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Dietary Vitamin K and Association with Hepatic Vitamin K Status in a Yup'ik study population from Southwestern Alaska

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Abstract

Scope—We investigated the relationship between dietary vitamin K and plasma PIVKA-II concentration, a biomarker of hepatic vitamin K status, in a Yup'ik study population in southwestern Alaska.

Methods and Results—A total of 659 male and female, self-reported Yup'ik people, 14 years of age, were enrolled. Blood was collected for genotyping and plasma PIVKA-II biomarker analysis. A Yup'ik-specific dietary food frequency questionnaire was used to assess vitamin K

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AUTHOR CONTRIBUTIONS

N.A., T.R., M.R., S.H., B.B., J.P., M.A., A.R. and K.T. designed the study; J.B., B.B., S.H., J.P. and T.R. recruited study participants and gathered and collated FFQ data. S.H. collected and identified tundra green samples. N.A. and M.R. conducted PIVKA-II and Vitamin K measurements. A.F. and J.Y. conducted the genetic analysis. T.R. M.A. and T.T. conducted the statistical analyses. All authors contributed to writing of the manuscript.

CONFLICTS OF INTEREST

Kenneth E. Thummel - no conflicts of interest,
Allan E. Rettie - no conflicts of interest,
Tove Ryman - no conflicts of interest,
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intake. Among the participants, 22% reported not consuming foods rich in vitamin K during the past year and 36% had a PIVKA-II concentration ≥ 2 ng/mL, indicating vitamin K insufficiency. The odds of an elevated PIVKA-II concentration were 33% lower in individuals reporting any versus no consumption of vitamin K rich foods. The association was significant after adjusting for *CYP4F2*3* genotype. Tundra greens were high in vitamin K1 content, but an exploratory analysis suggested that subsistence meat sources had a greater effect on vitamin K status.

Conclusions—A substantial proportion of the Yup'ik population exhibits vitamin K insufficiency, which was associated with low consumption of vitamin K rich foods and which might affect an individual's response to anticoagulant drugs such as warfarin that target the vitamin K cycle.

Keywords

Alaska Native; Vitamin K; Coagulation; PIVKA-II; CYP4F2

INTRODUCTION

The generic term “vitamin K” encompasses a group of lipid-soluble vitamins that are well recognized for their role in coagulation, but also are important for bone and blood vessel health, renal function and cell growth [1–3]. The reduced (hydroquinone) form of vitamin K is a cofactor to the enzyme γ -glutamyl carboxylase (GGCX), which catalyzes the posttranslational carboxylation of glutamic acid (Glu) to γ -carboxy glutamic acid (Gla) on several proteins, including vitamin K-dependent clotting factors – Factors II (prothrombin), VII, IX and X [4]. Vitamin K exists naturally in two main forms, either as phyloquinone (vitamin K1), commonly found in plants, or as menaquinones (vitamin K2), which are found in meats and fermented foods such as soybeans, fish heads and seal flippers [5–8]. Humans cannot synthesize vitamin K and must obtain it from dietary sources. Phyloquinone (VK1) is the predominant form of vitamin K present in the diet of westernized cultures [9]. The Institute of Medicine has recommended a daily intake of 120 and 90 μ g for men and women, respectively [6]. An inadequate intake of vitamin K will result in a decline in hepatic VK1 concentration and increased concentrations of undercarboxylated proteins, such as PIVKA-II (undercarboxylated form of prothrombin) [10]. Vitamin K status is typically assessed in plasma through measurement of VK1 or PIVKA-II. Because of its relatively long half-life (~60 hr), plasma PIVKA-II concentration is considered a better measure of past long-term vitamin K status, with values ≥ 2 ng/mL taken as an indicator of subclinical deficiency of vitamin K [11, 12]. An important variable influencing vitamin K status in humans is the *CYP4F2*3* allele [13–15], which is associated with decreased enzyme function and increased vitamin K concentrations, because CYP4F2 initiates catabolism of the vitamin [16, 17].

The main dietary source of vitamin K is green leafy vegetables, which contain VK1 and may only be intermittently available to circumpolar populations that still rely on traditional foods for much of their diet and/or who lack access to affordable and regularly available commercial sources of green vegetables. Fermented foods (e.g., fermented fish heads, walrus and seal flipper) are consumed by indigenous circumpolar populations [18] and can be an additional source of vitamin K (as menaquinones). However, these may not be eaten

regularly in these communities and so also may not be a reliable source of vitamin K. Thus, it is plausible that some circumpolar populations may be prone to a hypocoagulable state because of low intake of vitamin K, particularly during seasons when possible traditional sources such as “tundra greens” and seaweed are unavailable and fermented foods are not regularly consumed. Historical accounts of increased bleeding tendency in Greenlandic people were summarized by Dyerberg and Bang in the 1980’s, and included episodes of epistaxis, haemophysis, urinary tract bleeding, obstetrical bleeding and submucosal bleeding [19, 20]. Though the investigators attributed these symptoms to the high consumption of ω 3 polyunsaturated fatty acids (PUFAs) in this population that led to antagonism of platelet activity, vitamin K status was not explored. To date, there is little information about vitamin K status among indigenous populations of Arctic regions.

The Yukon-Kuskokwim Delta in southwestern Alaska is home to approximately 23,000 people, 85% of whom self-identify as Yup’ik Alaska Native (AN) and who live predominantly in remote communities [21]. Investigators from the University of Alaska Fairbanks, Center for Alaska Native Health Research have been working in partnership with members of the Yup’ik communities in the YK Delta since 2001. As part of this ongoing health research collaboration, we initiated a multi-faceted NIH-funded study in 2010 to identify genetic and environmental sources of inter-individual differences in warfarin response, with the goal of providing basic knowledge to the population and to their health care providers (principally the Yukon Kuskokwim Health Corporation) and of informing future decisions on the use of precision medicine tests to improve the health of the population [15, 22, 23].

In the present study, we used results of a Yup’ik specific food frequency questionnaire (FFQ) to ascertain consumption of vitamin K rich foods, to test for an association between vitamin K intake and plasma PIVKA-II concentration, controlling for *CYP4F2**3 variation and other demographic factors. Specifically, we characterized the association between diet and long-term vitamin K status, as indicated by PIVKA-II measurements, in the Yup’ik population. In addition, we analyzed the VK1 and MK4 contents of tundra greens and representative meats that are regularly consumed in the traditional Yup’ik diet, as potentially important sources of vitamin K in the population. Of note, we did not study fermented foods, as they are consumed infrequently by Yup’ik people living in the YK Delta [24].

MATERIALS AND METHODS

Research approval

This study was conceived through a partnership between the University of Washington, the Center for Alaska Native Health Research, the Yukon Kuskokwim Health Corporation, and Yup’ik communities in the Yukon-Kuskokwim Delta. The study was approved by the University of Alaska Fairbanks IRB, the University of Washington IRB and the Yukon Kuskokwim Health Corporation Executive Board of Directors.

Study population

A total of 733 male and female self-reported Yup'ik research participants, 14 years of age, enrolled in the parent study through written and oral advertisements between September 2009 and December 2013. Subsets of 661 participants had complete FFQs, and plasma PIVKA-II data and 646 had *CYP4F2**3 genotype data available. Two participants in each subset were removed from statistical analysis after determining that they had a very high plasma PIVKA-II concentration (1708 and 1618 ng/mL) as a result of warfarin therapy. The final subsets of 659 for dietary study and 644 participants for genetics study represent convenience sampling from 10 of 58 communities in the Yukon-Kuskokwim Delta.

Food frequency questionnaire

Dietary data were collected using a Yup'ik specific FFQ developed by Center for Alaska Native Health Research researchers and local community research assistants [24]. Participants reported how frequently they typically consumed each food during the previous 12 months. Frequency of consumption was measured on a 9-point scale, the lowest frequency option was “never or less than once per month,” and the highest frequency option was “2+ times per day”. For individuals who had participated in more than one research visit, we used food consumption data from their most recent visit for analysis. A complete description of the methodology employed can be found in references [24, 25].

Reagents and standards

Standards of phylloquinone and menaquinone-4 (MK4) were commercially available from Supelco. Deuterated phylloquinone (VK1-d7) was purchased from Aldrich. Deuterated menaquinone-4 (MK4-d7), a gift from Dr. Matthew McDonald (University of Washington, Seattle), was synthesized by the method of Suhara [26]. All standards were prepared in methanol and characterized spectrophotometrically and chromatographically before use. VK1 and MK4 concentrations were confirmed using a molar extinction coefficient of 19000M^{-1} at 248 nm [27, 28].

Assessment of vitamin K status

Long-term vitamin K status of the study participants was assessed by measurement of plasma PIVKA-II concentrations, determined by the UW Department of Laboratory Medicine, using a commercially available kit from Diagnostica Stago (Parsippany, NJ). The limit of quantitation for the PIVKA-II assay was 2 ng/mL. Hemolyzed samples were excluded from data analysis. Internal quality control samples were run in parallel with all of the experimental plasma samples. These were drawn from two archived lots of pooled plasma (A1138 and A1143) obtained from Precision BioLogic (Dartmouth, Nova Scotia, Canada). Over the course of the study, the mean PIVKA-II concentrations ($\pm$ SD) of these two lots were 2.23 ± 0.55 and 2.65 ± 0.70 ng/mL.

CYP4F2 genotyping

Detection of the *CYP4F2**3 allele (C>T, V433M, rs2108622) in study participant DNA was performed using a TaqMan-based genotyping assay (Applied Biosystems, Inc.), as reported previously [23]. A χ^2 goodness-of-fit test was used to determine if the observed genotype

frequencies for *CYP4F2*3* differed significantly from the expected frequency under the assumptions of Hardy-Weinberg Equilibrium.

Tundra Green Preparation

All samples of tundra greens were obtained and gifted to us by community members from areas near the Yup'ik community study sites where we enrolled participants. A list of the plants identified by their local and botanical names is found in Table 1. Because different parts of tundra greens may be consumed by the Yup'ik people, the leaf and stems (where available) were analyzed separately as two sets of duplicates, before and after boiling for 15 minutes in water, to reflect different methods of preparation prior to consumption. For comparison, we also measured vitamin K content of a set of commercially available 'reference' vegetables, which included broccoli, spinach, iceberg lettuce, celery, basil, red onion, and carrot. Samples were stored at -80°C after collection and then freeze-dried under vacuum prior to extraction. This was done to control for variation in the degree of desiccation that might occur between the time samples were collected and received in our research lab. To each 20 mg aliquot of freeze-dried tundra greens, 10 ng of VK1-d7 was added. After the addition of 0.5 mL of water, each sample was homogenized with a Bertin Technologies Precellys 24 tissue homogenizer utilizing 2.8 mm ceramic beads (MO BIO Laboratories, Inc.). Water (4.0 mL) and 4.0 mL of 2-propanol/hexane (3:2, v/v) were added and, after vigorous vortexing for 1 minute, samples were centrifuged at 1500 *g* for 5 minutes. The upper organic layer was removed and the extraction step was repeated with 4 mL of hexane. After drying the combined organic layer under nitrogen, samples were reconstituted in 500 μL of hexane and applied to Bond Elut 500 mg silica SPE columns (Varian). These cartridges were conditioned with 4.0 mL of ethyl ether/hexane (4:96, v/v) and then with 4.0 mL of hexane. After loading the concentrated green extracts, the columns were washed with 4.0 mL of hexane, and eluted with 8.0 mL of ether in hexane (4:96, v/v). The eluate was evaporated under nitrogen and the residue was dissolved in 100 μL of methanol solution for analysis of 15 μL aliquots by LC-MS/MS.

In addition to tundra and commercial greens, we tested three different subsistence meats – seal liver, lush fish (aka Burbot or freshwater cod) liver, and smoked salmon (skin and meat) – that are also part of the traditional Yup'ik diet and that have a relatively high fat content in which vitamin K might accumulate. Meat samples were received as gifts from the Yup'ik people, who harvested them as food sources from the wild. Samples were analyzed for VK1 and MK4. Approximately 50–100 mg of each sample was weighed (in duplicate, wet-weight), combined with 2 ng of VK1-d7 and 2 ng of MK4-d7 in 0.5 mL of water, and homogenized with a Bertin Technologies Precellys 24 tissue homogenizer utilizing 2.8 mm ceramic beads (MO BIO Laboratories, Inc.). The homogenate was combined with 2 mL of ethanol and vortexed vigorously for 1 minute. The mixture was centrifuged at 1500*g* for 5 min, the supernatant removed and dried under nitrogen. The remaining solid was dissolved in 0.5 mL of hexane and applied to the head of a Bond Elut silica-based solid phase extraction column (Varian) that had been conditioned with 4 mL of 4% diethyl ether in hexane and then 4 mL of 100% hexane. After loading the column was washed with 4 mL of 100% hexane, then eluted with 8 mL of 4% diethyl ether in hexane, dried under nitrogen and then reconstituted in 100 μL of methanol in preparation for LC-MS/MS analysis.

LC-MS/MS analysis of greens and meat extracts

All standards and samples were analyzed on a Waters TQ-S mass spectrometer connected to a Waters Acquity I-Class UPLC system with an APCI source operating in negative mode. The APCI probe temperature was set to 650° C. For VK1 and VK1d7, the cone voltage was set at 58 and 50 V, respectively, and the collision voltage was set at 30 V and 28 V, respectively. For MK4 and MK4d7, the cone and collision voltages were 56 V and 28 V, respectively. The corona voltage was set to 30 μ A and the desolvation gas flow was 500 L/hr. MRM transitions for VK1 and VK1d7 were m/z 450.6 >185.1 and 457.7 >185.1, respectively. MRM transitions for MK4 and MK4d7 were m/z 444.6 >185.2 and 451.7 >192.2, respectively.

The temperature-controlled UPLC column compartment was set to 50°C. Chromatographic separation of analytes was achieved on a Waters Acquity UPLC BEH phenyl 1.7 μ m, 2.1 $\times$ 50 mm column with a Phenomenex SecurityGuard Cartridge System equipped with a 4.0 $\times$ 2.0 mm C8 SecurityGuard cartridge (Phenomenex Inc., Torrance, CA). The mobile phase consisted of solvent A (water) and solvent B (0.05% formic acid in methanol). A linear gradient was run as follows: 85% solvent B at 0 minutes to 100% solvent B at 3 minutes with a flow rate of 0.35 mL/min. Elution continued with Solvent B at 100% for 3 more minutes before re-equilibration back to 85% solvent B over a final 2 minutes. The cycle was complete after 8 minutes. Under these conditions, VK1 eluted at 3.6 minutes and MK4 at 3.0 minutes.

The linearity of response for VK1 in tundra greens was assessed by spiking VK1 into samples containing 0.5 mL water with 20 mg of freeze-dried red onion in concentrations that ranged from 5 ng to 500 ng for VK1. Linear regression was used to compare measurements obtained by LC-MS/MS to expected concentrations of calibration standards. Standard curves for both PBS and FBS were linear with R^2 values of 0.99. For the VK1 standard curve derived from red onions, the R^2 value was 0.99.

For meat samples, standard curves were generated using phosphate-buffered saline (PBS). Stock solutions ranging from 0.5 to 3.0 ng of VK1 and MK4, were spiked into 500 μ L of PBS. In creating a standard curve to assess matrix effects, a human liver homogenate with known low endogenous levels of VK1 was spiked with stock solutions ranging from 0.5 to 3.0 ng of VK1 and MK4. Standard curves for both PBS and liver homogenate were linear with R^2 values > 0.99 for VK1, >0.98 for MK4 in PBS and >0.97 in liver homogenate.

VK1 content in each plant source (leaves and stems) and VK1 and MK4 content each meat source were analyzed from 4 separately weighed samples. Values reported represent the mean $\pm$ SD for all sources except boiled plant leaves and stems, which are the average measurements of two sample measurements.

Statistical Analysis

Statistical analysis was performed using STATA (version 11.0 SE). A logistic regression model was used to estimate the odds ratio (OR) of the association between diet and long-term vitamin K status (PIVKA-II). The outcome was dichotomous and categorized as either PIVKA-II $\geq$ 2 ng/mL (low vitamin K status) or PIVKA-II < 2 ng/mL (normal vitamin K

status). Frequencies of consumption of foods with vitamin K were non-normally distributed and, thus, were dichotomized into any consumption of vitamin K foods or no consumption. Foods with vitamin K were classified into three groups: (1) green vegetable sources, which included wild tundra greens, spinach, and broccoli and cauliflower; (2) liver sources, which included seal liver, wild game liver, beef liver, and fish liver; and (3) any source, which included all green and liver sources. Other predictor variables of interest were *CYP4F2* genotype (additive model, with 0, 1, or 2 copies of variant allele *3), age (continuous), sex (binary) and whether the study community was located in a coastal or inland region (binary). The OR and confidence interval (CI) of 95% were reported. The overall fit of the model to the data was evaluated using the likelihood ratio test (χ^2) and goodness-of-fit test (Pearson χ^2). A two-tailed p-value less than 0.05 was considered significant for all statistical tests, except for exploratory analyses where $p < 0.1$ was considered significant.

RESULTS

Population demographics and summary statistics

Demographic information and descriptive statistics for the 659 participants for whom complete dietary and vitamin K status information was available are shown in Table 2. The study cohort exhibited approximately equal distribution of gender and slightly more residents of coastal than inland communities. With regard to observed allele frequencies at the *CYP4F2**3 locus, there was no significant deviation from Hardy-Weinberg Equilibrium ($p = 0.41$).

FFQ – vitamin K rich foods

Descriptive statistics for the frequency of consumption of vitamin K rich foods is shown in Table 3. Green vegetables or tundra greens (any green source) were consumed most frequently, followed by animal or fish liver (any liver source). Approximately 79% of the study population consumed any vitamin K source at least once a month in the prior year. However, the annual mean and median frequency of consumption of any vitamin K source was 7.1 and 2.4 times a year, with a range between 0 and 124.7. Closer inspection of the frequency distribution revealed that those who consumed vitamin K rich foods less frequently consumed them rarely at all, resulting in a bimodal pattern of consumption, even after log transformation (Figure 1).

PIVKA-II status and *CYP4F2**3 genotype

Among the 659 study participants evaluated, 36% had a plasma PIVKA-II concentration > 2 ng/mL (Table 2). The mean plasma PIVKA-II concentration for those participants with a quantifiable value > 2.0 ng/mL was 2.9 ± 0.9 . Interestingly, women were less likely to exhibit an elevated plasma PIVKA-II level than were men ($p = 0.003$). The *CYP4F2**3 allele frequency was 0.47 for participants for which genotype information was available (644/659).

Regression analysis

A logistic regression model was used to evaluate the association between vitamin K intake and PIVKA-II. When comparing the group reporting dietary consumption of any source of vitamin K to those that reported no consumption of vitamin K (Table 4), after adjusting for

age, sex and community location, the odds of having an elevated plasma PIVKA-II concentration was 33% lower in the group reporting any vitamin K source in the diet, as compared to those with no dietary vitamin K ($p = 0.038$). After adjusting for *CYP4F2*3* status, in addition to age, sex and community location, the odds of having an elevated plasma PIVKA-II concentration were 32% lower in the group reporting any dietary vitamin K source, as compared to those with no dietary vitamin K ($p = 0.050$). In the regression model adjusted for *CYP4F2*3*, the p-value for the variant allele was 0.036 and associated with a reduced odds of an elevated plasma PIVKA-II concentration. We also found from the logistic regression model that the odds of having an elevated plasma PIVKA-II concentration was 37% higher in men than in women ($p = 0.006$) after adjusting for dietary vitamin K source, *CYP4F2*3* status, age, and community location.

In an exploratory analysis, we further tested separately for associations between any green or liver sources of vitamin K and elevated plasma PIVKA-II concentration. When comparing the group reporting consumption of any liver source to those that reported no consumption (Table 4), after adjusting for age, sex and community location, the odds of having an elevated plasma PIVKA-II concentration was 28% lower in the group reporting liver in the diet, as compared to those who did not ($p = 0.055$). After adjusting for *CYP4F2*3* status, in addition to age, sex and community location, the odds of having an elevated plasma PIVKA-II concentration was 26% lower in the group reporting liver in the diet, as compared to those that did not ($p = 0.084$). Consumption of green plants was not associated with elevated plasma PIVKA-II concentrations without or with *CYP4F2*3* adjustment ($p > 0.10$).

Vitamin K content of selected tundra greens and commercially available vegetables

A list of tundra greens that were collected and gifted to us by community members are found in Table 1. The measured VK1 contents in micrograms per gram of freeze-dried plant material are listed in Table 5. The values we report are generally higher than that cited in the USDA National Nutrition database and other publications [29], but preparation methods differed; greens analyzed in this study were freeze dried before weighing and analysis, which would concentrate VK1 compared to raw or frozen greens. Leaves were found generally to have higher VK1 content compared to stems. Boiling decreased the phylloquinone content by an average of 46.4% (5.49–71.0%) for leaves and 58.0% (45.1–75.3%) for stems. The VK1 levels in tundra greens were similar to amounts found in raw, freeze-dried reference vegetables (Table 6).

Vitamin K content in selected marine-derived foods

VK1 contents in selected marine-derived Yup'ik foods are found in Table 7. VK1 was detected in seal and lush fish (burbot) liver, but not salmon, whereas MK4 was found in all three foods. However, the levels of VK1 found in those foods (nanograms per gram wet wt) was notably less than that seen in most traditional and commercial green plants (micrograms per gram dry wt), even after consideration of a water dilution effect. The content of MK4 in marine foods was also relatively low (7–52 ng/g wet wt), in comparison to the VK1 content of green plants. Analytes with masses consistent with higher degrees of vitamin K unsaturation (e.g. MK7 and MK9) were detected in marine foods, but were not quantified.

DISCUSSION

It is well recognized that a consistent amount of vitamin K in the diet is important for warfarin treated patients to achieve a stable target International Normalized Ratio (INR) during anticoagulation therapy. Many Yup'ik people in southwest Alaska incorporate a subsistence-based diet that is high in marine animals, fish and local tundra greens into their lifestyle. We recently reported that consumption of foods rich in ω 3 PUFAs is associated with hemostasis biomarkers [15]. Specifically, high PUFA consumption was associated with reduced platelet activation, as indicated by lower plasma sP-selectin concentrations compared to control. However, the relationship between consumption of subsistence food from the Yup'ik diet and vitamin K status and hemostasis has not previously been explored.

In this study, we showed that, in a cross-section of the Yup'ik population living in the Yukon-Kuskokwim Delta, consumption of foods known to contain vitamin K (including commercial green vegetables, tundra greens and meats such as seal/walrus liver, wild game liver, beef liver or fish liver), was associated with a reduced risk of having an elevated PIVKA-II concentration, an accepted retrospective biomarker of vitamin K status. Those who reported some annual consumption of foods containing vitamin K exhibited a lower frequency of an elevated plasma PIVKA-II concentration. However, of the population tested, 36% had an elevated PIVKA-II level at the time of the study, suggesting that consumption of vitamin K rich foods is low in a substantial proportion of the population. Indeed, approximately 21% of the study population reported no consumption of vitamin K rich foods during the prior year. This may reflect challenges in accessing affordable commercial greens and seasonal subsistence greens, reduced harvesting of vitamin K rich subsistence foods, such as tundra greens and liver, or reduced consumption of all subsistence foods with movement away from Yup'ik dietary traditions [30–32].

All of the traditional plant foods tested were rich sources of VK1, with measured contents equivalent to that of a panel of reference vegetables (e.g, spinach, celery and lettuce). The VK1 content was higher in leaves compared to stems, which is consistent with greater green pigmentation in leaves and VK1 concentrated in chloroplasts that are found in greater abundance in leaves [33, 34]. However, among the traditional sources of vitamin K, one might speculate, based on our exploratory regression analyses with food subgroups, that animal and fish liver are more important than green vegetables for hemostasis in the population and that reduced traditional food intake predisposes an individual to vitamin K insufficiency and an elevated plasma PIVKA-II level. As noted in the introduction above, consumption of fermented foods, such as fermented fish heads and seal and walrus flippers, can be an additional source of vitamin K (i.e., menaquinones) for indigenous circumpolar populations. The physiological and health impact of vitamin K2 from fermented foods can be considerable, as noted by Kaneki et al in their investigation of fermented soybean consumption, circulating phyloquinone and menaquinone levels and hip fractures in the populations of eastern and western Japan [8]. We did not formally evaluate this potential source of vitamin K and, accordingly, this represents a limitation of the study. However, to our knowledge, these fermented foods and others are consumed only sporadically by Yup'ik people living in the YK Delta [24] and thus might not have contributed much to an individual's vitamin K status in this study.

The association between PIVKA-II and dietary liver sources is intriguing, as the VK1 and MK4 contents in those foods were much lower than that of green vegetables and tundra greens. In this case, 'sufficient' γ -carboxylation activity may be conferred by longer-chain menaquinones, which are thought to be concentrated in liver but were not quantified. To circumvent this study limitation, future investigations should include quantitation of these vitamin K analytes, in view of the liver-elevated PIVKA-II association trend. While the availability of these traditional foods and tundra greens may be seasonal, they could still be cost-effective sources of vitamin K in people who cannot afford or get access to commercial greens, particularly in remote Alaskan communities, and if frozen and consumed year round. Increased consumption of safely prepared fermented fish and marine mammal flippers represents another possible traditional source of vitamin K for the community.

The health consequences of vitamin K insufficiency are unclear, but it possibly could influence the dose-response of oral anticoagulants such as warfarin that act by reducing the hepatic availability of vitamin K to support the activation of vitamin K dependent clotting factors such as prothrombin. Reduced hepatic availability of vitamin K might influence initial warfarin dose requirements [35], as well as resiliency against bleeding events during chronic therapy [36, 37]. It might also affect other biological processes and health outcomes dependent on protein γ -carboxylation, such as bone strength. Interestingly, INR values for nearly all study participants (excluding the two receiving warfarin) were within the reference range and did not correlate with the plasma PIVKA-II level. However, it has been reported that prothrombin time becomes prolonged only when plasma prothrombin concentrations fall below 50% of normal [38], a threshold apparently not achieved with the modest elevations in PIVKA-II seen in this study.

As reported previously, inheritance of the *CYP4F2**3 allele was associated with long-term (PIVKA-II) vitamin K status in our study population [15]. *CYP4F2* has been shown to act as an ω -hydroxylase to VK1 and MK4, and the V433M coding variant defining the *3 allele is associated with a higher warfarin dose requirement of approximately 1 mg/day [17, 39] and a reduced risk of a major bleeding event in patients receiving warfarin therapy chronically [36]. The *CYP4F2* variant protein encoded by the *CYP4F2**3 allele exhibits reduced capacity to metabolize VK1, secondary to a decrease in steady-state levels of the hepatic enzyme [16]. Interestingly, the frequency of the *CYP4F2**3 allele in the Yup'ik population of the Yukon-Kuskokwim Delta (0.47 in this cohort) is higher than that reported for nearly all other world populations studied to date [23]. This may represent an adaptive response to episodic access to vitamin K in the Yup'ik diet. Despite the apparent benefit of a relatively high population-level frequency of *CYP4F2**3, a high fraction of the population exhibited vitamin K insufficiency, which may be an important consideration for the initiation and maintenance of warfarin therapy.

There are several limitations to this research study. We employed a cross-sectional design and thus cannot assign causality between the consumption of vitamin K rich foods and plasma PIVKA-II concentrations. There are other reasons why an individual might exhibit an elevated PIVKA-II concentration besides vitamin K deficiency, including liver disease, consumption of alcohol, treatment with VKOR inhibitors like warfarin, and use of broad-spectrum antibiotics [6, 40]. We excluded data from two individuals receiving warfarin

therapy, but liver function, alcohol and antibiotic use was not controlled for in the statistical analysis and thus might introduce study bias. In addition, we did not measure absolute amounts of vitamin K foods consumed, which may have reduced our power to detect true associations. Although the sample size was substantial, it was not sufficient to test for associations between PIVKA-II and individual food groups. The data suggests that animal and fish liver are important sources of vitamin K in the Yup'ik population, but we would need a larger sample size or a different study design to test that hypothesis. Moreover, we did not quantify most of the menaquinones that are thought to accumulate in liver and that might also support the γ -carboxylation of prothrombin and other vitamin K-dependent clotting factors and we did not assess the consumption (albeit sporadic) of fermented foods. A final limitation of the study is the lower limit of PIVKA-II quantitation (2 ng/ml) and the fact that most study participants had a plasma level below this threshold, thus hindering our evaluation of the association between dietary vitamin K intake and long-term vitamin K status. We also could not identify, for comparative purposes, the range of plasma PIVKA-II concentrations for a comparable untreated control population. It is possible that elevated plasma PIVKA-II concentrations might also be found in some individuals from a non-Alaska Native population, because of low dietary vitamin K intake. Future studies should employ more robust and specific measurements of under-carboxylated clotting factors, such as mass spectrometric-based protein quantitation, and a study of multiple 'control' populations should be undertaken to establish baseline data.

In summary, our data suggest that differences in the consumption of vitamin K rich foods, including tundra greens and animal and fish liver, contribute to long-term variation in vitamin K status. Specifically, the consumption of vitamin K rich foods in the Yup'ik diet was associated with reduced odds of a physiological measure of vitamin K insufficiency – elevated plasma PIVKA-II. This association was independent of the protective effect of the *CYP4F2*3* allele on vitamin K status. For the Yup'ik people living in the Yukon-Kuskokwim Delta, consumption of traditional foods rich in vitamin K may be an affordable and reliable means to avoid the adverse health consequences of vitamin K insufficiency.

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Abbreviations

(non-standard)

GGCX	γ -glutamyl carboxylase
Glu	glutamic acid
Gla	γ -carboxy glutamic acid
VK1	vitamin K1

PUFAs	ω 3 polyunsaturated fatty acids
FFQ	food frequency questionnaire
MK4	menaquinone-4

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Figure 1. Frequency distribution for consumption of vitamin K foods

Log transformed annual frequencies for consumption of any source (A), any green source of (B) and any liver source (C) of vitamin K are shown.

Table 1

A list of traditional tundra greens consumed in the diet of Yup'ik people in the Yukon-Kuskokwim Delta.

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

^{a)} Samples were collected from the same rural communities as study participants.

^{b)} Tundra greens are eaten either raw or boiled and are gathered seasonally.

Table 2Demographic and *CYP4F2* genotype information for study participants.

	All ^a	Men	Women
Sample size	659	337	322
Age (yr; mean $\pm$ SD)	37 $\pm$ 18	35 $\pm$ 18	39 $\pm$ 18
Range of ages (yr)	14 – 93	14 – 85	14 – 93
Range of PIVKA-II (ng/mL)	<2 – 9.5	<2 – 9.5	<2 – 5.8
Plasma PIVKA-II (< 2 ng/mL)	421 (63.9%)	197 (58.5%)	224 (69.6%)
Plasma PIVKA-II ($\geq$ 2 ng/mL)	238 (36.1%)	140 (41.5%)	98 (30.4%) ^b
<i>CYP4F2</i> *1/*1 ^c	184 (28.6%)	94 (28.4%)	90 (28.8%)
<i>CYP4F2</i> *1/*3 ^c	309 (48.0%)	160 (48.3%)	149 (47.6%)
<i>CYP4F2</i> *3/*3 ^c	151 (23.4%)	77 (23.3%)	74 (23.6%)
	Coastal^d	Inland^d	
Number of men	183 (51.0%)	154 (51.3%)	
Number of women	176 (49.0%)	146 (48.7%)	

^a) Data for this group of 659 study participants is a subset of the larger cohort previously reported [15].

^b) Women were less likely to exhibit an elevated PIVKA-II level (PIVKA-II $\geq$ 2 ng/mL) than were men ($p = 0.003$)

^c) *CYP4F2**3 data was not available for 15 participants (6 men and 9 women). The *CYP4F2**3 allele frequency for the remaining 644 participants was 0.47. Subgroup genotype percentages are shown in parentheses.

^d) Coastal communities were considered to be within 5 miles from the coast and inland communities were located upriver greater than 5 miles away from the coast.

Table 3

Descriptive FFQ statistics (n=659)

Source of vitamin K ^a	No. of participant with detailed Vit K source data	% of study participants reported consuming at least once a month per FFQ	Mean ($\pm$ SD) annual frequency of consumption	Median annual frequency of consumption
Any source ^b	651	79	7.1 $\pm$ 14	2.4
Any green source ^c	657	67	12.7 $\pm$ 29.2	4.0
Any liver source ^d	653	43	2.8 $\pm$ 9.4	0
Wild game liver	657	21	1.5 $\pm$ 12.0	0
Beef liver	658	4	0.8 $\pm$ 5.6	0
Marine mammal liver	657	12	2.9 $\pm$ 16.4	0
Fish liver	658	26	5.8 $\pm$ 23.4	0

^a) Detailed data for a small number of participants was not available: sample sizes for each vitamin K source category reported.

^b) Includes green sources and liver sources.

^c) Includes tundra greens, spinach, and cauliflower/broccoli.

^d) Includes wild game liver, beef liver, marine mammal liver, and fish liver.

Table 4

Associations between vitamin K consumption and odds of plasma PIVKA-II level at or above 2 ng/mL^a

Source of vitamin K	Unadjusted for <i>CYP4F2*3</i>		Adjusted for <i>CYP4F2*3</i> ^b	
	Odds Ratio (95% C.I.)	P-value	Odds Ratio (95% C.I.)	P-value
Any source	0.67 (0.46–0.98)	0.038	0.68 (0.46–1.00)	0.050
Any green source	0.91 (0.64–1.28)	0.57	0.90 (0.64–1.28)	0.56
Any liver source	0.72 (0.51–1.01)	0.055	0.74 (0.52–1.04)	0.084

^a) Associations were evaluated by logistic regression. PIVKA-II level was dichotomized into < 2 ng/mL or ≥ 2 ng/mL. Vitamin K consumption was dichotomized into any consumption or no consumption. All regressions were adjusted for age, sex and community location.

^b) In the regression models that included *CYP4F2*3* (additive model, with 0, 1, or 2 copies of variant allele), the variant allele p-value was 0.036 for any vitamin K source, 0.036 for any green source, and 0.058 for any liver source associations. C.I., confidence interval.

Table 5

Vitamin K1 (VK1) content of tundra greens from the Yukon-Kuskokwim Delta.

Latin Name	VK1 (µg/g) ^a			
	Leaves	Stem	Boiled Leaves ^b	Boiled Stem ^b
<i>Angelica lucida</i>	2.73 ± 0.51	-	2.58	-
<i>Arenaria peploides L.</i>	11.0 ± 1.21	2.46 ± 0.81	6.78	0.83
<i>Artemisia tilesii</i>	21.7 ± 4.66	5.13 ± 0.79	6.29	1.97
<i>Ledum palustris ssp groenlandicum</i>	9.23 ± 1.32	1.86 ± 0.91	5.85	0.46
<i>Hippuris vulgaris</i>	27.2 ± 4.52	10.5 ± 2.94	13.3	4.48
<i>Rumex arcticus</i>	31.2 ± 3.29	-	13.0	-
<i>Ranunculus Pallasii</i>	37.7 ± 8.90	3.76 ± 0.36	18.1	2.16
<i>Caltha palustris</i>	48.2 ± 8.50	9.71 ± 1.67	19.9	5.33

^a) Where applicable, the content of both leaves and stems from each freeze-dried plant were measured. The lower limit of VK1 quantitation was 0.75 µg/g sample. Values shown are mean ± SD of 4 separate plant samples, except for boiled samples, which are the average of two samples.

^b) Separate measurements were made on samples that had been boiled for 15 minutes in water prior to freeze-drying to provide a comparison to the vitamin K content of raw samples.

Table 6

VK1 content of raw, freeze-dried reference vegetables.

Vegetable Type ^a	VK1 ($\mu\text{g/g}$) ^b
Broccoli (bud)	17.6 ± 10.6
Broccoli (stalk)	6.59 ± 3.44
Spinach (leaves)	25.6 ± 7.55
Spinach (stem)	6.13 ± 0.63
Lettuce (leaves)	7.87 ± 5.19
Celery (leaves)	25.0 ± 3.72
Celery (stalk)	4.94 ± 0.56
Basil (leaves)	64.8 ± 11.3
Basil (stem)	5.57 ± 0.26
Carrot (root)	1.65 ± 0.06
Red Onion (whole)	0.15 ± 0.01

^a) Samples were obtained from a commercially available market in the Seattle area.

^b) Data shown are mean $\pm$ SD of 4 separately weighed samples.

Table 7

Vitamin K content of selected marine-derived Yup'ik foods.

	VK1 ^a	MK4 ^a
Tissue Sample	(ng/g wet wt)	
Seal Liver	3.73 ± 0.68	7.03 ± 1.07
Lush Fish Liver	107 ± 6.73	52.1 ± 7.18
Smoked Salmon Meat	BLQ	14.3 ± 4.23
Smoked Salmon Skin	BLQ	12.8 ± 2.34

^a) Data presented are mean ± SD of measurements from 4 samples from a single source of each food type. BLQ – below limit of quantitation (1 ng/g wet wt).