abundance of these bacterial

species [2**,3*]. Evidence supporting the absorption and metabolism of bacterially-synthesized menaquinones from the gut is limited to work conducted several decades ago. A limiting factor to previous approaches to studying the microbiome is the inability of certain anaerobic bacteria to be cultured. With the development and application of pyrosequencing and next-generation sequencing techniques, the genetic and functional capacity of the microbiome can now be more effectively characterized [10]. These techniques will provide insight into the preponderance of MK-n producing bacteria, the factors promoting MK-n synthesis, and menaquinones functions. For the reasons described above, the contribution of MK-n to vitamin K status remains controversial and the role of MK-n in vitamin K function is as yet incompletely elucidated.

Primary sources of phylloquinone are dark green leafy vegetables [11], along with phylloquinone-rich plant oils that are incorporated into many food products [2**]. A comprehensive database containing information on the phylloquinone content of foods can be found through the U.S. Department of Agriculture National Nutrient Database [12]. In contrast to phylloquinone, which is abundant in the food supply, MK-n are found in animal based foods such as dairy and meats, as well as fermented foods [2**,12]. The food composition data for the menaquinones are not-well characterized hence it is plausible that there are multiple dietary sources that are not being captured in dietary assessment studies. However with the development of sensitive assays for measurement of menaquinones in a variety of food matrices [13], it is anticipated that there will be more accurate databases available in the near future for assessment of menaquinone intakes.

The current U.S. Dietary Reference Intakes for vitamin K are represented by Adequate Intake (AI) values since a Recommended Dietary Intake (RDA) cannot be established due to the

insufficient knowledge regarding vitamin K bioavailability, transportation, excretion, and biomarkers, which are critical to establishing requirements for humans. The current AI for adult males and females are 120 micrograms and 90 μg per day respectively which by definition represents 50 percent of the general U.S. adult population meeting the recommendation [2**]. However, vitamin K intake varies among age groups and geographic location [2**,14].

In the absence of a robust biomarker to evaluate vitamin K status that is accepted universally, international dietary recommendations vary by 2-fold. For example the current recommendation for New Zealand, Australia, Germany, Switzerland, and Austria is 60 μg per day for women and 70 μg per day for men, which is about half the recommendation in the United States [15**]. The variability in dietary recommendations indicates the need for a better understanding of vitamin K bioavailability and metabolism in order to establish evidence-based dietary recommendations for vitamin K.

Vitamin K Function

Vitamin K is required for post-translational modifications of glutamic acid to form γ-carboxyglutamate (Gla) residues present in certain proteins. These proteins are referred to as vitamin K-dependent proteins (VKDP) [16]. The number of VKDP identified continues to increase, and it is now evident that these proteins have important roles in physiological processes beyond coagulation, such as bone metabolism and vascular calcification [2**,16]. All vitamin K forms function as an enzyme cofactor for posttranslational modification of VKDP. However, the identification of UBIAD1 as an enzyme involved in both vitamin K and cholesterol metabolism provides indirect evidence for potential additional novel physiological roles for vitamin K [7*,8].

Vitamin K's Role in Clinical Conditions

Pediatric Vitamin K Prophalaxis: Vitamin K-deficient bleeding (VKDB), formerly called hemorrhagic disease of the newborn, is an acquired condition in infants due to insufficient activation of vitamin K-dependent coagulation proteins. VKDB presents as intracranial and gastrointestinal hemorrhage. Infants are predisposed to VKDB because of poor placental transfer, limited liver stores, reduced vitamin K production due to immature or altered gut microbiota, and low content of vitamin K in breast milk [17*,18**,19]. As a cost-effective preventive strategy, the American Academy of Pediatrics recommends vitamin K prophylaxis of a single 0.5-1mg intramuscular vitamin K injection at birth. A number of other countries, including Australia, Canada, and New Zealand have adopted recommendations for vitamin K prophylaxis of VKDB by parenteral or oral regimens. However, many developing countries still do not have a national prophylaxis program, so while preventable, VKDB is still prevalent globally. Furthermore, a recent increase in infant VKDB has been observed in some regions within U.S. [17*] and can be attributed to parents declining vitamin K prophylaxis at birth. Reasons for refusal include the perceived (but undocumented) notion of an increased risk of childhood cancer, that prophylaxis is an unnecessary treatment indicating lack of parental VKDB knowledge, and a desire to limit child's exposure to "toxins" [17*]. Concerns regarding childhood cancer were based on an earlier study that associated the intramuscular delivery of vitamin K with leukemia [20*], observed in a limited geographical region in the United Kingdom that has not been has not been replicated. The American Academy of Pediatrics has summarized the data refuting this relationship, in a statement of reaffirmation for the use of

vitamin K prophylaxis [21*]. Given its cost-effectiveness and the lack of compelling evidence of side effects, intramuscular vitamin K prophylaxis continues to be the most effective preventive strategy for VKDB.

Vitamin K-Dependent Oral Anticoagulants (VKA): Vitamin K-dependent oral anticoagulants or vitamin K antagonist (VKA) therapy is widely prescribed for thrombosis and to decrease thromboembolic risk factors, the most common agent being warfarin. Warfarin inhibits vitamin K recycling (metabolic regeneration of vitamin K capable of acting as a co-factor), hence reduces the amount of vitamin K available for carboxylation of coagulation factors II, VII, IX, X and protein C and S [22].

Current dosing guidelines were developed by the American College of Chest Physicians and the American Heart Association-American College of Cardiology and support an 'induction' dose of 2 to 5mg warfarin per day when starting therapy [23**]. This dose is then adjusted according to the INR with continual monitoring. Warfarin dosing is influenced by the genetic variability of *CYP2C9* and *VCORC1,* enzymes involved in the metabolism of vitamin K. Three recent randomized controlled trials studied the impact of genotyping on VKA dose prediction and optimization of oral anticoagulant stability [24–26]. Whereas, one trial showed that genetic-based dosing was associated with increased time spent in the therapeutic range, fewer incidences of excessive anticoagulation, and shorter time to a stable therapeutic INR [24], the two other trials did not [25,26]. Overall, none of the trials showed a benefit in terms of bleeding and thrombosis.

Inconsistent dietary VK intake is a risk factor for anticoagulant instability. Dietary VK can interfere via a warfarin-insensitive alternative pathway that is not to regenerate vitamin K capable of carboxylating VK-dependent coagulation proteins. Alternatively, over-anticoagulation can be induced by reduction in VK intake. While consistent intake of VK by attention to diet is generally associated with more stable coagulation parameters [27*], variability may occur due to issues for which little data exist. Additionally, is unknown if MK-n intake or factors that promote MK-n synthesis influence anticoagulation.

No formal guidelines for vitamin K intake at the onset of warfarin therapy exist. Instead it is a widely accepted practice to instruct patients to limit dietary vitamin K intake, and/or maintain a constant vitamin K intake. The effect of vitamin K on anticoagulation stability during onset of therapy is critical to balance risk of adverse bleeding and thromboembolic events [27*]. A recent study demonstrated that moderate levels of dietary vitamin K intake (up to 125μg/day) were associated with a lower risk of non-therapeutic international normalized ratio (INR) during initiation of warfarin therapy [27*]. As a result, recommendations for patients on warfarin should be instructed to maintain their usual dietary habits and normal vitamin K intake similar to that of the AI for adults [27*]. Although the mechanisms of warfarin action are well understood, fluctuations in INR necessitating dose changes illustrate the need for not only initial but continued monitoring of dietary VK.

In situations of over-anticoagulation, vitamin K in doses above that achieved from the diet is a safe and effective treatment for the reversal of warfarin. Current recommendations for non-therapeutic INR without major bleeding are: an INR within the therapeutic range but below 4.5 requires no vitamin K dose, an INR 4.5 to 10 in patients at an increased risk for bleeding

requires 1-2mg vitamin K orally, and an INR greater than 10 requires 2.5 to 5mg vitamin K orally or as one dose [23**]. Guidelines for warfarin reversal recommend oral doses for non-bleeding patients with an INR >10, and intravenous doses for bleeding patients [23**]. Subcutaneous administration may have a delayed response and may be unpredictable, thus oral and intravenous are preferred delivery methods. A recent observational study concluded an intravenous vitamin K dose results in a more rapid response in correction of over-anticoagulation compared to an oral vitamin K dose [28]. Both administration routes are found to reverse excessive anticoagulation effectively, and the choice of delivery method dependent on the clinical situation.

Newer oral anticoagulants that are not based on vitamin K antagonism may have advantages over warfarin such as fewer food and drug interactions, improved pharmacokinetics, fixed dosage, and less monitoring, and may result in warfarin-based oral anticoagulants becoming less widely prescribed [22].

Cystic Fibrosis: Vitamin K is a fat soluble nutrient whose absorption is facilitated by even a modest amount of fat in the small intestine. Conditions that decrease dietary fat digestion or absorption can diminish absorption of VK. Cystic fibrosis resulting in pancreatic insufficiency and lipid malabsorption is associated with fat-soluble vitamin (FSV) deficiencies [29**,30]. Additional risk for FSV insufficiency in the setting of cystic fibrosis may result from long-term antibiotic use. While FSV supplementation has become more common, a recent retrospective study of cystic fibrosis patients revealed that despite a doubling in FSV supplementation dose, vitamin K concentrations did not change significantly over the five year

period. Contributing factors to the non-response may have been suboptimal dosage, low adherence, or low bioavailability of supplements due to malabsorption [31]. Specific recommendations for vitamin K supplementation range from 300 to 500 μg/day for patients with cystic fibrosis and pancreatic insufficiency. However, the efficacy of vitamin K supplementation to normalize blood concentrations similar to that of a healthy adult remains unclear. A recent study examining a lipid supplement that is absorbable without pancreatic enzymes, in aiding FSV absorption demonstrated that 12 months of supplementation with the lipid supplement improved vitamin K status [32]. Thus, in order to improve vitamin K status in patients with cystic fibrosis and pancreatic insufficiency, therapeutic goals may include both correcting fat malabsorption and providing higher doses of vitamin K.

Bariatric Surgery: Bariatric surgery is now popular due to the prevalence of obesity and its associated co-morbidities. The goal of bariatric or weight loss surgery is to reduce the risk of illness and death associated with obesity [33,34]. The type of bariatric surgery (restrictive and/or malabsorptive) and the corresponding procedure can result in different nutrient deficiencies [34]. Restrictive procedures, including adjustable gastric banding, may reduce intake but should not impact micronutrient status by altering absorption. However, due to post-operative dietary restrictions, supplements may be required [33]. Roux-en-Y gastric bypass and biliopancreatic diversion are both restrictive and malabsorptive approaches in bariatric surgery that limit stomach capacity and bypass sections of the small intestine [35,36*]. These forms of bariatric surgery require micronutrient supplementation, with the more malabsorptive biliopancreatic diversion resulting in greater risk for FSV deficiency. The topic has been recently reviewed [37]

and standard supplementation regimens have been recommended [38], doses required to maintain normal blood concentrations may be patient-specific [39]. A recent prospective study examined post-operative concentrations of FSV and followed up over a 2-year period. They concluded that the initial prescription for supplementation was insufficient to normalize blood concentrations, thus monitoring and multiple adjustments are necessary [36*].

The increased popularity of bariatric surgery and variety of treatment options reinforce the need to better understand longer term outcomes that are age and sex-specific, such as pregnancy. Weight reduction from surgical intervention results in lower incidence of obesity-associated complications including hypertension, gestational diabetes, large-for-gestational age, and need for caesarian section [40,41]. Yet, increased incidence of premature delivery and growth restriction among these patients has been observed, and the malabsorption and malnutrition associated with bariatric surgery may increase risk for adverse neonatal outcomes [40,41]. A case report examined maternal malnutrition and incidence of chondrodysplasia punctata in patients who underwent bariatric surgery [42]. Maternal vitamin K deficiency or exposure of warfarin can result in this phenotype of abnormal chondrogenesis and calcification [42]. In both cases, women were deficient throughout pregnancy secondary to bariatric surgery [42]. Evaluation of micronutrient status pre and post conception in women following bariatric surgery by a practitioner knowledgeable in management of micronutrient disorders associated with bariatric surgery is strongly recommended [43]. Additional studies for specific FSV are necessary in order to provide short and long term recommendations for the increasing number of individuals who have bariatric surgery.

Abnormal Soft Tissue Calcification: Vascular calcification is an indication of atherosclerosis and predictive of cardiovascular events and overall mortality independent of traditional cardiovascular disease (CVD) risk factors [44**]. Research has linked the VKDP osteocalcin, matrix Gla protein (MGP), and Gla rich protein (GRP) to mechanisms of vascular calcification [1]. These proteins regulate mineralization, calcium crystal formation, and differentiation or proliferation of vascular smooth muscle cells, which control the pathological calcification of vascular tissues. Limited data exist on the impact of vitamin K-dependent carboxylation on soft tissue calcification. Population-based observational studies of vitamin K intake and vascular calcification have been summarized elsewhere and are inconclusive [44**]. Few controlled trials have explored the relationship between vitamin K supplementation, VKDP carboxylation, and the progression of vascular calcification. Two intervention studies of vitamin K supplementation and vascular calcification have associated supplementation and better arterial elasticity and distensibility [45,46]. These finding support a potential role for vitamin K in modulation of vascular calcification. Ongoing clinical trials including VitavasK (NCT01742273), VitaK-CAC (NCT01002157), VITAKANDOP (NCT01232647), SAFEK (NCT01533441), OVWAK VII (NCT00990158), will provide more insight into the therapeutic potential of vitamin K in soft tissue calcification [1]. These trials are need to confirm benefit, define target populations, and identify the most effective supplementation regimens.

A clinical population susceptible to soft tissue calcification is chronic kidney disease (CKD) patients. The prevalence of vascular calcification and cardiovascular mortality is greater in patients with CKD. The role of vitamin K in the inhibition of calcification has been established through animal models of CKD [47*,48]. Dietary supplementation of vitamin K

increased tissue concentrations of phylloquinone (kidney, liver, serum, heart, aorta) and reduced the severity of vascular calcification [47*]. A therapeutic dose of warfarin depleted tissue stores of phylloquinone and increased susceptibility to vascular calcification, which further suggests vitamin K status may alter risk of calcification when CKD is present [47*,49*]. A recent examination of the NHANES III data suggested that low vitamin K intake may be associated with all-cause mortality in individuals with CKD; however, vitamin K intake may simply be a reflection of a healthy diet that is independent of CKD and calcification [50*]. To test this, two ongoing clinical trials the European VitaVasK study and the Canadian iPack-HD study are examining supplementation of 5mg phylloquinone, three times per week for 18 months, and 10mg phylloquinone, three times per week for 12 months respectively [49*]. The results of these studies will provide insight into the therapeutic potential of vitamin K in CKD, and future recommendations for this population.

Diabetes: Observational and intervention studies have shown a benefit of increased phylloquinone intake on improving measures of glucose homeostasis [51*], and a protective role for vitamin K against insulin resistance (IR) has been proposed [51*]. The mechanisms by which vitamin K influences IR are unclear, but evidence suggests that it may act through modulating inflammation [2**]. A recent analysis of the PREDIMED study reported higher dietary phylloquinone intake improved inflammatory markers associated with IR [52], further supporting this hypothesis. Another proposed mechanism may be through the action of VKDP, specifically osteocalcin. Data are limited, however, and secondary analyses of human data linking osteocalcin and IR have been inconclusive [51*,53–56]. More research is required to

confirm a role for vitamin K on glucose and insulin homeostasis and to determine if vitamin K supplementation should be recommended.

Conclusion:

Vitamin K has evolved from a nutrient thought to only be involved in coagulation to a multifunctional enzyme cofactor for various VKDP and their diverse functions. Current recommendations for vitamin K have been adapted for the prevention of VKDB and for the reversal of VKA. Although vitamin K may have therapeutic potential for chronic diseases including CVD, CKD, and IR, understanding of the role of vitamin K in these conditions is limited. Gaps in understanding about optimal forms, doses, and relative bioavailability limit dietary recommendations for the entire population as well as subsets that are at risk for specific conditions.

Key Points:

- Vitamin K has shifted from being solely characterized as a hemostatic vitamin to a multi-functional vitamin due to the different forms of vitamin K and its role in carboxylation of VKDP.
- Established guidelines for use of therapeutic vitamin K exist for prevention of VKDB in infants, and reversal of VKA.
- Altered lipid absorption may impact vitamin K status due to impaired FSV absorption and metabolism, cystic fibrosis and bariatric surgery are specific clinical conditions where vitamin K status may be affected and require supplementation.
- Vitamin K may impact soft tissue calcification and IR through the modulation of VKDP carboxylation, but more research is required prior to making specific dietary recommendations for vitamin K.

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There are no conflicts of interest.

References and Recommended Reading:

Papers of interest, published within the annual period of review have been highlighted as:

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Table 1. Summary of vitamin K biomarkers and methods of analysis.

Biomarker	Method	Strengths and Limitations	Clinical Use	Research Use	Ref
Serum or Plasma PK	HPLC	- Global measure of vitamin K status - Responds to changes in vitamin K intake - Validated assay with international quality assurance scheme (KEQAS) - Large variability - Must adjust for triglycerides	Yes	No	[14]
%ucOC	RIA or ELISA	- Responds to changes in vitamin K intake - May reflects bone vitamin K status - Must adjust for total OC - Cannot differentiate between the number of glutamate residues that are uncarboxylated - Clinical relevance unknown	No	Yes	[15]
Dp-ucMGP	Dual antibody-ELISA	- Responds to changes in vitamin K intake - May reflect smooth vascular muscle vitamin K status - Clinical relevance unknown	No	Yes	[16]
PIVKA-II	ELISA	- Useful as a measure of vitamin K deficiency - Low volume is useful for pediatric samples - Lacks sensitivity to reflect differences in vitamin K status in a healthy cohort	No	Yes	[17]
INR	$(Patient_{PT}/Control_{PT})^{ISI}$	- Validated method to monitor patients on warfarin or related oral anticoagulant therapy, patients with coagulation disorders - Lacks sensitivity for use as a measure of vitamin K status	Yes	No	[18]

Legend: HPLC: high performance liquid chromatography; %ucOC: percent undercarboxylated osteocalcin; RIA: radioimmunoassay; ELISA: enzyme-linked immunosorbent assay; dp-ucMGP: desphospho-uncarboxylated matrix gla-protein; PIVKA: protein induced vitamin K absence; INR: international normalized ratio; PT: prothrombin time; ISI: international sensitivity index.

The role of vitamin K in chronic aging diseases: inflammation, cardiovascular disease and osteoarthritis

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Conflicts of Interest

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Abstract

Vitamin K is an enzyme cofactor required for the carboxylation of vitamin K dependent proteins, several of which have been implicated in diseases of aging. Inflammation is recognized as a crucial component of many chronic aging diseases and evidence suggests vitamin K has an anti-inflammatory action that is independent of its role as an enzyme co-factor. Vitamin K-dependent proteins and inflammation have been implicated in cardiovascular disease and osteoarthritis, which are leading causes of disability and mortality in older adults. The purpose of this review is to summarize observational studies and randomized trials focused on vitamin K status and inflammation, cardiovascular disease, and osteoarthritis. Although mechanistic evidence suggests a protective role for vitamin K in these age-related conditions, the benefit of vitamin K supplementation is controversial because observational data are equivocal and the number of randomized trials is few.

Introduction

Vitamin K is a fat soluble vitamin found in two natural forms: phylloquinone (vitamin K1) and menaquinones (collectively known as vitamin K2), which differ from phylloquinone in length and saturation of the side chain [1*] (**Figure 1**). Phylloquinone, found predominantly in dark green leafy vegetables and vegetable oils is the primary dietary form of vitamin K in Western diets [2,3]. Menaquinones, which are found in animal based foods such as dairy and meats, as well as in fermented foods, are thought to contribute less than phylloquinone to overall vitamin K intakes in Western diets [4–6]. The current recommended Adequate Intake for vitamin K set by the United States' Institute of Medicine is 120 micrograms and 90 micrograms per day for adult males and females respectively [3]. Vitamin K intake has shown great variation among age groups and geographic location [7]. Among those at greater risk for low vitamin K status are older adults [8,9].

The only known function of vitamin K is as an enzymatic co-factor for the post-translational carboxylation of certain proteins (called vitamin K-dependent proteins). Carboxylation confers function to these proteins. While the most common vitamin K-dependent proteins are clotting proteins, vitamin K dependent proteins have been discovered in several extra-hepatic tissues and have important physiological functions, for example in soft-tissue calcification and bone metabolism [1*,10–12]. Additionally, vitamin K has been shown to have anti-inflammatory effects, through mechanisms that appear to be independent of its role as an enzymatic co-factor [13–15]. Vitamin K and vitamin K-dependent proteins have been linked to several age-related diseases in observational and intervention studies. The purpose of this review is to summarize key population-based studies and randomized trials focused on vitamin K nutritional status and age-related health outcomes in community-dwelling adults, namely

inflammation, cardiovascular disease and osteoarthritis (**Table 1**). Vitamin K has been implicated in age-related bone loss and studied extensively in that regard. This body of literature was reviewed extensively in 2014 [16], so studies focused on bone loss and fractures are not included here. Additionally, kidney function declines with age and a unique role for vitamin K in kidney disease has been proposed, which has been thoroughly reviewed [17]. Therefore studies focused on kidney disease are not included here.

Vitamin K and inflammation

Aging is characterized by a chronic low-grade pro-inflammatory state [18]. Age related increases in C-reactive protein (CRP), interleukin- (IL-6), and tumor necrosis factor-α (TNF-α) resulting in the low-grade inflammation appear to contribute to the onset and progression of chronic aging diseases including cardiovascular disease, osteoarthritis and other chronic diseases [19–22].

In vitro and animal experiments have found vitamin K suppresses production of pro-inflammatory cytokines [13–15]. At this time, however, the relevance of vitamin K nutritional status to inflammation in humans remains unclear. In a cross-sectional analysis of the Framingham Offspring (n= 1,381; mean age = 59±8 years), higher vitamin K intake was associated with lower inflammation overall and with lower concentrations of several individual pro-inflammatory biomarkers [11]. In a secondary analysis of the PREDIMED trial, a Mediterranean diet intervention being conducted in Spain, (n= 510; mean age = 67.2±6 years) participants who increased their dietary phylloquinone intake the most (≥70 mcg/d) had the greatest reductions in IL-6, and TNF-α over 1 year. In this analysis, the intervention and control groups were combined, which is not consistent with the RCT design [23]. In both studies [11,23] vitamin K intake was estimated using self-report measures, which are inherently limited (as

recently reviewed [24**]). Phylloquinone is found in generally healthy foods, so it cannot be discounted that the reported associations reflect a healthy diet rather than phylloquinone intake specifically.

Nutritional biomarkers are considered more objective measures of nutrient status and reflect nutrient intake, metabolism, and absorption [25]. There are multiple biomarkers indicative of vitamin K status, but no single biomarker is considered the 'gold-standard' [24**]. Circulating phylloquinone, a global indicator of vitamin K status, has been evaluated in relation to inflammation. In the Framingham Offspring higher plasma phylloquinone was also associated with lower inflammatory-burden cross-sectionally, consistent with the findings of vitamin K intake [11]. Higher serum phylloquinone was also associated with lower inflammation cross-sectionally in a multi-ethnic cohort of adults without clinically apparent CVD (the Multi-ethnic Study of Atherosclerosis, MESA, n= 662; mean age=62±10 years) [26].

Vitamin K status can also be estimated by measuring the uncarboxylated fractions of certain vitamin K dependent proteins in circulation, as recently reviewed [24**]. Osteocalcin (OC) is a vitamin K dependent protein synthesized in bone and higher circulating uncarboxylated OC (ucOC) reflects low vitamin K status [24**]. In the Framingham Offspring, ucOC, however, was overall not associated with inflammation [11]. That plasma phylloquinone, but not ucOC, was associated with an inflammatory burden, suggests vitamin Ks role in inflammation is independent of its role as an enzyme factor. Since this has not been well studied, it is premature to draw conclusions about the mechanism underlying the apparent anti-inflammatory effects of vitamin K. Additionally, these studies are cross-sectional [11,26], so causal relationship between vitamin K status and inflammation is uncertain.

Randomized trials can address causality and inflammatory measures have been evaluated as secondary outcomes in two trials conducted in older adults. However, neither phylloquinone [27] nor menaquinone-7 [28] supplementation reduced circulating inflammatory biomarkers in older men and women over 3 years. Both of these studies enrolled generally healthy older adults, who are less likely to have substantial increases in inflammation, so any ability to see an effect of vitamin K (or any nutrient) in reducing inflammation may have been blunted. It is plausible the anti-inflammatory effects of vitamin K may be more relevant to groups with a higher inflammatory burden.

Vitamin K, arterial calcification, and cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of mortality in adults ≥ 65 years old [29]. Coronary artery calcification (CAC) is indicative of subclinical CVD and predicts clinical cardiovascular events and all-cause mortality [30–33]. A role for vitamin K in CVD has been proposed, based on the presence of vitamin K-dependent proteins, such as matrix Gla protein (MGP), in vascular tissue [34,35*]. When MGP is carboxylated, which requires vitamin K, it inhibits calcification in arterial and other soft tissues.

The association between vitamin K status, CAC, and CVD has been evaluated, with equivocal results, as reviewed in 2012 [36*]. Results of more recent studies are also inconclusive. In a case-cohort analysis of the MESA, low serum phylloquinone was associated with a 34% higher odds of CAC progression over 3 years, but statistical significance was not reached [OR(95%CI) 1.34(0.94-1.90)] [37]. In secondary analysis, low serum phylloquinone was associated with a 2-fold higher odds of CAC progression in persons treated for hypertension [OR (95% CI): 2.37 (1.38 - 4.09)] but was not associated with CAC progression in persons not treated for hypertension. Although this was not hypothesized a priori, it was replicated in a post hoc

analysis of a phylloquinone supplementation trial [37]. The circulating concentration of phylloquinone considered sufficient is not yet defined clinically, so in this study low plasma phylloquinone was defined as < 1.0nmol/L, which is the concentration achieved when Adequate Intakes are met [8,38]. In a sub-study of the Dutch Prospect cohort, post-menopausal women (n=508 mean±SD age 57±5 years), with higher plasma phylloquinone, defined as >0.7 nmol/L, had a *higher* prevalence of CAC [39]. However in this study, CAC was defined as absent or present at a single time point measured 7 - 11 years *after* plasma phylloquinone was measured, so it is not known if CAC was actually prevalent at the time phylloquinone status was assessed. Furthermore, this analysis did not account for triglycerides, which may have confounded the findings. Phylloquinone is transported on triglyceride-rich lipoproteins [40], so adjustment for triglycerides is imperative in studies utilizing circulating phylloquinone as a biomarker, especially in relation to CVD since elevated triglycerides are a CVD risk factor [41].

Assays that measure uncarboxylated MGP in plasma have been developed [42] and are now commercially available. The dephosphorylated uncarboxylated MGP ((dp)ucMGP) responds to changes in vitamin K intake [43,44] and is thought to be a functional indicator of vitamin K status in tissues that use MGP. Higher plasma (dp)ucMGP reflects lower vitamin K status. (Dp)ucMGP has been evaluated in relation to cardiovascular outcomes in clinical observational studies. (There are other circulating forms of MGP, some of which have been evaluated in relation to CVD, but do not reflect vitamin K status [42], so are not considered here.) In 195 post-menopausal women analyzed cross-sectionally, higher (dp)ucMGP was associated with more CAC, although statistical significance was borderline (p=0.065) [45]. In a secondary analysis of the randomized controlled trial that found phylloquinone supplementation reduced CAC progression in older men and women, (dp)ucMGP was reduced by phylloquinone

supplementation, but the change in (dp)ucMGP did not correlate with change in CAC [43]. In type II diabetics in the Dutch European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, each standard deviation increase in (dp)ucMGP (reflecting lower vitamin K status) was associated with a 21% higher risk for CVD over 11 years of follow-up (HR(95%CI) 1.21 (1.06-1.38) [46]. However, a case-cohort analysis of the same cohort, did not find (dp)ucMGP to be associated with coronary heart disease or stroke [47]. In contrast, a longitudinal study conducted in 577 community dwelling older men and women in the Logitudinal Aging Study Amersterdam (LASA) who were free of CVD at baseline found that after a mean follow-up of 5.6+1.2 years, individuals with the highest circulating (dp)ucMGP had a 2-fold significantly higher risk of CVD [HR(95%CI) 2.69(1.09-6.62)] [48].

A growing body of evidence suggests arterial calcification is also implicated in arterial stiffness [49–51]. Arterial stiffness increases with age and is also an independent risk factor for CVD [52-54]. Examination of data from the National Health and Nutrition Examination Surveys 2007-2008, 2009-2010 of older adults (n=5296, age >50 yrs) showed that inadequate phylloquinone intake was a significant independent predictor of high arterial stiffness [55], although it is plausible this association is due, in part, to participants reporting low phylloquinone intake also consuming a less healthy diet. A cross sectional analysis of the Czech post-MONICA study found individuals in the highest dp-ucMGP quartile had a higher risk of elevated aortic stiffness [OR(95% CI): 1.73 (1.17-2.5)] [56]. Similar findings were obtained in a cross-sectional analysis of a family based study in Switzerland, in which (dp)ucMGP was positively associated with arterial stiffness after adjusting for various confounders [57]. Menaquinone-7 supplementation improved some parameters of arterial stiffness over 3 years in post-menopausal women (n=244; mean±age = 60±3) in a randomized trial designed test the

effect of menaquinone supplementation on bone strength, but measured arterial stiffness as a secondary outcome [28]. It is not known if these findings are generalizable to men or other groups.

There are two systematic reviews examining the association between vitamin K status and CVD. The first one, published in 2010, suggested a beneficial effect of menaquinone intake, but not phylloquinone intake, in lowering CVD risk [58]. This is primarily due to three studies conducted in the Netherlands that all reported inverse associations between menaquinone intake and CVD [59–61]. Whether or not these findings generalize to other countries or nationalities is not known. Menaquinone intake was assessed using food frequency questionnaires, which carry inherent limitations [62]. At the time these studies were conducted food composition databases for menaquinones were limited. (They are currently being expanded) [24**]. It is therefore premature to draw conclusions regarding the relative importance of phylloquinone and menaquinones with respect to CVD based on the available studies. A systematic review of vitamin K and CVD published in 2015 sought to include only vitamin K supplementation trials of at least 3 months in duration and conducted in healthy adults or adults at high risk for CVD [63]. Ultimately only one trial that tested the effect of menaquinone-7 supplementation on blood pressure and lipid levels over 12 weeks 60 men and women 45-60 years old was included in the review [63,64]. Overall, the authors concluded there is insufficient evidence to concludevitamin K affects CVD [63]. Of note, this review did not include the only randomized trial designed to test the effect of phylloquinone supplementation on CAC progression in older adults (n=388, mean±SD; age = 68±6 yrs) because the intervention and control groups both received calcium and vitamin D (to assure all participants were replete in those nutrients); hence the trial lacked a pure placebo group [27,63]. This trial found older adults who adhered to the 3-year

phylloquinone supplementation intervention had significantly less CAC progression compared to adherent participants in the control group, suggesting a protective effect of phylloquinone against subclinical CVD. In the intent-to-treat analysis, however, phylloquinone supplementation did not significantly affect CAC progression [27]. In a subsequent post-hoc analysis of this trial it was found that phylloquinone supplementation reduced CAC progression in participants treated for hypertension but did not affect CAC progression in participants not treated for hypertension [37]. While this suggests vitamin K may be particularly beneficial to hypertension therapeutic regimens, these findings need to be confirmed in studies designed to study treated hypertensives.

Although some observational studies suggest improving vitamin K status may reduce subclinical and clinical CVD, data are conflicting [27,37,39,59,61]. Additional prospective studies are necessary to determine whether increasing vitamin K intake decreases risk for cardiovascular events and subclinical CVD and whether this is modulated by (dp)ucMGP.

Vitamin K and osteoarthritis

Osteoarthritis is the leading cause of lower-extremity disability in older adults, and there is currently no therapy known to reduce osteoarthritis progression. Osteoarthritis is characterized by pathological changes in all joint tissues, including cartilage and bone. Vitamin K has been implicated in osteoarthritis because vitamin K dependent proteins are found in cartilage and bone [65–67]. MGP is among the most studied vitamin K dependent protein expressed in human cartilage. Uncarboxylated MGP (the nonfunctional form) is elevated in human arthritic cartilage, while carboxylated (functional) MGP is more abundant in healthy cartilage [65]. Gla rich protein (GRP), which is another vitamin K dependent implicated in calcification, has recently identified in human articular cartilage [68]. Similar to MGP, GRP from arthritic cartilage is primarily uncarboxylated, whereas GRP from healthy cartilage is primarily carboxylated [69].

In a cross-sectional analysis of older men and women from Japan, low vitamin K intake was associated with a higher prevalence of radiographic knee osteoarthritis [70]. In a cross-sectional analysis of the Framingham Offspring, low plasma phylloquinone, was associated with higher knee and hand osteoarthritis prevalence [71]. In the Multicenter Osteoarthritis (MOST) Study (mean±SD age = 62±8 yrs), participants with subclinical vitamin K deficiency (defined as plasma phylloquinone < 0.5 nmol/L) were 1.5 – 2 times more likely to develop radiographic knee osteoarthritis and cartilage damage over 30 months (risk ratio (95%CI) 1.56(1.08-2.25) and 2.39(1.05-5.40) respectively, compared to those without subclinical deficiency) [72]. In the Health Aging and Body Composition (Health ABC) Study (mean±SD age = 74±3 yrs), older community-dwelling adults with very low plasma phylloquinone (defined as below the assay limit of detection, <0.2 nmol/L) had a 1.7-and 2.6-fold higher odds of worsening cartilage damage and meniscal damage over 3 years. This analysis also suggested participants with very low phylloquinone were more likely to have bone attrition, subarticular cyst, and osteophyte progression, but statistical significance was not reached [OR (95% CI): 1.9(0.9–3.6); 1.5(0.8–2.7); 1.5(0.8–2.8) respectively] [73]. In this same study, individuals with elevated (dp)ucMGP (reflecting low vitamin K status) were more likely to have osteophytes, bone marrow lesions, subarticular cysts, and meniscus damage cross-sectionally, but this was not associated with progression of any structural abnormalities in the longitudinal analysis [73]. This may suggest vitamin Ks role in these pathologies is independent of its function as an enzymatic co-factor in the carboxylation of MGP. Neogi et al assessed the effect of phylloquinone supplementation on radiographic hand osteoarthritis using x-rays obtained at the end of study and did not find any effect of 3 years phylloquinone supplementation [74]. No baseline measures of hand osteoarthritis were available in this study. When participants with baseline plasma phylloquinone

<1.0nM were analyzed separately, there was a trend towards less joint space narrowing (a measure of radiographic osteoarthritis) in the phylloquinone supplemented group, suggesting persons with low vitamin K status are more likely to benefit from vitamin K supplementation, but this finding needs to be confirmed in trials designed specifically to test the effect of vitamin K on osteoarthritis development and progression. In this same cohort, MGP genotype was found to be associated with hand OA, but circulating total MGP concentrations were not [75].

While collective data suggest a protective role for vitamin K in osteoarthritis, several questions remain. Low circulating phylloquinone was associated with more OA in three cohorts, but low circulating phylloquinone has not been consistently defined [72,73,75]. It is not clear what level is 'sufficient' in terms of joint health. Vitamin Ks role in osteoarthritis was proposed because vitamin K dependent proteins are present in cartilage and bone. However, alternate mechanisms may exist. Osteoarthritis has been characterized as having an inflammatory component with measureable cytokines and inflammatory mediators present in the synovium of the joint resulting in joint pain, swelling, and stiffness [22]. Vitamin K appears to have anti-inflammatory effects [13–15], suggesting an alternate pathway through which vitamin K may affect joint health – an area of research that merits attention. Clinical trials are needed to evaluate the efficacy of vitamin K supplementation in OA development and progression.

Conclusion

There is accumulating evidence to support a protective role for vitamin K in chronic aging conditions and diseases, such as inflammation, cardiovascular disease, and osteoarthritis, but there are also inconsistencies in the studies conducted to date. It is therefore premature to make recommendations regarding vitamin K's efficacy in improving inflammation, and cardiovascular and joint health. Clinical trials designed to test the effect of vitamin K

supplementation on these chronic age-related conditions are needed. As the aging population continues to grow, the prevalence of these diseases will rise dramatically. Identifying and understanding nutritional factors that impact the progression or treatment of these diseases is imperative to address the health and function of older adults worldwide.

References

(*) of importance

(**) of major importance

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Table 1. Observational studies of vitamin K, inflammation, cardiovascular disease, and osteoarthritis					
Participants	Study Design	Vitamin K status exposure	Outcome(s)	Overall finding	Reference
Inflammation					
PREDIMED N=510 Age: (yrs) 67.2±6 55% female	Cross-sectional	Dietary phylloquineon intake (mcg/d)	Inflammatory biomarkers (12+ including TNF-a, IL-6)	Individuals who increased dietary phylloquinone intake had reduced IL-6.	[23]
Framingham Offspring Study N=1,381 Age: 59±8 52% female	Cross-sectional	Plasma phylloquinone (nmol/L) Dietary phylloquinone intake (mcg/d) %ucOC	Inflammation summary statistic Inflammatory biomarkers (13+)	Plasma phylloquinone inversely correlated with grouped inflammatory markers, and CD40 ligand, IAM-1, IL-6, OPG, TNF-α individually.	[11]
MESA N=662 Age: 62 ±10 46% female	Cross-sectional	Plasma phylloquinone (nmol/L)	Inflammatory biomarkers (6+ including IL-6, CRP)	Individuals with highest plasma phylloquinone had lower IL-6 levels.	[26]
N=388 Age: 86±6 60% female	RCT	phylloquinone supplementation (500mcg/d) MGP (ng/mL)	IL-6, CRP	No effect of supplementation on inflammatory markers.	[27]
N= 244 Post-menopausal women Age: 59.5 ±3.3	RCT	Menaquinone-7 supplementation (180mcg/d) Dp-ucMGP (pmol/L)	IL-6, CRP, TNF-a	No effect of supplementation on inflammatory markers.	[28]
Arterial calcification, stiffness, and CVD					

MESA N=857 Age: 64±10 45% female	Case-cohort	Plasma phylloquinone (nmol/L)	CAC Agatston score category: 0 AU 1-400 AU >400 AU	Low phylloquinone status is associated with greater CAC progression in antihypertensive users.	[37]
N=508 post-menopausal women Age: 56±6	Longitudinal	Plasma phylloquinone (nmol/L)	Aortic valve, mitral valve, or aortic artery calcification	Detectable circulating phylloquinone was not associated with reduced vascular calcification.	[39]
N=388 Age: 86±6 60% female	RCT	phylloquinone supplementation (500mcg/d) MGP (ng/mL)	CAC Agatston score	Intent to treat analysis: no difference in supplemented group. In those who adherence >85% had slowed progression.	[27]
N=564 Post-menopausal women Age: 67±5	Cross-sectional	Dietary menaquinone intake (mcg/d)	CAC Agatston score	High dietary menaquinone intake is associated with decreased coronary calcification.	[59]
Rotterdam Study N=4807 Age: 67±8 60% female	Longitudinal	Dietary menaquinone intake (mcg/d)	Aortic calcification	Menaquinone intake in inversely associated with severe aortic calcification.	[60]
Prospect-EPIC study N=16,057 post menopausal women Age: 57±6	Longitudinal	Dietary phylloquinone intake (mcg/d) Dietary menaquinone intake (mcg/d)	CVD	Higher menaquinone intake associated with lower incidence in CVD.	[61]

N=60 Age 60±3 60% female	RCT	Menaquinone-7 supplementation: 180 μg/d, 360 μg/d MK-7 or placebo for 12 weeks	Plasma dp-ucMGP, dp-cMGP, HDL, TGs	Menaquinone-7 decreased dp-ucMGP in a dose dependent manner. No effect on cardiovascular risk factors.	[64]
N=374 Age: 86±6 60% female	RCT	phylloquinone supplementation (500mcg/d)	Plasma ucMGP (ng/mL)	Phylloquinone supplementation significantly reduced plasma ucMGP over 3 years, but 3 year changein ucMGP was not associated with CAC	[43]
N=200 Post menopausal women Age: 66.9±5.5	Cross-sectional	Plasma dp-ucMGP	CAC	Trend towards high ucMGP associated with decreased CAC.	[45]
EPIC-NL N= 518 Type 2 diabetics Age: 58.1±7.1 82% female	Longitudinal	Plasma dp-ucMGP	CVD risk	Higher circulating dp-ucMGP was associated with significant increased risk of CVD in type 2 diabetics with peripheral artery disease (PAD) and heart failure.	[46]
EPIC-NL N=2985 Age: 49.5±11.8 75% female	Case-cohort	Dp-ucMGP	CVD and stroke risk	No association between circulating dp-ucMGP and stroke risk or CVD risk.	[47]
LASA N=577 Age: > 55	Longitudinal	Plasma dp-ucMGP	CVD incidence	Increased risk of CVD in highest tertile of dp-ucMGP indicative of vitamin K insufficiency.	[48]
NHANES N=5296 Age: >50	Cross-sectional	Dietary phylloquinone intake (mcg/d)	Arterial stiffness by Pulse pressure	Inadequate dietary phylloquinone intake was a strong and significant predictor of higher arterial pulse pressure.	[55]

Czech MONICA study N=1087 Age: 54.8±13 53% female	Cross-sectional	Dp-ucMGP	Arterial stiffness by aortic and distal pulse wave velocities	Individuals with highest circulating dp-ucMGP had highest risk of elevated aortic pulse wave velocity, indicating aortic stiffness.	[56]
N=1001 Age: 46.5±17.2 52% female	Cross-sectional	Dp-ucMGP	Arterial stiffness by aortic pulse wave velocity	Circulating dp-ucMGP were positively associated with dp-ucMGP before and after adjusting for lifestyle and health factors	[57]
N= 244 Post-menopausal women Age: 59.5 ±3.3	RCT	Menaquinone-7 supplementation (180mcg/d)	Aterial stiffness by aortic and arm pulse wave velocity	Menaquinone-7 supplementation improved arterial stiffness in individuals with higher baseline stiffness index.	[28]
Osteoarthritis					
ROAD study N=719 Age: >60 60% female	Longitudinal	Dietary phylloquinone (mcg/d)	Radiographic knee OA	Dietary intake was inversely associated with presence of knee OA and JSN	[70]
Framingham Offspring Study N=672 Age: 65.6±8.5 53% female	Cross-sectional	Plasma phylloquinone (nmol/L)	Osteoarthritis (OA) prevalence Osteophytes Joint space narrowing (JSN)	Low plasma phylloquinone associated with increase prevalence of OA in the hand and knee	[71]
MOST study N=1180 Age: 62±8 62% female	Longitudinal	Plasma phylloquinone (nmol/L)	Radiographic knee OA	Individuals with subclinical circulating phylloquinone were more likely to develop knee OA	[72]
Health ABC study N=791	Cross-sectional	Plasma phylloquinone (nmol/L) Dp-ucMGP	Knee OA structural features by MRI	Longitudinally, adults with low plasma phylloquinone more likely to have articular and meniscus cartilage	[73]

Age: 74±3 67% female	Longitudinal			damaged. Cross sectionally, higher plasma dp-ucMGP was associated with increased odds of OA features including osteophytes, and lesions.	
N=376 Age: 71±5.5 60% female	RCT	Serum MGP (ng/mL)	Radiographic hand OA	No association between circulating MGP and hand OA	[75]

a. PREDIMED: PREvención con DIeta MEDiterránea study
b. MESA study: Multiethnic Study on Atherosclerosis
c. Prospect-EPIC study: The European Prospective Investigation into Cancer and Nutrition Prospect cohort
d. EPIC NL- The European Prospective Invesigation into Cancer Dutch cohort
e. LASA: Longitudinal Aging Study in Amsterdam
f. NHANES: National Health and Nutrition Examination Surveys
g. ROAD study: Research on Osteoarthritis Against Disability
h. MOST study: The Multicenter Osteoarthritis Study
i. Health ABC: Health, Aging, and Body Composition study

Mixed dishes and restaurant foods are an unexpected source of dietary vitamin K

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Abstract

Background: Vitamin K's role in coagulation has nutrition therapy implications for those taking coumarin-based oral anticoagulation medications. Variable vitamin K intake can result in unanticipated interactions with stability of these medications. Current recommendations emphasize a consistent intake of vitamin K-rich foods while taking these medications. Comprehensive and current data on vitamin K content of mixed dishes and restaurant foods are lacking.

Objective: To characterize and quantify the amount of vitamin K per serving of various mixed dishes and restaurant foods in a representative U.S. sample.

Design: Food samples were obtained from the USDA Nutrient Data Laboratory as part of the National Food and Nutrient Analysis Program, and analyzed for three vitamin K forms (phylloquinone, 2',3'-dihydro-phylloquine, and menaquinone-4) using high performance liquid chromatography. Foods were classified into high or moderate vitamin K containing in accordance with the Nutrition Care Manual.

Results: Out of the 65 mixed dishes and restaurants foods analyzed, 21 were high or moderate in vitamin K content.

Conclusion: Vitamin K-containing oils and animal products in mixed dishes and restaurant foods can contribute substantially to a person's total daily vitamin K intake. This has important clinical implications for those prescribed oral anticoagulants that are vitamin K-antagonists.

Introduction

Vitamin K (VK) is a fat-soluble vitamin that has two natural forms: phylloquinone (PK) and menaquinones (MKn), a family of several similar molecules. All forms are characterized by a unique ring structure but differ in the length and saturation of the side chain[1,2]. Plant-synthesized PK is the primary source of VK in the diet. Dark green leafy vegetables and certain plant oils, including canola, soybean, and olive are the predominant sources.[3,4] Another form, 2',3'-Dihydro-phylloquine (dK) is formed by hydrogenation of PK during commercial processing of these oils.[3] The biological role of dK is unclear. Compared to PK, MKn are typically present in food in a much lower concentration. Animal-products are the major source of menaquinone-4 (MK4), which originates from either the animals' diet or through tissue conversion of PK.[5–7] Although bacterially produced, long-chain MKn (MK7 through MK14) are found in select fermented products.[8,9] MK4 was the only MKn analyzed in this study.

There is no established recommended daily allowance for VK due to the insufficient knowledge regarding metabolism and function of VK and its metabolites, all of which are critical for establishing dietary requirements. Instead, the adequate intake (AI) for VK is based upon dietary intake of PK observed in healthy individuals.[4,10] The AI is 120 micrograms (µg) and 90 micrograms (µg) per day for adult men and women, respectively.[4,10]

All VK forms participate in a post-translational modification of vitamin K-dependent proteins (VKDP). There are many VKDP that have varying important roles in physiological processes including coagulation, bone metabolism, and vascular calcification. [2,11] However, the most well-characterized VKDP are those essential to coagulation.[12] In the U.S., 4.2 million people are prescribed anticoagulant medication.[13] The most commonly prescribed, warfarin, a coumarin-based anticoagulant, directly counter-acts VK ability to act on VKDP involved in

coagulation.[14] This has important nutrition therapy implications as inconsistent VK intake can result in over anticoagulation and bleeding or inefficient antagonism resulting in instability of oral anticoagulation therapy. [14–17] MK4 have also been shown to effect coagulation, though it is unclear the relative contribution compared to PK when obtained in the diet.[18,19]

The National Institute of Health's Drug-Nutrient Interaction Task Force, The Academy of Nutrition and Dietetics, and other health professional organizations recommend consistent VK intake while on anti-coagulation therapy.[15,20–25] In order for dietitians to better instruct patients and clients, The Academy's Nutrition Care Manual categorizes whole foods into high, moderate, low, or free VK containing foods.[20] The Nutrition Care Manual does not attempt to classify mixed dishes by VK content. However, mixed dishes and restaurant foods with their larger portion size, use of plant oils, and frequent consumption, have the potential to contribute substantially to a person's daily VK intake. This is important as a dietitian or consumer may not recognize mixed dishes that do not contain high or moderate VK vegetables as a potential dietary source of VK. The purpose of this study was to characterize and quantify the amount of VK per serving of mixed entrees, side dishes, restaurant foods, and other related food products in a representative U.S. sample. To our knowledge, the PK, dK, and MK4 content of these foods has not been previously reported.

Materials and Methods

The analysis of VK in U.S. food samples is an ongoing collaborative agreement between the Vitamin K Laboratory at Tufts University and the U.S. Department of Agriculture (USDA). Food samples were obtained from the USDA Nutrient Data Laboratory as part of the National Food and Nutrient Analysis Program (NFNAP) conducted between 2008 and 2014 .[26] Samples

were collected in 12 cities in the U.S. and combined to form either brand specific or subnational composites appropriate to each food item. This food-sampling plan provides aliquots of homogenized foods that are representative major foods consumed in the U.S. [27,28] Food samples were shipped to the Food Analysis Control Center at Virginia Polytechnic Institute and State University in Blacksburg, Virginia, for aliquot preparation. Quality-control materials are also added to the sample stream. Aliquots were then shipped to the Vitamin K Laboratory at Tufts University, Boston, Massachusetts, and stored for up to 13 months at -80˚C prior to analysis.

The PK, dK, and MK4 contents of the food samples were determined by a high-performance liquid chromatography (HPLC) procedure, as previously described. [29] The assay is continually monitored for stability through the use of in-house control samples (baby food chicken vegetable dinner, Beechnut®, Amsterdam, NY) and control samples provided through the NFNAP program. All samples were analyzed in duplicate. For the purpose of this study, data were selected for those mixed entrees, side dishes, restaurant foods, and other related food products that have yet to be reported in the literature but whose PK content has been incorporated in the USDA National Nutrient Database for Standard Reference Release 28. (http://ndb.nal.usda.gov)**.**

The 65 foods are presented as the mean ± standard deviation (SD) PK, dK, and MK4 content per serving size for each food product. Ranges are also provided for food products with at least two samples per group. PK, dK, MK4 contents per serving size were summed and then categorized as high (greater than 100µg per serving), moderate (25-100µg per serving), low (5-25µg per serving), or free (less than 5µg per serving) VK containing in accordance with categorizations currently used in the Nutrition Care Manual. [20]

For each specific food or dish, serving size was based on the amount listed as 1 serving on the USDA Nutrient Database for Standard Reference.[30] If no serving size was listed, serving size was based upon the Food and Drug Administration (FDA) and USDA-FSIS reference amounts customarily consumed per eating occasion (RACC). RACC are household and/or gram measurements of food groupings that correspond to the amount customarily eaten at a time by U.S. persons over the age of 4.[31] RACC are the basis for serving sizes listed on the Nutrition Facts Panel.[26] For each specific food presented, the weight (in grams) and corresponding household measure, if available, was based on units used in the USDA Nutrient Database for Standard Reference. [30]

Results

All dishes and foods analyzed contained some amount of VK. Using the categories from the Nutrition Care Manual, we assessed 10 foods as VK-free, 34 foods as low in VK, and 20 foods as moderate in VK.[20] Only tuna submarine sandwich had a high level of VK, all of which was in the PK form. The food sources of PK were likely mayonnaise, lettuce, and any other PK-containing oil used in preparation.

PK was the predominant form of VK in these mixed dishes and other foods analyzed. However, 44 dishes also contained MK4. The majority of foods containing MK4 were animal-based. The remaining foods with MK4 likely contained animal-based fats such as butter or lard used in preparation. Only one food, chicken pot pie, contained >20µg/serving of MK4.

Eight foods analyzed contained dK. All foods had <15µg per serving. This aligns with prior data demonstrating dK decline in U.S. foods post 2006, after implementation of *trans* fat labeling.[32] The dK content of foods will likely continue to decline in response to the 2015 regulation removing partially hydrogenated oils (PHO) from the generally recognized as safe list,

which will result in further reductions in the *trans* fatty acids content of the food supply and concomitant reduction in dK. [33]

Discussion

The Nutrition Care Manual lists only vegetables, fruits, and spinach noodles in its categorization of high and moderate VK containing foods.[20] However,10 foods analyzed in this study (orange chicken, spaghetti with meatballs, chicken parmigiana, cheese filled ravioli with or without tomato sauce, cheese enchilada, cheese quesadilla, fried cheese sticks, frozen onion rings, and meat submarine sandwich) had moderate VK levels, even though they did not contain high or moderate VK-rich fruits or vegetables. Instead, the VK content of these mixed dishes presumably originates from plant oils and animal products. These foods would likely be overlooked in nutrition education for patients taking VK antagonist medication.

Current dietary recommendations for patients on vitamin K antagonist anticoagulation treatment focus on consistent intake of fruits and vegetables. However, the majority of Americans have suboptimal fruit and vegetable intake, consuming below the recommended guidelines..[34] In contrast, given the frequency of consumption of restaurant foods and mixed dishes, the proportion of VK obtained in the diet from these foods could contribute substantially to total daily VK intake compared to fruits and vegetables. Americans consume over one third of their calories on food outside the home and over three quarters consume one or more commercially prepared meals per week.[34,35] NHANES data from 2005-2006 showed mixed dishes represented five out of the top ten sources of calories for Americans.[36]

Additionally, studies have shown that, in the past two decades, the portion sizes Americans customarily consume are higher than those presented by the USDA guidelines or FDA RACC.[37] Young and Nestle, demonstrated that the portion size of ready-to-eat, take-out,

fast-food, and family-type restaurant foods are two to eight fold higher than government standard serving sizes.[37] RACC data are primarily obtained from national surveys conducted in 1978 and 1988.[31] The standard portion sizes used in this paper may not be reflective of current U.S. consumption patterns. Larger portion sizes result in larger VK ingestion. Hence, our classification based on the Nutrition Care Manual may reflect an underestimation of VK consumed.

Lastly, the bioavailability of VK in mixed dishes that contain PK-rich oil or another fat source is higher than unprocessed fruits and vegetables. The bioavailability of VK is related to the complexity of the food matrices and the fat content of the meal. Studies have demonstrated that post-prandial serum concentration of PK, is significantly higher after intake of PK-fortified oil or vegetables with added fat compared to vegetables alone.[38,39] Jones et al. showed the bioavailability of PK in "convenience" meals is more than twice the PK bioavailability in "cosmopolitan" meals. Convenience meals reflect dietary patterns with a higher than average intake of fast food and refined cereals and lower than average intake of fruits, vegetables, and whole grains whereas cosmopolitan reflects meal patterns with higher than average intake of fruits, vegetables, whole grains, fish, and dairy. Collectively these suggest that the PK obtained from a meal with PK-rich oil has greater bioavailability than PK obtained from fruits and vegetables.

The limitations of this study are that it did not attempt to quantify longer-chain MKn and thus the amount total amount of VK may be higher than represented here. Secondly, it was outside of the scope of this study to attempt to quantify the bioavailability and absorption of VK for each food. Given the wide variety of foods presented, bioavailability may vary considerably. However, these limitations were offset by the strengths of this study, which include the unique

aliquot preparation of samples, which results in a food composite that is indicative of that dish or food across the U.S. By translating data obtained from current foods available in the US food supply into serving sizes, and then further categorizing the foods in accordance with the Nutrition Care Manual, these data are applicable to clinical practice and can be easily understood by a wide variety of audiences.

Conclusion

This study demonstrates that 21 mixed dishes and restaurant foods analyzed are a high or moderate source of VK. This has important clinical practice implications for dietitians and other health care providers who counsel patients to consume a diet with consistent VK intake. Providers should be aware that other foods besides PK rich vegetables and fruits are good sources of VK. Mixed dishes and restaurant foods made with plant oils and animal products do contribute VK in the U.S. diet. Their frequent consumption, larger portion sizes, and increased bioavailability, could lead a person to consume more of their daily VK intake from these foods compared to fruits and vegetables. Areas of further research include the bioavailability of multiple VK forms in conjunction with varied meal composition as well as the biological activity of each of the forms of VK.

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Multiple vitamin K forms exist in dairy foods

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A running title: Vitamin K content of dairy foods

Footnotes:

Abbreviations: AI, adequate intakes; LAB, lactic acid bacteria; MK, menaquinones; PK, phylloquinone; USDA, United States Department of Agriculture.

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Abstract

The plant-based form of vitamin K (phylloquinone, PK, vitamin K1) has been well-quantified in the U.S. diet. Menaquinones (MK, vitamin K2) are another class of vitamin K compounds that differ from PK in the length and saturation of their side chain but have not been well characterized in foods. The objectives of this study were to: 1) quantify, using mass spectrometry technology, PK and the different forms of MK (MK4 through MK13) in milk, yogurt, Greek yogurt, creams and cheeses; and 2) compare the MK contents of full-fat, reduced-fat and non-fat dairy products. All dairy samples were either obtained from USDA National Food and Nutrient Analysis Program or purchased from retail outlets. Full fat dairy products contained appreciable amounts of MK, primarily in the forms of MK9, MK10 and MK11. We also measured modest amounts of PK, MK4, MK8 and MK12. In contrast, there was little MK5-7 or MK13 detected in the majority of dairy products. The total vitamin K contents of soft cheese, blue cheese, semi-soft cheese and hard cheese were 506±63, 440±41, 289±38 and 282±5.0 μg/100 g, respectively. Non-fermented cheeses, like processed cheese, contained lower amounts of vitamin K (98±11 μg/100 g). Reduced fat or fat free dairy products contained ~5-22% of the vitamin K found in full fat equivalents. For example, total vitamin K contents of full fat milk, 2% milk, 1% milk and non-fat milk were 38.1±8.6, 19.4±7.7, 12.9±2.0 and 7.7±2.9 μg/100 g, respectively. To the best of our knowledge, this is the first report of MK contents of U.S. dairy products. Findings indicate that the amount of vitamin K contents in dairy products is high and proportional to the fat content of the product. If biological activity of MK from foods is established, dairy products could have an important dietary role in vitamin K nutrition.

Key words: vitamin K, menaquinones, dairy products, fermented, reduced fat, phylloquinone

Introduction

Dietary sources of vitamin K are found in two natural forms: phylloquinone (PK; vitamin K1) and menaquinones (MK; vitamin K2). All forms of this fat-soluble vitamin share the common structure, 2-methyl-1,4-napthoquinone. The MK differ in structure from PK in their 3-substituted lipophilic side chain, and are designated by the number of isoprenoid units, i.e. MK-n. MK with up to 13 isoprenoid units have been identified (1). Whereas PK is widely distributed in the food supply, MK forms appear to be limited to animal products and fermented foods(2). As an essential vitamin, vitamin K plays a role as an enzyme co-factor necessary for the modification of glutamic acid residues to γ-carboxyglutamic acid residues in specific proteins, referred to as vitamin K-dependent proteins (3). Through the vitamin K-dependent γ-carboxylation of coagulation proteins, matrix Gla-protein, osteocalcin and gas-6, vitamin K has been implicated in hemostasis, tissue calcification, bone metabolism and cell cycle regulation (4-6). Vitamin K has multiple roles independent of its known biochemical function as an enzyme cofactor, like anti-inflammation (7), a ligand for steroid and xenobiotic receptor(8).

The current U.S. recommendation for intakes of vitamin K are 90 and 120 μg/day for women and men, respectively. These guidelines are termed adequate intakes (AI) due to insufficient data regarding vitamin K metabolism and lack of a robust biomarker to generate a precise dietary recommendations (9). The AI is based on usual PK intakes, and does not take into account the potential dietary contribution of other forms of vitamin K. Very little is known about the contribution of dietary MK to overall vitamin K nutrition, and although it has been stated that approximately 50% of the daily requirement for vitamin K is supplied by gut bacteria through the production of MK (1), there is little evidence to support this estimate. Estimated intakes of PK and MK in dairy-producing countries in Western Europe suggest that between 10% and 25%

of total vitamin K intake are provided by MK, primarily from dairy sources (10, 11). However, MK have not been systematically analyzed in U.S. foods nor have their intakes been estimated in the U.S. population so these observations have yet to be substantiated outside of Western Europe.

The need to analyze MK in commonly consumed foods is timely because observational data from dairy-producing countries in Europe suggest that intakes of MK present in dairy products have stronger associations with heart health benefits compared with PK intakes (12). MK data for commonly consumed foods from other countries are critical for determining if these observations are generalizable. Furthermore, the food composition data applied to these few observational studies, almost exclusively from the Netherlands, predominantly represent full-fat dairy products. Low fat and non-fat dairy products are recommended as part of a healthy diet in the U.S., to reduce risk of cardiovascular disease and associated co-morbidities (13). The impact of reducing the fat content of dairy products on MK content is unknown.

Advances in mass spectrometry methodology have provided an ability to quantify multiple forms of vitamin K (PK and MK) in various matrices, allowing us to explore the MK content in the U.S. food supply (14). The purpose of this study was to quantify the content of multiple forms of vitamin K content in various dairy products including yogurt, cheeses, milk, and milk-based products, and examine the effect of fat content on the distribution and concentration of vitamin K forms in those products.

Methods

Fifty (50) of the dairy samples used in this study were provided by United States Department of Agriculture (USDA) Nutrient Data Laboratory, which conducts the National Food and Nutrition Analysis Program (15). The nationally collected dairy samples were first delivered

to the Food Analysis Laboratory Control Center at Virginia Tech in Blacksburg, Virginia, for preparation of aliquots, and then delivered frozen on dry ice to the Vitamin K Laboratory at Tufts University and stored at -80^{o}C until analysis. The National Food and Nutrition Analysis Program infrastructure incorporates a nationally-representative sampling approach (15, 16), approved analytical methods, and a rigorous quality assurance scheme. In addition, 148 dairy samples used in this study were purchased in 2016 from retail outlets that have substantial annual sales in order to capture the diversity of products available in Boston (MA, USA) area. Appropriate containers were used to maintain refrigeration during the transport to the laboratory. All samples collected by our laboratory were composited, aliquoted and stored at -80^{o}C before analysis. Shelf life date, analysis date, brand name and fat content were recorded. We used available information from the manufacturers to determine fat content (i.e. full fat, reduced fat, etc.).

The dairy products were grouped in categories based on dairy types and fat content (**Table1**): milk, yogurts, Greek yogurts, kefirs, creams, processed cheeses, fresh cheeses, blue cheeses, soft cheeses, semi-soft cheeses and hard cheeses. Aside from processed cheese, all other types of cheeses included at least two different brands and different lots.

All cheese sample aliquots (about 10g) were frozen by liquid nitrogen and manually ground into a powder using a mortar and pestle. Approximately 0.05-0.2g of sample was used for analysis. The procedures for vitamin K extraction and sample purification have been previously described (14). PK and MK4-13 concentrations were measured by LC-MS, using deuterium-labeled PK as an internal standard (Sigma Aldrich, St. Louis, MO) and synthesized PK, MK4-MK13 as calibration standards (14).

The effect of dairy product fat content (full fat, fat-free/reduced) on concentrations of total vitamin K, PK, and all detectable MK were analyzed by two-sample T-test. Given the smaller sample size, the vitamin K content of cream dairy products (heavy/whipping cream, half and half, and light cream) was examined by general linear model, with heavy/whipping cream as the reference group. Significance was determined by $P < 0.05$, and all analyses were carried out using SAS v 9.4 (Cary, NC). Data are reported as means (expressed as µg/100g wet weight) ± SEM.

Results

Dairy products obtained from the USDA Nutrient Data Laboratory and those purchased from retail outlets contained appreciable amounts of MK, primarily in the forms of MK9, MK10 and MK11. Together these three MK account for about 90% of total vitamin K in dairy foods.

The vitamin K content of different cheeses had significant variability in total vitamin K concentrations, ranging from 40 µg/100g to values up to 850 µg/100g (**Figure 1**). All forms of cheese contained MK9, MK10 and MK11. We also measured modest amounts of PK, MK4, MK7, MK8 and MK12. In contrast, there was little MK5, MK6 or MK13 detected in the majority of cheese products. The total vitamin K content varied by cheese type, with soft cheese having the highest concentration, followed by blue cheese, semi-soft cheese and hard cheese (506±63, 440±41, 289±38 and 282±5.0 µg/100 g, respectively) (**Supplemental Table 1**). Non-fermented cheeses, like processed cheese, contained lower amount of vitamin K (98±11 µg /100 g). There was considerable diversity in vitamin K forms among fresh, semi-soft, blue and soft cheeses, but not in hard and processed cheeses. Soft cheeses and hard cheeses had a similar vitamin K pattern with high MK9 and MK10, with blue and semi-soft cheeses sharing a similar pattern dominated by MK9 and MK11.

Milk and yogurt products were also measured. The vitamin K concentrations of full fat, 2% fat, 1% fat and fat free milk varied by fat content (**Figure 2**). Mean total vitamin K content of full fat milk, 2% milk, 1% milk and fat free milk was 38.1±2.7, 19.4±2.4, 12.9±0.6 and 5.1±0.9 µg/100 g, respectively. Both total vitamin K and individual MK concentrations in the full fat milk was significantly higher than 2% milk products ($P<0.05$). PK was only detected in the full fat milk. MK5-8 and MK12-13 were not detected in any milk samples. Fat free milk only contained a minimal amount of MK9 and MK11.

Regular and Greek yogurt with full fat had similar vitamin K concentrations as full fat milk (**Table 2**). Surprisingly, neither MK or PK were detected in fat free yogurt. Low fat kefir (n=4) contained 10.2±0.3µg total vitamin K/100g, of which only MK9 and MK11 were detected.

Additional dairy products were examined including cottage cheese, cheddar cheese and cream (full fat and reduced fat) and found to have unique distribution of vitamin K forms (**Table 3**). 4% cottage cheese had significant higher MK8, MK9 and MK11 concentrations than reduced-fat cottage cheese. Reduced-fat cheddar cheese contained only 17% of total vitamin K content when compared to full fat cheddar cheese. MK9, MK10 and MK11 in reduced-fat cheddar cheeses decreased by 12.9%, 3.5% and 38.6% of full fat cheddar cheeses, respectively. Reduced fat cream products had less vitamin K content overall.

Discussion

Current dietary guidelines recommend a diet containing high quality dairy foods (17). Dairy does not contain appreciable amounts of PK, hence dairy has not historically been considered a rich dietary vitamin K source. However, our data indicate that U.S. dairy products are a good dietary source of MK. MK9 was the major form quantified in the dairy samples, which is consistent with the findings of others (18, 19). However, through use of a sensitive LC-

MS assay (14), we were able to extend that analysis to include measurement of MK11 through MK13, and our data indicated five to ten-fold higher MK9 and MK10 content in dairy products than previously reported (19), albeit in different dairy products, and in particular, artisan cheeses. We do not currently have an explanation for the higher concentrations reported here.

The large diversity of vitamin K forms among dairy products may be related to the microbial species used in the production of fermented dairy products. MK are synthesized by bacteria, including many found in the fermented foods. In particular, lactic acid bacteria (LAB) are widely used in dairy and fermented food industries(20, 21). LAB include a large number of *cocci* and *bacilli*, such as species of the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (22). Most of the cheese products contained LAB species as starters, which are reported to be the source of various MK forms (23). *Staphylococcus, Hafnia*, and *Arthrobacte* and other bacteria that are used in the surface ripening of certain cheeses may be the reason for their corresponding high MK values (2). However, the presence of MK in non-fermented products such as milk are largely unexplained, and could relate to the microbial content of the highly specialized ruminant digestive system (24). Further investigation of the microbial composition of the different fermented dairy products is needed to interpret the diversity of MK forms.

Our study demonstrates that dairy products are a significant source of MK, and that the MK content varies by fat content of the dairy product. This differs from the conclusions of Manoury *et al* (19), who did not find an overall association between MK9 content and fat content of fermented dairy products. Whereas the latter study conducted a single correlation analysis between MK9 content and fat content across all fermented dairy products, we compared the

individual MK contents with different levels of fat within the same dairy product, which may explain why we were able to detect a consistent reduction in MK content with reduction in fat content. Currently reduced-fat milk, and yogurt is the most commonly consumed dairy product in the U.S. In 2015, whole fat milk accounted for 33% of milk sales with the remaining 67% of milk purchased as reduced-fat milk (2%, 1% and nonfat) (25). There is a recent trend of increasing full fat milk and cheese consumption (25, 26), fueled by recent evidence that individuals consuming full-fat dairy products (measured by plasma dairy fat biomarkers) had at least 43% lower risk of developing diabetes over the course of 15 years, compared with people who opted for low-fat dairy products (27, 28). Moreover, they found greater intake of high-fat dairy products, but not low-fate dairy products, was associated with less weight gain in the Women's Health Study (29). The nutrient components contributing to these beneficial effects have yet to be identified, and our observations suggest that MK warrants consideration.

Although MK bioavailability has not been studied using stable isotopes, bacterially-produced MK7 isolated from a food source (natto, a fermented soybean product) can be absorbed and is attributed to multiple health benefits, including bone and cardiovascular health (30, 31). More recently, studies have demonstrated that consumption of dairy products fortified with individual MK forms are absorbed and may have greater bioactivity than MK delivered in supplement form (30, 31). However, current understanding of fat-soluble MK absorption, transport, and bioactivity is limited. As reviewed elsewhere (32), most MK forms are not normally detected in circulation unless administered in supplement form. As our data indicate MK forms are more abundant in commonly-consumed foods in the U.S. diet than previously recognized. It is critical that a more complete understanding of MK absorption and transport be developed in order to refine dietary recommendations for this nutrient. Collectively these data

highlight major gaps that still existing in our understanding of the role of MK forms in vitamin K metabolism and its contribution to human health.

Our study was limited by the reliance on food labels for fat content instead of direct measurement of fat content. Whereas the samples obtained from the National Food and Nutrition Analysis Program were geographically representative of the U.S. diet, those purchased in the Boston region were not. However, those purchased locally were selected from retail outlets that had national representation. Strengths of the study included using a highly sensitive and validated LC-MS method to quantify all MK forms in a variety of dairy product types, and direct comparison of full fat and reduced fat dairy products of the same brand and food type. Future studies are needed to compare relative bioavailability and contribution of these individual MK to health outcomes.

In summary, our results demonstrate that commonly-consumed dairy products in the U.S. diet contain appreciable amounts of multiple vitamin K forms which are directly related to fat content. Additional research is necessary to determine the role of microbes used in production of dairy products, and their impact on MK content. There is also a need to determine the relative bioavailability of all MK forms given their abundance in the U.S. diet.

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X.F. and X.S. conducted research; X.F. and S.G.H analyzed data; X.F. and S.G.H. wrote the paper; X.F., S.G.H., D.B.H., J.P.K., B.E.F., and S.L.B. reviewed the data, aided in interpretation of results and reviewed manuscript; S.L.B had primary responsibility for final content. All authors read and approved the final manuscript.

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Table 1. The analyzed dairy products.

Dairy	n	Type
Milk	43	Full fat, 2% fat, 1% fat, nonfat
Yogurt	16	Full fat and nonfat
Greek yogurt	16	Full fat and nonfat
Kefirs	4	Low fat
Cream	5	Heavy, light, half/half
Processed cheese	9	American cheese
Fresh cheese	12	Goat, Feta, Ricotta, Cotija, cottage and mozzarella cheeses
	12	Mozzarella part skim
	8	Reduced-fat cottage cheese
Blue cheese	10	Gorgonzola and blue cheeses
Soft cheeses	14	Brie, camembert, crème fraiche, limburger, mascarpone
Semi-soft cheeses	10	Montery jack, Havarti, fontina, gouda, Swiss and cream cheeses
Hard cheeses	12	Cheddar and parmesan
	10	Reduced-fat cheddar

Table 2. Vitamin K content of regular yogurt and Greek yogurt varies by fat content.

Vitamin K (μg/100g)	Yogurt		Greek yogurt	
	Full fat n=9	Fat free n=7	Full fat n=6	Fat free n=10
PK	0.4±0.1*	ND	0.3±0.1*	ND
MK4	0.7±0.3	ND	0.8±0.1*	ND
MK5	ND	ND	ND	ND
MK6	ND	ND	ND	ND
MK7	ND	ND	ND	ND
MK8	ND	ND	ND	ND
MK9	13.2±4.8*	ND	14.8±2.2*	ND
MK10	1.6±0.6*	ND	1.8±0.6*	ND
MK11	8.4±0.8*	ND	8.7±0.8*	ND
MK12	ND	ND	ND	ND
MK13	ND	ND	ND	ND
Total	26.3±6.4*	ND	28.2±2.7*	ND
Fat content (%)	4.6±0.5*	0.0	4.0±0.2*	0.0

5. Values are mean ± SEM.
6. ND: non-detectable. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 μg/100g).
7. * Significant difference between full fat and fat free within yogurt group at $P < 0.05$.
8. Total: Sum of PK and MK4 to MK13

Table 3. Vitamin K content of dairy products comparing fat and fat free or reduced fat products.

Dairy products	n	PK	Menaquinones										Total
			MK4	MK5	MK6	MK7	MK8	MK9	MK10	MK11	MK12	MK13	
Cottage cheese													
4% fat	6	0.3±0.1	0.3+0.1	0.5±0.2	0.5±0.1	0.6±0.2	2.5±0.7*	8.0±1.4*	0.4±0.2	39.1±3.0*	ND	ND	52.7±3.4*
Reduced-fat	8	ND	ND	ND	ND	ND	0.8±0.4	2.3±0.6	0.3±0.2	5.0±1.6	ND	ND	10.3±1.4
Cheddar cheese													
Full fat	12	2.4±0.1*	9.5±0.4*	0.4±0.1	0.9±0.2*	0.8±0.2	5.6±0.8	175±12.1*	42.9±7.8*	42.2±3.5*	1.3±0.1*	ND	281±11.9*
Reduced-fat	10	0.5±0.1	1.8+0.5	ND	ND	0.7±0.1	4.0±0.7	22.6±4.2	1.5±0.7	16.3±3.7	ND	ND	49.0±7.9
Cream													
Heavy	2	2.4±0.1	9.3±0.8	ND	ND	ND	ND	442±30.2	85.2±10.9	44.3±9.4	2.6±0.1	ND	587±27.8
Light	1	1.2	5.3	ND	ND	ND	ND	103†	13.0†	24.5	1.0	ND	149†
Half/half	2	0.8±0.1†	2.3±0.4†	ND	ND	ND	ND	40.4±17.3†	4.5±2.5†	35.5±11.3	ND	ND	85.1±3.8†

1. Values are mean ± SEM. ND: non-detectable. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 µg/100g).
2. Total: Sum of PK and MK4 to MK13.
3. *Significant difference between full fat and fat free/reduced fat within each dairy product category. Significance at $P < 0.05$.
4. † Significance determined by general linear model, with heavy cream as the reference group. Significance at $P < 0.05$.

Figure legends

Figure 1. Vitamin K content of different cheeses.

Figure 2. Vitamin K concentrations of full fat, 2%, 1% and fat free milk.

Values are mean ± SEM. X: non-detectable. MK5-8 and MK12-13 were not detected in any milk samples. Total: Sum of PK and MK4 to MK13. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 μg/100g). $^{a-c}$ means with different letters are significantly different ($p < 0.05$). Total vitamin K indicates the sum of PK and all MK forms.

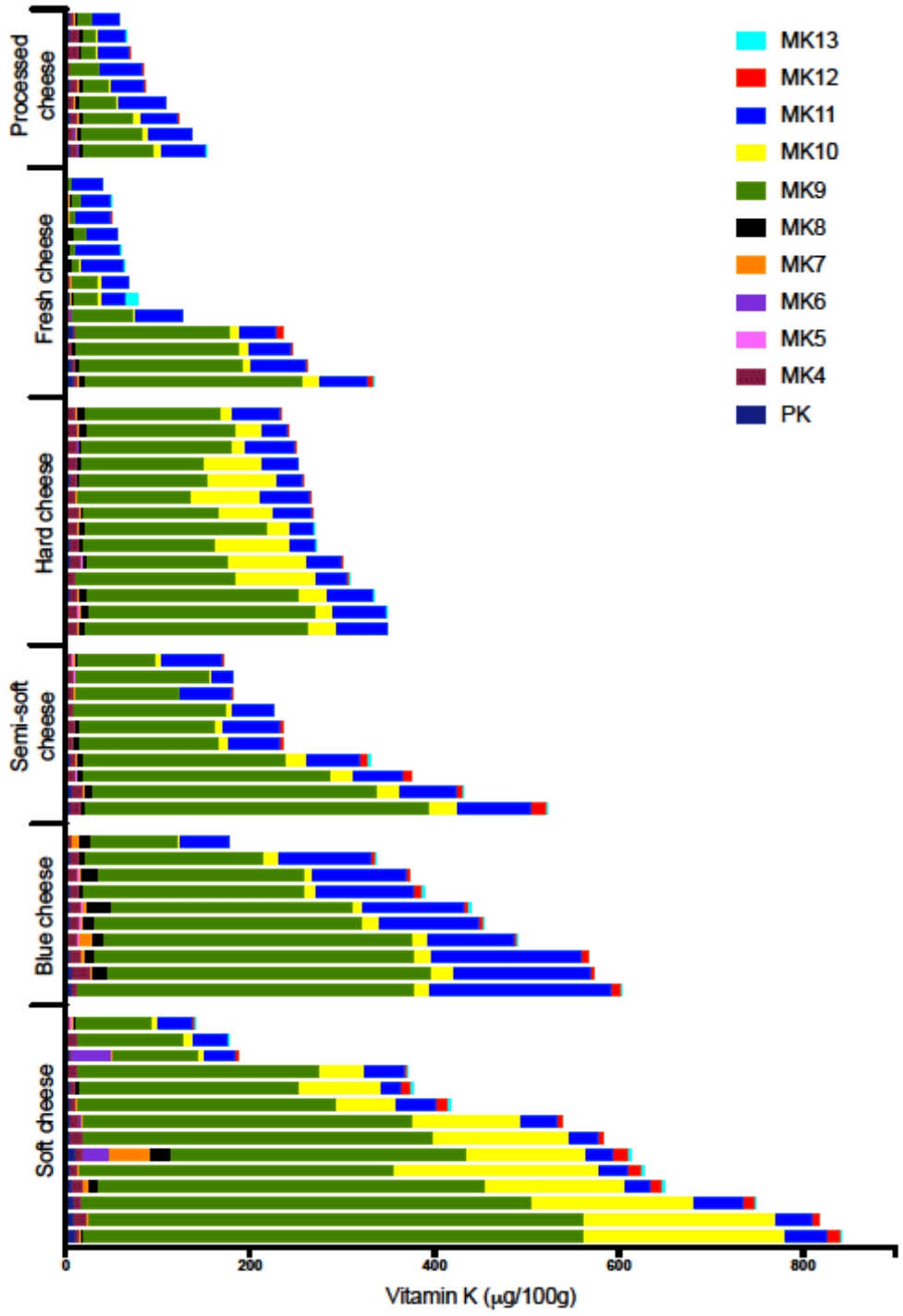
Processed cheese
Fresh cheese
Hard cheese
Semi-soft cheese
Blue cheese
Soft cheese
MK13
MK12
MK11
MK10
MK9
MK8
MK7
MK6
MK5
MK4
PK
0
200
400
600
800
Vitamin K (μg/100g)

Figure 1.

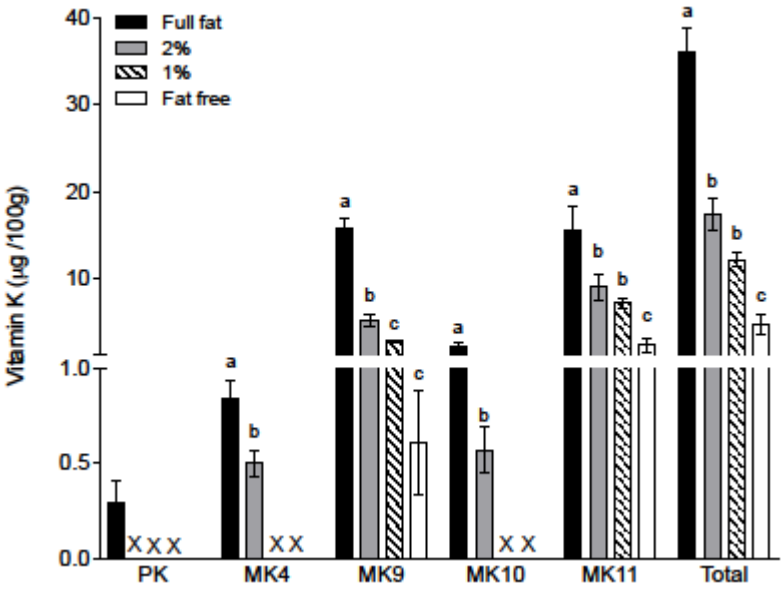
Full fat
2%
1%
Fat free
Vitamin K (µg /100g)
40
30
20
10
1.0
0.5
0.0
a
b
c
X X X
X X
PK
MK4
MK9
MK10
MK11
Total

Figure 2.

Supplement Table 1 Vitamin K content of cheese products (µg/100g).

Cheeses	n	PK	Menaquinones										Total
			MK4	MK5	MK6	MK7	MK8	MK9	MK10	MK11	MK12	MK13	
Blue cheese	10	3.2±0.5	10.3±1.3	0.6±0.2	ND	4.0±1.3	11.6±2.5	271±27.2	14.3±1.8	119±12.8	5.0±0.9	1.3±0.5	440±40.7
Fresh cheese	13	2.3±0.8	1.3±0.4	ND	ND	0.8±0.2	2.3±0.6	71.4±23.5	4.5±1.6	41.7±28.0	1.6±0.6	1.7±1.1	128.4±28.4
Soft cheese	14	4.5±0.7	8.1±1.0	ND	5.3±3.5	4.7±3.1	2.6±1.5	319±40.8	114±20.9	37.7±2.2	8.7±1.4	1.9±0.4	506±62.5
Semi-soft cheese	10	2.4±0.4	7.1±0.7	ND	ND	0.6±0.1	3.8±0.8	198±29.4	13.3±3.4	56.9±4.6	5.1±1.6	1.0±0.3	289±38.1
Processed cheese	9	2.5±0.2	7.0±10	ND	ND	1.2±0.3	2.8±0.4	38.6±7.7	3.7±1.0	40.8±2.8	ND	ND	98.1±11.2
Hard cheese	14	2.3±0.1	9.1±0.5	ND	ND	0.8±0.2	4.8±0.7	172±5.4	48.3±7.7	42.5±3.2	1.5±0.1	ND	282±5.0
Mozzarella part skim	12	1.4±0.1	1.4±0.3	ND	ND	0.5±0.1	ND	51.8±7.5	7.7±2.5	37.9±5.3	0.9±1.1	ND	106±10.6

Values are mean ± SEM. ND: non-detectable. Concentrations were below lower limit of detection using an LC-MS assay (LLOD: PK=0.2, MK4=0.2, MK5=0.4, MK6-9=0.6, MK10=0.1, MK11=0.7, MK12-13=0.8 µg/100g). Total: Sum of PK and MK4 to MK13.

A race-specific interaction between vitamin K status and statin use during warfarin therapy initiation

Manuscript in preparation for submission to the Journal of Thrombosis and Haemostasis.

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Running Title: Vitamin K and Statins during Warfarin Initiation

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Nomenclature and Abbreviations
PK: Phylloquinone; BMI: Body mass index; INR: International normalized ratio; IN-RANGE: International Normalized Ratio Genetics and Adherence study; PTTR: Percent time in therapeutic range; CI: Confidence Interval

Dear Editor,

Vitamin K regulates coagulation by modulating the post-translational modification of various clotting factors (1). The vitamin K antagonist, warfarin, is prescribed for the prevention and treatment of thromboembolism (2). Warfarin is highly efficacious, but has a very narrow therapeutic range (3). In addition to vitamin K, warfarin therapy is influenced by multiple factors including race (4) and drug-drug interactions (2,5,6). According to case-reports and small clinical trials, co-administration of statins and warfarin results in reduced warfarin dose, increased international normalized ratio (INR), and/or bleeding (7–12). The magnitude of these effects vary by and within statin type (5). Due to the lipophilic properties of vitamin K, and the lipid-lowering effect of statins, we hypothesized that vitamin K and statins could interact during warfarin therapy initiation. The objective of this study was to examine the inter-relationship of vitamin K and statins and its role in warfarin therapy initiation in a large cohort of Caucasians and African Americans.

Data were obtained from the INR Adherence and Genetics Study (IN-RANGE), a U.S. prospective cohort study designed to determine the associations between clinical and genetic factors in warfarin adherence and maintenance dose**.** The 687 patients in this cohort who were initiating warfarin treatment were recruited between 2009 and 2013 from three outpatient anticoagulation clinics (Hospital of the University of Pennsylvania, the Corporal Michael J. Crescenz Veterans Affairs Medical Center, and Johns Hopkins University) (13)**.** African Americans and Caucasians were specifically recruited to investigate whether racial differences in warfarin dosing could be attributed to genetic background (14) so all of our analyses were race-specific. Maintenance dose was ascertained by two consecutive INRs in the therapeutic range, at least a week apart, without a dose change (13).

Serum phylloquinone (PK), the primary form of vitamin K in circulation, has been used to rank individuals' vitamin K status (15). In this cross-sectional analysis, serum PK was categorized into evenly distributed quartiles (1: PK<0.6, 2: 0.6≤PK≤1.0, 3: 1.0<PK≤1.7, 4: PK>1.7nmol/L). Participants reported statin use at baseline. Multinomial logistic regressions were used to determine if statins influenced serum PK. Outcome measures of anticoagulation therapy stability, days to maintenance and percent time in the therapeutic range (PTTR), were modeled with Cox proportional hazard and linear regression models, respectively. Primary exposures included serum PK and statin use. Covariates in all analyses included age, sex, smoking status (current or non-smoker), body mass index (BMI) (kg/m^2), site, and triglycerides (mg/dL). Triglycerides were log transformed as needed to satisfy assumptions of linearity and normality of errors. Statistical analyses were performed using RStudio Version 1.0.44. Significance was determined at $p < 0.05$.

Of the 687 participants, 471 had data for all primary exposures, outcomes, and covariates and were included in these analyses. Serum PK (included fasting and non-fasting) ranged from non-detectable to 10.6 nmol/L in Caucasians, and non-detectable to 16.7 nmol/L in African Americans. Statin use was similar between races (**Supplementary Table 1**).

In African Americans, statin users were more likely to have high PK than low PK, (PK Q4 relative to PK Q1 odds ratio: 2.6, 95% confidence interval (CI): 1.16-5.70), but neither statin use nor serum PK were significant determinants of days to maintenance or PTTR. In Caucasians, statin use was not associated with serum PK, and neither measure was found to influence PTTR. However, a minor interaction was detected between serum PK and statin use with respect to days to maintenance such that statin users with moderate serum PK took longer to reach maintenance than statin users with low serum PK (**Table 1**). Dietary vitamin K has been shown to influence

serum PK status (16). The predominant dietary source of PK is green vegetables (17). We posit that those with consistent high vegetable intake or that completely avoid vegetables would have stable serum PK, at high and low levels, respectively. Those with sporadic vegetable intake would have moderate, but instable serum PK, and therefore more difficulty determining a warfarin dose that consistently results in proper anticoagulation. This would require further study.

Strengths of this study include examination of serum PK and statin use together during warfarin therapy initiation, the most sensitive phase of warfarin treatment, in a well-characterized cohort. The study was limited in sample size, some blood samples were non-fasting, and dietary vitamin K intake was not collected for most subjects. Secondary analyses by statin type were conducted in statin users, but interpretations were prohibitively limited by sample size. Overall, we found that the influence of vitamin K status and statin use on anticoagulation therapy varies by race. The time to reach maintenance may be prolonged by moderate serum PK in Caucasian statins users, but serum PK and statin use were not important factors with respect to anticoagulation stability in African Americans. Further examination of statin use in conjunction with dietary and fasted serum PK during anticoagulation therapy, in larger, multi-racial cohorts are warranted.

Table 1: Associations of phylloquinone and statin use with days to maintenance in the 2009-2013 IN-RANGE cohort												
	Caucasians						African Americans					
	All		Statins				All		Statins			
			Yes		No				Yes		No	
	N=140		N=60		N=80		N=331		N=140		N=191	
	Exp (Beta)	p-value	Exp (Beta)	p-value	Exp (Beta)	p-value	Exp (Beta)	p-value	Exp (Beta)	p-value	Exp (Beta)	p-value
PK Q2[a]	1.31	0.62	0.09	0.00	1.05	0.93	1.47	0.11	1.21	0.55	1.58	0.06
PK Q3[a]	1.00	0.99	0.17	0.00	0.68	0.52	1.42	0.19	1.07	0.83	1.61	0.09
PK Q4[a]	0.87	0.80	1.40	0.60	0.51	0.27	1.46	0.19	1.36	0.34	1.67	0.11
Statin Use	2.68	0.08	-	-	-	-	1.12	0.69	-	-	-	-
Triglycerides (mg/dl)	1.00	0.58	0.99	0.03	1.00	0.06	1.00	0.90	1.00	0.80	1.00	0.94
BMI (kg/m^2)[b], 25-<30	1.92	0.02	4.03	0.02	1.85	0.11	0.91	0.64	1.05	0.89	0.95	0.85
BMI (kg/m^2)[b], ≥ 30	1.56	0.11	1.76	0.25	1.49	0.34	1.11	0.58	1.37	0.30	1.18	0.47
Site-VA[c]	1.08	0.84	2.23	0.16	0.62	0.41	1.36	0.15	1.26	0.52	1.79	0.04
Site-HUP[c]	1.24	0.44	1.56	0.40	1.24	0.55	1.96	0.00	3.49	0.00	1.43	0.15
Sex[d]	1.37	0.25	0.80	0.70	1.27	0.49	0.68	0.02	0.59	0.05	0.61	0.03
Smoking	1.45	0.38	2.45	0.23	1.06	0.92	0.82	0.23	0.88	0.58	0.88	0.60
Age[e], 45-54 years	0.78	0.50	1.60	0.66	0.50	0.11	1.17	0.47	3.68	0.02	0.72	0.26
Age[e], 55-64 years	0.88	0.72	0.55	0.56	1.01	0.98	1.56	0.04	4.10	0.01	1.05	0.87
Age[e], 65-74 years	1.75	0.13	1.27	0.81	2.03	0.17	1.26	0.31	3.27	0.04	1.13	0.68
Age[e], ≥ 75 years	2.77	0.01	1.77	0.61	3.10	0.02	1.20	0.55	3.90	0.03	0.92	0.84
PK*Statin Use		0.01						0.91				
PK Q2*Statin Use	0.15	0.01					0.76	0.47				
PK Q3*Statin Use	0.17	0.01					0.91	0.82				
PK Q4*Statin Use	0.65	0.51					0.86	0.69				
[a]Quartile reference group: PK<0.6nmol/L, PK Q2: 0.6 ≤ PK ≤ 1nmol/L, PK Q3: 1< PK ≤ 1.7nmol/L, PK Q4: > 1.7nmol/L												
[b]BMI group reference <25 kg/m^2												

[c]Site reference group = Johns Hopkins Medical Center, HUP: Hospital of the University of Pennsylvania, VA: Corporal Michael J. Crescenz Veterans Affairs Medical Center
[d]Sex is defined as female or male. Male is the reference group.
[e]Age reference group: <45 years

Supplementary Table 1: Demographics of the 2009-2013 IN-RANGE sample examined for associations of statin use with plasma PK and coagulation			
	All	Caucasian	African American
	N = 471	N=140	N=331
	%	%	%
PK Quartile			
Q1	23	17	25
Q2	25	25	25
Q3	25	31	23
Q4	27	27	27
[a]Age, years			
< 45	22	19	23
45 - 54	19	16	21
55 - 64	33	31	34
65 - 74	16	17	15
≥ 75	10	16	8
Sex, % female	39	36	40
[a]Smoking Status, % smoking	20	12	23
[ab]Triglycerides, mg/dl	146.2 (95.6)	170.3 (98.9)	136.1 (92.5)
[a]BMI, kg/m^2			
< 25	27	29	27
25 - <30	29	33	27
≥ 30	44	39	46
Statin use, % on statins	42	43	42
[c]Statin Type (N=60, 140)			
Atorvastatin Calcium	17	22	15
Rosuvastatin Calcium	14	17	12
Simvastatin	53	43	56
Other	17	18	16
[abd]Days to Maintenance, days (N=110, 240)	73 (74)	63 (47)	78 (83)
[b]PTTR	36.7 (19.1)	37.0 (19.1)	36.6 (19.1)
[ae]Site	-		
HUP	45	46	45
VA	30	29	31
John Hopkins	24	26	24
Censoring			
Reached maintenance	74	79	73
Lost to follow up	11	6	12

Stopped warfarin	14	14	14
End of study	0.4	0.7	0.3
Reason unknown	0.6	0.7	0.6
[a]Significantly different by race at the $p<0.05$ level based on t-test or chi-square			
[b]Mean (standard deviation)			
[c]Other includes: Atorvastatin/Amlodopine, Ezetimibe/Simvastatin, Fluvastatin, Lovastatin, Pravastatin, and undefined.			
[d]Includes only those that reached maintenance phase			
[e]HUP: Hospital of the University of Pennsylvania, VA: Corporal Michael J. Crescenz Veterans Affairs Medical Center			

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7.3 Abstracts

An effective approach to create subclinical vitamin K deficiency in C57BL/6 mice

Stephanie Harshman, Donald Smith, Xiaohua Shen, Sarah Booth, Xueyan Fu.

USDA HNRCA at Tufts University, Boston, MA

Presented at Experimental Biology Boston, MA 2013

The response of vitamin K (VK) in tissues subsequent to VK manipulation has not been well studied in mice, which limits effective use of genetically-modified mouse models in VK nutritional studies. To address this concern, two studies were performed: Study #1: To determine the least amount of time required to reduce VK tissue concentrations without clinical signs of deficiency (including abnormal bleeding). Female C57BL/6 mice (n=12) were acclimated with AIN-93 G for 1 wk and subsequently fed a VK-deficient diet (21.4±3.0 µg phylloquinone/kg) for 0, 7, 14 and 28d while limiting coprophagy. Study #2: To determine if minimizing coprophagy makes a substantial contribution to VK tissue concentrations. C57BL/6 mice (n=64) were housed in conventional or suspended wire cages and fed AIN-93 or VK-deficient diet for 28d. Phylloquinone and menaquinone-4 concentrations were measured in serum, liver, and extra-hepatic tissues. Bleeding times were measured every 2 wks. There was a reduction of VK concentrations in all analyzed tissues within 7d ($P<0.05$), with the exception of body fat. VK was not detectable in liver by d28, yet there were no clinical signs of VK-deficiency when housed in suspended wire cages. In summary, a VK deficient diet that is also menadione-free can be used to induce low VK tissue concentrations within 7d, which will facilitate the study of VK metabolism.

Comparison of tissue concentrations in male and female C57BL/6 mice in response to vitamin K manipulation

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Presented at Experimental Biology San Diego, CA 2014

The tissue-specific response to dietary vitamin K (VK) manipulation has not been well studied in mice. This limits the use of genetically modified mouse models in VK studies. The objective of this study was to determine the sex-specific effects of dietary VK manipulation on serum, liver and extra-hepatic tissue VK concentrations in C57BL/6 mice (n=64). Mice were weight-matched and pair-fed a control diet (1.4±0.08 mg phylloquinone (PK) /kg) or VK-deficient diet (31±0.45 µg PK/kg) for 28 days. Liver, kidney, brain, pancreas, adipose tissue, and serum PK and menaquinone-4 (MK-4) concentrations were measured by HPLC. Data were log transformed and analyzed by a general linear model, stratified by diet. Male and female mice responded differently to dietary manipulation of VK in a tissue-dependent manner (for all sex by diet interactions p<0.031). Serum PK concentrations did not differ between males and females within each diet (p>0.95); no serum MK-4 was detected. Females on the control diet had significantly higher PK and MK-4 concentrations in adipose tissue, kidney, liver, pancreas, and brain compared to males (p<0.01). Only MK-4 concentrations in adipose tissue, liver, and pancreas did not differ between males and females on the deficient diet (p>0.44). Sex-specific differences in response to VK manipulation need to be considered when using animal models of VK metabolism.

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Tissue-specific proportions of phylloquinone to menaquinone-4 concentrations differ in response to dietary phylloquinone manipulation in lean male Zucker rats

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Presented at Experimental Biology Boston, MA 2015

Phylloquinone (PK) and menaquinone (MK) are naturally-occurring forms of vitamin K (VK).There is selective tissue distribution and conversion of dietary PK to MK4, providing indirect evidence of unique MK4 functions beyond those established for PK.
We determined the effect of dietary PK manipulation on the ratio of PK:MK4 in tissues of male lean Zucker rats (n=24, 4mo). Rats were pair-fed a PK-supplemented (S) (10±0.5 mg PK/kg) or PK-deficient (D) (0.03±0.003 mg PK/kg) diet for 12 weeks. PK was the sole dietary VK form provided. Liver, kidney, brain, pancreas, mesenteric adipose tissue, serum and diet PK and MK4 concentrations were measured by HPLC. Data were analyzed by ANOVA, with $p<0.01$ considered significant based on a Bonferroni adjustment. Data presented as mean±SD.
PK:MK4 was significantly higher in kidney, liver and mesenteric adipose tissue on the S diet (1.41±0.14 ; 78.8±15.5 and 21.8±4.8 pmol/g, respectively) compared to D diet (0.54±0.3, 7.4±4.9 and 12.2±5.4 pmol/g, respectively) ($p<0.01$). In contrast, PK:MK4 in brain (0.34±0.03, S; 0.55±0.3, D) and pancreas (0.86±0.3, S; 0.87±0.26, D) did not significantly differ between diet groups (both $p>0.147$).
PK supplementation resulted in an increase in PK:MK4 in liver, kidney and adipose tissue. In brain and pancreas, tissues that have a preferential conversion of PK to MK4, there was concomitant increase in MK4 such that the PK:MK4 ratio is maintained. The implications of this ratio merits further investigation in order to elucidate the roles of MK4.

Support: USDA ARS under Cooperative Agreements No. 58-1950-7-707; NIH/NIDDK T32 DK062032

Proportions of phylloquinone to menaquinone-4 differ in a depot specific manner in response to dietary phylloquinone manipulation in the Zucker rat model

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Presented at FASEB Summer Research Conference Chicago, IL Summer 2015

Phylloquinone (PK) and menaquinone (MK) are naturally-occurring forms of vitamin K (VK).There is selective tissue distribution and conversion of dietary PK to MK4, providing indirect evidence of unique MK4 functions beyond those established for PK. PK and MK4 are found in extra-hepatic tissues, including adipose tissue. Adipose tissue is heterogeneous tissue composed of multiple cell types that vary in conjunction with depot location and metabolic output. We determined the effect of dietary PK manipulation on the absolute concentrations and the ratio of PK:MK4 in adipose tissue depots including subcutaneous (SUBQ) and mesenteric, of male lean and fatty Zucker rats (n=48, 4mo). Rats were pair-fed a PK-supplemented (10±0.5 mg PK/kg) or PK-deficient (0.03±0.003 mg PK/kg) diet for 12 weeks. PK was the sole dietary VK form provided. Tissue PK, MK4, and long chain MK (6-13) concentrations were measured by HPLC and LC-MS. PK and MK4 concentrations are reported, no other VK forms were detected. Data were analyzed by a repeated measures general linearized model, with $p<0.05$ considered significant. Data presented as mean±SD.

Overall there was a diet effect in both depots ($p<0.001$). In the mesenteric depot, there was no effect of genotype in PK or MK4 concentrations ($p>0.065$), however there was a genotype effect observed in the subcutaneous depot ($p<0.001$) with lean rats having significantly higher absolute concentrations compared to fatty rats. A genotype by diet interaction exists for PK concentrations in the mesenteric and subcutaneous depots ($p<0.05$) and MK4 concentrations in the subcutaneous depot only ($p<0.001$).

		Mesenteric		Subcutaneous	
		PK(pmol/g)	MK4(pmol/g)	PK(pmol/g)	MK4(pmol/g)
Deficient Diet	Fatty	123+76.5	12.9+8.5	53.2+30.4	1.9+1.9
	Lean	151+75	14.3+9	186+66.7	5.5+2.6
Supplemented	Fatty	4272+1480	217+92	2565+1390	43+25.3
	Lean	3340+720	160+51	5395+1220	88+28

The ratio is an effective value to evaluate the conversion of PK to MK4. Examining the ratio, there was no effect of genotype or a genotype by diet interaction ($p>0.15$). PK:MK4 was significantly higher in the SUBQ depot compared to mesenteric ($p<0.001$) in both the deficient diet (lean 38.02±14.2 and 12.9±5.4; fatty 23.9±11.5 and 10.32±3.1 respectively), and the supplemented diet (lean 63.6±13.2 and 21.8±4.8; fatty 63.2±11.8 and 21.4±21.4). PK uptake and conversion to MK4 is not uniform amongst adipose tissue depots. Differences in the ratio and absolute concentrations may be indicative of preferred sites for storage, or different physiological needs of the depots thus requiring different VK forms. The results require further investigation to elucidate the roles of PK and MK4 at different adipose tissue depots.

Support: USDA ARS under Cooperative Agreements No. 58-1950-7-707; NIH/NIDDK T32 DK062032

Mixed dishes and restaurant foods are an unexpected source of dietary vitamin K

Presented at National Nutrient Database Conference, Washington D.C. May 2016.

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Abstract and poster to be considered for the Student Poster Award Competition.
Advisor's Name: ***Dr. Sarah Booth***
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Abstract
Background: Variable intake of foods containing vitamin K can result in unanticipated interactions with stability of coumarin-based oral anticoagulation medications. Current recommendations emphasize consistent intake of vitamin K-rich fruits and vegetables while taking these medications. However, comprehensive and current data on vitamin K content of mixed dishes and restaurant foods are lacking.
Objective: To examine the amount of vitamin K (phylloquinone, 2',3'-dihydro-phylloquine, and menaquinone-4) per serving of various representative restaurant foods and mixed dishes in the U.S. food supply.
Description: Food samples were obtained from the National Food and Nutrient Analysis Program and were analyzed using standardized high performance liquid chromatography methods. Vitamin K forms were summed and classified as high (>100g/μserving), moderate (>25-100μg/serving), low (5-25μg/serving), or free (<5μg/serving) in vitamin K according to classification used in the Academy of Nutrition and Dietetics *Nutrition Care Manual*. Of the 65 mixed dishes and restaurant foods, 1 was high, 20 were moderate, 34 were low, and 10 were free in vitamin K. Of the 21 high or moderate vitamin K foods, 13 foods (orange chicken, chicken parmigiana, spaghetti with meatballs, cheese-filled ravioli, cheese-filled ravioli with tomato sauce, meat submarine sandwich, cream of mushroom soup, fried cheese sticks, onion rings, nachos with cheese sauce, nachos with meat, cheese, and sour cream, cheese enchilada, and cheese quesadilla) contained no vitamin K-rich fruits or vegetables.
Conclusion: Mixed dishes and restaurant foods composed of vitamin K-containing oils and animal products can be moderate sources of vitamin K. These foods have the potential to be overlooked as sources of vitamin K but they could contribute substantially to a person's total

daily vitamin K intake when considering bioavailability, portion size, and the dietary pattern within which these foods are consumed. This has important clinical and nutrition therapy implications for those prescribed coumarin-based oral anticoagulation medications.

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A race-specific interaction between vitamin K status and statin use during warfarin therapy initiation

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Vitamin K (VK) is required for the post-translational modification of several clotting factors. Warfarin is a vitamin K antagonist and anticoagulant. The most common dietary and circulating form of VK is phylloquinone (PK). PK is lipid soluble, carried by triglyceride-rich lipoproteins, and shares a metabolic pathway with cholesterol. Thus, there is biological plausibility for an interaction between serum PK and lipid-lowering medications (statins) in warfarin therapy.

The objective of this study was to examine if serum PK and/or statin treatment was associated with anticoagulation control in African Americans and Caucasians initiating warfarin therapy. Warfarin sensitivity differs between African Americans and Caucasians, so analyses were race-specific.

Cox proportional hazards and linear regression models were used in a cross-sectional analysis of the 2009-2013 International Normalized Ratio Adherence and Genetics II cohort of African Americans (n=331; 40% female) and Caucasians (n=140; 36% female) initiating warfarin therapy. Primary exposures included serum PK (categorized as quartiles 1: PK<0.6, 2: 0.6≤PK≤1.0, 3: 1.0<PK≤1.7, 4: PK>1.7nmol/L) and statin use. Outcomes included number of days to maintenance dose and percent time in the therapeutic range (PTTR). Covariates included age, sex, smoking status, serum triglycerides, BMI, and clinic site.

A significant interaction between serum PK quartile and statin use with respect to days to maintenance was detected in Caucasians (p=0.04), but not African Americans (p=0.81). In Caucasians taking statins (n=60), the rate to reach maintenance was 88% (HR=0.12, 95%CI 0.04-0.39) and 81% (HR=0.19, 5%CI 0.06-0.53) slower for those in the second and third serum PK quartiles, respectively, compared to those in the lowest quartile. The rate to reach maintenance did not differ between the highest and lowest serum PK quartiles (HR=0.84, 95% CI 0.3-2.7) or between any quartiles among Caucasians not taking statins (n=80, p=0.76). In African Americans, serum PK was not associated with the rate to reach maintenance whether they used statins (n=140, p=0.98) or not (n=191, p=0.23). Serum PK and statin use were not associated significantly with PTTR in either race.

Statin use appears to slow the rate to establish maintenance warfarin dose among Caucasians with moderate serum PK. This apparent drug-drug-nutrient interaction suggests PK status and lipid-lowering medication may influence anticoagulation for Caucasians during warfarin therapy

initiation. The role of vitamin K with statin use in anticoagulation therapy in different races requires further examination.

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Mixed dishes are a top contributor to vitamin K intake in US adults: data from the 2011-2012 NHANES

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The primary dietary form of vitamin K in US diets is phylloquinone (PK), which is found in green vegetables and vegetable oils. PK content of foods are provided in the USDA National Nutrient Database for Standard Reference, which helps guide and estimate current dietary recommendations. As our food supply diversifies and expands, food groups contributing to dietary PK intake are also expanding. It is important to identify the contributors to dietary vitamin K estimates to guide healthcare professionals and recommendations on food sources of vitamin K.

The purpose of this study was to estimate: (1) the PK consumed in the diet of US adults; (2) the contribution of different food groups to PK intake in individuals with high or low vegetable intake (≥ 2 cups < 2 cups vegetables/day); and (3) to characterize the contribution of different mixed dishes to PK intake.

We used data from adults participating in NHANES 2011-2012 (≥ 20 y; 2092 men and 2214 women) to determine usual PK intake utilizing a complex, stratified, multistage probability cluster sampling design (National Cancer Institute Method). Food group classifications were determined according to the USDA What We Eat in America (WWEIA) food groupings. The proportion of PK supplied by each food group and mixed dishes was compared between low and high vegetable consumers, using multiple t-tests with Bonferroni-Dunn correction for multiple comparisons.

On average, 43.0% of men and 62.5% of women met the AI of 120 and 90 μg/day of PK respectively. Vegetables were the highest contributor to PK intake, contributing 60.0% and 36.1% in high and low vegetable intake groups respectively. Mixed dishes were the second highest contributor, contributing approximately 16% and 28% of total PK intake in high and low vegetable intake groups respectively (all $p<0.001$ between low and high vegetable intake groups). Within mixed dishes, overall grain based dishes including macaroni and cheese, pasta, and rice dishes contributed to 25% of dietary PK, followed by sandwiches (9.7%), and pizza (10%) in the low vegetable intake group. In individuals with high vegetable intake, primary sources of dietary PK in mixed dishes were soups (23%), Asian dishes (such as fried rice with soy based sauce mixtures, 21.5%), and meat and poultry (17.9%).

Our findings suggest mixed dishes are a significant contributor to PK intake in the United States. Unique to mixed dishes is the large contribution of grain-based foods that often lack PK-rich vegetables. Additional research is required to further quantitate other vitamin K forms in the US food supply, and to identify all dietary sources of vitamin K to better define our current recommendations.

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Atorvastatin decreases menaquinone-4 formation in C57Bl6 male mice

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Vitamin K is a fat-soluble vitamin found in two forms, phylloquinone (PK) and menaquinones. The two forms differ in saturation and length of their side chain. Menaquinone-4 (MK4) is a unique menaquinone because it is: (1) formed from dietary PK by means of tissue-specific conversion; and (2) has unique functions beyond the traditional coagulation role of vitamin K such as providing protection against oxidative damage. Geranylgeranyl pyrophosphate, an intermediate in the endogenous cholesterol synthesis pathway, has been indirectly implicated in the synthesis of MK4. Statins (HMG-CoA reductase inhibitors) are effective suppressants of endogenous cholesterol synthesis, hence can be used to elucidate the role of geranylgeranyl pyrophosphate in the conversion of PK to MK4.

The purpose of this study was to determine the effect of statin treatment on endogenous production of MK4 in both young and old male mice given a diet supplemented with PK.

Four month (n=32) and 20-month old (n=32) C57BL6 male mice were randomly assigned to a diet containing 300mg atorvastatin/kg diet and 2.67±0.33 mg PK/kg or a control diet containing 2.77±0.02 mg PK/kg for 8 weeks. During week 8, all mice received deuterium-labeled PK in the diet. Labeled and unlabeled PK and MK4 in tissues were measured by HPLC-MS. HMG-CoA reductase expression gene expression in liver, kidney, brain, and intestine was quantified by reverse transcriptase-PCR. Tissue MK4 and PK concentrations were compared between statin treatment groups using general linear models. We also examined the effect of statins in young and old mice.

Mice given statins had less total MK4 and deuterium-labeled MK4 tissue concentrations compared to mice not given statins ($p=0.02$ and 0.04, respectively, age-adjusted). In statin-treated mice, kidney MK4 concentration was reduced by 41% ($p\leq0.05$), and percent deuterium-labeled MK4 was reduced by 46.5% compared to mice not given statins ($p=0.001$). However, total and deuterium-labeled MK4 were not affected by statin treatment in brain and intestine ($p>0.31$). The effect of statins on MK4 did not differ by age. Liver PK was not affected by statins ($p=0.43$). In liver, HMG-CoA reductase expression increased in response to statin treatment by 1.5-fold and 4-fold in both young and old mice respectively (all p-values<0.05). There were no differences in HMG-CoA expression in kidney, brain, or intestine.

In male mice, statin treatment effectively reduced endogenous MK4 formation in the kidney, but not other organs. Our results support previous studies demonstrating that liver is not a site of PK to MK4 conversion. In the brain MK4 is the sole form but the specific statin used for the intervention does not cross the blood brain barrier. Therefore, brain MK4 concentrations are not expected to be effected. These observations are consistent with our hypothesis that

geranylgeranyl pyrophosphate is a key factor in the generation of MK4. Further research is needed to understand potential regulatory mechanisms and the unique functions of MK4.

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