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The association between vitamin K_2 intake and glucose homeostasis in individuals with and without chronic kidney disease

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Abstract

The association between vitamin K_2 intake and glucose homeostasis in individuals with and without chronic kidney disease

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Background: Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder worldwide. Subclinical micronutrient deficiencies have been found to contribute to glucose intolerance and insulin resistance. Vitamin K_2, in particular, possesses extrahepatic activities that may influence glucose metabolism. Individuals with chronic kidney disease (CKD) have poorer vitamin K status as well as impaired insulin sensitivity and glucose tolerance. However, whether or not the poorer metabolic outcome correlates with intakes of vitamin K_2 is unclear.

Objective: The objective was to investigate whether the intake of vitamin K_2 is associated with glucose tolerance and key factors determining glucose tolerance.

Design: A cross-sectional investigation was undertaken using data from the Study of Glucose and Insulin in Renal Disease (SUGAR) study that involved individuals with (n=53) and without CKD (n=39). Three-day dietary records were used to estimate intakes of vitamin K_2, using values of vitamin K_2 for foods in nutrient databases and relevant publications. Intravenous-glucose-tolerance tests (IVGTT), oral glucose-tolerance test (OGTT) and hyperinsulinemic-euglycemic clamps were performed to assess glucose tolerance, β-cell function and insulin sensitivity.

Results: In multivariate analyses adjusted for age, sex, BMI, race/ethnicity, eGFR and vitamin D status, higher vitamin K_2 intake was associated with better glucose tolerance (P = 0.047). Higher vitamin K_2 intake was also associated with insulin sensitivity (P = 0.017) in a crude model, but this association was attenuated and no longer significant after adjusting for classical risk factors. Vitamin K_2 intake was not associated with β-cell function.

Conclusion: Higher vitamin K_2 intake was associated with better glucose tolerance.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

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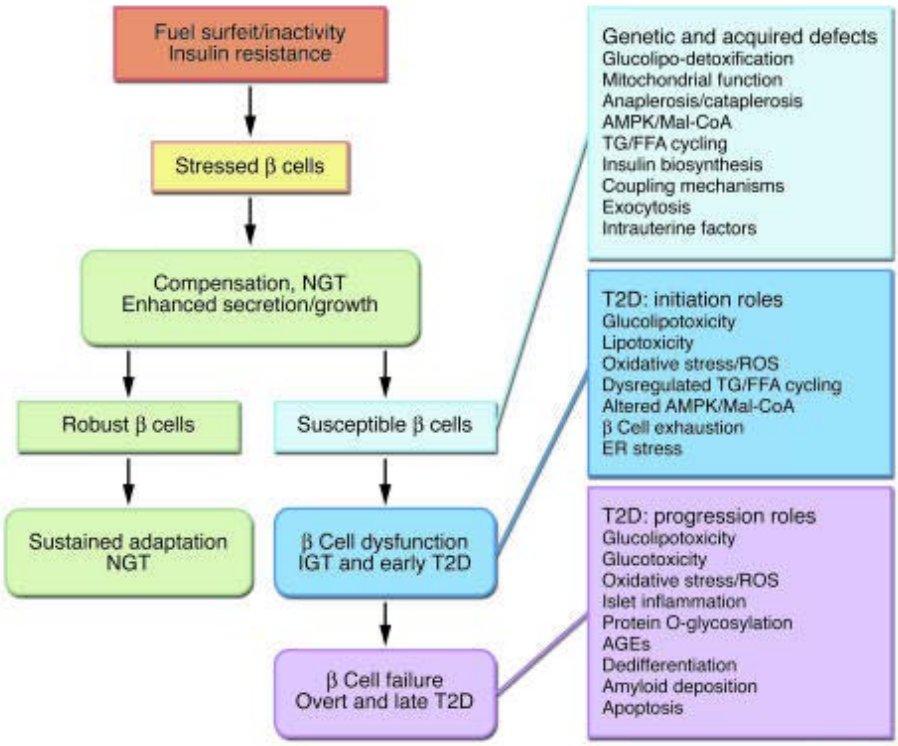
INTRODUCTION

Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder worldwide[1], with a prevalence growing at an alarming rate in both developed and developing countries[2,3]. In the United States, approximately 9.3% of the adult population has diagnosed T2DM, and an additional 86 million individuals have pre-diabetes[4]. By 2050, the number of people with T2DM in the US is expected to increase to 48.3 million[5]. Due to its associated morbidity and mortality, T2DM not only presents a serious challenge to the US health care system, but also an economic burden, with associated direct and indirect costs that were conservatively estimated at $254 billion in 2012[6,7].

Insulin resistance (IR) is defined as an attenuated biological response to insulin in target tissues, such as skeletal muscle, liver and adipose tissue[8]. In healthy individuals, glucose homeostasis is maintained by insulin secretion that is adequate for any given degree of insulin sensitivity, such that in the event of insulin resistance (IR), the pancreatic β-cell augments its insulin secretion to compensate for defects in insulin action[9-11]. The ability of individuals to secrete more insulin to compensate for IR makes them normal glucose tolerant (NGT). Conversely, individuals with "susceptible" pancreatic β-cells tend to develop impaired glucose tolerance (IGT) when they become insulin resistant, and they develop hyperglycemia due to the inability of their β-cells to fully compensate for IR[12,13]. Although these β-cells may initially attempt to restore euglycemia in many ways, such as expanding cell mass, enhancing insulin biosynthesis, and/or increasing the responsiveness of nutrient-secretion coupling[13], persistent stimulus

to these "susceptible" β-cells induces oxidative and endoplasmic reticulum (ER) stress[14]. This in turn, leads to a progressive decline in β-cell mass and function[15]. Although the actual cause of β-cell dysfunction remains unclear, some have proposed that chronic stress to β-cells impairs their inherent ability to appropriately respond to the constantly fluctuating metabolic demands for insulin. As a result, glucose intolerance and T2DM ensue (**Figure A**).



Prentki M and Nolan C. J Clin Invest. 2006; 116(7): 1802-1812

Figure A. Mechanisms of ß-cell failure in type 2 diabetes (T2DM)[13]

Islet ß-cell compensation for IR is sustained provided that ß-cells are robust, resulting in the long-term maintenance of normal glucose tolerance. However, compensation processes fail if there are genetic or acquired factors that result in susceptible ß-cells. The defects create weak links in the compensation process that promote ß-cell dysfunction through mechanisms with initiator roles. This results in impaired glucose tolerance and early T2DM. Hyperglycemia, once established, further catalyzes a set of mechanisms related to glucotoxicity, which causes severe ß-cell failure and overt and late T2DM.

Understanding the metabolic and structural derangements induced by chronic hyperglycemia in T2DM reveals a significant public health impact. Although T2DM is a difficult chronic illness on its own, people with T2DM often have common comorbidities of both microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (ischemic heart disease, peripheral vascular disease, and cerebrovascular disease) complications[16]. While some complications are episodic (e.g., foot ulcers or infections), others are progressive (e.g., nephropathy, neuropathy), and can potentially damage organs and result in permanent loss of organ function[17]. Cardiovascular disease (CVD) for instance, accounts for 65% of all deaths in people with T2DM[18]. Therefore, it remains crucial to identify modifiable lifestyle factors that improve glucose tolerance, insulin sensitivity, and β-cell function.

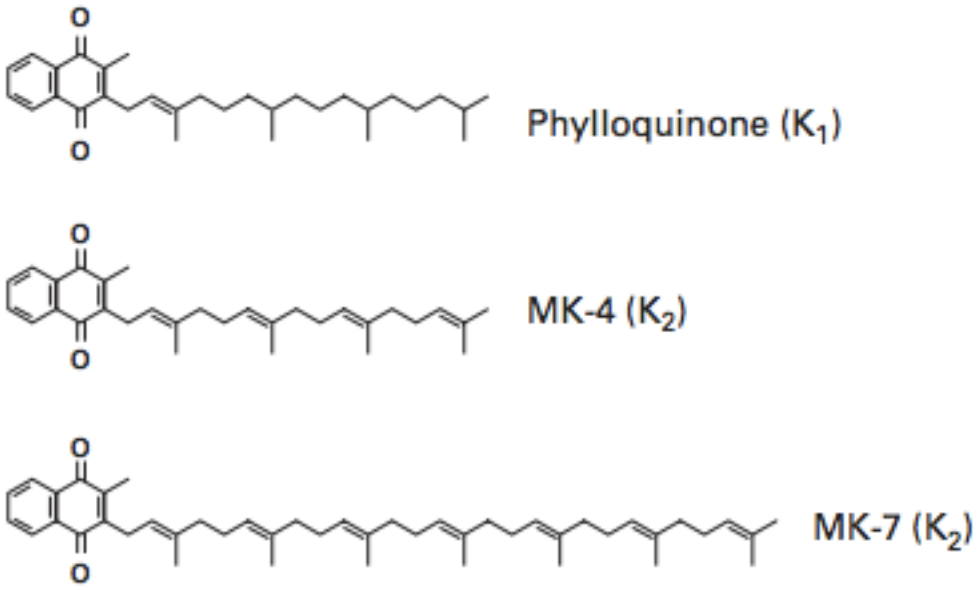
Dietary patterns and nutritional interventions may mitigate the risk of developing T2DM[19, 20]. While conventional nutrition therapy for the management of glycemic control places an emphasis on reducing total and saturated fat intake while increasing complex carbohydrate consumption, numerous findings suggest that micronutrients also play a role in the regulation of glucose homeostasis. One such nutrient, vitamin K, has been implicated in the regulation of insulin sensitivity and glucose homeostasis[21].

I. VITAMIN K

A. CHARACTERISTICS OF DIFFERENT MOLECULAR FORMS

Vitamin K is a fat-soluble vitamin known to activate not only blood coagulation factors, but also tissue-specific extrahepatic vitamin-K-dependent proteins (VKDP) through post-translational modification of γ-carboxylation, which converts glutamate (Glu) residues to γ-carboxy-glutamate (Gla)[22]. Examples of VKDP include bone proteins (e.g., matrix Gla protein, osteocalcin), cell-signaling proteins (e.g., Gas-6, protein S), and receptor proteins (e.g., conopeptides, PRGP1)[23]. The crucial role of vitamin K as a cofactor is reflected in the way that insufficient γ-carboxylated VKDP are tissue-specific sensitive markers of vitamin K insufficiency[24].

Vitamin K encompasses a group of related, fat-soluble nutrients, all of which share the common 2-methyl-1,4-napthoquinone structure[25]. The two naturally-occurring forms of vitamin K are phylloquinone (vitamin K_1) and menaquinones (MKs; collectively known as vitamin K_2)[26], which differ in length and in the degree of saturation at the aliphatic side chains. Whereas vitamin K_1 contains a side chain of four isoprenoid residues, three of which are saturated, vitamin K_2 has side chains of varying length between four and thirteen isoprene residues, most of which are unsaturated[26]. Vitamin K_2 is generally denoted as MK-*n*, where *n* stands for the number of isoprenyl side chains[25] (**Figure B**).



Beulens et al. British Journal of Nutrition. 2013; 110: 1357-1368

Figure B. Chemical structures of different forms of vitamin K[27]

Other distinctions can be seen in the dietary sources of vitamin K_1 and vitamin K_2. Primary sources of vitamin K_1 in the diet include green leafy vegetables and crucifers, and some vegetable oils[28 29]. Among the menaquinones of vitamin K_2, MK-4, which originates from animal products, is the most abundant form found in the human diet. Vitamin K_2 with longer isoprene units are derived mainly from bacterial fermentation[30, 31], with fermented foods such as cheese and sauerkraut being particular rich sources[32, 33]. The traditional Japanese food, *natto*, (made from fermenting soybeans with *Bacillus subtilis*), is an exceptionally rich source of menaquinones, where a 3.5oz serving may contain up to 1,000µg MK-7 and 84µg MK-8[34]. However, because *natto* is not commonly consumed in the US, the main sources of vitamin K_2 for the majority of the population are cheese, dairy, and meat products[35]. Human gut flora also produces vitamin K_2, but absorption is likely limited [35, 36].

Despite having similar biological functions, different molecular forms of vitamin K exhibit different cofactor activities. They also behave differently in processes such as absorption, transport, cellular uptake, tissue distribution, and turnover[37] (**Table 1).** For instance, only 15% of dietary vitamin K_1 is absorbed due to its tight binding to chloroplast membranes in plant cells, while only a slight increase in absorption is found when the vitamin is paired with vegetable oil[38]. In contrast, vitamin K_2, which is primarily derived from animal-based sources, is consumed in food matrices containing more fat, likely leading to improved absorption and higher bioavailability than vitamin K_1[38].

Following intestinal absorption, vitamin K_1 is primarily transported with the triacylglycerol (TAG)-rich lipoprotein fraction, which is quickly cleared by the liver. This results in the activation of vitamin K-dependent clotting factors within the hepatocytes[39], while only a small amount is released into the circulatory system for use in extrahepatic tissues[40]. Vitamin K_2, on the other hand, due to its more lipophilic character, enables the liver to redistribute it via low density lipoproteins (LDL) and very low density lipoproteins (VLDL), making the vitamin more accessible for extrahepatic tissues[39]. This results in much slower hepatic clearance, longer half-life in the blood, and more stable blood levels during prolonged intake[39, 41].

As with rats, humans also have the ability to convert dietary vitamin K_1 into MK-4 for storage in specific tissues[42]. It has been suggested that the conversion is preceded by the removal of the phytyl tail of vitamin K_1 to produce menadione as an intermediate, which is then condensed with an activated geranylgeranyl moiety to produce vitamin K_2 in the MK-4 form[43]. Despite our body's capability to convert dietary vitamin K_1 to vitamin

K_2, some evidence suggests that humans may benefit from preformed vitamin K_2 in the diet to reach and maintain optimal health[38, 41].

Taken together, the higher absorption, greater bioavailability, and longer half-lives of vitamin K_2 render it more capable of performing functions not related to hemostasis[40], such as the carboxylation of extrahepatic proteins at various target tissues, and the regulation of gene expression[24, 37]. Furthermore, it is unlikely that a metabolic pathway for the conversion of vitamin K_1 to MK-4 would have evolved unless the latter had a unique biological role[44], suggesting that vitamin K_2 has unique implications for health[27, 45-47].

Table 1. Differences in characteristics between molecular forms of vitamin K

Characteristics	Vitamin K_1 (phylloquinone) versus vitamin K_2 (Menaquinones, MK)
Structure	Length and degree of saturation of side chain
Function	MK-4 has functions that may be unrelated to its role as an enzyme cofactor, including the regulation of inflammation, oxidative stress and apoptosis[27].
Dietary intake	Due to the limited availability of food composition tables for vitamin K_2, its contribution to total vitamin K intake is difficult to estimate and likely varies between populations with different dietary patterns[48]. In Germany and the Netherlands, vitamin K_2 accounts for approximately 10-25% of total vitamin K intake[27]. In the average US diet, MK-4 accounts for about a third or more of all vitamin K_2 due to its presence in eggs and meats[32, 49]. The amount of vitamin K_1 contributing to total vitamin K intake is also difficult to estimate due to limited data on the contributions made from dietary vitamin K_2. Nonetheless, it likely varies between countries due to variations in dietary patterns. In Germany and the Netherlands, vitamin K_1 accounts for 75-90% of total vitamin K intake. Although no information is available for the US with regard to vitamin K_1 intake, it has been estimated that 60% of total vitamin K_1 intake comes from green leafy vegetables[48], with the remainder coming from plant oils and margarine in spreads and salad dressings[48 50 51].
Absorption	Absorption of longer chain vitamin K_2 is higher than vitamin K_1 due to differences in modes of absorption: vitamin K_1 is absorbed by an energy-mediated saturable transport system, and is more active in the duodenum than in the ileum,[52] whereas vitamin K_2 is absorbed via a passive non-carrier-mediated diffusion process in both the small and large bowels, with the proximal small bowel having higher rates of absorption than the ileum. Absorption rates of vitamin K_2 also decrease as the number of isoprenoid units in the side-chain increase.
Metabolism	Following intestinal absorption, both vitamin K_1 and K_2 are taken up in the TAG-rich lipoprotein fraction from which they are rapidly cleared by the liver. Nonetheless, longer chain vitamin K_2 also has the potential to be redistributed via LDL and VLDL due to its lipophilic character.
Bioavailability	Some longer-chain vitamin K_2 have longer plasma half-life times: While both vitamin K_1 and MK-4 have a half-life of approximately 8 hours[39], half-lives of MK-7 and MK-9 are 72-96 hours and 60 hours, respectively[39, 41]. The longer half-life times of the latter render them more readily available than vitamin K_1 for uptake by extrahepatic tissues. If expressed as area-under-the-curve over 96 hours (AUC_{96}), the availability of MK-7 is 6-fold better than that of vitamin K_1[39].

B. IMPLICATIONS IN METABOLIC HEALTH

Originally discovered as an anti-hemorrhagic factor, vitamin K is now known to be involved in a variety of physiological processes [35, 53-54], including the regulation of tissue calcium content[21,35], the mediation of inflammation[55] and the non-classical anti-oxidative processes[54]. Additionally, vitamin K can also directly and indirectly regulate the

expression of genes encoding proteins involved in processes such as growth[56] and cancer development[57-58].

While a substantial body of evidence supports the beneficial effects of vitamin K on bone and cardiovascular health[41,59, 60], its association with other diseases is less robust. For example, studies on the effect of dietary and supplemental vitamin K_1 and/or K_2 on different measures of insulin sensitivity and glucose metabolism in humans have yielded inconsistent results[61-63] (**Table 2).** These mixed findings are further undermined by the fact that studies on animals are limited. Nonetheless, the available literature suggests a potential beneficial effect of vitamin K on determinants of glucose homeostasis. For instance, when compared to rats fed a high vitamin K-containing diet, rats fed a diet deficient in vitamin K had higher glucose concentrations and a delayed insulin response to a glucose infusion[64]. In diabetic animal models, vitamin K treatment has also prevented pancreatic cell death, increased islet area, and enhanced insulin secretion, resulting in normoglycemia and lower-glycosylated hemoglobin[65]

Table 2. Studies on the association between dietary or supplemental vitamin K intake and glucose metabolism in humans

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Sakamoto[66]	2000	Japan	Analysis of efficacy of intervention with MK-4 (90mg/d) for 1 week on glucose metabolism-related parameters among same study subjects	Healthy male college students categorized into tertiles by serum level of DCP (n=12), mean age 21.4 yr	FPG, IRI, HbA1c, fructosamine, OGTT	FPG unchanged in all groups. IRI response to 75g OGTT improved in high baseline DCP group after MK-4 treatment.
Yoshida[62]	2008	USA	Cross sectional assessment of 12-month vitamin K_1 intake using FFQ	Framingham Offspring Cohort: Men (n=1247) and women (n=1472), mean age 54.0 yr	Insulin sensitivity (FPI, 2-hr post OGTT insulin, HOMA-IR, SI), and glycemic status (fasting glucose, 2-hr post-OGTT glucose, HbA1c)	Higher vitamin K_1 intake was associated with greater insulin sensitivity (as measured by 2-hr post OGTT insulin and SI) and improved glycemic status (as measured by 2-hr post OGTT glucose concentrations). Lower vitamin K_1 intake was associated with lower insulin and higher glucose concentrations 30 min after oral glucose loading in men, but no association was observed between vitamin K_1 intake and fasting glucose or insulin concentrations
Yoshida[67]	2008	USA	RCT with vitamin K_1 (500µg/d) vs. placebo for 36 months	Healthy non-DM men and women (n=355), mean age 68.4 yr	HOMA-IR (FPG, FPI)	HOMA-IR and plasma insulin levels reduced in men but not in women.
Pan[68]	2009	USA	Cross-sectional assessment of dietary vitamin K_1 intake based on a single 24-hr recall categorized into quartiles	NHANES 1999-2004: Men (n=2,867) and women (n=2,933), mean age 33.0 yr	FPG, FPI, CRP	Lower dietary vitamin K_1 intake was associated with hyperglycemia and higher CRP.

Table 2 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Kumar[63]	2010	USA	RCT with vitamin K_1 (1mg/d) vs. placebo for 12 months	Healthy, community-dwelling, postmenopausal women (n=42), mean age 62 yr	HOMA-IR (FPG, FPI)	Daily supplementation with 1mg of vitamin K_1 for 12 months decreased serum ucOC concentrations, but did not alter serum total OC, FPG, FPI and HOMA-IR.
Beulens[69]	2010	The Netherlands	Cross-sectional assessment of dietary vitamin K_1 and K_2 intakes based on FFQ, with 10.3 years of follow-up	Dutch EPIC cohort 1993-1997: Men (n=9,740) and women (n=28,354), mean age 49.1 yr	hs-CRP, HbA1c, incident DM at follow-up (detected via urinary glucose strip tests and verified via medical records)	Both vitamin K_1 and vitamin K_2 intake were associated with reduced T2DM risk; for vitamin K_1 intake, risk reductions occurred at higher levels of intake, whereas vitamin K_2 intake was inversely associated with risk of T2DM, with HR of 0.93 (95%CI: 0.87-1.00) for each 10ug increase of vitamin K_2 intake. Higher dietary vitamin K_2 intake was associated with lower CRP concentrations.
Knapen[70]	2011	The Netherlands	Three studies: 1. Cross-sectional measures of serum ucOC and cOC concentrations 2. Dose-response intervention with MK-7 (0, 10, 20, 45, 90, 180 or 360µg/d) for 12 weeks 3. RCT with MK-4 (45mg/d) vs. placebo for 3 years	Three studies: 1. Healthy postmenopausal women (n=244), mean age 60 yr 2. Healthy men (n=20) and women (n=22), mean age 27.5 yr 3. Healthy postmenopausal women (placebo n=75; intervention n=89), mean age 65.5 yr	Adiponectin	MK-4 and MK-7 supplementation (at doses >90ug/d) significantly decreased serum ucOC and increased cOC, but did not affect circulating adiponectin concentrations.
Choi[71]	2011	South Korea	RCT with MK-4 (30mg/d) vs. placebo for 4 weeks	Healthy young men: MK-4 (n=18) and placebo (n=15), mean age 29 yr	FS-OGTT, IVGTT (SI_{index}, AIRg, DI), adiponectin, IL-6, CRP	Vitamin K_2 supplementation was associated with increased cOC, better glucose tolerance, SI_{index} and DI but did not affect AIRg, FPG, IL-6, CRP, or adiponectin.

Table 2 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Ibarrola-Jurado [72]	2012	Spain	Cross-sectional assessment of dietary vitamin K_1 intake based on FFQ, with 5.5 years of follow-up	PREDIMED cohort: Community-dwelling men and women with (n=855) and without (n=1068) T2DM, mean age 67 yr	Plasma glucose, incident T2DM (assessed by FPG and 2hr OGTT)	Dietary vitamin K_1 intake was associated with reduced T2DM risk: Baseline dietary vitamin K_1 intake was associated with a 17% reduced risk of incident T2DM with HR=0.83 (95%CI: 0.71, 0.96, p=0.017) for each additional 100µg vitamin K_1/d. 51% lower risk of incident T2DM in subjects who increased their dietary intake of vitamin K_1 during 5.5 years of follow-up compared to those who decreased or did not change their intake.
Rasekhi[73]	2015	Iran	RCT with vitamin K_1 (1000µg/d) vs. placebo for 4 weeks	Premenopausal and pre-diabetic women: vitamin K_1 (n=39) and placebo (n=43), mean age 40.2 yr	OGTT (FPG, FPI), HOMA-IR, HOMA- ß, SI_{index}	Vitamin K_1 supplementation decreased %ucOC, decreased 2hr-post OGTT glucose and insulin, increased SI_{index}, but had no effects on serum total OC, FBG, FBI, HOMA-IR, HOMA- ß.
Dam[74]	2015	The Netherlands	Cross-sectional and longitudinal analysis of plasma dp-ucMGP, dietary vitamin K_1, vitamin K_2 and total vitamin K intake based on FFQ categorized into tertiles	Men from the PROFIEL cohort (n=400), and postmenopausal women from the EPIC cohort (n=402), mean age 63.3 yr	FPG, self-reported T2DM	Higher intakes of vitamin K_2 and vitamin K status (dp-ucMGP) were associated with lower FPG and reduced occurrence of MetS in both cross-sectional and longitudinal analyses. However, vitamin K_1 was not associated with MetS prevalence.

Abbreviations: AIRg: acute insulin response to glucose; cOC: carboxylated osteocalcin; CRP: C-reactive protein; DCP: decarboxy prothrombin (higher levels indicate subclinical vitamin K deficiency), DI: disposition index, DM: diabetes mellitus, Dp-ucMGP: desphospho-uncarboxylated matrix Gla protein, EPIC: European Prospective Investigation into Cancer and Nutrition; FBG: fasting blood glucose, FFQ: food frequency questionnaire; FPG: fasting plasma glucose; FPI: fasting plasma insulin; FS-OGTT: frequently sampled oral glucose tolerance test; HbA1C: glycated hemoglobin, HOMA- ß: homeostatic Model Assessment of β-cell function; HOMA-IR: homeostatic Model Assessment of insulin resistance; hs-CRP: high sensitivity C-reactive protein; IFG: impaired fasting glucose; IL-6: interleukin 6; IRI: immune-reactive insulin; IVGTT: intravenous glucose tolerance test; MetS: metabolic syndrome; MK: menaquinones; Non-DM: non-diabetic; OC: osteocalcin; OGTT: oral glucose tolerance test; PG: plasma glucose; PREDIMED: Primary Prevention of Cardiovascular Disease with a Mediterranean Diet; PROFIEL: Preservation of Function in Elderly; PROSPECT: RCT: randomized controlled trial; SI_{index}: insulin sensitivity index; T2DM: type 2 diabetes mellitus; yr: years

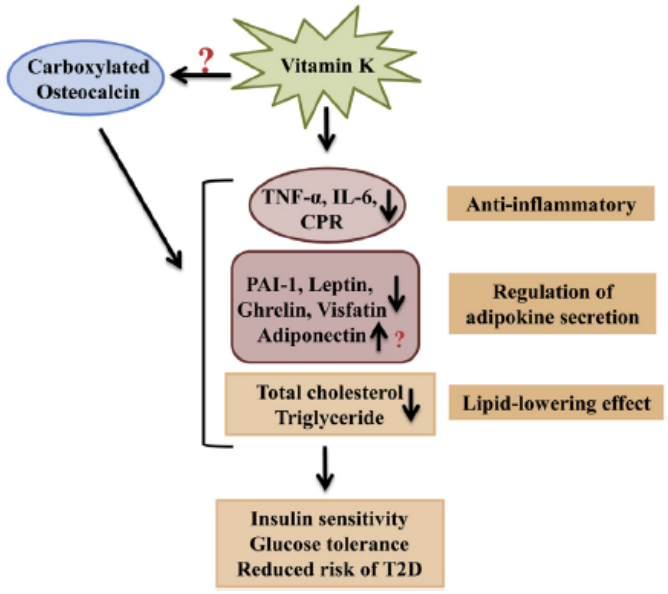
The relation between vitamin K_1 intake and glucose homeostasis was evident in an observational study involving 2,719 postmenopausal women, where an inverse association was observed between vitamin K_1 intake with 2-hour glucose concentrations and insulin sensitivity[62]. A subsequent trial among non-diabetic men and women showed that for men only, a reduced progression of IR was found after 3 years of supplementing their diets with 500µg vitamin K_1[67]. A large prospective cohort study involving 38,094 Dutch men and women showed that both dietary vitamin K_1 and vitamin K_2 intake were associated with a reduced risk of T2DM[69]. Interestingly, this association had a linear, inverse relationship for vitamin K_2, where a significant risk reduction for vitamin K_1 was only observed at higher levels of intake. In this study, high dietary vitamin K_2 intake was also associated with lower concentrations of C-reactive protein (CRP), an indicator of general levels of inflammation in the body, and an improved blood lipid profile. Together, this suggests that relative to vitamin K_1, vitamin K_2 may have greater potential to affect metabolic health. Unfortunately, due to the limited availability of food composition data for vitamin K_2, few studies have looked into the effects of dietary vitamin K_2 on metabolic health.

To our knowledge, only one study has investigated the effect of supplemental vitamin K_2 on glycemic status. In this study, investigators found that supplementing diets with 30mg/d of vitamin K_2 for four weeks improved glucose tolerance and increased insulin sensitivity in healthy young men[71]. Although this finding was promising, further

studies are warranted to confirm the beneficial effects of vitamin K_2 on glucose homeostasis.

C. MODULATION OF GLUCOSE HOMEOSTASIS & PROPOSED MECHANISMS

Several mechanisms have been proposed to explain the effects of vitamin K on insulin sensitivity and glucose metabolism. These include the role of vitamin K as a cofactor for vitamin K-dependent proteins (VKDP) such as osteocalcin (OC) and growth arrest-specific 6 protein (Gas6), its potential in modulating cytokines that influence inflammation[35], its efficacy in regulating lipid metabolism, and its anti-oxidative properties (**Figure C**).



Manna P & Kalita J. Nutrition. 2016; 32(7); 732-739

Figure C. Schematic diagram of proposed mechanism for vitamin K effects on insulin sensitivity, glucose homeostasis, and the reduced risk of T2DM[75]

i. Vitamin K-dependent protein – osteocalcin

Osteocalcin (OC) is the most abundant non-collagen VKD bone matrix protein that regulates the size and shape of hydroxyapatite via its γ-carboxylated (activated) form[76]. Its major feature is the presence of three Gla residues within its helical structure, which are the sites of carboxylation[77]. The extent to which Gla residues are carboxylated depends on the availability of vitamin K. In circulation, OC is detectable in both the carboxylated and undercarboxylated forms. The proportion of carboxylated (cOC) to undercarboxylated OC (ucOC) is used as a functional indicator of vitamin K status[76], as it is responsive to dietary vitamin K depletion, repletion, and supplementation[78-80]. Although both vitamin K_1 and vitamin K_2 have equal capacity of carboxylating OC[27], the longer residency time of long-chain vitamin K_2 in the circulation renders it more effective than vitamin K_1 at carboxylation[41,120]. A study conducted in the Netherlands showed that supplemental vitamin K_2 was three-times more effective than vitamin K_1 in carboxylating OC over a 40-day period[41]. Available studies that investigated the association between dietary and/or supplemental vitamin K and OC carboxylation status in humans are summarized in **Table 3.**

Although positive associations were observed between OC concentrations and determinants of glucose tolerance in both human and animal models, most studies do not distinguish between cOC and ucOC, likely as a result of the current limitations in standardizing techniques and measuring circulating ucOC[81]. Nonetheless, there appear to be close associations between total OC and glucose metabolism. For instance, OC knockout mice (OC -/-) accumulated body fat and exhibited dramatic impairments in

glucose metabolism[82]. They had elevated glucose and lipid concentrations, reduced insulin levels and reduced β-cell numbers, and became glucose intolerant and insulin resistance[82]. Conversely, a subcutaneous infusion of recombinant OC into wild-type mice enhanced glucose tolerance, increased insulin expression and secretion, and improved β-cell proliferation[82, 83]. Cross-sectional and longitudinal studies of metabolically healthy people and people with T2DM also indicate inverse associations between OC concentrations and fasting plasma glucose[84, 85]. Furthermore, interventions that improve glycemic control concomitantly increase serum total OC concentrations in diabetic subjects[86, 87 88]. Human studies investigating the association between OC and glucose homeostasis related parameters are summarized in **Table 4.**

While the precise mechanism by which OC affects glucose and insulin metabolism remains to be elucidated, several postulations have been made. First, OC may act through an effect on adiponectin[83], an adipocyte-derived hormone known to serve as an important regulator of insulin sensitivity. In animal models, OC significantly stimulates the expression of cyclin D1 and insulin in islets and adiponectin in adipocytes[89]. Among the few cross-sectional studies that have measured adiponectin and total OC serum concentrations, positive associations were found between the two[84, 90-95], although this relationship has not always been consistent[70, 96]. Second, OC has the potential to stimulate the release of glucagon-like peptide-1 (GLP-1), an incretin released by intestinal endocrine cells that simulates insulin secretion[97, 98]. GLP-1 achieves its insulinotropic effect by binding to its specific receptor thereby increasing cytosolic concentrations of cAMP and Ca^{2+} in β-cells[99]. GLP-1 also stimulates β-cell proliferation and protects the

cells from apoptosis[100]. Third, OC may reverse autophagic dysfunction and endoplasmic reticulum stress that results from diet-induced obesity, as intermittent administration of OC in mouse models of IR restored impaired insulin sensitivity[101, 102].

If the effect of vitamin K on glucose homeostasis were mediated through OC, one would only expect effects in the "active", carboxylated (cOC) form. However, inconsistencies exist between human data and experimental animal models regarding the nature of OC involvement in glucose metabolism. Whereas improvement in glucose tolerance was associated with only undercarboxylated osteocalcin (ucOC) in animal models[82, 83], clinical studies with humans reported that serum total OC concentrations, rather than ucOC, were inversely associated with measures of glucose metabolism[91, 103,104]. Additionally, higher vitamin K intake, which causes a decrease in the relative proportion of ucOC vs total OC, is reported to reduce IR among individuals at high risk for T2DM[62 73]. Among the few studies that measured serum ucOC, inverse associations were observed with β-cell function, while positive associations were seen with IR and T2DM risk[105, 106].

In contrast to the role of OC in bone, in which its carboxylated form is thought to confer functionality to the protein[35], the hormonal function of OC in regulating glucose homeostasis and energy metabolism is only thought to involve ucOC. If this hypothesis were true, it would imply that high intake of vitamin K would be detrimental to glucose homeostasis, which is the opposite of what has been observed in human studies. Species-specific differences in OC are thought to be the underlying reason for discrepancies between the forms of OC (carboxylated vs. undercarboxylated) that are possibly involved in the regulation of glucose metabolism. Given these uncertainties, possible connections

between OC and glucose metabolism are still an area of research that requires further investigation.

ii. Vitamin K-dependent protein – Gas6

Growth arrest-specific 6 (Gas6) is a ubiquitously expressed growth factor-like molecule that interacts with receptor tyrosine kinases of the TAM family (tyrosine kinase receptors including TYRO3, AXL, and MERTK) to regulate functions such as cell proliferation and survival[22]. Vitamin K activates Gas6, which in turn inhibits Toll-like receptor driven cytokine production that plays a role in inflammation[131], homeostasis of cultured cells and adiposity[22, 132]. However, the direct relationship between dietary vitamin K status and the function of Gas6 is difficult to study because serum free ucGas6 readily binds to proteoglycans on the surface of stromal cells, membrane receptors on mesenchymal cells, or remains anchored to the membrane-bound carboxylase within the endoplasmic reticulum[133].

iii. Anti- inflammatory effects of vitamin K

Vitamin K may influence glucose homeostasis through the modulation of pro-inflammatory cytokines. *In vitro*, vitamin K reduces lipopolysaccharide-challenged fibroblasts' secretion of pro-inflammatory cytokines[134-136]. In mice and human studies, respectively, vitamin K suppresses the expression of genes involved in an acute inflammatory response (e.g. NFKB) and lessens inflammatory responses in certain disease states, as evidenced by significantly lower levels of a number of systemic pro-

inflammatory biomarkers (e.g. leptin, TNG, IL-6, visfatin) in subjects with higher vitamin K plasma concentration or vitamin K intake[40,137].

iv. Lipid- modulating effects of vitamin K

Elevated total cholesterol and low-density lipoprotein cholesterol (LDL), and reduced high density lipoprotein cholesterol (HDL) are associated with insulin resistance and impaired glucose metabolism[138]. Vitamin K has a beneficial role on dyslipidemia, where its supplementation reduced the plasma levels of total cholesterol in hypercholesterolemic animal models[139]. When compared to control diet-fed rats, rats fed a vitamin K-rich diet had significantly decreased total fat accumulation and lower serum triacylglycerol (TAG) levels[140]. However, current literature on the effects of vitamin K on plasma lipid profile and measures of glucose homeostasis is scarce. Thus, the function of vitamin K in modulating glucose metabolism via alteration in lipid profile deserves further investigation.

v. Anti-oxidative properties of vitamin K

The reduced form of vitamin K, hydroquinone, has potential anti-lipid peroxidation activities, where it may block free radical accumulation and cell death by a mechanism independent of γ-carboxylation[141,142]. As IR is associated with oxidative stress, vitamin K may modulate insulin metabolism through its antioxidative properties[143].

Taken together, the role of vitamin K as an essential micronutrient is underscored from a large body of evidence linking it to various metabolic processes that could affect glucose metabolism.

Table 3. Association between dietary or supplemental vitamin K intake and osteocalcin carboxylation in humans

Reference	Year	Country	Study design	Study population	Results
Jie[107]	1995	The Netherlands	Cross-sectional assessment of daily dietary vitamin K intake based on FFQ [% contributed from vitamin K_1 or K_2 was not distinguished]	Population-based study of women (n=113), mean age 66.6yr	Lower vitamin K intake was associated with higher levels of ucOC and lower levels of cOC.
Booth[108]	2003	USA	Cross-sectional assessment of plasma phylloquinone, serum levels of total and ucOC	Framingham Offspring cohort: Men (n=741) and women (n=863), mean age 59 yr	Higher plasma vitamin K_1 was associated with lower %ucOC in postmenopausal women regardless of estrogen use, but such association was not observed in men or premenopausal women.
Braam[109]	2003	The Netherlands	36-month RCT with 3 intervention arms: 1) Placebo 2) Mineral + Vitamin D (8µg/day) 3) Mineral + Vitamin D (8µg/day) + K_1 (1mg/day)	Healthy post-menopausal women, mean age 55.2 yr: • Placebo (n=60) • Mineral + Vitamin D (n=58) • Mineral + Vitamin D + vitamin K (n=63)	In the vitamin K supplemented group, serum levels of ucOC were significantly decreased at 12 and 36 months, and serum levels of cOC were significantly increased at 3, 12 and 36 months. Such association was not observed in the other intervention arms.
Conway[110]	2005	UK	Cross-sectional assessment of serum vitamin K_1, PIVKA-II total OC, %ucOC	Boys (n=52) and girls (n=54) with cystic fibrosis, mean age 11.0yr	Higher serum vitamin K_1 levels were associated with lower levels of ucOC and %ucOC.
Schurgers[41]	2007	The Netherlands	Longitudinal analysis of OC carboxylation status during 6 weeks of supplemental vitamin K_1 or MK-7 intake (both at 0.22µmol) in a crossover design with a 12-week washout period.	Healthy men and women volunteers (n=18), mean age 34.2 yr	While both vitamin K_1 and MK-7 increase cOC at day 3, the effect of MK-7 on cOC inclined during the entire 6 weeks of the treatment whereas the effect of vitamin K_1 remained constant after day 3. Change of cOC/ucOC ratio was 3 times higher for MK-7 than for vitamin K_1.

Table 3 (cont.)

Reference	Year	Country	Study design	Study population	Results
Bolton-smith[111]	2007	UK	24-month RCT with 4 intervention arms 1) Placebo 2) Vitamin K_1 (200 µg/d) 3) Calcium (1000 mg/d) + Vitamin D_3 (10 µg/day) 4) Vitamin K_1 and Vitamin D_3 plus Calcium	Healthy non-osteoporotic women, mean age 68.2: • Placebo (n=61) • Vitamin K_1 (n=60) • Vitamin D_3 + Calcium (n=62) • Vitamin K_1 + vitamin D_3 + Calcium (n=61)	Vitamin K_1 supplementation increased serum concentrations of vitamin K_1, which corresponded with a significant decrease in absolute concentration as well as % of ucOC at 12 and 24 months. Vitamin K_1 + D_3 supplemented group had a greater decrease in %ucOC compared to vitamin K_1-only supplemented group.
Cheung[112]	2008	Canada	24-month RCT (extended to 48 months) with 2 intervention arms: 1) Placebo 2) Vitamin K_1 (5mg/d)	Postmenopausal women with osteopenia, mean age 59.1yr: • Placebo (n=223) • Vitamin K_1 (n=217)	Daily vitamin K_1 supplementation increased serum vitamin K_1 levels by 10-fold but did not increased circulating vitamin K_2 (MK-4 and MK-7). Vitamin K_1 supplementation decreased ucOC and %ucOC at 2 years.
Tsugawa[113]	2008	Japan	Cross-sectional assessment of plasma vitamin K (K_1, MK-4 and MK-7)	Japanese women (n=379), mean age 63.0 yr	Circulating K_1 and MK-7 concentrations were negatively correlated with serum ucOC concentrations.
Booth[114]	2008	USA	36-month RCT with 2 intervention arms: 1) Placebo 2) Vitamin K_1 (500 µg/day)	Healthy men and postmenopausal women, mean age 68.5yr: • Placebo (n=223) • Vitamin K_1 (n=229)	Daily vitamin K_1 supplementation for 36 months significantly increased plasma vitamin K_1 concentration and decreased %ucOC.
Binkley[115]	2009	USA	12-month RCT with 3 intervention arms: 1) Placebo 2) Vitamin K_1 (1mg/day) 3) MK-4 (15mg/day)	Community-dwelling postmenopausal women, mean age 62 yr • Placebo (n=129) • Vitamin K_1 (n=126) • MK-4 (n=126)	Daily supplementation with vitamin K_1 and MK-4 for 12 months resulted in significant reductions in %ucOC.

Abbreviations: cOC: carboxylated osteocalcin; MK: menaquinones; OC: osteocalcin; PIVKA-II: proteins induced by vitamin K absence (indicator of subclinical vitamin K deficiency); RCT: randomized controlled trial; ucOC: undercarboxylated osteocalcin; yr: year

Table 4. Association between carboxylated and/or undercarboxylated osteocalcin and glucose and fat metabolism in humans

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Im[116]	2008	South Korea	Cross sectional analysis of serum OC in quartiles vs. glucose metabolism-related measures	Postmenopausal women divided into NG (n=259), IFG (n=49), and T2DM (n=31), mean age 57.2 yr	HOMA-IR (FPG, FPI), HbA1c	Serum total OC level was negatively correlated with FPG, FPI, HbA1C and HOMA-IR and was significantly lower in T2DM than NG. No associations were observed between OC level and lipid profile, but OC level was negatively correlated with BMI.
Kindblom[117]	2009	Sweden	Cross-sectional analysis of OC and parameters of glucose homeostasis	T2DM (n=857) and non-DM (n=153) elderly men from the MrOS Swedish cohort, mean age 75.3 yr	HOMA-IR (FPG, FPI); leptin, TG, TC, LDL, HDL	T2DM subjects had lower plasma OC than non-DM subjects. In both groups, total OC was associated with BMI, fat mass, serum leptin, FPG but not FPI.
Shea[96]	2009	USA	Cross-sectional and longitudinal (3 yr follow up) analyses of serum OC (total, cOC, ycOC) vs. HOMA-IR	**Cross-sectional:** Non-DM men (n=142) and non-DM postmenopausal women (n=206) from a vitamin K supplementation study, mean age 69 yr **Longitudinal:** Participants randomly assigned to the "control group" and completed the 3-year intervention	HOMA-IR (FPG, FPI), adiponectin; % body fat	**Cross-sectional:** HOMA-IR and FPG were lower and adiponectin was higher across higher tertiles of total OC and cOC, but no difference in IR measures was observed across tertiles of ucOC - neither ucOC concentration nor % ucOC was associated with FPG, FPI or adiponectin. **Longitudinal**: No association was observed between baseline ucOC concentration and 3-yr change in HOMA-IR, but lower concentration of ucOC at baseline was associated with a greater increase in HOMA-IR at follow-up.

Table 4 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Hwang[105]	2009	South Korea	Cross-sectional analysis of serum OC and ucOC (in tertiles) vs. β-cell function and insulin sensitivity	South Korean men, mean age 47 yr (n=199)	OGTT, 2hPG, HOMA-IR and HOMA-ß (FPG, FPI), SI_{index}, fasting plasma C-peptide; TC, HDL, LDL	Both ucOC and cOC improved glucose tolerance. Higher ucOC levels were associated with higher HOMA-β levels (improved β-cell function). Higher cOC levels were associated with lower HOMA-IR (improved insulin sensitivity). FPI and C-peptide levels were not associated with either ucOC or cOC levels.
Pittas[118]	2009	USA	Cross-sectional analysis of serum total OC vs. parameters of glucose homeostasis, with a prospective analysis of association between serum OC vs. FPG at 3 yr of follow-up	**Cross-sectional:** Men (n=199) and women (n=246) involved in a RCT trial on vitamin D and calcium supplementation, mean age 71yr **Longitudinal:** Participants randomly assigned to the "placebo arm" of the trial	HOMA-IR (FPG, FPI); hs-CRP, IL-6, fat mass	**Cross-sectional:** Serum total OC was inversely associated with FPG, FPI, HOMA-IR, hs-CRP, IL-6, BMI and body fat. **Longitudinal**: Higher serum total OC was associated with lower increase in FPG at 3 yr follow-up.
Kanazawa[84]	2009	Japan	Cross-sectional analysis of serum total OC vs. parameters of glucose homeostasis	T2DM men (n=179) and postmenopausal women (n=149), mean age 65.8 yr	HbA1c, c-peptide, adiponectin, FPG; fat mass, HDL, LDL, TC	Serum total OC was negatively associated with FPG and HbA1c in T2DM subjects. No association was found between serum total OC and fasting C-peptide, a surrogate marker for endogenous insulin secretion. Serum total OC was negatively associated with % fat in T2DM men and positively associated with serum total adiponectin level in T2DM postmenopausal women.

Table 4 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Kanazawa[88]	2009	Japan	Cross-sectional analysis of effectiveness of dietary intervention on glycemic control	Poorly controlled T2DM, men (n=31) and women (n=19), mean age 63.6 yr	HbA1c, FPG, c-peptide, adiponectin	Improved glycemic status (FPG) in T2DM was associated with an increase in total OC and a decrease in %ucOC. This corresponded with an increase in serum adiponectin levels and a decrease in HbA1c.
Fernandez-Real[95]	2009	Spain	**Cross-sectional** analysis of serum total OC vs. insulin sensitivity and secretion. **Longitudinal studies:** **A:** Effects of 16-week intervention study (diet and/or exercise) on the same exposure and outcome measures **B:** Effects of 16-week dietary intervention (energy deficit diet of 500-1000kcal/d) on the same exposure and outcome measures	**Cross sectional study**: Men (n=149), mean age 50.2yr **Intervention study A:** Sedentary obese women, mean age 50.2yr •Control (n=7) •Weight-loss diet (n=8), •Diet & exercise (n=11) **Intervention study B:** Caucasian obese volunteers: Men (n=9) and women (n=11), mean age 43.3 yr	Adiponectin, HOMA-IR (FPG & FPI), IVGTT (SI_{index}, DI)	**Cross-sectional study**: Serum total OC was positively correlated with insulin sensitivity, with strong associations observed in lean subjects (BMI <25kg/m^2). Among lean subjects, serum total OC was also positively associated with insulin secretion and DI. Serum total OC was positively associated with adiponectin concentrations. **Intervention study A:** Diet & exercise group had an increase in total OC, with improvements in fat metabolism. This association was not observed in the control or diet alone group. In diet and diet + exercise group, total serum OC were associated with IR and fasting TG. **Intervention study B:** Baseline total OC was not significantly associated with insulin sensitivity but is negatively associated with total fat mass. Mean OC level increased after weight loss, but there was no change in IR, circulating adiponectin or TG.

Table 4 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Zhou[119]	2009	China	Cross-sectional analysis of serum total OC vs. glucose tolerance, lipid profile, insulin secretion and insulin sensitivity	Men (n=254) and women (n=246) with and without T2DM, mean age 53.0 yr	OGTT, 2hPG, HOMA-IR and HOMA-ß (FPG, FPI), HbA1c; TG, TC, HDL, LDL	Serum total OC was significantly lower in T2DM. Serum total OC was inversely correlated with FPG, 2hPG, HbA1C, and positively correlated with TG and HOMA-ß. No association was found between serum total OC and HOMA-IR
Saleem[91]	2010	USA	Cross-sectional analysis of serum total OC vs. IR, adiponectin levels and presence of MetS	Participants from the Genetic Epidemiology Network of Arteriopathy Study: Blacks (n=1284) and non-Hispanic whites (n=1209)	Adiponectin, HOMA-IR (FPG, FPI), T2DM, leptin	Serum total OC levels were inversely correlated with BMI, FPG, FPI, HOMA-IR, T2DM, serum TG, plasma leptin, and positively correlated with adiponectin levels.
Prats-Puig[92]	2010	Spain	Cross-sectional analysis of serum ucOC and cOC vs. adiponectin and insulin secretion (HOMA-β)	Population-based healthy pre-pubertal children (n=103)	Adiponectin, HOMA-IR and HOMA-ß (FPG, FPI), IRI	Higher ucOC was associated with lower adiponectin levels. Higher ucOC-to-cOC ratio was associated with higher HOMA-β in leaner children, and associated with higher adiponectin levels.
Winhofer[121]	2010	Austria	Case-control study investigating associations between OC and glucose metabolisms in GDM during pregnancy and after delivery	NGT (n=52) and GDM (n=26) pregnant women matched for age and BMI and postpartum women (n=34)	Fs-OGTT (FPG, FPI), C-peptide, HbA1c, hs-CRP, SI_{index}, DI, glucose AUC; TC, LDL, HDL	Serum total OC was higher in GDM vs. NGT pregnant women but no difference was observed between the two groups at 12 weeks postpartum, where OC levels were increased in all women. OC was significantly associated with basal and total insulin secretion, but not correlated with HbA1c.
Yeap[122]	2010	Australia	Cross-sectional analysis of serum total OC vs. MetS	Men from the Health in Men Study, HIMS (n=2765), mean age 76.4 yr	HOMA-IR (FPG, FPI); TC, TG, HDL, LDL	Serum total OC was inversely associated with glucose, TG levels and HOMA-IR and was lower in men with MetS.

Table 4 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Kanazawa[123]	2011	Japan	Longitudinal analysis of serum OC and atherosclerosis-related risk factors during a 6-month treatment for hyperglycemia	T2DM men (n=28) and women (n=22), mean age 64.5 yr	HbA1c; TG, LDL, HDL	Serum total OC was associated with serum levels of HbA1c, TG and HDL.
Iglesias[124]	2011	Spain	Case-control study that assessed serum levels of OC vs. varying degrees of glucose tolerance and associated metabolic markers	Obese men and women: NGT (n=20), pre-diabetes (n=20), T2DM (n=24), mean age 58.0 yr	OGTT, 2hPG, HOMA-IR (FPI, FPG); TC, LDL, HDL, TG	Serum total OC decreases with worsening glucose tolerance. Mean serum OC levels were higher in NGT group vs. pre-DM and T2DM. Serum total OC levels were negatively associated with parameters of glucose tolerance (FPG, 2hPG, glucose AUC after OGTT). No association was found between OC and insulin, HOMA-IR, TG, HDL
Tan[125]	2011	China	Cross-sectional analysis of serum total OC and risk factors for MetS	Fangchenggang Area Male Health and Examination Survey Cohort: Men with (n=297) and without MetS (n=2047), mean age 39.6 yr	FPG; TG, HDL	Higher serum total OC was associated with hyperglycemia and other components of MetS.
Kanazawa[93]	2011	Japan	Cross-sectional analysis of bone turnover markers (OC, ucOC), and diabetes-related parameters	T2DM men (n=180) and postmenopausal women (n=109), mean age 59.1 yr and 65.2 yr respectively	FPG, HbA1C, fasting C-peptide, serum adiponectin	Serum ucOC concentration was negatively correlated with FPG and HbA1c in men, and negatively correlated with HbA1c in postmenopausal women. ucOC/OC ratio was negatively correlated with HbA1c in T2DM men and positively correlated with serum adiponectin.

Table 4 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Kanazawa[90]	2011	Japan	Cross-sectional evaluation of serum total OC concentrations (classified in tertiles) and parameters of IR and insulin secretion	T2DM men (n=152) and postmenopausal women (n=101), mean age 55.6 yr and 62.4 yr respectively	HbA1c, HOMA-IR and HOMA-ß (FPG, FPI), OGTT, fasting C-peptide, adiponectin	Serum total OC was negatively associated with FPG, HOMA-IR, and positively associated with insulin sensitivity (HOMA-B) in both men and postmenopausal women. Serum total OC was positively associated with serum adiponectin in postmenopausal women. Insulin resistance based on OGTT was associated with lowest serum OC tertile group: postmenopausal women in lowest tertile of serum OC showed hyperglycemia and hyperinsulinemia vs. the highest tertile after oral glucose loading; and men in lowest tertile indicated hyperinsulinemia. No association was found between serum OC and IGI in OGTT examinations.
Shafer[94]	2011	USA	12-month RCT using alendronate (10mg/d) or PTH (100µg/d)	Participants from the PaTH study: Postmenopausal women on PTH (n=64) or Alendronate (n=33), mean age 70.1 yr	Adiponectin, leptin, (non- fasting) insulin, glucose, insulin: glucose	Increase in ucOC was associated with increased adiponectin, decreased body weight & fat mass, but not with changes in (non-fasting) insulin, glucose or insulin: glucose concentrations.
Hwang[126]	2012	South Korea	Nested case-control study (with 8.4 years of follow-up) investigating serum OC vs. incidental T2DM	Men with NGT (n=622) or IFG (n=607), mean age 47.4yr	HbA1c, HOMA-IR (FPI, FPG), HOMA-β, C-peptides, T2DM; TC, TG, HDL, LDL, Lp(a)	Serum total OC was not associated with development of T2DM in middle-aged men: although levels of HOMA-IR decreased from lower to upper OC tertiles, no differences were observed in FPG and HbA1c levels across OC tertiles. Serum total OC was negatively correlated with FPG and HbA1c in T2DM individuals only.

Table 4 (cont.)

Reference	Year	Country	Study design	Study population	Outcome measures	Results
Hwang[106]	2012	South Korea	Cross-sectional analysis of OC vs. glucose tolerance, insulin secretion and sensitivity	Men and women with NGT (n = 23); pre-diabetes (n = 150), and T2DM (n = 252), mean age 53 yr	HOMA-IR and HOMA-β (FPG and FPI), OGTT (IGI, AUC insulin & glucose, DI)	Total OC was associated with improvements in a) Glucose tolerance: glucose and HbA1c levels were inversely associated with serum OC tertiles. b) Insulin sensitivity and β-cell function: AUC insulin/glucose, SI_{index}, HOMA-β, IGI and DI were positively correlated with OC tertiles, *independent of adiponectin.*
Iki[127]	2012	Japan	Cross-sectional analysis of ucOC vs. parameters of glucose metabolism	Community-dwelling elderly men from the FORMEN cohort: men (n=2174), mean age 73yr	HOMA-IR (FPG, FPI), HbA1c	Serum ucOC but not total OC, was inversely associated with glucose metabolism indices including FPG, FPI, HOMA-IR and HbA1c.
Bullo[128]	2012	Spain	Cross-sectional analysis of total OC and ucOC vs. measures of IR, with 2 years of follow-up	Community-dwelling men from the PREDIMED cohort: men with (n=38) and without (n=41) T2DM, mean age 68.5 yr	FPG, FPI, HOMA-IR, HOMA-β; HDL, TC, TG, LDL	Increase in serum total OC was associated with increased β-cell function as determined by HOMA- ß. Increase in serum ucOC was associated with improvements in insulin action as determined by a decline in HOMA-IR.
Paldánius[129]	2013	Finland	Cross-sectional analysis of OC and cOC vs. markers of glucose and insulin metabolism in OGTT	Non-DM adults born preterm with VLBW (n=163) or at term (N=169), mean age 22.5 yr	OGTT, HOMA-IR (FPG, FPI)	Total OC and cOC were negatively associated with FPI and HOMA-IR, and (weakly) negatively associated with FPG.
Hu[130]	2014	China	Cross-sectional analysis of OC and markers of glucose and lipid metabolism	Healthy postmenopausal women (n=636), mean age 62.8 yr	HOMA-IR (FPG, FPI); TG, TC	Serum total OC concentration was inversely associated with FPG.

Abbreviations: 2hPG: 2-hour postprandial glucose; BMI: body mass index; cOC: carboxylated osteocalcin; DI: disposition index; FORMEN: Fujiwara-kyo Osteoporosis Risk in Men; FPG: fasting plasma glucose; FPI: fasting plasma insulin; fs-OGTT: frequently sampled oral glucose tolerance test; GDM: gestational diabetes mellitus; Glucose AUC: glucose area-

under-the-curve; HbA1c: glycated hemoglobin; HDL: high-density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment of insulin resistance; HOMA-β: homeostatic model assessment of β-cell function; hs-CRP: high sensitivity C-Reactive Protein; IFG: impaired fasting glucose; IGI: insulinogenic index; IL-6: interleukin 6; IR: insulin resistant; IRI: immunoreactive insulin; IVGTT: intravenous glucose tolerance test; Lp(a): lipoprotein (a); MetS: metabolic syndrome; MrOS: Osteoporotic Fractures in Men; NG: normal glucose; normal glucose tolerance; OC: osteocalcin; OGTT: oral glucose tolerance test; PaTH: PTH and Alendronate study; PREDIMED: Prevención con Dieta Mediterránea OR Primary Prevention of Cardiovascular Disease with a Mediterranean Diet; SI_{index}: insulin sensitivity index; T2DM: type 2 diabetes mellitus; TC: total cholesterol; TG: triglycerides; ucOC: undercarboxylated osteocalcin; yr: years

II. VITAMIN D

A. ROLE IN GLUCOSE HOMEOSTASIS

Vitamin D is a key regulator of bone and mineral metabolism[144]. Its signaling is mediated by the binding of the physiologically active form 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D$] to its intracellular receptor (VDR) which, after translocation to the nucleus, binds to vitamin D response elements (VDREs) of target genes involved in different pathways, such as cell proliferation, differentiation, and immunomodulation[144, 145]. The presence of VDR in different organs and tissues such as the brain, breast, immune cells, and pancreas underlines the extra-skeletal effects of vitamin D[144].

The association between vitamin D and glucose metabolism is highlighted in both animal and human models, where vitamin D deficiency is associated with IR in non-diabetic models, and with reduced insulin production in diabetics[146]. Additionally, T2DM rats and humans have lower serum $1,25(OH)_2D$ levels and circulating 25(OH)D concentrations, respectively, compared to their healthy control counterparts[147-150].

Biological evidence of the association between vitamin D and glucose homeostasis includes the presence of specific VDR on pancreatic β-cells and skeletal muscles [151, 152], expression of 1-α-hydroxylase enzyme in pancreatic β-cells [153], and the presence of a VDRE in the human insulin gene promoter region[154]. Additionally, $1,25(OH)_2D$ may directly activate transcription of the human insulin receptor gene and peroxisome proliferator activator receptor-δ (PPAR- δ) [155,156], stimulate the expression of insulin receptor, and enhance insulin-mediated glucose transport[157]. Vitamin D also possesses anti-inflammatory properties[158], where $1,25(OH)_2D$ can modulate the release of inflammatory

cytokines (eg. TNF-α), regulate the activity of NF-KB, and modulate the expression of genes encoding pro-inflammatory cytokines[159-161]. Because low-grade chronic inflammatory processes, particularly in the adipose tissue, are also thought to be involved in the etiology of IR, the anti-inflammatory effects of vitamin D could potentially counter such conditions, by decreasing the expression of pro-inflammatory mediators[162].

Along with its role in the synthesis and regulation of calbindin (a vitamin D-dependent calcium-binding protein in pancreatic β-cells[163]), vitamin D may also influence insulin secretion indirectly through the regulation of calcium flux. Since adequate calcium flux is essential for the dephosphorylation of glycogen synthase and glucose transporter type 4 (GLUT-4)[164], deficiencies of vitamin D may result in poor glucose uptake. Additionally, vitamin D also affects various components of the innate and adaptive immune system that could protect β-cells from fatal immune attacks, or programmed cell death[165].

While several long-term epidemiological cohort studies showed a strong association between vitamin D deficiency and the incidence of T2DM[166], population data are confounded, as vitamin D status is a marker of "good" health and healthy lifestyle habits[167]. In a pooled analysis from clinical trials, eight studies in patients with normal glucose tolerance (NGT), and three small trials of patients with T2DM, there was no demonstrated improvement in parameters of glucose homeostasis when treated with supplemental vitamin D[168]. Nonetheless, it is to be noted that the lack of an effect in these studies may be attributed to the low doses used (<2000 IU/d), which may have been too low to induce significant metabolic changes.

Despite overall views being in favor of the negative impact of hypovitaminosis D on glucose homeostasis and β-cell function, the available literature is inconsistent. Thus, further investigation is needed to clarify the role of vitamin D in glucose metabolism.

B. POTENTIAL INTERACTION WITH VITAMIN K TO MODULATE GLUCOSE HOMEOSTASIS

The synergy between vitamin D and vitamin K in generating functional OC may influence glucose homeostasis, since OC synthesis and activation require vitamin D and vitamin K, respectively[169-170]. As mentioned previously, when circulating concentrations of vitamin K are low, a greater proportion of OC is undercarboxylated. Interestingly, lower vitamin D status has also been associated with an elevated percentage of ucOC, although the biological mechanisms underlying this association are not well understood[171]. On the other hand, vitamin K may also influence OC synthesis because the addition of vitamin K to bone cells augmented vitamin D-induced OC production in osteoblasts[172].

Vitamin D also has a direct effect on the vitamin K-dependent γ-carboxylase system, where $1,25(OH)_2D$ increases the conversion of vitamin K_1 to MK-4[173-174]. As mentioned previously, vitamin K_2 is more readily available for non-coagulation functions in extrahepatic tissues. Thus, the synergistic effects of vitamin D and K on metabolic health may be attributed to this property of vitamin D.

While it is known that vitamin D increases intestinal calcium absorption, and vitamin K directs calcium to its appropriate sites, the increase in inactive VKDP as a result of vitamin K deficiency may facilitate the release of calcium from the bone matrix into circulation. Since consistent high calcium flux may impact insulin sensitivity and glucose

uptake, it is plausible that vitamin D and vitamin K may influence glucose metabolism via this pathway.

To our knowledge, no study has assessed the effects of both vitamin K and vitamin D on osteocalcin and measures of glucose homeostasis. However, studies that investigated serum total OC in response to supplementation showed a significant increase in serum total OC in subjects who received a combination of vitamins D and K, whereas no effect was observed in those who received vitamin K supplementation alone[175-177]. Since vitamin D and vitamin K are known to interact in various metabolic pathways, it may be worth investigating the synergistic effects of both vitamin K and vitamin D on glucose homeostasis.

III. VITAMIN K AND VITAMIN D – POTENTIAL LINK WITH ALTERED GLUCOSE HOMEOSTASIS IN CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) patients have abnormal mineral metabolism. Subclinical vitamin K deficiency is highly prevalent in these individuals, reflected by their significantly elevated levels of undercarboxylated VKDPs in their circulation[178-179]. Additionally, over 50% of stage 3-5 CKD patients consume less than the recommended adequate intake for vitamin K[180 181]. While the percentage of ucOC is used as a sensitive measure of vitamin K status in the non-CKD population, it may not be an ideal marker of vitamin K status in advanced CKD due to the combination of bone resorption that occurs with underlying secondary hyperparathyroidism and the potential for the retention of OC fragments when there is reduced kidney function[181]. Nonetheless, strong correlations have been found between the percentage of ucOC and with severity of CKD, parameters of mineral metabolism, and urinary loss of proteins[181].

Vitamin D metabolism is also profoundly disordered in all stages of CKD, evident by a decline in circulating calcitriol as a result of diminished 1-α hydroxylase substrate, mass, and activity[182-184]. Although CKD is not an independent risk factor for 25(OH)D insufficiency, low 25(OH)D concentrations are common in all CKD stages[185-187]. Contributing factors include decreased cutaneous synthesis (due to older age, comorbidities, and decreased outdoor physical activity), decreased dietary intake of fortified dairy products, and increased renal 25(OH)D losses, which are exacerbated in proteinuria[184 188 189].

Glucose metabolism is also frequently impaired in CKD, where defects in insulin sensitivity and/or insulin secretion are common[182, 190 191]. Nonetheless, it is not clear whether impaired glucose metabolism contributes to the pathogenesis of CKD and its progression (in the absence of overt diabetes), whether impaired renal function causes impaired glucose metabolism, or both[182]. In animals, induction of IR in podocytes leads to glomerulosclerosis. In observational human studies, IR is associated with albuminuria and the development of CKD[192]. On the other hand, the kidney is crucial for the catabolism of insulin in the systemic circulation, such that the glomerular filtration rate (GFR) influences insulin clearance[193]. Regardless, impaired glucose homeostasis in CKD further contributes to poor health outcomes.

Given that vitamin K and/or vitamin D may have the potential to influence glucose homeostasis in individuals with or without CKD, it is of interest to investigate the effects of both vitamins on measures of glucose metabolism in these individuals. If an association exists between these measures, it is likely that the IR observed in CKD is associated with suboptimal micronutrient states for vitamin D and K.

Current literature only focuses on the independent effects of vitamin D *or* vitamin K on glucose homeostasis, with even less emphasis on the CKD population. Because T2DM is associated with comorbidities, with health outcomes being worst in people with CKD, it is critical to examine the potential supporting role of both vitamin K and vitamin D on modulating glucose homeostasis so that healthcare professionals can be informed whether implementing non-traditional dietary interventions can improve health

outcomes in the population (e.g., by increasing the adequate intake level for vitamin K, or recommending a vitamin K supplement).

The objective of this study is to investigate if vitamin K (vitamin K_2 in particular) intake is associated with glucose tolerance and its major determinants in individuals with and without CKD. We hypothesize that higher intake of vitamin K_2 (and total vitamin K) will be associated with better indices of glucose metabolism, and such associations will present after adjustment for serum 25(OH)D levels and CKD status.

METHODS

I. STUDY POPULATION

Data were obtained from the *Study of Glucose and Insulin in Renal Disease* (SUGAR), a cross-sectional study aimed at (1) characterizing insulin sensitivity, β-cell function, and glucose tolerance in moderate-severe CKD, and (2) describing determinants and correlates of insulin resistance. Between 2011-2014, participants were recruited from Nephrology and Primary Care clinics associated with the University of Washington (UW), Harborview Medical Center (HMC), and the Puget Sound Veterans Affairs Medical Center (PSVAMC) in Seattle, Washington. Potentially eligible patients were approached at clinic visits or by mail, and study brochures were placed in nephrology clinics. Participants of two preexisting observational studies involving individuals with and without CKD from the same patient base were also screened and contacted for SUGAR. All interested and potentially eligible individuals were invited to attend a screening visit, where their eligibility was assessed.

A. ELIGIBILITY CRITERIA

Eligibility was determined at the screening visit, at which eGFR was calculated from serum creatinine measured at a clinical laboratory. Participants with moderate to severe CKD, defined as eGFR <60 mL/min/1.73m^2, were included in the study. To ensure that a full spectrum of GFR within stage 3-5 CKD would be available for analysis, recruitment was further stratified by estimated GFR (45-59 mL/min/1.73m^2, 30-44 mL/min/1.73m^2 and <30 mL/min/1.73m^2). Control subjects, frequency matched on age, sex, race and BMI,

were selected within the same provider networks as the targeted CKD population with the following criteria: 1) eGFR ≥60 mL/min/1.73m², and 2) spot urine albumin-creatinine ratio <30 mg/g. Exclusion criteria for both groups included age <18 years, a clinical diagnosis of diabetes, the use of anti-diabetic medications or insulin, maintenance dialysis or fistula in place, history of kidney transplantation, use of medications known to reduce insulin sensitivity (including corticosteroids and immunosuppressants), fasting serum glucose ≥126 mg/dL, and hemoglobin <10 g/dL (see **Table 5** for eligibility criteria and **Table 6** for target enrollment numbers in the different strata).

Table 5. Eligibility criteria

Inclusion criteria
Estimated GFR as targeted in **Table 6**
Age ≥ 18 years

Exclusion criteria
Clinical diabetes mellitus:
- Fasting glucose ≥ 126 mg/dL
- Use of hypoglycemic agents or insulin

Active systemic inflammatory disease
History of maintenance hemodialysis or kidney transplant
History of Deep Venous Thrombosis or Hypercoaguable state
Disease processes independently associated with insulin resistance:
- HIV
- Lipodystrophy
- Polycystic ovary syndrome

Use of medications known to have large effects on insulin sensitivity:
- Corticosteroids
- Immunosuppressive agents (e.g. calcineurin inhibitors, rapamycin)
- Antiretrovirals
- Certain psychotropic medications

Hemoglobin < 10 g/dL
Hemodialysis access in place
Current pregnancy
Active hepatitis or cirrhosis of the liver
Unable to ambulate without assistance
Unable to provide informed consent

Table 6. Targeted recruitment by eGFR

eGFR (mL/min/1.73m²)	N
≥ 60 (control subjects)	48
45-59	32
30-44	32
< 30	32
Total	144

B. ESTIMATED GFR AND ALBUMINURIA

Serum creatinine and cystatin C (Gentian) were measured in fasting serum collected immediately before the clamp using a Beckman DxC Automated Chemistry Analyzer (Beckman Coulter, Inc., Brea CA). Creatinine and cystatin C concentrations are traceable, respectively, to isotope dilution mass spectrometry and ERM-DA471 from the International Federation of Clinical Chemistry. Inter-assay coefficients of variation were 1.5%–3.0%. GFR was estimated from creatinine and cystatin C concentrations using the Chronic Kidney Disease Epidemiology Collaboration formula. Albumin excretion rate (AER) was measured using 24-hour urine samples. Urine albumin was measured using a turbidimetric method on a Beckman DxC Automated Chemistry Analyzer (Beckman Coulter, Inc.; inter-assay coefficient of variation =0.8%–1.7%). The eGFR calculated from creatinine and cystatin C was used for all analyses. With this measurement, compared with screening, four participants recruited as patients with CKD were reclassified as controls (three had normal AER, one had AER=52 mg/d, and none had a documented cause of CKD), and one control (with AER 39 mg/d) was reclassified as having CKD.

C. STUDY PARTICIPANTS

Of 157 participants providing informed consent, 34 did not qualify for SUGAR, and 24 others did not attend the first study visit. One participant developed fasting hyperglycemia between screening and study visits and was excluded after participation

but before analyses, leaving a final analytic sample of 98 participants. Among 98 participants in SUGAR, 59 had CKD and 39 did not.

D. COVARIATES

Demographics and medical history reported by participants were retrieved from the database. Prevalent cardiovascular disease was defined as a physician diagnosis of myocardial infarction, stroke, resuscitated cardiac arrest, or heart failure or a history of coronary or cerebral revascularization. Medications were ascertained by the inventory method. The Human Activity Profile maximum activity score was used to quantify physical activity. The adjusted activity score was highly correlated with the maximum activity score (r=0.91), and similar results were observed when analyses used the adjusted activity score. Food intake was recorded using 3 days of prospective food diaries analyzed with Nutrition Data System for Research software (Nutrition Coordinating Center, Minneapolis, MN). Body composition was measured by dual-energy x-ray absorptiometry, DXA (GE Lunar or Prodigy and iDXA; EnCore Software versions 12.3 and 14.1; GE Healthcare, Waukesha, WI).

II. CALCULATION OF VITAMIN K_2 INTAKES

Estimation of vitamin K_2 contents in foods

MEDLINE (www.ncbi.nlm.nih.gov/pubmed/) and Google Scholar (scholar.google.com) databases were utilized to conduct systematic literature searches for studies with information on vitamin K_2 contents in foods. Search terms used were "vitamin K_2" or "menaquinones" in combination with "nutrient composition", "nutrient content", "nutrient database" or "food composition". References of all papers identified were examined for additional relevant studies. A total of 10 English articles with details of vitamin K_2 contents were identified (**Table 7**).

Table 7. Published literature with available vitamin K_2 or menaquinone contents in foods

Reference	Country	Vitamin K_2‡	Units	Measures	Foods	Food source
Elder et al[32]	USA	MK-4	µg/100g µg/100ml	mean, SD, range	Meat, seafood, dairy, eggs, fast food	USA (12 retails; 24 fast food restaurants)
Schurgers et al [194]	The Netherlands	MK-4 through MK-10 * MK-10 not detectable in any food	µg/100g µg/100ml	Mean, range	meat, fish, fruit, vegetables, cereals, cheese, eggs, oils	Dutch local market
Schurgers et al [195]	The Netherlands	MK-4 & MK-n (MK-n's were unspecified)	µg/100g µg/100ml	Range	Broad category (meat, fish, fruit, vegetables, cereals, cheese, eggs, oils)	Dutch local market
Tikkanen et al[196]	Finland	MK-4 through MK-10	ng/g	Range	Meat, fish, dairy	Finland food chains around Helsinki area
Hirauchi et al[197]	Japan	MK-4 through MK-13	ng/g	Mean	Animal meat and organs	Source not specified but samples from Holstein bovines (5-6 yrs); cross-breed pigs (2-3 yrs); white leghorn chickens (27-28 wks)
Hojo et al[30]	Japan	MK-4 and MK-9	ng/g	Mean, SD	Various cheese types	Retail stores in Japan (cheese from Switzerland, France, Norway)
Kamao et al[29]	Japan	MK-4 and MK-7	µg/100g µg/100ml	Mean, SD	Various food (vegetables, meat, dairy, beverages, seasonings etc)	Retail stores in Japan from three major food chains in Kobe area
Walther et al[198]	USA	MK-4 through MK-10	µg/100g µg/100ml	Mean, range	Meat and dairy	N/A Review article
Manoury et al[199]	France	MK-6 through MK-10	ng/g	Mean, SD	Cheese	France, Germany, Denmark, England, Poland
Ferreira et al[49]	USA	MK-4	µg/100g µg/100ml	Mean, SD, range	Breakfast foods, grains, baked goods	USDA nutrient data laboratory

‡All studies used reversed phase HPLC and fluorometric detection for quantifying vitamin K2 content.
Abbreviations: MK, menaquinones; SD, standard deviation; N/A, not applicable; wks, weeks; yrs; years

The Network of Food Data Systems (www.fao.org/infoods/infoods/tables-and-databases/en/) was used to identify nutrient databases of countries where vitamin K_2 information was provided. Altogether, four nutrient databases with vitamin K_2 content in foods were identified (**Table 8).**

Table 8. Nutrient databases with vitamin K_2 contents in foods

Country	Database and URL	Type of vitamin K_2 data
France	Table Ciqual 2012 afssa.fr/TableCIQUAL/	Vitamin K_2 unspecified
Turkey	Turkish Food Composition Database turkomp.gov.tr/component_results/list/VITK	Vitamin K_2 unspecified
USA	USDA National Nutrient Database ndb.nal.usda.gov/ndb/search	MK-4 only
The Netherlands	NEVO-online rivm.nl/Onderwerpen/N/Nederlands_Voedingsstoffenbestand	Total vitamin K_2: Sum of MK-4 through MK-10

An Excel spreadsheet was used to record foods with vitamin K_2 concentration information (or individual menaquinone, if specified) based on all relevant articles and databases. Where applicable, vitamin K_2 concentrations were converted to micrograms per 100 milliliter (µg/100ml) for fluids or micrograms per 100 grams (µg /100g) for solid food to facilitate comparison across different literature sources. The mean was taken for foods with vitamin K_2 concentration given as a range. Hyphens (-) were designated for foods with vitamin K_2 concentrations labeled as "non-detectable", "traces", "unknown" or "zero" to signify no available data. Total vitamin K_2 concentration for each food item provided by individual studies was estimated by summing all values of the menaquinones for foods that were provided with information on individual menaquinone content.

To facilitate the comparison of vitamin K_2 contents in similar food items across different studies and databases, food items were categorized into the following food groups and subgroups:

- Meat (deli/processed, beef, chicken, pork, others)
- Seafood (fish, crustacean, mollusks)
- Dairy (egg, milk, yogurt, cheese)
- Vegetable & Fruits
- Oils and fats
- Grains
- Beverages
- Miscellaneous (seasonings, spices, dressings)
- Pastries (cakes, pies, desserts)
- Fast food (burgers, pizza, sandwiches)
- Others (snacks, mixed food, soups, egg-based food, meat-based food, vegetable-based food, pasta)
- Food chains/restaurant foods

Accounting for inter-study variations

Vitamin K_2 values used in this project were estimated for each food by taking the mean of all of the measured concentrations of the same food derived from the independent studies. For example, a single vitamin K_2 concentration was estimated for "egg yolks" to account for the variability in vitamin K_2 concentrations across studies and databases (8µg /100g from Turkish database, 15.5µg/100g from USDA, 31.3µg/100g from a study conducted in the Netherlands, and 64µg/100g from a study conducted in Japan). Because it was not possible to assess the quality of each individual study, or determine which figure was the most representative for the food that our study participants consumed, we used the average from all relevant studies. In the egg yolk example, the value used in this project was 29.7µg/100g.

Accounting for inter-sample variations

Foods where vitamin K_2 concentrations are largely dependent on higher menaquinones may have huge variations in vitamin K_2 values even of the same type (e.g. cheese), due to differences in the fermentation processes. Thus, for these food items, mean vitamin K_2 concentrations were estimated by factoring all measured vitamin K_2 values. As an example, Manoury et al[199] studied six Cheddar cheese samples from England, with vitamin K_2 concentrations ranging from 5.1µg/100g to 42.9µg/100g. The investigators also noted a 7-fold difference in vitamin K_2 concentration for two different blue cheese samples from France, and a 14-fold difference in vitamin K_2 concentrations of the same cheese-type (blue cheese) from different countries (France and England). Taking the mean of estimated vitamin K_2 concentrations across all studies provided the best available estimate of vitamin K_2 contents, and helped minimize the intake assessment error.

Accounting for various cuts and fat contents

Since vitamin K_2 concentrations do not vary drastically between different cuts and fat contents of the same meat type, these were grouped together as a generic food item and assigned a single vitamin K_2 concentration value by taking the mean of their measured values from different studies. For instance, vitamin K_2 concentration derived for food item "beef" included those values for beef chuck, beef round, roast beef and canned beef, and vitamin K_2 concentration for "ground beef" included those values for low fat, medium fat and high fat varieties. Using this approach allowed us to estimate vitamin K_2

concentrations of all foods consumed by the study participants, especially if they had not specified the cut and fat content of any meat consumed.

Estimation of vitamin K_2 for foods known to provide low vitamin K_2 concentrations

Although information on vitamin K_2 concentrations for certain foods such as grains, fruits and vegetables is limited, it was consistent across studies that these foods provide very little to no vitamin K_2. Nonetheless, the same approach used to estimate vitamin K_2 concentrations for meat products discussed above was used here, for the same reasons.

Maximizing precision of vitamin K_2 concentration estimation in foods rich in vitamin K_2

In contrast to the more generic vitamin K_2 concentrations assigned to meats, seafood, fruits and vegetables, individual vitamin K_2 values were estimated for milk and yogurt categorized by their fat contents (whole, 2%, 1%, non-fat), because the amount of menaquinones provided by these dairy foods depends on the amount of fat they contain, and because participants are more likely to accurately report the fat content of milk and yogurt than that of meat. However, since values on specific types of yogurts (i.e., Greek, Bulgarian, Turkish) are limited, we did not distinguish between them and regular yogurts. The vitamin K_2 values provided for these types of yogurt were still factored in into our estimation of vitamin K_2 concentrations.

Individual vitamin K_2 values were assigned to each cheese for which we had information, because the amount of vitamin K_2 is highly dependent on production methods, starting ingredients, starter bacteria and age, etc. For instance, different types of

starter bacteria in cheese contribute to varying degrees of ripening as well as different amounts of menaquinones. As mentioned previously, vitamin K_2 concentrations can vary significantly, even among cheese of the same type. Thus, it seemed reasonable to estimate a single vitamin K_2 value for each type of cheese for which we had information.

Because we did not have information on vitamin K_2 concentration for all cheeses, and because participants did not always know or report the specific type of cheese consumed, it was necessary to establish estimates for categories of cheese. As degrees of cheese ripening can roughly be judged based on their textures, we first categorized the cheese for which we had information into hard, semi-hard, semi-soft, soft, and processed cheese. Next, a single vitamin K_2 concentration was estimated for each category by taking the mean of all vitamin K_2 values of cheeses within that particular group, such that each cheese category had its own mean vitamin K_2 concentration. Using this approach, we could estimate the vitamin K_2 content of a type of cheese that was specified in the food record, but was not in our database. For instance, if a subject had reported consuming Havarti cheese, we would use the mean vitamin K_2 value derived for semi-soft cheese, as Havarti cheese would be grouped in this category. In order to account for uncertainties of foods consumed by study participants, a single vitamin K_2 value for cheese was derived by taking the mean of all cheese types, such that if a subject had reported eating cheese without specifying any type, we would utilize this value.

Estimation of vitamin K_2 for food substitutes

For dairy food substitutes such as soy milk, soy yogurt, soy cheese, rice milk, and almond milk, vitamin K_2 was estimated for these by considering the original ingredient that they were made out of. For example, the vitamin K_2 value for soy was used to estimate that for soy milk, soy yogurt and soy cheese; the value for rice was used to estimate that for rice milk, the value for almonds was used to estimate that for almond milk, etc.

Derivation of vitamin K_2 for mixed food items

Since vitamin K_2 values for mixed food items were limited and potentially under- or over-estimated in the available literature and nutrient databases, vitamin K_2 concentrations of these items were estimated by summing the amount of vitamin K_2 contributed by each ingredient for which we had finalized values, using available online recipes. This maximized the accuracy and consistency for us to determine the participants' dietary vitamin K_2 intake, especially for those food items known to contain ingredients with substantial vitamin K_2 concentrations (e.g., milk, cream, egg, and butter in pastries; cheese and meat in pizzas, etc.). In cases where there was a lack of specificity in the reported foods, we estimated vitamin K_2 concentrations for the unspecified food items by summing the vitamin K_2 values of the listed ingredients for which we had information. For instance, if a subject had reported eating a *cheese* sandwich rather than a *Cheddar cheese* sandwich, we would simply calculate the amount of vitamin K_2 contributed by the sandwich by summing the value of the bread and the general value for cheese.

Certain miscellaneous items such as salad dressings, condiments, or soups may have varying vitamin K_2 contents depending on their preparation methods and the ingredients involved in their production. For example, ranch dressing may have very different vitamin K_2 concentration compared to vinegar-based dressing, because ranch dressing includes dairy as a main ingredient. As another example, clam chowder may have very different vitamin K_2 content compared to minestrone soup, because it utilizes vitamin K_2-rich cream as an ingredient while the latter does not usually include dairy. Because information on vitamin K_2 concentrations for these specific foods is limited, it was necessary to derive estimates for these items, as they may contribute substantially to an individual's vitamin K_2 consumption. Using the same approach as we did for mixed food items, vitamin K_2 concentrations for these miscellaneous items were estimated based on established recipes online. These were then categorized into dairy-based and non-dairy based subgroups, with a single vitamin K_2 value estimated for each subgroup by taking the mean of the vitamin K_2 values for all items categorized under the respective subgroups.

Established vitamin K_2 data for mixed food items in restaurants and food chains measured by the USDA were utilized if subjects had reported eating in those specific restaurants.

III. ASSESSMENT OF VITAMIN K_2, VITAMIN K_1 AND TOTAL VITAMIN K INTAKE

Participants were provided with verbal and written instructions for the accurate collection of prospective food records over three 24-hour periods. These food records

were returned during the oral glucose tolerance test (OGTT) visits (**Table 9**). Individual food item listed on the three-day food records for all participants were reviewed, and vitamin K_2 concentration contributed by individual food items was estimated by multiplying the derived vitamin K_2 concentrations by the gram or milliliter amount consumed. Sum and mean of vitamin K_2 intake over three days were calculated for each subject. Three-day vitamin K_1 intake analyzed using the Nutrition Data System for Research software was summed and averaged to micrograms consumed per day (μg/d) for each subject. Mean of total vitamin K (vitamin K_1 and K_2) consumed over three days for each subject was calculated by averaging the means of vitamin K_1 and K_2 intake. To account for differences in energy intake, the amount of individual vitamin K intake was further transformed into μg/1000kcal by dividing derived vitamin K values (μg/d) by energy intake (kcal/d) and multiplied by 1000.

IV. DETERMINATION OF ENDPOINT MEASURES

A. MEASUREMENTS OF GLUCOSE AND INSULIN HOMEOSTASIS

Each participant was admitted to the University of Washington Clinical Research Center (UW CRC) after an overnight fast for oral glucose tolerance (OGTT), intravenous glucose tolerance (IVGTT), and a hyperglygemic-euglygemic clamp in two separate visits (**Table 9**). Between the Clamp Visit and the OGTT Visit, at home, participants were asked to collect a 24-hour urine sample and to wear an accelerometer.

Table 9. Study visits and measurements for the SUGAR study	
Clamp visit	*OGTT visit*
Complete medical history	Fasting blood draw
Physical examination	OGTT
Verify eligibility	Physical activity questionnaire
Fasting blood draw	DXA
Urine collections (x2)	CT
IVGTT	Physical/Cognitive measurements
Euglycemic clamp	Pulse amplitude tonometry
Food record instructions	Return food records
24 hour urine instructions	Return 24 hour urine
Accelerometer instructions	Return accelerometer

Abbreviations: CT, computerized axial tomography scan; DXA, dual-energy X-ray absorptiometry; IVGTT, intravenous glucose tolerance test; OGTT, oral glucose tolerance test

Intravenous-glucose-tolerance test

A short intravenous-glucose-tolerance test (IVGTT) was performed in the first visit to assess the acute insulin response to glucose (AIRg), the gold standard measure of pancreatic β-cell function. Three fasting plasma samples were drawn 5 minutes apart. The IVGTT was then initiated with an infusion of unlabeled 20% dextrose (11.4 g/m^2 over 60 seconds), after which plasma was frequently sampled for insulin and glucose concentrations. Thirty minutes after commencing the dextrose infusion, an insulin infusion was initiated as a prime (160 mU/m^2 per minute for 5 minutes) followed by a constant rate (80 mU/m^2 per minute). A variable rate infusion of unlabeled 20% dextrose was administered to maintain blood glucose (measured every 5 minutes) at approximately 90 mg/dl. Beginning 120–150 minutes after initiation of the insulin infusion, the dextrose infusion rate was held constant for 30 minutes, over which time three steady–state plasma samples were obtained 15 minutes apart. Plasma concentrations of insulin (two–site immune–enzymometric assay; Tosoh 2000 Autoanalyzer) and glucose (glucose

hexokinase method; Roche Module P Chemistry Autoanalyzer; Roche, Basel, Switzerland), as well as the dextrose concentration of the infusate, were measured at the Northwest Lipid Research Laboratories (Seattle, WA). AIRg was calculated from the IVGTT as the AUC insulin response above basal from 0 to 10 min. AIRg was adjusted for insulin sensitivity measured by the clamp method to estimate β-cell function. The glucose disappearance constant, a measure of intravenous glucose tolerance, was calculated as the slope of the natural log of glucose from 10 to 30 min during the IVGTT.

Hyperinsulinemic-euglycemic clamp

Immediately following the IVGTT, a hyperinsulinemic-euglycemic clamp procedure as described by Matsuda and DeFronzo[200] was performed to measure insulin clearance and sensitivity. Intravenous catheters were placed in peripheral veins in each upper extremity and kept patent with a slow infusion of normal saline. One arm was warmed to allow for the sampling of arterialized blood. A primed infusion of insulin (160 mU/m^2/min x 5 minutes) followed by a continuous infusion (80 mU/m^2/min) commenced at time 0. Blood glucose was monitored every 5 minutes using an iStat machine, and a 20% dextrose infusion was titrated according to established protocol to clamp glucose concentrations at 90±5 mg/dL regardless of baseline glucose. If steady state was not reached by 120 minutes, the study was extended for an additional 30 minutes. Plasma for glucose and insulin concentrations was collected for laboratory analysis during minutes 120-150, and a sample from the intravenous dextrose preparation was sent to the clinical laboratory for definitive calculation of the glucose infusion rate. After collection of the final blood sample, insulin infusion was discontinued, and the dextrose infusion was titrated off over

approximately 80 minutes to avoid hypoglycemia. Blood glucose was checked approximately 15 minutes after cessation of dextrose infusion to ensure euglycemia was maintained.

Glucose disposal rate was calculated as the glucose infusion rate during the last 30 minutes of the clamp, adjusted for the drift in plasma glucose concentration using the Steels non-steady-state equations. Insulin sensitivity was calculated as the insulin sensitivity index (SI_{index})[201]:

$$SI_{index} = M / (G \times \Delta I)$$

where M is the steady state glucose infusion rate (mg/min during minutes 120-180), G is the steady state blood glucose concentration (mg/dL), and ΔI is the difference between basal and steady state plasma insulin concentration (μU/mL). Body size parameters were adjusted rather than incorporating lean mass into SI_{index} to evaluate the relationships of different body size metrics with SI.

Oral-glucose-tolerance-test

A standard 75-g oral-glucose-tolerance test (OGTT) was performed approximately one week after the IVGTT and clamp. Seventy-five grams of glucose were consumed within 5 min, and blood samples were drawn at -10, -5, -1, 10, 20, 30, 60, 90 and 120 minutes relative to the start of glucose ingestion to measure plasma glucose and insulin concentrations. These were used to calculate the Matsuda-Defronzo insulin sensitivity index[200]. The insulinogenic index (IGI)[202], a measure of the early insulin response to oral glucose, was calculated as the difference in insulin concentration divided by the difference in glucose concentration from fasting to 30 minutes. Glucose tolerance was

measured by calculating total area under the curve (AUC) for glucose and insulin, and the AUC for incremental glucose and insulin above basal from 0 to 120 min using the trapezoidal method. The oral disposition index (oral DI) was calculated as the insulinogenic index × 1/fasting insulin.

B. Quantification of serum 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3

Immunoaffinity extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to quantify the amount of 25-hydroxyvitamin D_2 [25(OH)D_2] and 25-hydroxyvitamin D_3 [25(OH)D_3] in participants' serum or plasma samples. Patient serum or plasma, calibrators, and controls (400 µL) were spiked with deuterated internal standards and immunoaffinity purified using anti-1α,25-dihydroxyvitamin D beads from ALPCO. After incubation, the beads were washed and bound analytes were eluted with organic solvent. The eluent was dried down and the residue reconstituted with the derivatizing agent PTAD in acetonitrile. After incubation at room temperature, the reaction was quenched with water. A portion of the mixture was analyzed on a Waters Xevo TQ tandem mass spectrometer equipped with an Acquity UPLC. Analytes that were enriched during the immunoaffinity purification include 25(OH)D_2 and 25(OH)D_3, with deuterated internal standards for each analyte included. Standards were prepared in stripped human serum.

V. STATISTICAL ANALYSES

All statistical analyses were conducted with SPSS for Macintosh (version 20; IBM Corporation). Normal distribution was confirmed by checking histograms and normal plots and by conducting Shapiro-Wilk and Kolmogorov-Smirnov tests. Variables that were not consistent with a normal distribution were log(10)-transformed before statistical analyses. Descriptive data are shown as means ± standard deviations for normally distributed variables, and as medians (25^{th} percentile; 75^{th} percentile) for non-normally distributed variables. Baseline characteristics of study participants, including demographics, physical characteristics, lifestyle factors and laboratory measures, were compared based on quartiles of vitamin K_1, K_2, or total vitamin K intake measured in µg/1000kcal using the Student's t-test for trend. Subjects with missing data on any dietary variable, given endpoint measure, or covariate were excluded from those analyses.

We conducted linear regression analyses on the following dependent variables: oral glucose tolerance (glucose area-under-the-curve, glucose AUC) insulin sensitivity [insulin sensitivity index (SI_{index}) and Matsuda-DeFronzo insulin sensitivity index (Matsuda-ISI)], and β-cell function [insulinogenic index (IGI), acute insulin response to glucose (AIRg) and oral disposition index (oral DI)]. Besides glucose AUC, all other endpoint measures were log(10)-transformed prior to regression analyses. Independent variables were quartiles of vitamin K_1 intake, quartiles of vitamin K_2 intake, and quartiles of total vitamin K intake, categorized based on the intakes measured in µg/1000kcal (each separately). For all analyses, we computed 4 models:

- Model 1: Crude unadjusted model
- Model 2: Adjusted for age, sex, BMI and race/ethnicity
- Model 3: As model 2, further adjusted for eGFR status;
- Model 4: As model 3, further adjusted for vitamin D status and any potential confounding dietary variables

We tested model 4 to assess whether any observed associations between vitamin K intake and metabolic endpoints may be attributable to confounding by vitamin D status (indicated by serum 25-hydroxyvitamin D concentrations) and other dietary variables by performing linear regression analyses adjusted for each dietary variable separately in addition to age, sex, BMI, race/ethnicity and eGFR status (i.e. model 3). The dietary variables tested were those that were significantly associated with the specific exposure variable (quartiles of vitamin K_1, K_2, or total K intake), and included % calories from protein, % calories from carbohydrate, % calories from fat and total fiber intake (g/1000kcal). The final model 4 shown in the results tables includes all of the covariates the inclusion of which changed the β-coefficient describing the relationship between the primary exposure of interest and the endpoint by more than 10%. *P* values <5% were considered statistically significant.

RESULTS

I. SUBJECT CHARACTERISTICS

Baseline characteristics of participants from the SUGAR study categorized by CKD status are shown in **Table 10.** Of 98 participants enrolled in the study, six subjects from the CKD group were excluded due to missing data for one or more covariates (*n*=5) and implausibly low energy intake of <750kcal/d (*n*=1). In the final analytical sample of 92 subjects, CKD status was positively associated with BMI (*P* = 0.036) and systolic blood pressure (*P* < 0.001). CKD patients also tended to have history of cardiovascular disease (*P* < 0.001), and were less physically active (*P* < 0.001). As expected, CKD patients had lower eGFR (*P* < 0.001), elevated parathyroid hormone concentrations (*P* < 0.001) and higher urinary albumin excretion rates (*P* = 0.005). However, CKD patients in this study had better vitamin D status (*P* = 0.001). In terms of parameters of glucose metabolism, CKD patients had lower insulin sensitivity based on the insulin sensitivity index (*P* = 0.015) and Matsuda-DeFronzo insulin sensitivity index (*P* = 0.004), and higher insulin resistance as assessed by HOMA-IR (*P* = 0.019). They also tended to have lower energy intake (*P* = 0.021), as well as intakes of fiber (*P* = 0.009), vitamin K_1 (*P* = 0.010) and total vitamin K (*P* = 0.014).

When participants were categorized into quartiles of vitamin K_2 intake in μg/1000kcal (**Table 11**), higher vitamin K_2 intake was negatively associated with age *(P* = 0.005), % energy from carbohydrate (*P* < 0.001), fiber intake (*P* = 0.001), and total serum 25-hydroxyvitamin D (*P* = 0.026), while positively associated with BMI (*P* = 0.003), %

energy from fat (P = 0.035) and % energy from protein (P < 0.001). When categorized based on quartiles of vitamin K_1 intake (**Table 12**), higher vitamin K_1 intake was positively associated with eGFR status (P = 0.002) and fiber intake (P = 0.014). Similarly, higher total vitamin K intake was positively associated with eGFR status (P = 0.001) and fiber intake (P = 0.018) (**Table 13**).

While insulin sensitivity index data were available for all 92 participants, data on glucose AUC and Matsuda-DeFronzo insulin sensitivity index were missing from two subjects (n=90), and data on AIRg, IGI and oral DI were missing from one subject (n=91).

II. RELATION BETWEEN VITAMIN K INTAKE AND MEASURES OF GLUCOSE TOLERANCE

We assessed the relation between vitamin K_2, vitamin K_1 or total vitamin K intake and glucose tolerance in linear regression models that were either unadjusted (model 1), or adjusted for classical risk factors including age, sex, BMI, and race/ethnicity (model 2), plus eGFR status (model 3), as well as vitamin D status and potential dietary confounders (model 4) (**Table 14**). Higher vitamin K_2 intake was associated with better glucose tolerance (P = 0.047) after adjusting for vitamin D status and fiber intake (g/1000kcal), % energy from protein and % energy from carbohydrate. While vitamin K_1 was marginally associated with glucose tolerance prior to adjusting for covariates (P = 0.070), such association was attenuated after adjusting for classical risk factors, eGFR and vitamin D status, and dietary covariates (P= 0.129). No association was observed between total vitamin K intake and glucose AUC.

III. RELATION BETWEEN VITAMIN K INTAKE AND INSULIN SENSITIVITY

Higher vitamin K_2 intake was associated with lower insulin sensitivity (*P* = 0.017*)* as measured by the Matsuda-DeFronzo insulin sensitivity index in a crude, unadjusted model (**Table 14**). However, such association was attenuated after adjusting for BMI, age, sex and race/ethnicity and no longer statistically significant. No association was detected between vitamin K_2 intake and insulin sensitivity as measured by the clamp method, and neither vitamin K_1 intake nor total vitamin K intake was associated with indices of insulin sensitivity.

IV. RELATION BETWEEN VITAMIN K INTAKE AND B-CELL FUNCTION

No association was found between any form of vitamin K intake and any measure of β-cell function (**Table 15**). Adjustment for vitamin D status and dietary covariates in addition to BMI, sex, age, race/ethnicity and eGFR status had very little effect on the relation between any vitamin K intake and indices of β-cell function, including AIRg, IGI and oral DI.

Table 10. Baseline characteristics of study population from the SUGAR study categorized by eGFR[1]

Covariates	All subjects *n* = 92[‡]	Categories of eGFR (mL/min/1.73m^2) > 60 *n* = 39	< 60 *n* = 53	*P*-value[2]
Demographics				
Age (year)	63 ± 13	61 ± 2	65 ± 2	0.253
% Male sex	52%	58%	51%	0.491
% Race				
White	79.3 %	87.2%	73.6%	0.111
Black	15.2%	10.3%	18.9%	
Others	5.4%	2.6%	7.5%	
Physical characteristics				
BMI (kg/m^2)	29.1 ± 6.2	27.3 ± 1.0	30.0 ± 0.7	0.036*
Systolic BP (mmHg)	130 ± 16	123 ±2	135 ± 2	< 0.001*
Diastolic BP (mmHg)	79 ± 10	77 ± 2	80 ± 1	0.115
Medial history & lifestyle				
% Smoker	11%	8%	14%	0.407
% History of CVD[◊]	21%	5%	31%	< 0.001*
Max PA Score (0- 100)	80 ± 10	83 ± 1	77 ± 1	< 0.001*
Adjusted PA Score (0-100)	72 ± 15	78 ± 2	68 ± 2	< 0.001*
Laboratory measures				
Urine AER (mg/24 hour)	12.7 (5.7; 95.9)	5.7 (3.5; 8.7)	39.2 (13.2; 210.6)	0.005*
eGFR (mL/min/1.73m^2)	50.7 (36.4; 81.7)	84.7 (73.8; 101.6)	37.7 (26.0; 47.1)	< 0.001*
hs-CRP (µg/mL)	0.22 (0.09; 0.57)	0.15 (0.06; 0.25)	0.26 (0.15; 0.77)	0.829
PTH (pg/mL)	62 (38; 82)	43 (35; 64)	70 (55; 97)	< 0.001
Total serum 25(OH)D (ng/mL)	28 (22; 38)	24 (21; 30)	33 (24; 41)	0.001*

Table 10 (cont.)

Covariates	All subjects $n = 92^{‡}$	Categories of eGFR (mL/min/1.73m^2) > 60 $n = 39$	< 60 $n = 53$	*P*-value[2]
Parameters of glucose metabolism				
Glucose AUC (min*mg/dL)	19,505 ± 3,435	19,053 ± 584	19,723 ± 453	0.494
Insulin sensitivity index	4.0 (2.9; 5.4)	5.0 (3.2; 6.5)	3.4 (2.5; 4.5)	0.015*
Matsuda-DeFronzo index	4.5 (2.6; 6.9)	5.51 (2.96; 8.91)	3.50 (2.45; 5.49)	0.004*
Insulinogenic index	0.8 (0.5; 1.3)	0.68 (0.38; 1.08)	0.84 (0.58; 1.39)	0.571
AIRg (uU*min/mL)	337 (237; 549)	343 (199; 607)	337 (242; 535)	0.905
Oral disposition index	3.4 (2.0; 5.2)	3.8 (2.0; 6.6)	3.3 (2.1; 4.8)	0.149
HOMA-IR	1.7 (1.0; 2.7)	1.3 (0.7; 2.7)	2.3 (1.4; 2.9)	0.019*
Dietary variables				
Energy (kcal/d)	1,897± 541	2,019 ±86	1,786 ± 72	0.021*
Fiber (g/1000kcal)	10.9 ± 4.3	12.4 ± 40.7	10.1 ± 0.6	0.009*
% Energy from fat	34.8 ± 6.8	34.1 ± 7.2	34.5 ± 0.9	0.672
% Energy from carbohydrate	46.4 ± 8.1	46.7 ± 1.4	46.3 ± 1.1	0.875
% Energy from protein	16.3 ± 4.5	15.7 ± 0.6	16.7 ± 0.7	0.232
Vitamin K_1 (µg/1000kcal)	48 (28; 82)	63 (30; 123)	38 (28; 65)	0.010*
Vitamin K_2 (µg/1000kcal)	13 (9; 18)	11 (9; 19)	14 (10; 18)	0.363
Total vitamin K (µg/1000kcal)	62 (41; 103)	80 (42; 134)	55 (40; 79)	0.014*

[1] Data are means ± SD, or medians (25th percentile; 75th percentile), or percentages.

[2] Significant difference set at $P < 0.05$ for independent samples *t*-test. * Statistically significant association, $P < 0.05$

[‡] Analyses were based on a total sample population of *n*= 92 besides urinary AER (*n*=91), HOMA-IR (*n*=91), Matsuda-DeFronzo index (*n*=90), glucose AUC (*n*=90) and insulinogenic index (*n*=90) due to missing data

[◊] CVD (Cardiovascular disease) is defined as myocardial infarction, chronic heart failure, stroke, cardiac arrest, CABG (Coronary Artery Bypass Grafting surgery), or coronary/cerebral revascularization.

Abbreviations: adjusted PA score, adjusted physical activity score; AIRg, acute insulin response to glucose; BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; glucose AUC, glucose area-under-the-curve; HOMA-IR, homeostasis model assessment for insulin resistance; hs-CRP, high sensitivity C-reactive protein; max PA score, maximum physical activity score; PTH, parathyroid hormone; total serum 25(OH)D, total serum 25-hydroxyvitamin D; urine AER, urinary albumin excretion rate

Table 11. Distribution of potential confounding variables across quartiles of vitamin K_2 intake measured in µg/1,000kcal[1]

	Quartiles of vitamin K_2 intake (ranges, in µg/1,000kcal), n=92				
	Q1 (4 – 9)	Q2 (9 – 13)	Q3 (13 – 18)	Q4 (18 – 45)	Test for trend *P* value [2]
Vitamin K_1 (µg/1000kcal)	30 (22; 54)	63 (38; 129)	60 (29; 93)	45 (30; 83)	0.676
Vitamin K_2 (µg/1000kcal)	7 (6; 8)	11 (10; 11)	15 (14; 16)	21 (19; 25)	< 0.001*
Vitamin K_2 (µg/d)	13 (10; 20)	19 (16; 24)	25 (21; 34)	41 (21; 34)	< 0.001*
Total vitamin K (µg/1000kcal)	38 (29; 61)	73 (48; 139)	74 (42; 106)	67 (52; 104)	0.267
Age (year)	67 ± 9	65 ± 15	64 ± 14	55 ± 13	0.005*
BMI (kg/m^2)	28.2 ± 6.8	26.9 ± 6.1	30.0 ± 6.1	31.3 ± 5.1	0.033*
eGFR (mL/min/1.73m^2)	59.6 ± 28.4	58.9 ± 31.5	55.4 ± 32.1	60.6 ± 29.0	0.988
% Smoker	13%	9%	9%	13%	1.00
Max PA Score (0- 100)	79 ± 9	82 ± 8	79 ± 11	79 ± 11	0.679
Adjusted PA Score (0-100)	71 ± 14	75 ± 14	71 ± 17	71 ± 14	0.760
Energy intake (kcal/d)	2,075 ± 656	1857 ± 472	1,763 ± 504	1,893 ± 499	0.205
Fiber intake (g/1000kcal)	12.6 ± 5.2	12.1 ± 4.5	10.3 ± 3.4	8.8 ± 3.1	0.001*
% Energy from fat	31.8 ± 7.4	35.1 ± 8.2	36.5 ± 5.7	35.8 ± 4.8	0.035*
% Energy from carbohydrates	50.6 ± 9.4	47.7 ± 6.9	44.3 ± 7.0	42.8 ± 6.7	< 0.001*
% Energy from protein	14.6 ± 3.4	14.1 ± 2.7	16.8 ± 4.7	19.6 ± 4.9	< 0.001*
Total serum 25(OH)D (ng/mL)	29.9 (26.4, 47.2)	32.1 (19.9; 40.4)	25.6 (21.7; 38.7)	25.9 (21.0; 34.3)	0.026*

[1] Data are means ± SD, medians (25th percentile; 75th percentile), or percentages (95% CI).

[2] Significant difference set at $P < 0.05$ (test for trend). * Statistically significant association, $P < 0.05$.

Abbreviations: adjusted PA score, minimum physical activity score; BMI, body mass index; eGFR, estimated glomerular filtration rate; max PA score, maximum physical activity score; total serum 25(OH)D, total serum 25-hydroxyvitamin D; 95% CI, 95% confidence interval.

Table 12. Distribution of potential confounding variables across quartiles of vitamin K_1 intake measured in µg/1,000kcal[1]

	Quartiles of vitamin K_1 intake (ranges, in µg/1,000kcal), n=92				
	Q1 (10 – 28)	Q2 (28 – 48)	Q3 (48 – 81)	Q4 (81 – 651)	Test for trend *P* value [2]
Vitamin K_1 (µg/d)	48 (33; 55)	66 (49; 74)	114 (80; 135)	261 (191; 305)	< 0.001*
Vitamin K_1 (µg/1000kcal)	22 (19; 27)	34 (30; 39)	75 (63; 75)	135 (100; 187)	< 0.001*
Vitamin K_2 (µg/1000kcal)	21 (14; 34)	26 (20; 38)	22 (15; 25)	23 (19; 34)	0.420
Total vitamin K (µg/1000kcal)	32 (29; 38)	51 (47; 56)	74 (67; 91)	148 (115; 199)	< 0.001*
Age (year)	61 ± 11	64 ± 14	62 ± 16	64 ± 12	0.523
BMI (kg/m^2)	29.8 ± 7.3	30.6 ± 5.4	28.6 ± 6.0	27.5 ± 5.8	0.132
eGFR (mL/min/1.73m^2)	53.7 ± 29.3	45.8 ± 19.0	57.3 ± 29.3	77.7 ± 31.7	0.002*
% Smoker	22%	4%	13%	4%	0.136
Max PA Score (0- 100)	80 ± 10	77 ± 11	80 ± 7	83 ± 9	0.203
Adjusted PA Score (0-100)	71 ± 15	68 ± 16	74 ± 9	76 ± 17	0.221
Energy intake (kcal/d)	2,053 ± 605	1,882 ± 442	1,770 ± 585	1,882 ± 514	0.218
% Energy from fat	34.2 ± 7.1	33.1 ± 5.0	35.9 ± 6.9	36.1 ± 8.0	0.196
% Energy from carbohydrates	46.7 ± 8.7	48.4 ± 8.1	45.1 ± 9.3	45.2 ± 5.8	0.318
% Energy from protein	15.6 ± 5.4	16.8 ± 3.9	16.5 ± 5.9	16.3 ± 2.3	0.645
Fiber intake (g/1000kcal)	10.0 ± 3.8	10.1 ± 4.4	10.8 ± 3.5	13.0 ± 4.9	0.014*
Total serum 25(OH) D (ng/mL)	27.3 (23.3; 40.7)	31.1 (21.9; 47.7)	27.1 (19.9; 39.3)	25.6 (22.0; 34.2)	0.104

[1] Data are means ± SD, medians (25th percentile; 75th percentile), or percentages (95% CI).

[2] Significant difference set at $P < 0.05$ (test for trend). * Statistically significant association, $P < 0.05$.

Abbreviations: adjusted PA score, minimum physical activity score; BMI, body mass index; eGFR, estimated glomerular filtration rate; max PA score, maximum physical activity score; total serum 25(OH)D, total serum 25-hydroxyvitamin D; 95% CI, 95% confidence interval.

Table 13. Distribution of potential confounding variables across quartiles of total vitamin K intake measured in µg/1,000kcal[1]

	Quartiles of total vitamin K intake (ranges, in µg/1,000kcal), n=92				
	Q1 (19 - 40)	Q2 (42 - 62)	Q3 (63 - 100)	Q4 (100 - 661)	Test for trend *P* value [2]
Vitamin K_1 (µg/1000kcal)	22 (19; 27)	35 (31; 44)	63 (52; 76)	135 (100; 187)	< 0.001*
Vitamin K_2 (µg/1000kcal)	8 (6; 14)	14 (10; 20)	15 (11; 19)	13 (11; 19)	0.003*
Total vitamin K (µg/1000kcal)	32 (29; 38)	52 (48; 57)	74 (67; 91)	148 (115; 199)	< 0.001*
Total vitamin K (µg/d)	71 (51; 81)	93 (76; 110)	141 (103; 166)	292 (218; 345)	< 0.001*
Age (year)	63 ± 10	62 ± 18	65 ± 12	62 ± 14	0.986
BMI (kg/m^2)	28.9 ± 7.0	30.4 ± 5.8	29.8 ± 5.8	27.3 ± 6.0	0.358
eGFR ($mL/min/1.73m^2$)	52.3 ± 30.3	49.9 ± 19.8	51.0 ± 26.3	81.2 ± 30.8	0.001*
% Smoker	17%	4%	17%	4%	0.371
Max PA score (0- 100)	79 ± 10	78 ± 11	78 ± 7	83 ± 9	0.151
Adjusted PA score (0-100)	70 ± 16	69 ± 16	73 ± 9	77 ± 17	0.070
Energy intake (kcal/d)	2,071 ± 611	1,859 ± 477	1,790 ± 548	1,867 ± 513	0.180
% Energy from fat	33.9 ± 7.2	34.5 ± 6.1	35.2 ± 6.6	35.8 ± 7.6	0.326
% Energy from carbohydrates	48.8 ± 8.1	45.8 ± 9.2	45.9 ± 8.2	44.9 ± 6.4	0.124
% Energy from protein	14.4 ± 2.9	17.1 ± 4.4	16.9 ± 6.7	16.8 ± 2.6	0.094
Fiber intake (g/1000kcal)	10.4 ± 4.4	9.3 ± 3.9	11.1 ± 3.3	12.9 ± 5.0	0.018*
Total serum 25(OH)D (ng/mL)	28.2 (23.8; 40.7)	31.1 (19.3; 47.7)	30.6 (22.3; 40.4)	24.7 (22.0; 31.2)	0.065

[1] Data are means ± SD, medians (25th percentile; 75th percentile), or percentages (95% CI).

[2] Significant difference set at $P < 0.05$. * Statistically significant association, $P < 0.05$.

Abbreviations: adjusted PA score, minimum physical activity score; BMI, body mass index; eGFR, estimated glomerular filtration rate; max PA score, maximum physical activity score; total serum 25(OH)D, total serum 25-hydroxyvitamin D; 95% CI, 95% confidence interval.

Table 14. Multiple linear regression analyses of the relation between quartiles of vitamin K intake (μg/1,000kcal) and oral glucose tolerance and indices of insulin sensitivity[1]

	Glucose AUC (mg/dL/min) *n* = 90		Indices of insulin sensitivity			
			Log (SI_{index}) *n* = 92		Log (Matsuda-ISI index) *n* = 90	
		P-value[2]		*P-value*[2]		*P-value*[2]
Vitamin K_1						
Model 1	-587 (-1,222; 48)	0.070	-0.012 (-0.048, 0.025)	0.526	0.008 (-0.047, 0.062)	0.779
Model 2	-589 (-1,244; 66)	0.077	-0.011 (-0.047, 0.024)	0.528	-0.007 (-0.051, 0.037)	0.752
Model 3	-600 (-1,300; 98)	0.092	-0.014 (-0.052, 0.024)	0.425	-0.014 (-0.060, 0.032)	0.550
Model 4	-549 (-1,252; 154)	0.129	N/A[†]		-0.017 (-0.064, 0.031)	0.488
Vitamin K_2						
Model 1	-349 (-998; 300)	0.288	-0.020 (-0.056, 0.016)	0.274	-0.065 (-0.118, -0.012)	0.017*
Model 2	-337 (-1,057; 384)	0.356	-0.026 (-0.062, 0.010)	0.158	-0.019 (-0.066, 0.028)	0.426
Model 3	-336 (-1,060; 388)	0.358	-0.026 (-0.062, 0.010)	0.160	-0.019 (-0.066, 0.028)	0.426
Model 4	-817 (-1,624; -106)	0.047*	-0.017 (-0.058, 0.024)	0.413	-0.035 (-0.086, 0.015)	0.170
Total vitamin K						
Model 1	-507 (-1,145, 131)	0.118	-0.013 (-0.049, 0.023)	0.468	-0.002 (-0.057, 0.052)	0.937
Model 2	-465 (-1,122; 192)	0.163	-0.018 (-0.053, 0.017)	0.298	-0.010 (-0.053, 0.034)	0.657
Model 3	-459 (-1,159; 240)	0.196	-0.022 (-0.059, 0.015)	0.245	-0.017 (-0.063, 0.029)	0.468
Model 4	-408 (1,111; 295)	0.252	N/A[†]		-0.020 (-0.067, 0.028)	0.412

[†] We identified no other potential confounders that changed the β-coefficient in model 3 by more than 10%.

[1]Data are unadjusted β coefficients (95% Confidence intervals).

Model 1, unadjusted;

Model 2, adjusted for age, sex, BMI, and race/ethnicity;

Model 3, adjusted as for model 2 plus eGFR, a measure of kidney function;

Model 4, adjusted as for model 3 plus total 25-hydroxyvitamin D concentration and dietary confounding variables:

Glucose AUC: fiber (g/1000kcal) for vitamin K_1; fiber (g/1000kcal), % energy from protein and % energy from carbohydrate for vitamin K_2, fiber (g/1000kcal) for total vitamin K.

Log (SI_{index}): fiber (g/1000kcal) for vitamin K_1; % energy from carbohydrate, % energy from protein, and % energy from fat for vitamin K_2; fiber (g/1000kcal) for total vitamin K.

Log (Matsuda-ISI index): fiber (g/1000kcal) for vitamin K_1; % energy from carbohydrate and fiber (g/1000kcal) for vitamin K_2; and fiber (g/1000kcal) for total vitamin K.

[2] Significant difference set at $P < 0.05$. * Statistically significant association, $P < 0.05$.

Abbreviations: SI_{index}, insulin sensitivity index; N/A, not applicable; Matsuda-ISI index, Matsuda-DeFronzo insulin sensitivity index; glucose AUC, glucose area-under-the-curve.

Table 15. Multiple linear regression analyses of the relation between quartiles of vitamin K intake (μg/1,000kcal) and indices of beta-cell function and compensation[1]

	Indices of beta-cell function					
	Log (AIRg) *n*=91		Log (IGI) *n*=91		Log (oral DI) *n*=91	
		P-value[2]		*P*-value[2]		*P*-value[2]
Vitamin K_1						
Model 1	-0.001 (-0.064, 0.062)	0.974	0.039 (-0.024, 0.102)	0.222	0.050 (-0.006, 0.105)	0.077
Model 2	0.014 (-0.049, 0.077)	0.664	0.052 (-0.008, 0.113)	0.090	0.046 (-0.012, 0.103)	0.117
Model 3	0.012 (-0.056, 0.079)	0.734	0.055 (-0.009, 0.120)	0.093	0.045 (-0.016, 0.106)	0.147
Model 4	0.007 (-0.062, 0.076)	0.848	0.050 (-0.014, 0.115)	0.125	0.038 (-0.022, 0.099)	0.213
Vitamin K_2						
Model 1	0.036 (-0.026, 0.098)	0.257	0.034 (-0.030, 0.097)	0.294	-0.022 (-0.079, 0.034)	0.436
Model 2	0.013 (-0.052, 0.079)	0.686	-0.005 (-0.071, 0.061)	0.876	-0.015 (-0.079, 0.048)	0.637
Model 3	0.013 (-0.052, 0.079)	0.686	-0.005 (-0.071, 0.060)	0.883	-0.015 (-0.078, 0.048)	0.637
Model 4	0.028 (-0.052, 0.108)	0.485	0.028 (-0.047, 0.104)	0.456	0.019 (-0.053, 0.091)	0.604
Total vitamin K						
Model 1	-0.001 (-0.064, 0.061)	0.963	0.036 (-0.027, 0.099)	0.264	0.036 (-0.019, 0.092)	0.197
Model 2	0.011 (-0.051, 0.074)	0.718	0.043 (-0.018, 0.103)	0.166	0.033 (-0.024, 0.091)	0.254
Model 3	0.009 (-0.058, 0.076)	0.792	0.044 (-0.020, 0.109)	0.126	0.031 (-0.030, 0.092)	0.315
Model 4	0.004 (-0.064, 0.072)	0.906	0.039 (-0.025, 0.104)	0.228	0.024 (-0.036, 0.851)	0.428

[1]Data are unadjusted β coefficients (95% CIs).

Model 1, unadjusted;

Model 2, adjusted for age, sex, BMI, and race/ethnicity;

Model 3, adjusted as for model 2 plus eGFR, a measure of kidney function;

Model 4, adjusted as for model 3 plus total 25-hydroxyvitamin D concentration and dietary confounding variables:

Log (AIRg): fiber (g/1000kcal) for vitamin K_1; % energy from protein, % energy from fiber, % energy from fat, and fiber (g/1000kcal) for vitamin K_2; fiber (g/1000kcal) for total vitamin K.

Log (IGI): fiber (g/1000kcal) for vitamin K_1; % calories from protein, % calories from carbohydrate, and fiber (g/1000kcal) for vitamin K_2; fiber (g/1000kcal) for total vitamin K.

Log (oral DI): fiber (g/1000kcal) for vitamin K_1; % calories form protein, % calories from carbohydrate, % calories from fat and fiber (g/1000kcal) for vitamin K_2; fiber (g/1000kcal) for total vitamin K.

[2] Significant difference set at $P < 0.05$.

Abbreviations*:* AIRg, acute insulin response to glucose; IGI, insulinogenic index; oral DI, oral disposition index.

DISCUSSION

In this cross-sectional study, we found some evidence for an association between vitamin K_2 intake and measures of glucose homeostasis. In support of our hypothesis, higher intake of vitamin K_2 was associated with better glucose tolerance in response to a standardized oral glucose load. Although higher vitamin K_2 intake was also associated with insulin sensitivity as measured by the Matsuda-DeFronzo insulin sensitivity index in a crude, unadjusted model, the relation was attenuated and no longer statistically significant after we adjusted for classical risk factors (i.e., age, sex, BMI, and race/ethnicity), eGFR status, vitamin D status, and dietary confounders. Similarly, vitamin K_2 intake was not associated with insulin sensitivity based on assessments from the gold standard, hyperinsulinemic-euglycemic clamp. We did not find any association between vitamin K_2 intake and indices of β-cell function. Neither vitamin K_1 nor total vitamin K intake was associated with any parameters of glucose homeostasis, including glucose tolerance, insulin sensitivity, and β-cell function.

To date, only two observational studies have investigated the association between dietary vitamin K_2 intake and glucose homeostasis[69, 74]. In both studies, higher dietary vitamin K_2 intake had beneficial effects on glucose homeostasis. Beulens and colleagues found that higher vitamin K_2 intake was associated with lower T2DM risks (P = 0.060), with a HR of 0.95 (95% CI: 0.91- 1.01) for each 10μg increment in an age-, sex-, and waist-adjusted model[69]. In a multivariate model, the inverse association was even more robust (P = 0.038), with a HR of 0.93 (95%CI: 0.87-1.00) [69]. Using quartiles of vitamin K_2 intake

also produced similar results[69]. Dam and colleagues, on the other hand, correlated higher dietary vitamin K_2 intake with lower fasting plasma glucose and reduced occurrence of the metabolic syndrome[74]. At baseline, vitamin K_2 intake was inversely associated with the prevalence of metabolic syndrome (P_{trend}=0.08) with a prevalence ratio of 0.74 (95%CI: 0.54 – 1.03)[74]. At ten-year follow-up, the highest tertile of vitamin K_2 intake was associated with a lower occurrence of metabolic syndrome (P_{trend}=0.01), with a prevalence ratio of 0.62 (95%CI: 0.40-0.95) for the highest vs. the lowest tertile group[74]. It is important to note, however, that neither study included comprehensive assessments of glucose metabolism as endpoint measures, thereby making it difficult to compare these data to our study.

Of the few intervention studies conducted on the effects of supplemental vitamin K_2 on selected measures of glucose homeostasis[66,70,71], only one study employed gold-standard measures of glucose tolerance, insulin sensitivity, and β-cell function to assess changes in glucose tolerance and its determinants. In this study, Choi and colleagues showed that supplemental vitamin K_2 (30mg/d) for 4 weeks was associated with improved glucose tolerance, increased insulin sensitivity, IGI, and oral DI, but not with AIRg[71]. Results from our study were only partially consistent with the findings from this clinical trial, as vitamin K_2 intake was associated with glucose tolerance but not with insulin sensitivity or β-cell function. The discrepancies between our findings may be attributed to the high dose used in this intervention study (30mg/d), in comparison with the relatively low dietary vitamin K_2 intake of our study population (mean=27μg/d).

Current literature suggests that vitamin K_1 may be associated with greater insulin sensitivity[67, 73], improved HOMA-IR[62], lower incidences of hyperglycemia[68], and T2DM[69, 72]. Nonetheless, findings of these existing studies were not entirely consistent, and commonly were not based on gold-standard measures of insulin sensitivity and β-cell function. The lack of an association between vitamin K_1 intake and glucose tolerance, insulin sensitivity, and β-cell function in our study that did include gold-standard measures is therefore a valuable contribution to the literature. Finally, we did not find any association between total vitamin K intake and any parameter of glucose metabolism. This is not surprising, given that total vitamin K intake is largely driven by the amount of vitamin K_1 (bivariate correlation with total vitamin K intake: $r = 0.824$) rather than vitamin K_2 ($r = 0.069$) in the diet [data not shown], and suggests that the impact of vitamin K_2 may be greater or fundamentally different than that of vitamin K_1.

Strengths of our study include the enrollment of a diverse population ranging from healthy individuals to those with moderate-severe CKD, the use of gold standard measures of glucose tolerance and its determinants, and the collection of relevant and high-quality covariate data[203]. The major limitation of this study is the limited availability of high quality food composition data for vitamin K_2 to be used for estimating its intake in our study population. To our knowledge, food composition databases for vitamin K_2 are only available in four countries (**Table 8**), with some made available by experimental studies (**Table 7**). Nonetheless, most of these are limited in quality and quantity of detail, such as the lack of a comprehensive measurement of all menaquinones (MK-4 through MK-14) in foods. The measurement error is further increased by the varying amounts in

each food, which is attributable to regional and/or seasonal differences[48]. For instance, meat from animals grazed on grass will accumulate vitamin K_2 in their tissues in direct proportion to the amount of vitamin K_1 in their diet. On the other hand, meat from those fed soy and corn may not accumulate as much vitamin K_2 due to the relatively low vitamin K_1 concentrations in soy and corn compared to grass.

The amount of vitamin K_2 can also vary between batches of the same food item, especially if the total vitamin K_2 content is highly dependent on fermentation processes. One example is cheese, where the same type of cheese could have as much as a 14-fold difference in vitamin K_2 content[193]. As stated by Walther et al[198], different bacterial starters used in the dairy and meat fermentation industries could also contribute to varying contents of vitamin K_2. **Table 16** shows some of the most commonly used MK-producing species in industrial food fermentation applications[198].

Table 16. Menaquinones produced by bacteria species commonly used in industrial food fermentations[1,2]

Species, subspecies	Food use	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10
Lactococcus lactis subsp. *lactis*	Cheese, buttermilk, sour cream, cottage cheese, cream cheese, kefir	√		√	√	√√	
Lactococcus lactis subsp. cremoris	Cheese, buttermilk, sour cream, cottage cheese, cream cheese, kefir			√	√	√√	
Leuconostoc lactis	Cheese			√	√	√√	
Brevibacterium linens	Cheese				√		
Brochontrix thermosphacta	Meat	√	√	√√			
Hafnia alvei	Cheese				√		
Staphylococcus xylosus	Dairy, sausage		√	√√	√		
Staphylococcus equorum	Dairy, meat		√	√√	√		
Arthrobacter nicotinae	Cheese			√	√√	√	
Bacillus subtilis "natto"	Natto			√√		√	
Propionibacterium shermanii	Cheese						

[1] MK, menaquinones; √, minor form; √√, major form.

[2] Danisco internal data. Note that most species within the genera *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* commonly used in fermentation or added to foods as probiotics are not known to produce MK.

Manoury et al. Journal of dairy science. 2013; 96(3): 1335-46

Adding to the complexity of having incomplete and variable data on vitamin K_2 composition in foods, assessment of food intake in our study was only based on a single three-day dietary record. One previous study showed that a minimum of six days of diet recording is needed to assess usual vitamin K intake among older adults[204]. Taken together, it is possible that our vitamin K_2 intake data based on three-day dietary records and imperfect nutrient databases may have led to misclassification of the exposure, i.e., that our measured vitamin K_2 intake may not be representative of the actual long-term intake of vitamin K_2 in the participants.

Other limitations include our inability to assess specific sites or mechanisms of insulin sensitivity (liver vs. peripheral tissues); the relatively small study size, which reduces power to detect small associations independent of numerous covariates; and the cross-sectional nature of the study design, which precludes causal inference and the evaluation of health outcomes over time[203]. Additionally, enrollment of our study necessarily involved some degree of participant self-selection[203]. For example, individuals interested in participating in a clinical study may be more health-conscious, and our results may therefore not generalize to all healthy individuals and patients with moderate-severe CKD[203]. As in any observational study, it is important to point out that our findings may have been affected by residual and unmeasured confounding by factors not considered in this analyses, or factors that are hard to measure accurately, such as that of energy intake.

In conclusion, in this cross-sectional cohort of healthy individuals and patients with stage 3-5 CKD, vitamin K_2 intake was associated with glucose tolerance in

multivariate analyses, but not with insulin sensitivity or β-cell function. Together with the previous observational studies and the clinical trial conducted by Choi et al[71], our results suggest that vitamin K_2 intake may have beneficial effects on glucose tolerance. However, our data do not clearly suggest whether improvements in glucose tolerance in individuals consuming more vitamin K_2, if causal, are due to a primary effect on insulin sensitivity or pancreatic β-cell function. Thus, follow-up studies with better assessment of vitamin K_2 intake status and a larger sample size are necessary.

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